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A Comparison of the Oncogenicities of 3-Hydroxyxanthine, Guanine 3-*N*-Oxide, and Some Related Compounds[1]

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SUMMARY

Assays of the oncogenic *N*-oxide derivatives of xanthine and guanine, which have now been proven to be 3-hydroxyxanthine and guanine 3-*N*-oxide, continue to show about equal activities. Parallel titrations at 1.0, 0.5, and 0.1 mg/week for 26 weeks, administered subcutaneously, in female Wistar rats show that, for these conditions, the 50% tumor incidence doses lie between the two lower dose levels, or between a total of 2 and 10 mg of the free bases.

The isomeric 1-hydroxyxanthine elicits a different response. While being administered it induces a severe inflammatory and granulomatous condition, but it leads to only a small incidence of tumors. This, coupled with the confirmed low incidence of tumors from 6-mercaptopurine 3-*N*-oxide, indicates that the oncogenicity of purine *N*-oxide derivatives is influenced both by the position of the *N*-oxide and by other substituents.

Although the assay response to adenine 1-*N*-oxide has been variable, a sufficient incidence of tumors has been observed to indicate that it is at least a moderately oncogenic purine *N*-oxide.

The inactivities of the parent purines and of a few other purine derivatives are recorded.

INTRODUCTION

Assays of the oncogenicity of certain purine *N*-oxides have now progressed to a point which permits an assessment of the quantities required to cause various tumor incidences in rats and a reasonable comparison with the oncogenicities of some other chemical oncogens. The most oncogenic were derived from guanine by oxidation with a peroxy acid and were first referred to as x-*N*-oxides of guanine and xanthine (7). For a time they were thought to be 7-*N*-oxides (8, 25), but it has now been unequivocally demonstrated (29) that they are 3-*N*-oxide derivatives, namely guanine 3-*N*-oxide and 3-hydroxyxanthine[2] (Chart 1). When these were considered to be 7-*N*-oxides we also reported (25) an "xanthine 3-*N*-oxide" to be nononcogenic, but it is now known (29) that an unusual rearrangement had converted it to uric acid, which was the product found to be nononcogenic.

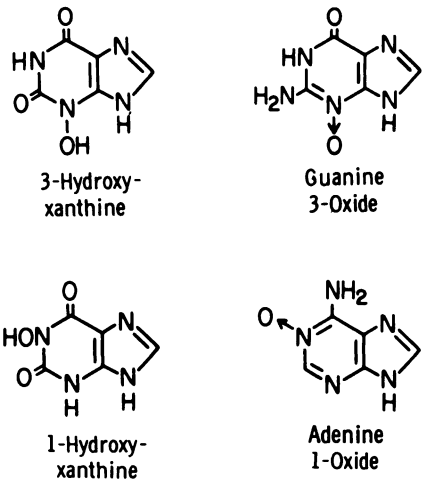

Chart 1. Formulae.

The complication in the determination of the structures was an entirely unexpected formation of 8-hydroxy derivatives in the course of acetylation, and of other reactions, of certain purine 3-*N*-oxide derivatives (29, 30). This is an apparent rearrangement of the oxygen from N-3 to C-8 of the purine ring. A possible intermediate in this reaction, the *O*-acetyl derivative of 3-hydroxyxanthine, has now been obtained. This 3-acetoxy derivative can readily undergo nucleophilic attack at the 8-position, with elimination of the acetoxy substituent from the 3-position (Chart 2) (28). In water at room temperature uric acid is formed. In an aqueous solution of methionine, 3-acetoxyxanthine reacts preferentially with the methionine to yield a sulfonium derivative that decomposes to 8-methylmercaptoxanthine.

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[2]The nomenclature is based on the tautomer predominant in the neutral species (3, 29).

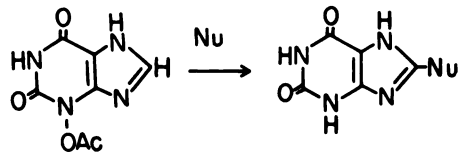

Chart 2. Reaction of 3-acetoxyxanthine with a nucleophile. Nucleophiles (Nu) with which reaction in aqueous solutions has been demonstrated include water, methionine, chloride, and nitrite and, in a nonaqueous medium, other nucleophils such as pyridine, nicotinic acid, and nicotinamide (28).

The 3-acetoxyxanthine is so reactive that it is even attacked by the weakly nucleophilic chloride ion in 1% aqueous NaCl to yield considerable amounts of 8-chloroxanthine (28).

