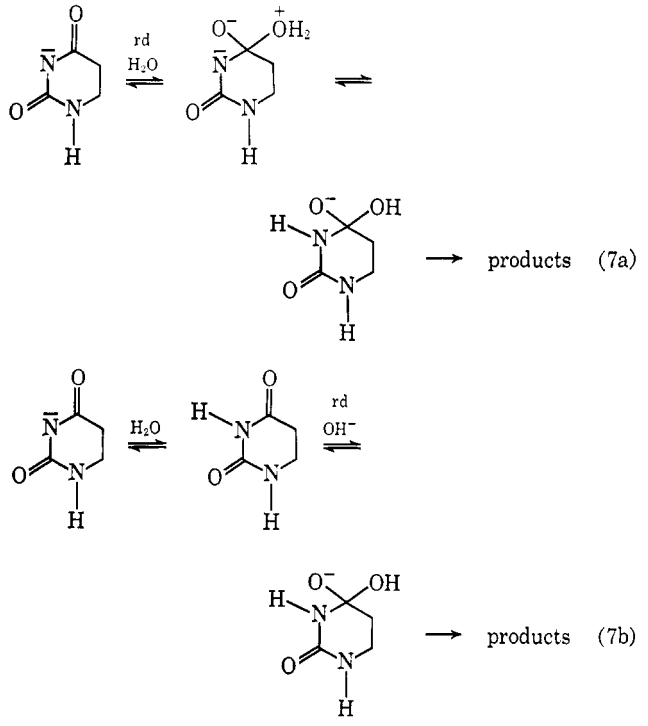

anion. For these compounds, the rates of hydrolysis change from a second- to a zero-order dependence on OH^- which like the case of 3-methyldihydrouracil indicates a change in the rate-determining step of a multistep pathway. Since at low OH^- concentration the hydrolysis of the unsubstituted dihydropyrimidines obeys a rate law which is second order in OH^- (eq 2) it can be concluded that under these conditions the rate-determining step of the reaction is the breakdown of the tetrahedral intermediate. Some of the rate measurements reported were conducted at pH values near and in several cases below the pK_a of the proton on the 3 position of the dihydropyrimidine ring. Consequently, some of the tetrahedral intermediate is likely formed by the attack of OH^- on the undissociated dihydropyrimidine as is the case with 3-methyldihydrouracil. As the OH^- concentration is increased the predominant dihydropyrimidine species becomes the anion and the rate of hydrolysis becomes invariant with OH^- concentration. This observation would be compatible with a change in the rate-determining step of the reaction from breakdown of the tetrahedral intermediate to either the attack of H_2O on the dihydropyrimidine anion (eq 7a) or the kinetically indistinguishable attack of OH^- on the protonated dihydropyrimidine preceded by proton donation to the dihydropyrimidine anion (eq 7b). Mader[11] has invoked a similar explanation for the analogous alkaline hydrolysis of 2,2,2-trifluroacetanilide.

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Isolation and Characterization of a Pyrimidine Sulfenic Acid *via* Scission of the Sulfur–Sulfur Bond in the Methyl Analog of Bis(4-thiouridine) Disulfide[1,2]

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Abstract: It has been established that bis(1-β-D-ribofuranosyl-4-thiouracil) disulfide and its methyl analog are hydrolyzed almost quantitatively in alkali to the corresponding thiones and sulfenic acids in 1:1 molar ratios. 1-Methyluracil-4-sulfenic acid was isolated and characterized as its silver salt. Uridine-4-sulfenic acid was identified by the similarity of its uv absorption spectra with those of its 1-methyl analog. Some of the properties of the sulfenic acid derivatives are reported. The cleavage of the disulfides in acid, on the other hand, gives quantitative formation of the corresponding thiones and uracil derivatives.

The recent discovery of four thiopyrimidines[3–5] and one thiopurine[6] as minor bases in tRNA has stimulated considerable interest in these compounds, particularly in 4-thiouridine, which is known to constitute a major portion of these thionucleosides in bacterial tRNA. Although the presence of bis(4-thiouridine) disulfide has not yet been demonstrated in native tRNA, it has been claimed to be artificially formed therein by iodine oxidation.[7,8] The potential significance of these thio bases in tRNA has been discussed by Irie, *et al.*[9] Since acid and alkali are very often used in degrading nucleic acids, their action on these disulfides is impor-

(1) Research sponsored by the U. S. Atomic Energy Commission under contract with the Union Carbide Corp.

