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# The Reactions of 2-Thiouracil with Permanganate and with Bisulfite-Oxygen

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As an extension of the work on the permanganate oxidation and the oxygen-mediated bisulfite reaction of 4-thiouridine, the reactions of 2-thiouracil were studied. On treatment with a stoichiometric amount of permanganate at pH 7 and 0°, 2-thiouracil yielded uracil-2-sulfonate. The sulfonate was susceptible to hydrolysis with weak acid but was stable in alkali. Kinetic studies of the acid hydrolysis were carried out. 2-Thiouridine also underwent a rapid oxidation on treatment with permanganate under similar conditions. Uridine and a small amount of uracil were detected as the products of the reaction of the nucleoside, suggesting that the intermediate uridine-2-sulfonate is more susceptible to hydrolysis compared with uracil-2-sulfonate.

Treatment of 2-thiouracil with dilute bisulfite at pH 7 and room temperature under oxygen-bubbling resulted in quantitative formation of uracil-2-sulfonate. The mechanism of the reaction involves action of the free radicals generated during the autoxidation of bisulfite, a mechanism already had been described in the bisulfite reaction with 4-thiouridine. Oxygen-mediated bisulfite reaction with 2-thiouridine was shown to be complex, yielding not only uridine but also other products including an appreciable amount of uracil.

Since the discovery of 4-thiouridine as a constituent of certain transfer RNAs,[2] chemical reactions of thiouracils have received considerable attention.[3,4] Methods to transform a thiouridine in aqueous media under mild conditions may be useful in elucidating biochemical significance of the modified nucleoside in tRNA molecules. Aiming at developing such methods we have investigated the reaction of 4-thiouridine with permanganate[5] and with the bisulfite-oxygen system.[6,7] 4-Thiouridine is rapidly oxidized with permanganate at pH 7 and 0° giving uridine-4-sulfonate, the sulfonate group of which can readily be displaced by

1) Location: *Bunkyo-ku, Tokyo, 113, Japan.*
2) M.N. Lipsett, *J. Biol. Chem.*, **240**, 3975 (1965).
3) *a*) H. Hayatsu, *Protein, Nucleic Acid and Enzyme, Supplement*, ("Chemical Synthesis of Nucleic Acids"), **1968**, 158 (in Japanese); *b*) N.K. Kochetkov and E.I. Budovskii, "Organic Chemistry of Nucleic Acids," Plenum Press, New York, 1972; *c*) R.H. Hall, "The Modified Nucleosides in Nucleic Acids," Columbia University Press, New York, 1971.
4) More recent papers not cited in refs 3 involve, *a*) K. Burton, *Fed. Eur. Biochem. Soc. Lett.*, **6**, 77 (1970); *b*) R. Faulkner and M. Uziel, *Biochem. Biophys. Acta*, **238**, 464 (1971); *c*) A. Favre, M. Yaniv and A.M. Michelson, *Biochem. Biophys. Res. Commun.*, **37**, 266 (1969); *d*) H. Hara, T. Horiuchi, M. Saneyoshi and S. Nishimura, *ibid.*, **38**, 305 (1970); *e*) S. Iida and H. Hayatsu, *ibid.*, **43**, 163 (1971); *f*) N.J. Leonard, D.E. Bergstrom and G.L. Tolman, *ibid.*, **44**, 1524 (1971); *g*) M. Pleiss and P.A. Cerutti, *Biochemistry*, **10**, 3093 (1971); *h*) B.C. Pal, L.R. Shugart, K.R. Isham and M.P. Stulberg, *Arch. Biochem. Biophys.*, **150**, 86 (1972); *i*) M. Saneyoshi, *Biochem. Biophys. Res. Commun.*, **40**, 1501 (1079); *j*) M. Saneyoshi and S. Nishimura, *Biochem. Biophys. Acta*, **204**, 389 (1970); *k*) M. Saneyoshi and S. Nishimura, *ibid.*, **246**, 123 (1971); *l*) E. Sato and Y. Kanaoka, *ibid.*, **232**, 213 (1971); *m*) K.H. Scheit, *ibid.*, **195**, 294 (1969); *n*) J.A. Secrist, S.R. Barrio and N.J. Leonard, *Biochem. Biophys. Res. Commun.*, **45**, 1262 (1971); *o*) L. Shugart and M.P. Stulberg, *J. Biol. Chem.*, **244**, 2806 (1969); *p*) L. Shugart, *Arch. Biochem. Biophys.*, **148**, 488 (1972); *q*) R.T. Walker and U.L. RajBhandary, *Biochem. Biophys. Res. Commun.*, **38**, 907 (1970).
5) *a*) H. Hayatsu and M. Yano, *Tetrahedron Letters*, **1970**, 755; *b*) M. Yano and H. Hayatsu, *Biochim. Biophys. Acta*, **199**, 303 (1970).
6) H. Hayatsu, *J. Am. Chem. Soc.*, **91**, 5693 (1969).
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hydroxyl or amino group under mild conditions. In the reaction of 4-thiouridine with bisulfite in the presence of oxygen, uridine-4-sulfonate is again the product. Studies of the mechanism of the reaction have revealed that uridine-4-thiosulfate is the intermediate and the intermediate formation involves an action of the free radicals generated from oxygen and bisulfite.[7] As an extension of the work, we have now investigated the oxidation of 2-thiouracil with per-