Such reactions, occurring *in vitro* under physiological conditions of temperature and pH, are reminiscent of the recent demonstrations by J. A. and E. C. Miller and their coworkers (13, 16, 20) of the reactivities *in vitro* of esters of *N*-hydroxyacetylaminofluorene and other *N*-hydroxyarylamines. In fact the reaction of 3-acetoxyxanthine with methionine is reminiscent of the attack *in vivo* by *N*-hydroxyacetylaminofluorene on the methionine residues of proteins and of the reactivity of the acetoxy derivative with methionine *in vitro* (13, 16).

MATERIALS AND METHODS

Assays were conducted as previously described (7, 25). Subcutaneous injections of 0.5 ml of suspensions in 0.5% carboxymethyl cellulose in 0.85% NaCl solution into the interscapular area were made once weekly for 26 or 22 weeks. Groups of, usually, 15 Wistar (Carworth Farms, New City, N. Y.). or Sprague-Dawley (Blue Spruce Farms, Altamont, N. Y.) young adult female rats (80 to 100 g) were used, but 1 group of 25 male Wistar weanling rats was included.

The guanine 3-*N*-oxide hemihydrochloride (mol. wt. 185.4) (7, 8), 3-hydroxyxanthine hydrate (mol. wt. 186.1) (7, 8), adenine 1-oxide (mol. wt. 151.1) (22), 1-hydroxyxanthine•$2H_2O$ (mol. wt. 168.1) (18), 6-mercaptopurine 3-*N*-oxide•H_2O (mol. wt. 186.1) (6), and 6-hydroxylaminopurine (mol. wt. 151.1) (11) have been described. The 6-mercaptopurine (mol. wt. 152.1) was kindly furnished by Dr. G. H. Hitchings of the Burroughs Wellcome Laboratories, Tuckahoe, N. Y. The 2-aminopurine (mol. wt. 135.1) was purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y. and some guanine 3-*N*-oxide was from Zellstofffabrik Waldhof, Mannheim, Germany.

The stability of samples prepared for injections has been confirmed by paper or ion-exchange chromatography of samples of 6-mercaptopurine 3-*N*-oxide and adenine 1-*N*-oxide stored for 1 to 3 months. Because of known light sensitivity of many of the derivatives (5, 6) samples were stored in dark or foil-wrapped bottles.

RESULTS AND DISCUSSION

It was last reported (25) that the purine derivatives now known to be 3-hydroxyxanthine and guanine 3-*N*-oxide had induced tumors at the site of subcutaneous injection in essentially 100% of the rats when either was administered at a dose of 3 mg/week for 26 weeks. Numerous additional assays have now been completed (Table 1). The cumulative totals for 193 rats at the 3 mg/week or higher doses include 88 rats previously reported in detail (7, 25). Parallel titrations of the 2 compounds at 1.0-, 0.5-, and 0.1-mg doses now show (Table 1) that the weekly dose required for 50% tumor incidence on this experimental regimen lies between 0.1 and 0.5 mg, or total quantities of between 2 and 10 mg of the free bases. Although the 3-hydroxyxanthine might appear to be somewhat more potent than guanine 3-*N*-oxide, and its latent period to be slightly less, any differences between them based upon the present numbers of rats on the lower dose levels cannot be considered significant. This titration was carried out with female Wistar rats; other evidence (26) suggests that males may be even more susceptible.

The incidences of liver tumors shown in Table 1 include both hyperplastic nodules and hepatomas and are not wholly indicative of the toxicity to the livers, since many animals were killed early to study the tumor at the s.c. site. In addition to the tumors, the livers of the majority of the animals showed focal necrosis, venous stasis, and/or parenchymatous degeneration. There was no other consistent appearance of tumors in these animals.

In our initial assay (7) of adenine 1-*N*-oxide no tumors were produced. Subsequent assays have led to tumors in

Table 1

Tumors induced by 3-hydroxyxanthine and guanine 3-oxide

Rat	Sex	Dose (mg/wk for 22 or 26 wk)	Latent period (mo.) Median	Latent period (mo.) Range	No. with tumors by 15 mo. At site[a]	No. with tumors by 15 mo. Livers[b]
3-Hydroxyxanthine						
W[c,d]	♀♂	3–7	7	4–15	131/134	22
S-D	♀	3–7	7	4–10	30/30	4
S-D	♀	1.0	8	6–11	17/18	
W	♀	1.0	8	5–11	15/15	
W	♀	0.5	10	6–14	14/14	
W	♀	0.1	12	9–15	9/30	
Guanine 3-oxide						
W	♀	3.0	11	7–15	15/15	1
S-D	♀	3.0	8	7–12	14/14	
W	♀	1.0	12	8–15	15/15	
W	♀	0.5	12	6–15	10/15	
W	♀	0.1	12	8–15	4/15	

[a]Histological diagnoses with 3-hydroxyxanthine included 191 fibrosarcomas, 16 fibromas, 12 liposarcomas, 8 rhabdomyosarcomas, 3 carcinomas, and 4 miscellaneous tumors; and with guanine 3-oxide included 44 fibrosarcomas, 5 fibromas, 6 liposarcomas, 1 carcinoma, and 6 miscellaneous tumors. Serveral animals had multiple tumors at the site of the injections, sometimes of more than 1 type.