(2) Presented at the 156th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 8–13, 1968, Abstracts, BIOL-213.

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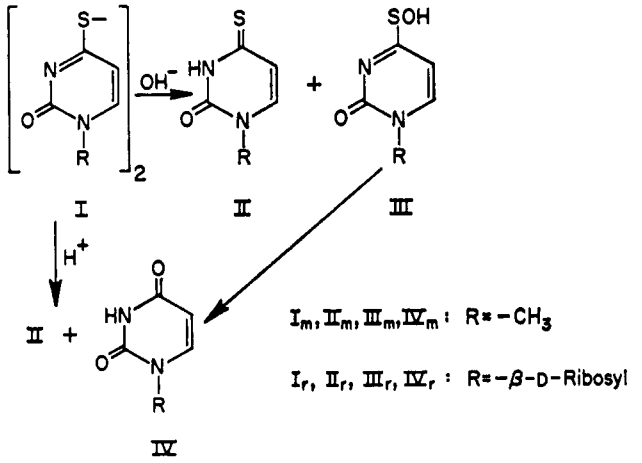
tant. The formation of thiol and sulfenic acid in the first step of the hydrolytic cleavage of both aliphatic and aromatic disulfides has been postulated by a number of investigators from time to time. The unstable sulfenic acid supposedly served as a precursor for the various reaction products isolated and characterized.[10] Uziel reported earlier the hydrolysis of bis(4-thiouridine) disulfide in alkali with the formation of two major products, 4-thiouridine and an unidentified compound X. On the basis of this observation, he postulated that initially the disulfide undergoes predominantly –S–S– cleavage to 4-thiouridine and its sulfenic acid.[11] In contradiction with this observation, Lipsett[8] reported the formation of a single product T, also unidentified, as a result of the treatment of bis(4-thiouridine) disulfide with alkali.



For identification and characterization of the unknown product, we synthesized the methyl analog of bis(4-thiouridine) disulfide (I_m), a compound more soluble in organic solvents and lacking the glycosyl linkage. 1-Methyl-4-thiouracil (II_m), prepared after Fox, *et al.*,[12] was oxidized by iodine to form the desired methyl analog in fair yield. The spectrophotometric behavior of bis(4-thiouridine) disulfide (I_r) and its methyl analog (I_m) are very similar. The spectra at pH 2 are irreversibly and almost instantaneously altered by alkali and are not regenerated on reacidification in either case (Figure 1). This indicates that the ribose moiety does not participate in this reaction, and that the results obtained from the study of the transformation of I_m in alkali should be applicable to I_r. After alkaline treatment,

(10) Aromatic disulfides such as diphenyl disulfide have been reported to decompose according to the following equations [R. Schiller and R. Otto, *Ber.*, **9**, 1937 (1876); E. Fromm, *ibid.*, **41**, 3403 (1908)]

$$KOH + C_6H_5SSC_6H_5 \longrightarrow C_6H_5SH + C_6H_5SOH$$
$$2C_6H_5SOH \longrightarrow C_6H_5SH + C_6H_5SO_2H$$

The following course of reactions was postulated for the action of alkali on aliphatic disulfides such as cystine [A. Schoberl and P. Rambacher, *Ann. Chem.*, **538**, 84 (1939)]

$$[HOOCCH(NH_2)CH_2S-]_2 \xrightarrow{OH^-} HOOCCH(NH_2)CH_2SH + [HOOCCH(NH_2)CH_2SOH]$$
$$\downarrow$$
$$HOOCCH(NH_2)CHO + H_2S$$

Other examples are cited in N. Kharasch, S. J. Potempa, and H. L. Wehrmeister, *Chem. Rev.*, **39**, 269 (1946), and in D. S. Tarbell and D. P. Harnish, *ibid.*, **49**, 1 (1951).

