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 $(CH_3)_3CO^+$ ions rather than OH^+ ions because of the electron-releasing nature of the alkyl substituent. It was therefore suspected that the bulky $(CH_3)_3CO^+$ ion attacks the –SS– bond more slowly than 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.[11]

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.[6] (2) The equilibrium of the reaction, *i.e.*, about 80% interchange, was unchanged by 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.

TABLE II

EFFECT OF SULFENYL CHLORIDES ON DISULFIDE INTERCHANGE

Cystine, 10^{-3} *M*; bis-DNPcystine, 10^{-4} *M*; HCl, 9.5 *M*; trichloromethanesulfenyl chloride, 1.1×10^{-5} *M*, prepared by diluting a 10^{-2} *M* stock solution in ethanol with 9.5 *N* HCl; 2,4-dinitrobenzenesulfenyl chloride, 1.1×10^{-5} *M*, prepared by diluting a 5×10^{-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.	Interchange, % Control	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

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WOODS HOLE, MASS.

[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-β-D-ribofuranosylpurine ("Thioguanosine") and Related Purine Nucleosides[1]

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

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Syntheses are described for the preparation of 2-amino-6-mercapto- and 6-mercapto-9-β-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-mercaptopurine nucleosides results in a relatively facile synthesis of 2-amino-9-β-D-ribofuranosylpurine and 9-β-D-ribofuranosylpurine (nebularine). The spectra of 6-mercaptopurine and 2-amino-6-mercaptopurine along with those of their 9-β-D-ribofuranosyl derivatives are reported at various *p*H values. Spectral shifts are correlated with the particular functional group(s) which ionize in these *p*H regions. The pK_a 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-mercaptopurine nucleosides in experimental tumors and in tissue cultures is given.

The importance of 6-mercaptopurine (6MP) as a bacterial growth antagonist[2] and as an anti-tumor agent[3,4] and the indication that this purine analog interferes with polynucleotide biosynthesis[5] suggests that nucleoside or nucleotide analogs of 6MP are worthy of investigation as potential chemotherapeutic agents. Johnson and Thomas[6] have prepared 6-mercapto-9-β-D-ribofuranosylpurine ("thioinosine"), Ia, by treatment of the synthetic nucleoside, 6-chloro-9-β-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.

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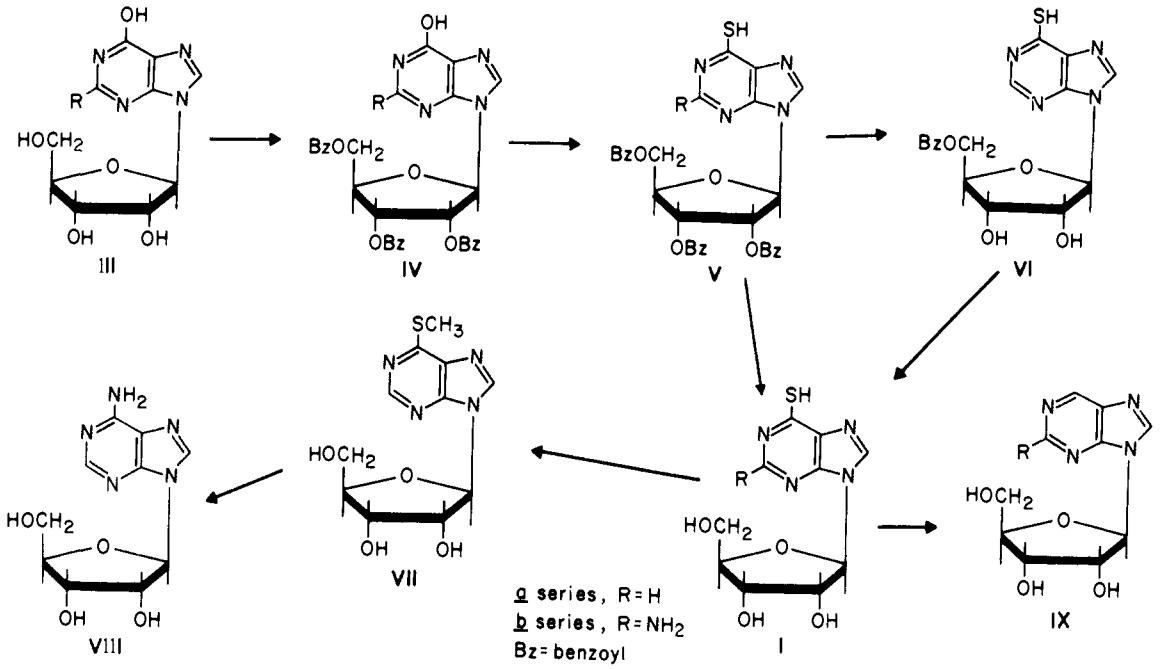

Scheme I.

rine,[7] (II), with methanolic hydrogen sulfide. Nucleoside II was first synthesized by Brown and Weliky[7] by the condensation of the chloromercuri derivative of 6-chloropurine[8] with tri-*O*-acetyl-D-ribofuranosyl chloride followed by removal of the protecting acetyl groups. The corresponding 2'-deoxyribofuranosyl analog of Ia has also been prepared, albeit in small amount, by Friedkin[9a] by the use of enzymic procedures. Lukens and Herrington[9b] have reported recently a reaction between 5-phosphoribosyl pyrophosphate and 6MP in the presence of inosinic acid phosphorylase to give a nucleotide of 6MP. Skipper and coworkers[10] have reported that Ia is approximately as active as 6MP against adenocarcinoma 755 in mice.

