Reaction of Sodium Bisulfite with Uracil, Cytosine, and Their Derivatives*

Hikoya Hayatsu, Yusuke Wataya, Kazushige Kai, and Shigeru Iida

ABSTRACT: Quantitative addition of bisulfite to uracil and cytosine in hot aqueous solution at pH ~6 produced 5,6-dihydrouracil-6-sulfonate (**1a**). Compound **1a** was obtained in a crystalline form as monohydrate. Compound **1a** regenerates uracil on treatment with alkali. The structure of **1a** was elucidated by elemental analysis, and nuclear magnetic resonance, ultraviolet, infrared, and Raman spectroscopies. The position of the sulfonate group in the dihydrouracil ring was confirmed to be 6-C on the basis of deuterium incorporation experiments.

The reaction between uridine and bisulfite to give 5,6-dihydrouridine-6-sulfonate (**1b**) is optimal at pH 7. For example, it comes to a completion within 1 hr when carried out at 22° and pH 7, in 1 M sodium bisulfite solution. The formation of **1b** from cytidine is optimal at pH about 6. A quantitative conversion of cytidine into **1b** can be brought about by treatment with 1 M sodium bisulfite for 21 hr at pH 6 and 37°. The intermediate product, 5,6-dihydrocytidine-6-sulfonate (**2b**), was isolated in a crystalline form. Regeneration of uridine from **1b** comes to a completion within 4 min at pH 10.6. Compound **2b** regenerates cytidine at neutral pH very rapidly. 5-Methylcytosine is converted directly into thymine on reaction with bisulfite. Uridylyl(3′–5′)adenosine and cytidylyl(3′–5′)adenosine can be quantitatively transformed into the dihydrouracil-6-sulfonate derivative of uridylyl-adenosine on treatment with sodium bisulfite. The modified uridylyl(3′–5′)adenosine is susceptible to the digestion with pancreatic ribonuclease. These findings may imply a potential genetic hazard of sulfur dioxide and bisulfite salts, which are present in the environment, toward living organisms.

Studies on the interactions between chemicals and nucleic acids are of importance because they may be useful as a means for the chemical modification of biologically active nucleic acids, and also because the presence of such interactions implys potential hazards of those chemicals toward living organisms.

Currently, we have investigated the permanganate oxidation of nucleic acids and their components (Hayatsu and Ukita, 1967; Hayatsu and Iida, 1969; Hayatsu and Yano, 1969; Yano and Hayatsu, 1970). In these studies we have discovered that 4-thiouridine affords uridine-4-sulfonate when it is treated with permanganate (Hayatsu and Yano, 1969; Yano and Hayatsu, 1970). This oxidation has been terminated by addition of bisulfite. It has then been found that the bisulfite used there does react with 4-thiouridine. Subsequent studies have revealed that this reaction is oxygen requiring and brings about the formation of uridine-4-sulfonate (Hayatsu, 1969). In this research, it has also been noted that 4-thiouridine forms a complex with bisulfite, which lacks the characteristic ultraviolet absorption of 4-thiouridine at 330 mμ. These discoveries have led us to investigate the effects of bisulfite on other nucleic acid bases. We have now discovered that uracil and cytosine, and their nucleosides, undergo the Bücherer-type reactions with bisulfite under mild conditions. This paper deals with the conditions and specificities of the reaction, and the structure and the properties of the products. These studies form a basis for the elucidation of the mechanism of the recently discovered mutation of phage λ which occurs when the phage is treated with bisulfite (Hayatsu and Miura, 1970). They are also of importance for the possible use of this reaction to the selective chemical modification of uracil or cytosine residues in nucleic acids. A part of the chemical reaction described in this paper has been independently and simultaneously investigated by Shapiro and coworkers and a brief report of their research has been recently published (Shapiro *et al.*, 1970). Most of their results are consistent with those reported in the present paper.

Results

Materials and Methods. 1-Methyluracil was prepared by chemical synthesis (Hilbert and Johnson, 1930). UpA[1] and CpA were kindly supplied by Mr. M. Imazawa of our laboratory. Sodium bisulfite and sodium sulfite solutions were always freshly prepared before use. Bisulfite solutions having various pH values were prepared by admixture of the sodium bisulfite and the sodium sulfite solutions in varying ratios. For example, the pH 7 solution was prepared by admixture of 1 M $NaHSO_3$ and 1 M Na_2SO_3 in 1:3 (v/v) ratio. The bisulfite solutions of pH 5–8 are strong buffers. [^{35}S]Na_2SO_3 was purchased from The Radiochemical Centre, Amersham, England. Pancreatic ribonuclease, which had been purified according to Hirs *et al.* (1953), was a gift from Mr. T. Tsuruo of our laboratory. Deuterium oxide was purchased from Merck Chemical Industries. Paper chromatography was carried out ascendingly on Toyo filter paper no. 53, using the following solvent systems; solvent A, 1-butanol–acetic acid–water (5:3:1, v/v); solvent B, *t*-amyl alcohol–formic acid–water (3:2:1, v/v); solvent C, 1-butanol–water (86:14, v/v);

* From the Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan. *Received March 9, 1970.* A part of the work has been published in a preliminary report (Hayatsu *et al.*, 1970).

[1] UpA and CpA stand for uridylyl(3′–5′)adenosine and cytidylyl-(3′–5′)adenosine, respectively.

TABLE I: Chromatographic and Electrophoretic Data.

