Spectrophotometric and Chemical Studies of 5-Mercaptouracil, 5-Mercaptodeoxyuridine, and Their *S*-Substituted Derivatives

By THOMAS J. BARDOS and THOMAS I. KALMAN

5-Mercaptouracil (I) and 5-mercaptodeoxyuridine (II), structural analogs of thymine and thymidine, respectively, are effective growth inhibitors in various biological systems and under study as potential antineoplastic and antiviral agents. Both compounds were found to be extremely unstable in aqueous solutions as they undergo rapid autoxidation to the corresponding disulfides. Determination of the ultraviolet spectra and pKa values of the thiols was possible only by special techniques, using dithiothreitol (DTT) as a "protecting" agent. Both I and II have very low pKa's, and their anionic forms show characteristic absorption maxima in the 330-mμ region. These results are discussed in comparison with the spectra and ionization equilibria of related compounds including some new *S*-substituted derivatives of I and II. DTT was found to be also a uniquely suitable reagent for the preparation of pure I and II by stoichiometric reduction of the corresponding disulfides. A special technique was developed for the quantitative determination of the free thiols.

A STRUCTURAL analog of thymine (1), 5-mercaptouracil (I), has been under biological and preclinical investigation during the last few years as an experimental antineoplastic agent (2). Its 2′-deoxyriboside (II) was recently synthesized enzymatically (3) and chemically (4), and it was shown to have high inhibitory activity in various biological test systems (3). Both compounds, however, undergo rapid autoxidation in aqueous solution, and this property has presented a major problem in their preparation and biological testing. In fact, the autoxidation of I in dilute aqueous buffer solutions proceeds so rapidly that the ultraviolet spectrum originally reported (1) for this compound was actually that of the corresponding disulfide (III). In view of the continuing interest in the biological activities and possible chemotherapeutic applications of these compounds, a careful study was undertaken to determine their correct spectra and dissociation constants, and to establish the conditions of their stability to autoxidation.[1] This study was greatly aided by two recent developments, *i.e.*, (*a*) the availability of Cleland's reagent (5), dithiothreitol (DTT), and (*b*) the excellent method of Klotz and Carver (6) for the determination of sulfhydryl groups.

EXPERIMENTAL

Materials.—5-Mercaptouracil (I) and 5-uracilyl-disulfide (III) were prepared by previously described methods (1, 7). Both compounds were purified by repeated crystallizations from water, and the free thiol content of each preparation was determined by the titrimetric method described below. 5-Mercaptodeoxyuridine disulfide[2] (IV) was a sample of the enzymic preparation (3). The synthesis of 5-methylmercaptouracil (V) and 5-acetylmercaptouracil (VI) will be reported.[3]

Reagents.—DTT (dithiothreitol, Cleland's reagent)[4] and glutathione (reduced)[5] were used as indicated. Mersalyl acid (*o*-{[3-(hydroxymercury)-2-methoxypropyl]carbamyl}-phenoxyacetic acid)[6] was dissolved at 10^{-3} *M* concentration in 0.2 *M* phosphate buffer, pH 6.3, containing 8×10^{-3} *M* NaCl. Indicator dye, pyridine-2-azo-*p*-dimethylaniline,[7] was dissolved in absolute EtOH and used at 10^{-3} *M* concentration. Both the mercurial and the dye solutions were stored in the refrigerator and freshly prepared after 3 days.

Buffers.—The following buffers were used: between pH 3 and 5, 0.025 *M* acetate buffers; between pH 5 and 8, 0.015 *M* phosphate buffers; between pH 8 and 12, Sorensen's glycine buffers. Below 3 and above 12, dilute HCl and NaOH solutions were used, respectively.

Ultraviolet Absorption and pKa Determinations.—Stock solutions of compounds having sulfhydryl groups (I, II) were prepared in the following manner to prevent their rapid air oxidation. I was dissolved in 5×10^{-4} *N* HCl (0.250 mg./ml.) in the presence of 1.0 mg./ml. of DTT. The disulfide (IV) was reduced to II at 0.500 mg./ml. concentration (pH 7–9) by the addition of DTT (1 mg./ml.), then the pH was adjusted to 3–4 with HCl. Stock solutions of the other compounds were freshly prepared in distilled water, except for the disulfide (III) which was dissolved in 0.01 *N* NaOH.