Similarly, 2-amino-6-mercaptopurine ("thioguanine") is also a bacterial growth inhibitor.[2,5] This purine was found to be about twenty times more effective in producing 50% inhibition of Sarcoma 180 in the mouse than was 6MP, although the anti-tumor action of thioguanine was accompanied by adverse toxic effects, *i.e.*, nearly complete depletion of bone marrow.[11] The 9-β-D-ribosyl derivative of 2-amino-6-mercaptopurine, hitherto unreported, is therefore of chemotherapeutic interest.

Since the first report of the direct thiation of pyrimidines,[12] several studies have appeared dealing with the preparation of mercapto derivatives of pyrimidines and purines.[13-18] Earlier reports[12-14] involved the treatment of pyrimidinones with phosphorus pentasulfide in inert solvents, and in this fashion 6MP was prepared from hypoxanthine.[15] Later studies[16-18] used the modification of Klingsberg and Papa[19] for the thiation of pyridones in which pyridine was substituted for inert solvents in the reaction. By this procedure, 2-amino-6-mercaptopurine was prepared from guanine.[18]

The applicability of the thiation procedure to nucleosides has not been reported hitherto. The availability of the naturally-occurring nucleosides warrants investigation of this class of compounds as source materials for relatively simple routes for the preparation of their mercapto analogs (and derivatives thereof). In this paper, the successful thiation of suitably-blocked inosine and guanosine is reported. Results dealing with the thiation of pyrimidine nucleosides will be reported later.

Treatment of inosine[20] (IIIa), with benzoyl chloride in pyridine yielded the 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl derivative IVa (Scheme I). This blocked nucleoside[21] was then thiated with phosphorus pentasulfide in boiling pyridine to give Va, 6-mercapto-9-(2',3',5'-tri-*O*-benzoyl-β-D-ribofuranosyl)-purine, which was deacylated with so-

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(20) Schwarz Laboratories, Inc.
(21) Direct thiation of IIIa gave tarry products from which the desired product could not be isolated.

dium methoxide to Ia, 6-mercapto-9-β-D-ribofuranosylpurine, in 35% over-all yield from IIIa.

When Va was treated with alcoholic ammonia in a sealed tube at 120°, extensive decomposition occurred and no identifiable products were obtained. When the reaction was carried out at 60°, however, two products were obtained in approximately equal amounts. One of these was Ia while the other, VI, analyzed for a *mono-O*-benzoylated derivative of Ia. VI gave a positive spray test on paper chromatograms for vicinal hydroxyls[22] which would indicate that part, if not all, of this substance consisted of 6-mercapto-9-(5′-*O*-benzoyl-β-D-ribofuranosyl)-purine. De-benzoylation of VI with sodium methoxide afforded Ia.

Treatment of Ia with methyl iodide and alkali yielded VII, 6-methylmercapto-9-β-D-ribofuranosylpurine, which was indistinguishable from a synthetic sample prepared previously[23] by another route. When VII was allowed to react with alcoholic ammonia in a sealed tube, a product (VIII) was obtained which proved to be identical with the natural nucleoside adenosine. It must be concluded, therefore, that the 9-β-D-ribofuranosyl structure was maintained throughout the reaction scheme of IIIa → VIII.

The fact that Ia may be methylated to VII in high yield would indicate that the assignment of the sulfhydryl rather than disulfide structure at the 6-position of Ia is correct. Further substantiation of this assignment is the fact that Ia possesses an acidic *pK* of 7.71 ascribable only to ionization at position 6 (see Table II). Similar conclusions apply to "thioguanosine" (see below).

Compound Ia also may be converted directly into 9-β-D-ribofuranosylpurine (IXa) (nebularine[24]), a naturally-occurring nucleoside found in certain mushrooms and active against Mycobacteria.[24] Compound IXa has shown a high degree of toxicity in tissue culture and a differential toxicity *in vitro* toward Sarcoma 180 cells, mouse embryonic fibroblasts and epithelial cells.[25] This substance, the structure of which was proved by synthesis from II,[7] also has been prepared by Hampton, *et al.*,[23] by reduction of VII. In the present work, dethiolation of Ia with Raney nickel gave 9-β-D-ribofuranosylpurine in 50% yield. This product was identical with a synthetic sample[7] of nebularine.

In similar fashion guanosine[20] (IIIb) was utilized as starting material for the synthesis of 2-amino-6-mercapto-9-β-D-ribofuranosylpurine (Ib). IIIb was converted into its tri-*O*-benzoyl derivative IVb by the method of Weygand and Sigmund[26] and IVb was treated with phosphorus pentasulfide in pyridine to give the thiated analog Vb. De-benzoylation of Vb with sodium methylate gave Ib ("thioguanosine") in 43% over-all yield from IIIb.

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Compound Ib was reduced with Raney nickel to IXb, 2-amino-9-β-D-ribofuranosylpurine.[27]

Spectrophotometric Studies.—The spectra of these 6-mercaptopurine nucleosides at different *p*H values along with the spectra of their free bases are given in Figs. 1–4. The very close similarity

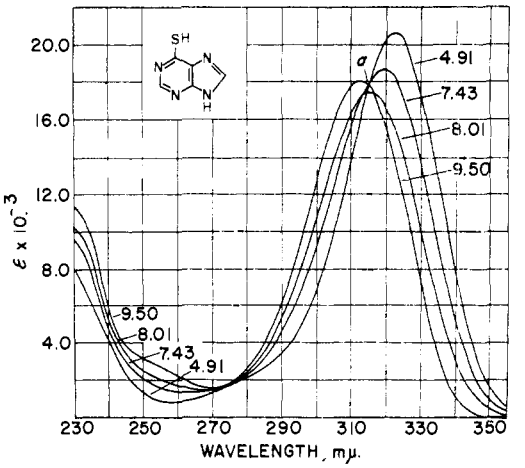

Fig. 1.

