Science Foundation (CHE76-08534) for an equipment grant for the Bruker WH 180 NMR spectrometer.

Registry No.—**1**, 15413-34-0; **2**, 66702-00-9; **2d**, 66674-96-2; methanol, 67-56-1.

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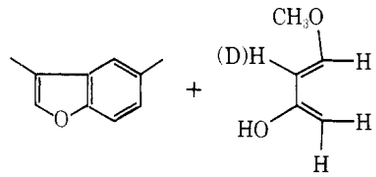
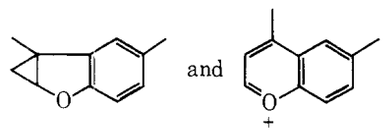
(8) The shift study was easier to interpret for **2d** than for **2**, because of the absence of H_D.
(9) The mass spectrum shows, in addition to M^+ (246; for **2d**, 247) and $M^+ -$ methanol (214; for **2d**, 215 and 214), two sets of interesting fragmentation peaks. A retro-Diels–Alder[10] of the enol can give



m/e 146 *m/e* 100 (101)

accounting for the same base peak in **2** and **2d**. The peaks at *m/e* 160 and 159 in both compounds may arise from α-carbonyl and benzylic cleavage to give



m/e 160 *m/e* 159

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Determination of p*K* Values for the Bisulfite Adducts of Cytidine 5′-Monophosphate by Carbon-13 Nuclear Magnetic Resonance

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Addition of bisulfite to carbon 6 of biologically important pyrimidines is a well-studied reaction,[1-3] having been investigated for nucleosides,[4] nucleotides,[5] and nucleic acids.[6] From a bioorganic standpoint the most intriguing event is the bisulfite-catalyzed deamination of cytidine to form uridine, the biological implications of which have been previously demonstrated.[7,8] Shapiro et al.[9] have advanced a mechanistic rationale (Scheme I) which includes both the protonated and nonprotonated cytidine–bisulfite adducts. In this mechanism the assumption was made that there is only one adduct formed. The present communication characterizes the two diastereomeric bisulfite adducts of cytidine 5′-monophosphate (CMP, 4) (Scheme II) and reports the p*K* values for the N-3 proton dissociation of these two adducts.

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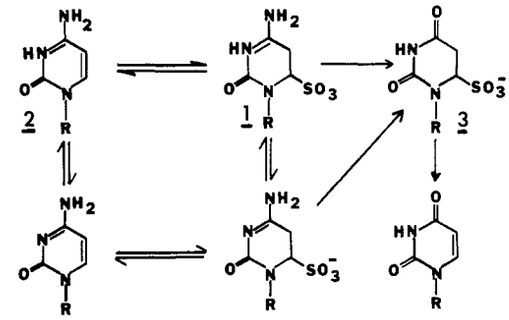
Scheme I



^{13}C NMR spectroscopy of an aqueous solution of CMP (**4**) yields a nine-line spectrum (Table I). Upon addition of bisulfite, a spectrum is obtained which is a composite of the original spectrum and those of two new compounds (Table I). Significantly, the signals corresponding to the sp^2 carbons of CMP (C-6, 142.7 ppm, and C-5, 97.3 ppm) are diminished. The CMP–bisulfite adducts (**5A** and **5B**) each display a set of signals of unequal intensity which includes carbon 2, carbon 4, and the sugar carbons. Based on their relative intensities, the signals can be grouped into two sets (CMP/HSO_3^- A and CMP/HSO_3^- B) and assigned to the appropriate carbons of the adducts[10] (Table I). In addition, two new sets of signals corresponding to the sp^3 carbons at positions 5 and 6 of the adducts are observed at 28.8 and 28.5 ppm (C-5) and 68.1 and 66.1 ppm (C-6). These are readily assigned by analogy with the known spectra for the bisulfite adducts of uracil and uridine.[4,11] When the sample is allowed to stand for longer periods (24 h), two more nine-line spectra are observed. The new carbon signals are assigned (Table I) as the diastereomeric bisulfite adducts of uridine monophosphate (**6A** and **6B**, Scheme II). This assignment is made on the basis of data previously reported from our laboratories in which uridine[4] and uracil[11] were substrates of similar bisulfite addition.

Shapiro et al.[9] reported a p*K* value of 5.3 for the N-3 proton dissociation in the cytidine–bisulfite adduct. This value was determined by ^1H NMR spectroscopy with deuterium oxide as the solvent. Thus, corrections were made to account for the effect of the deuterated solvent on the observed pH values. In light of the evidence for the existence of two diastereomeric bisulfite adducts of CMP, and because one of the parameters in the kinetically derived mechanism is the p*K* of these species, we were prompted to determine the p*K* values for each adduct.