A Leeds and Northrup pH indicator was used for the pH measurements.

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[1] A study of the kinetics of autoxidation and its dependence on several variables will be presented in a subsequent publication.

[2] The authors are grateful to Mrs. K. Baranski for a sample of this compound.

[3] The authors thank Mr. M. Kotick for the preparation of these compounds.

[4] Purchased from Calbiochem, Inc., Los Angeles, Calif.

[5] Purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

[6] Purchased from Winthrop Laboratories, New York, N. Y.

[7] Purchased from Sigma Chemical Co., St. Louis, Mo.

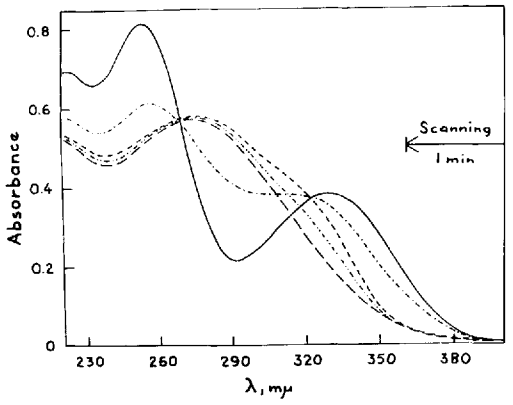


Fig. 1.—Change of ultraviolet absorption spectrum of 8.68×10^{-5} *M* solution of 5-mercaptouracil (I) at pH 7.4 (in 0.05 *M* phosphate buffer). Time lapse: from the dilution of the stock solution with the buffer until the beginning of the recording. Key: – · – · – ·, 2 min.; – – – –, 8 min.; — · · — · ·, 16 min.; — — —, 60 min.; ——, stabilized control (contains DTT). The scanning speed is indicated in the graph.

The ultraviolet absorption curves were obtained on a Beckman DB recording spectrophotometer, but a Gilford model 2000 photometer was used for the accurate determination of the $\lambda_{max.}$, $\lambda_{min.}$, ϵ, and pKa values. Aliquots of stock solutions were pipeted and diluted with buffers directly in the cells (10 mm. light path) to a final concentration of 12.5 or 25 mcg./ml.

Absorbance values measured at fixed wavelengths when plotted against pH gave the pKa values as midpoints of the curves. These pKa values were compared to the calculated ones obtained by the following formula (after Gage) (8):

if $A(a) < A(b)$:

$$\text{pKa} = \text{pHx} + \log \frac{A(b) - A(x)}{A(x) - A(a)}$$

if $A(a) > A(b)$:

$$\text{pKa} = \text{pHx} - \log \frac{A(a) - A(x)}{A(x) - A(b)}$$

where $A(a)$ is the absorbance value of the acidic (unionized) form, $A(b)$ is the value of the basic (ionized) species, and $A(x)$ is the value measured at a pH close to the apparent pKa. The pKa values obtained from the curves and by calculation agreed within 0.1 pH unit.

Reduction of the Disulfide with DTT.—The disulfide (III) (recrystallized from H_2O), 65 mg., and DTT, 130 mg., were dissolved in 30 ml. of 10^{-2} *N* NaOH and brought to pH 8. After a few minutes of stirring under N_2, the solution was acidified with HCl to pH 1.6, then 10 ml. of absolute ethanol was added, and the product was allowed to crystallize at −5 to −10°. The crystals were washed with 1:3 ethanol–water mixture (adjusted to pH 2 with hydrochloric acid), followed by absolute ethanol and, finally, by a few drops of acetone, then they were dried *in vacuo* at 110°. The infrared spectrum of the product showed a sharp peak at 2550 cm.$^{-1}$, characteristic for the S-H bond, and quantitative sulfhydryl group determination by the method described below proved that the reduction of the disulfide to the free thiol was complete.

