

TABLE IV

R	M.p., °C.	Yield, %	Nitrogen, %	
			Calcd.	Found
Methyl	191.5-193.5	50	15.05	14.97
Ethyl	168.5-170.0 <sup>a</sup>	76	13.08	13.06
Propyl	122.5-124.0	79	11.56	11.35
Isopropyl	205-206	82	11.56	11.49
Butyl	106.0-107.5	81	10.36	10.44
Isobutyl <sup>b</sup>	130-131	87	10.36	10.28
sec-Butyl	163.5-164.0	84	10.36	10.28

<sup>a</sup> T. B. Johnson and W. T. Caldwell [THIS JOURNAL, 51, 873 (1929)] reported m.p. 168°. <sup>b</sup> Calcd. for C<sub>13</sub>H<sub>22</sub>N<sub>2</sub>O<sub>4</sub>: mol. wt., 270.4. Found: mol. wt., 275.

(B).—A mixture of 5 g. of this thioracil was refluxed for 4 hr. with 75 g. of concentrated hydriodic acid; the crust of brown solid, which appeared during the initial half-hour of refluxing, subsequently disappeared. From the chilled reaction mixture was obtained 1.8 g. (59% yield) of 5-hydroxy-6-methyl-2-thiouracil as white needles melting above 310°. The product was added to a dilute, neutral, aqueous solution of ferric chloride and produced immediately an intense, blue coloration.

*Anal.* Calcd. for C<sub>6</sub>H<sub>8</sub>N<sub>2</sub>SO<sub>2</sub>: N, 17.71. Found: N, 17.64.

The same product (1.3 g., 55% yield) was obtained after 4 g. of 5-isopropoxy-6-isopropoxymethyl-2-thiouracil had been heated for 4 hr. with 65 g. of hydriodic acid.

*Anal.* Calcd. for C<sub>8</sub>H<sub>8</sub>N<sub>2</sub>SO<sub>2</sub>: C, 37.95; H, 3.80; N, 17.71. Found: C, 37.77; H, 3.86; N, 17.76.

Interaction of 5-Isobutoxy-6-isobutoxymethyluracil with Concentrated Hydriodic Acid. (A).—Three grams of this uracil derivative was heated for 2.5 hr. with 35 g. of concentrated hydriodic acid. After being chilled, the reaction mixture was filtered, and the brown, fluffy solid was recrystallized as white needles from diluted methyl alcohol; m.p. 238-240°, 1.2 g. (55% yield) of 5-isobutoxy-6-methyluracil; the latter did not impart color to a ferric chloride solution.

*Anal.* Calcd. for C<sub>9</sub>H<sub>14</sub>N<sub>2</sub>O<sub>3</sub>: N, 14.14. Found: N, 14.04.

(B).—A mixture of 4.1 g. of the diisobutoxyuracil was refluxed for 4.5 hr. with 60 g. of concentrated hydriodic acid. Filtration of the chilled mixture left a dark-brown solid. The latter was first boiled with diluted alcohol, then was dissolved in concentrated, ammonium hydroxide solution; reprecipitation, by means of concentrated hydrochloric acid, gave 1.1 g. (51% yield) of white, granular crystals of 5-hydroxy-6-methyluracil. The product,<sup>8</sup> when heated began to decompose at 220°, but did not melt below 310°. The material produced an intense blue coloration in a dilute, aqueous solution of ferric chloride.

*Anal.* Calcd. for C<sub>8</sub>H<sub>8</sub>N<sub>2</sub>O<sub>3</sub>: N, 19.71. Found: N, 19.53.

(8) This compound had previously been prepared, by oxidation of 6-methyluracil with potassium permanganate in dilute acetic acid solution, by R. Behr and R. Grünwald (*Ann.*, **323**, 186 (1902)), who reported that it "decomposed from 220° on."

