Effect of Amines on the Bisulfite-Catalyzed Hydrogen Isotope Exchange at the 5 Position of Uridine†

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ABSTRACT: A new method to label uracil derivatives at the 5 position was developed. When uridine 5′-phosphate was treated with a D_2O solution of 1 M ammonium sulfite at pD 9.2 and 37° for 24 hr, 35% of the hydrogen at the 5 position of uracil was replaced by deuterium. pD 9 was optimal for this exchange to occur. Both ammonia and sulfite are required for this reaction, since neither sodium sulfite nor ammonium carbonate alone exhibited the catalytic effect. Methylamine, dimethylamine, trimethylamine, ethylamine, diethylamine, quinuclidine, and imidazole were also effective as ammonia, whereas triethylamine or guanidine was not. Kinetic studies have shown that the rate of the hydrogen–deuterium exchange is linearly dependent on both bisulfite and amine concentrations. Since it is known [Shapiro, R., Servis, R. E., and Welcher, M. (1970), *J. Amer. Chem. Soc. 92*, 422; Hayatsu, H., Wataya, Y., Kai, K., and Iida, S. (1970), *Biochemistry 9*, 2858] that uridine forms a stable addition compound with bisulfite, it was considered that the role of amine in this labeling reaction is to subtract hydrogen from the 5 position of the uridine–bisulfite adduct. It was also found that amines accelerate the rate of the reversible addition of bisulfite to uridine. The acceleration was noted both for the addition and the elimination, and the equilibrium was shifted to the adduct side by the presence of amines. The exchange reaction mechanism is discussed on the basis of these findings. Uridylyl-(3′–5′)-cytidine was labeled with tritium by treatment with T_2O in the presence of ammonium sulfite. When the labeling was carried out at pH 9.0, the uracil residue was preferentially labeled, whereas at pH 7.5 both the uracil and cytosine moieties were labeled; results expected from the present data and the previously reported bisulfite-catalyzed hydrogen isotope exchange of cytidine [Kai, K., Wataya, Y., and Hayatsu, H. (1971), *J. Amer. Chem. Soc. 93*, 2089]. The mildness of the reaction conditions required for the labeling of uridine suggests applicability of the present method to polynucleotides.

Considerable interest has been focused upon chemically induced hydrogen isotope exchange at the 5 position of uracil derivatives (Fink, 1964; Chambers, 1968; Santi and Brewer, 1968; Santi *et al.*, 1970; Heller, 1968; Cushley *et al.*, 1968; Wechter, 1970; Kalman, 1971). The exchange reaction has been investigated in order to elucidate the mechanism of action of thymidylate synthetase, since this enzymic reaction involves not only the methylation at the 5 position of uracil moiety but also simultaneous hydrogen isotope exchange at the same position (Lomax and Greenberg, 1967). Further, search for efficient methods to bring about the exchange reaction is important for the preparation of isotopically labeled uracil derivatives of biological interest.

Nucleophiles, notably SH compounds, have been shown to be effective in carrying out this isotope exchange. In this process, intermediate formation of unstable adduct across the 5,6-double bond of uracil has usually been postulated. Bisulfite adds across the 5,6-double bond of pyrimidine nucleosides producing stable 5,6-dihydro-6-sulfonate derivatives (Shapiro *et al.*, 1970a; Hayatsu *et al.*, 1970a,b). The addition compounds regenerate the parent pyrimidines under mild conditions. Bisulfite therefore has been expected to be useful for the isotope exchange at the 5 position of uridine and cytidine. Recent experiments from our laboratory have indeed shown that bisulfite catalyzes the isotope exchange at the 5 position of cytidine under mild conditions of pH 7.5 and 37° (Chart I), where the deamination (**3** → **2**) does not appreciably take place (Kai *et al.*, 1971). On the other hand, hydrogen–deuterium exchange was not observed for uridine, when it was treated first with sodium bisulfite in D_2O to form the adduct **2** and subsequently with OD^- to regenerate the 5,6-double bond (Hayatsu *et al.*, 1970a,b). We have now found that an efficient hydrogen isotope exchange can be achieved by the presence of amines in the uridine–bisulfite system. In this paper we report comparison of effectiveness of various amines, investigation on the mechanism of the reaction, and use of this method for labeling of UpC.[1] Previous data on effective chemicals for the isotope exchange of uridine are discussed in the light of the present finding. The mildness of the reaction conditions required in the catalysis of bisulfite to label uridine, as well as cytidine (Kai *et al.*, 1971), indicates that the method could be extended to the labeling of pyrimidines at the polynucleotide level.