Compounds	R_F Values Solvent A	B	C	D	Rel Mobility in Electrophoresis pH 4.0	pH 2.2
Uridine	0.46	0.44	0.18		0	
Uridine 5′-phosphate					1.00	
Uracil	0.56	0.58	0.29	0.23		
1-Methyluracil	0.68	0.66	0.45			
Cytosine		0.52	0.17	0.19		
Cytidine		0.49				−1.76
Thymine			0.44	0.42		
5-Methylcytosine			0.20	0.26		
Sodium 5,6-dihydrouracil-6-sulfonate	0.23	0.36	0.00		1.90	
Sodium 1-methyl-5,6-dihydrouracil-6-sulfonate	0.31	0.40	0.02		1.90	
5,6-Dihydrocytosine-6-sulfonate		0.28				
Sodium 5,6-dihydrouridine-6-sulfonate	0.19	0.29	0.00		1.50	
5,6-Dihydrocytidine-6-sulfonate		0.26				
UpA		0.24				
CpA		0.28				−1.0
U*pA[a]		0.12			1.33	1.0
C*pA[a]		0.12				0.0

[a] U* and C* represent 5,6-dihydrouridine-6-sulfonate and 5,6-dihydrocytidine-6-sulfonate residues, respectively.

and solvent D, water-saturated 1-butanol–concentrated ammonia (100:1, v/v). Paper electrophoresis was performed at pH 4.0 using 0.1 M sodium acetate buffer or at pH 2.2 using 0.1 M glycine–HCl buffer as the solvent. R_F values in paper chromatography and the relative mobilities in paper electrophoresis of the compounds are summarized in Table I. Solvents were evaporated under reduced pressure. Nuclear magnetic resonance spectra (100 MHz) were recorded by Jeol-NM4H-100 nuclear magnetic resonance spectrometer. As a standard for the spectra of D_2O solutions, the signal of HOD was adjusted to 5.00 ppm and the chemical shifts of the other signals were read against it. In some experiments, sodium 2,2-dimethyl-2-silapentane-5-sulfonate was added as a standard into the D_2O solution, and the signals of compounds were read against that of this standard. Me_4Si was employed as an internal standard of the spectra of d_6-dimethyl sulfoxide solutions. Raman spectra were taken by Perkin-Elmer CRI spectrometer, the excitation beam used being the He–Ne gas laser. Spectral data are summarized in Tables II and III.

Addition of Bisulfite to Uracil and to Cytosine. When uracil (2 mmoles) was treated with aqueous sodium bisulfite solution (12 mmoles of $NaHSO_3$ plus 3 mmoles of Na_2SO_3 in 10 ml of H_2O) at 66° and at pH about 6, a rapid reaction took place and a crystalline product, **1a**, precipitated in the reaction mixture. The reaction was completed within 30 min and paper chromatographic analysis showed that only **1a** was the product. Colorless prisms of **1a** (recrystallized from 0.05 N acetic acid) were obtained in 80% yield. *Anal.* Calcd for $C_4H_4N_2O_2 \cdot NaHSO_3 \cdot H_2O$: C, 20.51; H, 3.01; N, 11.96; S, 13.69. Found: C, 20.73; H, 3.00; N, 11.94; S, 14.00. Figure 1 shows the ultraviolet spectra of **1a**. In neutral and acid solutions, **1a** exhibited only an end absorption, whereas in alkali it showed a spectrum which was identical with that of uracil (curve 2). Subsequent acidification gave a spectrum of undissociated uracil (curve 3). When **1a** was chromatographed after prior treatment with alkali, it gave a single spot whose R_F value was identical with that of uracil. The alkaline solution of **1a** reduced malachite green, an indication that sulfite ions had been produced from **1a**. These facts have shown that **1a** is an addition compound produced from uracil and sodium bisulfite.

It has been found that cytosine also undergoes a facile addition of bisulfite. Thus, when an aqueous solution of cytosine was treated with $NaHSO_3$ in the same manner as uracil (reaction temperature, 80°), **1a** was obtained in 72% yield. Since the formation of **1a** from cytosine should involve two steps, namely, the addition of sodium bisulfite and the subsequent hydrolysis of the amino group, detection and isolation of the intermediate, **2a**, were attempted. When a solution of cytosine was treated with sodium bisulfite at 0°, and was analyzed by paper chromatography, a spot corresponding to **2a** was detected. In 0.1 N HCl solution, **2a** exhibited an end absorption in the ultraviolet region. In neutral or alkaline solution, however, it showed a spectrum identical with that of cytosine, an indication that **2a** had regenerated cytosine in these solutions. All the attempts to obtain **2a** in a pure form have failed. Successful isolation of the intermediate compound of the type **2** was achieved in the reaction between sodium bisulfite and cytidine, which will be described below.

TABLE II: Infrared and Raman Data.

		ν_{CO} (cm^{-1})	ν_{SO} (cm^{-1})	ν_{CS} (cm^{-1})	δ_{SO} (cm^{-1})
Infrared (KBr)	Sodium 5,6-dihydrouracil-6-sulfonate (**1a**)	1729, 1692	1223, 1164, 1059	738	525
	Pyridinium 5,6-dihydrouracil-6-sulfonate	1740–1682	1227, 1164, 1059	728	518
	Trimethylammonium 5,6-dihydrouracil-6-sulfonate	1739–1683	1226, 1174, 1054	733	516
	Sodium 1-methyl-5,6-dihydrouracil-6-sulfonate	1732–1690	1056	732	516
	Pyridinium 1-methyl-5,6-dihydrouracil-6-sulfonate	1730–1665	1152, 1048	724	
Raman (H_2O)	Trimethylammonium 5,6-dihydrouracil-6-sulfonate	1710	1055	740	520

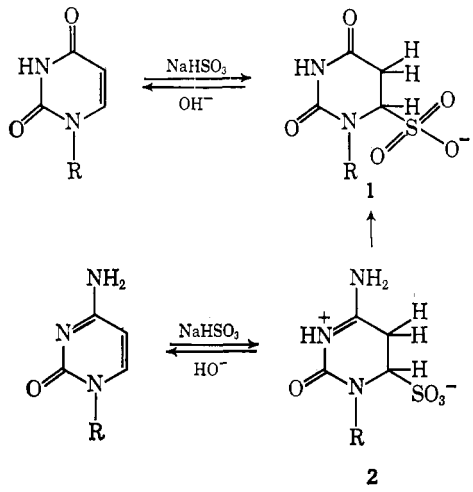
TABLE III: Nuclear Magnetic Resonance Data.