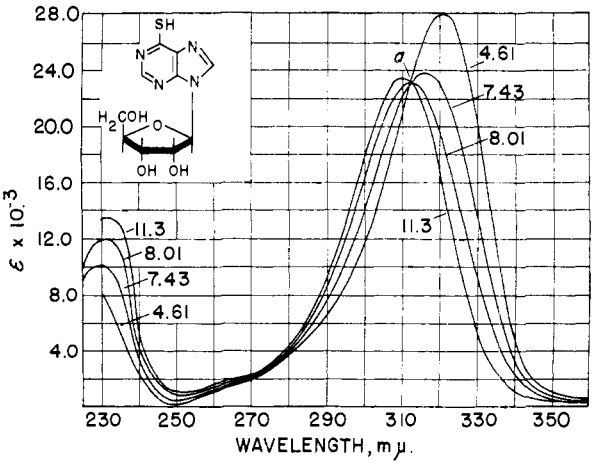

Fig. 2.

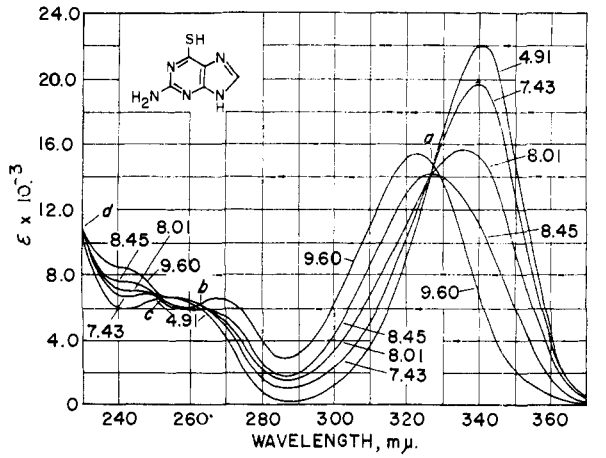

Fig. 3.

(27) It has come to our attention during the course of these investigations that Dr. H. J. Schaeffer of the Southern Research Institute has prepared this nucleoside by another route.

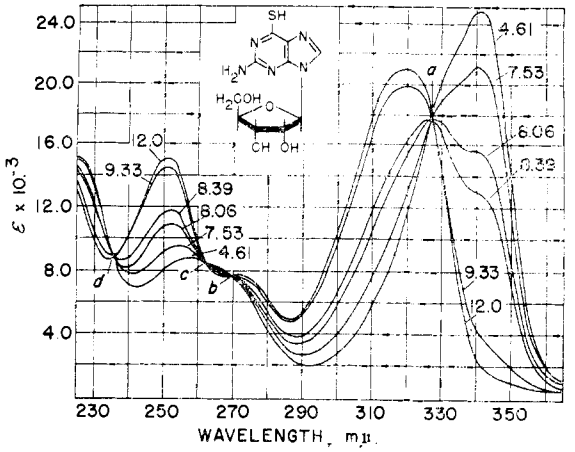

Fig. 4.

of the spectral pattern of 6MP (first acidic pK_a 7.7, curves taken in the pH range of 4.9 to 9.5) to that of its 9-β-D-ribofuranosyl analog Ia (pK_a 7.7, in the range of pH 4.9 to 11) shows that the same group is ionizing in each case. Since proton removal from the imidazole ring is excluded in the case of the nucleoside Ia, the spectral patterns must in each case refer to ionization of the 6-mercapto function. The second acidic dissociation of 6MP (pK_a 11.17, determined potentiometrically, see Table II) is hence attributable to ionization at the imidazole ring.

A similar situation obtains when the spectra of 2-amino-6-mercaptopurine and its ribosyl derivative are compared. In the long wave length region (*ca.* 340 mμ) the maximum for the neutral species of both compounds undergoes a hypochromic shift as the pH of the medium is raised, with the appearance (at *ca.* pH 10) of a new maximum at 320 mμ due to the *mono*-anionic species. Both substances exhibit four isosbestic points. By virtue of these similarities in spectral behavior, it is concluded that in the aforementioned pH range the ionization also refers to the 6-mercapto group.

The curves denoting proton addition to the purine moiety (basic pK_a) were not determined.

Instability in Dilute Alkali.—A slow decomposition was noted for these mercaptopurines in dilute alkali (0.01 *N* sodium hydroxide) as evidenced by the decrease in optical density of their respective long wave length maxima (see Table I).

Table I

Treatment of 6-Mercaptopurines with 0.01 *N* NaOH at 23°

	Decrease in opt. density at 320 or 340 mμ,[a] %		
	1.5 hr.	24 hr.	96 hr.
6-Mercaptopurine	0	37	85[b]
2-Amino-6-mercaptopurine	0	32	65
"Thioinosine" (Ia)	0	9	85[c]
"Thioguanosine" (Ib)	0	9	60

[a] 6MP and its ribosyl derivative were measured at 320 mμ. Their 2-amino derivatives were measured at 340 mμ. [b] A new maximum appears at 275 mμ. [c] A new maximum appears at 270 mμ.

