then acidified with excess (*ca.* 80 ml.) of concentrated hydrochloric acid. The product was then extracted with ether and the ether solution was dried with Drierite, filtered, then concentrated. The residue was distilled under reduced pressure to give IV, 19 g. (57%), b.p. 82–85° (0.45–0.5 mm.), $n^{25}D$ 1.4826 to 1.4833, for the four fractions collected; neut. equiv. 170, 171 (theory 168.2).

The dicyclohexylamine salt of IV, m.p. 163–164°, was prepared from this distillate in petroleum ether and recrystallized from ether.

Anal. Calcd. for $C_{17}H_{36}NO_2PS$: C, 58.41; H, 10.38; N, 4.01. Found: C, 58.5; H, 10.5; N, 4.2.

O-Methyl Ethylphosphonothioic Acid (V).—O,O-Dimethyl ethylphosphonothioate[10] (30 g., 0.20 mole), $n^{25}D$ 1.4753 (reported 1.4722), was hydrolyzed by stirring with 165 ml. of 1 *N* base (0.165 mole, 85%) at *ca.* 45° for 24 hours. About 4 ml. of unreacted starting material was still present as a second phase after this time. The reaction mixture was worked up as described[3]; the residue thus obtained was distilled to give V, 10 g., b.p. 66–70° (0.18 mm.), $n^{25}D$ 1.4976, neut. equiv. 145 (theory 140).

Anal. Calcd. for $C_3H_9O_2PS$: C, 25.68; H, 6.47. Found: C, 25.8; H, 6.4.

The dicyclohexylamine salt of V, m.p. 185°, was prepared in petroleum ether and recrystallized from acetone–ether.

Anal. Calcd. for $C_{15}H_{32}NO_2PS$: C, 56.04; H, 10.03; N, 4.36. Found: C, 55.8; H, 9.6; N, 4.4.

Resolutions.—The acids were resolved by recrystallization of their diastereoisomeric quinine (though in one case, brucine) salts from acetone solution until constant melting points were obtained. Because a variation in the melting points of these alkaloid salts could generally be obtained by varying the rate of heating, the melting point of a recrystallized crop was customarily determined simultaneously with that of the previously recrystallized crop in order to determine whether a change had occurred. The acids were liberated from their alkaloid salts as previously described,[2] except that the aqueous solutions of the acids were saturated with sodium chloride before extraction with ether. Except as indicated, the free acids were not isolated, but were converted into their dicyclohexylamine salts for characterization. The latter were obtained by distilling off the ether from the O-alkyl alkylphosphonothioic acid solution, taking up the residue in petroleum ether, and adding a slight excess of dicyclohexylamine. Occasionally, the amine was added before the distillation of the ether. The dicyclohexylamine salts thus obtained were recrystallized from ether or acetone–ether.

The (−)-antipodes of these acids, with the exception of VI, were obtained apparently optically pure from the corresponding quinine salt head crops. Of the levorotatory acids, only that of III was isolated as the free acid: b.p. 74–81° (0.3–0.5 mm.), $n^{25}D$ 1.4801 to 1.4806 (range of four fractions collected), α_{obsd} −13.92 ± 0.02° (neat, 1 dm.).

Of the dextrorotatory acids in this series, that of II was obtained from the head crop of its brucine salt, those of III, IV and V from the tail crops, VI from the head crop, of their respective quinine salts, all from acetone solution. In this manner, the (+)-antipodes of II and of III were obtained apparently optically pure; that of IV in a nearly (*ca.* 93%) optically pure state; that of V in an approximately 52% optically pure state. In this series, only the (+)-antipodes of II and III were isolated as the free acids: (+)-II, b.p. 79–84° (0.9–1.1 mm.), $n^{25}D$ 1.4920, $\alpha^{28.5}_{obsd}$ +9.57° (neat, 1 dm.); (+)-III, b.p. 59–64° (0.22–0.25 mm.), $n^{25}D$ 1.4802, α^{30}_{obsd} +13.59 ± 0.01° (neat, 1 dm.). Unlike as in the cases of I and II, the (+)-IV and (+)-V acids could not be conveniently obtained by resolution as a brucine salt from acetone solution. With IV, the diastereoisomeric brucine salts were too similar in their acetone solubilities to yield an effective separation, while with V, brucine formed a more insoluble salt with the (−)-antipode.

The resolution of VI was unique in two respects. First, both quinine and brucine formed a more insoluble salt with the *dextro* enantiomorph in acetone solution. Secondly, the head and the tail crop quinine salts, though of different crystalline character, had the same melting and mixed melting points, and their specific rotations were very similar. Therefore, neither the melting points nor the specific rotations of these quinine salts could be used to follow the extent of the separation of the head and tail crops in this system. The resolution was followed, however, by converting the various fractions to dicyclohexylamine salts for determination of their specific rotations. The highest specific rotation of a dicyclohexylamine salt obtained from this system was +6.6°. This derivative was prepared from a quinine salt obtained after seven recrystallizations of the original head crop. That this rotation represents the maximum obtainable for resolved VI is uncertain, since insufficient quinine salt remained for an eighth recrystallization. No attempt was made to carry the tail crop through to the same state of optical purity as was attained here for the head crop. Attempts to resolve VI with brucine in acetone or benzene–ether solutions, though not as carefully investigated, led to the same experimental difficulties.

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(10) F. W. Hoffmann, D. H. Wadsworth and H. D. Weiss, THIS JOURNAL, **80**, 3945 (1958).

[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, THE HEBREW UNIVERSITY—HADASSAH MEDICAL SCHOOL, JERUSALEM, ISRAEL, AND THE DEPARTMENT OF MEDICAL CHEMISTRY, AUSTRALIAN NATIONAL UNIVERSITY, CANBERRA]

The Action of Mammalian Xanthine Oxidase on N-Methylated Purines

BY FELIX BERGMANN, HANNA KWIETNY,[1] GERSHON LEVIN[1] AND D. J. BROWN

RECEIVED JUNE 9, 1959

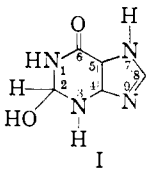
N-Methylation may alter either the rate or the course of the enzymatic oxidation of purines. 2-Hydroxypurine and its 1-methyl derivative are both attacked at carbon atom 8, while the 3-methyl compound gives 3-methylxanthine. 8-Hydroxypurine and its 7-methyl derivative are oxidized in position 2, while the 9-methyl isomer is refractory. These and other, related observations on N-methylated mono- and dihydroxypurines lead to an explanation of the exclusive pathway of oxidation of hypoxanthine *via* xanthine to uric acid. A new hypothesis on the mode of combination of xanthine oxidase with purine substrates has been based on the assumption that the enzyme binds a specific mesomeric or tautomeric form of the substrate, regardless of whether this form represents the major structure, present in solution.

