feature of the parotid and especially of the submaxillary secretion. Lysozyme is a basic protein known to be present in salivas. The PE_{11} and PE_{12} components move rapidly enough possibly to correspond to acidic polysaccharides or glycoproteins.

It is generally considered that the parotid gland has purely a serous secretion, the sublingual a mucus secretion and the submaxillary a mixed secretion. The electrophoretic and ultracentrifugal patterns of the submaxillary and parotid secretions showed many similarities. In contrast, the single sample of sublingual secretion had more numerous and generally quite different ultracentrifugal components from those of the other secretions.

The only electrophoretic or ultracentrifugal component that has been identified is the amylase of parotid saliva. The high degree of complexity of the secretions and the relative lack of knowledge of the macromolecules will require considerable additional work before the major components are identified.

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[CONTRIBUTION FROM THE NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

6-Thiouric Acid—a Metabolite of 6-Mercaptopurine

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6-Thiouric acid has been successfully synthesized by the direct thiation of uric acid. Its structure was established by comparison with the isomeric 2- and 8-thiouric acids synthesized unequivocally. Its identity with a specimen isolated from the urine of patients treated with 6-mercaptopurine confirms that 6-thiouric acid is one of the metabolites of 6-mercaptopurine in humans.

The metabolism of 6-mercaptopurine (6-purinethiol) has been of interest because of its clinical application in the chemotherapy of leukemia. Using S^{35}-labeled 6-mercaptopurine, Elion[1,2] stated that one of the routes of metabolic transformation of 6-mercaptopurine in humans and mice was through oxidation to 6-thiouric acid. Unfortunately Elion's papers were more or less in the form of preliminary reports and no experimental details were published. It is the purpose of this paper to report for the first time the preparation, by means of the direct thiation[3] of uric acid, and characterization of 6-thiouric acid. That the thiation did indeed take place preferentially to give the desired 6-thiouric acid was established by a comparison of the thiated product with the remaining two possible isomeric thiouric acids synthesized unequivocally. Further, the synthetic 6-thiouric acid was found to be identical with a specimen isolated from the urine of patients treated with 6-mercaptopurine, thus proving conclusively that 6-thiouric acid is one of the metabolites of 6-mercaptopurine in humans.

Experimental

Preparation of 6-Thiouric Acid.—To a solution of 2.8 g. of phosphorus pentasulfide[4] in 100 ml. of dry pyridine at its refluxing temperature was added 1.7 g. of uric acid. The mixture was refluxed under constant agitation for 24 hours, care being exercised to exclude moisture. The mixture was then cautiously poured into an equal volume of boiling water and gently boiled for about five minutes. The unchanged uric acid (about 0.5 g.) that separated upon cooling to 0° was removed by filtration and the filtrate was evaporated *in vacuo* (water aspirator) to dryness at about 50°. To the residue was added 50 ml. of a saturated solution of sodium bicarbonate and the mixture was heated to 90° for a few minutes. A second crop of unreacted uric acid (about 0.9 g.) was removed at this stage by centrifugation. The supernatant, containing principally sodium 6-thiourate in solution, was again evaporated to dryness *in vacuo* (water aspirator) at about 50°. The residue was suspended in 20 ml. of water and 5 ml. of concentrated hydrochloric acid. The crude 6-thiouric acid which separated as a brown precipitate was once again warmed to 90° with 25 ml. of a saturated sodium bicarbonate solution, and evaporated to dryness *in vacuo* at 50°. This residual material was triturated with 15 ml. of water and acidified to *p*H 7.6 with 1 *N* hydrochloric acid. The 6-thiouric acid, obtained as a brown solid, was washed with water and dried on a porous plate; yield 0.22 g., 83% based on the uric acid consumed. The final purification by means of ion-exchange chromatography is the same as the procedure described below for the purification of 6-thiouric acid isolated from patients' urine.

Pure 6-thiouric acid crystallizes from water in yellow microcrystals as a hydrate, m.p. above 300°.

Anal. Calcd. for $C_5H_4N_4O_2S.H_2O$: C, 29.70; H, 2.99. Found: C, 30.29; H, 2.79.

Drying in a vacuum desiccator gave the hemihydrate.

Anal. Calcd. for $C_5H_4N_4O_2S.0.5H_2O$: C, 31.15; H, 2.59; S, 16.64. Found: C, 30.89; H, 2.67; S, 16.91.

The anhydrous compound was obtained only after drying at 120° *in vacuo* for several hours.

Anal. Calcd for $C_5H_4N_4O_2S$: C, 32.60; H, 2.19; loss of weight (from hemihydrate to anhydrous), 4.66. Found: C, 32.39; H, 2.68; loss of weight, 4.31.

