per 102 mg. of this substance, this would calculate to 36 μmole split per 0.3 μmole blood group substance or about 110 galactoses per molecule. There is no necessity for all of these to be B specific groupings and, in addition, if chains of α-linked galactoses occurred, these would be split sequentially and the estimate of terminal galactose would be reduced. The value thus provides a theoretical upper limit for the number of B groups per molecule. A relatively small number of B specific groupings, however, would adequately account for precipitation and hemagglutination inhibition.

The present study opens numerous avenues for further investigation. The cross reactivity and residual precipitating power for anti-B of the enzyme-treated B and BP1 substances should be studied by oligosaccharide inhibition techniques. Further degradation with other enzymes may yield additional information about the sequences involved in the various specificities.

[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, THE HEBREW UNIVERSITY-HADASSAH MEDICAL SCHOOL JERUSALEM, ISRAEL]

The Enzymatic Oxidation of 6-Mercaptopurine to 6-Thiouric Acid[1]

BY FELIX BERGMANN AND HANNA UNGAR

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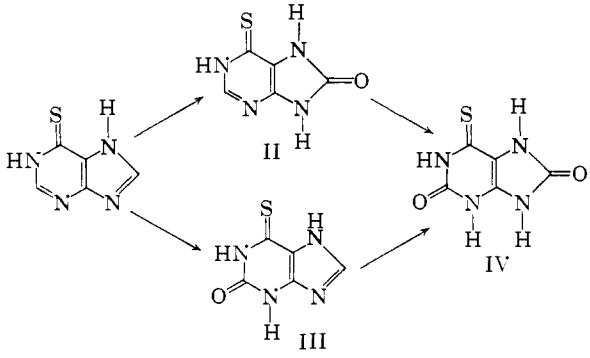
6-Mercaptopurine is attacked by xanthine oxidase first at carbon atom 8 and then at 2. The intermediate, 6-mercapto-8-hydroxypurine (II), cannot be isolated, as a result of its low concentration during the steady state of the reaction. However, II can be identified unequivocally by the use of isosbestic points. The pathway of oxidation of 6-mercaptopurine is different from that of hypoxanthine but corresponds to that of 4-hydroxypteridine.

From the urine of humans and mice, treated with 6-mercaptopurine (I), 6-thiouric acid (IV) has been isolated.[2] The metabolite is formed also *in vitro* under the catalytic influence of xanthine oxidase (XO).[3] The oxidation of I thus may appear to be in complete analogy with the conversion of hypoxanthine to uric acid. However, the pathway of the over-all reaction I → IV so far has not been elucidated.

In early experiments on this problem, we were unable to detect on paper chromatograms, developed at intermediate stages of the oxidation by XO, either of the two potential intermediates, *viz.*, 6-mercapto-8-hydroxypurine (II) or 6-thioxanthine (III).



The reason for our failure to isolate the intermediate became clear, when the enzymatic oxidation of synthetic II and III[4] was studied. In Fig. 1, the absorption spectra of both monohydroxy-6-thiopurines and of 6-thiouric acid are shown together. The conversion of II into IV can conveniently be measured at 305, 311 and 350 mμ. The results of a representative experiment are shown in Fig. 2, from which these various initial rates are derived: at 305 mμ, 101 μmole/h. ml.; at 311 mμ, 100 μmole/h. ml.; at 350 mμ, 115 μmole/h. ml. From these figures, the average relative rate, reported in Table I, was calculated.

TABLE I

PROPERTIES OF 6-MERCAPTOPURINE AND ITS DERIVATIVES

Compound	λ_{max} (mμ) at pH 8.0	Relative rate (%)[a]	R_F value in solvent[b] 1	2	3	Fluorescence
6-Mercaptopurine (I)	316	3.8	0.48	0.65	0.52	Yellowish
6-Mercapto-8-hydroxypurine (II)	311	23	.42	.58	.33	Pale blue-violet[c]
6-Thioxanthine (III)	341	46.5	.42	.53	.31	Yellow to white-blue
6-Thiouric acid (IV)	348		.08			Sky-blue

[a] All rates were measured with substrate concentrations of $6.5 \times 10^{-5} M$ and expressed as percentage of the rate of xanthine oxidation. [b] For composition of the solvents, see Experimental. [c] Compound II exhibits very weak fluorescence and thus cannot be visualized if less than 50γ is present.