Since the absorption at 320 or at 340 mμ is ascribable in large measure to the presence of the 6-mercapto function in the molecule (hypoxanthine or guanine or their respective nucleosides possess no selective absorption in this region), it is reasonable to conclude that the decrease in optical density is due at least in part to the degradation of this functional group. The question of the identity of the product(s) is currently under investigation. It would be advisable to use only freshly-prepared solutions of these mercaptopurines for biological studies.[28]

Dissociation Constants.—The potentiometrically and/or spectrophotometrically determined acidic pK_a values of these 6-mercaptopurines along with those of their 6-hydroxy analogs are given in Table II. Replacement of the 6-hydroxyl group by mercapto lowers, in every case, the pK_{a1} by approximately one pH unit, as expected. The introduction of a ribofuranosyl moiety at position 9 of hypoxanthine or guanine exerts no appreciable effect upon the pK_{a1}. This type of substitution likewise has little effect upon the acidic strength of the 6-mercapto group in 6MP or in 2-amino-6-mercaptopurine. In the case of hypoxanthine or guanine, replacement of the 6-OH by 6-SH has the effect of also increasing the acidity of the second dissociating group.

Table II

Acidic Dissociation Constants of 6-Hydroxy- and 6-Mercaptopurines

	pK_{a1}		pK_{a2}
	Spectrophotometric	Potentiometric	Potentiometric
Guanine	.....	9.2[30]	12.3[30]
2-Amino-6-mercaptopurine	8.2 (±0.1)	.....	11.6 (±0.1)
Guanosine	.....	9.16[31]	.....[a]
"Thioguanosine" (Ib)	8.35 (±0.05)	8.33 (±0.05)	.....[b]
Hypoxanthine	.....	8.94 (±0.02)[29]	12.10 (±0.03)[29]
6-Mercaptopurine	7.7 (±0.1)	7.77 (±0.02)[29]	11.17 (±0.06)[c]
Inosine	.....	8.75[31]	.....[a]
"Thioinosine" (Ia)	7.56 (±0.05)	7.71 (±0.02)	.....[b]

[a] The pK_{a2} for the purine nucleosides refer to ionization of the sugar moiety. The naturally occurring nucleosides have been shown to possess pK_{a2} values of approximately 12.3.[31] [b] The pK_{a2} due to the dissociation of the sugar hydroxyl(s) was not determined. [c] Albert and Brown[29] report 10.88.

At the pH of blood (7.45), 33% of 6MP and of its ribosyl derivative Ia exist in the mono-anionic species, while 2-amino-6-mercaptopurine and its ribofuranosyl derivative are approximately 15% ionized.

Screening Studies.[32]—In preliminary studies, 2-amino-6-mercapto-9-β-D-ribofuranosylpurine (Ib) was found to be an inhibitor of Sarcoma 180; its activity was approximately equal to that of 2-amino-6-mercaptopurine itself on an equimolar basis. 6-Mercapto-9-β-D-ribofuranosylpurine like-

(28) Analytically-pure samples of "thioinosine," which gave only one spot on paper chromatograms, developed a new fluorescent spot after standing for one week in aqueous solution (pH 6.5-7.0).

(29) A. Albert and D. J. Brown, *J. Chem. Soc.*, 2060 (1954).

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(32) The authors are indebted to Dr. Donald A. Clarke for preliminary data on the screening of these compounds with Sarcoma 180 in the mouse and to Dr. John J. Biesele for data on the effects of these compounds in tissue culture.

wise inhibited the growth of Sarcoma 180 but was considerably less active then 6MP.[33a]

In mouse tissue cultures, by methods previously described,[33b] thioguanosine inhibited cell division and caused much cell death over a concentration range from 0.1 to 5.0 micromoles per milliliter. The nucleoside agreed with 2-amino-6-mercaptopurine in this respect and also in the absence of a marked differential inhibition of Sarcoma 180 cells. Cells of Human Epidermoid Carcinoma #2 suffered only slight mitotic inhibition from thioguanosine but were somewhat more sensitive to the parent purine.

Acknowledgments.—The authors are indebted to Drs. Aaron Bendich and George Bosworth Brown for helpful discussions and continued interest.

Experimental[34]

9-(2′,3′,5′-Tri-*O*-benzoyl-β-D-ribofuranosyl)-hypoxanthine (IVa).—A well-stirred suspension of 10 g. (0.037 mole) of inosine[20] in 250 ml. of anhydrous pyridine was warmed to 55° and treated in dropwise manner with 18 ml. (0.156 mole) of benzoyl chloride so that the temperature never exceeded 65°. The solid gradually dissolved and the pale-amber solution was heated for 2 hours at 60–65°, then for 3 hours at 40–45° and finally allowed to remain overnight at room temperature. The reaction mixture was separated from pyridine hydrochloride and the solution concentrated *in vacuo* to a thin sirup which was subjected to steam distillation. After removal of most of the pyridine, the hot aqueous layer was decanted and the residual sirup again subjected to steam distillation. After decantation from hot water, the cooled sirup was taken up into methylene chloride, washed once with cold bicarbonate solution, twice with water and dried over sodium sulfate. After removal of the solvent *in vacuo*, the glassy solid was triturated with ether containing a few drops of ethanol. A cream-colored solid separated, 17.6 g. (82%), m.p. 120–125°. This product is sufficiently pure for the thiation reaction. A small sample was recrystallized from ethyl acetate from which tiny tapered prisms separated, m.p. 133–136°, $[\alpha]^{25}D$ −86° (*c* 1.1 in $CHCl_3$).

Anal. Calcd. for $C_{31}H_{24}N_4O_8$: C, 64.13; H, 4.17; N, 9.65. Found: C, 63.88; H, 4.27; N, 9.56.

