matographed on a DEAE-cellulos ecolumn (0.5 × 80 cm) preequilibrated with 7 *M* containing 0.02 *M* Tris–HCl, pH 7.5. The elution was carried out using a linear gradient of NaCl (0.03–0.3 *M*) of total 700 ml. The hexanucleotide CpCpApCpCpAp was eluted at a salt concentration of 0.22 *M*. The combined fractions were desalted by gel filtration technique using Bio-Gel P-2 column (2 × 80 cm). R_f values of the hexanucleotide are shown in Table II. Bacterial alkaline phosphatase treatment of the hexanucleotide gave the dephosphorylated compound, which was shown to have the same mobility with the compound derived from VIb in solvent C.

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The Oxygen-Mediated Reaction between 4-Thiouracil Derivatives and Bisulfite. Isolation and Characterization of 1-Methyluracil 4-Thiosulfate as an Intermediate in the Formation of 1-Methyluracil-4-sulfonate

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Abstract: The titled reaction was investigated in detail in order to clarify its mechanism. 1-Methyl-4-thiouracil was treated with [^{35}S]bisulfite and oxygen at pH 7 and room temperature, and the reaction mixture was analyzed by paper electrophoresis. It was found that the radioactive sulfur was incorporated into the reaction product, 1-methyluracil-4-sulfonate. Another anionic compound, also radioactive, was observed in the electrophoresis. This compound is an intermediate of the reaction, and was identified as 1-methyluracil-4-thiosulfate (I). The thiosulfate I, which can also be prepared by sulfitolysis of bis(1-methyl-4-thiouracil) disulfide, is readily hydrolyzed by acid to give 1-methyl-4-thiouracil. On treatment with bisulfite at pH 7, I yields 1-methyluracil-4-sulfonate. Compound I is attacked by glycine at pH 10.4, giving rise to 1-methyl-N^4-carboxymethylcytosine. A powder of the sodium salt of I is rapidly decomposed by sunlight, whereas its aqueous solution is stable. An aqueous solution of the light-exposed powder of I gives a single spot corresponding to 1-methyl-4-thiouracil in paper chromatography. In analogy to the adduct formation between bisulfite and uracil, both 1-methyl-4-thiouracil and 1-methyluracil-4-sulfonate appear to add bisulfite at their 5,6-double bonds. Nmr spectroscopy supports the 5,6-dihydro-6-sulfonate structure of these addition compounds. Since hydroquinone inhibits the oxygen-mediated reaction between 4-thiouridine and bisulfite, it is proposed that the sulfite radical is participating in the reaction. A procedure to transform 1-methyl-4-thiouracil into 1-methyluracil on a preparative scale is described.

Following the discovery of 4-thiouridine in tRNA of *E. coli*,[1] many methods have been developed for the chemical transformation of the thio base. These methods may be of value for the elucidation of the biochemical role of this minor nucleoside as a constituent of tRNA. Oxidation of 4-thiouridine with iodine results in the formation of bis(4-thiouridine) disulfide.[1] Treatment of 4-thiouridine with cyanogen bromide brings about the formation of uridine 4-thiocyanate,[2] which in turn can be converted into uridine under certain mild conditions.[3] Photochemical oxidation converts thiouridine into uridine.[4] Oxidation with hydrogen peroxide[5] or with osmium tetroxide followed by treatment with acid[6] yields uridine. In these oxidations, the intermediate oxygenated sulfur compounds have not been identified. In the course of our studies on the permanganate oxidation of nucleosides,[7,8] we have become aware that 4-thiouridine undergoes a facile reaction with permanganate, giving rise to an oxygenated sulfur compound in a quantitative fashion. This compound has been identified as uridine-4-sulfonate.[9] Ziff and Fresco have independently shown that 2′,3′-isopropylideneuridine-4-sulfonate is produced by the oxidation of 2′,3′-isopropylidene-4-thiouridine with periodate.[10]

When the time course of the permanganate oxidation of 4-thiouridine was determined, bisulfite was added to the reaction mixture in order to destroy the permanganate. It was found that the bisulfite itself reacts with 4-thiouridine. In a previous note, we described briefly the nature of this reaction.[11] The present paper reports the detailed investigation of this reaction, including the isolation and characterization of an intermediate, uracil-4-thiosulfate derivative, and the discussions on the mechanism of the reaction.

As for the reaction of bisulfite with nucleotide bases, it should be noted that bisulfite undergoes ionic addi-

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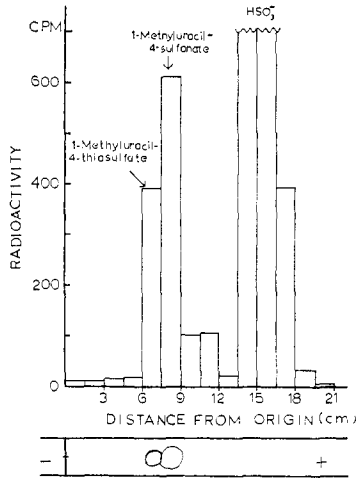

Figure 1. Incorporation of [^{35}S]bisulfite into 1-methyluracil-4-sulfonate and 1-methyluracil-4-thiosulfate fractions, as detected by paper electrophoresis. The bottom figure indicates spots as detected by ultraviolet absorption. See Experimental Section for detailed methods.