[b]Including 9 with hepatomas and 18 with hyperplastic nodules.

[c]Line 1 includes results on both young adult and weanling rats. All others were young adults.

[d]W, Wistar; S-D, Sprague-Dawley.

both Sprague-Dawley and Wistar rats; at a dose level of 10 mg/week for 26 weeks, tumors have been induced at the site of injection in 33 of 41 rats. Unexplained variations in the assay response to adenine 1-*N*-oxide are under study. Assays are complicated, particularly at higher levels, by the characteristic "adenine kidney" syndrome (2), which is brought about by both adenine and its 1-*N*-oxide (4). Although the activity of adenine 1-*N*-oxide is less than those of 3-hydroxyxanthine and guanine 3-*N*-oxide it must now be included among the significantly oncogenic purine *N*-oxide derivatives.

Additional control groups injected with the 3 parent purines (Table 2) have shown no tumors. Enough groups of controls treated with the vehicle alone, carboxymethyl cellulose (Table 2), have now been accumulated to permit a statistical consideration of the significance of 1 or 2 tumors per group of 15 animals. The significance of even 2 tumors in 15 rats may be questioned since, despite a low incidence of 2.2% in 180 Wistar rats, 2 of the 4 tumors observed were in a single group of 15 (p = 0.04 by chance alone). The incidence of tumors at other than the s.c. site or the livers was less in the experimental Wistar rats than in the controls, probably because the controls lived longer.

Table 2

Controls with vehicle and parent purines

Rat	Sex	Compound, once wk for 26 wk	Latent period (mo.) Median	Range	No. with tumors by 15 mo., at site
W	♀	Vehicle[a]	14	12–15	4/180
S-D	♀	Vehicle[a]	13	11–15	2/45
W	♀	Xanthine[b]			0/31
W	♀	Guanine[b]			0/30
W	♀	Adenine[b]			0/45

[a]Vehicle was 0.5% carboxymethylcellulose in 0.85% NaCl solution, 0.5 ml/week. The incidence of random tumors at sites other than that of injections was 12 in 180, 6.7%, in Wistar rats, and in Sprague-Dawley rats there were 2 in 45.
[b]Dose, 10 mg/injection.

Of the 4 possible structurally isomeric *N*-oxides of xanthine (*i.e.*, the 1-, 3-, 7-, or 9-), 1 more in available, 1-hydroxyxanthine (Chart 1) (1, 18). In its initial assay masses were observed in all animals in 2 to 6 weeks. Injections were discontinued at 13 weeks and the masses regressed. Of those rats 2 in 15 developed fibrosarcomas (Table 3). In subsequent experiments several animals with similar masses were killed at 1 to 6 months and in each case granulomatous tissue and inflammation were the only histological findings. In 45 rats given 13 to 26 weekly injections, the inflammations persisted until treatment was discontinued. It has also been observed that 3-hydroxyxanthine and adenine 1-*N*-oxide can lead to a granulomatous condition during the injection period, but the inflammatory response is less obvious. Despite the fact that 1-hydroxyxanthine shows a

Table 3

Tumors induced by other purine derivatives

Rat	Sex	Dose (mg/wk for 26 wk)	Latent period (mo.) Median	Range	No. with tumors by 15 mo., at site
1-Hydroxyxanthine[a]					
W	♀	10[b]	9	9–10	2/15
W	♀	10	14		1/15
W	♂	10	14	6–15	11/15[c]
W	♀	3	8		1/15
W	♀	1	14		1/15
6-Mercaptopurine 3-oxide					
W	♀	50	12	10–14	3/15
S-D	♀	50			0/15
6-Mercaptopurine					
W	♀	5			0/11
W	♀	2.5	13		1/15
6-Hydroxylaminopurine					
W	♀	10	11		1/15[d]
S-D	♀	10			0/15
2-Aminopurine					
W	♀	10			0/15

[a]All rats showed inflammation (inflammatory granulomas) which regressed when the injections were discontinued.
[b]Administered for only 13 weeks.
[c]In this group of male rats 4 tumors, 3 fibrosarcomas, and 1 histiocytoma weighing 29 to 162 g were palpable. At autopsy, by microscopic examination 7 additional tumors, 6 fibromas and 1 fibrosarcoma, were detected. This result parallels the greater sensitivity of males to 3-hydroxyxanthine (26).
[d]A second tumor started at the axilla and grew to include the site.

decided toxicity toward the subcutaneous tissues it is not strongly oncogenic; in females a 100-fold greater dosage (Table 3) led to fewer tumors than did the isomeric 3-hydroxyxanthine (Table 1). In males a much higher incidence was observed (Table 3). The actions of 1-hydroxyxanthine in combination with other agents will be of interest.