In a recent study on the specificity of mammalian xanthine oxidase (XO),[2] a hypothetical mechanism for the oxidation of purines was derived from the behavior of hydroxypurines and certain of their N-methyl derivatives. It was assumed that purines become adsorbed onto the isoalloxazine ring of the prosthetic group in such a way that direct transfer of two hydrogens from the system $HN^{3}—C^{4}=C^{5}—NH^{7}$ (as, *e.g.*, in the hydrated form of hypoxanthine [I]) to the corresponding positions N-1 and N-10 in the flavin moiety can take place. This hypothesis required that—when necessary—a hydrogen atom be placed on N-3 of the substrate by a hydration step, prior to the hydrogen transfer.

(1) Part of Ph.D. theses, submitted to the Faculty of Science, The Hebrew University, Jerusalem, Israel, 1960.

(2) F. Bergmann and S. Dikstein, *J. Biol. Chem.*, **223**, 765 (1956).

Extension of the experiments to 8-azapurines[3] and pteridines[4] raised doubt as to the general validity of the above reaction scheme. Especially in the pteridine series the corresponding, central, unsaturated system, comprising the chain $\overset{1}{N}—C=C—\overset{5}{N}$ (II), does *not* participate in the enzymatic reaction, since carbon atom 6 is the only position, resistant to oxidation. As the original hypothesis thus proved inapplicable to other heterocycles, it became questionable whether it was correct even for the purines themselves. Re-investigation of the mechanistic problem with new methyl derivatives of purines revealed indeed that in this series, too, examples can be found, the behavior of which toward XO is incompatible with the assumption of a central, hydrogen-transferring structure, common to all substrates. By systematic substitution of all NH-groups of a given structure, it became possible to elucidate the factors, which determine the susceptibility of purines to enzymatic attack and direct the latter toward certain positions, in preference to others.



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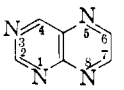


II

(hydrated form of hypoxanthine)

Experimental

Substrates. Purine.—The 7-methyl derivative[5] was a gift from Dr. A. Bendich, Sloan-Kettering Institute for Cancer Research, New York. The 9-methylpurine was made according to Albert and Brown.[6]

2-Hydroxypurine.—The 1- and 9-methyl derivatives were made according to Johns.[7,8] The 3-methyl derivative, originally described by Tafel and Weinschenk,[9] was synthesized by a new method to be reported later.

8-Hydroxypurine.—The 7- and 9-methyl derivatives were prepared by known methods.[10]

Hypoxanthine.—The 3-methyl derivative[11] was obtained through the courtesy of Dr. G. B. Elion, Wellcome Research Laboratories, Tuckahoe, N. Y.

2,8-Dihydroxypurine.—The 1- and 9-methyl derivatives were synthesized according to Johns[8,12] and the 3-methyl isomer by a method, described previously.[13] The hitherto-unknown 7-methyl derivative was obtained as the oxidation product of 7-methyl-8-hydroxypurine (see under Results).

6,8-Dihydroxypurine.—The 9-methyl derivative was made by a method, described previously.[14] The preparation of the 1-methyl isomer will be published later.

Enzymes.—Highly purified xanthine oxidase from cow's milk (XO) was a gift of Prof. F. Bergel and Dr. R. C. Bray, Chester-Beatty Institute of Cancer Research, London. This preparation, at a dilution of 1:800, produced 1 γ of uric acid per ml. per min., when xanthine, 6.5×10^{-5} *M*, served as substrate. The *p*H in all runs was 8.0 (0.01 *M* phosphate buffer).

To protect XO against inactivation by H_2O_2, catalase (Worthington Biochemicals Corp.), 1:500, was added.

The dilutions of XO, actually used for each substrate, are recorded in Table II. However, the rates in the table are calculated for a standard dilution of 1:800 to permit comparison with the reference substrate xanthine.

Method.—Four Beckman quartz cells were kept in a thermospacer at 28°. The first contained substrate ($6–7 \times 10^{-5}$ *M*) and buffer and the second in addition catalase. These two vessels served as controls. The third cell was charged with buffer and catalase, while the fourth one received in addition substrate. At zero time, XO was pipetted into vessel 3 and 4 and spectral measurements were made at predetermined wave lengths (see spectra in Figs. 1–8). Changes in optical density were recorded as the difference in readings between vessel 3 and 4. After the reaction had subsided, the enzymes were inactivated by immersion into boiling water for 10 min. The solution was then concentrated *in vacuo* and the concentrate spotted on Whatman paper No. 1.

Paper chromatography was carried out by the descending method, using either acid solvent A (95% ethanol, 85 vol.; water, 10 vol.; glacial acetic acid, 5 vol.) or basic solvent B (95% ethanol, 70 vol.; pyridine, 20 vol.; water, 10 vol.). The spots were located with the aid of a Mineralight ultraviolet lamp, emitting light of about 255 mμ. The purines were then extracted from the paper and their absorption spectra measured. Whenever necessary for unequivocal identification, the shift of λ_{max} as function of *p*H was also determined.[15]

The relevant physical properties of the compounds used in the present study are collected in Table I. All reactions described here consist in a single oxidation step. Therefore, not more than 2 spots were found in any paper chromatogram.

Determination of Initial Rates.—For qualitative comparison, the initial rates are very useful. They were measured by following the changes of optical density at a suitable wave length at which starting material and product exhibit a large difference in extinction. No difficulty was encountered with this simple method, because in the present investigation all reactions stopped after a single oxidation step. By plotting optical density as function of time, curves were obtained with initial straight line portions. From the latter, "initial rates" were derived. The dependence of rate on substrate concentration has been determined only for a small number of substrates so that in most cases K_M values are not available at present for comparison.

Results

1. **Purine.**—Both the 7- and 9-methyl derivatives are converted by XO into the corresponding hypoxanthines. Since the ultraviolet absorption spectra of 7-methylpurine and 7-methylhypoxanthine are very similar (see Fig. 1), measurements were taken at three wave lengths to evaluate the initial rate of oxidation (see Table II). The conversion of 9-methylpurine into 9-methylhypoxanthine was measured at 251 mμ only (Fig. 2). While substitution at N-9 decreases the rate to about $^1/_6$, a methyl group at N-7 has a much more pronounced effect, lowering the rate of purine to $^1/_{170}$!