Materials and Methods

UpC was prepared according to Michelson (1959). 5,6-Dihydrouridine-6-sulfonate and 5-deuterio-6-hydrouridine-6-sulfonate were prepared as described previously (Hayatsu *et al.*, 1970b). 3-Methyluridine was synthesized by the method of Szer and Shugar (1968). Reagents including isotopically labeled compounds were commercially available reagent grade chemicals. Radioactivity of tritium was measured by use of Kinard's scintillator (Kinard, 1957) on a Packard Tri-Carb 3320 liquid scintillation spectrometer. Nuclear magnetic resonance (nmr) spectra (100 MHz) were recorded by Jeol-NM 4H-100 nmr spectrometer and ultraviolet (uv) spectra by Hitachi 124 spectrophotometer.

Measurement of Hydrogen–Deuterium Exchange. Before

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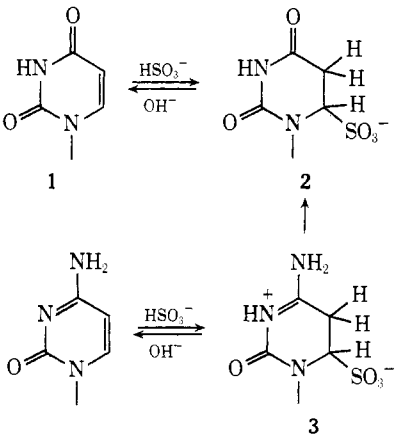
[1] Abbreviations used were: UpC, uridylyl-(3′–5′)-cytidine; UMP, uridine 5′-phosphate; CMP, cytidine 5′-phosphate.

CHART I



use, uridine and Na_2UMP were dissolved in D_2O and freeze-dried. The pD values of reaction mixtures were the uncorrected readings on a pH meter which had been normalized with aqueous buffers. The pH of reaction mixture was measured before and after incubation and the value obtained after the incubation was employed as the pH representing the reaction. The change of pH during the incubation was usually less than 0.1. For incubation, the reaction mixture was tightly sealed in a glass tube for nmr measurement and allowed to stand usually at 37°. After the desired time of incubation, the tube was rapidly cooled and stored frozen until nmr measurement was carried out. The nmr spectra were taken with a 100-MHz oscillator at 22°. For the determination of the hydrogen–deuterium exchange at the 5 position of uracil moiety, the signal at ~8 ppm of 6-H was utilized. The 5-deuterated species gives a singlet 6-H signal at the middle of the doublet 6-H of 5-H species (Heller, 1968). An example of the signals is given in Figure 1. The three-peak pattern was cut out of the recorded chart and further cut at the bottom of the troughs between the peaks, and the incorporation extent was estimated by the ratio of weight of the center peak sheet to that of the total three-peak sheet. Average of five such estimations was taken as representing the incorporation extent. The example shown in Figure 1 gave a value of 37% deuterium exchange at the 5 position. In the reactions of UMP with $NaDSO_3$ at pD 5–8, nmr recording was performed after making the reaction mixture strongly alkaline with NaOD in order to regenerate UMP from the bisulfite adduct. In a

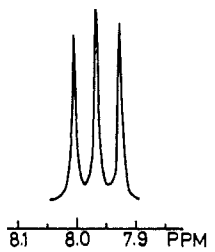


FIGURE 1: A typical nmr signal of the proton at position 6 of uridine 5′-phosphate observed after treatment of the nucleotide with trimethylammonium bisulfite in D_2O. To a mixture of Na_2UMP (100 mg) and Na_2SO_3 (126 mg) was added 4.0 M $(CH_3)_3N \cdot HCl$ in D_2O (250 μl), 1 N NaOD (50 μl), and D_2O (700 μl). The resulting solution, of which pD was 9.1, was incubated at 37° for 24 hr, and nmr spectrum was recorded.