The oxidation of 6-thioxanthine (III) was measured at 325 mμ (Fig. 3). The rate obtained, *viz.*, 200 μmoles/h. ml., is twice that of the oxidation of II.

As shown in Table I, the two potential intermediates are attacked by XO at rates about 6 and 12 times higher than the oxidation of 6-mercaptopurine. Therefore, already in the early part of the reaction a stage is reached where consumption of the intermediate keeps pace with its formation in the first step and thus prevents its accumulation.

However, the pathway of oxidation of I can be determined unequivocally by the proper use of isos-

(1) This work was supported in part by a grant from the U. S. Public Health Service, National Institutes of Health.

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

(3) (a) T. L. Loo, M. E. Michael, A. J. Garceau and J. C. Reid, THIS JOURNAL, **81**, 3039 (1959); (b) G. B. Elion, S. Mueller and G. H. Hitchings, *ibid.*, **81**, 3042 (1959).

(4) E. C. Moore and G. E. Le Page, *Cancer Research*, **18**, 1075 (1958), reported the enzymatic conversion of III into 6-thiouric acid but did not measure the rate.

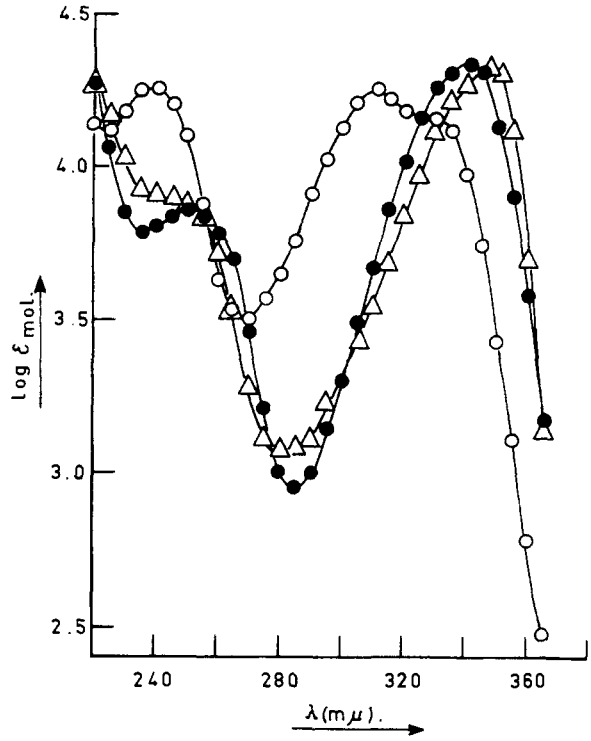


Fig. 1.—Ultraviolet absorption spectra of 6-mercapto-8-hydroxypurine (II), (O—O); 6-thioxanthine (III), (●—●); and 6-thiouric acid (IV), (△—△); *p*H 8.0 (10^{-2} *M* phosphate buffer).