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[CONTRIBUTION FROM THE MARINE BIOLOGICAL LABORATORY]

## The Mechanism of Disulfide Interchange in Acid Solution; Role of Sulfenium Ions

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RECEIVED OCTOBER 10, 1957

Evidence is presented to show that disulfide interchange in strongly acid solution proceeds through sulfenium ions, which arise from the acid hydrolysis of the disulfide bond. A number of substances which can be regarded as precursors of sulfenium ions were found to catalyze this interchange.

### Introduction

Disulfide interchange (RSSR + R'SSR' = 2 RSSR') recently has attracted the attention of a number of investigators, notably Huggins and his collaborators,<sup>2</sup> Calvin,<sup>3</sup> Kauzmann, *et al.*,<sup>4</sup> and Sanger.<sup>5,6</sup> From the results obtained by these workers it has become clear that disulfide interchange in neutral and alkaline solution probably occurs through an anionic attack of the mercaptide ion, RS<sup>-</sup>, on a disulfide bond. Thus, addition of a thiol to a mixture of disulfides, at pH values which allow significant concentrations of RS<sup>-</sup>, results in disulfide interchange. The unexpected finding of Ryle and Sanger<sup>6</sup> that the addition of thiols to disulfide mixtures in *acid solution* has exactly the opposite effect, *i.e.*, suppression of spontaneous

interchange, suggested, of course, that in this medium the mechanism was quite different. It is the purpose of this paper to explain this difference and to propose a mechanism for disulfide interchange in strongly acid solution.

### Methods and Materials

The system used to study disulfide interchange was that employed by Ryle, *et al.*,<sup>6</sup> *i.e.*, the reaction between cystine and bis-(2,4-dinitrophenyl)-cystine (bis-DNPcystine) to form mono-DNPcystine. Since bis-DNPcystine is soluble in ether, but mono-DNPcystine is not, the extent of interchange can be followed very conveniently by measuring the absorption at 355 m $\mu$  in the aqueous phase after extraction with ether. The reaction mixtures were prepared from stock solutions of 2  $\times$  10<sup>-4</sup> M bis-DNPcystine in 12 N HCl and 4  $\times$  10<sup>-4</sup> M cystine in 9 N HCl in such a way that the bis-DNPcystine was mixed with the catalyst to be tested (in aqueous solution except where otherwise indicated) and the interchange then started by adding cystine. The final concentrations of reactants were: bis-DNPcystine, 10<sup>-4</sup> M; cystine, 10<sup>-3</sup> M; and HCl, in the majority of experiments, 9.5 N. All reactions were carried out at 35°. Under these conditions the spontaneous interchange between the two disulfides was sufficiently slow to permit the observation of catalytic effects.

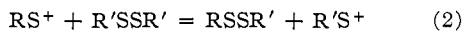
L-Cystine was a commercial sample standardized by optical rotation. The sample of 2,6-dichlorophenolindophenol used (Harleco) had a molar extinction coefficient of 1.88  $\times$

- (1) Established Investigator of the American Heart Association.
- (2) C. Huggins, D. F. Tapley and E. V. Jensen, *Nature*, **167**, 592 (1951).
- (3) M. Calvin in "Glutathione," Academic Press, Inc., New York, N. Y., 1954, p. 9.
- (4) W. Kauzmann and R. G. Douglas, Jr., *Arch. Biochem. and Biophys.*, **65**, 106 (1956).
- (5) F. Sanger, *Nature*, **171**, 1025 (1953).
- (6) A. P. Ryle and F. Sanger, *Biochem. J.*, **60**, 535 (1955).

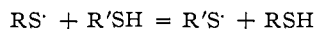
$10^4$  at  $600 \text{ m}\mu$ .<sup>7</sup> Cysteine hydrochloride (Merck) was found to contain 86% -SH by the specific proton displacement method.<sup>8</sup> Bis-DNPcystine was prepared according to Porter and Sanger.<sup>9</sup> *t*-Butyl hydroperoxide was obtained from the Lucidol Corporation. We are deeply indebted to Dr. T. Lavine for highly purified samples of cystine disulfide and cysteine sulfinic acid. Lipoic acid monosulfide (Protogen B) was prepared by oxidation of DL- $\alpha$ -lipoic acid with ammonium persulfate.<sup>10</sup> Trichloromethanesulfonyl chloride was a gift from the Stauffer Chemical Co. and 2,4-dinitrobenzenesulfonyl chloride was a commercial sample.

