Conversion of the Oncogenic 1-Methylguanine 3-Oxide to 3-Hydroxy-1-methylxanthine

Jerome J. McDonald, Gerhard Stöhrer, and George Bosworth Brown
Memorial Sloan-Kettering Cancer Center, New York, New York 10021

SUMMARY

Deamination of the oncogenic 1-methylguanine 3-oxide occurs to a significant extent in rats to yield 3-hydroxy-1-methylxanthine and its metabolites. When 3-hydroxy-1-methylxanthine is administered, 1-methyl-8-methylthioxanthine can be recovered from urine and released from hepatic protein. No 1-methyl-8-methylthioguanine was detected in urine or bound to protein. There is no evidence of significant activation of 1-methylguanine 3-oxide by sulfotransferase, but deamination to the oncogenic 3-hydroxy-1-methylxanthine suffices to explain its oncogenicity.

INTRODUCTION

Two oncogenic purine N-oxides, 3-hydroxyxanthine and 3-hydroxyguanine, have been extensively studied in vivo. Many metabolites of 3-hydroxyxanthine have been characterized as products resulting from the action of xanthine oxidase and from the reaction of nucleophiles with chemically reactive esters. 3-Hydroxyguanine also functions as a substrate for xanthine oxidase and yields 8-substitution products resulting from nucleophilic attack on an activated intermediate, and may be an oncogen by a mechanism similar to that of 3-hydroxyxanthine. In addition, 3-hydroxyguanine in vivo does undergo some deamination to 3-hydroxyxanthine. In parallel assays, the 1-methyl derivatives of each of the 1st 2 showed the same order of oncogenicity as did their parent 3-hydroxypurines. The 3-hydroxy-1-methylxanthine may well be expected to be oncogenic by the same mechanism as the 1st 2, since the chemical reactivity of 3-acetoxy-1-methylxanthine is similar to that of 3-acetoxyxanthine and the 1-hydrogen is not implicated in the intermediate chemical reactions thought to be involved. However, 1-methylguanine-3-oxide is not analogous to the 1-methylxanthine derivative in that it cannot yield a 3-hydroxy derivative by tautomerism of a 1-H, as does 3-hydroxyguanine.

Of additional interest to the present study are the observations that the deamination of 3-hydroxyguanine to 3-hydroxyxanthine could be demonstrated in vitro with commercial guanine deaminase, while the comparable transformation of 1-methylguanine 3-oxide was not observed. This observation, combined with the very low activity of 1-methylguanine 3-oxide as a substrate for sulfotransferase in an in vitro assay for activation, caused us to compare the metabolic fates of 1-methylguanine 3-oxide and 3-hydroxy-1-methylxanthine with their unmethylated parent 3-hydroxypurines to provide a better understanding on the oncogenic properties of these purine N-oxides.

MATERIALS AND METHODS

Animals. Male CD rats, 150 to 350 g body weight, were used. Test compounds in 0.4 ml of buffer were administered i.p., and the animals were allowed food pellets and 0.5% NaCl solution to drink during the course of the experiment.

Radioactivity Determinations. A Packard scintillation counter with external standard was used with a scintillation flow cell coupled to an analog rate meter to monitor the effluent from the column chromatography of urine samples. Determinations on liquid samples were made in a dioxane-based scintillation cocktail. Protein samples were first solubilized in Soluene 100 (Packard, Chicago, Ill.) and counted in 3a70B cocktail (Research Products International, Elk Grove Village, Ill.). Paper chromatograms were quantitated with a paper-strip scanner of 4π geometry (Atomic Accessory Inc., Chicago, Ill.).

[2,14C]1-Methylguanosine. In an adaptation of a procedure described in the literature, [2,14C]1-Methylguanosine, 46.7 mCi/mmole (Schwarz/Mann, Orangeburg, N. Y.) and 3.5 mg (25 μmole) of well-powdered anhydrous potassium carbonate in 0.3 ml of dry dimethyl sulfoxide were stirred in a closed vial with a magnetic stirring bar. After 1.5 μl of methyl iodide (~25 μmole) were added, the reaction was allowed to proceed at room temperature for 1 hr. The reaction mixture was poured onto a 1.2- x 23-cm column of Bio-Rex 9 (Bio-Rad Laboratories, Richmond, Calif.) previously equilibrated with the eluting buffer of 0.1 M (NH4)2CO3 at pH 8.2. The [2,14C]-1-methylguanosine fractions comprised 50% of the total radioactivity. Unreacted guanosine could be recovered pure upon further elution. Pooled 1-methylguanosine fractions were radiochemically pure on paper chromatography (No. 597; Schleicher and Schuell, Keene, N. H.) in isopropyl alcohol:water:concentrated ammonia, 70:20:10 (Rf 0.62).

