Mutagenicity of Derivatives of the Oncogenic Purine N-Oxides

Robert W. McCuen, Gerhard Stöhrer, and Francis M. Sirotnak

SUMMARY

Acetoxy esters of purine N-oxides inactivate and induce mutations in Bacillus subtilis-transforming DNA. The esters were the chemical models available for the sulfate esters believed to be formed in vivo. When metabolic susceptibilities were taken into consideration, there is a reasonable correlation between the mutagenicity of various acetoxy esters and the oncogenicity of the parent N-oxide derivatives. The acetoxy esters of 3-hydroxyxanthine and 3-hydroxy-1-methylguanine (both strong oncogens in rats) were the most potent mutagens. The acetoxy ester of 7-hydroxyxanthine was also a strong mutagen. Acetoxy esters of the 7-methyl- and 8-aza-substituted 3-hydroxyxanthine derivatives, as well as 1-acetoxyadenine, 3-acetoxypurine, and 3-acetoxyhypoxanthine, were weak mutagens or not mutagenic. Most of the 3-acetoxyxanthine-induced mutations spontaneously reverted to wild type. The frequency of reversion was increased to the greatest extent with ethylmethane sulfonate, but was also increased with the base analogs 2-aminopurine and 5-bromodeoxyuridine. Only a few of the mutations were reverted by frame-shift mutagens. It was concluded that mutation induction in transforming DNA by the acetoxy esters of purine N-oxides occurs by both transition and transversion base-pair substitution.

INTRODUCTION

A possible basis for tumor induction by chemicals is via a direct interaction with chromosomal DNA that results in mutation. A number of chemical oncogens (1, 2, 11, 12, 42) or their metabolic derivatives (1–3, 23, 25, 28, 29, 35) have been shown to be mutagenic in a variety of systems. Although the data on mutagenicity provide no direct proof that the initial events leading to tumor induction by chemicals are genetic, the frequent correlations between mutagenicity and oncogenicity do have important biological implications.

Tumor induction by certain purine N-oxides occurs following s.c. injection in rats (8, 9, 35, 36, 38, 39). One of these, 3-hydroxyxanthine, has been studied chemically in some detail (6, 7). It has been postulated (27, 32, 33) that this compound is enzymatically converted in vivo to a sulfate or phosphate ester, which is a proximate oncogen. This metabolic activation is similar to that reported for the arylhydroxyamines and amides (13, 19, 28–31).

This investigation was undertaken to assess the mutagenic activity of a group of activated purine N-oxides. The N-acetoxy derivative, a chemical model of the reactive sulfate ester (27, 32) formed in vivo, was allowed to react with transforming DNA from Bacillus subtilis. This type of study offers distinct advantages in that the mutagenic activity can be determined in the absence of permeability barriers or metabolic alteration of the test compound.

MATERIALS AND METHODS

Bacterial Strains. Wild-type donor DNA was extracted from B. subtilis strain SB19 provided by Dr. I. Takahashi, McMaster University, Hamilton, Ont., Canada. The recipient during transformation was the tryptophan-requiring B. subtilis strain T3 which bears a mutation (trp-) in the tryptophan synthetase B gene (obtained from Dr. B. S. Strauss, University of Chicago, Chicago, Ill.).

Media and Preparation of DNA. Following transformation by the method of Anagnostopoulos and Spizizen (5), the trp+ transformants that no longer require tryptophan were counted on the modified (24) minimal medium of Vogel and Bonner (41) containing 2% Bacto agar (Difco Laboratories, Detroit, Mich.). The same medium lacking indole was used for counting indole revertants (ind+) in the back-mutation studies described below. Purified DNA was prepared by a modification (16) of the method of Marmur (26).

Compounds Used for Mutagenicity Testing. All purine N-oxides used were analytically and chromatographically pure. Special attention was given to ensure that the preparations were free of related purine N-oxides. References for all compounds are found in the paper of Brown et al. (9).

Treatment of DNA with Test Compounds. To 0.1 to 0.3 mg of DNA in 0.5 ml of 0.01 M NaCl at pH 6.5 were added 7 mg of the acetoxypurine derivative in 0.05 ml of dimethyl sulfoxide. A drop in pH due to the decomposition of the active ester was avoided by continuous addition of 1 N NaOH to the stirred solution at 37°. In some experiments, the acetoxy derivative was generated³ in the presence of

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²To whom reprint requests should be sent.