### Results and Discussion

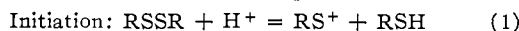
On the assumption that disulfide interchange is preceded by fission of a disulfide bond, it could, in principle, occur by three reaction paths, as



Reactions 1 and 2 would occur as a consequence of heterolytic splitting of a disulfide bond, *i.e.*,  $\text{RSSR} = \text{RS}^+ + \text{RS}^-$ , while reaction 3 would be the result of homolytic dissociation into free radicals, *i.e.*,  $\text{RSSR} = 2 \text{RS}\cdot$ . Neither reaction 1 nor 3 could explain the interchange in strongly acid solution. Reaction path 1 is obviously unlikely on kinetic grounds, in view of the very low concentration of  $\text{RS}^-$ . Reaction path 3 cannot account for the inhibition of interchange by thiols,<sup>6</sup> since the addition of  $\text{R}'\text{SH}$  in this case could merely replace one thiol free radical by another one



The experimental evidence obtained in the present paper supports the suggestion that disulfide interchange in acid solution occurs *via* sulfenium ions, in accord with steps 1 and 2



Addition of an external thiol would, of course, lower the concentration of  $\text{RS}^+$  (equation 3) and hence inhibit interchange.



**Effect of Acid Concentration on the Rate of Disulfide Interchange.**—It was noted already by Ryle and Sanger<sup>6</sup> that the rate of spontaneous interchange is extremely sensitive to acid concentration. It was found to be quite fast in concentrated HCl, but fell off rapidly at concentrations below  $9 \text{ N}$ . This is in agreement with the proposed mechanism. The concentration of  $\text{RS}^+$  ion would decrease rapidly with decreasing acid concentrations, since protons are required to generate the  $\text{RS}^+$ , and at higher  $p\text{H}$  values the rate of reaction with water would also lower their concentration.<sup>11</sup> These considerations also account for the variation in spontaneous interchange found in different series of experiments when the stock solutions are made

(7) R. E. Basford and F. M. Huennekens, *THIS JOURNAL*, **77**, 3873 (1955).

(8) R. Benesch and R. E. Benesch, *Biochim. et Biophys. Acta*, **23**, 643 (1957).

(9) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).

(10) J. A. Bartrop, P. M. Hayes and M. Calvin, *THIS JOURNAL*, **76**, 4365 (1954).

(11) N. Kharasch, C. M. Buess and W. King, *ibid.*, **75**, 6035 (1953).

up in  $12 \text{ N}$  HCl as recommended by Ryle and Sanger.<sup>12</sup>

Prolonged storage of one or both of the disulfides in strong acid leads to greater rates of interchange, presumably because of the increase in concentration of  $\text{RS}^+$  *via* the initiation reaction of the proposed interpretation. Conversely, when fresh solutions of disulfides are used, a definite lag period is observed (*cf.* Fig. 1) which reflects the time required for the formation of a sufficient concentration of  $\text{RS}^+$  ions to initiate interchange. Stock solutions of the two disulfides were therefore stored for only short periods and that of cystine was made up in  $9 \text{ N}$  instead of  $12 \text{ N}$  HCl. In this way low and more reproducible blanks were obtained.