The system under study using ^{13}C NMR spectroscopy initially consists of an aqueous solution containing only CMP (**4**) and its bisulfite adducts (**5A** and **5B**). However, after 24 h it was found to contain five discrete chemical species (Scheme II): CMP, its two diastereomeric bisulfite adducts (**5A** and **5B**), and two diastereomeric bisulfite adducts of uridine 5′-monophosphate (UMP) (**6A** and **6B**). These five species have a total of 18 possible p*K* values. Theoretically it is possible,

Scheme II

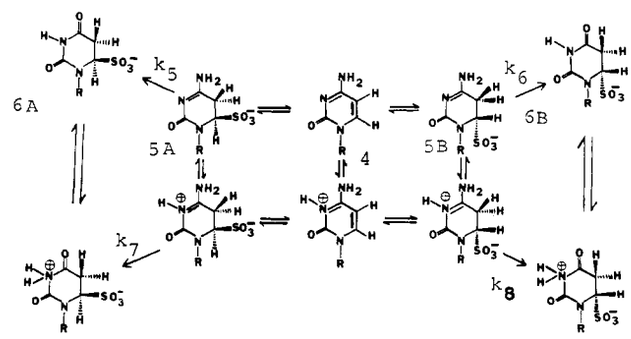


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Table I. Chemical Shift Assignments (ppm)[a]

Compd	Registry no.	C-2	C-4	C-5	C-6	C-1′	C-2′	C-3′	C-4′	C-5′
CMP	63-37-6	157.1	165.9	97.5	142.6	90.3	70.5	75.3	84.4[b]	64.6[b]
CMP/HSO_3^- A	66687-68-1	156.8	170.1	29.0	70.30	94.27	71.05	75.3	83.16[b]	64.71[b]
CMP/HSO_3^- B	66687-69-2	155.8	169.8	29.0	65.88	91.97	69.83	72.57	83.16[b]	64.00[b]
UMP/HSO_3^- A	66748-91-2	153.70	172.93	33.28	66.77	92.45	71.17	74.89	83.08[b]	64.82[b]
UMP/HSO_3^- B	66748-92-3	154.58	172.93	33.66	65.88	94.69	70.78	72.45	83.48[b]	65.57[b]

[a] Assignments for CMP, the two diastereomeric bisulfite adducts of CMP, and the two diasteromeric bisulfite adducts of UMP which result from deamination; pH 5.3. [b] Denotes signals which are split into doublets by the ^{31}P nucleus.

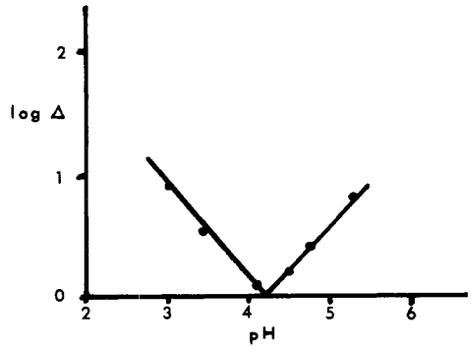

Figure 1. A plot of log Δ vs. pH for cytidine 5′-monophosphate, where Δ is $(\delta_{max} - \delta)/(\delta - \delta_{min})$. The intercept (log Δ = 0) is that point where the pH value is equal to the pK value. In this instance the pK value of CMP is found to be 4.2.

Table II. pK Values for the Components Found in a Reaction Mixture of CMP and Bisulfite

	pK (obsd)	pK[a]
Uridine[b]	9.20	9.2
CMP	0.6–1.0	0.8[a]
	4.20	4.2
	6.08	5.97
CMP/HSO_3^- A	5.20	
CMP/HSO_3^- B	4.82	

[a] See ref 14. [b] Registry no.: uridine, 58-96-8.

using ^{13}C NMR spectroscopy, to determine each and every pK value for the components of this complex mixture. This is accomplished by plotting the ^{13}C chemical shifts for each species as a function of pH. As an initial test of this approach and in order to simplify the results observed for the reaction mixture, the pH dependence of the uridine and CMP ^{13}C NMR spectra was measured. Large shifts are observed for C-2 and C-4 and to a lesser extent for C-6 upon removal of the proton from N-3. Not as obvious are two smaller changes in the chemical shift for C-5′ (and to a lesser extent C-4′) of CMP corresponding to the successive removal of protons from the phosphate moiety. Accurate numerical values for the four pK_a's can be obtained from the Henderson–Hasselbach equation as described by Dorman and Roberts[12]

$$\mathrm{pH} = \mathrm{p}K + \log \left| \frac{(\delta_{max} - \delta)}{(\delta - \delta_{min})} \right| \quad (1)$$

where δ is the observed chemical shift in ppm for a particular carbon atom and δ_{max} and δ_{min} are the maximum and minimum observed chemical shifts, respectively.