(11) M. Uziel, *Biochem. Biophys. Res. Commun.*, **25**, 105 (1966).

(12) J. J. Fox, D. V. Praag, I. Vempen, I. Doerr, L. Cheong, J. E. Knoll, M. L. Edinoff, A. Bendich, and G. B. Brown, *J. Am. Chem. Soc.*, **81**, 178 (1959).

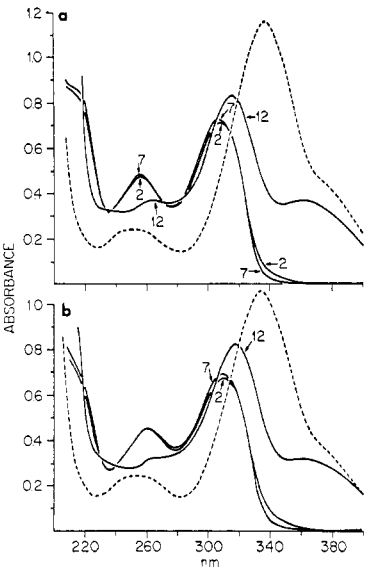


Figure 1. (a) The uv absorption spectra of bis(1-methyl-4-thiouracil) disulfide (I_m) at pH 2, 7, and 12 (———). The attempted regeneration of the spectrum at pH 2 (– – – – –). (b) The uv absorption spectra of bis(1-β-D-ribosyl-4-thiouracil) disulfide (I_r) at pH 2, 7, and 12 (———). The attempted regeneration of the spectrum at pH 2 (– – – – –).

the reaction mixtures in both cases can be reduced quantitatively at pH 7 to the 4-thio derivatives, as noted earlier[8,11] in the case of I_r. The alkaline treatment of the disulfides I_m and I_r did not alter the pyrimidine ring.

Separation of the products of alkaline treatment of I_m has been achieved by (1) paper chromatography, (2) column chromatography on Dowex 50 (H^+), (3) chromatography on cation-exchange columns,[13] and (4) precipitation as the silver salt. Only two major products were found: 1-methyl-4-thiouracil (II_m) and the hitherto unknown compound, herein identified and characterized as 1-methyluracil-4-sulfenic acid (III_m). In the first two methods, recovery of the reaction products was about 90%. The amount of sulfenic acid formed was estimated spectrophotometrically after reducing it to 1-methyl-4-thiouracil by dithiothreitol. The sulfenic acid (III_m) was retained by the Dowex 50 (H^+) column; apparently it behaves as a cation due to protonation at low pH. Compound II_m was not retained by the column under these conditions. After separation, the sulfenic acid was eluted by neutralizing the column with dilute ammonia. Attempts to isolate the acid or its ammonium salt were not successful.

The chemical method enabled us to obtain and characterize the sulfenic acid in the form of a stable silver salt. The disulfide (I_m) was treated with dilute alkali followed by ammoniacal silver acetate solution, whereupon the thione (II_m) formed a gelatinous precipitate. It was centrifuged, and the supernatant was evaporated until crystallization of the silver salt began. The silver sulfenate was obtained in pure form in about 30% over-all yield. The free sulfenic acid could be liberated from the silver salt by carefully acidifying with hydrochloric acid to pH 2 and filtering the silver chloride formed. The sodium salt can be obtained by dissolving the silver salt in aqueous ammonia and passing the ammoniacal solution through a column of Dowex 50 (Na^+).

(13) M. Uziel, C. Koh, and W. E. Cohn, *Anal. Biochem.*, **25**, 77 (1968).