It is quite stable in boiling water; solubility at 25° about 17 mg. per liter. It gives an R_f value of 0.22 by ascending chromatography[5] on Whatman no. 1 paper, using a saturated ammonium sulfate–water–isopropyl alcohol system (79:19:2)[6]; ultraviolet absorption in aqueous media (see Fig. 1) at *p*H 2.1; λ_{max} 255 mμ, log ε 3.85; 354 mμ, log ε 4.36; at *p*H 10.9; λ_{max} 233 mμ, log ε 4.11; 343 mμ, log ε 4.20.

2-Thiouric acid, a known compound, was prepared by the fusion of 4,5-diamino-2-thiouracil[7,8] with urea according to Johns and Hogan[9]; brownish-yellow crystals, m.p. above 300°, insoluble in most solvents; R_f in the ammonium sul-

(1) G. B. Elion, S. Bieber and G. H. Hitchings, *Ann. N. Y. Acad. Sci.*, **60**, 297 (1954).

(2) L. Hamilton and G. B. Elion, *ibid.*, **60**, 304 (1954).

(3) The use of the term "thiation" to mean the direct replacement of oxygen by sulfur finds precedent in G. B. Elion and G. H. Hitchings, THIS JOURNAL, **69**, 2138 (1947).

(4) Distilled grade, Victor Chemical Works, Chicago, Ill.

(5) It should be noted that throughout this work, the paper chromatograms were visualized under ultraviolet illumination of 253.7 mμ.

(6) A. Deutsch and R. Nilsson, *Acta Chem. Scand.*, **7**, 858 (1953).

(7) W. Traube, *Ann.*, **331**, 64 (1904).

(8) G. B. Elion, E. Burgi and G. H. Hitchings, THIS JOURNAL, **74**, 411 (1952).

(9) C. O. Johns and A. G. Hogan, *J. Biol. Chem.*, **14**, 299 (1913).

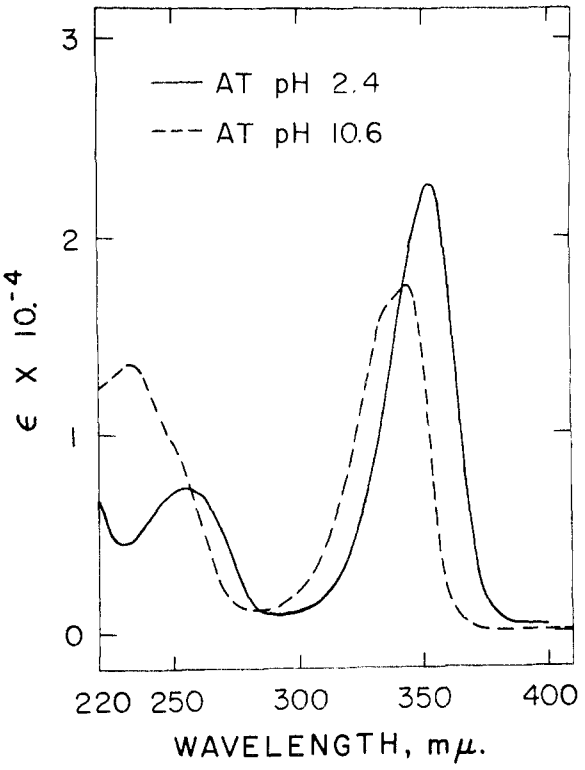

Fig. 1.—Ultraviolet absorption spectra of 6-thiouric acid.

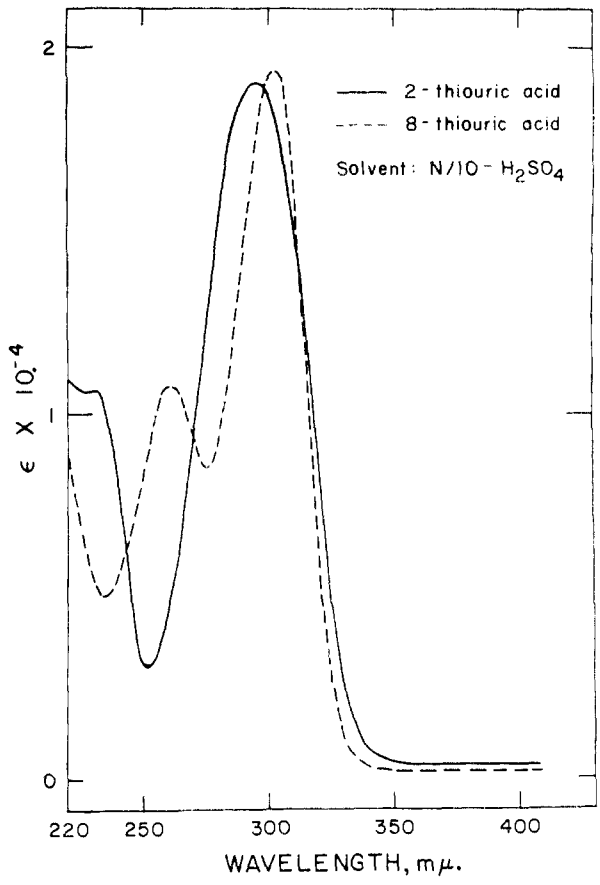

Fig. 2.—Ultraviolet absorption spectra of 2- and 8-thiouric acids.