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³G. Stöhrer, in preparation.

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DNA from 6 mg of N-hydroxypurine and 0.005 ml of acetic anhydride with the pH controlled as described above. After treatment by either method for 10 min, the reaction mixture was chromatographed over 40 ml of Sephadex G-50 equilibrated with 2 M NaCl and 0.02 M citrate at pH 6.5. This procedure separated DNA from most of the low-molecular-weight products so that on storage at 4° in 2 M NaCl there was no loss in activity over a period of several months. DNA was treated with NaNO₃ in the manner described by Strack et al. (34).

**Assay for Mutation Induction and DNA Inactivation.** Mutation induction was determined in the system of Freese and Strack (15) where (ind⁻) mutant transformant colonies requiring indole for growth are detected by their UV fluorescence. Transformation of the T3 strain was done at a limiting concentration of DNA (less than 0.5 μg/ml). Transformant (trp⁺) colonies obtained with treated and untreated DNA provide a measure of biological inactivation for each sample. All DNA samples were routinely checked for sterility.

**Reversion of trp⁺ ind⁻ Mutants to Wild Type by Base Analogs.** trp⁺ ind⁻ cultures were grown overnight at 37° with shaking in a minimal medium (40) plus indole (20 μg/ml) and either AP (50 μg/ml) or BUdR (20 μg/ml). Control cultures were prepared in the same medium without the base analogs. Each culture was centrifuged, washed, and resuspended at 10-fold concentration in minimal medium (40) and plated on the supplement Vogel-Bonner medium (41) without indole. For total viable cell count, an aliquot of cells was diluted and plated on the same medium with indole. All plates were incubated for 48 hr at 37°.

**Reversion of trp⁺ ind⁻ Mutants to Wild Type by EMS.** Revertants were obtained by platting 0.1 ml of an overnight culture grown in minimal medium (40) plus indole on Vogel-Bonner medium (41) without indole. EMS (Eastman Organic Chemicals, Rochester, N. Y.) at 1 spot on each plate prior to incubating for 48 hr at 37°. Reversion frequencies were estimated by comparing the number of colonies observed in the vicinity of the mutagen to the colony number observed in a similar size area on the control plates.

**Reversion of trp⁺ ind⁻ Mutants to Wild Type by Frame-Shift Mutagens.** Mutant cultures were grown overnight as described above in minimal medium (40) plus indole with one of the following mutagens: hycanthone monomethane sulfonate (35.6 μg/ml) from Sterling-Winthrop Research Institute, Rensselaer, N. Y., Miracil D (3.4 μg/ml), or the acridine mustard, ICR 191 (35 μg/ml). The treated culture and a control culture were centrifuged and resuspended at a 10-fold concentration in minimal medium (40) and plated on Vogel-Bonner medium (41) without indole. Viable counts were obtained as described above.

**RESULTS AND DISCUSSION**

**Reactions of Acetoxypurines with DNA.** In experiments in which DNA was incubated with acetoxypurines, the addition of ethanol gave an appreciably bulkier precipitate than was observed when the untreated DNA was reprecipitated. The precipitate from the treated DNA was deep blue, and in solution it showed large spectral differences from the DNA. This apparent physical binding of reaction products was investigated by the use of radioactively labeled purines followed by chromatography over Sephadex G-50. To 0.5 ml of solution containing 70 μg of DNA were added 5 mg of xanthine-8-¹⁴C or of 3-hydroxyxanthine-8-¹⁴C. The mixtures were stirred at pH 8.0 for 30 min and then clarified by centrifugation. The solution of DNA and xanthine contained 0.48 μmole of xanthine per ml, with an activity of 6.41 x 10⁴ cpm/μmole. That of DNA and 3-hydroxyxanthine contained 2.3 μmoles of 3-hydroxyxanthine per ml, with an activity of 9.5 x 10⁴ cpm/μmole. An aliquot of the solution of labeled 3-hydroxyxanthine and DNA was also treated with acetic anhydride as described in “Materials and Methods.” The 3 mixtures were each precipitated with ethanol and processed for radioactivity measurements which, expressed as moles of purine per mole of nucleotide, were 1 in 400 for xanthine, 1 in 40 for 3-hydroxyxanthine, and 1 in 3 for the 3-hydroxyxanthine incubated with acetic anhydride.