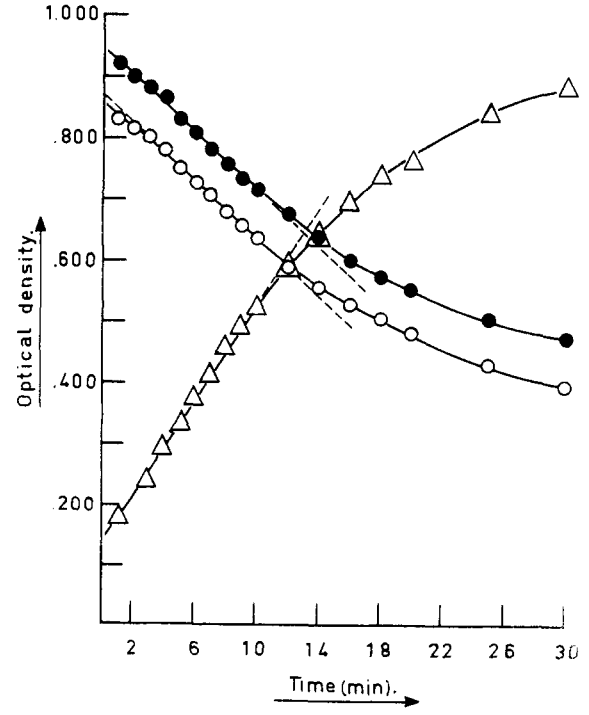


Fig. 2.—Changes of optical density during the oxidation of 6-mercapto-8-hydroxypurine to thiouric acid: XO, 1:4000; substrate, 6.5×10^{-5} *M*; *p*H, 8.0; temp., 28°: ●—●, 311 mμ; O—O, 305 mμ; △—△, 350 mμ.

bestic points. It is seen in Fig. 4 that at 308 mμ, the isosbestic point of I and II, any initial *decrease*

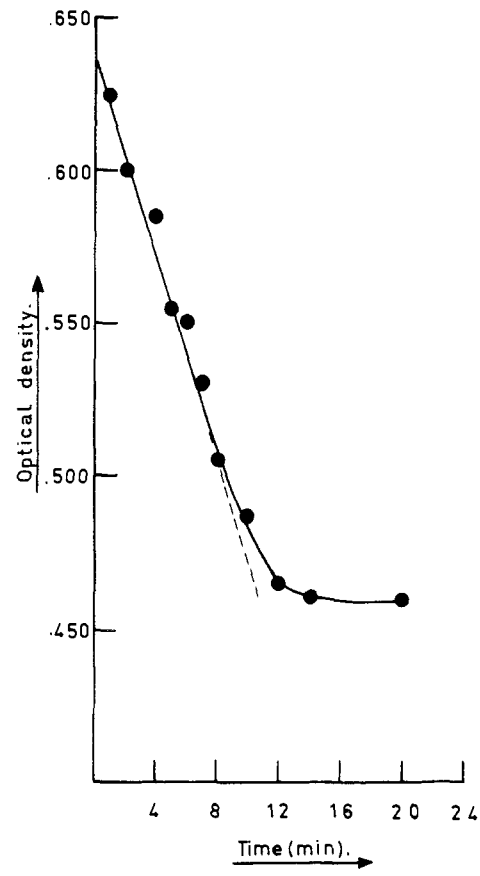


Fig. 3.—Changes of optical density at 325 mμ during the conversion of 6-thioxanthine to 6-thiouric acid. Conditions are as in Fig. 2.

in optical density (o.d.) will measure the formation of III. On the other hand, at 240 mμ, the isosbestic point of I and III, the appearance of II can be recognized by an initial *rise* in optical density. Finally, at 256 mμ, the two potential intermediates II and III possess an isosbestic point. Here, therefore, any initial increase in o.d. measures the rate of disappearance of I, *irrespective of the pathway used*. The results of such experiments are represented in Fig. 5. Curve 1 demonstrates that during the first 20 minutes 6-thioxanthine is absent from the reaction mixture. From the initial slope of curve 2, we can derive the initial rate of formation of II, while curve 3 gives the corresponding information on the initial rate of disappearance of 6-mercaptopurine. As shown in Table II, these two rates are practically identical, thus supporting the conclusion that most—if not all—of the substrate is oxidized by XO along the pathway I → II → IV.

From the maximal increase of o.d. in curve 2, it is possible to calculate the amount of II that is present during the steady state, *i.e.*, at the horizontal portion of curve 2, as about 1.5×10^{-6} *M* or approximately 0.25 γ/ml. This explains our failure to detect the intermediate on paper chromatograms, when the reaction was interrupted after 20 min.