Sulfhydryl-Group Determination.—Essentially, the method of Klotz and Carver (6) was employed with some modifications. An excess of the mercurial was immediately added in order to effect rapid and quantitative combination with the very unstable mercapto compounds. This was followed by the addition of a known excess of glutathione which was then titrated with additional portions of the mercurial in the described manner. Weighed samples were dissolved in 10^{-2} *M* HCl under N_2, from which aliquots were pipeted into a 18 × 150 mm. test tube. After 5 ml. of 10^{-3} *M* mersalyl acid was added and mixed, 0.1 ml. of 5×10^{-2} *M* glutathione and 0.6 ml. of indicator were added. The mixture was titrated with 10^{-3} *M* mersalyl acid, the color change was followed with a Bausch & Lomb photometer at 550 mμ, and the end point of the titration was determined as described (6).

RESULTS AND DISCUSSION

Figure 1 shows the rapid change of the absorption spectrum of I in a dilute aqueous solution (8.68×10^{-5} *M*) buffered at a neutral pH. Even when the spectrum was taken immediately after dissolving the compound in the buffer, it already showed a vast shift in comparison to the spectrum of a "stabilized" solution of I (*i.e.*, a freshly prepared solution containing DTT, see below) and within 16 min. became nearly identical with that of the disulfide (III). The same process was observed to occur at a comparable rate under alkaline conditions, while acid pH decreased the rate of oxidation, and the compound appeared to be relatively stable at pH < 3.7. Thus, solutions of I in 5×10^{-4} *N* hydrochloric acid did not show appreciable change within several hours and could be used as stock solutions in the spectrophotometric studies. (See *Experimental.*)

DTT was found to be an effective protecting

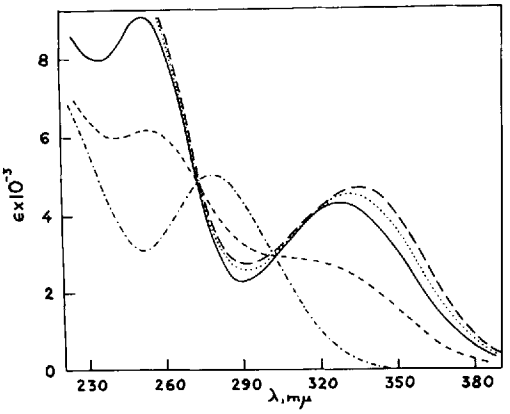


Fig. 2.—Ultraviolet absorption spectra of 5-mercaptouracil (I) stabilized by the addition of DTT (see text) at various pH values. Key: – · – ·–, pH = 2.0; – – – – –, pH = 5.3; ——, pH = 7.7; . . . . . , pH = 10.6; — — —, pH = 11.8.

TABLE I.—SPECTROPHOTOMETRICALLY DETERMINED, APPARENT pKa VALUES[a]

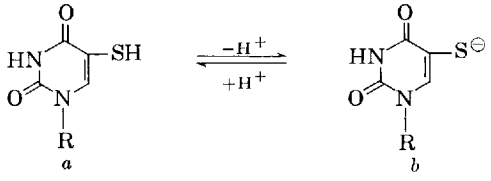
Compd.	pKa_1	pKa_2	pKa_3
I	5.3	10.6	>13
II	5.0	10.5	..
III	8.0	>13	..
IV	8.1	>13	..
V	8.5	>13	..
VI	>8[b]	...	..

[a] Limits of accuracy: ±0.05 pH units. [b] Hydrolysis occurs at pH > 8.

agent by virtue of its low redox-potential (5). This compound not only prevented the air oxidation of I, but it was also capable of reducing, rapidly and quantitatively, the disulfides (III and IV) to the corresponding thiols (I and II), respectively. Thus, in the presence of excess DTT, it was possible to determine the ultraviolet absorption spectra of the free thiols at various pH values and to estimate their dissociation constants by spectrophotometric methods. (See *Experimental.*) The ultraviolet absorption of DTT itself interfered with the spectra of I and II only at pH > 9, and then only in the range below 260 mμ. The spectra of I at various pH values in the presence of DTT are shown in Fig. 2; in the pH 2-7 range, two isosbestic points appear, at 273 and 302 mμ.