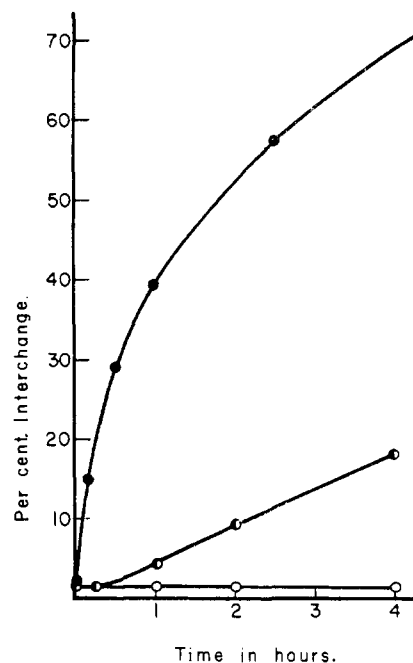


Fig. 1.—Catalysis of interchange by oxidized cysteine: cystine,  $10^{-3} \text{ M}$ ; bis-DNPcystine,  $10^{-4} \text{ M}$ ; HCl,  $10 \text{ M}$ ;  $\bullet-\bullet$ , control;  $\bullet-\bullet$ ,  $7.5 \times 10^{-6} \text{ M}$  "oxidized cysteine" prepared as described in the text;  $\circ-\circ$ ,  $7.5 \times 10^{-6} \text{ M}$  cysteine.

**Catalysis by a Two-electron Oxidation Product of Cysteine.**—Basford and Huennekens<sup>7</sup> found that 2,6-dichlorophenolindophenol reacts with cysteine in an approximately 1:1 molar ratio and therefore concluded that the dye oxidizes cysteine to a compound of the oxidation level of the sulfenic acid (" $\text{RS}^+$ ").

In view of this finding,  $5 \times 10^{-5} \text{ M}$  cysteine was allowed to react with  $5 \times 10^{-5} \text{ M}$  2,6-dichlorophenolindophenol in  $0.033 \text{ M}$  acetate/phosphate buffer,  $p\text{H}$  7.0, until the dye was almost completely bleached (about five minutes). In order to free the cysteine oxidation product from the dye (in both the oxidized and reduced form), the solution was brought to  $p\text{H}$  4 and extracted three times with ether. An aliquot of the aqueous layer was incorporated in the interchange mixture to give a

(12) This variation was evidently also encountered by these workers, as can be seen from a comparison of Figs. 2 and 3 in their paper.<sup>6</sup>

final concentration of  $7.5 \times 10^{-6} M$ . The catalytic effect of this substance is shown in Fig. 1.

Some additional observations concerning this catalysis deserve further comment: (1) In spite of the well-known instability of sulfenic acids,<sup>13</sup> the solution containing the cysteine oxidation product (after removal of the dye) can be stored at pH 4 at 0° for several days without any loss of catalytic activity. It is thus likely that the primary product of the reaction between cysteine and 2,6-dichlorophenolindophenol is a relatively stable compound which can give rise to sulfenium ions in strongly acid solution. (2) When cysteine is oxidized with 2,6-dichlorophenolindophenol at pH 4 (*i.e.*, acid to the *pK* of the dye), instead of at pH 7, the product is completely devoid of catalytic activity. A comparison of the rate and extent of bleaching likewise indicates that the reaction is quite different at the two pH values.

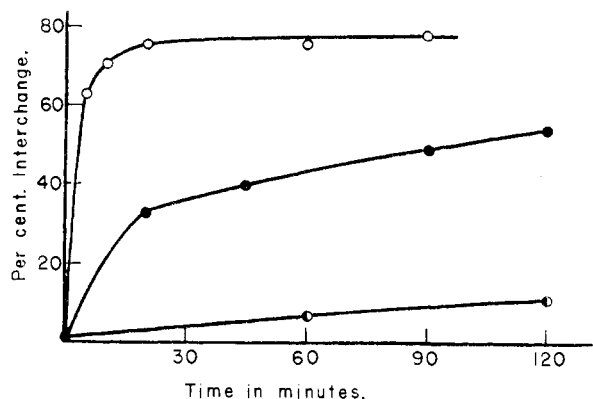
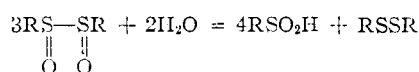
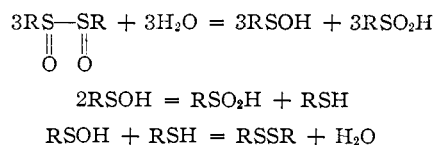


Fig. 2.—Catalysis of interchange by hydrogen peroxide: cystine,  $10^{-3} M$ ; bis-DNPeystine,  $10^{-4} M$ ; HCl, 9.45  $M$ ; ●—●, control; ●—●,  $1.1 \times 10^{-3} M H_2O_2$ ; ○—○,  $1.1 \times 10^{-5} M H_2O_2$ .