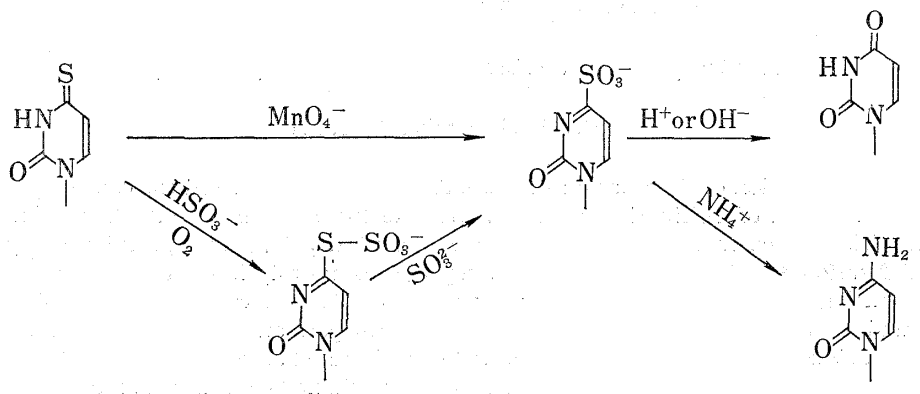


Chart 1

manganate and with the bisulfite-oxygen system. Preliminary experiments using 2-thiouridine for the substrate were also performed. It should be noted that 5-methylaminomethyl-2-thiouridine,[8,9] 2-thiouridine-5-acetic acid methyl ester[10–12] and 5-methyl-2-thiouridine[13] have recently been isolated from some tRNA species.

**Permanganate Oxidation of 2-Thiouracil**

When 2-thiouracil was treated with two equivalents of potassium permanganate at pH 7 and 0°, uracil-2-sulfonate was produced as expected. Use of an excess of permanganate was not attempted to avoid possible extensive degradation of the pyrimidine derivatives.[14] The sulfonate was isolated as an alkali metal salt which was paper-chromatographically and paper-electrophoretically pure. The compound was characterized by nuclear magnetic resonance and infrared spectroscopy. Further, uracil-2-sulfonate can be hydrolyzed with acid giving uracil as a single ultraviolet-absorbing product. The presence of sulfite ion in the hydrolyzate was demonstrated by decoloration of Malachite green and by precipitation with $Ba^{2+}$ ion.

