Oxidizing Action of Purine N-Oxide Esters

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SUMMARY

A technique involving O-acetylation of purine N-oxide derivatives in buffered aqueous solutions has permitted studies of the reactivity of many compounds for which the O-acetyl derivatives are not otherwise available. The oxidizing properties of a variety of N-acetoxypurines have been measured through their ability to oxidize iodide ion to iodine, a reaction which is representative of a more general oxidizing ability. Those esters that oxidize iodide ion also catalyze the autooxidation of sulfate, a property characteristic of radicals. The same esters also oxidize cysteine to cysteic acid and tryptophan, tyrosine, and uric acid to yet uncharacterized products. Their oxidizing reactivity was compared with the ability of the same esters to react as electrophiles in another assay that measured the rate of formation of pyridine substitution products. The sulfate ester of 3-hydroxyxanthine has been synthesized. Its reactivity is qualitatively the same as that of 3-acetoxyxanthine but proceeds at a higher rate. Syntheses of S-(8-xanthyl)-N-acetylcysteine, 8-(2-hydroxyethylthio)xanthine, and 1-methyl-8-methylmercaptoguanine are also described.

INTRODUCTION

Several N-oxide derivatives of purines have proven to be strong oncogens (6, 29). The chemistry of one of the most potent members of this group, 3-hydroxyxanthine, has been studied. Its highly reactive N-acetoxy ester has been shown to undergo a reaction that results in the substitution at position 8 by nucleophiles (5). In metabolic studies in rats, it has been demonstrated that 8-substitution products are derived from methionine and chloride (26) and possibly from tryptophan (28), and the intermediary formation of a sulfate ester has been implicated for the “activated oncogen” in vivo (27). During the reaction of 3-acetoxyxanthine in water at pH 7, some xanthine is formed spontaneously (4). When KI is present, the iodide is oxidized to iodine, and the production of xanthine is quantitative (5). It was suggested that these reactions could be explained by the formation of a radical from 3-acetoxyxanthine (4).

This paper presents a method for the preparation in situ of O-acetyl esters of purine N-oxides, many of which are not otherwise available. The method has been utilized in studies of the general oxidizing reactivity of a series of purine N-oxide esters and in a comparison of that with their reactivity as electrophiles. The reactivity of the newly prepared sulfate ester of 3-hydroxyxanthine is compared with that of 3-acetoxyxanthine.

MATERIALS AND METHODS

Purine N-oxide derivatives (references in Table 2) usually contained less than 1 ppt of UV-absorbing impurities by column chromatographic analysis, with special attention paid to contamination by other purine N-oxide derivatives. Radioactive materials were obtained commercially and were chromatographically pure. [2-14C]Uric acid was purified by paper chromatography in System A, and [3,5-3H]tyrosine was twice lyophilized from a solution in 1 ml of H2O before use. Dowex 50 AG 50W-X8 cation exchange resin, 200 to 400 mesh, 1.7 mEq/ml, was purchased from Bio-Rad Laboratories, Richmond, Calif.

Paper Chromatography and Radiopaper Chromatography. Schleicher and Schuell No. 597 paper was used with the following solvents, ascending: Solvent A, 3% NH4HCO3 in water containing 10−4 M EDTA; Solvent B, 1-butanol:acetic acid:water, 5:1:4, upper phase; Solvent C, Whatman P81 cellulose phosphate paper prewashed with 1 N HCl and water and dried, with 1.3 N formic acid, ascending. Retardation factors and corresponding chromatographic systems in the solvents used were: deoxyxycytidine, 0.25 B; N-acetyldoxyctydine, 0.60 B; all 8-methylmercaptopurines, 0.20 to 0.40 A; methionine, 0.93 A; methionine sulfonium derivatives of purines, ~0.80 A; S-(8-xanthyl)-N-acetylcysteine, 0.75 A; 8-(2-hydroxyethylthio)xanthine, 0.48 A; cysteine, 0.35 B, 0.25 C; cystine, 0.10 B, 0.05 C; cystine disulfoxide, 0.14 C; cysteic acid, 0.10 B, 0.75 C; cysteinesulfinic acid, 0.60 C; uric acid, 0.42 A; and uric acid oxidation products, 0.75 to 0.95 A. Radioactivity on paper chromatograms was scanned and quantitated with a 4π Geiger-Müller-type scanner.

Transacetylation by 3-Acetoxyxanthine Derivatives. In a total of 0.2 ml of buffer (0.1 M potassium phosphate; 0.1 M methionine, pH 7.4), 10,000 cpm [8-14C]-3-hydroxyxanthine (26) and 1.0 mg of 3-acetoxyxanthine (3) were dissolved at 37° for 10 min with stirring. In a control experiment the [14C]-3-hydroxyxanthine was added after 10 min of preincubation at 37°, by which time the 3-acetoxyxanthine derivative is known to be decomposed. After an additional 10 min at 37°, the samples were heated to 100° for 10 min to decompose the methionine-sulfonium adduct, which is then

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initial product, to 8-methyl-mercaptoxanthine which was determined by paper chromatography in System A.

**Reaction of Acetoxypurines with \[^{14}C\]Methionine.** The purine N-oxide derivative, 1 mg, 5000 cpm [methyl-\[^{14}C\]]methionine, and 0.005 ml of acetic anhydride in 0.5 ml 0.1 M phosphate buffer, pH 7.4, were incubated for 10 min at 37° and for an additional 10 min at 100° and examined by paper chromatography in System A.