	Solvent	Std	ppm (δ)
Sodium 5,6-dihydrouracil-6-sulfonate (**1a**)	Dilute DCl[a]	HOD	3.75 (5-H_2, octet)
Sodium 5-deuterio-6-hydrouracil-6-sulfonate (**1a–1**)	Dilute DCl[a]	HOD	3.56 (5-HD, s), 5.10 (6-H, s)
Trimethylammonium 5,6-dihydrouracil-6-sulfonate	d_6-Me_2SO	Me_4Si	2.78 (5-H_2, m), 2.78 $N^+(CH_3)_3$ s), 3.95 (6-H, m), 7.81 (1-NH, d, $J_{1,6} \sim 3$ Hz), 9.80 (3-NH, s)
Pyridinium 1-methyl-5,6-dihydrouracil-6-sulfonate (**1c**)	d_6-Me_2SO	Me_4Si	2.77 (5-H_2, m), 3.02 (1-N-CH_3, s), 4.07 (6-H, m), 9.76 (3-NH, s)
Sodium 5,6-dihydrouridine-6-sulfonate (**1b**)	0.1 N DCl	DSS[c]	3.17 (5-H_2, octet), 3.80 (5′-H, m), 3.99 (4′-H, m), 4.26 (3′-H, m), 4.56 (2′-H, m), 5.00 (6-H, m), 5.38 (1′-H, d, J = 3.1 Hz), 5.67 (1′-H, d, J = 5.6 Hz)
Sodium 5-deuterio-6-hydrouridine-6-sulfonate (**1b–1**)	0.1 N DCl	DSS[c]	3.05 (5-HD, s), 5.00 (6-H), 5.38 (1′-H, d, J = 3.1 Hz), 5.67 (1′-H, d, J = 5.6 Hz)
Sodium 5,6-dihydrocytidine-6-sulfonate (**2b**)	0.1 N DCl[b]	HOD	4.28 (5-HD, s), 5.82 (6-H, s), 6.42 (1′-H, d)
Sodium 5-deuterio-6-hydrocytidine-6-sulfonate (**2b–1**)	0.1 N DCl[b]	HOD	4.28 (5-HD, s), 5.82 (6-H, s), 6.42 (1′-H, d)

[a] Recorded at 55°. [b] Recorded at 80°. [c] Sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

The following evidence has established the structure of **1a** to be sodium 5,6-dihydrouracil-6-sulfonate. Infrared spectrum of **1a** has shown two carbonyl bands indicating that 2- and 4-keto groups are present in this molecule. Therefore **1a** possesses a 5,6-dihydrouracil-type structure. Nuclear magnetic resonance spectrum of the pyridinium salt of this compound taken in d_6-dimethyl sulfoxide revealed a multiplet signal centered at 2.76 ppm corresponding to two protons. Another multiplet signal corresponding to one proton was present at 3.92 ppm. In the light of the nuclear magnetic resonance data of a number of 5- or 6-substituted 5,6-dihydrouracil derivatives (Wechter and Smith, 1968; Smith and Aplin, 1966; Katritzky *et al.*, 1969), the 2.76- and the 3.92-ppm signals were assigned to 5-H_2 and 6-H, respectively. The following facts strongly supported this assignment. The signal at 7.79 ppm, which can be assigned to 1-NH, is a doublet with $J \sim 3$ Hz.

CHART I



R = H (a), β-D-ribofuranosyl (b), CH_3 (c)

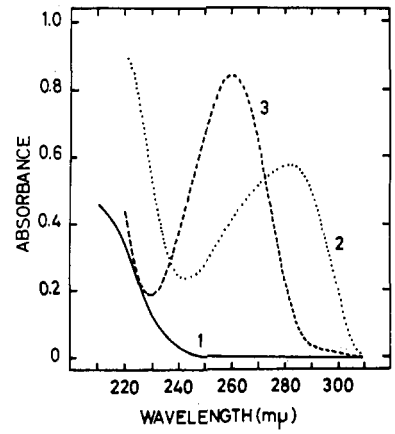


FIGURE 1: Ultraviolet spectrum of sodium 5,6-dihydrouracil-6-sulfonate (**1a**). Compound **1a** (153.8 mg) was dissolved in 0.1 N HCl (50 ml). Curve 1: 1 ml of the solution was diluted with 0.1 N HCl to 100 ml. Curve 2: 1 ml of the solution was diluted with 0.1 N NaOH to 100 ml. Curve 3: 1 ml of the solution was mixed with 5 ml of 0.1 N NaOH, allowed to stand at room temperature for about 30 min, and then diluted with 0.1 N HCl to 100 ml.

Upon addition of D_2O in the d_6-dimethyl sulfoxide solution, the 1-NH signal disappeared and there was no deformation observed in the multiplet signal of 5-H_2 as expected, although the 6-H signal was not detectable because, under these conditions, it was hidden by the strong signal of HOD. Furthermore, decoupling studies have substantiated these assignments.