9-(2′,3′,5′-Tri-*O*-benzoyl-β-D-ribofuranosyl)-6-mercaptopurine (Va).—A well-stirred suspension of IV (10.0 g., 0.017 mole) and 14.3 g. (0.065 mole) of phosphorus pentasulfide in 250 ml. of reagent-grade pyridine was treated dropwise with 2.5 ml. of water[35] and the reaction flask heated at reflux temperature for 4 hours. The yellow-orange turbid mixture was chilled and the liquid portion decanted. (Both the residue and the decantate contain the desired product.) The residue was added in portions to boiling water and stirred at boiling temperature until the residue became granular. (A large beaker should be used since considerable frothing occurs.) The decantate was concentrated to a thin sirup *in vacuo* and added in a thin stream to the stirred, boiling mixture. Stirring of the boiling solution was continued for an additional 0.5 hour. The precipitate was removed by filtration of the hot mixture and washed repeatedly with hot water, triturated with 50% alcohol–ether until the filtrates were colorless and finally with ether. The filtrates were discarded. The yield of dried material was 88% (8.9 g.). An aliquot was recrystallized from methyl ethyl ketone–ethanol, m.p. 206–214°, $[\alpha]^{25}D$ −130° (*c* 0.6 in CH_2Cl_2).

Anal. Calcd. for $C_{31}H_{24}N_4O_7S$: C, 62.41; H, 4.12; N, 9.39; S, 5.37. Found: C, 62.93; H, 4.20; N, 9.52; S, 5.26.

6-Mercapto-9-β-D-ribofuranosylpurine (Ia).—Fifteen ml. of a freshly-prepared 2 *N* solution of sodium methylate in methanol was added to a stirred suspension of Va (10.2 g.) in 750 ml. of anhydrous methanol. The solution was refluxed for 4.5 hours during which time the *p*H was maintained at approximately 9 (when tested with moist "*p*Hydrion" paper) by further additions of sodium methylate. The methanol was removed *in vacuo* and the residue taken up in 50 ml. of water. The *p*H was adjusted to 8.5 with acetic acid and the solution subjected to steam distillation to remove methyl benzoate. The aqueous residue was cooled, extracted 3 times with methylene chloride (to remove starting material), and concentrated under vacuum to a final volume of approximately 35 ml. The warm solution was acidified with glacial acetic acid to *p*H 4–5, cooled, and the precipitated solid triturated with ether–ethanol (90:10). Upon filtration and drying, 3.76 g. (77%), of Ia was obtained. This product was recrystallized from 50 ml. of hot water plus sufficient ammonium hydroxide to effect solution. Upon filtration and acidification with glacial acetic acid and cooling, tiny cream-colored needles were obtained, m.p. 208–210° dec. Further recrystallization may be carried out from hot water or hot ethanol; $[\alpha]^{25}D$ −73° (*c* 1 in 0.1 *N* NaOH); (Johnson and Thomas[6] report $[\alpha]D$ −73° (*c* 2 in 0.1 *N* NaOH), m.p. 207–210° dec.; ultraviolet absorption properties: *p*H 4–5, maximum at 322 mμ, ϵ_{max} 27,900; at *p*H 11–12, maxima at 232 and 312 mμ, ϵ_{max} 13,500 and 23,350. Paper chromatography showed the product to be free from 6-mercaptopurine (butanol–H_2O, 86:14).

This product was identical both chromatographically and spectrophotometrically with an authentic sample kindly provided by Dr. J. A. Johnson.

6-Mercapto-9-(5′-*O*-benzoyl-β-D-ribofuranosyl)-purine (VI).—Six grams of Va was heated with alcoholic ammonia (previously saturated at 0°) in a sealed tube at 60° for 10 hours. The resulting dark-yellow solution was concentrated *in vacuo* to a residue which was subjected to steam distillation to remove ethyl benzoate. The aqueous residue was cooled and the solid mass filtered (the filtrate contains Ia). The precipitate was washed with ether, dried and weighed. The dry precipitate was suspended in methylene chloride, stirred or shaken vigorously for several minutes, filtered, dried and weighed. This process was repeated until no loss of weight was observed (usually two resuspensions sufficed). The combined methylene chloride washings contained starting material Va. The precipitate was then triturated with alcohol, washed thoroughly with ether, yield 1.8 g. (45%), m.p. 225–226° (eff.).

Anal. Calcd. for $C_{17}H_{16}N_4O_5S$: C, 52.57; H, 4.15; N, 14.43; S, 8.25; benzoyl no., 1.0. Found: C, 52.20; H, 4.24; N, 14.79; S, 7.93; benzoyl no., 1.1.

Upon concentration of the original aqueous filtrate and working it up in the usual manner, 1.05 g. (38%) of Ia was obtained.

6-Methylmercapto-9-β-D-ribofuranosylpurine (VII).—A solution of 6-mercapto-9-β-D-ribofuranosylpurine (330 mg.) in 0.4 *N* sodium hydroxide (2.5 ml., 0.86 equiv.) was shaken at room temperature for 10 minutes while methyl iodide (0.073 ml., 1 molec. equiv.) was added in portions. Sodium hydroxide (0.4 ml. of 0.4 *N*) was added and the solution again shaken with methyl iodide (0.073 ml.). The solution was kept at room temperature for 2 hours during which time white needles separated. After refrigeration overnight, the solid was collected, dried over sodium hydroxide and refluxed for several minutes with absolute ethanol (2 ml.). Filtration of the chilled suspension gave white micro-needles (257 mg., 74%, m.p. 163–164°). The melting point was not depressed by admixture with 6-methylmercapto-9-β-D-ribofuranosylpurine[23] of m.p. 163–164°. The product was indistinguishable from the latter nucleoside by its absorption maxima in 0.1 *N* HCl and in water or by paper chromatography in 1-butanol–water–acetic acid (5:3:2) and in 1-butanol–H_2O.

(33) (a) D. A. Clarke, F. S. Phillips, S. S. Sternberg, C. C. Stock G. B. Elion and G. H. Hitchings, *Cancer Research*, **13**, 593 (1953); (b) J. J. Biesele, M. C. Slautterback and M. Margolis, *Cancer*, **8**, 87 (1955).