fate–water–isopropyl alcohol system, 0.18; ultraviolet absorption in aqueous media (see Fig. 2) at *p*H 1.2; λ_{max} 230 mμ (inflection), log ε 3.97; 295 mμ, log ε 4.17; at *p*H 11.7; λ_{max} 226 mμ, log ε 4.33; 284 mμ, log ε 4.08.

8-Thiouric Acid.—The synthesis of 8-thiouric acid was effected by two different methods. **Method 1.**—Analogous to the above synthesis of 2-thiouric acid, 200 mg. each of 4,5-diaminouracil sulfate[10] and thiourea were fused at 180–190° (bath temperature) in a silicone oil-bath for one hour. The fused mass was warmed with 2 ml. of 2 *N* sodium hydroxide and filtered. The insoluble material was washed with a little water and the washings were combined with the alkaline filtrate. The combined solution was acidified with 2 ml. of glacial acetic acid. The crude 8-thiouric acid that separated was centrifuged, washed with water, and then dissolved in 5 ml. of a saturated solution of sodium bicarbonate. The brownish material that was insoluble in sodium bicarbonate was removed by filtration. Upon treatment with 5 ml. of glacial acetic acid, the 8-thiouric acid was obtained from the golden yellow filtrate as a brownish-yellow microcrystalline precipitate. It was purified simply by washing thoroughly with water followed by ethanol; yield 100 mg. (56%). This material appeared to be hydrated.

Anal. Weight loss after drying *in vacuo* at 120°. Calcd. for $C_5H_4N_4O_2S \cdot H_2O$: 8.9. Calcd. for $C_5H_4N_4O_2S \cdot 1.5H_2O$: 12.8. Found: 10.1.

However, the powder gave a satisfactory analysis for anhydrous 8-thiouric acid.

Anal. Calcd. for $C_5H_4N_4O_2S$: C, 32.60; H, 2.19. Found: C, 32.98; H, 2.34.

Method 2.—4,5-Diaminouracil sulfate (100 mg.) was dissolved in 10 ml. of water in a pressure bottle. To the solution was added 1 ml. of 2 *N* potassium hydroxide, 15 ml. of ethanol and 0.5 ml. of carbon disulfide. The pressure bottle was tightly closed and carefully heated in a steam-bath at 100° for one hour, then cooled to room temperature. It was cautiously opened and its contents were filtered. The filtrate was acidified with 5 ml. of glacial acetic acid and chilled. The slightly brown crystalline precipitate was removed by filtration, washed with water and dried; yield 70 mg., 78%. It was identical with the product of method 1.

8-Thiouric acid, in common with its 2- and 6-isomers, is sparingly soluble in most solvents; m.p. also above 300°; R_f in the ammonium sulfate–water–isopropyl alcohol system, 0.18; ultraviolet absorption characteristics in aqueous solutions (see Fig. 2), at *p*H 1.6: λ_{max} 261 mμ, log ε 4.02; 303 mμ, log ε 4.26; at *p*H 12.4; λ_{max} 240 mμ, log ε 4.19; 305 mμ, log ε 4.12.

It is clear that the three monothiated uric acids could easily be characterized by virtue of their distintive ultraviolet spectra.

Isolation[11] and Identification of 6-Thiouric Acid from the Urine of Patients Treated with 6-Mercaptopurine.—After an intravenous injection of 6-mercaptopurine (sodium salt, 1.0 to 1.5 g. per patient, *p*H adjusted to 8), the 24-hour urine specimen of the patient was collected, filtered, and treated with boric acid (about 1 g. per 100 ml.). The acidic constituents of the urine were adsorbed on a column (inside diameter 4.5 cm.) packed with Dowex 1- X8 resin, chloride form, 200–400 mesh, to a height of 14 cm. The free bases passed through the column unadsorbed, while the weaker acid fraction was eluted with 5 resin volumes of 1% acetic acid. The stronger acids, including 6-thiouric acid, were stripped off the column with 2% hydrochloric acid, and this eluate was freeze-dried and reconstituted to 100–200 ml. with water. After adjustment of *p*H to 7 with sodium hydroxide, it was then placed on a Dowex 1- X8, chloride, 200–400 mesh, resin column (inside diameter 2 cm., height of wet resin, 15 cm.). The final elution was accomplished with 14 liters of dilute hydrochloric acid in a gradient system varying from 0.001 to 0.010 *N*. Spectrophotometric analysis indicated that the last fractions, roughly the 11th and the 12th liters of eluate

(10) M. T. Bogert and D. Davidson, This Journal, **55**, 1667 (1933).