Discussion

From these experiments the important fact emerges that 6-mercaptopurine differs from hypo-

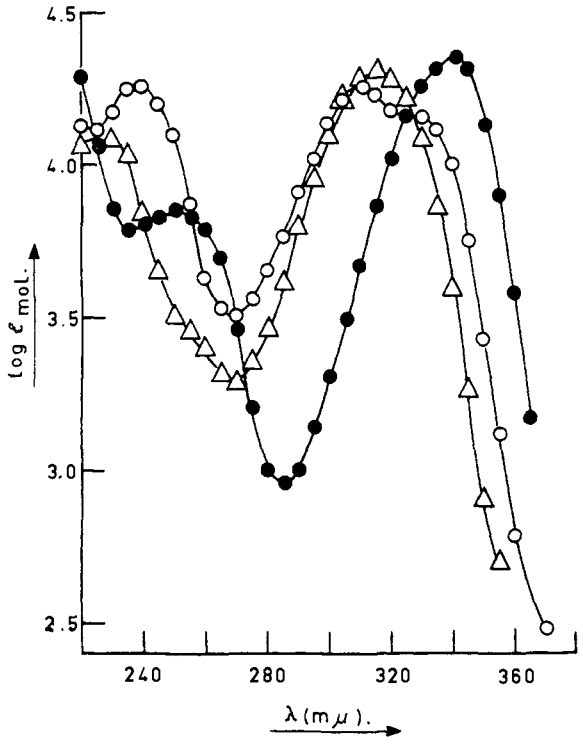


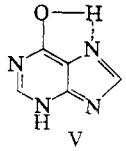
Fig. 4.—Ultraviolet absorption spectra of 6-mercaptopurine and its monohydroxy derivatives at *p*H 8.0: △—△, 6-mercaptopurine; ○—○, 6-mercapto-8-hydroxypurine; ●—●, 6-thioxanthine.

TABLE II

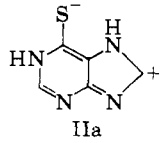
RATE OF THE ENZYMATIC OXIDATION OF 6-MERCAPTOPURINE (I) TO 6-MERCAPTO-8-HYDROXYPURINE (II)
XO, 1:4000; substrate, 10γ/ml.; *p*H, 8.0; temp. 28°

	Wave length (mμ) 240	256
Measured change of optical density (Fig. 5)	0.186/h	0.06/h
Change of o.d. per mole	1.17×10^4	0.38×10^4
Rate (μmole/h ml.)	15.9	15.8
Relative rate (%) (xanthine = 100)	3.7	3.7

xanthine in its behavior toward XO. The structures assumed by these purines when entering the activated ES-complex must therefore also differ. The behavior of 6-mercaptopurine may be ascribed to the specific properties of the sulfur atom which exhibits only a weak tendency to form hydrogen bonds and thus resists transformation into a tautomeric form, analogous to the activated form of hypoxanthine (V), postulated previously.[5] In the absence of such an activation process, the polarity of the molecule in the ground state, exemplified in IIa, becomes the decisive factor for orienting the enzymatic attack, *i.e.*, for the primary attachment of an hydroxyl ion.



V



IIa

(5) F. Bergmann, H. Kwietny, G. Levin and D. J. Brown, THIS JOURNAL, **82**, 598 (1960).

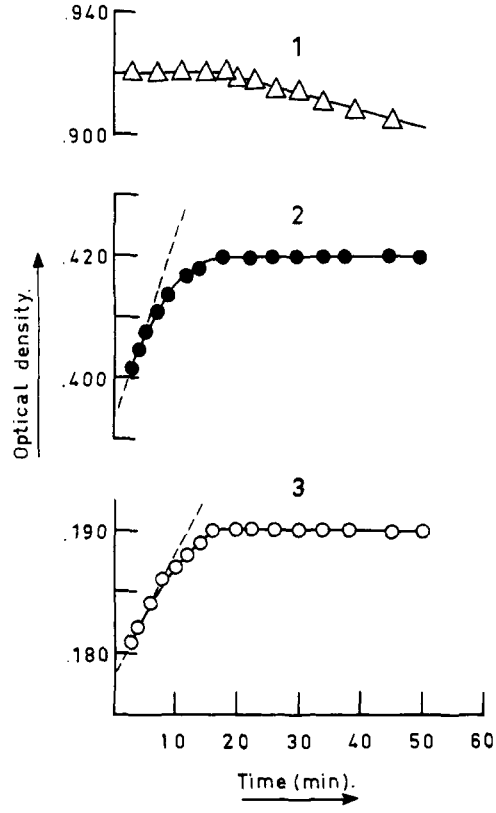


Fig. 5.—Initial changes of optical density during the oxidation of 6-mercaptopurine; conditions as in Fig. 2: curve 1, at 308 mμ; curve 2, at 240 mμ; curve 3, at 256 mμ. The decline in curve 1 after 20 min. is due to the increasing concentration of 6-thiouric acid.