**Catalysis by Cystine Disulfoxide.**—It was shown by Toennies and Lavine<sup>14</sup> that cystine disulfoxide<sup>15</sup> breaks down in acid solution according to the equation



They suggested that this breakdown occurs *via* the intermediates



(13) N. Kharasch, S. J. Potempa and H. L. Wehrmeister, *Chem. Revs.*, **39**, 269 (1946).

(14) G. Toennies and T. F. Lavine, *J. Biol. Chem.*, **113**, 571, 585 (1936).

(15) It should be pointed out that although we have retained both the name and the structure of this compound as used by Toennies

and Lavine in their paper, the thioisulfonate structure,  $-\text{R}-\overset{\text{O}}{\parallel}{\text{S}}-\text{S}-\text{R}$  is more likely. For a literature survey of this question cf. Cymerman and Willis, *J. Chem. Soc.*, 1332 (1951).

According to the above scheme, three compounds are formed during the acid decomposition of cystine disulfoxide, *i.e.*, cysteine sulfenic acid, cysteine sulfinic acid and cystine. The data in Table I show that cysteine sulfinic acid has no catalytic activity. The marked catalysis by cystine disulfoxide, shown in the same table, therefore can be ascribed to the sulfenium ions formed from the disulfoxide during its acid decomposition. In contrast to cystine disulfoxide, lipoic acid monosulfoxide was found not to catalyze disulfide interchange. This is to be expected because of its known stability in acid solution.<sup>16</sup>

TABLE I  
EFFECT OF CYSTINE DISULFOXIDE ON DISULFIDE INTERCHANGE

Cystine,  $10^{-3} M$ ; bis-DNPeystine,  $10^{-4} M$ ; HCl, 10  $M$ ; cystine disulfoxide,  $7.5 \times 10^{-6} M$ , added as a solution in 4  $N$  HCl; cysteine sulfinic acid,  $7.5 \times 10^{-6} M$ .

Time, min.	Control	Cysteine sulfinic acid	Interchange, % Cystine disulfoxide
30	26.5	22.5	79.0
60	38.2	34.3	83.0

**Catalysis by Hydroperoxides.**—In strongly acid solution, hydrogen peroxide can give rise to  $\text{OH}^+$  ions<sup>17</sup> by heterolytic fission:  $\text{HOOH} + \text{H}^+ = \text{H}_2\text{O} + \text{OH}^+$ . The addition of  $\text{H}_2\text{O}_2$  to a mixture of disulfides in strongly acid solution could therefore be expected to catalyze interchange, since sulfenium ions might be formed by the reaction

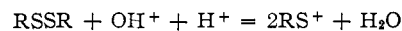


Figure 2 shows that hydrogen peroxide is indeed a powerful catalyst of disulfide interchange, since a marked catalysis is still evident when the hydrogen peroxide concentration is only 1/1000 of the total disulfide concentration.