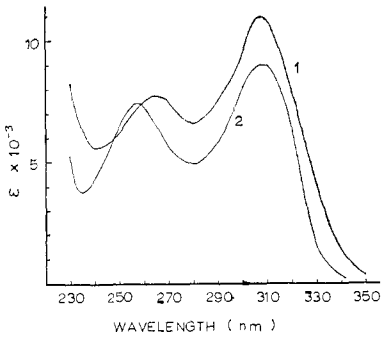

Figure 2. Ultraviolet spectrum of 1-methyluracil-4-thiosulfate (1) and bis(1-methyl-4-thiouracil) disulfide (2); solvent, water. The ϵ value of 1-methyluracil-4-thiosulfate was determined by transforming it into 1-methyl-4-thiouracil. Spectrum 2 is represented on a half-molar basis.

tion to the 5,6-double bond of pyrimidines, such as uracil and cytosine, to give 5,6-dihydro 6-sulfonate derivatives.[12,13]

As described in the previous paper,[11] the characteristics of the reaction between 4-thiouridine and bisulfite[14] are as follows. (1) Oxygen is required for the reaction to proceed, and the product is uridine-4-sulfonate. (2) For the optimal rate of reaction, the concentration of bisulfite in the reaction mixture must be around 10^{-2} *M*. Higher concentration of bisulfite inhibits the reaction. (3) The reaction proceeds at room temperature and at neutral pH, and comes to completion within several hours. (4) The yield of uridine-4-sulfonate from 4-thiouridine is quantitative. Since uridine-4-sulfonate is readily convertible into uridine by mild acid or alkali treatment, this reaction offers an excellent means to transform 4-thiouridine into uridine.

Several pertinent questions arise as to the mechanism of the reaction. (1) What is the origin of the sulfur atom of the product, uridine-4-sulfonate? Is it the sulfur of the starting material, 4-thiouridine, or of the reagent, bisulfite? (2) What is the role of the oxygen? (3) Why is the reaction inhibited at a high concentration of bisulfite? In order to answer these questions and to clarify the total mechanism of the reaction, we carried out experiments employing 1-methyl-4-thiouracil as a model for 4-thiouridine.

Reaction Products. The answer to the first question was provided by the oxygen-mediated reaction of 1-methyl-4-thiouracil with [^{35}S]bisulfite. Through a solution, consisting of 12 m*M* 1-methyl-4-thiouracil, 20 m*M* [^{35}S]sodium sulfite (0.683 Ci/mol), and 0.2 *M* sodium phosphate buffer, pH 7.0, oxygen was bubbled at a speed of 5 ml/sec. The progress of the reaction was followed by use of paper electrophoresis. Figure 1 shows a pattern obtained at 70 min. A similar result was also obtained at 10 min. Two zones were present which were not only radioactive but also ultraviolet absorbing. The compound contained in the faster travelling zone showed identical properties with authentic 1-methyluracil-4-sulfonate[9] in paper electrophoresis, paper chromatography, ultraviolet absorption, and the hydrolysis with acid or with alkali. The radioactivity present in this zone was found to be 84% of the theoretical value expected for the incorporation of the bisulfite into the sulfonate group. The corresponding value for 1-methyluracil-4-sulfonate of the 10-min reaction was 76%. From these results, we conclude that the sulfonate group of 1-methyluracil-4-sulfonate originates from the reagent, bisulfite. A possibility exists that a direct oxygenation of [^{32}S]1-methyl-4-thiouracil might have taken place giving 1-methyluracil-4-[^{32}S]sulfonate, and then the sulfonate group of this compound might have undergone an exchange reaction with the nucleophilic [^{35}S]sulfite present in the solution, resulting in the formation of 1-methyluracil-4-[^{35}S]sulfonate. This possibility is excluded by the following experiment. 1-Methyluracil-4-[^{32}S]sulfonate, in place of 1-methyl-4-thiouracil, was subjected to the same treatment as described above. It was found that, although the exchange reaction appeared to take place, the rate of the incorporation of the radioactive sulfur was low. Thus, after 70 min of the treatment, only about 30% of the original [^{32}S]sulfonate group was substituted by the [^{35}S]sulfonate.