The rate of the hydrolysis of uracil-2-sulfonate at 37° was determined at several pH values by utilizing the change in absorbance at 260 mμ (see Fig. 1). The hydrolysis proceeded by pseudo-first order kinetics and the half lives of the starting material found were; 1.5 min at pH 2; 11 min at pH 3; 90 min at pH 4; and, 16 hr at pH 5. At pH's 6, 7 and 10, the sulfonate was stable. The stability of uracil-2-sulfonate at pH 10 contrasts to the susceptibility of uridine-4-sulfonate to hydrolysis under similar conditions. The presence of a dissociable NH group in uracil-2-sulfonate is apparently responsible for this stability. The

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13) F. Kimura-Harada, M. Saneyoshi and S. Nishimura, *Fed. Eur. Biochem. Soc. Lett.*, **13**, 335 (1971).
14) *a*) H. Hayatsu and T. Ukita, *Biochem. Biophys. Res. Commun.*, **29**, 556 (1967); *b*) H. Hayatsu and S. Iida, *Tetrahedron Letters*, **1969**, 1031; *c*) S. Iida and H. Hayatsu, *Biochim. Biophys. Acta*, **213**, 1 (1970); *d*) *Idem*, *ibid.*, **228**, 1 (1971); *e*) *Idem*, *ibid.*, **240**, 370 (1971).

p*K*a value for this proton is between 5 and 6 as judged by ultraviolet absorption spectra at different pH values (Fig. 1). This means that uracil-2-sulfonate is a dianion at pH's above 6.

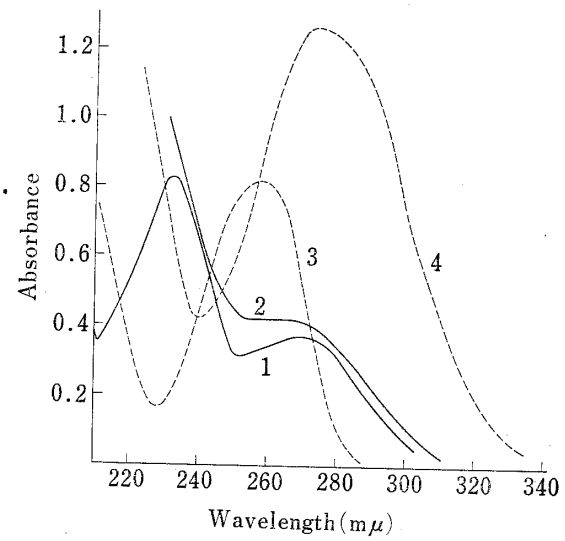


Fig. 1. Ultraviolet Spectra of Uracil-2-sulfonate

spectrum 1: $10^{-4}$M uracil-2-sulfonate in 0.1M sodium phosphate buffer, pH 7.0. 2: $10^{-4}$M uracil-2-sulfonate in 0.1M sodium acetate buffer, pH 5. 3: $10^{-4}$M uracil at pH 7. 4: $10^{-4}$M 2-thiouracil at pH 7. At pH 6 or pH 10, the spectrum of uracil-2-sulfonate is the same as 1, and at pH 4 it is the same as 2. Therefore 1 represents the spectrum of the dianionic form of uracil-2-sulfonate, and 2 that of the monoanionic form.

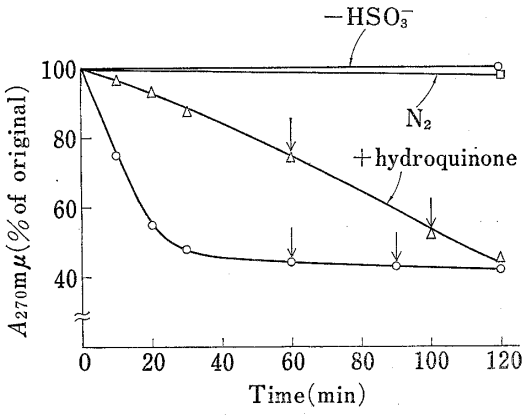


Fig. 2. Conversion of 2-Thiouracil into Uracil-2-sulfonate by the Oxygen-dependent Action of Bisulfite

Detailed explanation is given in Experimental section.