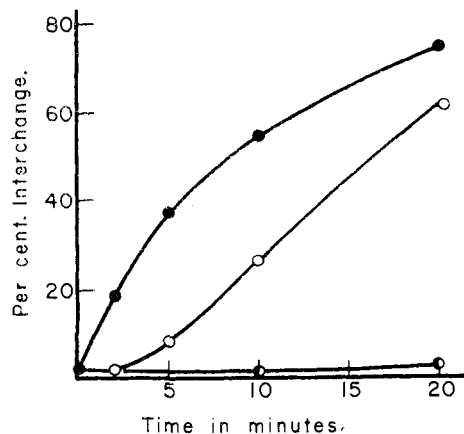


Fig. 3.—Effect of *t*-butyl hydroperoxide on disulfide interchange: cystine,  $10^{-3} M$ ; bis-DNPeystine,  $10^{-4} M$ ; HCl, 9.5  $M$ ; ●—●, control; ○—○,  $1.1 \times 10^{-5} M t$ -butyl hydroperoxide; ●—●,  $1.1 \times 10^{-3} M t$ -butyl hydroperoxide incubated with cystine for 20 minutes at 35° before the addition of bis-DNPeystine.

(16) L. J. Reed, I. C. Gunsalus, G. H. F. Schnakenberg, O. F. Soper, H. E. Boaz, S. F. Kern and T. V. Parke, *THIS JOURNAL*, **75**, 1267 (1953).

(17) W. C. Schumb, C. N. Satterfield and R. L. Wentworth, "Hydrogen Peroxide," Reinhold Publishing Corp., New York, N. Y., 1955, p. 370.

As can be seen from Fig. 3, *t*-butyl hydroperoxide also catalyzes disulfide interchange, except that, in this case, a distinct lag period was observed (Fig. 3, curve 2). This hydroperoxide would be expected to form  $(\text{CH}_3)_3\text{CO}^+$  ions rather than  $\text{OH}^+$  ions because of the electron-releasing nature of the alkyl substituent. It was therefore suspected that the bulky  $(\text{CH}_3)_3\text{CO}^+$  ion attacks the  $-\text{SS}-$  bond more slowly than  $\text{OH}^+$ . In order to test this idea, one of the disulfides, *i.e.*, cystine, was first incubated with the *t*-butyl hydroperoxide for 20 minutes before the interchange reaction was started by the addition of the other disulfide. As can be seen from Fig. 3, curve 3, this preincubation did, indeed, eliminate the lag period.

**Catalysis by Sulfenyl Chlorides.**—Perhaps the most conclusive evidence that sulfenium ions are involved in disulfide interchange in acid solution is the demonstration that both an aliphatic and an aromatic sulfenyl chloride catalyze this reaction (Table II). The latter has actually been shown to form a sulfenium ion in strongly acid solution.<sup>11</sup>

All the above conclusions rest, of course, on the assumption that an increase in the rate of formation of water-soluble DNP derivatives is only due to catalysis of disulfide interchange. This was ascertained in the following manner: (1) The only yellow product found in both the uncatalyzed and catalyzed reaction mixtures (after extraction with ether) was mono-DNPcystine, which was identified chromatographically as described by Ryle and

Sanger.<sup>6</sup> (2) The equilibrium of the reaction, *i.e.*, about 80% interchange, was unchanged by

TABLE II

EFFECT OF SULFENYL CHLORIDES ON DISULFIDE INTERCHANGE

Cystine,  $10^{-3} M$ ; bis-DNPcystine,  $10^{-4} M$ ; HCl, 9.5 *M*; trichloromethanesulfenyl chloride,  $1.1 \times 10^{-6} M$ , prepared by diluting a  $10^{-2} M$  stock solution in ethanol with 9.5 *N* HCl; 2,4-dinitrobenzenesulfenyl chloride,  $1.1 \times 10^{-6} M$ , prepared by diluting a  $5 \times 10^{-3} M$  stock solution in ethanol with 9.5 *N* HCl. It was found essential to add the solution of trichloromethanesulfenyl chloride to the reaction mixture *immediately* after diluting the ethanolic stock solution with acid, since after this treatment the solution becomes turbid rapidly, indicating dismutation to disulfide.

Time, min.	Control	Interchange, %	
		Trichloromethanesulfenyl chloride	2,4-Dinitrobenzenesulfenyl chloride
7.5	..	12.4	24.0
15	..	26.1	40.5
20	5.6	..	46.9
30	..	41.0	63.2
60	19.2	64.0	76.5

any of the compounds added. (3) All the catalysts were active in concentrations 1/100 to 1/1000 that of the total concentration of disulfides.