For the unequivocal proof of the position of the sulfite group in **1a**, an introduction of deuterium at the 5 position of uracil was attempted through the use of the reverse reaction, **1a** to uracil. The sulfite addition to uracil was carried out in D_2O. The product, **1a–1**, was isolated and then it was treated with alkali, regenerating uracil. Nuclear magnetic resonance spectrum (in D_2O) of **1a–1** showed that the signal of AB protons of an ABX system, which had been observed for **1a**, was substituted by a signal of one proton, indicating that one deuteron was introduced in either one of the AB protons. Uracil obtained from **1a–1** showed, however, normal 5-H and 6-H signals having an intensity ratio 1 to 1. This result indicated that both the addition and elimination proceeded with equal stereospecificity. Successful introduction of a deuteron at 5 position of uracil was achieved in experiments performed with cytosine. The 5,6-dihydrouracil sulfonate (**1a–2**), obtained on treatment of cytosine with sodium bisulfite in D_2O, exhibited a signal near 3.5 ppm, whose intensity was about half of that of the one-proton signal at 5.1 ppm. This can be interpreted as deriving from an extensive double deuteration of the AB protons. When the nuclear magnetic resonance spectrum of uracil that was regenerated from **1a–2**, was examined, it was found that the ratio of signal intensity of the 6-H to that of the 5-H was approximately 2 to 1.

These results have clearly indicated that the position of the sulfite group in **1a** is the 6-C, and that the AB protons of the ABX system are the 5-H_2. An alternative structure in which the sulfite is attached to the 5 position does not explain the facts described above.

There are two possible modes of linkage between the 6-C and the sulfite group. One is the sulfonic acid type and the other the sulfite ester type. A strong support for the sulfonic acid structure was provided by infrared and Raman spectra. Infrared spectrum of the trimethylammonium salt derived from **1a** gave absorptions characteristic of a $CS(O_2)O^-$ group. The sulfite ester structure, $COS(O)O^-$, is not consistent with this spectrum. The Raman spectrum of this compound gave a set of strong bands assignable to the CSO_3^- group, the ν_{CS} band being the strongest among all of the absorptions in this spectrum. These Raman bands have been reported to be characteristic of alkylsulfonates and have been employed for the distinction of such sulfonates from isomeric sulfite esters (Houlton and Tartar, 1938). Compound **1a** traveled toward the anode in paper electrophoresis and the mobility was equal to that of 1-methyluracil-4-sulfonate (Yano and Hayatsu, 1970), a fact consistent with the sulfonate structure. From these experiments we have concluded that **1a** is sodium 5,6-dihydrouracil-6-sulfonate. 1-Methyluracil reacts with sodium bisulfite in the same manner as uracil.

Addition of Bisulfite to Uridine. When uridine was dissolved at 22° in 1 M $NaHSO_3$–Na_2SO_3 solution (pH 7) a rapid drop in its $A_{260\,m\mu}$ was observed and the reaction was complete after 60 min. At 37°, the reaction was complete in 20 min. The reaction with uridine 5′-phosphate was somewhat slower than with uridine.

The nature of this reaction is quite analogous to that of the uracil–bisulfite addition. Thus, uridine forms a product which possesses only an end absorption in the ultraviolet and which regenerates uridine upon treatment with alkali. The addition and elimination of bisulfite proceed in a quantitative fashion, as revealed by spectroscopic and paper chromatographic studies. From the analogy to the reaction of uracil, this reaction product is believed to be 5,6-dihydrouridine-6-sulfonate (**1b**). By addition of ethanol into the reaction mixture, **1a** was isolated as a precipitate which was free of uridine. Compound **1b** behaved as an anion in paper electrophoresis. Uridine was treated with [^{35}S]$NaHSO_3$ and the product isolated by paper electrophoresis (Figure 2). It was found that, as expected, one molecule of the product contained one ^{35}S atom.

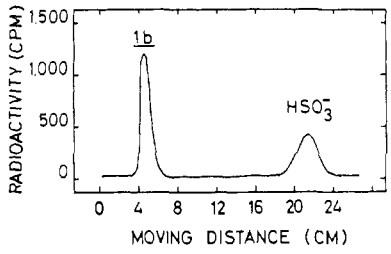


FIGURE 2: Paper electrophoresis of [^{35}S]-5,6-dihydrouridine-6-sulfonate (**1b**). Uridine (112 mg) was mixed with 2 M [^{35}S]$NaHSO_3$ (0.5 ml, containing 3.73 μCi of ^{35}S) and the solution was heated at 90–95° for 30 min. To the reaction mixture another 0.5 ml of the 2 M [^{35}S]$NaHSO_3$ solution was added and the solution was heated at 90–95° for additional 10 min. The solution was allowed to come to room temperature and was stood for 30 min. It was then chilled in ice, and cold ethanol (5 ml) was added to it. The precipitate was collected by centrifugation and washed with ethanol and ether, successively. The white material was further purified by reprecipitation twice from 0.05 N acetic acid–ethanol. The product was subjected to paper electrophoresis (0.1 M sodium acetate buffer, pH 4.0; 17 V/cm; 80 min). The paper was scanned for the radioactivity using Packard radiochromatogram scanner Model 7200, the scanning speed being 1 cm/10 min. The zone corresponding to [^{35}S]-5,6-dihydrouridine-6-sulfonate was eluted with water. After the solution had been treated with alkali, the A_{260} value and the ^{35}S radioactivity of this solution were determined. It was revealed that this solution contained 0.140 μmole of uridine and 0.138 μmole of ^{35}S.

The pH optimum of the reaction between uridine and bisulfite was found to be around 7. Essentially no reaction takes place at pH below 4, at room temperature. In a more dilute sodium bisulfite solution, the reaction was slower. Thus, whereas in 1 M sodium bisulfite solution at pH 7, the reaction extent was 86% after 30 min at 22°, in 0.5 M solution it was 59% and in 0.1 M solution it was only 12%.

The conditions for the regeneration of uridine from **1b** were examined, spectrophotometrically. The regeneration was more rapid in solutions of higher pH values. At pH above 12, it was instantaneously complete. At pH 10.6 (1 M NH_4Cl–NH_4OH) and 20°, the regeneration was complete in 4 min. At 36°, the regeneration extent by 2-hr treatment was found to be 100%/pH 9.0, 98%/pH 8.6, 18%/pH 7.0, 3%/pH 6.0, and 0%/pH 5.0.