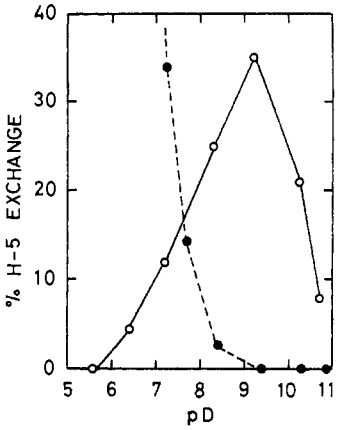


FIGURE 2: pD profile of incorporation of deuterium into uridine 5′-phosphate and cytidine 5′-phosphate. Na_2UMP (100 mg) or Na_2CMP (100 mg) was dissolved in a solution (1 ml) of following composition and incubated for 24 hr at 37°. pD after dissolving nucleotide:$(NH_4)_2SO_3 \cdot H_2O$:$NaHSO_3$: for UMP (○), pD 5.7, 0.05 mmole:0.95 mmole; pD 6.5, 0.4:0.6; pD 7.2, 0.8:0.2; pD 8.3, 1.0:0; for CMP (●), pD 7.2, 0.8 mmole:0.2 mmole; pD 7.7, 0.98:0.02; pD 8.4, 1.0:0. pD higher than 8.4 was fixed by addition of NaOD.

separate experiment it was confirmed that there was no change in the shape (and consequently in the value of the incorporation extent) of the signal of 6-H before and after the alkali treatment.

Time Course of Bisulfite Addition to UMP. A solution (5 ml) containing 50 μmoles of Na_2UMP, 1.0 M sodium bisulfite–sodium sulfite, and 1.0 M amine was incubated at 20°. The pH was 7.5. An aliquot (50 μl) was withdrawn at the desired time, diluted with 0.1 M sodium phosphate buffer of pH 6.0, and the OD at 260 nm was determined. Decrease in the OD was taken as representing the extent of the adduct formation since the adduct does not absorb uv light at 260 nm.

Results

pD Dependence of Ammonium Bisulfite Catalyzed Incorporation of Deuterium into UMP and CMP. The exchange, hydrogen at the 5 position with deuterium, occurs at 37° at a moderate speed, as detected by nmr spectroscopy. For example, incubation of UMP in D_2O solution containing 1 M ammonium sulfite at 37° and pD 9.2 resulted in 35% hydrogen–deuterium exchange at position 5 after 24 hr and 60% after 72 hr. No isotope exchange was observed for hydrogen at position 6. The pD profile of the exchange is shown in Figure 2. A bell-shaped pD dependence was obtained and the pD maximum was about 9. In this figure is also shown the pD profile of the hydrogen exchange in CMP observed under the same conditions. It is seen that there is a great difference between these two substrates in the pD dependence.

Ammonium Bisulfite Catalyzed Incorporation of Tritum into UpC. From Figure 2 it was anticipated that the exchange would occur selectively to uracil moiety at pH 9 when both uracil and cytosine moieties are simultaneously present in a reaction mixture. UpC was treated with 1 M ammonium bisulfite in T_2O at pH 9.0 and 37° for 24 hr. UpC was recovered from the reaction mixture by means of paper chromatography, and tritium incorporated into the uracil and cytosine moieties was measured after digesting the dinucleoside monophosphate with RNase T_2 into uridine 3′-phosphate and cytidine. All the easily exchangeable tritium was replaced by hydrogen during the work-up. As Table I shows, the uracil

TABLE I: Ammonium Bisulfite Catalyzed Incorporation of Tritium into Uridylyl-(3′–5′)-cytidine.

pH of Reaction	Radioactivity after RNase T_2 Digestion (cpm/0.1 μmole)		% of Complete Exchange	
	Uridine-3′-P	Cytidine	Uridine-3′-P	Cytidine
9.0[a]	93,200	3,040	23.1	0.8
7.5[b]	24,700	45,200	6.1	11.5

[a] pH 9.0 reaction. To a solution of UpC (37.5 OD units at 263 nm) in 50 μl of 2 M $(NH_4)_2SO_3$, which had been adjusted to pH 9.2 with NaOH, was added 50 μl of T_2O (1 Ci/ml). The resulting solution was incubated at 37° for 24 hr. In a separate experiment it was found that dilution of the 2 M $(NH_4)_2SO_3$ with an equal volume of water gave a pH value of 9.0. After the incubation, the mixture was cooled in ice–water and 1 M $BaCl_2$ (100 μl) was added to precipitate barium sulfite. The precipitate was removed by centrifugation and washed with water (three times with each 200 μl). The supernatant and washings were combined, and the solution evaporated again to dryness. The evaporation was repeated further twice in order to remove traces of T_2O. The residue was then subjected to paper chromatography using the solvent, isopropyl alcohol–concentrated NH_4OH–H_2O (7:1:2, v/v). Weak alkalinity of this chromatographic solvent served to regenerate UpC from its bisulfite adduct. Single spot of UpC was observed on the chromatogram. The UpC recovered from the chromatogram accounted for 70% of the starting material used. About 1 OD unit of the UpC was treated with RNase T_2 (0.75 unit) in 0.05 M sodium acetate buffer (pH 4.5) at 37° for 2.5 hr. The completely digested UpC was submitted to two-dimensional cellulose thin-layer chromatography using isobutyric acid–0.5 N NH_4OH (10:6, v/v) for the first dimension and isopropyl alcohol–concentrated HCl–H_2O (75:17:8, v/v) for the second dimension. Spots corresponding to uridine 3′-phosphate and cytidine were eluted with 0.01 N HCl, and ultraviolet spectra and radioactivity were determined. The molar ratio, uridine 3′-phosphate to cytidine, was found to be 1.04:1.00. (b) pH 7.5 reaction. The procedure was similar to that for the pH 9.0 reaction. The 2 M aqueous bisulfite solution used was a mixture of $(NH_4)_2SO_3$ and $NaHSO_3$ (20:1, mole/mole), pH 7.4. UpC recovered after the paper chromatographic step amounted to 67%. The molar ratio, uridine 3′-phosphate to cytidine, found after RNase T_2 digestion was 1.05:1.00.