2. **2-Hydroxypurine.**—Both the 3- and 9-methyl derivatives were attacked at carbon atom 6, giving the corresponding xanthines, as evidenced by the continuous shifting of λ_{max} during the reaction to shorter wave lengths (Figs. 3 and 4). The oxidation products were identified by comparison with authentic materials.[16,17] Rate measurements at the

(3) F. Bergmann, G. Levin and H. Kwietny, *Arch. Biochem. Biophys.*, **80**, 318 (1959).

(4) F. Bergmann and K. Kwietny, *Biochim. Biophys. Acta*, **33**, 280 (1959).

(5) E. Fischer, *Ber.*, **31**, 2550 (1898).

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

(7) C. O. Johns, *J. Biol. Chem.*, **11**, 73 (1912). We wish to thank Dr. J. J. Fox of the Sloan-Kettering Institute for a gift of the 1-methyl derivative of 2-hydroxypurine.

(8) C. O. Johns, *ibid.*, **9**, 161 (1911).

(9) J. Tafel and A. Weinschenk, *Ber.*, **33**, 3369 (1900).

(10) D. J. Brown and S. F. Mason, *J. Chem. Soc.*, 682 (1957).

(11) W. Traube and F. Winter, *Arch. Pharm.*, **244**, 11 (1906); G. B. Elion in "Chemistry and Biology of Purines," Ciba Found. Symp., 1957.

(12) C. O. Johns, *J. Biol. Chem.*, **11**, 393 (1912).

(13) D. J. Brown, *J. Appl. Chem.*, **9**, 203 (1959).

(14) E. Fischer and F. Ach, *Ber.*, **32**, 250 (1899).

(15) F. Bergmann and S. Dikstein, THIS JOURNAL, **77**, 691 (1955).

(16) W. Traube, *Ber.*, **33**, 3035 (1900).

(17) R. A. Baxter and F. S. Spring, *J. Chem. Soc.*, 232 (1945).

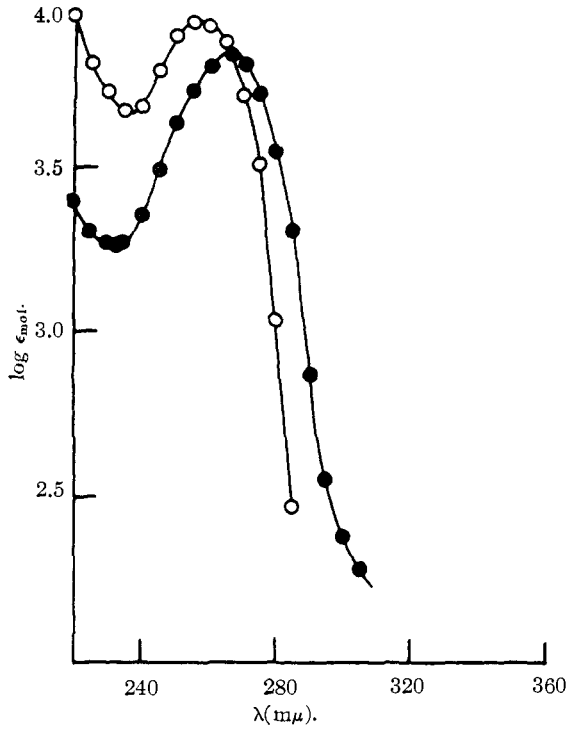


Fig. 1.—Ultraviolet absorption spectra at *p*H 8.0 of 7-methylpurine (●—●) and its oxidation product, 7-methylhypoxanthine (O—O). Isosbestic point at 267 mμ.

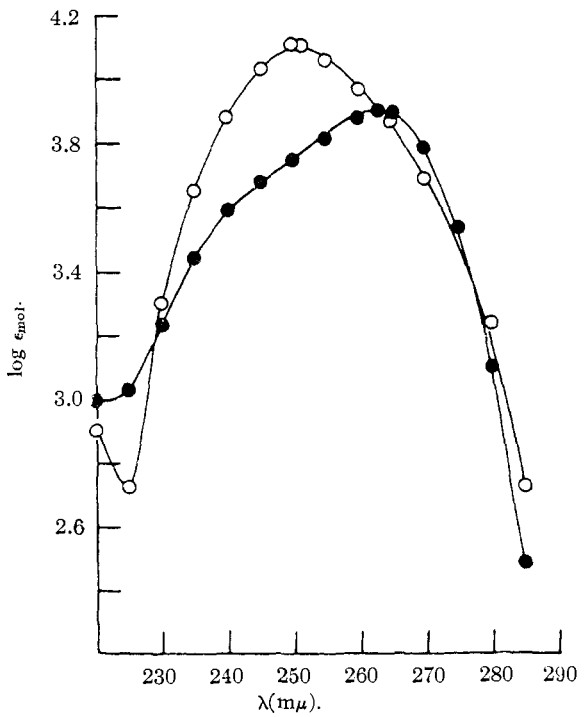


Fig. 2.—Ultraviolet spectra at *p*H 8.0 of 9-methylpurine (●—●) and its oxidation product, 9-methylhypoxanthine (O—O). Isosbestic points at 228, 262 and 277 mμ.

absorption maximum of either the starting materials (315 mμ) or the oxidation products (272 and 275 mμ, respectively) gave identical results. On the other hand, the 1-methyl derivative was oxidized in position 8 (see Fig. 5), similar to the mother substance, but at a considerably higher rate (Table II).

TABLE I

PHYSICAL PROPERTIES OF METHYLATED PURINES

	Purine derivative	λ_{max} (mμ) of neutral molecule	*Rf* Solvent A	*Rf* Solvent B	Fluorescence under UV light
1.	Purine	263	0.6	0.6	Black-violet
	7-methyl	266	.65	.55	Black-violet
	9-methyl	264	.68	.62	Black-violet
2.	2-Hydroxypurine	314	.27	.24	Blue
	1-methyl	318	.36	.30	Blue
	3-methyl	318	.5	.4	Blue
	9-methyl	314	.43	.36	Blue-violet
3.	8-Hydroxypurine	275	.6	.63	Black-violet
	7-methyl	280	.65	.6	Black-violet
	9-methyl	278	.67	.65	Black-violet
4.	Hypoxanthine	251	.52	.44	Black-violet
	3-methyl	264 ?[a]	.4		
	7-methyl	256	.54	.49	Black-violet
	9-methyl	251	.5	.44	Black-violet
5.	6,8-Dihydroxypurine	256	.34	.35	Black-violet
	1-methyl	258	.41	.46	Black-violet
	9-methyl	258	.45	.45	Black-violet
6.	2,8-Dihydroxypurine	309	.28	.28	Blue-violet
	1-methyl	311	.34	.38	Blue
	3-methyl	311	.43	.42	Blue
	7-methyl	315	.35	.10	Blue-violet
	9-methyl	308	.4	.4	Blue-violet
7.	Xanthine	267	.35	.4	Black-violet
	3-methyl	271	.43	.52	Black-violet
	9-methyl	264	.37	.16	Black-violet
8.	Uric acid	292	.26	.31	Black-violet
	3-methyl	292.5	.32	.52	Black-violet

[a] Unexpectedly, 3-methylhypoxanthine possesses 3 *pK* values. This behavior will be discussed at a later occasion.