**Reaction of Other Radioactive Substrates with Acetoxypurines.** In Procedure A, 1 mg of acetoxypurine together with the radioactive substrate in 0.5 ml of 0.1 M phosphate buffer, pH 7.4, were incubated at 37° for 10 min. In Procedure B, to 1 mg of a purine N-oxide and the radioactive substrate in 0.5 ml of 0.1 M phosphate buffer, pH 7.4, 37°, were added 3 μl of acetic anhydride, and the mixture was incubated at 37° for 10 min.

**Rate Determination of N-Acetoxy Ester Formation, KI Oxidation, and Substitution by Pyridine.** A Beckman DK-2 spectrophotometer equipped with a constant wavelength time drive of 10-cm/min speed was used to determine the wavelengths to follow for loss of the parent generation of the ester in situ. Formation of pyridine derivative in 2.0 ml of 0.1 M phosphate buffer, pH 7.4, at 37° and for an additional 10 min at 1000 and examined by paper chromatography in System A.

**Adduct Formation.** For KI oxidation, a buffer of 1 M KI:0.1 M H\(_3\)PO\(_4\):0.1 M H\(_3\)BO\(_3\):0.1 M hydroxyacetate, pH 7.4, was used, and the pH was adjusted to values between pH 3 and 10, and its rate of formation was measured spectrophotometrically at that wavelength after generation of the ester in situ. Formulation of pyridine substitution products was similarly measured, arbitrarily, at 352.5 nm. Similar concentrations of purine N-oxides were used in a buffer containing 1 mM pyridine and 0.1 mM phosphate, pH 7.4, and the acetoxypurine was also generated in situ.

**pH Dependence of KI Oxidation and of Pyridinium Adduct Formation.** For KI oxidation, a buffer of 1.0 mM KI:0.1 mM K\(_2\)HPO\(_4\):0.1 mM H\(_4\)BO\(_4\):0.1 mM acetic acid was used, and the pH was adjusted to values between pH 3 and 10 with NaOH. For these measurements, 3 mg of 3-acetoxyxanthine was dissolved in 1.0 ml of dry dimethyl sulfoxide, and 0.02 ml of this solution was blown, for stirring, into 2.5 ml of buffer at 23°. For other compounds, the esters were generated in situ.

**For pyridinium adduct formation a buffer of 1 mM pyridine:0.1 mM H\(_2\)PO\(_4\):0.1 mM H\(_4\)BO\(_4\):0.1 mM hydroxyacetate was used, and the pH was adjusted, with NaOH, to values between pH 3 and 10. The long-wavelength spectrum of 8-pyridinium xanthine is strongly pH dependent (wavelength in nm, ε \(\times 10^{-4}\) in parentheses): pH 1.0, 237 shoulder (10.0), 255 shoulder (7.6), 350 (8.0); pH 7.4, 241 (11.0), 266 (7.0), 374 (9.6); pH 10.8, 249 (10.5), 274 (8.6), 414 (8.0). ε ext in parentheses for various pH's: 3.4 (7.7), 4.7 (7.6), 6.0 (7.2), 7.0 (7.1), 8.2 (6.9), 9.4 (5.0), 10.8 (2.45).

**S-(8-Xanthyl)-N-acetylcyesteine.** 3-Acetoxyxanthine, 200 mg, was added to a solution of 500 mg of N-acetylcyesteine in 5 ml of H\(_2\)O over a period of 10 min at room temperature, with the pH held at 6.0 by the addition of 1 N NaOH. After the mixture was stirred for an additional 30 min, 5 ml of Dowex 50 (H\(^+\)) were added and stirred for 10 min, and the slurry was placed on a 200-ml Dowex 50 (H\(^+\)) column, preheated to 50°. Elution with H\(_2\)O at 50° yielded uric acid between 121 and 147 ml, N-acetylcysteine between 148 and 182 ml, and S-(8-xanthyl)-N-acetylcysteine between 183 and 245 ml of the eluate. Evaporation in a vacuum and recrystallization from H\(_2\)O at pH 6 yielded 45 mg of crystals.

\[
\text{C}_{16}\text{H}_{11}\text{N}_{5}\text{O}_{3}\text{S}
\]

Calculated: C 38.35, H 3.54, S 10.21
Found: C 38.58, H 3.35, S 10.01

**8-(2-Hydroxyethylthio)xanthine.** 3-Acetoxyxanthine, 200 mg, and mercaptoethanol, 1 ml, were reacted as described previously. The reaction mixture was fractionated over 80 ml of Dowex 50 with 1 N HCl to yield uric acid between 50 and 125 ml, 3-hydroxyxanthine between 175 and 350 ml, and 50 mg of 8-(2-hydroxyethylthio)xanthine between 350 and 425 ml. The latter was recrystallized from 95% methanol.

\[
\text{C}_{7}\text{H}_{12}\text{N}_{4}\text{O}_{3}\text{S}
\]