residue was indeed selectively labeled with tritium. Similar treatment of UpC at pH 7.5 resulted in labeling of both the uracil and cytosine moieties as expected. During these treatments, neither phosphodiester-bond cleavage nor deamination of the cytosine residue was observed. As far as we know this is the first example in which labeling at the 5 position of the pyrimidine bases was achieved at the oligonucleotide level without concomitant decomposition of the substrate. Since the addition of bisulfite to uracil and cytosine does not occur if the pyrimidines are the constituents of a hydrogen-bonded region of polynucleotides (Furuichi *et al.*, 1970; Shapiro *et al.*, 1970b), the bisulfite-catalyzed labeling technique should be a useful means to detect pyrimidines located in the exposed region of a polynucleotide.

TABLE II: Effect of Amines on the Incorporation of Deuterium into Uridine 5′-Phosphate.[a]

Expt No.	Additions	pD	Hydrogen–Deuterium Exchange at Position 5 (%)
1	None	8.9	Nd[c]
2	NH_4Cl	8.6	23
3	NH_4Cl[b]	8.4	31
4	NaCl	8.8	Nd
5	$(NH_4)_2CO_3$	9.4	19
6	$(NH_4)_2CO_3$, but sulfite subtracted	9.4	Nd
7	$CH_3NH_2 \cdot HCl$	8.9	21
8	$(CH_3)_2NH \cdot HCl$	8.9	37
9	$(CH_3)_3N \cdot HCl$	8.9	35
10	$C_2H_5NH_2 \cdot HCl$	8.9	17
11	$(C_2H_5)_2NH \cdot HCl$	8.9	15
12	$(C_2H_5)_3N \cdot HCl$	9.0	Nd
13	Imidazole	9.3	23
14	Guanidine·HCl	8.8	Nd
15	Quinuclidine·HCl	8.6	13

[a] Amine salt (1 mmole, unless otherwise noted) was mixed with a D_2O solution (1 ml) containing 1 mmole of Na_2SO_3 and 0.1 g of Na_2UMP and the resulting solution was incubated at 37° for 24 hr. [b] 2 mmoles, instead of 1 mmole, of NH_4Cl was used. [c] Not detectable.

Temperature Dependence of Incorporation of Deuterium into UMP. UMP was treated in D_2O with 1 M ammonium bisulfite at pD 9.2 for 24 hr at various temperatures. As the temperature rose, the incorporation became greater: temperature per per cent of exchange: 0°/3%, 21.5°/9%, 37°/35%, and 60°/78%.

Requirement of Amines for the Incorporation of Deuterium into UMP. We carried out the exchange reaction in 1 M Na_2SO_3 at 37° for 24 hr, supplementing various amines (Table II). No incorporation was detected without added amine (expt 1).[2] In the presence of 1 M NH_4Cl (expt 2), a significant incorporation was observed, and the incorporation became greater as the NH_4Cl concentration was increased (expt 3). Ammonia was essential, but the anion, chloride, or carbonate was not (expt 2–5). Sulfite was also essential (expt 6), indicating that the bisulfite adduct 2 is the intermediate of this reaction. Organic amines can replace ammonia (expt 7–15); methylamine, dimethylamine, trimethylamine, ethylamine, diethylamine, imidazole, and quinuclidine were effective. Interestingly, triethylamine was not effective (exp 12). It is known that triethylamine does not form a complex with trimethyl boron whereas quinuclidine forms a stable complex (Brown and Taylor, 1947; Brown and Sujishi, 1948), although these two amines behave similarly in approaching protons (pK_a's of triethylamine and quinuclidine are 10.9 and 10.6, respectively). This fact has been reasonably explained in terms of steric hindrance generated by freely moving ethyl groups in the triethylamine molecule, which inhibit the nitrogen to approach the trimethyl boron molecule whose size is

[2] After incubation at 37° for 7 days, 9% exchange was observed.