The slower moving zone contained a new compound. This compound readily gave 1-methyl-4-thiouracil on treatment with acid. Therefore the molar quantity of this substance can be determined by its absorbance (330 nm) in acid. In this way the mole ratio of the incorporated radioactive sulfur to the pyrimidine base was determined and found to be 0.91:1. This compound is apparently an intermediate of the reaction, because (1) it disappears as the reaction proceeds, and (2) it readily yields 1-methyluracil-4-sulfonate, when it is treated with sodium bisulfite at pH 7. The mobility in the electrophoresis indicates that this compound is monoanionic. The ultraviolet spectrum of this compound resembles that of bis(1-methyl-4-thiouracil) disulfide (Figure 2). This compound is also produced when bis(1-methyl-4-thiouracil) disulfide is treated with an equimolar amount of bisulfite at pH 7. Thus, the sulfitolysis of the disulfide yields, besides 1-methyl-4-thiouracil, a compound which shows identical behavior

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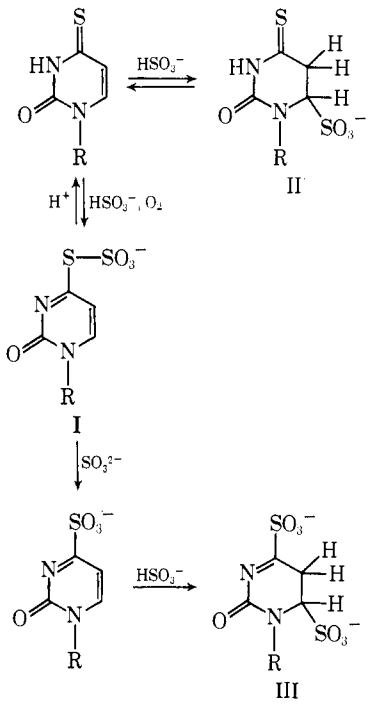
(14) In this paper, the term "bisulfite" is used representing a mixture of bisulfite and sulfite. For example, a bisulfite solution, pH 7, is a 1:3 (mole/mole) mixture of aqueous bisulfite and sulfite solutions. This mixture has a strong buffer action.

with the compound described above, in paper electrophoresis, in ultraviolet spectroscopy, and in reactions with acid and with bisulfite. In protein chemistry it is well known that a disulfide undergoes sulfitolysis in the following manner.[15] The thiosulfate structure I (see

$$RSSR + SO_3^{2-} \longrightarrow RS^- + RSSO_3^-$$

Chart I) for this compound explains all of the experi-

Chart I



R = CH_3 or ribofuranosyl

mental results described above. Evidence that the oxygen-mediated bisulfite reaction with 4-thiouridine and its methyl analog follows a similar course was provided by observing the expected similarity of the ultraviolet spectra of uridine-4-thiosulfate and its methyl analog. Thus, paper electrophoresis of a reaction mixture in which 4-thiouridine had been treated with bisulfite in the presence of oxygen gave a monoanionic spot of uridine-4-thiosulfate, in addition to a spot corresponding to uridine-4-sulfonate. An aqueous solution of this compound showed λ_{max} at 308 nm at pH 7, and exhibited λ_{max} at 330 nm at pH 1, the $A_{308}^{pH\ 7}/A_{330}^{pH\ 1}$ value being 0.5 as expected.

We undertook preparative isolation of this compound I in order to further investigate its properties. For this purpose bis(1-methyl-4-thiouracil) disulfide was treated with bisulfite at pH 7. The 1-methyluracil-4-thiosulfate and 1-methyl-4-thiouracil thus produced were fractionated by extraction with chloroform. From the chloroform-insoluble fraction, the thiosulfate was isolated as a sodium or triethylammonium salt. This compound undergoes hydrolysis even under slightly acidic conditions, giving 1-methyl-4-thiouracil. Thus, at pH 4.0 (0.1 *M* acetate buffer) and 40°, compound I is quantitatively converted into 1-methyl-4-thiouracil in 30 min. At pH 5.4 (0.1 *M* acetate buffer), this conversion takes 150 min for completion. In this hydrolysis the generation of the bisulfate ion makes the solution more acidic. Therefore, during the preparation described above, the thiosulfate solution should always be kept slightly alkaline. When the faintly yellow powder of the sodium salt of I was exposed to sunlight, it rapidly changed color to bright yellow. The resulting product by its ultraviolet spectrum and by paper chromatography was identical with 1-methyl-4-thiouracil. This light-induced decomposition does not occur when the thiosulfate I is in solution.

A compound having the structure, $RSSO_3^-X^+$ (R = alkyl or aryl), is called a "Bunte salt." The chemistry of Bunte salts has been recently reviewed.[16,17] Bunte salts in general undergo acid hydrolysis to give thiols and bisulfate, a property that compound I also possesses. It is known[18] that treatment of a Bunte salt with sulfite results in an exchange reaction of the following type. As described above, the thiosulfate I

$$*SO_3^{2-} + RSSO_3^- \longrightarrow RS*SO_3^- + SO_3^{2-}$$

differs from the general Bunte salts in this respect. Compound I did not react with cyanide at room temperature or even at 90°, in spite of the fact that Bunte salts generally yield thiocyanate derivatives by this treatment. Thiosulfate I did not give bis(1-methyl-4-thiouracil) disulfide on treatment with 1-methyl-4-thiouracil in alkaline solution. A method[17] for the preparation of Bunte salt consists of treatment of a thiol with *N*-pyridiniumsulfonate.[19] We tried this reaction using 1-methyl-4-thiouracil, but recovered the starting material.