An example of these plots is illustrated in Figure 1 for C-4 of CMP. Agreement between the values measured here and those previously reported in the literature is excellent as

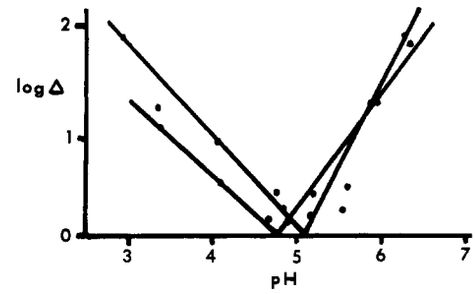

Figure 2. A plot of log Δ vs. pH for diastereomeric bisulfite adducts of CMP_x, where Δ is $(\delta_{max} - \delta)/(\delta - \delta_{min})$. The graphs intercept the x axis at 4.82 and 5.2, indicating that these are the pK values of the adducts.

shown in Table II. This is particularly interesting in light of the potential deviations expected from operating with relatively concentrated solutions at high ionic strength.[13]

With the data for CMP available, the more complex reaction mixture is readily interpreted. Samples containing equimolar amounts of CMP and bisulfite were prepared, their pH and ionic strength were adjusted, and after standing for exactly 24 h their ^{13}C spectra were obtained. The pK's of the phosphate groups are not expected to change appreciably, and they are not involved in the proposed deamination mechanism. Therefore, attention was focused on the pH region corresponding to the deprotonation of N-3 in the adducts. Unfortunately, desulfonation of the uridylate adducts (**6A** and **6B**) to form UMP occurs at pH below the pK of the deprotonation of N-3 in the UMP adducts. The CMP adducts are more stable, and the corresponding signals (C-2, C-4, and C-5) display the expected pH dependence. The final results are listed in Table II and plotted in Figure 2. Henderson–Hasselbach plots, as in Figure 1, yield values for the pK_a's of each individual adduct.

It is most significant that the pK values for the diastereomeric bisulfite adducts of CMP are different. This would imply that if the deamination step for CMP follows that of cytidine (Scheme I), being pH dependent, it may proceed at significantly different rates for the individual diastereomers. For example, at pH 4.5 the diastereomer with a pK value of 4.82 will be 48% dissociated while the other diastereomer (pK value 5.20) will only be 20% dissociated. Thus, if in Scheme II the rate constants for the deamination of the nonprotonated diastereomers (k_5 and k_6) are much smaller than those for the protonated species (k_7 and k_8), then the rates will be reflected by the concentration of the protonated species. Therefore, the rate of deamination of the diastereomer with the lower pK value will be faster than for the diastereomer with the larger pK value. In light of the data presented here, it appears that the mechanism for the bisulfite-catalyzed deamination of CMP is far more complicated than the one previously advanced.[9]

Experimental Section

Sample Preparation. Uridine was purchased from Aldrich Chemical Co. (Milwaukee, Wis.), and cytidine 5′-monophosphate was purchased from Sigma Chemical Co. (St. Louis, Mo.). These compounds were used without further purification. Typically, samples were prepared as aqueous solutions containing either 100 mg per mL or 200 mg per mL of the nucleoside or nucleotide, with or without equimolar sodium bisulfite. Concentrated HCl or sodium hydroxide (8 M) was used to obtain specific pH values. Sufficient solid potassium chloride was added to maintain the final ionic strength at a fixed value of 1.0; pH measurements were carried out at 30 °C with an IL Delta Matic pH meter (Perkin-Elmer).

NMR Measurements. All carbon-13 spectra were measured on samples in 8 mm sample tubes. A capillary tube containing 20% of 1,4-dioxane and 80% of deuterium oxide was used as a lock signal and an external reference. The observed chemical shifts were converted to the Me_4Si scale using δ(C − dioxane) = 67.40 ppm. Spectra were recorded on a Varian CFT-20 (16K) spectrometer equipped with a single side-band crystal filter for signal to noise ratio improvement and a Sykes diskette unit for storage. All ^{13}C NMR spectra were measured corresponding to a 4000 Hz (200 ppm) spectral width in 4096 data points.