3. Hypoxanthine.—The 1- and 7-methyl derivatives have been found previously to be refractory.[2] The same has now been observed for 9-methylhypoxanthine. On the other hand, the 3-methyl isomer is attacked very slowly at carbon atom 2. Since the spectrum of 3-methylxanthine is close to that of the original substrate (Fig. 6), the rate was again measured at three different wave lengths (see Table II). The values obtained were identical within 10%. It is noteworthy that 3-methylxanthine can be obtained by oxidation of two different substrates.

4. 8-Hydroxypurine.—Only the 7-methyl derivative was attacked, the reaction taking the same pathway as with the mother substance. The oxidation product, 2,8-dihydroxy-7-methylpurine, is unknown. The assignment of structure is based on the fact that the absorption maximum is above 300 mμ (Fig. 7) at any *p*H and that the *pK* values are similar to those of the other members of the 2,8-dihydroxypurine series (Table III). The alternative pathway to 7-methyl-6,8-dihydroxypurine would have yielded a compound with λ_{max} near to 260 mμ, as shown in Table I.

5. 2,8-Dihydroxypurine.—Out of the four methylated products tested, only the 3-methyl deriva-

TABLE II

PATHWAYS AND RELATIVE RATES OF OXIDATION OF METHYLATED PURINES

All substrates were used at concentrations of $6–7 \times 10^{-5}$ *M*. In column 3 are reported the actual enzyme dilutions, used for each substrate. However, the experimental rates were recalculated for a standard enzyme dilution of 1:800, assuming a linear relationship between enzyme concentration and rate of oxidation, and expressed as percentage of the rate of conversion of xanthine, 6.5×10^{-5} *M*, at an enzyme dilution of 1:800.

	Purine derivative	λ_{max} (mμ) at *p*H 8.0	Enzyme diln. used	Oxidn. at position no.	Wave lengths (mμ), at which rate was measured	Relative initial rate, %
1.	Purine	263	1:200	6		20
	7-methyl	266	1:50	6	240; 250; 280	0.1
	9-methyl	264	1:100	6	251	3.4
2.	2-Hydroxypurine	314	1:400	8	303; 317	16
	1-methyl	318	1:800	8	265; 290; 310	180
	3-methyl	315	1:800	6	272; 315	100
	9-methyl	313	1:400	6	275; 315	53
3.	8-Hydroxypurine	280	1:100	2	320	1.5
	7-methyl	284	1:100	2	285; 310	2.2
	9-methyl	278	Not attacked			
4.	Hypoxanthine	252	1:800	2	270	70
	1-methyl	251	Not attacked			
	3-methyl	264	1:50	2	250; 280; 285	0.15
	7-methyl	256	Not attacked			
	9-methyl	251	Not attacked			
5.	6,8-Dihydroxypurine	261	1:800	2	292	100
	1-methyl	261.5	Not attacked			
	9-methyl	260	Not attacked			
6.	2,8-Dihydroxypurine	304	1:100	6	320	0.2
	1-methyl	311	Not attacked			
	3-methyl	320	1:100	6	285; 319	2.1
	7-methyl	312	Not attacked			
	9-methyl	309	Not attacked			
7.	Xanthine	272	1:800	8	300	100
	1-methyl	271	1:800	8	300	45
	3-methyl	272	Not attacked			
	7-methyl	270	Not attacked			
	9-methyl	277	Not attacked			

TABLE III

PHYSICAL CONSTANTS OF 2,8-DIHYDROXYPURINE AND ITS METHYL DERIVATIVES

Compound	λ_{max} at *p*H 5.0	λ_{max} at *p*H 10.0	*pK* values[a] Cation	Mono-anion	Bis-anion
2,8-Dihydroxypurine	309	304	0.5	7.4	12.3
1-Methyl derivative	315	310	−0.5	7.0	13.0
3-Methyl	311	328	1.25	8.0	13.0
7-Methyl	315	310	0	7.7	12.0
9-Methyl	308	314	0.3	8.5	..

[a] Determined by the spectrophotometric method.[15]

tive is susceptible to enzymatic oxidation, forming 3-methyluric acid (Fig. 8).

6. **6,8-Dihydroxypurine.**—Neither of the two available methyl derivatives was attacked by XO (Table II).

7. **Xanthine.**—The inertness of all methyl derivatives—with the exception of 1-methylxanthine, which yields the corresponding uric acid—has been established previously.[2]

Discussion

Some of the data in Table II are clear evidence that dehydrogenation of purines cannot involve the central portion HN—C=C—NH. *E.g.*, 7-methyl-8-hydroxypurine (VII, R = CH_3) is oxidized at C-2, in spite of the blockage of the 7-position by a methyl group.

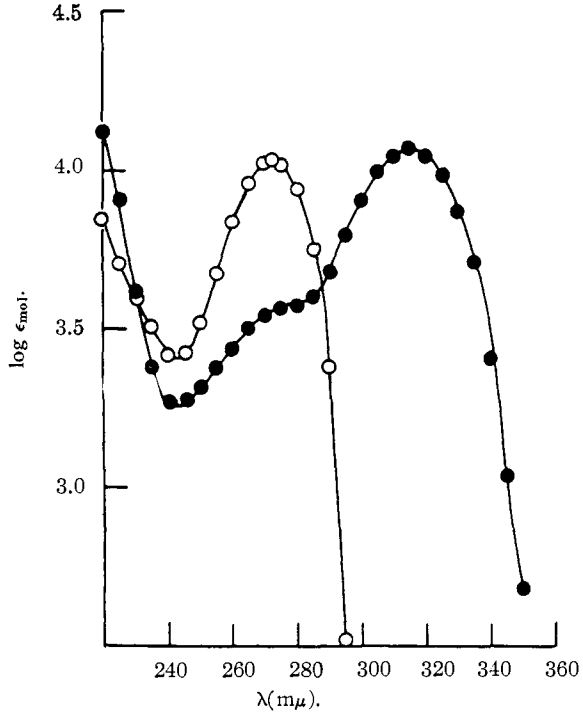


Fig. 3.—Ultraviolet spectra at *p*H 8.0 of 2,3-dihydro-2-oxo-3-methylpurine (●—●) and its oxidation product, 3-methylxanthine (○—○). Isosbestic points at 230 and 288 mμ.