It should be recalled that similar considerations have been applied to 4-hydroxypteridine which is attacked first at position 7, *i.e.*, in the pyrazine ring, before being converted to 2,4,7-trihydroxypteridine.[6] This behavior was ascribed to the lack of an NH-group in peri position to the carbonyl group. In 6-mercaptopurine, on the other hand, where an NH-group is potentially available in the proper location, it cannot be utilized efficiently by the sulfur atom.

Similar modifying effects of the 6-mercapto group also should be expected in 6-thioxanthine and 6-mercapto-8-hydroxypurine. The former reacts at about half the speed of xanthine, while with the latter the rate is lowered to one quarter of that of the oxygen analog. For a better understanding of the mechanism of these reactions, it is necessary to determine whether in the 6-thio series N-methyl substitution exerts the same pronounced influence on the susceptibility of these substrates to attack by XO, as has been found for hydroxypurines.[5]

Experimental

6-Mercaptopurine was a commercial product (California Foundation for Biochemical Research). **6-Thioxanthine** was a gift from the Upjohn Company, Kalamazoo, Michigan, while **6-mercapto-8-hydroxypurine** was obtained through the courtesy of Dr. G. B. Elion, Wellcome Research Labora-

(6) F. Bergmann and H. Kwietny, *Biochim. Biophys. Acta*, **33**, 29 (1959).

tories, Tuckahoe, N. Y. The latter product was not pure and had to be purified by paper chromatography, using solvent 1 (see below). Thereafter, this compound gave the same quantitative yield of 6-thiouric acid as 6-thioxanthine. IV was made according to the method of Loo, *et al.*[3a] The synthetic product was in all respects identical with the material isolated from the enzymatic oxidation of I, II or III.

Milk xanthine oxidase was a gift of Prof. F. Bergel and Dr. R. C. Bray, Chester Beatty Institute of Cancer Research, London, England. This preparation, when diluted 1:4800, produced at *p*H 8.0 and 28° 1γ of uric acid/ml. min., with 6.5×10^{-5} *M* xanthine as substrate. In order to prevent inactivation by H_2O_2, all solutions of XO contained also catalase (Worthington), 1:500.

The enzyme experiments were carried out in a Beckman ultraviolet spectrophotometer, equipped with a thermospacer. The changes in optical density represent the difference between the readings with the reaction mixture and a control vessel, containing all components besides XO. The *p*H was kept at 8.0 by the use of 0.01 *M* phosphate buffer. All purines used in the present investigation are stable in solution. Therefore, no change in the absorption of the controls was detected.

For paper chromatography on Whatman paper No. 1, the descending method was used with the following solvents:

Solvent 1—95% ethanol, 85 vols.; acetic acid, 5 vols.; water, 10 vols.

Solvent 2—2-propanol, 65 vols.; dimethyl formamide (DMF), 25 vols.; water, 10 vols.

Solvent 3—2-propanol, 65 vols.; DMF, 25 vols.; 25% ammonia, 10 vols.

The R_f values of the purines are included in Table I. For separation of I from II, solvent 3 is most suitable.

For location of the spots, a Mineralight ultraviolet lamp, which emits radiation of about 255 mμ, was used.