[2,14C]-1-Methylguanine. After concentration of the 1-methylguanosine fractions to dryness, hydrolysis was performed in 2 ml of 2 N HCl on the steam bath for 30 min. Again, the product was radiochemically pure on paper in the above solvent with an Rf of 0.50.

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2 To whom requests for reprints should be sent.

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[2-14C]-1-Methylguanine 3-Oxide. In an adaptation of a published procedure (4), the lyophliized 1-methylguanine preparation was redissoved in 1 ml of trifluoroacetic acid, cooled to 4°, and treated with 0.05 ml of 30% H2O2. After 24 hr at 4°, the mixture was lyophilized. Paper chromatography of an aliquot in 3% NH4HCO3 showed complete conversion of products with [2-14C]-1-methylguanine 3-oxide present in 34% yield. The N-oxide was isolated radiochemically of an aliquot in 3% NH4HCO3 showed complete conversion or laboratories, ammonium form, in 0.1 M ammonium acetate, pure after chromatography on 10 ml of AG-SO (Bio-Rad Laboratories), ammonium form, in 0.1 M ammonium acetate, pH 5.0.

[2-14C]-1-Methyl-3-hydroxyxanthine. A portion of the [2-14C]-1-methylguanine 3-oxide was made free of buffer on a 0.9- x 3-cm column of AG-50 [H+] by thoroughly washing with water. 1-Methylguanine 3-oxide was eluted with 1 N HCl, the fractions were combined, and hydrolysis was performed on the steam bath for 18 hr. After the solution was evaporated to dryness, the [2-14C]-1-methyl-3-hydroxyxanthine was readily separated from unhydrolyzed 1-methylguanine by elution with water from the short AG-50 column.

8-Chloro-1-methylxanthine. With the use of a recently developed method of in situ generation of an active ester (8), 200 mg of 3-hydroxy-1-methylxanthine were reacted in 30 ml of 2 n NaCl with periodic additions of a total of 1 ml of acetic anhydride. The temperature was kept at 35° and the pH was kept near 5 by the addition of 1 n NaOH. When the pH no longer changed, a blue precipitate was filtered, the filtrate was evaporated, and the residue was recrystallized to yield 8-chloro-1-methylxanthine identical to an authentic sample (6). It was eluted with 1 N HCl at 22 ml from 10 ml of AG-50 resin, and showed Rf's of 0.44 and 0.82 in Systems A and B (Table 1).

1-Methyl-8-methylthioxanthine. A mixture of 400 mg of 3-hydroxy-1-methylxanthine and 1.2 g of methionine was dissolved with the aid of NaOH in 30 ml of H2O at 70°. The pH was kept at 6 while 0.3 ml of acetic anhydride was added in portions. The hot, filtered solution deposited 250 mg of crystals on cooling which were again recrystallized from H2O to yield 1-methyl-8-methylthioxanthine identical to an authentic sample (5).

RESULTS

Metabolism of [2-14C]-1-Methylguanine 3-Oxide. After a male CD rat was given 13 × 10⁶ cpm (0.36 μmole) of [2-14C]-1-methylguanine 3-oxide by injection, the urine was collected at 6, 12, and 24 hr. In 5 experiments, 72 to 93% of the administered radioactivity was recovered at 6 hr in the urine; in a 6th experiment, only 40% was recovered. Up to an additional 7% was recovered at 12 hr and less than 2% at 24 hr. The variation in recoveries did not parallel body weights.

When aliquots of 6-hr urine, upon admixture with nonradioactive standards dissolved in 1 N HCl, were chromatographed on a 10-ml AG-50 column with 1 N HCl as eluent, the metabolites were well resolved. Appropriate fractions were concentrated in a vacuum over KOH, taken up in 1 N NH4OH, and chromatographed on paper in 2 systems. Table 1 lists the identified metabolites from a representative experiment along with chromatographic data on 2 unknowns. The appearance of 3-hydroxy-1-methylxanthine as a major metabolite required a search for its possible metabolites. For identification of the minor metabolite 1-methyl-8-methylthioxanthine, better separation in the presence of 1-methylguanine and other metabolites was achieved with a shorter column eluted with weaker acid (Table 1). Administration of a larger dose (0.7 μmole) of [2-14C]-1-methylguanine 3-oxide of the highest available specific activity increased the total amount of metabolite present in the urine, leading to clear identification of 1-methyl-8-methylthioxanthine by chromatography. This metabolite is analogous to the 8-methylthioxanthine detected as a urinary metabolite of 3-hydroxyxanthine (16). However, the analog of the 8-chloroxanthine, which also comes from 3-hydroxyxanthine (16), was not detected. The elution volume of 22 ml of 1 N HCl on the 10 ml column for 8-chloro-1-methylxanthine did not correspond to that of any radioactive metabolite. Both unlabeled reference materials were prepared from 3-hydroxy-1-methylxanthine.