Calculated: C 36.85, H 3.53, S 14.05
Found: C 36.89, H 3.60, S 13.85

**1-Methyl-8-methylmercaptoguanine.** 1-Methylguanine 3-oxide, 400 mg, (2) and methionine, 1.2 g, in 20 ml of H\(_2\)O were heated to 40°, and 1 N NaOH was added to pH 6. Acetic anhydride, 0.3 ml, was then added, the mixture was stirred for 10 min while at 40°, and the pH was maintained at 6.0. After this the mixture was heated to boiling for 10 min and evaporated in a vacuum. The mixture was separated on 5 ml of Dowex 50, and 1-methyl-8-methylmercaptoguanine was eluted between 160 and 225 ml of 1 N HCl. Evaporation and recrystallization from H\(_2\)O yielded 80 mg of crystals: UV 1 N HCl, λ max 291 nm (ε = 15,500); pH 7.0, λ max 291 nm (ε = 12,600); 1 N NaOH, λ max 291 nm (ε = 13,200).

\[
\text{C}_{7}\text{H}_{12}\text{N}_{4}\text{O}_{3}\text{S} \cdot \text{H}_{2}\text{O}
\]

Calculated: C 36.67, H 4.84, N 30.54, S 13.99
Found: C 36.61, H 4.30, N 30.28, S 13.88

**3-Hydroxyxanthine Sulfate Ester.** To 300 mg of neutral anhydrous 3-hydroxyxanthine (34) in 3 ml of dry dimethylformamide were added, 2 ml of 1.4 M dimethylformamide were added, 2 ml of 1.4 M dimethylform-
mamidase:sulfur trioxide complex (10), and the suspension was stirred for 4 hr at 25°. After the addition of 50 ml of ether, an oil separated which was treated with 5 ml of methanol. A small amount of unreacted 3-hydroxyxanthine remained undissolved. The sulfate ester, contained in the methanol, was precipitated with 50 ml of ether to yield 200 mg of analytically pure ester.

\[
\text{C}_4\text{H}_6\text{N}_4\text{O}_8\text{S}
\]

Calculated: C 24.20, H 1.62, N 22.58, S 12.92
Found: C 24.42, H 1.90, N 22.41, S 12.71

RESULTS AND DISCUSSION

3-Acetoxyxanthine reacts rapidly in buffered aqueous solution and yields 3 major UV-absorbing products, 3-hydroxyxanthine, uric acid, and xanthine (4). These 3 products are representative of 3 modes of spontaneous reaction which this ester can undergo. Reaction I to II, essentially hydrolysis, has now been shown to have some of the characteristics of the reaction of an anhydride, since the acetyl group can be transferred to substances other than water. Reaction I to III was suggested by Birdsall et al. (4) to explain the formation of xanthine, and additional evidence for a possible pair of oxidizing radicals is offered here. Reaction I to IV is the formation of a nitrenium ion, which is in equilibrium with a carbonium ion at C-8 that leads to the 3-acetylpyrimidine 8-substitution reaction previously described (5). With water this reaction is the source of the formation of uric acid, and with strong nucleophiles such as pyridine it can be the predominant mode of reaction to yield 8-pyridinium xanthine.

Transacetylation.

3-Acetoxyxanthine and some of its derivatives can acetylate suitable substrates (Chart 1, Reaction I to II) as do other O-acetylhydroxamic acids (19). Among the active esters of N-hydroxylated carcinogens, ethyl N-acetoxy-N-acetylcarbamate was shown by Nery (18) to N-acetylate cytosine. Acetylated guanosines are mentioned as by-products in the reaction of N-acetoxyacetaminofluorene with guanosine (22). With 3-acetoxyxanthine no acetylation of either guanosine or cytidine was observed after incubation with radioactive nucleosides. However, an acetyl transfer from nonradioactive 3-acetoxyxanthine to radioactive 3-hydroxyxanthine can be demonstrated. If the radioactive 3-acetoxyxanthine is allowed to react with methionine, radioactive 8-methylmercaptoxanthine is formed in 37% yield. Similarly, 3-acetoxy-7-methylxanthine and 3-acetoxy-1,7-dimethylxanthine activate radioactive 3-hydroxyxanthine to yield 46 and 60% of radioactive 8-methylmercaptoxanthine. In all 3 reactions, radioactive 3-hydroxyxanthine was converted to reaction products with more than 90% efficiency; while in control experiments in which 3-acetoxy- or 7-acetyl-3-acetoxyxanthine were allowed to decompose before the addition of radioactive 3-hydroxyxanthine, the latter remained unchanged. In addition to these cases of transacetylation between N-hydroxy derivatives, N-acetylation of methionine by 3-acetoxyxanthine has been observed.

Activation in Situ of N-Hydroxypurines and Purine N-Oxides

Comparative studies of esters of purine N-oxide derivatives have been limited because of the few N-acetoxy derivatives that are available (3). No O-acyl esters of the series of 3-hydroxyguanine derivatives have been isolated, and their reactions have been studied only in acetic anhydride or with esters formed from acyl chlorides in dimethylformamide (5). Samples of the N-acetoxy derivatives of xanthine derivatives are difficult to assay for purity, since solutions, even in dry dimethyl sulfoxide, are not stable for long periods. From adenine 1-oxide an O-acyl acetate, characterized through its analysis and spectrum, was isolated, but at that date only its reversion to the parent N-oxide in water or acid and its ring opening in acetic anhydride were studied (25).