(11) The modified ion-exchange chromatography procedure was adapted from W. E. Cohn in E. Chargaff and J. N. Davidson, "The Nucleic Acids," Academic Press, Inc., New York, N. Y., Vol. 1, 1955, p. 211.

that came off the column, contained exclusively 6-thiouric acid. These were combined and freeze-dried. The resultant yellow microcrystalline powder was recrystallized from boiling water; m.p. above 300°.

Anal. Calcd. for $C_5H_4N_4O_2S \cdot H_2O$: S, 15.86. Found: S, 15.65.

This material was entirely identical with the synthetic 6-thiouric acid with respect to its ultraviolet absorption in acid and alkali, and its chromatographic behavior but distinctly different in ultraviolet absorption from both the 2- and 8-thiouric acids described above.

Demonstration of the Occurrence of 6-Thiouric Acid in the Urine of Mice Treated with 6-Mercaptopurine.—The urine excreted by ten mice each of which had received an intraperitoneal injection of 1 mg. of 6-mercaptopurine (sodium salt, concentration about 1 mg. per ml., *p*H adjusted to 8)[1] was pooled, filtered, and concentrated to one-tenth volume at freezing point. The concentrated urine was chromatogrammed on Whatman no. 1 paper in the ammonium sulfate–water–isopropyl alcohol system. For comparison, the urine of ten untreated mice was likewise concentrated and chromatogrammed on paper. Under ultraviolet light, four spots present only in the chromatogram of the urine of treated mice were rendered visible; R_f, 0.19, 0.32, 0.45 and 0.54, respectively. The spot of R_f 0.19 was shown to be 6-thiouric acid spectrophotometrically, whereas the spot of R_f 0.32 had a spectrum quite similar to that of 6-mercaptopurine except at shorter wave length (below 250 mμ). The other two spots were not identified.

Discussion

The oxidation of 6-mercaptopurine to 6-thiouric acid appears to be a metabolic process common to humans and mice.[1,2] Leukemic patients who were treated with 50–200 mg. of 6-mercaptopurine per patient a day, regularly excreted about 15% of the daily dose as 6-thiouric acid.[12] The mice were found to behave similarly. Besides, it was observed by the present authors,[13] independently of Elion[1] and of Carey,[14] that the incubation of 6-mercaptopurine with xanthine oxidase in a *p*H 7.52 phosphate buffer solution at 37.5° resulted in the rapid formation of 6-thiouric acid. However, it was apparent that the enzymic oxidation followed a somewhat complex course as evidenced by the simultaneous formation of a number of other not yet defined products. These are currently still under investigation.

(12) A. J. Garceau, unpublished work based on the studies of more than 20 leukemic patients of all types; see also ref. 2.

(13) T. L. Loo, to be published.

(14) N. H. Carey and H. G. Mandel, Abstracts of Papers, Fall Meeting, American Society for Pharmacology and Experimental Therapeutics, Ann Arbor, September, 1958, p. 8.

In addition to 6-thiouric acid, at least two, possibly three, other metabolites were found together with unchanged 6-mercaptopurine in the urine of patients treated with this drug. It was speculated that perhaps 6-thioxanthine, a likely intermediate in the oxidation of 6-mercaptopurine to 6-thiouric acid, might be one of the metabolites. However, all attempts have thus far failed to substantiate such a conjecture.

The previous observation[15] that in xanthine the 6-hydroxyl group, in preference to the 2-hydroxyl group, is readily replaced by a mercapto group through the action of phosphorus pentasulfide could now be extended to uric acid. In other words, of the three hydroxyl groups in uric acid, the 6-hydroxyl is the only one directly replaceable by a mercapto group.

Considerable difficulties were encountered in the preparation of analytical specimens of the anhydrous forms of the thiouric acids. In view of the recent work of Albert,[16] it is conceivable that the thiouric acids as well as 6-mercaptopurine add water spontaneously to the ethylenic double-bond of the π-electron deficient purine ring, analogous to 6-hydroxypteridine. This would perhaps account for their tenacious retention of water.

Recently Loo and Michael[17] reported a colorimetric method for the determination of 6-mercaptopurine and related compounds in biological fluids. In pharmacological work, it is evident that the findings could not be meaningful unless the method is specific for the drug under study. It is therefore gratifying that 6-thiouric acid fails to demonstrate the color reaction reported for 6-mercaptopurine.

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(15) A. G. Beaman, THIS JOURNAL, **76**, 5633 (1954).

(16) A. Albert, D. J. Brown and G. Cheeseman, *J. Chem. Soc.*, 1620 (1952).

(17) T. L. Loo and M. E. Michael, *J. Biol. Chem.*, **232**, 99 (1958).