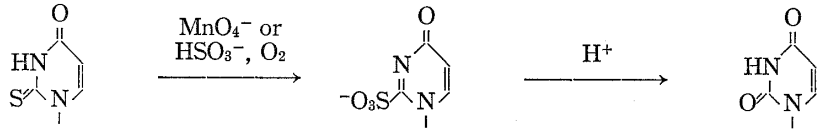


Chart 2

Indeed in paper electrophoresis at pH 7, the sulfonate traveled toward the anode about two times faster than 1-methyluracil-4-sulfonate.

In a preliminary experiment, 2-thiouridine was treated with permanganate (two equivalents) at pH 7 and 0° for 5 min. After removal of the manganese dioxide that precipitated, the reaction mixture was diluted with water and the solution was allowed to stand at room temperature for 20 hr and then was subjected to cellulose thin layer chromatography (two dimensional; solvent B for the first, and C for the second dimension. See Experimental for the solvents). Uridine and uracil were found on the chromatogram in a molar ratio 93: 7, and 2-thiouridine was not detectable. The formation of uridine suggests that the intermediate, probably uridine-2-sulfonate, is quite susceptible to hydrolysis. The presence of a small amount of uracil in the product implys that some cleavage of the glycosidic bond occurred during the reaction.

**Reaction of 2-Thiouracil and 2-Thiouridine with Bisulfite-oxygen**

2-Thiouracil was treated with dilute bisulfite at pH 7 and room temperature with oxygen-bubbling that permits the autoxidation of bisulfite. The reaction conditions were similar to those employed for the conversion of 4-thiouridine into uridine-4-sulfonate.[7] As Fig. 2 shows, the absorbance at 270 mμ decreased as a function of the time of treatment. The

reaction does not proceed under a nitrogen atmosphere, indicating that the reacting species involves the free radicals generated by the oxidation of bisulfite. In support of the free radical mechanism, hydroquinone strongly inhibited the progress of the reaction. When the concentration of 2-thiouracil in the reaction mixture was increased, the reaction rate became significantly lower, the reason for which is not clear.

The reaction product was identified as uracil-2-sulfonate by paper chromatography, paper electrophoresis and ultraviolet spectrum, as well as by the rate of hydrolysis at pH 3, comparing with the sample obtained by the permanganate oxidation of 2-thiouracil. The conversion of 2-thiouracil into uracil *via* uracil-2-sulfonate by the action of bisulfite-oxygen followed by the acidic hydrolysis was quantitative as judged by ultraviolet absorption spectroscopy and by paper chromatographic analysis. The rate of the bisulfite reaction with 2-thiouracil, however, was markedly lower than that of the reaction with 4-thiouridine. In addition, the intermediate thiosulfate formation was not detectable in the 2-thiouracil reaction.

The bisulfite-oxygen reaction with 2-thiouridine was found to be even slower and more complex than that with 2-thiouracil. $Mn^{2+}$ ion which catalyzes the autoxidation of bisulfite accelerated the reaction, while hydroquinone inhibited it (Fig. 3a). Analysis by paper chromatography of the reaction mixture has shown that several products are formed. Uridine, uracil and an unidentified compound traveling faster than uracil on the chromatogram (with solvent B) were detected. Further, the change in ultraviolet (UV) spectrum of the reaction mixture during the incubation was greater than that which can be expected by the formation of only uridine and uracil (Fig. 3b). As an attempt to make the reaction simpler, the effect of