Gel chromatography of the treated DNA preparations removed all of the xanthine or 3-hydroxyxanthine in 1 chromatographic step. The loss of the several purine reaction products from the DNA treated with 3-hydroxyxanthine and acetic anhydride required repeated recchromatography. The radioactivity decreased exponentially and approached background after 5 repeated chromatographies (Chart 1).

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*The abbreviations used are: AP, 2-aminopurine; BUdR, 5-bromodeoxyuridine; EMS, ethylmethane sulfonate.*
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An insoluble, unidentified, blue reaction product of 3-acetoxyxanthine (Ref. 6; J. Birdsall and G. Stöhrer, unpublished results) is apparently responsible for the strong physical binding of radioactivity to the treated DNA. With native DNA, the question of a trace of chemical binding cannot yet be settled because of the considerable and persistent physical binding. The blue reaction product is not mutagenic when mixed with *B. subtilis*-transforming DNA (R. McCuen, and F. Sirotnak, unpublished results). In addition, the level of mutagenicity induced by 3-acetoxyxanthine is not altered by the repeated chromatographies.

There is also no evidence for chemical binding to any of the nucleosides present in DNA. Even if some indication of chemical binding was obtained with isolated nucleosides, the results would still be ambiguous since the product of such interaction would not necessarily represent that obtained with native DNA. It has already been shown (21) that the reaction of N-acetoxyacetaminofluorene with native DNA can lead to products different from the ones obtained when the same ester is reacted with isolated nucleosides. Therefore, these nucleosides must be considered a poor chemical model for native DNA.

The Biological Effects of the Reaction of Acetoxypurine Derivatives on Transforming DNA. The results of treatment of transforming DNA with 3-acetoxyxanthine and with acetoxy esters of 3 related substituted derivatives are given in Table 1. All 4 compounds decreased transforming activity and increased the frequency of mutation. 3-Acetoxyxanthine was the most potent of this group. Transforming activity in this experiment was reduced to less than 10%, and the frequency of mutation was increased 10- to 15-fold over that obtained with the solvent control. In other experiments, the increase in mutation frequency with 3-hydroxyxanthine varied from 5-fold to as much as 45-fold. The 7- and 8-methyl- and 8-aza-substituted derivatives had a lesser effect on transforming activity and induced a level of mutation that was only 2- to 3-fold higher than that obtained with the solvent control. The parent compound, 3-hydroxyxanthine, had no effect on the DNA in this system. On the other hand, the strong mutagen, nitrous acid, reduced transforming activity of the same DNA to less than 1% and increased the mutation frequency 43-fold over the solvent control level. Overall, the mutagenic effect obtained with each compound was approximately proportional to the degree of inactivation also observed.

The extent of the mutagenic effect of 3-acetoxyxanthine was also evaluated by comparing mutation induction to inactivation with a number of treated DNA samples. As shown in Chart 2, the mutation frequency exhibits a linear relationship with inactivation (calculated as lethal hits). In accordance with prior studies (14, 24) on chemically induced mutation of DNA, this result is to be expected if both effects are caused by the same chemical alteration of the DNA but at different genetic regions. A value of approximately 10 induced mutations per lethal hit observed for 3-acetoxyxanthine is similar to that previously reported for the acetoxy and sulfate esters of *N*-hydroxy aromatic amines and amides (23, 24). The actual plot obtained with the 3-acetoxyxanthine data has a slope which is somewhat less, but approaches that obtained for samples of DNA treated with nitrous acid (Chart 2).