With the application of the current method for esterification in situ, N-acetoxy derivatives are formed from all N-hydroxypurines (Table 1). Even true N-oxides, such as adenine 1-oxide or 1,7-dimethylguanine 3-oxide, readily form acetoxy esters under these conditions. The esters are formed very rapidly and 1st-order rate constants for their formation at 37° and pH 7.4 have been obtained for 3-hydroxy-7-methylxanthine \( (k = 6.5/\text{min}) \) and for adenine 1-oxide \( (k = 4.4/\text{min}) \). The rates of acetylation must be even higher for some of the more reactive N-hydroxypurines, for which higher rates were measured for their reactions with KI or pyridine (Table 2). The UV spectra of the N-acetoxy derivatives (Table 1) are usually different from those of the parent compounds, and esterifications were followed spectroscopically. The extent of the acetylation in the equilibrium mixture of ester and purine N-oxide derivative is at least 95%. This is most readily observed in 3-hydroxy-7-methylxanthine, the ester of which undergoes only Reaction I to II (Chart 1). Here the absorption of the N-hydroxy derivative, at 308 nm, completely disappears.
Table 1

<table>
<thead>
<tr>
<th>Parent compound</th>
<th>UV spectral properties of N-acetoxy purines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-Acetoxy derivative</td>
</tr>
<tr>
<td>1-Hydroxyxanthine</td>
<td>275 (7.5)</td>
</tr>
<tr>
<td>3-Hydroxyxanthine</td>
<td>257 (6.5)</td>
</tr>
<tr>
<td>3-Hydroxy-7-methylxanthine</td>
<td>238 (5.6)</td>
</tr>
<tr>
<td>3-Hydroxy-1,7-dimethylxanthine</td>
<td>261 (3.7)</td>
</tr>
<tr>
<td>3-Hydroxy-8-phenylxanthine</td>
<td>261 (15.6)</td>
</tr>
<tr>
<td>3-Hydroxy-8-methylxanthine</td>
<td>260 (5.5)</td>
</tr>
<tr>
<td>7-Hydroxyxanthine</td>
<td>256 (7.1)</td>
</tr>
<tr>
<td>7-Hydroxy-1,3-dimethylxanthine</td>
<td>257 (7.4)</td>
</tr>
<tr>
<td>7-Hydroxy-1,3,8-trimethylxanthine</td>
<td>250 (7.8)</td>
</tr>
<tr>
<td>9-Hydroxyxanthine</td>
<td>272 (3.25)</td>
</tr>
<tr>
<td>3-Hydroxyuric acid</td>
<td>302 (9.35)</td>
</tr>
<tr>
<td>Guanine 3-oxide</td>
<td>252 (5.8)</td>
</tr>
<tr>
<td>1,7-Dimethylguanine 3-N-oxide</td>
<td>267 (5.2)</td>
</tr>
<tr>
<td>7-Methylguanine 3-N-oxide</td>
<td>251 (5.7)</td>
</tr>
<tr>
<td>9-Methylguanine 3-N-oxide</td>
<td>275 (7.4)</td>
</tr>
<tr>
<td>3-Hydroxyhypoxanthine</td>
<td>285 (12.0)</td>
</tr>
<tr>
<td>Adenine 1-oxide</td>
<td>261 (8.1)</td>
</tr>
<tr>
<td>Purine 3-oxide</td>
<td>296 (8.85)</td>
</tr>
<tr>
<td>7-Methyl-6-methoxypurine 3-oxide</td>
<td>286 (8.9)</td>
</tr>
</tbody>
</table>

### Oxidizing Action of Purine N-Oxide Esters

The spectra of those esters that undergo only the hydrolytic reaction (I to II of Chart 1) are maintained for about 10 min at pH 7.4 and 37°C, after which they begin to revert to the spectra of the starting materials. The spectra of a rapidly reacting ester, such as 3-acetoxyxanthine, starts to change immediately toward the spectrum of the mixture of reaction products, and with the most rapidly reacting esters no definite intermediate ester spectrum is found. No spectral shift occurs with purines, such as adenine or guanine, under acetylation conditions, but a decrease of absorption, 20% with adenine and 14% with guanine, indicates that reaction, probably N-acetylation, does take place. Xanthine undergoes no spectral change. Acetic anhydride has an absorption at about 240 nm, and to observe the spectrum of the ester of compounds like pyridine N-oxide (λ_max 253 nm) requires care in the preparation of a control cuvet. The activation of N-hydroxyxypurines in situ has also been used for preparative syntheses, such as that of 1-methyl-8-methylmercaptoguanine, and for the reaction with transforming DNA (17).

### Oxidation of KI

The formation of iodine from KI and acetoxyxanthine (5) has now been demonstrated to be representative of a general oxidative reactivity of several acetoxy purines. The reaction can be measured at 352 nm where iodine has a strong absorption, and in the presence of I⁻ iodide the rate of the reaction is approximately 1st order. First-order rate constants, as given in Table 2, are a result of combined rates of the formation in situ of the ester and its subsequent reaction with iodide. The rate of iodide oxidation was also measured with chemically prepared 3-acetoxyxanthine and with xanthine 3-O-sulfate at k = 4.2 min⁻¹, and >10 min⁻¹, respectively, at 37°C, under conditions at which the combined rate of esterification and oxidation is k = 0.75 min⁻¹ (Table 2). The rate of esterification appears to be the limiting factor in the combined rate, but the combined rate is the only rate that can be obtained for those compounds for which the acetoxy derivatives are not available.