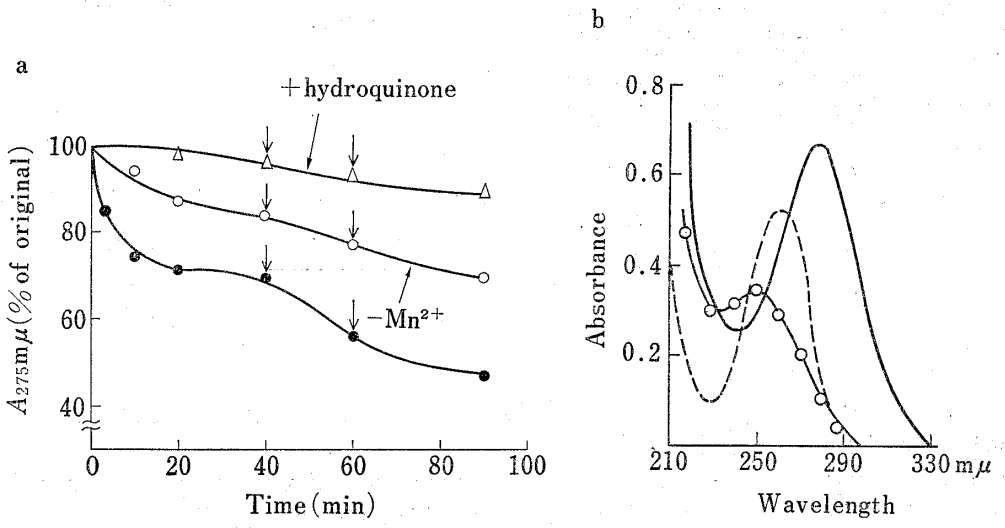


Fig. 3. Reaction of 2-Thiouridine with Bisulfite in the Presence of Oxygen at pH 7

Details are given in Experimental.

*a*: Oxygen bubbling was given at 5 to 10 min intervals for 1 min at each time. Continuous bubbling did not produce significant difference in the rate of the reaction. Arrows indicate additions of fresh bisulfite, each addition representing $2\times10^{-3}$M increase in the bisulfite concentration of the reaction mixture.

*b*: (——), spectrum of 2-thiouridine (0-time). (------); spectrum of uridine, the concentration of which being the same as the 2-thiouridine concentration at 0-time. (—○—); spectrum observed after 4 hr-reaction with bisulfite in the presence of $Mn^{2+}$ ions

changing the pH of the reaction mixture was studied. When the reaction was carried out at pH 4 and pH 10, however, spectral changes again greater than that expected were observed. In the pH 10-reaction, only uridine and uracil (7 : 3 in mole ratio) were the ultraviolet-absorbing products as detected by cellulose thin-layer chromatography. This indicates that some non-ultraviolet absorbing products have been generated. The sum of the amount of uridine and uracil recovered from the chromatogram accounted for only 60 % of the starting material used.

Although uridine-2-sulfonate was not detected, it is probably an intermediate of the bisulfite-catalyzed transformation of 2-thiouridine into uridine. The presence of a considerable amount of uracil as a product indicates either that the free radicals involved in this reaction can cleave the glycosidic bond of the pyrimidine nucleosides, or that the intermediate of the reaction inherently possesses a weak glycosidic bond. It should be noted that the bisulfite-oxygen can cause damage in double-stranded DNA leading to chain cleavage in an alkaline medium.[15]

Another aspect of bisulfite action is the ionic addition across the 5,6-double bond of pyrimidine nucleosides which occurs at high concentrations (*i.e.*, >1M) of bisulfite.[7,16] Such ionic reaction of bisulfite toward 2-thiouracil derivatives is an open problem, although the addition compound 5,6-dihydro-2-thiouracil-6-sulfonate has been prepared by a reaction of 5-carboxy-2-thiouracil with sodium bisulfite.[17]

## Experimental

**General**——2-Thiouracil was a commercial sample. 2-Thiouridine was synthesized according to Vorbrüggen, *et al.*[18] by condensation of O,S-bis(trimethylsilyl)-2-thiouracil with 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribose followed by treatment with sodium methoxide. 1-Methyluracil-4-sulfonate was prepared as described previously.[5] Potassium permanganate and sodium bisulfite solutions were always freshly prepared before use. Buffer used in the kinetic study of the hydrolysis of uracil-2-sulfonate were; 0.1M sodium acetate-HCl, pH 2 and 3; 0.1M sodium acetate, pH 4 and 5; 0.1M sodium phosphate, pH 6 and 7; 0.1M sodium carbonate, pH 10.