Strong oncogenicity is not a property common to all 3-*N*-oxides of purines, as indicated by the low incidence of tumors resulting from massive doses of 6-mercaptopurine 3-*N*-oxide (Table 3). The experiment with Wistar rats was previously reported in detail (25). A similar experiment with Sprague-Dawley rats did not lead to any tumors. The experiments with 6-mercaptopurine (Table 3) are difficult to compare with those with 3-*N*-oxide, since the maximum tolerated dose of the former on this regimen is about 20-fold less than that of the *N*-oxide; a dose of 2.5 mg/week was tolerated but 5 mg/week resulted in several early deaths. The single tumor appearing in the 6-mercaptopurine-treated rats is of doubtful significance. Through reduction *in vivo* (Murphy, unpublished data; Ref. 3), each 50-mg dose of

6-mercaptopurine 3-*N*-oxide should lead to more than 2.5 mg 6-mercaptopurine. Without additional assays, which would require prohibitively large amounts of 6-mercaptopurine 3-*N*-oxide, it is not possible to say whether the low and even questionable oncogenicity of 6-mercaptopurine 3-*N*-oxide is inherent in this *N*-oxide derivative.

The amounts of 3-hydroxyxanthine or of guanine 3-*N*-oxide which induce a high incidence of tumors at the site of subcutaneous injection are roughly of the order required of the oncogenic arylamines such as *N*-hydroxyacetylaminofluorene (12, 14–16), of 4-hydroxylaminoquinoline *N*-oxide (21), or of several common oncogenic hydrocarbons (12, 19).

From the present assays of a limited number of compounds it is obvious that there are specific structural requirements for oncogenicity. The importance of the particular *N*-oxide isomer of a specific purine is evident from the marked qualitative and quantitative differences between the 3- and 1-hydroxyxanthines. Purines with an *N*-oxide function at the 3-position are not universally oncogenic, as is shown by the very low incidence of tumors induced with massive doses of 6-mercaptopurine 3-*N*-oxide. Assays of additional structural variations are needed before a decision can be made between the importance of an *N*-oxide grouping at the 3- or 1-positions or of the presence of an oxygen where the parent purine is normally subject to chemical oxidation, that is, the 1-nitrogen of adenine or the 3-nitrogen of guanine.

Because of the oncogenicity of several *N*-hydroxy derivatives of arylamines (16), we have also tested 6-hydroxylaminopurine, a purine derivative with a formal structural analogy to the arylhydroxylamines. It is also a mutagenic agent toward phage T4 (10). Its oncogenicity is marginal at best (Table 3). Another purine known to be similarly mutagenic (9), 2-aminopurine, did not induce any tumors (Table 3). Neither the oncogenic 3-hydroxyxanthine nor guanine 3-*N*-oxide have shown a mutagenic effect toward phage T4 (10). Oncogenesis assays are complicated by the metabolism of the compounds in the animal and comparison of these 2 assays does not constitute a valid test of the possible relationship between oncogenicity and mutagenicity.

Metabolic studies of guanine 3-*N*-oxide (23) and of 3-hydroxyxanthine (17) in rats have included the identification of several urinary metabolites which result from either reducing or oxidizing actions of xanthine oxidase (24). Despite the very limited solubilities in water (10^{-3} to 10^{-4} M), these compounds are obviously rapidly dissolved in body fluids. The bulk of subcutaneously administered samples does not remain long at the site of injection, as indicated by the detection in the urine (17, 23) of 75 to 85% of the radioactivities within 6 hr.

A hypothetical "activated" 3-hydroxyxanthine or guanine 3-*N*-oxide, for which the 3-acetoxyxanthine (Chart 2) is a chemical model, and which can react with nucleophilic groups of any type of macromolecule, offers a potential explanation for chemical alterations which could eventuate in oncogenicity. However, this cannot now be considered as a universal explanation for oncogenicity by purine *N*-oxide derivatives since no comparable chemical behavior is shown for adenine 1-*N*-oxide.

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