From the reactions for which rate constants are given in Table 2, it is apparent that the oxidizing activity is associated with 3-acetoxy derivatives of the guanine and xanthine type. 7-Acetoxyxanthine also oxidizes KI, but 1-acetoxyxanthine and 9-acetoxyxanthine do not. Four effects of substituents on the reaction can be noted. Substitution of nitrogen for C-8 (Table 2, Compound 12) and of the 7-H of the imidazole ring with a methyl (compounds 7 and 9) each reduces the oxidation of iodide by those compounds. Substitution of N-1 with methyl and, particularly, of C-2 with amino enhances the oxidizing activity. Thus, all guanine derivatives tend to be more reactive than the corresponding xanthine derivatives (compare Compounds 5 with 13 and 6 with 14 in Table 2). There is a clear trend of increasing oxidizing activity toward iodide from Compounds 7 and 8 (both inactive) through 15 (weakly active) to 16 (strongly active), and with 1,7-dimethyl-
Table 2
Reactivities of O-acetyl esters of purine N-oxide derivatives and related compounds

<table>
<thead>
<tr>
<th>Compounds and references</th>
<th>k (KI)*</th>
<th>[min⁻¹]</th>
<th>k (pyridine)*</th>
<th>[min⁻¹]</th>
<th>[¹⁴C]Methyl derivative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 1-Hydroxyxanthine (20)</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>2. 1-Hydroxyguanine (33)</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>3. Adenine 1-oxide (25)</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>4. 8-Hydroxyadenine 1-oxide (25)</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>5. 3-Hydroxyxanthine (34)</td>
<td>0.75</td>
<td>6.5</td>
<td>0.40</td>
<td>4.0</td>
<td>85</td>
</tr>
<tr>
<td>6. 3-Hydroxy-1-methylxanthine (2)</td>
<td>1.1</td>
<td>&gt;10</td>
<td>0.52</td>
<td>4.7</td>
<td>62</td>
</tr>
<tr>
<td>7. 3-Hydroxy-7-methylxanthine (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8. 1,7-Dimethyl-3-hydroxyxanthine (2)</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>9. 7,9-Dimethyl-3-hydroxyxanthine (34)</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>10. 3-Hydroxy-8-methylxanthine (2)</td>
<td>1.3</td>
<td>&gt;10</td>
<td>0.06*</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>11. 3-Hydroxy-8-phenylxanthine</td>
<td>7.5</td>
<td>—</td>
<td>2.0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>12. 3-Hydroxy-8-azaxanthine (7)</td>
<td>0</td>
<td>&lt;0.02</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13. 3-Hydroxyguanine (34)</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>1.2</td>
<td>5.7</td>
<td>89</td>
</tr>
<tr>
<td>14. 1-Methylguanine 3-oxide (2)</td>
<td>9.8</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>27</td>
</tr>
<tr>
<td>15. 7-Methylguanine 3-oxide (2)</td>
<td>0</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16. 1,7-Dimethylguanine 3-oxide (2)</td>
<td>8.7</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>17. 1,N2,N4-Trimethylguanine 3-oxide</td>
<td>&gt;10</td>
<td>—</td>
<td>&gt;10</td>
<td>—</td>
<td>42</td>
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<tr>
<td>18. 6-Methylpurine 3-oxide</td>
<td>&gt;0.01</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>19. 6-Methoxyguanine 3-oxide</td>
<td>0.02</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>20. 6-Methoxy-7-methylguanine 3-oxide</td>
<td>0.32</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>41</td>
</tr>
<tr>
<td>21. Hypoxanthine 3-oxide (21)</td>
<td>0</td>
<td>0.19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>22. 3-Hydroxyuric acid (15)</td>
<td>8.0</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>23. 7-Hydroxyxanthine (35)</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>8.0</td>
<td>&gt;10</td>
<td>43</td>
</tr>
<tr>
<td>24. 7-Hydroxy-8-methylxanthine</td>
<td>8.5</td>
<td>—</td>
<td>6.0*</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>25. 9-Hydroxyxanthine (32)</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>26. 3-Hydroxy-7-deazaxanthine (14)</td>
<td>&gt;10</td>
<td>—</td>
<td>&gt;10</td>
<td>—</td>
<td>54</td>
</tr>
<tr>
<td>27. 1,2,4-Trihydroxy-5,6-diaminopyrimidine</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>28. Pyridine 1-oxide</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>29. N-Hydroxyacetylaminofluorene (22)</td>
<td>&lt;0.01</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>30. NH₂OH</td>
<td>0.3</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>31. (NH₃)₂S₂O₄</td>
<td>0.90</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
</tbody>
</table>

* First-order rate constants for the oxidations of KI and substitutions by pyridine.
\* Yields of 8-methylmercapto derivatives from [methyl-¹⁴C]methionine.
\* —, no measurement was made.
\* The k's for these 8-methyl derivatives are based upon an increasing absorption maximum at about 420 nm, rather than the maxima at about 370 nm common to the 8-pyridinium derivatives. No pyridine substitution products have been obtained from these 8-methyl derivatives (30).
\* D. R. Sutherland, unpublished material.
\* J. J. McDonald, unpublished material.
\* F. L. Lam and J. C. Parham, unpublished material.
\* G. Zvilichovsky and D. R. Sutherland, unpublished material.
\* The N-hydroxypyrimidine from which 3-hydroxyxanthine is derived.