Paper chromatography was run on Toyo filter paper 53A by ascending technique using the following solvent systems. Solvent A, isopropyl alcohol-concentrated ammonia–water (7:1:2, v/v); solvent B, *n*-butyl alcohol–water (86:14, v/v); solvent C, ethyl alcohol–1M ammonium acetate (pH 7.5) (7:3, v/v); Avicel plates were used for cellulose thin-layer chromatography. Nuclear magnetic resonance (NMR) spectra were recorded at 100 MHz with Jeol-NM 4H-100 spectrometer.

**Permanganate Oxidation of 2-Thiouracil**——A suspension of 2-thiouracil (1.28 g, 10 mmoles) in 0.1M sodium phosphate buffer (pH 7.0, 50 ml) was cooled in ice. While the suspension was being stirred, 0.1M potassium permanganate (200 ml) was added to it dropwise in a period of 20 min. The resulting brown mixture was concentrated to about 1/3 of the original volume by evaporation under reduced pressure at a temperature lower than 20°. Manganese dioxide that precipitated was removed by centrifugation and washed with water. The supernatant and the washings were combined and evaporated to dryness. The yellowish residue was dissolved in water (2 ml) and the solution was poured into methanol (50 ml) producing a white suspension. After concentration of the suspension to about a half volume by evaporation, the precipitate was collected by centrifugation, washed with methanol, and dried *in vacuo*. The white powder weighed 1.0 g. This material gave a single UV-absorbing spot in paper chromatography using solvent systems A, B and C (see Table I for *Rf* values). In paper electrophoresis (using 0.05M sodium phosphate buffer, pH 7.0, at an electric voltage 20 V/cm paper) this compound traveled to the anode 10.0 cm by a 45 min run, while 1-methyluracil-4-sulfonate concomitantly run on the paper traveled 6.5 cm. UV (at pH 7 and pH 10); $\lambda_{max}$, 232 and 270 mμ; $\lambda_{min}$, 212 and 252 mμ (see Fig. 1 for the spectrum). IR (KBr); 1044 $cm^{-1}$ ($SO_3^-$ of a sulfonic acid): 2-Thiouracil and uracil lack this absorption. NMR in $D_2O$ gave signals at 6.39 ppm (5-H) and at 8.02 ppm (6-H) (DSS as the internal standard), indicating the presence of the 5,6-double bond of the pyrimidine ring.

In another run of the oxidation, the phosphate buffer was not included in the reaction mixture, and the pH was found not markedly to deviate during the oxidation (pH 7 to 6.5). A result similar to that described above was obtained with a yield of the dipotassium salt of uracil-2-sulfonate 60%. *Anal.* Calcd. for $C_4H_2O_4N_2SK_2$; N, 11.10. Found; N, 11.07. The salt of uracil-2-sulfonate was highly hygroscopic.

**Reaction of 2-Thiouracil with Bisulfite in the Presence of Oxygen (*cf.* Fig. 2)**——The composition of the reaction mixture was $4\times10^{-2}$M sodium phosphate buffer (pH 7.0), $1.0\times10^{-2}$M $Na_2SO_3$–$NaHSO_3$ (6:5, mole/mole), and $5\times10^{-5}$M 2-thiouracil. At room temperature, oxygen was continuously bubbled into this solution

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17) *a*) K. Isono, S. Suzuki, M. Tanaka, T. Nanbata and K. Shibuya, *Tetrahedron Letters*, **1970**, 425; *b*) *Idem*, *Agr. Biol. Chem.*, **36**, 1571 (1972).
18) H. Vorbrüggen, P. Strehlke and G. Schulz, *Angew. Chem.*, **81**, 997 (1969).