Two unknowns (Table 1, A and B) were eluted in 1 N HCl from the 10-ml column as a shoulder preceding 1-methylguanine 3-oxide. Paper chromatography revealed them in mixture with the N-oxide and allowed only an estimate of their combined yield.

A small amount of 8-methylthioguanine does appear as a metabolite of 3-hydroxyguanine (8). The analogous 1-methyl-8-methylthioguanine was not detected in these experiments, even after pooling and concentration of the column fractions centered around its elution volume of 380 ml.

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**Table 1**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Elution volume (ml)</th>
<th>Rf a</th>
<th>Rf b</th>
<th>% 14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Methyluric acid</td>
<td>11</td>
<td>0.50</td>
<td>0.54</td>
<td>19</td>
</tr>
<tr>
<td>3-Hydroxy-1-methylxanthine</td>
<td>55</td>
<td>0.73</td>
<td>0.59</td>
<td>5</td>
</tr>
<tr>
<td>1-Methylxanthine</td>
<td>88</td>
<td>0.57</td>
<td>0.64</td>
<td>0.4</td>
</tr>
<tr>
<td>Compound A</td>
<td>~140</td>
<td>0.45</td>
<td>0.47</td>
<td>~6</td>
</tr>
<tr>
<td>Compound B</td>
<td>~260</td>
<td>0.87</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>1-Methylguanine 3-oxide</td>
<td>163</td>
<td>0.67</td>
<td>0.46</td>
<td>68</td>
</tr>
<tr>
<td>1-Methyl-8-methylthioxanthine</td>
<td>165c</td>
<td>0.40</td>
<td>0.77</td>
<td>0.2</td>
</tr>
</tbody>
</table>

a Three % aqueous ammonium bicarbonate.
b 1-Butanol:acetic acid:water, 4:1:5.
c Estimations from the scintillation flow cell chart and scintillation counting of fractions were in good agreement.
d Eluted from 5 ml of AG-50 with 0.1 N HCl. Administered 1-methylguanine 3-oxide elutes from this column only after development with 1 N HCl.

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**Urinary Metabolites from [2-14C]-1-methylguanine 3-oxide**

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The absence of 1-methylguanine as a metabolite is attributable to its susceptibility to guanase (1).

Metabolism of [2-14C]-3-Hydroxy-1-methylxanthine. In order to determine from which purine N-oxide the unknowns in Table 1 are derived, [2-14C]-3-hydroxy-1-methylxanthine was prepared. The metabolites found in urine collected 6 hr after administration of 6 \times 10^4 cpm (0.17 \mu mole) of the test material are listed in Table 2 along with chromatographic data. It is apparent that the unknowns contained in Peaks A and B of Table 1 must result from metabolic transformation of the 1-methylguanine 3-oxide and not from the metabolites related to the xanthine, for no radioactivity is eluted after the peak at 130 ml. However, a new metabolite (eluting at 79 ml) appears which is readily resolved from 1-methylxanthine.

The material eluting at 130 ml can be desulfurized with Raney nickel to yield a single product identical to 1-methylxanthine on paper chromatography, which furnishes additional proof of the identity of 1-methyl-8-methylthioxanthine. The same product could be released from hepatic protein prepared 24 hr after injection with [2-14C]-3-hydroxy-1-methylxanthine. Thus, boiling a dried protein preparation, with 222 cpm/mg, in water led to recovery of 8% of the bound radioactivity on the filtrate. Of this, 90% was identified as 1-methyl-8-methylthioxanthine. The corresponding 8-methylthioxanthine has been released from protein following in vitro activation experiments of 3-hydroxyxanthine (16). No radioactivity corresponding to an 8-methylthio derivative was obtained from hepatic protein following the metabolism of [2-14C]-1-methylguanine 3-oxide under the same conditions.

The identity of the 1-methyluric acid metabolite in each experiment was confirmed by concentration of the early fractions from the AG-SO column, and rechromatography on 10 ml of AG-1 resin (Bio-Rad Laboratories). After the AG-1 column was washed with 50 ml of 10% ammonium acetate, the 1-methyluric acid was eluted with 5% acetic acid in 10% ammonium acetate with the marker at 50 ml. The extensive accumulation of 1-methyluric acid (Tables 1 and 2) is attributed to its nonsusceptibility to uricase (3), an observation that we have verified.