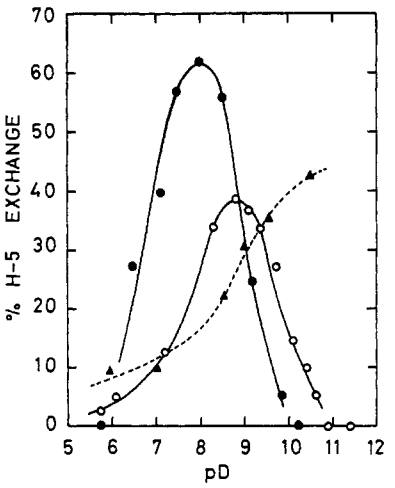


FIGURE 3: pD profile of incorporation of deuterium into uridine 5′-phosphate and 3-methyluridine. Effect of trimethylamine and imidazole. Solid lines: a mixture of Na_2UMP (100 mg) and Na_2SO_3 (and/or $NaHSO_3$) (1 mmole) was dissolved in 1 ml of 1 M $(CH_3)_3N\cdot HCl$ (○) or 1 M imidazole (●) in D_2O. Adjustment of pD was similar to that described in the legend to Figure 2, except that DCl was used to fix the pD of the imidazole solution at pD 5.8–6.9. Incubation was at 37° for 24 hr. Broken line: a mixture of Na_2SO_3 and $NaHSO_3$ was dissolved in 1.5 ml of a solution containing 3-methyluridine (0.1 g) and 0.75 M $(CH_3)_3N\cdot HCl$ and the resulting solution incubated at 37° for 24 hr. pD, Na_2SO_3:$NaHSO_3$: pD 5.9, 0.25 mmole:0.75 mmole; pD 7.0, 0.75:0.25; pD 8.6, 0.93:0.07; pD 9.0, 1.0:0. pD higher than 9.0 was fixed with NaOD.

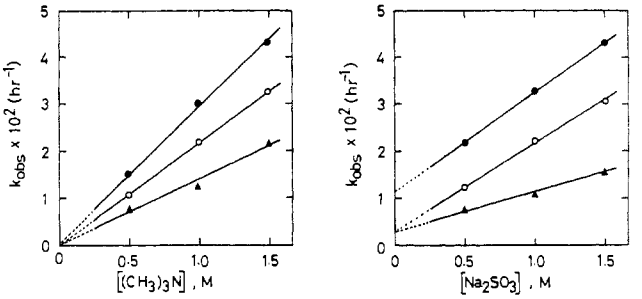


FIGURE 4: Dependence of the rate of the hydrogen–deuterium exchange on concentration of bisulfite and trimethylamine. (a, left) ●, 1.5 M bisulfite; ○, 1.0 M bisulfite; ▲, 0.5 M bisulfite. (b, right) ●, 1.5 M trimethylamine; ○, 1.0 M trimethylamine; ▲, 0.5 M trimethylamine.

much larger than that of a proton. The same argument may be applicable to the present case since quinuclidine is an effective agent in this exchange reaction (expt 15). That guanidine, a strong base having a pK_a of 13.6, did not show the catalytic activity (expt 14) indicates that the amines act in the unprotonated form.

Effects of Trimethylamine and Imidazole. The pD profile of incorporation of deuterium into UMP was determined using trimethylamine and imidazole as the amine (Figure 3). With trimethylamine, a maximum incorporation was obtained at pD 8.8. Imidazole was more effective than trimethylamine, exhibiting the maximum at pD 8.0. The shift of the maximum toward less alkaline region apparently reflects the smaller pK_a value (7.0) of imidazole than that of trimethylamine (9.9), again indicating the free base as the reacting species. The greater effectiveness of imidazole than trimethylamine at their maximum pD's may be explained by the fact that more bisulfite adduct of uracil is formed at lower pD region (Hayatsu *et al.*, 1970b).