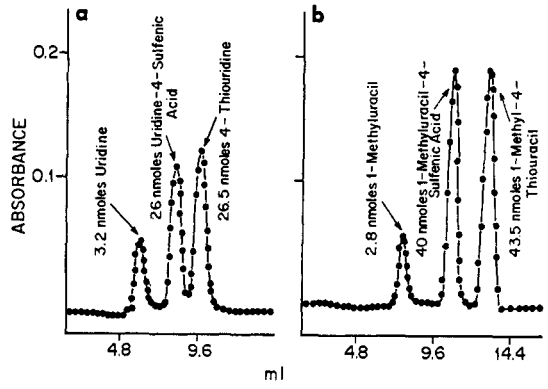


Figure 2. Chromatography of (a) alkali-treated bis(1-β-D-ribosyl-4-thiouracil) disulfide (I_r) and (b) its methyl analog (I_m). Column 0.6 × 23.0 cm, Bio-Rad A6. Eluent, 0.4 *M* formate buffer, pH 4.6, and 0.24 ml/min (48°), monitored at 250, 260, 280, and 310 nm. For clarity, only the absorbance at 260 nm is shown in the figures.

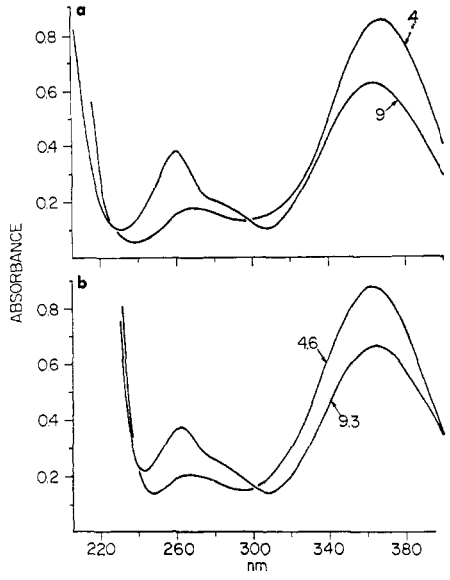


Figure 3. (a) The uv absorption spectra of 1-methyluracil-4-sulfenic acid (III_m) at pH 4 and 9. (b) The uv absorption spectra of 1-β-D-ribosyluracil-4-sulfenic acid (III_r) at pH 4.6 and 9.3.

All attempts to crystallize the sulfenic acid in free form were unsuccessful. It slowly decomposes, more readily in acid than in alkali. The silver salt was found to be stable for at least 4–6 weeks when stored in the freezer. The sulfenic acid on heating for 1 hr at 100° in 0.1 *N* hydrochloric acid was converted quantitatively into 1-methyl-4-thiouracil (II_m) and 1-methyluracil (IV_m) in 1:2 molar ratio. A control experiment indicated that II_m is not converted into IV_m under identical conditions. The form in which two-thirds of the sulfur is lost has not been investigated, and we are unable to present a reaction mechanism explaining the stoichiometry of the products formed.

The silver salt procedure was not successful in the case of the ribosyl compound (I_r); presumably because of the presence of the ribosyl group, the silver salt of the thione II_r did not precipitate. However, we were able to separate the thione (II_r) and the sulfenic acid (III_r) by chromatography on a cation-exchange column. The stoichiometry of the products formed is very similar to that of the methyl analog (Figure 2). From the similarity of the uv absorption spectra of III_m and III_r,

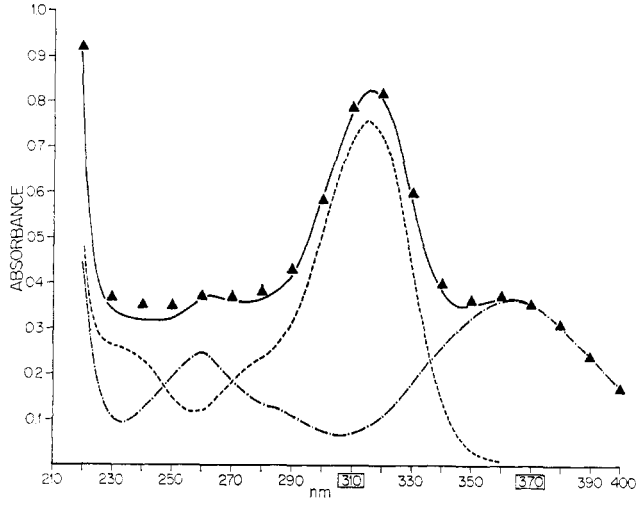


Figure 4. Spectral correlation among alkaline hydrolysis products of bis(1-methyl-4-thiouracil) disulfide (I_m), 1-methyl-4-thiouracil (II_m), and 1-methyluracil-4-sulfenic acid (III_m). Spectra at pH 12 of I_m (———), II_m (– – – –), and III_m (–·–·–·–), and addition spectrum at pH 12 of II_m and III_m (▲). The spectra of II_m and III_m are normalized with respect to A_{310} and A_{370}, respectively.