DISCUSSION

The metabolism of 1-methylguanine 3-oxide is less extensive than that of some other purine N-oxides. In urine collected at 6 hr, 68% of the radioactivity collected is unconverted 1-methylguanine 3-oxide, while in the metabolism of 3-hydroxyguanine, only 23% was unconverted by 5 hr (14). In contrast, comparison of the xanthine derivatives shows equally extensive metabolism in that, after 6 hr, only 12% of 3-hydroxyxanthine is unconverted (11) and only 8% of 3-hydroxy-1-methylxanthine is recovered (Table 2). The 4 identified metabolites of 1-methylguanine 3-oxide are products that have been deaminated: 1-methyluric acid, 3-hydroxy-1-methylxanthine, 1-methylxanthine, and 1-methyl-8-methylthioxanthine (Chart 1). Despite an earlier lack of success in detecting deamination in vitro (8), it is obvious that, in vivo, the major metabolic pathway leading to at least two-thirds of the products is deamination. The discrepancy may be due to species specificity, since the in vitro studies were carried out with commercial rabbit liver guanine deaminase. However, parallel comparisons of the deamination in vitro of 1-methylguanine and guanine show differences that are concentration dependent; the deamination of 1-methylguanine is barely detectable at 25 \mu M and, at 125 \mu M, proceeds at one-half the rate at which guanine does at 13.3 \mu M (1). The 2 metabolites, 1-methyluric acid and 1-methyl-8-methylthioxanthine, which have been isolated from the urine of rats given either 1-methylguanine 3-oxide or 3-hydroxy-1-methylxanthine, must result from reactions of the ester of the latter. The 8-substituted metabolites are directly analogous to those detected during the metabolism of 3-hydroxyxanthine (11, 16). Conversion of 3-hydroxyguanine in vivo results in formation of 8-methylthioguanine (8), while the analogous 1-methyl-8-methylthioguanine has not been detected in this study. The products formed in vivo and attributable to the chemical reactivity are the same from both oncogens studied here, but they can result from enzymatic activation of only the xanthine derivative. This evi-
idence indicates that deamination is the necessary and limiting step in the conversion of the methylguanine derivative to the actual oncogenic compound.

Both methylated purines are highly oncogenic (8), although the guanine displays insignificant activity (3%), compared with the xanthine (45%), in terms of the release of sulfate from 3'-phosphoadenylyl sulfate during the sulfotransferase activation assay (10). The sulfate ester of the N-hydroxypurines is believed to be the chemically reactive intermediate involved in oncogenesis (17), and the formation of 8-substituted derivatives has been taken as evidence for this enzymatic activation. The difference observed in the sulfotransferase assay probably reflects a difference in $K_m$, since both purines were used at the same 0.5 to 0.6 mM concentration. The detection of 1-methyl-8-methylthioguanine as a urinary metabolite, its release from hepatic protein, and the lack of detection of a comparable 1-methylguanine metabolite all suggest that activation of 1-methylguanine 3-oxide by sulfotransferase in vivo is not important to its oncogenesis. This contrasts with 3-hydroxyguanine, with which both deamination to 3-hydroxyxanthine and sulfate ester formation leading to 8-methylthioguanine are observed (8, 10, 14).

Since it is not affected by uricase, the formation of 1-methyluric acid represents the final transformation of a very large proportion of the 3-hydroxy-1-methylxanthine. A portion of the 1-methyluric acid could arise via 1-methylxanthine. However, 1-methylxanthine represents a very small proportion of the transformation products of 3-hydroxy-1-methylxanthine, while xanthine represents as much as 8.6% of the metabolites of 3-hydroxyxanthine (11). Since 1-methylxanthine is not oxidized faster than xanthine by either milk or human liver xanthine oxidase (2), a considerable portion of the 1-methyluric acid may arise via the alternative pathway of reaction of the intermediate sulfate ester of 3-hydroxy-1-methylxanthine with water. Rechromatography of 1-methyluric acid fractions failed to detect significant radioactivity in either uric acid or an "allantoin fraction," such as is observed from 3-hydroxyxanthine (11) and 3-hydroxyguanine (14). Accumulation of only 1-methyluric acid adds evidence that significant demethylation occurs.

The unidentified materials encountered as metabolites of 1-methylguanine 3-oxide could be oxidation products such as the 8-hydroxy derivative and 8-hydroxy-1-methylguanine, analogous to those resulting from 3-hydroxyguanine (14). The unknown compound C (Table 2) from 3-hydroxy-1-methylxanthine has chromatographic characteristics predictable for 3-methoxy-1-methylxanthine. A further examination of the metabolism of 3-hydroxyxanthine has resulted in the identification of a 3-methoxyxanthine as a significant metabolite (13). The lack of evidence for the formation of 1-methyl-8-

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