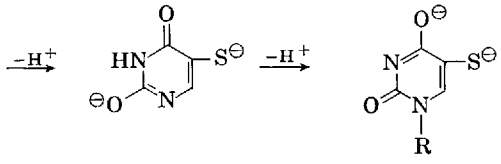
The apparent pKa values of I and its derivatives are given in Table I. The pKa_1 values corresponding to the first acid dissociation equilibria of I (5.3) and II (5.0), respectively, are considerably lower than the pKa of thiophenol (7.76) (9). 5-Nitrouracil has a similarly low pKa_1 as I, and in the case of the former this is due to the powerful electron-attracting inductive and conjugative effects of the nitro group both of which promote the ionization of the N-1 hydrogen (10). The relatively small, electron-releasing conjugative effect of the divalent sulfur (11) is essentially inoperative from the 5-position of the uracil nucleus (*meta* to both ring-nitrogens) (12) and the inductive (−I) effect of the C → S bond prevails. This would cause only a moderate increase in the acidity of the N-1, or N-3 hydrogen, corresponding to lowering of the pKa_1 value only by about 1 unit, as seen by comparison of the pKa_1 values (8.0–8.5) of the *S*-substituted derivatives, III, IV, V, and VI (Table I), with that of uracil (9.45) (10). Therefore, the much lower pKa_1 values of I and II clearly correspond to the dissociation of the sulfhydryl group, and the high acidity of this group must be attributed to the electron-attracting effect of the uracil nucleus. This effect seems to be slightly larger in the case of II. (Scheme I.)

Table II summarizes the spectrophotometric data of compounds I–V. The free thiols (I and II) are readily distinguished from their disulfides and other *S*-substituted derivatives by their characteristically large bathochromic shifts in neutral or basic solutions (*i.e.*, in their ionized forms). Compound II shows slightly higher wavelength and greater intensity absorption than I in both the "neutral" (*a*) (pH 2) and "mono-anionic" (*b*) (pH 7.7) form.