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A Carbon-13 Nuclear Magnetic Resonance Study of Dibenzoylcystine Gels

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Gels are semirigid colloidal systems rich in liquid. Protoplasm ranks as the most widespread example of this peculiar state of matter. Two not entirely distinct theories have been advanced to explain gelation. The first, championed by Bradford[1] in the 1920's, maintains that the sol-to-gel transformation is a type of crystallization (the gel consisting of two phases composed of microcrystalline forms surrounded by water[2]). Alternatively, a gel may be formed by noncrystalline aggregates which cross-link in solution so as to entrain the dispersing medium in the capillary spaces between them.[3,4]

Dibenzoylcystine (I) is conspicuous among those organic substances that gel (e.g., agar, gelatin, poly(2-hydroxyethyl methacrylate), etc.) because of its simple structure.[5,6] Dibenzoylcystine is unique for another reason; a stiff hydrogel is produced by only 3×10^{-3} M disperse phase! In contrast to gelatin which associates in solution,[7,8] dibenzoylcystine seems to be a "fibrillary crystalline gel".[9] We describe herein an examination of dibenzoylcystine gels by ^{13}C NMR spin–lattice relaxation times (T_1).

Table I. Carbon-13 Spin–Lattice Relaxation Times in Seconds (T_1) of Dibenzoylcystine Gels at 37 °C

conditions	physical state	T_1 ortho	T_1 para
0.62 M in Me_2SO	liquid	0.35	0.16
0.30 M in Me_2SO	liquid	0.49	0.16
0.30 M in 10% D_2O–Me_2SO	soft gel	0.41	0.14
0.30 M in 20% D_2O–Me_2SO	thick gel	0.41	0.15
0.30 M in 20% D_2O–Me_2SO (sonicated)	thick gel	0.42	0.16

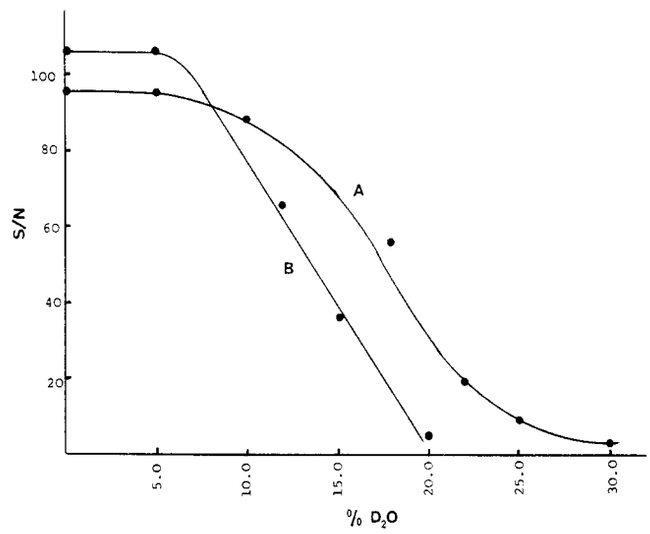
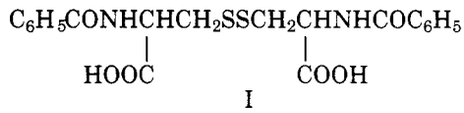


Figure 1. ^{13}C NMR signal-to-noise values for 0.32 M (curve A) and 0.63 M (curve B) dibenzoylcystine in Me_2SO with varying concentrations of D_2O at 37 °C using a constant number of transients (10 000 for A and 1000 for B).



I

T_1 studies provide information on molecular motion without the need for an external probe.[10,11] If a gel-forming compound associates in solution akin to surfactants, then T_1 values should decrease only modestly (two- to five-fold) relative to the monomeric state.[12] If a clear gel behaves as a two-phase system, then the ^{13}C NMR line widths and T_1's should be affected dramatically.[13] It is the purpose of this note to differentiate these alternatives with gels of a simple organic substance.

Since dibenzoylcystine in water does not form clear gels at the relatively high concentrations required for T_1 measurements, we used a dimethyl sulfoxide–water solvent system. A 0.30 M solution of I in pure Me_2SO is a fluid liquid; adding 10% water (v/v) produces a transparent semisolid gel. Further quantities of water (20%) give a thick white material. Thus, the gel consistency can be varied continuously by regulating the water content. The following T_1 values in seconds were found for 0.62 M I in pure Me_2SO at 37 °C: carbonyls (2.2 and 2.3); methine (0.15); C_1, C_2, C_3, and C_4 of aromatic ring (2.3, 0.35, 0.35, and 0.16, respectively).[14] These are quite ordinary values for an organic molecule the size of I.[15] In Table I we tabulate T_1 values for two aromatic carbons of I in Me_2SO-d_6 with and without D_2O. It is seen that gelation, in contrast to micellization,[12] need not alter the T_1's. Dibenzoylcystine apparently exists in two *non-exchanging* states: (a) a monomeric species which possesses normal T_1 values and line widths and (b) an aggregate whose ^{13}C resonances are broadened to the point of unobservability by ^{13}C–^1H dipolar

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