Reaction of Bisulfite with Cytidine. Here again an analogy is present between a nucleoside and a base. Cytidine undergoes an addition of sodium bisulfite followed by a hydrolytic deamination giving **1b**. A preliminary experiment has shown that the first step, the addition, is very quick and the second step, the hydrolysis, is relatively slow. The first-step product, **2b**, is stable only in acid. In a neutral solution, it rapidly regenerates cytidine. Since the second-step product, **1b**, is stable in the neutral solution, these two steps can be assayed separately. For this purpose, a portion of the reaction mixture was diluted with 0.1 M sodium phosphate buffer (pH 7.0) and the $A_{271\,m\mu}$ was determined, giving the extent of the formation of **1b**. Another portion of the reaction mixture was diluted with 0.1 N HCl and the $A_{280\,m\mu}$ was determined, which showed the extent of the formation of **2b** plus **1b**.

The formation of **1b** from cytidine in 1 M bisulfite solution proceeds at pH between 4 and 6.5 at 37°. No formation of **1b** was observed at pH 7 or at pH 8. In the initial stage, the pH 5 reaction appeared to be the fastest (50%/pH 6, and 65%/pH 5, at 4-hr period). In order to push the reaction to completion, however, pH 6 seemed to be the most favorable (more than 95%/pH 6, and 74%/pH 5, at 21-hr period). Effect of temperature on the pH 6 reaction was studied. At higher temperature the reaction is more rapid. Thus, after 6-hr reaction at 60°, the reaction extent was 83%, whereas the reaction at 0° afforded only 3% formation of **1b** after 6-hr treatment. In a more concentrated bisulfite solution, the formation of **1b** is more rapid. Thus, in 3 M $NaHSO_3$ (pH 6), the reaction is complete after 6 hr (50% reaction after 1.5 hr). It is noteworthy that the formation of the dihydrouridinesulfonate from cytidine is much slower than that from uridine.

The formation of **2b** from cytidine, in contrast, is an equilibrium reaction which is completed almost instantaneously. Slow decrease in $A_{280\,m\mu}$, which was observed after the equilibrium had been reached, was due to the gradual formation of **1b**. The following values were obtained representing the percentage of the dihydrocytidine compound being present at the equilibrium: 73%/pH 4, 69%/pH 5, 64%/pH 6, 41%/pH 6.5, 24%/pH 7, and 0%/pH 8. Effect of temperature on this equilibrium was remarkable. Thus, in 1 M $NaHSO_3$ (pH 6) the content of the dihydrocytidinesulfonate (**2b**) was higher at a lower temperature: more than 90% at 0°, 64% at 37°, and 43% at 60°. At 0°, the equilibrium was reached within 20 min.

Isolation and Properties of 5,6-Dihydrocytidine-6-sulfonate (**2b**). A solution of cytidine (2.43 g, 10 mmoles) in water (20 ml) was chilled in an ice bath. Sodium bisulfite (5.20 g, 50 mmoles) was added to this solution and was dissolved while the solution was being cooled. The solution was then filtered by suction to remove traces of impurities, and allowed to stand in the ice bath. Crystals began to precipitate after 1 hr. After the reaction mixture had been stood in the ice bath for 12 hr, the crystals that precipitated were collected by filtration and washed with cold water, ethanol, and ether, successively. The material was dried *in vacuo*, giving a pure sample of 5,6-dihydrocytidine-6-sulfonate (1.41 g; 43% of the theoretical yield). *Anal.* Calcd for $C_9H_{15}N_3O_8S$ (zwitterionic structure); C, 33.23; H, 4.65; N, 12.92; S, 9.85. Found: C, 33.19; H, 4.60; N, 13.23; S, 9.79. On heating at 146°, this material decomposed into cytidine with simultaneous evolution of a gas (probably SO_2). For a complete regeneration of cytidine, it was necessary to carry out the heating at 146–155° for 1 hr, or at 211° for several minutes. The material obtained after this treatment gave a single spot of cytidine when checked by paper chromatography (solvent B). Ultraviolet spectrum of an acid solution of **2b** gave an end absorption. In neutral solution, however, it showed a spectrum identical with that of cytidine. This crystalline compound is probably one of the diastereomers of 5,6-dihydrocytidine-6-sulfonate. Rates of regeneration of cytidine from **2b** were examined. At 13°, the half-time of the regeneration was as follows: 6 min/pH 10, 8 min/pH 7, and 2.5 hr/pH 4. In 0.01 N NaOH, the regeneration instantaneously came to a completion. At pH 2.2, the regeneration was only 2% after 8-hr standing at 13°, and 6% after 26 hr. At 37°, the time required for the complete regeneration was less than 10 min at pH 7, and about 40 min at pH 4. When **2b** was treated with 0.1 N HCl at 87° for 1 hr, it produced **1b** (10%) along with cytidine (90%). This experiment has shown that the deamination can occur in the absence of sodium bisulfite and has, in turn, confirmed the position of the sulfonate group in **2b** to be 6-C.

Deuterium-Exchange Reactions Performed with Uridine and Cytidine. The exchange reactions carried out with the nucleo-

sides have yielded results analogous to those obtained with bases. When 5,6-dihydrouridine-6-sulfonate was prepared in D_2O from uridine (**1b–1**), and its nuclear magnetic resonance spectrum was measured, it was found that the 3.17-ppm octet of 5-H_2 of **1b** was changed to a dull singlet which corresponded to one proton. Uridine regenerated in H_2O from **1b–1** showed absence of deuterium in its molecule. Compound **2b**, which had been prepared from cytidine in H_2O, was subsequently treated with $NaDSO_3$ in D_2O, yielding the dihydrouridinesulfonate (**1b–2**). It was found with this compound that the ratio of the signal intensity of 6-H to that of 5-H was about 2:1, as it had been the case with the dihydrouracilsulfonate, **1a–2**. Uridine regenerated from **1b–2** was a 1:1 mixture of 5-D and 5-H species.