I, R = H
II, R = deoxyribosyl

Scheme I

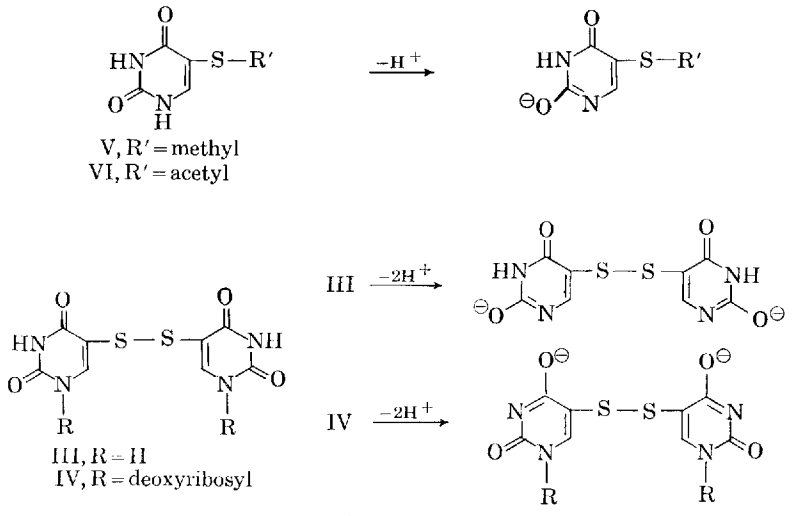


Scheme II

TABLE II.—ULTRAVIOLET ABSORPTION DATA

Compd.	pH	$\lambda_{max.}$[a]	$\epsilon \times 10^{-3}$[b]	$\lambda_{min.}$[a]	$\epsilon \times 10^{-3}$[b]
I	2.0	280	5.0	253.5	2.9
	7.7	253.5	9.4	239	8.3
		329.5	4.5	291	2.1
	11.8	(250–260)[c]	...[c]	...[c]	...[c]
		336	4.9	295	2.5
II	2.0	284	6.4	254	3.0
	7.7	253.5	9.4	242.5	8.4
		334	5.0	292	2.2
	11.8	(250–260)[c]	...[c]	...[c]	...[c]
		319.5	4.5	291	3.5
III	2.0	272	14.9	234	11.1
	10.6	292	18.5	241	9.0
IV	2.0	270.5	16.9	226	10.0
	7.0	270	16.5	231	10.7
	11.8	268	13.4	254	12.8
V	2.0	227 (infl.)	6.3		
		270 (infl.)	4.7		
	10.6	233.5	7.3	263	3.6
		294	7.7		
VI	2.0	269.5	7.4	242	3.8

[a] Absorption maxima and minima, wavelength in mμ. [b] Molar absorptivities at given wavelength. [c] Presence of DTT prevents correct reading below 260 mμ.



Scheme III

Ionization of the second proton (pH 11.8 in Table II) increases both the wavelength and the intensity of the absorption band in the 330 mμ region in the case of I, and decreases both values in the case of II; however, the dianions of I and II have of necessity different structures. (Scheme II.)

The *S*-substituted derivatives, III, V, and VI, show much smaller acid-base shifts in their ultraviolet spectra than the free thiols and rather resemble thymine in their spectral behavior (10), indicating that their first ionization involves the N-1 hydrogen. The disulfide (IV), in which both the sulfhydryl group and the N-1 position are blocked, shows no acid-base shift, being in this respect similar to 1-methyl-uracil (10) or thymidine (13). (Scheme III.)

Since the ionizations of the symmetrical disulfides (III and IV) probably proceed in two steps, *i.e.*, giving rise to intermediate structures in which only one of the pyrimidines is ionized, the spectra of these compounds do not give real "isosbestic points" (Fig. 3), and their pKa_1 values actually represent averages of *two* pKa's which are very close and cannot be clearly distinguished from each other.

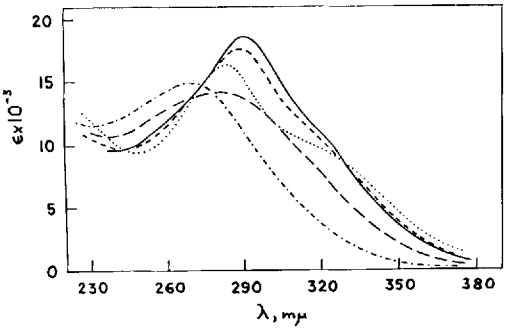


Fig. 3.—Ultraviolet absorption spectra of 5-uracilyl-disulfide (III) at various pH values. Key: -·-·-·, pH = 2.0; ———, pH = 7.7; ——, pH = 10.6; - - - - - -, pH = 13.0; . . . . . , pH = 14.0.

DTT was found to be a useful reducing agent for the preparative conversion of the disulfide (III) to the free thiol (I). (See *Experimental.*) This conversion was accomplished previously by a much less convenient procedure (using zinc and sulfuric acid) (1, 7), which cannot be applied to the reduction of the deoxyriboside (IV).

For the determination of the free thiol content of various preparations, the method of Klotz and Carver (6) was employed in a somewhat simplified form, without the elaborate precautions recommended for the exclusion of air. This, however, gave variable results in the case of 5-mercaptouracil since a considerable portion of the sample oxidized during the titration procedure. Glutathione, on the other hand, was found to be quite stable under the same conditions. Taking advantage of this fact, an "indirect method" was developed. (See *Experimental.*) The results[8] are given in Table III.