In Table 2, Compound 16 the 1-methyl group together with the 2-amino group overcome the blocking effect of the 7-methyl group. In a previous study (4) it was postulated that the imidazole anion of the acetoxy ester is the reactive species that undergoes either the rapid 8-substitution reaction or the reduction to xanthine. This study confirms the importance of the unsubstituted imidazole moiety for strong reactivity, but it also shows that an overall negative charge of the ester decreases its reactivity. The trimethyl derivative of guanine (Table 2, Compound 17) can only form a neutral zwitterion, and this ester has one of the highest oxidative and substitution reactivities.

Listed in Table 2 are some nonpurine compounds which serve as controls. Compound 26 is an analog of 3-hydroxy-
Oxidizing Action of Purine N-Oxide Esters

xanthine, and Compound 27 is a chemical precursor of 3-hydroxyxanthine lacking the imidazole ring. Hydroxylamine (Table 2, Compound 30) is the simplest functional group present in an N-hydroxypurine. Curiously, NH₂OH reduces iodine in neutral solution, but it has been observed that its acetyl derivative, perhaps N,O-diacetylhydroxylamine or N,N,O-triacetylhydroxylamine, oxidizes iodide. N-Hydroxy-N-acetyl-2-aminofluorene, a very extensively studied oncogen (22), was also measured (Table 2, Compound 29). Its ester is also readily formed in situ [noted by Bartsch et al. (1) for N-hydroxy-2-aminofluorene], and it does oxidize KI but at a rate very much lower than those of some of the acetoxy purines. Chart 2 (left) presents the influence of the pH on the rate of KI oxidation for 3 esters. It again demonstrates the generally greater rate constants for guanine derivatives, and the strong pH dependence of rates emphasizes the need for adequate buffering during the measurements. Chart 2 (right) depicts pyridinium adduct formation from 2 of those acetoxy derivatives.

Oxidation of Uric Acid, Cysteine, and Tyrosine

Uric Acid. The oxidative degradation of [8-'¹⁴C]uric acid, based upon a paper chromatographic separation of the mixture of oxidation products and the unchanged uric acid in Solvent A, was studied as a function of pH (Chart 3, bottom). The rapid oxidation of uric acid under mild conditions now permits an explanation of an earlier result (4) reproduced in Chart 3 (top). It shows a decrease in the overall recovery of UV-absorbing products of the reaction of 3-acetoxyxanthine with water at pH values above 3, while yields of uric acid reach a maximum near pH 5 and decrease at higher pH values. It is now apparent (Chart 3, bottom) that above pH 4 the previously observed decreasing recovery of uric acid, and the loss of UV-absorbing material, coincide with an increasing ability of 3-acetoxyxanthine (3-OAcXan) to oxidize uric acid according to the equation.

Uric + 3-AcOXan → Uric-OP + Xan + acetic acid

where Uric-OP is the mixture of uric acid oxidation products derived from uric acid after oxidation with 1 equivalent of oxygen. The material balance in Chart 3 (top) is essentially 100% if, for every equivalent of xanthine (Xan), an equivalent of uric acid oxidation product is added. Chart 3 (bottom) also shows a plot of the appearance of the blue by-product of unknown structure (4). Although deeply colored, its quantitative contribution to the material balance is marginal.

Ammonia is one of the products characterized in other oxidations of uric acid and, under reaction conditions as used for the lower part of Chart 3 (bottom), ammonia, 0.1%, was produced at pH 7.0. Traces of 8-aminoxanthine

Chart 3. Top, yield of products of reaction of 3-acetoxyxanthine with water as function of pH, reproduced from Ref. 4. Bottom, [2-'¹⁴C]uric acid oxidation by 3-acetoxyxanthine as a function of pH (O). [2-'¹⁴C]Uric acid autoxidation in a control experiment (Δ). Appearance of blue, insoluble by-product, arbitrary units, judged visually by comparison with a set of standard dilutions (□). 3-Acetoxyxanthine (3 mg) dissolved in 0.07 ml dimethyl sulfoxide was added to 0.2 ml of buffer which was 0.1 M with respect to sodium acetate, KH₂PO₄, and H₃BO₃, adjusted to pH values between 4.0 and 8.0 by 10 n NaOH. Previously added to the buffer were 10,000 cpm of [2-'¹⁴C]uric acid, and incubation was for 10 min at room temperature.
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occasionally observed among the reaction products from 3-acetoxyxanthine (N. J. M. Birdsall and U. Wölcke, unpublished material) can be attributed to this source of ammonia.

Esters of 3-hydroxyxanthine and 3 other derivatives that readily oxidize KI, and xanthine, were incubated with radioactive uric acid; the results are listed in Table 3, Column 1.