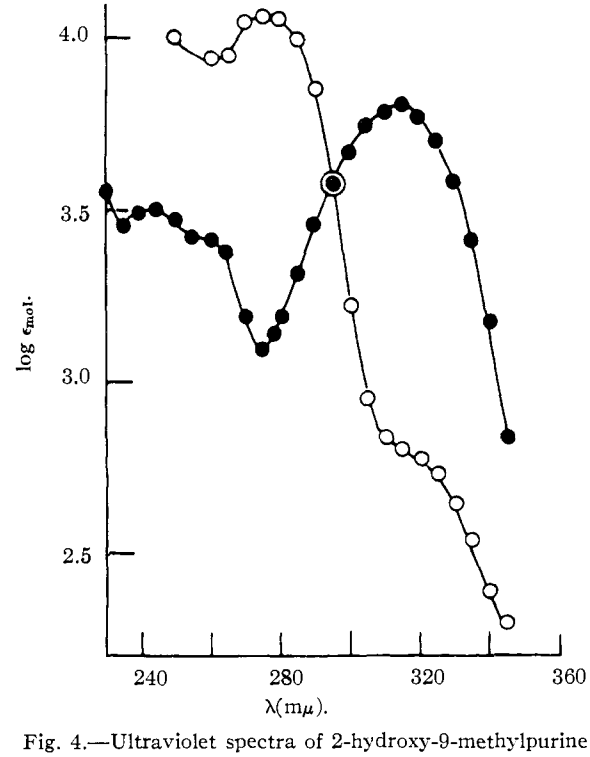


Fig. 4.—Ultraviolet spectra of 2-hydroxy-9-methylpurine (●—●) and its oxidation product, 9-methylxanthine (○—○), at *p*H 8.0. Isosbestic point at 295 mμ.

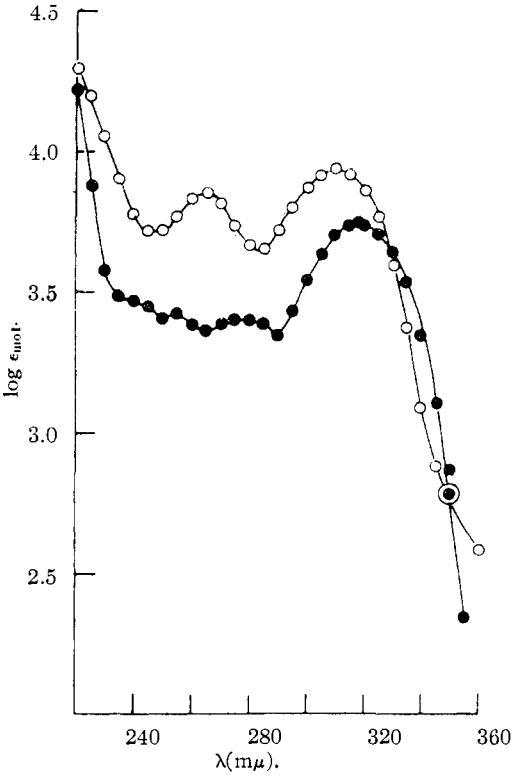


Fig. 5.—Ultraviolet absorption spectra at *p*H 8.0 of 1,2-dihydro-1-methyl-2-oxopurine (●—●) and also of its oxidation product, 1,2-dihydro-1-methyl-2-oxo-8-hydroxypurine (○—○). Isosbestic points at 329 and 350 mμ.

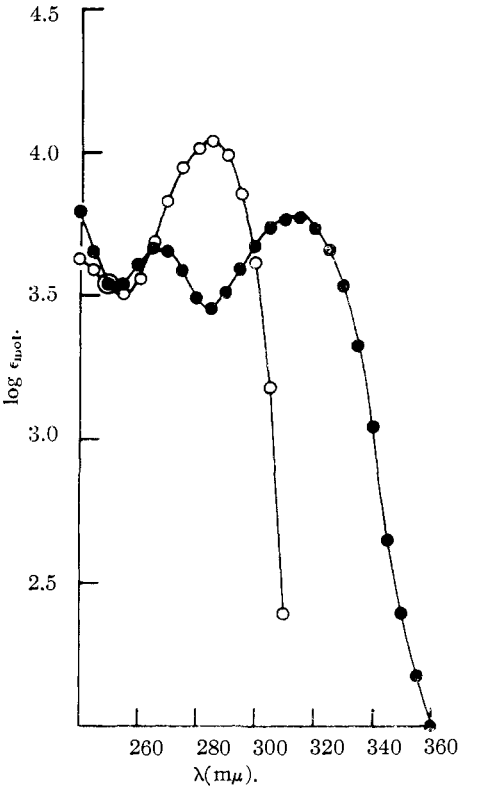


Fig. 7.—Ultraviolet spectra of 7-methyl-8-hydroxypurine (○—○) and its oxidation product, 7-methyl-2,8-dihydroxypurine (●—●), at *p*H 8.0. Isosbestic points at 251, 264 and 299 mμ.

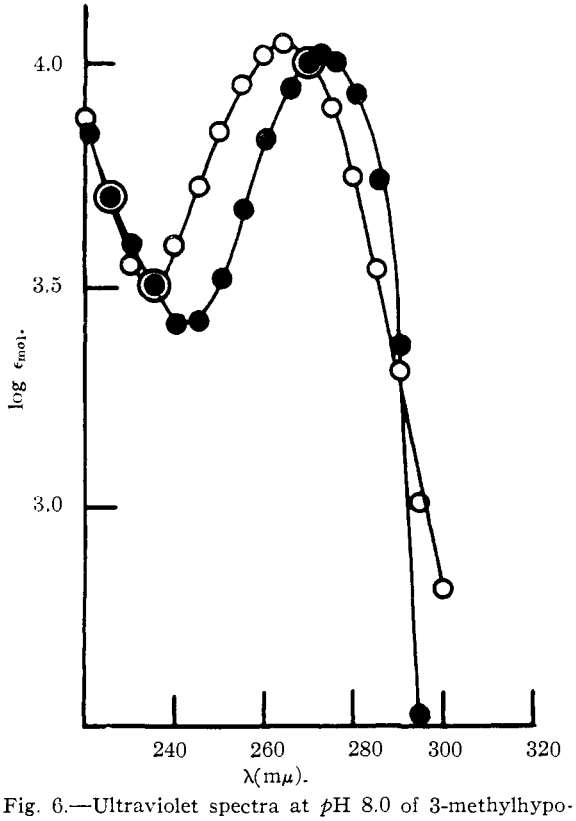


Fig. 6.—Ultraviolet spectra at *p*H 8.0 of 3-methylhypoxanthine (○—○) and its oxidation product, 3-methylxanthine (●—●). Isosbestic points at 236, 270 and 291 mμ.