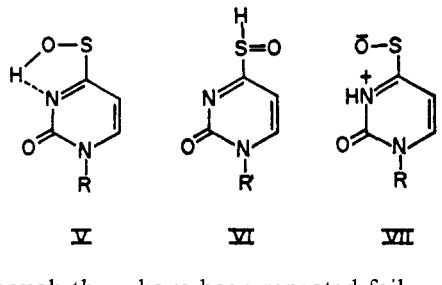
we identify III_r as 1-β-D-ribofuranosyluracil-4-sulfenic acid (Figure 3). The sulfenic acid (III_m), isolated by all four methods, had the same uv absorption spectra.

If 1-methyl-4-thiouracil and 1-methyluracil-4-sulfenic acid are the sole products of alkaline hydrolysis of the methyl analog, then the uv absorption spectrum of the disulfide at pH 12 should be the sum of the spectra of the two at pH 12, assuming complete hydrolysis of the disulfide. That this is so is indicated in Figure 4; the A_{360} of the uv absorption spectrum of the disulfide at pH 12 is solely due to the sulfenic acid (III_m). Hence, it is possible to calculate the ϵ_{360} at pH 12 of the sulfenic acid from the uv absorption spectrum of the disulfide at pH 12. This was found to be 10,660, a value in agreement with the ϵ_{360} (= 10,990) at pH 12 determined on the basis of the silver salt.

Although no effect of oxygen was reported by Lipsett[8] on the alkaline hydrolysis of the disulfide I_r, we observed a small peak, identified spectrophotometrically as due to uridine, in cation-exchange chromatography (Figure 2a). Uridine had been identified earlier by Uziel.[11] A similar peak, due to 1-methyluracil, appeared in the case of the methyl analog (Figure 2b). When the alkaline hydrolysis was carried out in the presence of nitrogen, analysis of the hydrolysate showed that the formation of IV_m was reduced by one-third as compared with the results of a similar experiment carried out without any special precaution to exclude the presence of air. Chromatographic analyses indicated that neither IV_m nor IV_r was present in the original disulfides as an impurity. Although the effect of oxygen is rather small (formation of the uracil derivatives is about 3–8% of the total products), they are nonetheless detectable by cation exchange and paper chromatography. They are probably formed as a result of the direct action of oxygen on the disulfide in alkali.

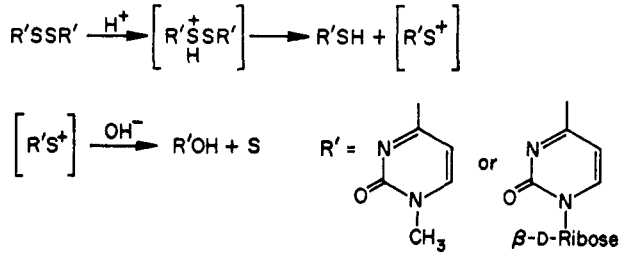
To our knowledge, this is the first time that a pyrimidine sulfenic acid has been characterized. The sulfenic acids themselves are rather rare; only three are known and all are mono- or diacids of anthraquinone.[14]

Recently the existence of an aliphatic sulfenic acid, namely *t*-butylsulfenic acid (*t*-butanesulfenic acid), has been demonstrated.[15] Following the first successful synthesis of 1-anthraquinonesulfenic acid by Fries, repeated attempts to prepare other sulfenic acid series were unsuccessful. This led to the suggestion that hydrogen bonding to the nearby quinone oxygen atom in 1-anthraquinonesulfenic acid may lead to the stabilization of the molecule.[16] A similar possibility of hydrogen bonding to the neighboring N_1 of the pyrimidine ring of these compounds also exists (V). In fact, three different tautomeric forms, V, VI, and VII, can be written for these sulfenic acids. Structural studies on these compounds are currently in progress in our laboratory.