The results suggest that, in this oxygen-mediated reaction between 1-methyl-4-thiouracil and bisulfite, bis-(1-methyl-4-thiouracil) disulfide is the immediate precursor of the intermediate I. Therefore detection of the disulfide during the course of the reaction was attempted. In any conditions tested, however, the disulfide was not detected (see Experimental Section). This fact suggests, although does not prove, that the disulfide is not the intermediate of the reaction.

In the previous paper,[11] it was briefly mentioned that 4-thiouridine forms an unstable complex with bisulfite. The complex formation can be detected by the change in the ultraviolet absorption, in the absence of oxygen. Figure 3 shows the spectra of 4-thiouridine (0.1 m*M*) dissolved (1) in water and (2) in 1 *M* sodium bisulfite, pH 6.9. The spectra suggest that about half of the 4-thiouridine molecules are in the form of a bisulfite adduct that does not absorb the light at 330 nm. When the 1 *M* bisulfite solution of 4-thiouridine was diluted with an equal volume of water, the resulting absorbance was found to be approximately two-thirds, but not half, of the original value, indicating that a considerable portion of the adduct was decomposed by the dilution, regenerating 4-thiouridine. Similar results have been obtained for 1-methyl-4-thiouracil.

The adduct formation was found to be strongly pH dependent. As Figure 4 shows, the absorbance of 4-thiouridine is minimal when the pH of the 1 *M* bisulfite solution is lower than 6. The midpoint of this curve is around pH 7. Since the pK_a value of bisulfite is 7.2, the results suggest that the effective species in the adduct

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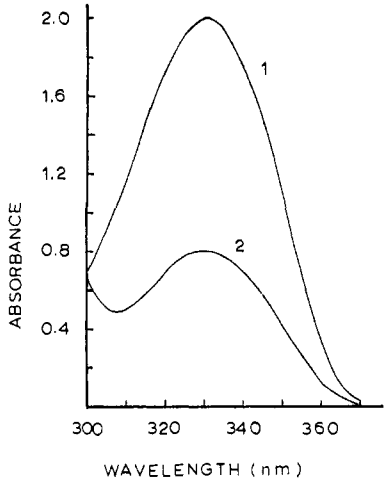

Figure 3. Complex formation between 4-thiouridine and bisulfite, as demonstrated by ultraviolet spectra: (1) spectrum of 10^{-4} *M* 4-thiouridine solution in water (reference, water); (2) spectrum of 10^{-4} *M* 4-thiouridine solution in 1 *M* sodium bisulfite, pH 6.9 (reference, 1 *M* sodium bisulfite, pH 6.9). Before dissolving 4-thiouridine, nitrogen was bubbled through the solvent for 3 min to remove dissolved oxygen, and the spectrum was recorded under the atmosphere of nitrogen.

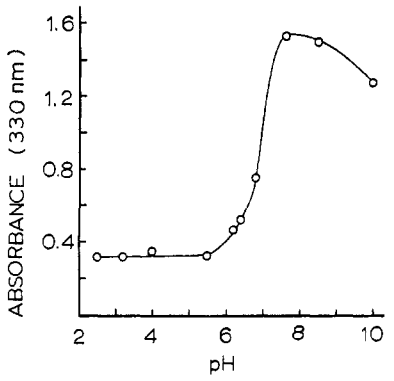

Figure 4. Effect of pH on the complex formation between 4-thiouridine and bisulfite. Each sample was prepared by mixing solutions of 4-thiouridine, $NaHSO_3$, and Na_2SO_3 to give final concentrations of 10^{-4} *M* in 4-thiouridine and 1 *M* in bisulfite plus sulfite. By mixing $NaHSO_3$ and Na_2SO_3 in various ratios, the pH of the solution can be fixed at an appropriate value. The pH lower than 4 was fixed by addition of concentrated HCl. The uv recording was carried out at 22°. Change in the order of mixing of the three components did not affect the result.

formation is HSO_3^- but not SO_3^{2-}. In addition to this pH effect on the absorbance, the temperature of the solution has a marked effect. The lower the temperature becomes, the lower is the value of the absorbance, *i.e.*, more adduct is formed at a lower temperature. This temperature effect is also of reversible nature. In analogy to uridine bisulfite and cytidine bisulfite adducts,[12,13] this unstable adduct may be postulated to be 5,6-dihydro-4-thiouridine-6-sulfonate (and its 1-methyl analog) (II). Nmr spectroscopy supports this structural assignment. Thus, the nmr spectrum of a D_2O solution of 1-methyl-4-thiouracil containing a 1 *M* sodium sulfite–sodium bisulfite (3:1, mole/mole) mixture gave signals assignable to 5-H (3.52 ppm) and 6-H (4.61 ppm) of the dihydropyrimidine in addition to the signal of 5-H (6.50 ppm) and 6-H (7.50 ppm) of 1-methyl-4-thiouracil. As judged by the signal strengths, the starting material and the adduct II were present in approximately 1:2 ratio under the conditions employed.