![Chart 2](chart2.png)

**Chart 2.** A Poisson distribution analysis of inactivation and mutation induction by 3-acetoxyxanthine. Inactivation is expressed as lethal hits calculated by the Poisson equation from the residual activities of 37, 13.5, 5, 1.9, 0.7, 0.27, and 0.1%.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Residual activity (%)</th>
<th>trp&lt;sup&gt;+&lt;/sup&gt; transformants *</th>
<th>Mutation frequency *&lt;sup&gt;x 10&lt;sup&gt;-4&lt;/sup&gt;&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent control</td>
<td>100</td>
<td>54,288</td>
<td>1.7</td>
</tr>
<tr>
<td>3-Hydroxyxanthine</td>
<td>100</td>
<td>24,953</td>
<td>1.6</td>
</tr>
<tr>
<td>3-Acetoxyxanthine</td>
<td>7.6</td>
<td>24,811</td>
<td>20.1</td>
</tr>
<tr>
<td>3-Acetoxy-7-methylxanthine</td>
<td>36.5</td>
<td>37,804</td>
<td>4.5</td>
</tr>
<tr>
<td>3-Acetoxy-8-methylxanthine</td>
<td>72.0</td>
<td>31,739</td>
<td>4.1</td>
</tr>
<tr>
<td>3-Acetoxy-8-azaxanthine</td>
<td>44.5</td>
<td>27,566</td>
<td>5.8</td>
</tr>
<tr>
<td>Nitrous acid</td>
<td>0.8</td>
<td>8,138</td>
<td>72.3</td>
</tr>
</tbody>
</table>

* Based on the number of trp<sup>+</sup> transformants obtained with treated samples versus that obtained with the solvent control obtained in a separate group of assays.

* Total number of colonies counted in all experiments.

*<sup>x</sup> The number of fluorescent (trp<sup>+</sup> ind<sup>+</sup>) colonies/10,000 trp<sup>+</sup> transformants.
The incubation time for the purine derivatives with DNA in these experiments is well beyond the lifetime of the active esters. The results presented in Table 2 show that the maximum biological effects were obtained with an incubation period as short as 1 min. This result agrees with rate studies which show a half-life for 3-acetoxyxanthine of about 10 sec under these conditions (7). In a 2nd experiment, we determined the effects of preincubating the 3-acetoxyxanthine prior to the addition of transforming DNA (Table 3). The results are interpretable in essentially the same manner as those presented in Table 2, i.e., inactivation and mutagenic effects occur almost immediately after mixing the DNA solution with 3-acetoxyxanthine. The data presented in Table 3 also show that none of the products (7) of spontaneous decay of 3-acetoxyxanthine in water are mutagenic. We also tested both major products from 3-acetoxyxanthine, xanthine and 8-chloroxanthine, and found no effects on transforming DNA.

Acetic anhydride in neutral aqueous solution almost instantaneously converts all of the N-hydroxypurine or purine N-oxide to the respective acetic acid ester. Generation of the esters in the presence of DNA has several advantages over the use of the preformed esters in cases where the esters are insoluble and tend to crystallize and also in permitting better quantitative data. The results of a dose-response experiment are shown in Chart 3. Maximal induction of mutation and inactivation occur at a concentration of 6 mg/ml, less than one-half the amount used for experiments discussed below (see Table 4).

A variety of acetoxy purines similarly generated in situ were also examined for their effects on DNA. The results are given in Table 4. 3-Acetoxyxanthine was again found to be the most potent mutagen within the group tested. Of the other xanthine derivatives tested, only 3-acetoxy-8-methylxanthine and 7-acetoxyxanthine exhibited a definite mutagenic effect. The 3-acetoxy-1-methylguanine derivative had mutagenic activity that was nearly as strong as that shown by 3-acetoxyxanthine. The 3-acetoxypurine derivative was only weakly mutagenic, and neither 1-acetoxyadenine nor 3-acetoxyhypoxanthine were mutagenic.