**Acknowledgments.**—This investigation was supported by grants from the National Heart Institute of the National Institutes of Health, Public Health Service, the National Science Foundation and Eli Lilly and Co.

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[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE]

## Thiation of Nucleosides. I. Synthesis of 2-Amino-6-mercapto-9- $\beta$ -D-ribofuranosylpurine ("Thioguanosine") and Related Purine Nucleosides<sup>1</sup>

BY JACK J. FOX, IRIS WEMPEN, ALEXANDER HAMPTON AND IRIS L. DOERR

RECEIVED OCTOBER 19, 1957

Syntheses are described for the preparation of 2-amino-6-mercapto- and 6-mercapto-9- $\beta$ -D-ribofuranosylpurine in good yields by thiation of suitably-blocked guanosine and inosine followed by removal of the protecting acyl groups. Reduction of these 6-mercapto-ribofuranosylpurine nucleosides results in a relatively facile synthesis of 2-amino-9- $\beta$ -D-ribofuranosylpurine and 9- $\beta$ -D-ribofuranosylpurine (nebularine). The spectra of 6-mercapto-ribofuranosylpurine and 2-amino-6-mercapto-ribofuranosylpurine along with those of their 9- $\beta$ -D-ribofuranosyl derivatives are reported at various *pH* values. Spectral shifts are correlated with the particular functional group(s) which ionize in these *pH* regions. The *pK<sub>a</sub>* values of these compounds were determined spectrophotometrically and/or potentiometrically and are compared with analogous 6-hydroxypurine derivatives. A preliminary report of the effects of these 6-mercapto-ribofuranosylpurine nucleosides in experimental tumors and in tissue cultures is given.

The importance of 6-mercapto-ribofuranosylpurine (6MP) as a bacterial growth antagonist<sup>2</sup> and as an anti-tumor agent<sup>3,4</sup> and the indication that this purine an-

alog interferes with polynucleotide biosynthesis<sup>5</sup> suggests that nucleoside or nucleotide analogs of 6MP are worthy of investigation as potential chemotherapeutic agents. Johnson and Thomas<sup>6</sup> have prepared 6-mercapto-9- $\beta$ -D-ribofuranosylpurine ("thioinosine"), Ia, by treatment of the synthetic nucleoside, 6-chloro-9- $\beta$ -D-ribofuranosylpu-

(1) This investigation was supported in part by funds from the National Cancer Institute, National Institutes of Health, Public Health Service (Grant No. CY-3190) and from the Ann Dickler League.

(2) G. B. Elion, G. H. Hitchings and H. Vanderwerf, *J. Biol. Chem.*, **192**, 505 (1951).

(3) D. A. Clarke, F. S. Phillips, S. S. Sternberg, C. C. Stock and G. B. Elion, *Am. Assn. Cancer Res.*, **1**, 9 (1953); K. Sugiura, *ibid.*, **1**, 9 (1953); J. H. Burchenal, D. A. Karnofsky, L. Murphy, R. R. Ellison and C. P. Rhoads, *ibid.*, **1**, 7 (1953).

(4) For a comprehensive review of the biological activities of this antipurine see G. H. Hitchings and C. P. Rhoads, *Ann. N. Y. Acad. Sci.*, **60**, 183 (1954).

(5) G. B. Elion, S. Singer, G. H. Hitchings, M. E. Balis and G. B. Brown, *J. Biol. Chem.*, **202**, 647 (1953); G. B. Elion and S. Singer and G. H. Hitchings, *ibid.*, **204**, 35 (1953); M. E. Balis, D. K. Levin, G. B. Brown, G. B. Elion, H. C. Nathan and G. H. Hitchings, *Arch. Biochem. and Biophys.*, **71**, 358 (1957).

(6) J. A. Johnson, Jr., and H. J. Thomas, *TETRAHEDRON JOURNAL*, **73**, 3863 (1957).