Adduct formation was also observed between 1-methyluracil-4-sulfonate and bisulfite. In this case the adduct is stable and can be isolated. A solution consisting of 0.2 *M* 1-methyluracil-4-sulfonate and 0.4 *M* sodium bisulfite, pH 7.0, was allowed to stand at room temperature for 4 hr. The solution was then submitted to paper electrophoresis (in 0.1 *M* acetate buffer, pH 4.0) and to paper chromatography (*n*-butyl alcohol–acetic acid–water, 5:3:2, by volume). 1-Methyluracil-4-sulfonate was completely converted into a new compound, which traveled about two times faster in electrophoresis and slower in paper chromatography than the starting compound. This behavior suggests a dianionic character for this compound and the compound is thus assumed to have the 5,6-dihydro-6-sulfonate structure, III. The product III was eluted from the paper chromatogram and its properties were investigated. The nmr spectrum of III gave three signals assignable to 5-H (4.30 ppm, d, J = 1 cps), 6-H (4.89 ppm, d, J = 1 cps), and 1-CH_3 (3.18 ppm, s). The ultraviolet spectrum showed a single peak with λ_{max} 230 nm. No absorption was observed above 280 nm. When compound III was treated with alkali, it yielded 1-methyluracil. 1-Methyluracil was also produced on treatment of III with ammonia–ammonium chloride buffer, pH 10. On such ammonia treatment, 1-methyluracil-4-sulfonate yields 1-methylcytosine.[9] This in turn indicates in the above ammonia treatment of III first the sulfonate group at the 4 position is attacked by hydroxide ion, but not by ammonia, to give 1-methyl-5,6-dihydrouracil-6-sulfonate,[13] and then this compound undergoes elimination[13] yielding 1-methyluracil. 1-Methyluracil-4-sulfonate is readily convertible into 1-methyl-4-thiouracil by treatment with sodium hydrogen sulfide.[9] When the compound III was treated with aqueous sodium hydrogen sulfide, however, it gave 1-methyluracil and 1-methyl-4-thiouracil in an 8:1 ratio. In contrast to the extreme lability of 1-methyluracil-4-sulfonate in acid, compound III is not degraded on treatment with 0.1 *N* HCl at room temperature for 90 min.

When 1-methyluracil-4-sulfonate was treated with 10–20 m*M* bisulfite, pH 7, no formation of the adduct III was observed. This is consistent with the fact that uridine-4-sulfonate is quantitatively produced from 4-thiouridine on treatment with 10 m*M* bisulfite in the presence of oxygen.

In the previous work[11] the reaction was carried out on a micromole scale. This time the reaction between 1-methyl-4-thiouracil and bisulfite was performed on a 2-mmol scale, and, after subsequent treatment with acid, 1-methyluracil was recovered in excellent yield (see Experimental Section). This experiment demonstrates the feasibility of the reaction for preparative purposes.

Reaction Mechanism and Discussion. It has been shown that both bisulfite and oxygen are necessary for the production of uridine-4-sulfonate from 4-thiouridine. When oxygen was bubbled through a solution of 4-thiouridine in the absence of bisulfite, no reaction occurred as judged by the ultraviolet absorption of the solution.[11]

Although the thiosulfate I is clearly an intermediate of the reaction, it is still possible that another pathway for the formation of uridine-4-sulfonate exists. A simple

route would be the direct substitution of the 4-thio group (in its thiol form) by sulfite. Since uridine-4-sulfonate is readily convertible into 4-thiouridine by treatment with hydrogen sulfide,[9] this substitution process would be an equilibrium reaction. The equilibrium could be pushed to the product side by removal of hydrogen sulfide that has been generated by the substitution. This removal of hydrogen sulfide could be achieved by oxidizing it with oxygen. This possible reaction mechanism, however, has been excluded. If the equilibrium mechanism is actually operating, the reaction should be reversed by addition of hydrogen sulfide during the reaction. This was found not to be the case. When sodium hydrogen sulfide (pH 7, 0.1 m*M* in the final concentration) was added to a solution containing 4-thiouridine (0.1 m*M*) which had been treated with sodium bisulfite (pH 7, 10 m*M*) in the presence of oxygen for 2 hr, the reaction was not reversed at all, as judged by the constancy of the absorbance (at 330 nm) of the reaction mixture.

Another possibility that sulfate, produced from sulfite by the action of oxygen, could be the reacting species has also been excluded. Thus, when sodium sulfate was used in place of sodium sulfite no change occurred in 4-thiouridine.