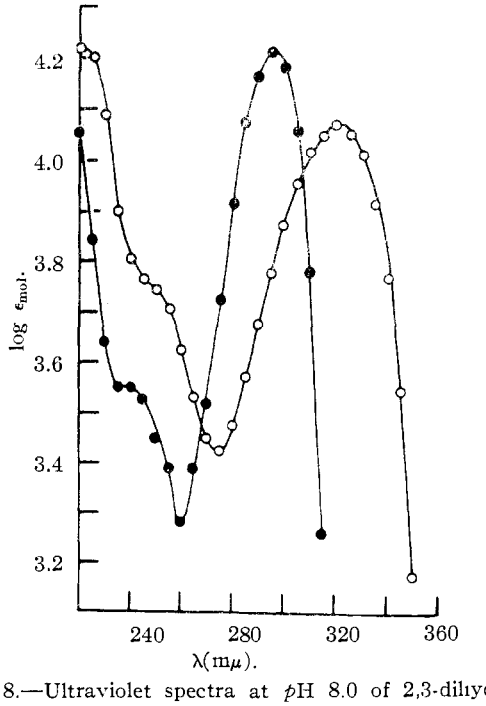
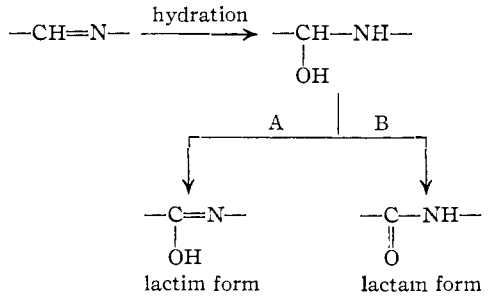


Fig. 8.—Ultraviolet spectra at *p*H 8.0 of 2,3-dihydro-2-oxo-3-methyl-8-hydroxypurine (⊙—○) and its oxidation product, 3-methyluric acid (●—●). Isosbestic points at 269 and 307 mμ.

The results in Table II also demonstrate a variety of effects, produced by methylation of an NH-

group. In some cases, such as 1-methylxanthine or 7-methyl-8-hydroxypurine, only a slight effect on the rate is observed, indicating that the corresponding free NH-group is not involved in the formation of the ES complex and that the methyl substituent is so far from the center of the reaction as not to exhibit steric interference. In other examples we find appreciable rate changes, the methylated derivative being either superior or inferior to the mother substance; the pathway, however, remains unchanged. In many cases, a single methyl group makes enzymatic attack impossible. Finally, of greatest theoretical importance are those examples, where methylation changes the course of the reaction.

In analogy to the oxidation of aldehydes by XO, it may be assumed that purines undergo hydration at one CH=N group, prior to or simultaneously with the dehydrogenative step. The latter may therefore involve either pathway A or B

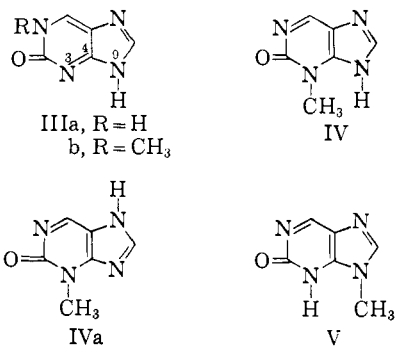


In a multistage oxidation, *e.g.*, in the conversion of purine to uric acid, the ES complex dissociates after each individual step to recombine in a different fashion. This is evident from the fact that the intermediates accumulate in solution to such a degree that they can be identified spectrophotometrically or chromatographically. They may therefore reach a concentration far above the equivalent of the enzyme concentration.

Although the enzymatic reaction involves only a single CH=N group at one time, more remote parts of the substrate molecule exert a pronounced influence either through their polar effects or by actual participation in the binding of enzyme and substrate. The mode of attachment of the two reactants may therefore be deduced from the effects of systematic substitution of NH-groups.

Let us consider first the 2-hydroxypurine series. In the 1-methyl derivative (IIIb), a single form of the pyrimidine ring has been fixed in such a way that C-6 no longer participates in a CH=N double bond and thus cannot attach a hydroxyl ion to participate in enzymatic oxidation (the assignment of a specific tautomeric form to the imidazole ring of IIIa and b is discussed below). Therefore, the reaction now can proceed only in the imidazole ring. We may conclude that 2-hydroxypurine itself reacts with XO in the tautomeric form IIIa. The fact that oxidation of IIIb proceeds about 9 times faster than the conversion of the mother substance to 2,8-dihydroxypurine may be explained by either one of the following assumptions: (a) The enzyme combines preferentially with a specific tautomeric form of 2-hydroxypurine, which represents only a small percentage of all molecules present in solution. In this way, the tautomeric equilibrium is shifted continuously. Brown and Mason[10] concluded from ultraviolet and infrared spectra that 2-hydroxypurine can be described by a structure analogous to IV. Their observations do not exclude the presence of a small percentage of tautomers such as IIIa. On this basis, the small rate of oxidation of 2-hydroxypurine is ascribed to the low concentration of the "active form" IIIa. (b) The enzyme may catalyze the tautomerization of 2-hydroxypurine into IIIa during the formation of the ES complex. In this case, the slow conversion of 2-hydroxypurine to the 2,8-dihydroxy derivative would be an expression of the rate of enzymatic *tautomerization* rather than of enzymatic *oxidation*.