Reversion of 3-Acetoxyxanthine-induced Mutations to Wild Type. The results of reversion to wild type of the indole-requiring (ind~) mutants by the mutagens 2-AP, 5-BUdR, and EMS are shown in Table 5. Most of the mutants examined also reverted spontaneously. Two types of revertants were observed on agar plates. One is wild type, appearing as large colonies that do not require indole for maximum growth, and the other is a group of partial revertants, which appear as very small, slightly fluorescent colonies and still require indole for maximum growth. A number of the latter revertant strains have been tested and were found to contain a 2nd (suppressor) mutation at a site somewhere outside of the tryptophan operon. The calculation for the induced reversion frequency was based only on the number of large (wild-type) colonies present on each agar plate. Most of the mutations were reverted (see Table 5) by the alkylating agent EMS (72%). Of this group, about 1 out of 3 was also reverted by AP and BUdR while 1 out of 10 was reverted only by BUdR. In view of the known mode of action of these mutagens (18), it would appear that the original mutation in these strains are base-pair substitutions of the transition type, probably A/T → G/C. In the same

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**Table 2**

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Residual activity (%)</th>
<th>trp* transfectants</th>
<th>Mutation frequency (× 10⁻⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.0</td>
<td>5,145</td>
<td>15.6</td>
</tr>
<tr>
<td>2</td>
<td>7.8</td>
<td>8,774</td>
<td>15.9</td>
</tr>
<tr>
<td>3</td>
<td>13.1</td>
<td>5,085</td>
<td>11.8</td>
</tr>
<tr>
<td>30</td>
<td>14.7</td>
<td>14,853</td>
<td>15.5</td>
</tr>
</tbody>
</table>

* Based on the number of trp* transformants obtained with the treated DNA samples versus that obtained with the solvent control obtained in a separate group of assays.

* The mutation frequency obtained with all solvent controls was 1.7 × 10⁻⁴. The frequency represents the number of fluorescent (trp* ind~) colonies/10,000 trp* transformants.

**Table 3**

<table>
<thead>
<tr>
<th>Preincubation time (min)</th>
<th>Residual activity (%)</th>
<th>trp* transfectants</th>
<th>Mutation frequency (× 10⁻⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.9</td>
<td>15,846</td>
<td>14.5</td>
</tr>
<tr>
<td>1</td>
<td>87.0</td>
<td>7,008</td>
<td>2.9</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>7,102</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>6,709</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>10,967</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* 3-Acetoxyxanthine was added simultaneously with or prior to the DNA at the times indicated above.

* Based on the number of trp* transformants obtained with the treated DNA samples versus that obtained with the solvent control.

* The mutation frequency obtained with all solvent controls was 1.7 × 10⁻⁴. The number of fluorescent (trp* ind~) colonies/10,000 trp* transformants.
group of mutants reverted by EMS, about 1 out of 10 was also reverted by AP but not with BUdR. The original mutations in these isolates also would seem to be base-pair substitutions of the transition type, but probably G/C → A/T. The remaining group of mutants (1 out of 5) in the class reverted by EMS was not reverted by either AP or BUdR. The original mutation here was probably a base-pair substitution of the transversion type, i.e., A/T → C/G or G/C → T/A. However, frame-shift mutations (18), since they are also reverted by EMS (10, 20, 22), could be present in these isolates. This latter possibility seems unlikely in this system (14, 18). Also, only 2 of the 12 mutations in this group were reverted by the frame-shift mutagens (14, 17) hycanthone monomethane sulfonate, Miracil D, or ICR 191.

A total of 17 mutations were not reverted by either AP, BUdR, or EMS. Also, 9 of these did not revert spontaneously or with any of the frame-shift mutagens and could bear deletions or extended multisite mutations in the tryptophan operon. Of the remaining 8 mutants in this group, only 2 were reverted by frame-shift mutagens. Many of these might also be multisite mutations. Possibly 4 to 6 of the mutants in this class arose spontaneously in the DNA-donor culture. This estimation is made from the comparison of the spontaneous and induced level of mutant frequencies (Tables 1 and 4). The origin of the other mutants in this class are unaccounted for and may have arisen as a result of minor, nonspecific, chemical alteration by 3-acetoxyxanthine.

Correlation of Mutagenic Activity with Chemical Reactivity of Acetoxypurines. 3-Acetoxyxanthine can undergo 3 different reactions in neutral aqueous solution (6, 7): (a) substitution of nucleophiles such as methionine or pyridine; (b) oxidation of suitable molecules such as uric acid, hydroquinone, or potassium iodide; and (c) acetylation of other purine N-oxides. The data in Table 4 provide an opportunity to examine the relevance of Reaction Modes a and b to mutation induction. Reaction Mode c is most likely not relevant, since acetic anhydride, a strong acetylating agent, does not affect transforming activity and produces no mutations as shown by the solvent control in Table 4.