Kharasch, May, and Mayo reported the oxygen-mediated addition of bisulfite to olefins.[20] Since the reaction is inhibited by radical scavengers, such as hydroquinone, they concluded that the sulfite radical is the reactive species in this reaction. In addition, the aerobic oxidation of bisulfite has been recognized as a free-radical chain reaction.[21]

$$HSO_3^- + O_2 \longrightarrow \cdot SO_3^- + \cdot HO_2$$

It is likely that the sulfite radical is also participating in the reaction of 4-thiouridine under discussion. Figure 5 shows the effect of hydroquinone on the reaction. Addition of hydroquinone in an amount $^1/_{200}$th of that of bisulfite ($\sim^1/_5$th of that of 1-methyl-4-thiouracil) blocks the reaction. A possible reaction scheme would be as follows

$$RSH + \cdot SO_3^- \longrightarrow RS\cdot + HSO_3^-$$

$$RS\cdot + \cdot SO_3^- \longrightarrow RSSO_3^-$$

It is difficult to judge whether the reacting species RSH in the process is the free 4-thiouracil derivative or its bisulfite adduct II. However, the free form is the more likely candidate, for a higher concentration of bisulfite, by which more adduct is produced, inhibits the reaction rather than accelerates it.

Hydrogen peroxide is known to be a product of the autoxidation of bisulfite.[22] Scheit has shown that uridine is produced from 4-thiouridine by treatment with hydrogen peroxide at pH 8.[5] It appears that hydrogen peroxide is not participating in the oxygen-mediated reaction of the 4-thiouracil derivatives with bisulfite, because no uracil derivatives were detected during this reaction.

The next step of the total reaction, $RSSO_3^- \rightarrow RSO_3^-$, is a nucleophilic substitution, for it proceeds more rapidly with a higher concentration of sulfite.

Having seen the radical nature of the step of the thiosulfate formation, we can think of a possible reason why

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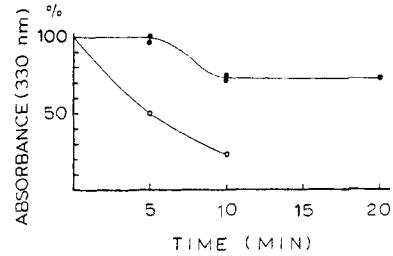

Figure 5. Effect of hydroquinone on the oxygen-mediated reaction between 1-methyl-4-thiouracil and bisulfite. Composition of the solutions: —●—, 0.06 m*M* in 1-methyl-4-thiouracil, 2.5 m*M* in sodium bisulfite, 0.0125 m*M* in hydroquinone, and 12.5 m*M* in sodium phosphate buffer, pH 7.0; —○—, same as above, except that hydroquinone is omitted.

in 1 *M* bisulfite solution the total reaction was highly inhibited. A sulfite radical that is produced in a bisulfite solution may be rapidly destroyed by the bisulfite (or sulfite) ions surrounding it. If the bisulfite ions are present in an amount far in excess of the thiouracil molecule, the sulfite radicals would be consumed exclusively by the bisulfite, before they could act to produce the thiosulfate I. With the concentration of the substrate, 4-thiouridine, high enough to compete with bisulfite ions to trap the sulfite radical, the formation of thiosulfate I should be observable. This is indeed the case. Thus, when oxygen was bubbled through a solution of 10^{-4} *M* 4-thiouridine in 1 *M* sodium bisulfite (pH 6.9), only a very slow decrease of $A_{330\,nm}$ was observable: 1% at 5 min, 4% at 15 min, and 10% at 60 min. On the other hand, when a similar reaction was carried out with 10^{-1} *M* 4-thiouridine in 1 *M* sodium bisulfite, the decrease in $A_{330\,nm}$ was 45% after 10 min. A similar phenomenon has been observed by Freese and Freese[23] in the concentration dependence of the oxygen-mediated peroxide production from hydroxylamine. Thus, the highest concentration of the peroxide is obtained when the concentration of hydroxylamine is 10^{-2} *M*, while in 1 *M* hydroxylamine solution the level of the peroxide is practically zero. The authors ascribed this to the consumption of the peroxide by hydroxylamine molecules.

Experimental Section

Materials. 1-Methyl-4-thiouracil and 4-thiouridine were prepared according to Fox, *et al.*[24] Bis(1-methyl-4-thiouracil) disulfide was synthesized by the method of Pal, *et al.*[25] $Na_2{}^{35}SO_3$ was purchased from The Radiochemical Centre, Amersham, England. Oxygen was supplied directly from an oxygen bomb. Unless otherwise noted, neutral bisulfite solutions were prepared by mixing $NaHSO_3$ and Na_2SO_3 solutions.

Methods. Nmr spectra of D_2O solutions were recorded on a Jeol-NM4H-100 spectrometer using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. Paper chromatography was carried out ascendingly on Toyo filter paper No. 53.