[Contribution from the Department of Chemistry of the University of Wisconsin]

Piperidine Derivatives. XXXI. Certain 6-Oxo-octahydro- and Decahydroisoquinolines and Related Compounds

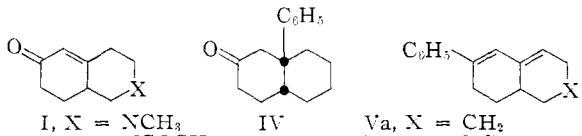
By S. M. McElvain and David C. Remy[1]

Received November 30, 1959

$\Delta^{1(9)}$-Octalone-2 (III) yields *cis*-9-phenyldecalone-2 (IV) by the 1,4-addition of phenylmagnesium bromide in the presence of catalytic amounts of cuprous salts. Under these conditions the 2-acetyl-6-oxo-octahydroisoquinoline II shows no 1,4 addition of the Grignard reagent; II is hydrogenated in acidic methanol to approximately equal amounts of *cis*- and *trans*-2-acetyl-6-oxo-decahydroisoquinoline XI and XII which show anomalous behavior with most of the ordinary carbonyl reagents. The amido ketone II shows an ultraviolet absorption maximum in ethanol at 241 mμ, which corresponds closely to the calculated value (244 mμ); the absorption maximum does not vary significantly with the polarity of the solvent. The amino ketone I yields an allylic chloride XVI that reacts readily with phenol to yield 2-methyl-6-(*o*-hydroxyphenyl)-1,2,3,4,6,7,8,9-octahydroisoquinoline (XVII).

Earlier work in this Laboratory showed that phenyllithium and phenylmagnesium bromide added to the carbonyl group of the 6-oxo-octahydroisoquinoline I with no indication of any 1,4-addition to give the 10-phenylated saturated ketone.[2] Subsequent experiments to promote 1,4-addition by use of catalytic amounts of cuprous salts[3] were unsuccessful due to complexing of the basic amino group with the catalyst.

It appeared that this deactivation of the catalyst might be circumvented by the use of the N-acetyl compound II, but before undertaking this project it seemed desirable to determine whether phenylmagnesium bromide could be made to add in the 1,4-manner to the octalone III, to which Birch and Robinson had added methylmagnesium iodide in the presence of cuprous bromide and obtained *cis*-9-methyl-2-decalone in 60% yield.[4]



I, X = NCH_3
II, X = $NCOCH_3$
III, X = CH_2

IV

Va, X = CH_2
Vb, X = NCH_3

It was found possible to phenylate the angular position of III with phenylmagnesium bromide in the presence of cuprous chloride to obtain *cis*-9-phenyl-2-decalone (IV) in 25% yield. The major product (66%) was the hydrocarbon Va, which had a broad band in its ultraviolet spectrum at 284 mμ (log ϵ 4.16) that is indicative of the phenylbutadiene chromophore.[5] The formation of Va doubtless results from the 1,2-addition of the Grignard reagent to III followed by an allylic rearrangement of the resulting carbinol and subsequent dehydration in the same manner that Vb was formed from I.[2] The similarity of the ultraviolet absorption maxima of the two dienes Va and Vb was quite close. No satisfactory analyses could be obtained for Va; on standing it became increasingly viscous and finally turned to a polymeric gel. During this process its weight increased probably by absorption of oxygen.

When the reaction of III with phenylmagnesium bromide was carried out in the absence of the cuprous salt, none of the ketone IV was obtained.

The ketone IV had a sharp melting point and was quite probably stereochemically homogeneous. It was reduced by the Wolff–Kishner reaction to one of the 9-phenyldecalins, whose homogeneity was shown by distillation into fractions of identical refractive index, and its conversion to a single crystalline sulfonamide, m.p. 196°. The *cis* structure VI was assigned to this hydrocarbon after a comparison of its properties with those of the 9-phenyl-

(1) Wisconsin Alumni Research Foundation Research Assistant 1956–1958; du Pont Summer Research Assistant 1958.

(2) S. M. McElvain and P. H. Parker, This Journal, **78**, 5312 (1956).

(3) M. S. Kharasch and O. Reinmuth, "Grignard Reactions of Nonmetallic Substances," Prentice-Hall, Inc., New York, N. Y., 1954, p. 219.

(4) A. J. Birch and R. Robinson, *J. Chem. Soc.*, 501 (1943).

(5) E. A. Braude, *et al.*, *J. Chem. Soc.*, 1087 (1947); O. Grummitt and F. J. Cristoph, This Journal, **73**, 3497 (1951).