pD Profile of Incorporation of Deuterium into 3-Methyluridine. In order to investigate the role of dissociable proton at the 3 position of uridine, 3-methyluridine was employed as the substrate, and incorporation of deuterium into the 5 position was studied using trimethylammonium bisulfite as the catalyst. As Figure 3 shows an efficient incorporation comparable to the case of UMP did take place and the incorporation increased as the pD was raised. Only a little incorporation was noted in the absence of either bisulfite or amine. Thus, incubation of 3-methyluridine in D_2O at 37° for 24 hr (a) in the presence of 1 M trimethylamine alone (pD 9.8), (b) in the presence of 1 M sodium bisulfite alone (pD 10.1), or (c) in dilute NaOD solution of pD 9.5 resulted in (a) 9.3%, (b) 5.7%, and (c) 3.2% exchange. Since 3-methyluridine forms a bisulfite adduct the mechanism of the hydrogen isotope exchange must be similar to that of uridine.[3] This in turn implies that the bisulfite-catalyzed hydrogen–deuterium exchange at position 5 of uridine does not require the dissociation of the proton at position 3; rather, the dissociation of the 3 proton of uridine inhibits the exchange as can be seen in the decrease of the incorporation above pD 9.

Dependence of Incorporation of Deuterium into UMP on Concentrations of Bisulfite and Amine. At various levels of bisulfite and trimethylamine concentration, the time course of the incorporation was determined. The conditions were pD 9.0 and 37° at UMP concentration of 100 mg/ml. Pseudo-first-order kinetics were obtained and apparent rate constants were found to be as follows—$k \times 10^2$ (hr^{-1}), concentration of bisulfite (M), concentration of trimethylamine (M): 2.18, 0.5, 1.5; 3.28, 1.0, 1.5; 4.33, 1.5, 1.5; 1.23, 0.5, 1.0; 2.21, 1.0, 1.0; 3.05, 1.5, 1.0; 0.747, 0.5, 0.5; 1.08, 1.0, 0.5; 1.53, 1.5, 0.5. Figure 4a,b shows that the incorporation is linearly dependent on both bisulfite and trimethylamine concentrations. In Figure 4b, the intercepts at zero concentration of bisulfite are not zero, suggesting that the reaction may proceed without bisulfite. However, with 1.0 M trimethylamine alone, the incorporation was extremely slow, giving less than 1% exchange after 16-days incubation at pD 9.0 and 37°. The reason why the intercepts do not cross the zero point is not clear. By use of 1.0 M sodium bisulfite without added amine, a k value of 6.21×10^{-4} hr^{-1} was obtained, indicating that the presence of 1.0 M trimethylamine increased the k value by by a factor of 30.

Isotope Effect on the Exchange. Using uridine and [5-^{3}H]-uridine, the rate of exchange with deuterium was compared. The reaction was carried out in D_2O containing 1.0 M Na_2SO_3 and 1.0 M $(CH_3)_3N\cdot DCl$, at pD 9.0 and 37°. $k_{5\text{-H}-5\text{-D}}$ was found to be 2.69×10^{-2} hr^{-1} and $k_{5\text{-T}-5\text{-D}}$ 6.03×10^{-3} hr^{-1}. The ratio, $k_{5\text{-H}-5\text{-D}}/k_{5\text{-T}-5\text{-D}}$, was thus 4.47. This positive isotope effect indicates that the rate-determining step is the cleavage of the hydrogen from position 5.

Effect of Amine on the Regeneration of Uridine from 5,6-Dihydrouridine-6-sulfonate. Effect of amines on the rate of regeneration of uridine from uridine–bisulfite adduct was studied. The regeneration was carried out at several pH values

[3] The adduct formation between 3-methyluridine and bisulfite can be observed by nmr. Thus, a D_2O solution of a mixture of 3-methyluridine and bisulfite (pD 6–8) gave signals of the adduct (diastereomers): 5-H, 3.14 ppm; 6-H, 4.94 and 4.98 ppm; 1′-H, 5.36 ppm (d, J = 3.0 Hz), 5.70 ppm (d, J = 5.5 Hz); N-CH_3, 3.10 and 3.08 ppm. The adduct regenerates 3-methyluridine on treatment with alkali.