The electrophoresis (Figure 1) was performed on No. 53 filter paper using 0.1 *M* acetate buffer, pH 5.35, as the solvent. The electrophoresis was run at 26 V/cm for 50 min. Since chloroform used as the cooling solvent dissolves the starting material, 1-methyl-4-thiouracil, the spot corresponding to this compound was not detectable on the paper. After the electrophoresis was completed, the paper was air-dried, and, while it was still wet, 0.1 *N* NaOH was sprayed on it in order to fix the bisulfite. The paper was then dried by air and cut into pieces of 1.5-cm width. Each piece of paper

(23) E. Freese and E. B. Freese, *Biochemistry*, 4, 2419 (1965).
(24) J. J. Fox, D. V. Praag, I. Wempen, I. L. Doerr, L. Cheong, J. E. Knoll, M. L. Eidinoff, A. Bendich, and G. B. Brown, *J. Amer. Chem. Soc.*, 81, 178 (1959).
(25) B. C. Pal, M. Uziel, D. G. Doherty, and W. E. Cohn, *ibid.*, **91**, 3634 (1969).

was soaked in 2 ml of water overnight. From each aqueous extract, 100 μl was taken up, placed on a planchet, evaporated to dryness by heating with an electric lamp, and counted on an Aloka Model TDC-307 gas-flow counter. From the aqueous extract which contained uv-absorbing component, another 100 μl was taken, mixed with 10 μl of 1 *N* HCl, and submitted to the recording of ultraviolet absorption. The counting efficiency was determined using known amounts of $Na_2{}^{35}SO_3$ and was found to be $36 \pm 1\%$.

1-Methyluracil-4-thiosulfate. (i) **Sodium Salt.** Bis(1-methyl-4-thiouracil) disulfide (850 mg) was suspended in a mixture of ethanol (150 ml) and water (330 ml). While the mixture was being stirred, 6 ml of a mixture of 0.5 *M* Na_2SO_3 and 0.5 *M* $NaHSO_3$ (3:1, v/v) was added dropwise. The resulting yellow solution was allowed to stand at room temperature for 20 min, and the solution was concentrated by evaporation under reduced pressure to remove most of the ethanol. From the residual aqueous solution, 1-methyl-4-thiouracil was extracted with chloroform (each *ca.* 60 ml, four times). The aqueous phase was evaporated to dryness under reduced pressure. During the evaporation, pyridine was frequently added into the solution in order to prevent the acid hydrolysis of the thiosulfate. The residue was triturated with ethanol and the ethanol removed by evaporation. A similar treatment was carried out using ether, giving a faintly yellow powder of sodium 1-methyluracil-4-thiosulfate (600 mg). The purity of this material was checked for its aqueous solution by use of paper electrophoresis, *n*-hexane being employed as the cooling solvent. The material was contaminated with a small amount of 1-methyl-4-thiouracil which could have been generated from the thiosulfate during the purity-check procedure. Since this powder is extremely sensitive to exposure to light, it must be kept in the dark. A portion of the powder was taken up and dissolved in D_2O as quickly as possible. This solution was submitted to nmr spectroscopy. Signals assignable to 1-methyluracil-4-thiosulfate were 3.53 (1-CH_3, s), 7.05 (5-H, d), and 8.05 ppm (6-H, d), the ratio of the signal strength being 3:1:1. Signals due to contaminating 1-methyl-4-thiouracil appeared at 3.42 (1-CH_3, s), 6.50 (5-H, d), and 7.38 (6-H, d). Comparison of the two 1-CH_3 signals indicated that the ratio of 1-methyluracil-4-thiosulfate to 1-methyl-4-thiouracil in this solution was 3:1 when the spectrum was recorded.

(ii) **Triethylammonium Salt.** An aqueous solution which contained 5 mmol of Na_2SO_3 was placed on a column of Dowex 50 (20–50 mesh, 2.5 × 10 cm, triethylammonium form). The column was eluted with 0.5 *M* triethylammonium bicarbonate (pH 7.2) until the eluate became negative to Malachite green. A portion (17 ml) of the resulting 0.033 *M* triethylammonium bisulfite solution was added dropwise into a stirred suspension of bis(1-methyl-4-thiouracil) disulfide (140 mg) in ethanol–water (30 ml:40 ml). The reaction was allowed to proceed at room temperature for 40 min. The solution was then loaded on a column of Dowex 1 (1 × 19 cm, bicarbonate form). The column was washed first with water (40 ml) and then with 0.015 *M* triethylammonium bicarbonate (pH 7.2, 60 ml) to elute 1-methyl-4-thiouracil. The column was then eluted with 2 *M* triethylammonium bicarbonate (350 ml) until no ultraviolet absorbing material was detectable in the eluate. This 2 *M* bicarbonate fraction was evaporated to dryness. During the evaporation, pyridine was frequently added into the solution. The residual triethylammonium salt of 1-methyluracil-4-thiosulfate was obtained as an oily substance. *Anal.* Calcd for $C_{11}H_{21}N_3O_4S_2$: N, 12.99. Found: N, 13.12. The ultraviolet absorption spectrum of this material in water gave λ_{max} 265 and 311 nm and λ_{min} 241 and 282 nm. In 0.1 *N* HCl solution, the ultraviolet spectrum of this material was the same as that of 1-methyl-4-thiouracil: λ_{max} 260 and 334 nm and λ_{min} 288 nm. These spectra also showed that the triethylammonium salt was free of pyridine, 1-methyl-4-thiouracil, and 1-methyluracil-4-sulfonate.