The crystalline dihydrocytidinesulfonates, that had been prepared in H_2O (**2b**) and in D_2O (**2b–1**), were subjected to nuclear magnetic resonance spectroscopy. Because of their poor solubility and of their labile nature, the spectra were taken immediately after the materials were dissolved in hot 0.1 N DCl. Compound **2b–1** gave a spectrum identical with that of **2b**. Cytidine regenerated in D_2O from either **2b** or **2b–1** contained no deuterium at their 5 or 6 positions.

Base Specificity of the Reaction. No reaction was observed with adenosine 5′-phosphate, guanosine 5′-phosphate, inosine 5′-phosphate, thymidine, pseudouridine, and 5,6-dihydrouridine, when they were treated with 1 M bisulfite (pH 7) at room temperature for 2 hr. In addition to this pH 7 treatment, the pH 6 treatment with 1 M $NaHSO_3$ appeared to have no effect upon adenosine 5′-phosphate, guanosine 5′-phosphate, and thymidine. In contrast, 5-methylcytosine, which is a minor base in DNA (Shapiro, 1968), does react with bisulfite. A treatment of 5-methylcytosine with 3 M $NaHSO_3$ (pH 5.6) at 37°, followed by a brief treatment with alkali brings about a quantitative conversion of the amino base into the keto base, thymine. Under these conditions the conversion was about 50% after 5 hr and essentially complete after 24 hr. It should be mentioned that even when the alkali treatment was omitted, a considerable portion of the 5-methylcytosine and of the thymine is present as free bases in the reaction mixture. This has suggested that the intermediates of this reaction, the bisulfite addition compounds of both 5-methylcytosine and thymine, are quite unstable.

Bisulfite Modification of UpA and CpA. UpA was derived into its bisulfite adduct on treatment with 2 M sodium bisulfite (pH 7.1) for 10 hr at 37°. No fission of the phosphodiester bond was observed during this treatment and the product was produced in a quantitative fashion. The modified UpA regenerated UpA quantitatively, on treatment with dilute ammonia (pH 8.6, 37°, 2 hr). When the modified UpA was digested with pancreatic ribonuclease at pH 5 (0.05 M sodium acetate buffer) at 37°, it was hydrolyzed giving adenosine and the modified uridylic acid in 1:1 ratio.

When CpA was treated with 1 M sodium bisulfite (pH 6.1) at 0°, its dihydrocytosinesulfonate derivative was produced. On additional incubation (24 hr) of the reaction mixture at 37° yielded the modified UpA quantitatively.

Discussion

It is known that a number of nucleophilic agents attacks the 5,6-double bond of pyrimidine nucleosides. Among them, the addition of hydroxylamine (Brown and Schell, 1965), the ultraviolet-catalyzed hydration (Wang *et al.*, 1956; Moore and Thomson, 1957; Wang, 1958), and the bromination (Wang, 1957, 1959) have been reported to cause the nucleophile to attack the 6 position of the pyrimidine ring (In the bromination, nucleophilic hydroxyl ion adds to the 6 position, while electrophilic bromine binds to the 5 position). It has now been demonstrated that bisulfite also undergoes the addition at 6 position of both uracil and cytosine derivatives. The ease with which the sulfite is eliminated from the addition compounds is analogous to, for instance, the facile regeneration of parent pyrimidines from the hydrates of them (Grossman *et al.*, 1965).

Although the addition of bisulfite to carbon–carbon double bonds has been reported previously (Cerfontain, 1968), the present work demonstrates the first example in pyrimidines. It should be mentioned that, while our research is under way, Isono *et al.* (1969) have reported that bisulfite catalyzes the decarboxylation of uracil-5-carboxylic acid, and they have suggested a mechanism in which the bisulfite attaches to the 6 position. Among the reactions in which bisulfite adds to a carbon–carbon double bond, the Bücherer reaction bears an apparent analogy to the bisulfite addition of uracil and cytosine. In the Bücherer reaction, the bisulfite undergoes a reversible addition to the 3 position of 1-naphthol or of 1-naphthylamine, producing a dihydrosulfonate derivative, and the dihydrosulfonate derivative of 1-naphthylamine readily undergoes hydrolytic deamination (Rieche and Seeboth, 1960).

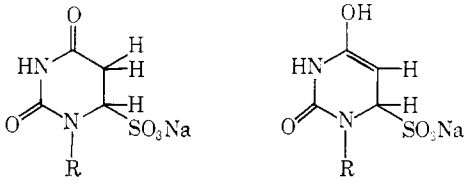
From the data presented here, there is little doubt about the structure of 5,6-dihydrouracilsulfonate. In order to provide a further confirmation of the structure, preparation of **1a** was attempted by catalytic hydrogenation of uracil-6-sulfonic acid, which is a known compound (Greenbaum and Holmes, 1954). This attempt, however, has failed because this sulfonic acid resists the hydrogenation catalyzed either by rhodium–aluminum or by Adams' platinum.

The pH profile of the conversion of cytidine into **1b** shows that the reaction is optimal at pH 6, and that at pH 4 or 5 it is slowed down after several hours' rapid reaction. This slowdown phenomenon was repeatedly observed and appeared to be significant. Some unidentified factors, such as a catalytic function of a specific base (for example, cytidine), may be involved in this process. The ease with which the dihydrocytosine-6-sulfonate is deaminated may be compared to that of 5,6-dihydrocytidine previously reported (Green and Cohen, 1958).