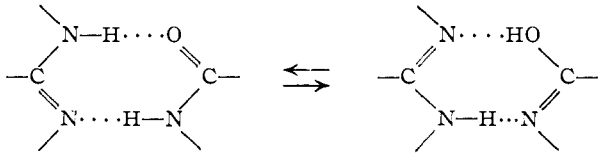
On the other hand, the conversion of IV or IVa to 3-methylxanthine demonstrates that here the $\overset{6}{\text{CH}}=\overset{1}{\text{N}}$ group serves as reactive center for hydration and dehydrogenation. As the 9-methyl derivative likewise is oxidized to 9-methylxanthine, it must be concluded that in this case a tautomeric form V, related to IV, combines with the enzyme. These considerations suggest that the assumption of structure IIIa by 2-hydroxypurine is dependent on the availability of a free NH-group in position 9 to form the partial arrangement $\overset{3}{\text{N}}=\overset{4}{\text{C}}-\overset{9}{\text{NH}}$ (see below).



Similarly in the 8-hydroxypurine series a free 9-NH group is necessary to make attack at C-2 possible (see Table II). On the basis of the foregoing discussion we may suggest that the mesomeric form VI and not VII is bound to the active center. Reaction therefore starts with hydration of the $\overset{2}{\text{C}}=\overset{1}{\text{N}}$ double bond as the only one polar group free of steric hindrance. Fixation of the mesomeric form VI in the ES complex evidently requires the presence of a hydrogen atom at N-9 to provide the partial structure $\overset{3}{\text{N}}=\overset{4}{\text{C}}-\overset{9}{\text{NH}}$ and thus reveals an important relationship to the ES complex of 2-hydroxypurine. This specific group is able to undergo a prototropic shift

$$\overset{3}{\text{N}}=\overset{4}{\text{C}}-\overset{9}{\text{NH}} \rightleftarrows \overset{3}{\text{HN}}-\overset{4}{\text{C}}=\overset{9}{\text{N}}$$

and this may be the feature promoting the binding to the active surface. *E.g.*, chelation may take place with an amide group in the enzyme surface as is shown below in the equilibrium scheme. Such ring formation would be shared by the "active forms" of both 2- and 8-hydroxypurine and would give an explanation of the enzymatic "coupling" of



positions 2 and 8 in the purine ring.[2] Chelation would stabilize the specific mesomeric or tautomeric form participating in the ES complex. The present study clearly shows that the coupling phenomenon is not a general one for all members of the 2- or 8-hydroxypurine series but appears only under specific structural conditions.

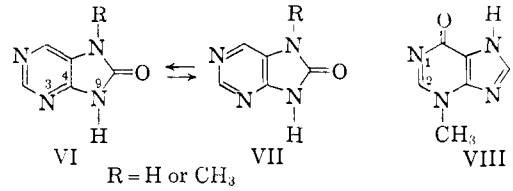
The above results led us to study derivatives of hypoxanthine with fixed tautomeric structures in order to approach an understanding of its oxidation to xanthine and not to 6,8-dihydroxypurine. The fact that 3-methylhypoxanthine (VIII) is susceptible to oxidation at C-2, whereas the 1-methyl derivative is resistant, makes it probable that the mother substance itself reacts in a tautomeric form, analogous to VIII, thus exposing a $\overset{2}{C}=\overset{1}{N}$ double bond. The requirement of a free 1-NH group in hypoxanthine thus finds a simple explanation.

In addition, both 7- and 9-methylhypoxanthine are refractory to XO. Clearly, a free NH-group in the imidazole ring is also indispensable for conversion of hypoxanthine into its "active form." We may recall here the curious observation that 4-hydroxypteridine (IX), an analog of hypoxanthine, is oxidized at carbon atom 7, while 4,6-dihydroxypteridine (X) is attacked at C-2, like hypoxanthine.[4] This comparison stresses the requirement for a free NH-group in the peri position to the carbonyl group to make attack at C-2 possible. We therefore suggest that the type of hydrogen bonding shown in XIa plays a decisive role in the tautomerization of the pyrimidine portion of the molecule and formulate the active form of hypoxanthine as XIb. In the ES complex, the structure XIb is again stabilized by chelation of the portion $HN\overset{3}{—}\overset{4}{C}=\overset{9}{N}$ with a corresponding group in the active surface. In VIII, on the other hand, the appropriate tautomeric form is chemically fixed. The observation that oxidation of VIII proceeds at a rate only about $^1/_{500}$ of the rate of conversion of hypoxanthine to xanthine (Table II), may be explained by steric hindrance, for in this substrate the enzyme is attached to a position very close to the methyl-bearing nitrogen. The rather unusual form of hydrogen bridge, shown in formulas XIa and b, has been discussed by Brown and Mason[10] on the basis of infrared evidence. Similar structures have been established for 8-hydroxyquinoline[18] and β-amino-alcohols.[19]

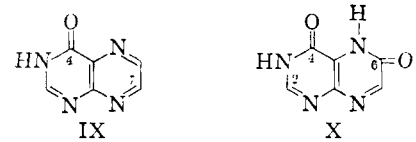
The problem of the enzymatic oxidation of dihydroxypurines may now be approached along similar lines. 6,8-Dihydroxypurine (XII) is a derivative of hypoxanthine as well as of 8-hydroxypurine, either of which is oxidized at C-2. We may therefore assume that enzymatic oxidation of XII involves the tautomeric form XIIa, in which the

(18) G. M. Badger and A. G. Moritz, *J. Chem. Soc.*, 3437 (1958).

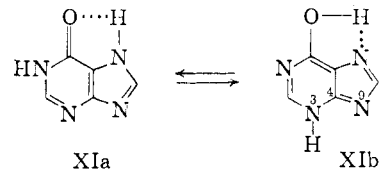
(19) E. D. Bergmann, E. Gil-Av and S. Pinchas, THIS JOURNAL, **75**, 68 (1953).



VI R = H or CH_3 VII VIII

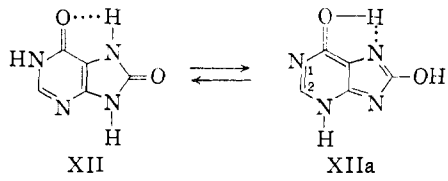


IX X



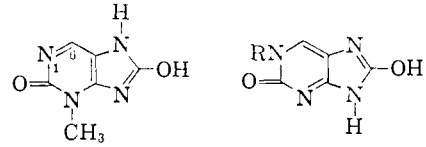
XIa XIb

$\overset{2}{C}=\overset{1}{N}$ double bond has been properly fixed. This formulation of the active form of XII explains at once the observations in Table II, *viz*, the adverse effect of methylation at N-1 and the requirement of a free NH-group at N-9 to make a proton available for lactimization in the imidazolone ring. The hypothesis advanced here can be tested experimentally by introducing methyl groups at either N-3 or N-7. This problem is presently under investigation.