Table I. *Rf* Values of Compounds

| | Solvent | | |
|---|---|---|---|
| | A | B | C |
| Uracil-2-sulfonate | 0.48 | 0.03 | 0.62 |
| 2-Thiouracil | 0.58 | 0.57 | 0.76 |
| Uracil | 0.55 | 0.36 | 0.72 |
| 2-Thiouridine | 0.51 | 0.34 | — |
| Uridine | 0.51 | 0.21 | — |

at the rate of 1.8 ml/sec. The reaction was traced by the change in absorbance at 270 mμ. In order to push the reaction to completion 1M sodium bisulfite solution (1/500 of the volume of the reaction mixture, at a time) was added from time to time (indicated by arrows in Fig. 2). In the reaction under a nitrogen atmosphere, nitrogen was bubbled into the solution before the reaction was started by addition of bisulfite, and the tube filled with nitrogen was tightly stoppered after the addition of bisulfite. $10^{-5}$M Hydroquinone was present in the inhibition experiment. In the absence of bisulfite, all the other conditions being the same as the test reaction, no change in 2-thiouracil was observed.

Paper chromatographic analysis (solvent A, B and C) of the 120 min-reaction mixture showed that a complete conversion of 2-thiouracil into uracil-2-sulfonate occurred. Upon acid treatment of the reaction mixture, uracil was produced as a single product. Comparison of the intensity of UV absorption spectrum of the starting material, 2-thiouracil, with that of the final product, uracil, indicated that the overall conversion proceeded quantitatively.

When the initial concentration of 2-thiouracil in the reaction mixture was increased to $2.5\times10^{-3}$M, the rate of the sulfonate formation became about half of the rate of the $5\times10^{-5}$M 2-thiouracil-reaction.

**Reaction of 2-Thiouridine with Bisulfite in the Presence of Oxygen (*cf.* Fig. 3)**——The composition of the reaction mixture was $10^{-1}$M buffer (sodium acetate for pH 4-, sodium phosphate for pH 7-, and sodium carbonate for pH 10-reaction), $10^{-2}$M sodium bisulfite, $2\times10^{-4}$M manganese chloride and $5\times10^{-5}$M 2-thiouridine. Incubation was at room temperature with occasional oxygen bubbling. In the inhibition experiment, manganese chloride was absent and $10^{-5}$M hydroquinone was present in the reaction mixture. At all the pH values tested, the decrease of the absorbance at 275 mμ was considerably greater than that which can be expected for the conversion of 2-thiouridine into uridine. The absorbance continued to decrease with elongation of the time of incubation. Paper chromatography (solvent B) of the mixture after 50 hr-reaction at pH 7 gave four spots corresponding to 2-thiouridine (trace), uridine, uracil, and an unidentified material (showing fluorescence on irradiation of UV light) traveling to the front of the solvent. With the pH 10-4 hr-reaction, only uridine and uracil were the UV-absorbing materials detected on the chromatogram. In order to achieve a better separation of the spots, this reaction mixture was desalted by passing through a column of active carbon and subjected to a two dimensional cellulose thin layer chromatography (solvent A for the first, and B for the second dimension). Determination of the UV absorbance of the spots corresponding to uridine and uracil showed that these compounds were present in 7 : 3 mole ratio and that the recovered amount of the sum of them corresponded to 30% of the starting material used. As a control, a known amount of uridine was subjected to the same treatment as above and the technical losses during the work-up were found to be 15% at the desalting step and 40% at the chromatography step. If one calculates the recovery of the uridine (plus some uracil) taking the losses into account, the net recovery was about 60% of the 2-thiouridine used as the starting material. When in the pH 10-reaction the initial concentration of 2-thiouridine was increased to $6.2\times10^{-3}$M, the rate of the reaction became markedly lower, and two unidentified UV-absorbing compounds having *Rf* values smaller than that of uridine were detected on the paper chromatogram using solvent B.

**Acknowledgement** We thank the late Professor T. Ukita of this Faculty for his encouragement. Professor T. Ueda of Hokkaido University is also thanked for giving us an authentic specimen of 2-thiouridine with which our sample was compared.