The quantitative regeneration of uridine from 5,6-dihydrouridine-6-sulfonate by treatment with alkali is in sharp contrast to the hydrolytic ring cleavage of 5,6-dihydrouridine (Batt *et al.*, 1954) which occurs very readily at its N^3–C^4 bond when the latter compound is treated with alkali. We have so far been unable to detect this type of ring cleavage in **1b** under any conditions. The facile regeneration of cytidine from **2b** either by heat or by alkali is to be compared to the regeneration of cytidine from 6-hydroxy-5,6-dihydrocytidine (Pietrzykowska and Shugar, 1969).

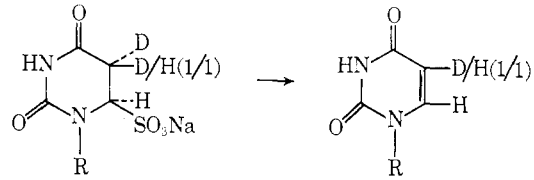
On the basis of the previously reported stereochemistry of ionic additions and eliminations (Newman, 1956), probably *trans* addition and *trans* elimination are occurring in the reactions, uracil → **1a** → uracil. This indicates that a tautomerism between **1** and **3** (Chart II) does not take place during these processes. This is in contrast to the ultraviolet-irradiated hydration of uracil derivatives in which this kind of tautomerization appears to occur (Wechter and Smith, 1968).

CHART II



1 3

CHART III



1a–2, and 1b–2

Compound **1a** has an assymmetric carbon at its 6 position. The crystalline **1a**, that was obtained, is, however, optically inactive and is apparently a racemic compound.

In order to explain the fact that the 5-H signal intensities of both **2b** and **2b–1** correspond to one proton and that the cytidine regenerated from either of them does not possess a deuterium at its 5 position, it has to be assumed that one of the two protons at the 5 position of **2** which is located only *trans* to the sulfonate group, is exchangeable with the deuterons in the solvent being used for the nuclear magnetic resonance recording.

The fact that the dihydrouracil compound which has been produced in D_2O from cytosine (or from cytidine) exhibits the 6-H:5-H value, 2:1, and that the uracil regenerated therefrom is a 1:1 mixture of the 5-H and the 5-D species, indicates the following situation for these compounds (Chart III). Since the cytidine which has been regenerated from crystalline **2b**, which in turn has been prepared on treatment of cytidine with bisulfite in the D_2O solvent, does not possess any deuterium at its 5 position, it is likely that the fission of a half of the 5-H atoms of cytidine takes place during the deamination.

It would be of interest to use the bisulfite reactions in the chemical modification of polynucleotides, because the reaction can be made highly specific for a single base. For example, the treatment with 1 M $NaHSO_3$–Na_2SO_3 at pH 7 followed by the dilution with the pH 7 buffer would bring about a uracil-specific transformation, whereas the treatment with 1 M bisulfite solution at pH 4 would produce a cytosine-specific modification. Furthermore, the pH 6 reaction coupled with a subsequent treatment with weak alkali would bring about the alteration of cytosine into uracil. The conditions under which cytosine is modified will also transform 5-methylcytosine into thymine, although this transformation is slower than that of cytosine.

The demonstration that UpA and CpA can be quantitatively transformed into their dihydrouracil-6-sulfonate derivatives without affecting the phosphodiester bond, and that this dihydro compound regenerates UpA upon treatment with weak alkali, has shown the potential usefulness of the reaction for the chemical modification of nucleic acids. The susceptibility of the modified UpA toward the treatment with pancreatic ribonuclease has diminished the usefulness of this reaction in the primary-sequence studies of RNA.

A preliminary experiment using DNA as the substrate of the bisulfite reaction has suggested that the reaction is specific to the single-strand region. The reaction of DNA with bisulfite is now under investigation in order to elucidate the mechanism of the mutation of phage λ which is inducible by the treatment with sodium bisulfite (Hayatsu and Miura, 1970). These studies may be of importance because sodium bisulfite is being used as food additives and sulfur dioxide is the main cause of the air pollution.

Acknowledgments

Thanks are due to Professor T. Ukita of the University of Tokyo for his encouragement throughout the present work. Dr. Y. Kyogoku of our Faculty is also gratefully acknowledged for his valuable discussions on the infrared, Raman, and nuclear magnetic resonance spectra. We thank Dr. M. Sano of Daiichi Seiyaku Co. Ltd., for his measurement of the nuclear magnetic resonance spectra.

References

Batt, R. D., Martin, J. K., Ploeser, J. M., and Murray, J. (1954), *J. Am. Chem. Soc. 76*, 3663.

Brown, D. M., and Schell, P. (1965), *J. Chem. Soc.*, 208.

Cerfontain, H. (1968), Mechanistic Aspects in Aromatic Sulfonation and Desulfonation, New York, N. Y., Interscience, Chapter 14.

Green, M., and Cohen, S. S. (1958), *J. Biol. Chem. 228*, 601.

Greenbaum, S. B., and Holmes, W. L. (1954), *J. Am. Chem. Soc. 76*, 2899.

Grossman, L., Ono, J., and Wilson, R. G. (1965), *Fed. Proc. 24*, S-80.

Hayatsu, H. (1969), *J. Am. Chem. Soc. 91*, 5693.

Hayatsu, H., and Iida, S. (1969), *Tetrahedron Lett.*, 1031.

Hayatsu, H., and Miura, A. (1970), *Biochem. Biophys. Res. Commun. 39*, 156.

Hayatsu, H., and Yano, M. (1969), *Tetrahedron Lett.*, 755.

Hayatsu, H., and Ukita, T. (1967), *Biochem. Biophys. Res. Commun. 29*, 556.

Hayatsu, H., Wataya, Y., and Kai, K. (1970), *J. Am. Chem. Soc. 92*, 724.

Hilbert, G. E., and Johnson, T. B. (1930), *J. Am. Chem. Soc. 52*, 2001.