TABLE III.—SULFHYDRYL GROUP ANALYSIS

Compd.	Free SH/S × 100[a] Direct[b]	Indirect[c]
I[d]	55–85	98
I[e]	. . .	98–100
III	<2	<2
Glutathione	100	100
DTT	. . .	98–100

[a] Per cent of total sulfur present in the form of free sulfhydryl-group. [b] As determined by direct titration with mersalyl acid (6). [c] As determined by the modified, indirect procedure. (See *Experimental.*) [d] Recrystallized and stored in the desiccator for a period of over 12 months. [e] Prepared from III by reduction with DTT. (See *Experimental.*)

The results indicate that I is quite stable for a period of at least 1 year when stored in solid dry state. It is also seen from Table III that the reduction of the disulfide (III) to the free thiol (I) by DTT has proceeded in a quantitative manner.

[8] The authors acknowledge the technical assistance of Mr. Peter Forgach in these determinations.

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Absorption, Distribution, and Elimination of a Long-Acting Vitamin B_{12} Preparation

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The prolonged effect of various long-acting vitamin B_{12} preparations has been examined on rabbits regarding absorption, distribution, and elimination after intramuscular injection.

RECENT investigations (13) have shown that patients with pernicious anemia have considerably greater requirements for vitamin B_{12} than have previously been assumed. More recently, there has been considerable interest in preparations that can meet the requirements more adequately and in a more satisfactory way than the aqueous solutions of vitamin B_{12} used previously. The authors have investigated the characteristics of various preparations made on the basis of various principles. A preparation containing cyanocobalamin–tannin complex suspended in aluminum monostearate oil gel[1] was studied in detail.

The clinical value of this preparation is reflected in papers by Bastrup-Madsen *et al.* (4, 5), Schwartz *et al.* (19, 20), Meulengracht (14, 15), an editorial (8), Nielsen and Vedsø (16), Schrumpf (18), and Gough *et al.* (10).

EXPERIMENTAL

Materials and Methods

Preparations A–G were investigated (Table I). After intramuscular injection (i.m.) in rabbits, the following were investigated: (*a*) liberation of vitamin $B_{12}{}^{60}Co$ from the site of injection; (*b*) absorption of vitamin $B_{12}{}^{60}Co$ by the liver; and (*c*) excretion of vitamin $B_{12}{}^{60}Co$ in urine and feces.

For preparations F and G the investigations were supplemented by radioactivity counts and microbiological determinations of vitamin B_{12} in liver, kidney, and femoral muscle about 3 months after the start of trial.

Preparations

A number of preparations were made using vitamin B_{12} labeled with ^{60}Co. Their composition is shown in Table I. The products were prepared according to methods described in a British patent (6).

The distribution and excretion of vitamin B_{12} after parenteral administration of preparation B and of aqueous solutions of cyanocobalamin were investigated in rats and in healthy subjects by Davis *et al.* (7), Thompson and Hecht (22), Astudillo *et al.* (3), and Glass *et al.* (9), but no records have been found of investigations on patients with pernicious anemia. The authors have not investigated any preparations of vitamin B_{12} suspended in oil or vitamin B_{12} suspended in 2% monostearate oil gel; the latter was described by Arnold *et al.* (1, 2), Sobell *et al.* (21), and Heinrich and Gabbe (11). It does not appear to possess any retarded action of interest for clinical use.

Preparations F and G correspond to a marketed suspension of cyanocobalamin–tannin complex in aluminum monostearate oil gel,[1] except that labeled vitamin B_{12} (^{60}Co) was used instead of ordinary cyanocobalamin. Spectrophotometric and microbiological checks were made on the preparations. The radiochemical purity of the labeled compounds was confirmed by paper chromatography and subsequent counts and by microbiological determinations of the vitamin B_{12} activity on agar plates with *Lactobacillus leichmannii*, as described by Winsten and Eigen (23), among others.

Animal Material.—Twelve white male rabbits were used. The initial weights were from 2.7 to 3.0 Kg. The rabbits were anesthetized with sodium amobarbital (30 mg./Kg.) supplemented by ether during the radioactivity counts.

Standards and Methods of Measurement.—The radioactivities above the site of injection and above the liver were determined on a scintillation detector shielded by 5 cm. of lead with a 30-mm. round opening upon which the object to be measured was placed. Feces and organs were homogenized before

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[1] Marketed as Betolvex by Dumex Ltd., Copenhagen, Denmark.