(34) Melting points were determined by the capillary method and are uncorrected. Analyses were performed by the Schwarzkopf Microanalytical Laboratory and by J. F. Alicino. Paper chromatograms were run by the ascending method using Schleicher and Schuell No. 597 paper.

(35) If water is omitted from the reaction, the contents darken considerably within 30 minutes. Tarry residues are produced from which isolation of desired product is extremely laborious and, most often, impossible. Attempts to correlate (stoichiometrically) the amount of water needed to produce the yellow-orange color in the reaction with the starting materials (phosphorus pentasulfide or IIIa) have been unsuccessful.

Synthesis of Adenosine (VIII).—A solution of 0.5 g. of VII in 15 ml. of methanolic ammonia (saturated at 0°) in a sealed tube was heated at 146–148° for 7 hours. After removal of the solvent *in vacuo* the white residual solid crystallized from ethanol (20 ml. concentrated to 10 ml.). The product (0.31 g., 69%), m.p. 214–218° (dec.) (unaffected by admixture with natural adenosine), migrated as a single spot of the same R_f as adenosine on paper chromatograms run in 1-butanol–water or in 1-butanol–water–acetic acid (5:3:2). The absorption maxima in water (257 mμ at *p*H 1, 259–260 mμ at *p*H 7) were identical with those of natural adenosine; $[\alpha]^{25}D$ −61° (*c* 1 in water) (reported[36] $[\alpha]D$ −61.7° in water).

Anal. Calcd. for $C_{10}H_{13}O_4N_5$: N, 26.21. Found: N, 26.44.

Synthesis of Nebularine (IXa).—One gram of thioinosine (Ia) was dissolved in 50 ml. of boiling water and small portions (1 to 2 g.) of Raney nickel[37] were added to the stirred solution. After 3 hours at reflux temperature, the ultraviolet absorption maximum at 318 mμ had disappeared. (Care was taken to avoid excess of catalyst since considerable adsorption of the product on the nickel may occur.) The reaction mixture was filtered and the catalyst leached several times with boiling water. The original filtrate and washings were combined and evaporated *in vacuo* to a sirup which was dissolved in hot ethanol and reconcentrated several times. The dried sirup was dissolved in hot absolute ethanol, charcoaled, filtered, concentrated to *ca.* 15 ml. and cooled slowly. A tan precipitate formed, 0.52 g., 58%, m.p. 173–176°. One recrystallization raised the melting point to 174.5–176.5°. A mixed melting point with an authentic specimen[7] showed no depression. Additional crops may be obtained from the mother liquors; $[\alpha]^{25}D$ −48° (*c* 1.5 in H_2O) (Brown and Weliky[7] report $[\alpha]^{25}D$ −48.6°). Light absorption properties also agreed with those reported.[7]

9-(2′,3′,5′-Tri-*O*-benzoyl-β-D-ribofuranosyl)-guanine (IVb).—The procedure of Weygand, *et al.*,[26] was followed with modifications. Benzoyl chloride (147 g., 1.05 moles) was added dropwise to a well-stirred suspension of 60 g. (0.21 mole) of guanosine[38] in 1500 ml. of dry pyridine. The temperature during this addition was maintained at 60–65°. After a total time of two hours at this temperature, the bright yellow solution was allowed to cool to 40–45°. After three hours at this temperature, the reaction mixture was allowed to remain at room temperature overnight. The mixture was separated from pyridine hydrochloride and the filtrate concentrated *in vacuo* to approximately 400 ml. and poured in a thin stream into 3 liters of well-stirred, boiling water. The sirup gradually solidified to a pale-yellow granular material. The precipitate was triturated with water until all lumps were broken up and treated twice with separate portions of boiling water to remove any occluded benzoic acid, residual pyridine or starting material. The precipitate was triturated with ethanol until no more color could be extracted,[39] washed with ether and dried at 100° for 2 hours; yield 100 g., 80%, m.p. 252–256° (sinters at 250°). Weygand and Sigmund[26] report a melting point of 259–261° (cor.) on analytically-pure material and a yield of 95%.

2-Amino-6-mercapto-9-(2′,3′,5′-tri-*O*-benzoyl-β-D-ribofuranosyl)-purine (Vb).—A well-stirred suspension of tri-*O*-benzoylguanosine (IVb, 40 g., 0.067 mole) and phosphorus pentasulfide (57 g., 0.25 mole) in 1500 ml. of reagent grade pyridine was treated dropwise with 4.2 ml. (0.23 mole) of water and heated at reflux temperature for 6 hours. The cautious addition of further drops of water was necessitated if the solution began to lose its turbidity. (Failure to maintain this inhomogeneity inevitably resulted in much reduced yields). At the end of the reflux period, the reaction mixture was chilled until heavy precipitation occurred. The solution was decanted into a distillation flask and most of the pyridine removed *in vacuo* to a thin sirup. Both the sirup and the solid were added slowly to vigorously-stirred boiling water (*ca.* 1500 ml.). Considerable foaming and copious evolution of hydrogen sulfide occurred. The stirring was continued (15–30 minutes) until the precipitate appeared to be completely granular. The hot suspension was filtered, the precipitate washed with boiling water, and the damp product triturated with alcohol–ether (50:50) until the filtrate was colorless. An homogeneous, cream-colored solid was obtained. After filtration and a final washing with ether (the filtrate and washings were discarded), the product was air-dried; yield 37 g. (90%), m.p. 212–217 (shrinks at 205°). A sample of the crude material was recrystallized from ethanol for analytical purposes and had a melting point of 223.5–227.5°, $[\alpha]^{22}D$ −77° (*c* 0.6 in pyridine).