TABLE III: Effect of Amine and Isotope on the Regeneration of Uridine from the Bisulfite Adduct.[a]

Starting Material	pH	Rate Constant for Regeneration (hr^{-1}) Without Amine	With Amine[b]
5,6-Dihydrouridine-6-sulfonate (**2**)	6.0	0.0168	0.0391
	6.5	0.0486	0.137
	6.5		0.0648[c]
	7.0	0.117	0.360
	7.9	0.769	1.97
5-Deuterio-6-hydro-uridine-6-sulfonate (**2-D**)	6.0	0.00144	0.0132
	6.5	0.0160	0.0900
	7.0	0.0494	0.237
	7.9	0.420	1.59

[a] The bisulfite adduct of uridine (0.8 μmole) was dissolved in 10 ml of 0.1 M sodium phosphate buffer and the solution was incubated at 37°. Aliquots, usually at five points in a run, were taken, diluted with 0.1 N HCl to stop the reaction, and the optical density at 260 nm was recorded. Increase in the optical density was taken to indicate the amount of uridine formed. Very good pseudo-first-order profile was generally obtained. [b] Trimethylamine hydrochloride had been added to the buffer solutions in 1.0 M concentration and the pH was adjusted to the desired value by addition of NaOH or HCl. [c] Triethylamine hydrochloride (in 1.0 M) was used instead of trimethylamine hydrochloride.

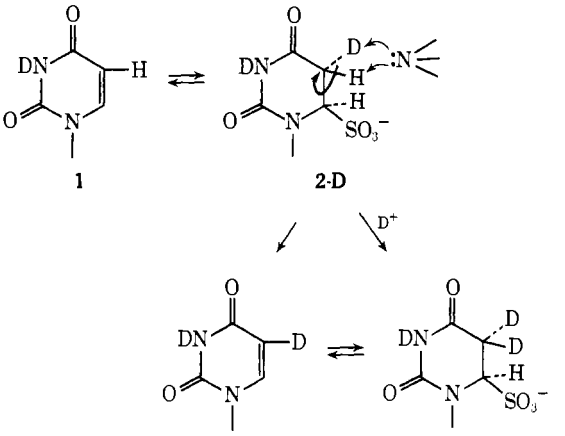
in the presence and absence of amines. As Table III shows the rate was greatly enhanced by trimethylamine. For example, at pH 6.5 the rate was 2.8 times greater in the presence of trimethylamine than in the absence. In contrast, triethylamine hydrochloride, which was not an effective amine in the hydrogen isotope exchange, did not show such a marked effect. The same effect of trimethylamine was noted at every pH value tested.

In order to study the isotope effect in the regeneration of uridine from the adduct, the rate of regeneration was similarly determined for *trans*-5-deuterio-6-hydrouridine-6-sulfonate (**2-D**) (Table III). It can be seen that the deuterated compound reacts more slowly than the nondeuterated counterpart. The acceleration of the reaction by trimethylamine was somewhat greater for the deuterated than for the nondeuterated compound at every pH value examined. These results indicate that the rate of regeneration of uridine from the adduct is dependent, at least partially, on the rate of cleavage of the hydrogen situated trans to the sulfonate group, and that trimethylamine helps cleaving this trans hydrogen.[4]

Effect of Amine on the Adduct Formation between UMP and Bisulfite. The time course of the addition of bisulfite to UMP, the forward reaction, was determined in the presence and absence of amines. The initial rates showed pseudo-first-order kinetics, and trimethylamine and imidazole were found

[4] After this paper was submitted for publication, we were informed of a recent report by Erickson and Sander (1972) which showed accelerating effect of amines in regeneration of 1,3-dimethyluracil from its bisulfite adduct.

CHART II



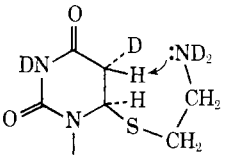
to accelerate the reaction. The apparent rate constants obtained were as follows: k (1.0 M bisulfite, pH 7.5 and at 20°) 1.57 hr^{-1} (1.0 M trimethylamine added), 1.29 hr^{-1} (1.0 M imidazole added), 0.922 hr^{-1} (no addition). In the presence of 1.0 M trimethylamine the apparent rate increased 1.7 times over that observed in the absence of the amine. It should be pointed out that during this process the backward reaction, the elimination of bisulfite from the adduct, is occurring concomitantly, thereby decreasing the apparent rate of the forward reaction. Since amines accelerate the backward reaction, actual enhancement of the forward reaction by amines must be greater than that which can be seen by the apparent rate constants. After allowing the reaction to proceed for an adequate time to reach the equilibrium, the equilibrium constant, [bisulfite adduct of UMP]–[UMP][bisulfite], was determined. The values obtained were 7.2 l. $mole^{-1}$ (in 1.0 M bisulfite plus 1.0 M imidazole at pH 7.5 and 37°), 4.0 l. $mole^{-1}$ (in 1.0 M bisulfite at pH 7.5 and 37°), 0.55 l. $mole^{-1}$ (in 1.0 M bisulfite plus 1.5 M trimethylamine DCl, at pD 9.0 and 37°), 0.43 l. $mole^{-1}$ (in 1.0 M bisulfite plus 1.0 M trimethylamine DCl at pD 9.0 and 37°), 0.30 l. $mole^{-1}$ (in 1.0 M bisulfite plus 0.5 M trimethylamine DCl, at pD 9.0 and 37°), and 0.23 l. $mole^{-1}$ (in 1.0 M bisulfite at pD 9.0 and 37°). It is obvious that the equilibrium shifts to the adduct side as a function of the concentration of amine. This means that the acceleration by the amine of the forward reaction is greater than that of the reverse reaction. It can thus be estimated that in the presence of the amine the rate of the turnover, **1** → **2-D** → **1** → **2-D** →, is larger by one order of magnitude than in its absence. This acceleration of the turnover would probably contribute to the overall effect of amine in this exchange reaction.