The weak mutagens 1-acetoxyadenine, 3-acetoxyhypoxanthine, and 3-acetoxypurine have no chemical reactivity for Reaction Mode a or b, while the strong mutagens 3-acetoxy-1-methylguanine, 3-acetoxypurine, and 7-acetoxanthine strongly react in both modes. The remaining derivatives shown in Table 4 (the 3-acetoxy-8-substituted derivatives of xanthine) represent cases of borderline chemical reactivity. The 7-methyl and 8-aza derivatives do not react in either Mode a or Mode b with a measurable rate. Both derivatives, under the experimental conditions shown in Table 1, were found to be weak mutagens. These same derivatives were shown to be nonmutagenic in the in situ experiments shown in Table 4. This difference in results remains unexplained.

3-Acetoxy-8-methylxanthine is a weak mutagen by both methods of DNA treatment (Tables 1 and 4). This derivative has an unusual chemistry (37). It undergoes the oxidative Reaction Mode b at a rate slightly higher than that of the parent 3-acetoxypurine, while Reaction Mode a, as
tested by interaction with methionine, is absent. This same derivative gives a weak positive reaction with pyridine that is indicative of Reaction Mode a, but the product of this reaction has not been identified.

As there were no definitive examples of purines giving either substitution (a) or oxidation (b) modes of reactivity, we used a nonpurine-oxidizing agent, peroxisulfate, that presumably represents Reaction Mode a. This agent leads to strong oxidizing radicals. At pH 7, its rate of reaction with potassium iodide is similar to that of 3-acetoxyxanthine. When tested in experiments such as those shown in Table 1, it was found to be a strong mutagen with a mutation frequency 10- to 15-fold greater than the control level at a concentration of 5 mg/ml. This suggests that the oxidative mode of reaction can induce mutations in native DNA. The mutations induced by peroxisulfate were similar to those obtained with 3-acetoxyxanthine, i.e., they were mainly a variety of base-pair substitutions. We also tested, as an oxidizing agent, an acetylated derivative of hydroxylamine derived from the reaction of hydroxylamine with acetanhydride at pH 7.0. However, hydroxylamine alone gave a strong mutagenic effect under these conditions, and therefore it is not possible to assess the effect of the acetyl derivative on transforming DNA. Another indication for the oxidative mode of reactivity of 3-acetoxyxanthine was obtained with the reducing agents hydroquinone and 2-mercaptoethanol. Both compounds, at a concentration of 20 mg/ml, nearly abolished the mutagenic effect obtained with 3-acetoxyxanthine.

**Correlation between Mutagenicity and Oncogenicity of Purine N-Oxide Derivatives.** There is a reasonably good correlation between the relative magnitude of the mutagenic and oncogenic effects of the purine N-oxides tested (see Table 4). Both 3-acetoxyxanthine and 3-acetoxy-1-methylguanine are the strongest mutagens in this system. The parent N-hydroxy derivatives of both compounds have also been shown (9) to be strong oncogens in rats. In addition, 7-hydroxyxanthine, the acetoxo derivative of which has been shown to be a moderately strong mutagen, has recently been demonstrated to be an oncogen (M. Teller, G. Zvili-chofsky, and G. B. Brown, personal communication). With 4 of the acetoxo esters exhibiting a weak effect or no effect with transforming DNA (3-acetoxy-7-methylxanthine, 3-acetoxy-8-methylxanthine, 3-acetoxy-8-axazanxanthine, and 3-acetoxyadenine), the parent hydroxy compounds were also found (9) to have only weak oncogenic activity. The N-oxide derivatives of the 2 other esters placed in the category of weak mutagens, purine 3-N-oxide and hypoxanthine 3-N-oxide, have been found to be fairly strong oncogens in rats. In these 2 cases, the discrepancy between mutagenic and oncogenic activity may be explicable by the fact that each of the N-oxides can be converted in the rat by xanthine oxidase to the strong oncogen 3-hydroxyxanthine (M. Teller, G. Stöhrer, and G. B. Brown, in preparation).

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