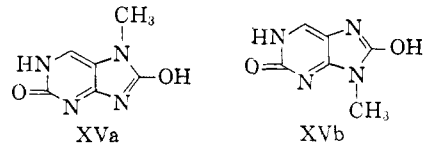


XII XIIa

All possible methyl derivatives of 2,8-dihydroxypurine have been examined as substrates of XO. Appropriate fixation of the $\overset{6}{C}=\overset{1}{N}$ double bond is accomplished only in the 3-methyl derivative XIII, which is oxidized to 3-methyluric acid at a rate about 10 times larger than the oxidation of the mother substance. The latter apparently exists predominantly in the "inactive" form XIV (R = H), containing an *o*-quinoid pyrimidine ring, analogous to the 1-methyl derivative (XIV, R = CH_3), in which C-6 is not part of a polar double bond and therefore does not attach a hydroxyl ion. The analogous structures XVa and XVb may be suggested for the refractory 7- and 9-methyl derivatives.

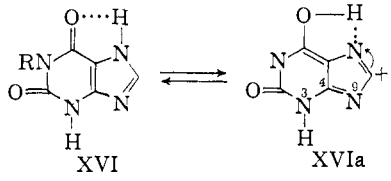


XIII XIV, R = H or CH_3



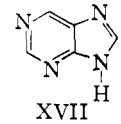
XVa XVb

Applying similar considerations to the xanthine series, we observe that only the 1-methyl derivative serves as substrate of XO (see Table II). Oxidation at C-8 thus requires free NH-groups in position 3 and in the imidazole ring. The first condition again suggests participation of the partial structure $HN^{3}—C^{4}=N^{9}$ in chelation with an appropriate part of the active surface. In addition, intramolecular hydrogen bonding as in hypoxanthine involves a free 7-NH group (XVI). Therefore, we propose formula XVIa as the active form of xanthine to explain all experimental observations in this series.



XVI XVIa

Finally, there remains the problem of oxidation of purine, where neither a 7- nor a 9-methyl group prevents enzymatic attack at C-6, although the rate is greatly reduced. Purine may assume the form XVII, in which again the grouping $N^{3}=C^{4}—NH^{9}$ is present.[20] This would probably lead to attack at C-2. Therefore, we may assume that XVII does not become stabilized in the absence of a carbonyl group. Further discussion of this problem will be deferred in view of recent results, obtained in the pteridine series.



XVII

In summarizing this discussion, it appears that attachment of purines to the active surface of XO may involve structures, which differ from the form prevailing in free solution. It is not so much the intrinsic polarity of a given purine derivative that decides its interaction with the enzyme; of much greater importance is the structure of the ES complex which depends on specific binding groups both in the enzyme and substrate and therefore may involve mesomeric or tautomeric forms, which represent only a small percentage of all molecules present. This view is supported by the observation that the same purine derivative can be oxidized along a different pathway by another xanthine oxidase, present in certain bacterial strains.[21] Thus, the fact that 3-methylxanthine is oxidized by the bacterial enzyme[22] indicates that here the type of chelation, assumed for the structures III, VI, XIb, XIIa and XVIa, is absent.

(20) A. Bendich, P. S. Russell, Jr., and J. J. Fox, THIS JOURNAL, **76**, 6073 (1954), place the mobile hydrogen atom in purine at N-9, because of the similarity of the absorption spectra of the cationic form of purine and its 9-methyl derivative.

(21) F. Bergmann, H. Kwietny, G. Levin and H. Engelberg, *Biochim. Biophys. Acta*, in press.

(22) S. Dikstein, F. Bergmann and Y. Henis, *J. Biol. Chem.*, **224**, 67 (1957).

[CONTRIBUTION FROM THE PHOTO PRODUCTS DEPARTMENT, RESEARCH DIVISION, E. I. DU PONT DE NEMOURS AND CO.]

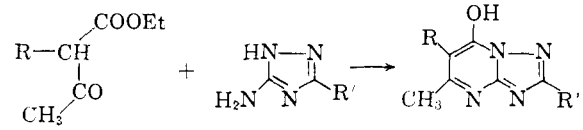
The Structure and Reactivity of 5-Hydroxy-7-methyl-*s*-triazolo[2,3-a]pyrimidine

BY V. C. CHAMBERS

RECEIVED JUNE 11, 1959

Physical and chemical evidence has been presented for the tautomeric equilibrium between an enol and three keto forms of 5-hydroxy-7-methyl-*s*-triazolo[2,3-a]pyrimidine (I). The structures of two products obtained by reaction of the silver salt of I with ethyl iodide were investigated. One of these was established by synthesis.

The product of the reaction of ethyl acetoacetate and 3-amino-1,2,4-triazole was first prepared by Bülow,[1] who ascribed to it the structure of 5-hydroxy-7-methyl-*s*-triazolo[2,3-a]pyrimidine (I). Since 1936, various triazolopyrimidines have been suggested as additions to photographic emulsions to inhibit the development of fog.[2] The structure was originally questioned by Birr, who later presented evidence favoring the structure of 5-hydroxy-7-methyl-*s*-triazolo[4,3-a]-pyrimidine.



I, R, R = H
II, R = C_2H_5, R′ = H
III, R = H, R′ = C_2H_5

The acidic character of this substance was demonstrated by Bülow.[1]

Recently Allen *et al.*,[3] showed that Birr's conclusions were incorrect and established the structure originally proposed by Bülow.

In the present research, the structure and reactivity of I were investigated in order to interpret its photographic behavior. As in the case of the hydroxypurines, tautomeric forms of I may be written in which the acidic proton is attached to nitrogen or oxygen.

Similar forms also contribute to the anion derived from I and this resonance stabilization probably accounts for the acidic character. In the case of the anion, an additional form may be written in which the negative charge is ascribed to carbon. That such a structure does not make an important contribution to the stabilization of the anion is

(1) C. Bülow and K. Haas, *Ber.*, **42**, 4642 (1909).

(2) (a) E. J. Birr, *Z. wiss. Phot.*, **47**, 2 (1952); (b) N. Heimbach and W. Kelly, Jr., U. S. Patent 2,444,605 (July 6, 1948) (assigned to General Aniline and Film Corp.).

(3) C. F. H. Allen, H. R. Beilfuss, D. M. Burness, G. A. Reynolds, J. F. Tinker and J. A. VanAllan, *J. Org. Chem.*, **24**, 787 (1959).