In summary, effect of amine is (1) to greatly accelerate the hydrogen isotope exchange at the 5 position of uridine, (2) to make both the forward and backward reactions between bisulfite and uridine more rapid, and (3) to shift the equilibrium of the reaction toward the adduct side. A possible mechanism is represented in Chart II.

Discussion

Most of the previously reported methods for the exchange of hydrogen at position 5 of uridine with isotopes have utilized conditions too vigorous to apply them to biochemical materials. Thus, the temperature at which the incorporation was

CHART III



carried out has usually been high, ranging from 60 to 95°, and in some cases extreme pH's have been used. An exception to this is the exchange catalyzed by 2-mercaptoethylamine which proceeds at 37° (Heller, 1968). The pH of the alkaline solution which was presumably about 10, however, was not described. From the studies reported here, it became clear that bisulfite, in the presence of amines, can catalyze the hydrogen exchange at a moderate rate under mild conditions of pH 7–9 and 37°. By choosing the kind of amine and the pH of the reaction (Figure 3), and by changing the concentration of both bisulfite and amine (Figure 4), the rate of the exchange could be controlled.

Now that the role of amine in the hydrogen isotope exchange of uridine has been disclosed, it seems possible to provide a new interpretation for some of previously reported experimental results and for mechanism of thymidylate synthetase. 2-Mercaptoethylamine appears to catalyze the exchange (Heller, 1968) as effectively as ammonium bisulfite. From the data presented here it seems reasonable to assume that the amino group of mercaptoethylamine that has been linked to the 6 position of uracil through the sulfur atom can attack intramolecularly the hydrogen atom at position 5, which is conveniently located cis to the mercaptoethylamino group (Chart III). 2-Mercaptoethanol was also reported by Heller to be an effective catalyst in the hydrogen–deuterium exchange of uridine under alkaline conditions. Although we also noted that the agent was effective at pD 8–10, the effectiveness was much less than that of 2-mercaptoethylamine. This is apparently explicable in terms of the lack of intramolecular abstraction of C-5 hydrogen by amino group. This was also supported by another experiment (unpublished work of Y. Wataya and H. Hayatsu) in which cysteine, but not N-acylated cysteine, was shown to effectively catalyze the hydrogen isotope exchange of UMP. Actually, most of reported procedures with which the hydrogen isotope exchange of uridine was brought about do not involve amines in the reaction system. In these instances, where the exchange is much slower than the present reaction, hydroxide ion may serve as a base to subtract the hydrogen at position 5 of the unstable adduct formed by the agents across the 5,6-double bond of uridine (Santi *et al.*, 1970).

Thymidylate synthetase which catalyzes the hydrogen isotope exchange as well as the methylation at position 5 of uracil derivatives has been shown to be an SH enzyme (Dunlop *et al.*, 1971). Santi and Sakai (1972) have suggested the presence of an amino group in the active center of the enzyme on the basis of inhibition studies. The action of amine in the bisulfite-catalyzed hydrogen isotope exchange may be taken as a model for the possible role of amine in the enzymic reaction. This chemical model is consistent with the assumption that the enzyme functions by a concerted action of SH and amino groups.

Another aspect of bisulfite reaction with nucleosides is the free radical reactions accompanying autoxidation of bisulfite. Such examples so far reported involve *S*-sulfonate formation from 4-thiouridine (Hayatsu and Inoue, 1971), the anti-Markownikoff-type addition of bisulfite to isopentenyladenine (Hayatsu *et al.*, 1972) and the damage of double-stranded DNA leading to chain cleavage (Hayatsu and Miller, 1972).

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