Light-Induced Decomposition of 1-Methyluracil-4-thiosulfate. The sodium salt of I is stable in the dark under the atmosphere of either oxygen or nitrogen. The sodium salt decomposes on exposure to light, changing its color into bright yellow in a few minutes. This occurs even under the atmosphere of nitrogen. Before the decomposition, an aqueous solution of the sodium salt exhibited pH 10.2. The slight alkalinity was probably due to the presence of a trace of NaOH in this material. After the sodium salt was exposed to light, the resulting powder was dissolved in water. The solution was found to be acidic (pH 2.7). It did not decolorize Malachite green in dilute phosphate buffer (pH 7), but it gave a white precipitate upon addition of an aqueous barium chloride solution. This precipitate was insoluble in concentrated hydrochloric acid but was soluble in concentrated sulfuric acid. These facts demonstrated the presence of sulfate in the test solution.

Reaction of 1-Methyluracil-4-thiosulfate with Nucleophiles. 1-methyluracil-4-thiosulfate was treated with bisulfite at pH 7 and room temperature. Paper electrophoretic analysis showed that a single compound was produced in this reaction mixture. This product was identical with 1-methyluracil-4-sulfonate with respect to its behavior in paper electrophoresis, its ultraviolet spectrum, and its facile production of 1-methyluracil on treatment with acid. The progress of the reaction can be followed by determining the absorbance at 330 nm after acidification of the reaction mixture. Thus, in a solution consisting of 1.7 m*M* 1-methyluracil-4-thiosulfate and 17 m*M* sodium bisulfite, pH 7, the reaction came to completion at 1 hr; in a solution consisting of 1.7 m*M* 1-methyluracil-4-thiosulfate and 170 m*M* bisulfite, it did so at 10 min.

When the thiosulfate was dissolved in 0.1 *M* glycine–sodium hydroxide buffer, pH 10.4, and the solution incubated at 37°, nucleophilic substitution of the thiosulfate group by the amino group took place, yielding 1-methyl-N^4-carboxymethylcytosine. This reaction came to completion in 19 hr. The ultraviolet spectra of this compound at pH 1.4 (λ_{max} 288 nm; λ_{min} 247 nm) and at pH 9.6 (λ_{max} 276 nm; λ_{min} 252 nm) were identical with those of an authentic 1-methyl-N^4-carboxymethylcytosine prepared from 1-methyluracil-4-sulfonate and glycine.[9] The product also showed identical paper chromatographic behavior with the authentic sample.

When the thiosulfate I was treated with strong alkali (2 *N* NaOH for 30 min at room temperature), it was converted into 1-methyluracil.

Attempted Detection of Bis(1-methyl-4-thiouracil) Disulfide as the Intermediate. The compositions of the reaction solutions prepared for this purpose were for 1-methyl-4-thiouracil–sodium bisulfite, pH 7: 10 m*M*:7 m*M*, 10 m*M*:14 m*M*, 20 m*M*:5 m*M*, and 20 m*M*:10 m*M*. Oxygen was bubbled through each solution, aliquots were withdrawn at 0, 1, 2, 5, and 10 min, and the aliquots examined by paper chromatography (solvent, *n*-butyl alcohol–water, 86:14, v/v). In all of these aliquots, no spot corresponding to the disulfide (R_f, 0.37) was observed. Only two spots corresponding to 1-methyl-4-thiouracil (R_f, 0.65) and to a mixture of 1-methyluracil-4-thiosulfate and 1-methyluracil-4-sulfonate (R_f, 0.0) were observed.

Conversion of 1-Methyl-4-thiouracil into 1-Methyluracil on a 2-mmol Scale. Oxygen (7 ml/sec) was bubbled through a solution of 1-methyl-4-thiouracil (284 mg) in water (200 ml). Na_2SO_3–$NaHSO_3$ (2 *M*, pH 6.7, 5 ml in total) was added portionwise into it. This addition took 7 hr, when the reaction came to a completion. Exactly one-fourth of the solution was taken and passed through a column of Dowex 50 (1 × 10 cm, H^+ form). The column was washed with water (50 ml) to elute 1-methyluracil. Paper chromatography of this fraction gave a single spot of 1-methyluracil. The optical density unit (265 nm) collected in this fraction was 4500, which corresponded to a 90% yield. The solution was evaporated to dryness and the residue extracted with hot ethanol (40 ml, 90°). The ethanolic solution was filtered to remove colored impurities and then concentrated to give crystalline 1-methyluracil (43 mg, 70%, mp 226–228°). A portion of this material was recrystallized from ethanol giving a pure sample (mp 230–232°), which did not show any depression of the melting point when mixed with an authentic specimen[26] of 1-methyluracil.

Reactivity of Major Nucleotide Bases toward the Sulfite Radical. A solution consisting of a nucleoside (or a base), 10^{-4} *M*, and sodium bisulfite, pH 7, 10^{-2} *M*, was subjected to oxygen bubbling at room temperature, and the absorbance at 260 nm of the solution was followed, up to 2 hr. There was no uv change observed, however, for thymidine, uracil, cytosine, adenine, or guanosine. These results are to be compared to the fact that in 1 *M* bisulfite solution uracil and cytosine readily add bisulfite to their 5,6-double bond.[12,13]

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