V VI VII

Although there have been repeated failures to isolate free sulfenic acids, they have often been assumed to be formed as an intermediate in various reactions, including those involving the hydrolytic scission of the disulfide bond.[10] However, this is the first time that it has been possible to prove the formation of sulfenic acid as a result of such cleavage in alkali. This remarkably fast reaction is probably initiated by the direct nucleophilic attack of the hydroxide ion on one of the sulfur atoms, as postulated by Danehy and Hunter[17] in the case of aliphatic disulfides. The absence of an easily ionizable hydrogen atom α or β to the sulfur atom and the formation of a good leaving group in the form of thiolate ion are important factors contributing to the high reactivity of the disulfide I_m and I_r in alkali. On the other hand, the action of acid on these disulfides follows a different course. There was no evidence for the formation of sulfenic acid as an intermediate when the reaction was followed spectrophotometrically in a cuvette. In this case we propose the following mechanism, which is in complete agreement with the stoichiometry of this reaction.



We may thus conclude that bis(4-thiouridine) disulfide and its methyl analog hydrolyze in alkali predominantly (more than 90%) to the corresponding thione and sulfenic acid in a 1:1 molar ratio. We have been unable to find any evidence for the formation of a single product like the T from bis(4-thiouridine) disulfide on alkali treatment, as reported previously by Lipsett.[8] In conflict with this earlier observation, neither I_m nor I_r showed any appreciable fluorescence in alkali. In agreement with the observation of Ulbricht, *et al.*,[18] the compound III_r showed the positive Cotton effect expected of all pyrimidine β-nucleosides.

Experimental Section

Melting points were observed in a Thomas-Hoover apparatus and are uncorrected. Thin layer chromatography was carried out using E. Merck tlc plates and cellulose F. Descending paper chromatography was carried out using Whatman No. 3MM paper and the following solvent systems: A, 1-butanol–water 86:14 (v/v); B, 2-propanol–water–ammonia 170:30:2.6 (v/v/v). Bis-(1-β-D-ribosyl-4-thiouracil) disulfide was purchased from Cyclo Chemical Corp., Los Angeles, Calif., and was purified by crystallizing twice from water. 1-Methyluracil[19] and 1-methyl-4-thiouracil[12] were synthesized as described in the literature. These two compounds have been identified in all cases by comparing their uv absorption spectra with those of synthetic materials. The sulfenic acid (III_m) was identified by comparing its uv absorption spectra with those of the material liberated from the silver salt as described below.

Bis(1-methyl-4-thiouracil) Disulfide (I_m). A fine suspension of 1-methyl-4-thiouracil, 1.14 g (8 mmol) in 250 ml of 0.025 *M* phosphate buffer, pH 6.8, was placed in a beaker cooled in ice water. The mixture was treated dropwise with 7.6 ml of 1 *N* iodine solution with vigorous stirring. The pH was maintained at 6.8 by addition of 1 *N* potassium carbonate solution during the course of the reaction. After the addition of iodine solution, the reaction mixture was allowed to stand for 2 hr in ice water and then was filtered. The crude material weighted about 1 g. It was recrystallized from 450 ml of oxygen-free water at 50°. The yield of chromatographically homogeneous (tlc, solvent A, R_f 0.41) bis(1-methyl-4-thiouracil) disulfide was 683 mg (60% of theory); mp 227° dec. Spectral characteristics: pH 5.6, maxima 257 and 307.5 mm, minima 236 and 277 nm, ϵ_{260} 11,820 and ϵ_{307} 18,120.

Anal.[20] Calcd for $C_{10}H_{10}N_4O_2S_2$: C, 42.54; H, 3.57; N, 19.85; S, 22.71. Found: C, 42.57; H, 3.55; N, 20.04; S, 22.90.