Hirs, C. H. W., Moore, S., and Stein, W. H. (1953), *J. Biol. Chem. 200*, 493.

Houlton, H. G., and Tartar, H. V. (1938), *J. Am. Chem. Soc. 60*, 544.

Isono, K., Asahi, J., and Suzuki, S. (1969), *J. Am. Chem. Soc. 91*, 7490.

Katritzky, A. R., Nesbit, M. R., Kurtev, B. J., Lyapova, M., and Pojarlieff, I. G. (1969), *Tetrahedron 25*, 3807.

Moore, A. H., and Thomson, C. H. (1957), *Can. J. Chem. 35*, 163.

Newman, M. S. (1956), Steric Effects in Organic Chemistry, New York, N. Y., Wiley.

Pietrzykowska, I., and Shugar, D. (1969), *Biochem. Biophys. Res. Commun. 37*, 225.

Rieche, A., and Seeboth, H. (1960), *Ann. Chem. 638*, 66.

Shapiro, H. S. (1968), *in* Handbook of Biochemistry, Sober, H. A., Ed., Cleveland, Ohio, Chemical Rubber Co., p H-30.
Shapiro, R., Servis, R. E., and Welcher, M. (1970), *J. Am. Chem. Soc. 92*, 422.
Smith, K. C., and Aplin, R. T. (1966), *Biochemistry 5*, 2125.
Wang, S. Y. (1957), *Nature 180*, 91.
Wang, S. Y. (1958), *J. Am. Chem. Soc. 80*, 6196.
Wang, S. Y. (1959), *J. Org. Chem. 24*, 11.
Wang, S. Y., Apicella, M., Stone, B. R. (1956), *J. Am. Chem. Soc. 78*, 4180.
Wechter, W. J., and Smith, D. C. (1968), *Biochemistry 7*, 4064.
Yano, M., and Hayatsu, H. (1970), *Biochim. Biophys. Acta 199*, 303.

Interactions between Cytidine and Its Cation in Polycytidylic Acid, Cytidylyl-3′-cytidine, and Cytidine Aggregates*

Thérèse Montenay-Garestier† and Claude Hélène

ABSTRACT: Fluorescence and phosphorescence titration curves of cytidine molecules dispersed in a water–propylene glycol glass (1:1, v/v) at 77°K have a classical sigmoidal shape. On the contrary, in cytidine aggregates formed in frozen aqueous solutions at 77°K, these titration curves exhibit an anomalous behavior. A luminescence intensity maximum is observed at a pH value close to the ground-state p*K*. The same phenomenon is observed at 77°K for poly C in ice and in a propylene glycol–water glass. Poly C exists in a double-stranded conformation at pH values close to the cytidine p*K*. The dinucleotide cytidylyl-3′:5′-cytidine dispersed as a single strand in this pH range behaves in the same way. At pH 4, the absorption spectrum of cytidine aggregates and poly C, deduced from low-temperature reflectance measurements, is shifted to longer wavelengths relative to that at pH 2 or 6. These observations are interpreted in terms of charge-transfer interactions between cytidine and its cation in stacked structures, where cytidine protonated on nitrogen 3 would behave as the acceptor and neutral cytidine as the electron donor.

A few homopolynucleotides, *e.g.*, poly A (Rich *et al.*, 1961; Adler *et al.*, 1969), and poly C (Fasman *et al.*, 1964; Hartman and Rich, 1965; Guschlbauer, 1967), form double-stranded structures in the acid pH range. Hydrogen bonding between bases and their cations as well as electrostatic forces are supposed to play a major role in maintaining these structures. However, other interactions such as those due to charge-transfer complex formation between a base and its cation could also help stabilize the double-stranded conformation.

Besides oligo- and polynucleotides, stacked bases can also be obtained in frozen aqueous solutions, the freezing of which induces the formation of microcrystalline aggregates (Wang, 1965; Bruice and Butler, 1965; Hélène *et al.*, 1968). These aggregates can be thought of as model structures for studies of electric interactions between nucleic acid bases (either neutral or ionized form) and have been used already to study excitation energy transfer (Hélène and Montenay-Garestier, 1968).

We have studied the pH dependence of the luminescence properties of the dinucleotide CpC, the polynucleotide poly C, and of cytidine aggregates in frozen solutions at 77°K. In all cases, a new fluorescence band appears for a pH value close to the ground-state cytidine p*K* that leads to anomalous luminescence titration curves. Reflectance spectra reveal the formation of molecular complexes between cytidine and its cation. The contribution of charge-transfer interactions to the stabilization of these complexes for both the ground state and the lowest excited singlet state is discussed.

Materials and Methods

Cytidine and cytidylyl-3′:5′-cytidine (CpC), were purchased from California Corp. for Biochemical Research. Poly C has been kindly supplied to us by Dr. M. Leng. Propylene glycol (B. D. H. Laboratory Reagent) has been distilled before use. The pH of the solutions was adjusted with microvolumes of hydrochloric acid or sodium hydroxide. All pH measurements were performed at room temperature with a Tacussel TS 40 N pH meter-titrimeter.

Spectroscopic studies were carried out with a Cary spectrophotometer Model 14. For low-temperature reflectance spectra, this apparatus was equipped with the total diffuse reflectance accessory adapted for low-temperature measurements as described elsewhere (T. Montenay-Garestier and C. Hélène, in preparation).

Thermal denaturation curves were obtained on a Cary Model 15 spectrophotometer.

Luminescence spectra at 77°K were recorded on a Jobin-Yvon spectrofluorimeter equipped with a xenon XBO 250-W lamp, two-quartz prism monochromators, and a 1P28

* From the Centre de Biophysique Moléculaire, La Source, 45-Orléans, France.
† Assistante au Muséum National d'Histoire Naturelle, Laboratoire de Biophysique 75-Paris-5ème, France.