Anal. Calcd. for $C_{31}H_{25}N_5O_7S$: C, 60.88; H, 4.12; N, 11.45; S, 5.24. Found: C, 60.89; H, 4.64; N, 11.46; S, 4.77.

2-Amino-6-mercapto-9-β-D-ribofuranosylpurine (Ib).—A freshly-prepared solution of 6.5 g. of sodium methylate (0.12 mole) in 300 ml. of methanol was added to a well-stirred suspension of 37 g. (0.06 mole) of unrecrystallized Vb in 1500 ml. of hot methanol. The *p*H of the resulting amber-colored solution was 10–11. At the end of two hours of refluxing, the *p*H dropped gradually to *ca.* 8.5. After removal of the solvent *in vacuo* the residue was taken up into 250 ml. of hot water and subjected immediately to steam distillation to remove the methyl benzoate. (If the *p*H of this aqueous mixture is higher than 9 it should be adjusted to this value with acetic acid. Once water has been added the subsequent steam distillation should not be delayed.) The hot aqueous residue was filtered and acidified with glacial acetic acid to *p*H 5 and cooled. The tan solid was collected, taken up in hot water, charcoaled, filtered and cooled slowly. The product, consisting of tiny tapered prisms was filtered and dried; yield 11.4 g. (63%), m.p. 224–227° (efferv.). Though the product is quite pure at this stage, it may be recrystallized by redissolving in dilute ammonia and acidification with glacial acetic acid. It also may be recrystallized from water; $[\alpha]^{22}D$ −64° (*c* 1.3 in 0.1 *N* NaOH); ultraviolet absorption properties: at *p*H 4–6, maxima at 257 and 342 mμ, ϵ_{max} 8,820 and 24,800, respectively; at *p*H 10.4–12.0, maxima at 252 and 319.5 mμ, ϵ_{max} at 14,700 and 21,000, respectively; shoulder at 270 mμ. The product was free from 2-amino-6-mercaptopurine. In some runs traces of guanosine may be present as noted by slightly lower extinction coefficient at 320 or at 340 mμ.

Anal. Calcd. for $C_{10}H_{13}N_5O_4S \cdot {}^1/_2H_2O$: C, 39.00; H, 4.54; N, 22.75; S, 10.39. Found: C, 39.06; H, 4.49; N, 22.26; S, 10.07.

2-Amino-9-β-D-ribofuranosylpurine (IXb).—Thioguanosine (1.0 g., 0.0033 mole) was dissolved in 50 ml. of boiling water and treated with approximately 2 g. of Raney nickel. After approximately 2 hours, the reaction was complete as evidenced by the loss of the absorption maximum at 340 mμ. The nickel was separated and leached several times with boiling water. The washings and the filtrate were combined, charcoaled, and concentrated *in vacuo* to a sirup which was dissolved in hot ethanol and reconcentrated. The residual sirup was azeotroped with hot benzene several times until a yellow amorphous solid remained, 0.62 g. (69%). The crude solid was recrystallized from absolute ethanol twice to give a cream-colored material. This product begins to melt at *ca.* 110° slowly to an opaque glass which commences to effervesce at 137° to a clear liquid at 165°; $[\alpha]^{23}D$ −39° (*c* 1.2 in water).

Anal. Calcd. for $C_{10}H_{13}N_5O_4$: C, 44.95; H, 4.90; N, 26.21. Found: C, 44.68; H, 4.94; N, 25.91.

The ultraviolet absorption spectrum of 2-amino-9-β-D-ribofuranosylpurine (*p*H range 1–7, see Fig. 5) is similar to that for 2-aminopurine reported by Mason.[40] Unlike 2-aminopurine, no spectral shifts are observed between *p*H 6.8 to 12.0 in accord with the absence of a dissociable proton in the imidazole moiety of the molecule. The spectrophotometrically-determined pK_a of IXb is 3.40 ± 0.05.

Spectrophotometric Studies.—Measurements were made with a Cary recording spectrophotometer, model 11, using

(36) J. Davoll, B. Lythgoe and A. R. Todd, *J. Chem. Soc.*, 967 (1948).

(37) D. J. Brown, *J. Soc. Chem. Ind. (London)*, **69**, 353 (1950).

(38) A pure grade of guanosine is necessary. A "chrome pure" grade (Schwarz Laboratories, Inc.) was found to be satisfactory.

(39) Unless this trituration with ethanol is carried out, the final product will melt much lower due to contamination with what is believed to be a tetrabenzoyl derivative of IIIb (Fox, Wempen and Doerr, unpublished observations). This contamination interferes with the subsequent thiation reaction.

(40) S. F. Mason *J. Chem. Soc.*, 2071 (1954).

techniques and buffers previously described.[41,42] The apparent pK_a values were determined spectrally by procedures previously employed.[43,44]

Potentiometric Titrations.—Potentiometric titrations were carried out at 23° with a glass electrode standardized at *p*H 4.00. Carbonate-free 0.100 *N* NaOH was added to a nitrogen-stirred solution of the particular mercaptopurine in pre-boiled water and the pK_a values calculated at seven equidistant points on the titration curve and averaged; 6MP, 2-amino-6-mercaptopurine and 6-mercapto-9-β-*D*-ribofuranosylpurine were titrated at concentrations of 0.01 *M*. 2-Amino-6-mercapto-9-β-*D*-ribofuranosylpurine was titrated at 0.0025 *M*.