Isolation of 1-Methyluracil-4-sulfenic Acid (III_m). a. Chemical Method. The disulfide (I_m), 282 mg (1 mmol), was stirred magnetically with 40 ml of 0.1 *N* sodium hydroxide solution for 1 hr at room temperature; the material gradually dissolved, forming a clear yellow solution. A solution of silver acetate, 334 mg (2 mmol) in 5 *N* ammonia, was added, whereupon the thione II_m came down immediately as a gelatinous precipitate. The reaction mixture was stirred for 30 min and then was centrifuged. The yellow supernatant was decanted, and the residue was washed with 25 ml of 5 *N* ammonia and separated by centrifugation. The combined supernatant was evaporated at room temperature in a rotary evaporator until the silver salt began to precipitate out. The evaporation was stopped, and the flask was cooled in ice for 1 hr. The yellowish green silver salt was removed, washed three times with water by centrifugation, and dried *in vacuo* (0.1 mm) at room temperature over phosphorus pentoxide. Exposure to light should be avoided as much as possible during the preparation of the silver salt. Yield of analytically pure silver 1-methyluracil-4-sulfenate, 80 mg (30% of theory). Spectral characteristics of the sulfenic acid liberated from the silver salt: pH 4.6, maxima 270 and 368 nm, minima 238.5 and 295 nm, ϵ_{260} 2610; pH 12, maxima 260 and 364 nm, minima 230 and 307.5 nm, ϵ_{360} 10,990; pK = 6.3, <2 dec.

(14) (a) K. Fries, *Ber.*, **45**, 2965 (1912); (b) T. C. Bruice and P. T. Markiw, *J. Am. Chem. Soc.*, **79**, 3150 (1957); (c) W. Jenny, *Helv. Chim. Acta*, **41**, 317 (1958).

(15) J. R. Shelton and K. E. Davis, *J. Am. Chem. Soc.*, **89**, 718 (1967).

(16) (a) See Kharasch, *et al.*, ref 10; (b) T. C. Bruice and A. B. Sayigh, *J. Am. Chem. Soc.*, **81**, 3416 (1959).

(17) J. P. Danehy and W. E. Hunter, Abstracts, 151st National Meeting of the American Chemical Society, Pittsburgh, Pa., March 22–27, 1966, ORGN-42.

(18) T. R. Emerson, R. J. Swan, and T. L. V. Ulbricht, *Biochemistry*, **6**, 843 (1967).

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

(20) Analyses by Galbraith Laboratories, Knoxville, Tenn.

Anal.[21] Calcd for $C_5H_5N_2O_2SAg$: C, 22.66; H, 1.9; N, 10.57; S, 12.10; Ag, 40.70. Found: C, 22.64; H, 1.96; N, 10.59; S, 11.80; Ag, 40.75.

The free acid can be regenerated from the silver salt by carefully acidifying with dilute hydrochloric acid to pH 2 and filtering off the silver chloride formed. The acid may also be regenerated by passing the ammoniacal solution of the silver salt through a column of Dowex 50 (Na^+) and washing the column with 5% ammonia. The combined eluate is evaporated to dryness in a rotary evaporator at 30°. The liberated sulfenic acid was found to be chromatographically pure when analyzed on the cation-exchange column. The purity of the silver salt can be checked by its complete solubility in dilute ammonia.

b. By Paper Chromatography. The disulfide (I_m), 6.41 mg, was treated with 1 ml of 0.1 *N* aqueous sodium hydroxide for 1 hr. An aliquot (0.1 ml) was chromatographed on paper in solvent system B. Three uv-absorbing bands (R_f's 0.22, 0.36, 0.52) were observed and identified as the sulfenic acid (III_m), 1-methyl-4-thiouracil (II_m), and 1-methyluracil (IV_m), respectively. The disulfide (2.27 μmol) yielded sulfenic acid (1.8 μmol), 1-methyl-4-thiouracil (2.1 μmol), and 1-methyluracil (0.02 μmol). The estimates were made spectrophotometrically using the following molar extinction coefficients: 1-methyl-4-thiouracil, pH 5.6, ϵ_{260} 2580, ϵ_{267} 1570, ϵ_{335} 20,190, ϵ_{350} 11,050; 1-methyluracil, pH 5.6, ϵ_{267} 9480, ϵ_{260} 8650. The sulfenic acid was reduced with dithiothreitol to 1-methyl-4-thiouracil and estimated spectrophotometrically. Dithiothreitol, in both reduced and oxidized forms, does not have appreciable absorption at 350 nm (pH 5.6).