Cysteine. The reaction of 3-acetoxyxanthine with N-acetylcysteine resulted in a moderate yield of the 8-substitution product, S-(8-xanthyl)-N-acetylcysteine. With mercaptoethanol an analogous 8-substitution product was obtained. From similar incubations with cysteine, however, no analogous substitution product was detected. Instead [3-14C]cysteine was oxidized, presumably via cystine, to cysteic acid. Radioactive cysteine, incubated with 3-acetoxyxanthine by Procedure A and chromatographed in System B, showed the conversion of 50% of the radioactivity to material with an RF of 0.0 to 0.1, characteristic for a number of oxidation products. In a control with xanthine instead of 3-acetoxyxanthine, cysteine remained unchanged. Cation exchange chromatography (System C) separates cysteic acid and cysteinesulfinic acid from cysteine, while cystine and cystine sulfoxides remain near the origin. In that system the foregoing reaction mixture shows the presence of 30% of an acidic derivative, which could be either cysteic acid or cysteinesulfinic acid. Cysteinesulfinic acid was added, and the mixture was reduced with 1 N HCl: 1 M KI for 1 hr at room temperature (31). In the resulting mixture the added carrier sulfinic acid had been reduced, but the radioactivity remained with the acidic derivatives, which suggests that it was cysteic acid. Cystine, the likely 1st product of cysteine oxidation, was similarly investigated using radioactive cystine. The products of reaction with 3-acetoxyxanthine were indistinguishable in Systems B and C from those of the reaction of 3-acetoxyxanthine with cysteine. This suggests that cystine is an intermediate in the oxidation of cysteine to cysteic acid. No attempt was made to identify all of the reaction products, but cystine disulfide is probably not a major product, since treatment with 1 N NH4OH, a reaction that converts cystine disulfide into cysteinesulfenic acid (31), did not increase the amount of acidic material. Treatment of the reaction mixture with 1 N HCl at 100° for 1 min, however, increased the amount of cysteic acid from 30 to 50%.

In this oxidation of cysteine, 3-acetoxyxanthine is acting as a quite strong oxidizing agent. Another oncogene, 4-hydroxylaminoquinoline N-oxide, also oxidizes cysteine to cystine, but it does so only catalytically in the course of its autooxidation (12).

The oxidation of cysteine by other acetoxypurines, generated in situ by Procedure B, was also investigated. Amino acids are N-acetylated, in part, under these conditions, which complicates the reaction mixtures. In addition, S-acetylation of cysteine apparently takes place, and this lowers the K13 uptake upon titration (8). However, brief treatment with hot NH4OH restores the K13 titration values and permits an assay of the extent of oxidative loss of sulfhydryl groups. Results of such an assay are included in Table 3, Column 2.

Tyrosine. The reaction of 3-acetoxyxanthine with tyrosine includes an oxidation of the tyrosine molecule. This oxidation involves the m-position, as demonstrated by incubating [3,5-3H]tyrosine with acetoxypurines generated in situ and measuring the liberated tritium that could be recovered by lyophilization of the water. The percentage of radioactivity released by 4 oxidizing acetoxypurines is given in Table 3, Column 3. Substitution at position 3 of tyrosine, a reaction known to occur with N-benzoyloxy-N-methyl-4-azobenzene (16), may also occur with the acyloxy derivatives of xanthine and guanine, and such a reaction would also contribute to the amount of tritiated water liberated.

Table 3

<table>
<thead>
<tr>
<th>Oxidation of various compounds by acetoxypurines</th>
<th>Oxidation of [8-14C]uric acid (%)</th>
<th>mEq cysteine, as oxidized by I2*</th>
<th>Tritium liberated from [3,5-3H]tyrosine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Hydroxyxanthine</td>
<td>88</td>
<td>0.65</td>
<td>19</td>
</tr>
<tr>
<td>3-Hydroxy-8-methylxanthine</td>
<td>94</td>
<td>0.45</td>
<td>48</td>
</tr>
<tr>
<td>3-Hydroxyguanine</td>
<td>96</td>
<td>0.40</td>
<td>10</td>
</tr>
<tr>
<td>1,7-Dimethylguanine 3-oxide</td>
<td>30</td>
<td>0.75</td>
<td>9</td>
</tr>
<tr>
<td>Xanthine (control)</td>
<td>12</td>
<td>1.30</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* Purified [2-14C]uric acid, 10⁶ cpm (84 x 10⁶ cpm/μmole) was reacted with acetoxypurines according to Procedure B, and the extent of oxidation was determined after chromatography in System A.

* To L-cysteine, 1.6 mmoles in 2.0 ml of 0.1 M phosphate buffer, pH 7.4, containing 10⁻³ M EDTA, plus 5 mg of a purine N-oxide in 1.0 ml of the same buffer were added 10 ml of acetic anhydride, and the mixture was allowed to react at 25° for 5 min. After that 3 drops of concentrated NH4OH were added; the mixture was then heated to 100° for 1 min, cooled, acidified by the addition of 7 drops of concentrated HCl, and titrated with 1 N KI₄ solution.

* [3,5-3H]Tyrosine, 1.38 x 10⁶ cpm (8.0 Ci/mmole), was incubated with acetoxypurines according to Procedure B in 10-ml test tubes. After completion each test tube was lyophilized into a glass trap with cotton wads preventing solids from reaching the trap. Tritium in the lyophilisate was then determined in a liquid scintillation counter.
However, the tritium liberated by the ester of 1,7-dimethylguanine 3-oxide, with which no 8-substitution reaction occurs, must be presumed to be solely the result of oxidation. The products from the reactions with tyrosine are under investigation, as are the products of oxidation of tryptophan that accompany the formation of an adduct, [3-(2-amino-2-carboxyethyl)-3-(8-xanthinyl)]indolenine (28).