For spectrophotometric and potentiometric studies, analytical samples of thioinosine (Ia) and thioguanosine (Ib) were used. 6-Mercaptopurine was purified as follows: A commercial sample of 6MP (0.15 g.) was dissolved in boiling pyridine (4.5 ml.) and cooled overnight in the refrigerator. The white prisms which formed were collected and washed with pyridine. The solid was triturated immediately with 4 ml. of water and the *p*H of the mixture adjusted to 5 with 1 *N* hydrochloric acid. The suspension was warmed to 50–60°, cooled, and the cream-colored solid collected and washed with 10 ml. of water. The recovery was 0.11 g. (dried at 15 mm. over NaOH). Paper chromatography in BuOH–AcOH–water (5:2:3) and in BuOH–HCOOH–water (85:0.2:14) using Schleicher and Schuell No. 597 paper showed only one spot. The pyridine mother liquor contained traces of a foreign spot.

2-Amino-6-mercaptopurine was recrystallized several times by methods previously described.[18] Paper chromatograms revealed the presence of a small impurity.

In spite of these purifications, it was impossible to establish sharp isosbestic points[45] in the spectra of 6MP and thioguanine, due probably to the slow decomposition with time (see Table I) already noted for these purines in dilute alkali. The relatively small errors in those two spectra do not alter the argument with regard to the allocation of ionization (between *p*H 4.9 to 9.6) to the dissociation of the 6-mercapto function; nor do they affect the accuracy of the spectrally-determined pK_a values (within the margin of error listed) in Table II.

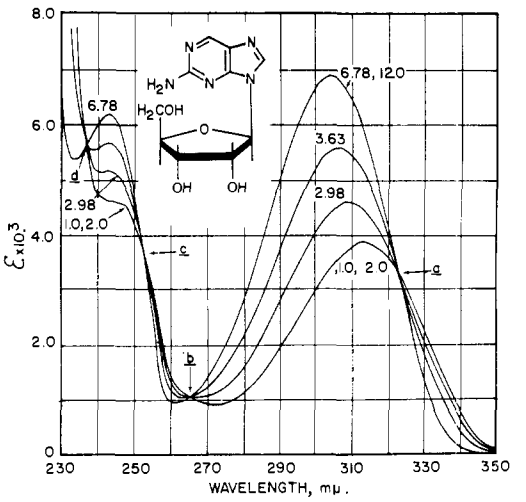

Fig. 5.

Key to Figures.—All the spectra listed were run in aqueous solutions at *p*H values indicated on the curves. The italicized letters refer to isosbestic points.

(41) J. J. Fox, L. F. Cavalieri and N. Chang, THIS JOURNAL, **75**, 4315 (1953).

(42) J. J. Fox, N. Yung, J. Davoll and G. B. Brown, *ibid.*, **78**, 2117 (1956).

(43) D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta*, **9**, 199 (1952).

(44) J. J. Fox and D. Shugar, *Bull. soc. chim. Belges*, **61**, 44 (1952).

(45) For a discussion of the significance of isosbestic points see J. J. Fox and D. Shugar, *Biochim. et Biophys. Acta*, **9**, 369 (1952). See also A. Bendich, in "The Nucleic Acids," Vol. I (Chargaff and Davidson, eds.), Academic Press, Inc., New York, N. Y., 1955, p. 81.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE OHIO STATE UNIVERSITY]

The Controlled Thermal Decomposition of Cellulose Nitrate. IV. C^{14}-Tracer Experiments[1,2]

BY F. SHAFIZADEH AND M. L. WOLFROM

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Radioassay of the products formed from the controlled ignition of cellulose-C^{14} nitrates, predominantly labeled at C1 and C6 of the anhydro-D-glucose units, indicates that C1 gives mainly carbon dioxide and lesser amounts of formic acid and glyoxal (from C1 and C2) and that the major product from C6 is formaldehyde with lesser amounts of formic acid and carbon dioxide.

Several aspects of the controlled thermal decomposition of cellulose nitrate have been discussed in the previous papers of this series.[2–4] Thus, it has been noted that the controlled ignition of cellulose nitrate under reduced pressure provides a mixture of carbon-containing volatile fragments consisting mainly of carbon dioxide (and carbon monoxide), formic acid, formaldehyde and glyoxal. The quantitative analysis of these compounds and their relation to the chemical nature of the ignition process has been reported. In order to obtain further information concerning the ignition of cellulose nitrate, it was deemed pertinent to recover the ignition products, or derivatives thereof, and to correlate them with the various positions of the anhydro-D-glucose units of the cellulose nitrate, from which they originate, through the use of isotopic tracer techniques. Thus, the first step toward this goal was the preparation of specifically labeled cellulose-C^{14}. Several samples of these materials[5]

(1) This work was carried out under contract (DA-33-019-ord-2042, technical supervising agency, Ballistic Research Laboratories, Aberdeen Proving Ground, Md.) between the Office of Ordnance Research of the U. S. Army Ordnance Corps and The Ohio State University Research Foundation (Project 679). Preliminary communication: *Abstracts Papers Am. Chem. Soc.*, **132**, 16D (1957).

(2) Previous communication in this series: M. L. Wolfrom, A. Chaney and P. McWain, THIS JOURNAL, **80**, 946 (1958).

(3) M. L. Wolfrom, J. H. Frazer, L. P. Kuhn, E. E. Dickey, S. M. Olin, R. S. Bower, G. G. Maher, J. D. Murdock, A. Chaney and Eloise Carpenter, THIS JOURNAL, **78**, 4695 (1956).

(4) M. L. Wolfrom, J. H. Frazer, L. P. Kuhn, E. E. Dickey, S. M. Olin, D. O. Hoffman, R. S. Bower, A. Chaney, Eloise Carpenter and P. McWain, *ibid.*, **77**, 6573 (1955).

(5) F. Shafizadeh and M. L. Wolfrom, *ibid.*, **77**, 5182 (1955).