c. Column Chromatography on Dowex 50 (H^+). The disulfide I_m, 7.6 mg (27 μmol), was treated with 1 ml of 0.1 *N* sodium hydroxide and allowed to stand at room temperature for 1 hr. An aliquot of 0.1 ml (2.7 μmol) was passed through a Dowex 50 (H^+) column (4 × 0.4 cm diameter) in a cold room at 2°. The column was washed with water until the A_{335} was negligible. The combined eluate contained 2.9 μmol of 1-methyl-4-thiouracil (neglecting the small amount of 1-methyluracil formed). The column was then eluted with 1 *N* ammonia until the uv absorbance in the eluate was negligible. The sulfenic acid thus obtained was reduced with dithiothreitol and estimated as 1-methyl-4-thiouracil (1.94 μmol). This accounts for about 90% of the total hydrolysis products of the disulfide. The relatively high yield of 1-methyl-4-thiouracil was probably due to the action of the highly acidic column on the sulfenic acid.

d. Chromatography on Cation-Exchange Column. Chromatography of the alkali-treated disulfide (I_m) on the cation-exchange column indicates the formation of the thione and sulfenic acid in approximately 1:1 molar ratio (Figure 2b). It also indicates the formation of a small amount of 1-methyluracil, identified spectrophotometrically. The recovery from the column was almost 100%. The alkaline hydrolysate of the disulfide (I_r) was analyzed in a like manner with similar results (Figure 2a).

Action of Acid on 1-Methyluracil-4-sulfenic Acid (III_m). The sulfenic acid in salt form, as prepared by the procedure described above, was dissolved in 0.1 *N* hydrochloric acid and the reaction mixture was heated for 1 hr at 100°. Only two products were formed, 1-methyl-4-thiouracil and 1-methyluracil, in 1:2 molar ratio. The conversion was almost quantitative. The form in which sulfur was lost was not investigated. The estimates were made spectrophotometrically using the molar extinction coefficients given before.

Action of Acid on Bis(1-methyl-4-thiouracil) Disulfide (I_m). The disulfide (I_m), 7.95 mg (28.2 μmol), was stirred with 5 ml of 1 *N* hydrochloric acid for 24 hr at room temperature. The precipitate was filtered off and washed with water until the filtrate showed negligible absorption at 335 nm. The residue on the filter paper was dried in a desiccator over calcium chloride and triturated with mercury. The mercury thus obtained gave a positive test for sulfur in the iodine–azide reaction.[22] The filtrate was made up to 1000 ml with water. Chromatographic (tlc, solvent B) and spectrophotometric examination of an aliquot showed the presence of only two uv-absorbing components, 1-methyl-4-thiouracil and 1-methyluracil, the amounts of which were calculated from the A_{335} and A_{267}, respectively, using the extinction coefficients reported before. The yield was found to be 26.8 μmol in each case.

Action of Acid on Bis(1-β-D-ribosyl-4-thiouracil) Disulfide (I_m). The experiment was carried out as described before for I_m. Starting from the disulfide (I_r) (11.85 μmol), 4-thiouridine (9 μmol) and uridine (11.1 μmol) were obtained. The following molar extinction coefficients were used for spectrophotometric estimation: II_r, pH 6.5, ϵ_{331} 21,000,[23] ϵ_{260} 3268; IV_r, pH6.5, ϵ_{260} 9950.

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(21) C, H, and N analyses by Galbraith Laboratories. Ag and S analyses by Dr. G. Goldstein (Analytical Chemistry, Oak Ridge National Laboratory), by flame emission spectrophotometry and by combustion to SO_2 followed by titration with iodine, respectively.

(22) F. Feigel, "Spot Tests in Organic Analysis," 6th ed, Elsevier Publishing Co., Amsterdam, 1960, p 508.

(23) Data from N. K. Kochetkov, E. I. Budowsky, V. N. Shibaev, and M. A. Grachev, *Biochim. Biophys. Acta*, **59**, 749 (1962).