**Evidence for Radicals Formed from Oxidizing Acetoxypurines**

The catalysis of the autoxidation of sulfite has been used to identify radical intermediates in chemical and enzymatic reactions (9). In that method the autoxidation of sulfite is followed with an oxygen electrode, and an abrupt drop of oxygen pressure upon admission of reactant is considered evidence for the presence of radicals. Three preformed acetoxypurines were tested in the apparatus shown in Chart 4. The 2 compounds showing oxidizing activity, 3-acetoxyxanthine and 3-acetoxy-8-methylxanthine, were positive (Chart 4); while 3-acetoxy-8-azaxanthine and xanthine did not catalyze sulfite autoxidation. These results suggest that the oxidizing agent formed from acetoxypurines may be a radical. Radicals from several esters of carcinogens and noncarcinogens have been demonstrated by the method of chemically induced dynamic nuclear polarization and are evidence for a radical pair formed by homolytic fission of the N—O bond of the active ester (13). An alternative structure has been attributed to radicals from hydroxamic esters of polycyclic hydrocarbons. Decoloration of dipicrylhydrazyl radicals by solutions of hydroxamic esters was used as evidence for those radicals (23), and the nature of the radicals was considered to be a nitrene triplet (10), formed by spin inversion of a nitrene and typically facilitated by heavy atoms. However, this heavy atom effect could not be demonstrated for 3-acetoxyxanthine. In an experiment to test for a heavy atom effect, acetoxyxanthine, prepared in situ, was allowed to decompose spontaneously in buffers of either 2 M sodium acetate or 2 M sodium bromoacetate, pH 7.4. In duplicate experiments no significant difference could be detected in the amounts of xanthine formed. It appears, as was the case in an experiment with the ester in dioxyanecarbon tetrachloride (4), that there is no evidence that a nitrene—carbon tetrachloride may be formed from 3-acetoxyxanthine.

**Oxidizing Activity Compared with Electrophilicity**

The nitrenium IV ion formed in Reaction 1 to IV (Chart 1) is in equilibrium with a carbonium ion at C-8 and results in electrophilic substitution of the purine at position 8 by nucloephiles (4, 5). For comparison of this mode of reaction with the oxidative mode, 2 representative substitution reactions were measured (Table 2). The reaction with pyridine, leading to 8-pyridinium xanthine from 3-acetoxyxanthine (5), was found to be a general reaction, and rate measurements were based on the absorption of the product at 352 nm, in a manner analogous to that of the iodide oxidation. The 2nd substitution reaction (Table 2) is that with methionine, followed by heat cleavage of the intermediate to yield 8-methylmercaptopurines. This reaction is measured by the extent rather than by the rate of the reaction. The results summarized in Table 2 indicate that iodide oxidation and the 2 substitution reactions generally parallel one another. The acetoxy derivatives of all compounds thus far found to be oncogenic show both properties (6), and the sulfate ester of 3-hydroxyxanthine resembles 3-acetoxyxanthine in this respect, but its reaction rates are >10 min⁻¹, at 37⁰, for both reactions. Two acetoxy derivatives derived from 1,7-dimethylguanine 3-oxide (Table 2, Compound 16) and 3-hydroxyuric acid (Table 2, Compound 22) oxidize only iodide. No case of the reverse, namely strong susceptibility to substitution without oxidizing ability, has yet been found. The nitrene formed from 3-hydroxyuric acid has no substitutable carbon atom, whereas 1,7-dimethylguanine 3-oxide has an unsubstituted C-8, but, like other 7-methyl derivatives (Table 2, Compounds 7, 8, 9, and 15), it does not substitute. An assay of it may test the oncogenic significance of the oxidative mode of reaction. 3-Hydroxyuric acid has been tested in rats, but the interpretation is complicated by metabolic ambiguity (15). Three cases of reaction with pyridine but not with methionine can be noted; 2 are 8-methyl derivatives (Compounds 10 and 24) and 1 is an 8-phenyl derivative (Compound 11). The pyridine derivatives of both 8-methyl derivatives, as well as 1 from 8-methylguanine 3-oxide (not listed in Table 2), have an absorption maximum around 415 to 425 nm, at pH 7.4; whereas all the other 8-pyridinium purines have an absorption maximum around 375 nm, at pH 7.4. None of the pyridinium derivatives of 8-methylpurines has yet been chemically characterized. 8-Methyl-3-hydroxyxanthine has been studied by Sutherland (30), and a rearrangement of its ester with water to yield 8-hydroxymethylxanthine was
described. However, this was the only nucleophilic substitution found, and the reaction is thus different from the more general reactivity of the ester of 3-hydroxyxanthine. Among this group of compounds only 3-hydroxy-8-methylxanthine has been tested and is now known to be a non-oncogen (6).

In conclusion, the significance of either or both of the 2 modes of reactivity to the oncogenicity of purine N-oxide derivatives cannot yet be determined. The present results suggest that oxidative destructions as well as substitutions may well take place in vivo. All examples that show a strong activity in the KI oxidation and in the reactions with pyridine and have been assayed are oncogens in the rat. Most examples yet assayed that are negative in both modes of reactivity are also nononcogenic or only weakly oncogenic (6). The present experiments suggest that 1,7-dimethylguanine 3-oxide, with solely the oxidative activity, is a prime candidate for a biological test of the importance of the oxidative type of activity. Adenine 1-oxide is definitely a weak oncogen (29), but its acetyl derivative reacts solely by Mode 1 and not at all by Modes 2 and 3 of Chart I. No chemical properties relating it to those of the potent oncogens, 3-hydroxyxanthine and 3-hydroxyguanine, have yet been observed.

ACKNOWLEDGMENTS

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1970.
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