Metabolic Activation of the Potent Mutagen, 2-Naphthohydroxamic Acid, in Salmonella typhimurium TA98

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ABSTRACT

The objective of the present study was to explore the mechanisms responsible for the strong, direct-acting mutagenicity of 2-naphthohydroxamic acid (NHA) for Salmonella typhimurium TA98. NHA was converted to its O-acetate (O-Ac-NHA) by acetyl-CoA, in the presence of competent or heat-treated cell-free bacterial preparations. O-Ac-NHA, which is more mutagenic than NHA, reacted nonenzymatically with tRNA in neutral solutions with retention of both the naphthyl and carboxyl groups in the products, but NHA did not react. Enzymatic sulfation conjugation was not demonstrated. TA98 cells converted NHA to 2-aminoanaphthelene, presumably through a Lossen rearrangement following O-acetylation or conjugation by other metabolic pathways. TA98 cells reduced O-Ac-NHA to 2-naphthamide, and NADH and NADPH were shown to be cofactors for reduction in the presence of a cell-free bacterial preparation. Although horseradish peroxidase and H2O2 catalyzed the binding of these compounds to tRNA, no evidence of oxidation of NHA or O-Ac-NHA was obtained with H2O2 and cell-free preparations of TA98 or the cells themselves, as judged by the lack of formation of the peroxidative product, 2-naphthoic acid. Both NHA and O-Ac-NHA reacted with DNA of TA98 with retention of both naphthyl group and carboxyl of the naphthyl moiety in the adduct(s). These results suggest that NHA may be activated in TA98 by esterification, and the resulting metabolites may amidate or carbamoylate nucleic acids.

INTRODUCTION

Hydroxamic acids (i.e., ArCONHOH, RCONHOH) are a group of widely distributed chemicals. Although some occur naturally in fungi, yeasts, bacteria, and plants (1-4), others have been manufactured for pharmaceutical, consumer, agricultural, and industrial concerns for possible use as antitumor agents, antibiotics, growth factors, and iron-chelating agents (reviewed in Ref. 5). Recently, it has been found that some hydroxamic acids are inhibitors of 5-lipoxygenase (6-8), and they can inhibit the biosynthesis of leukotrienes which are important mediators in a variety of diseases such as asthma, arthritis, and psoriasis. Thus, new and potentially more effective treatments for these conditions may be developed from these hydroxamic acids.

Certain hydroxamic acids are mutagenic to bacteria (3, 9-13), and acehydroxamic acid (14) and NHA4-5 are also mutagenic to V79 Chinese hamster cells. Some are clastogenic and induce sister chromatid exchange in cultured mammalian cells (5). However, in the only chronic toxicity study of which we are aware, NHA did not significantly increase the incidence of tumors in male or female Sprague-Dawley rats in a 75-wk observation period following 8 weekly injections of the maximum tolerated dose started at birth. It is generally believed that the covalent binding of a chemical with DNA is an early step in most mutagenic and carcinogenic processes (15, 16). However, very little is known about the chemical reactivity of the unsubstituted hydroxamic acids with macromolecules and their underlying mechanisms of activation.

It has been shown that NHA was highly mutagenic for Salmonella typhimurium TA98 (9, 13), its O-acetate ester being much more mutagenic than the parent compound (11, 13). Additionally, it was observed by Koga et al. (17) that O-acyl derivatives of the structurally analogous N-hydroxurethane were more mutagenic than the parent compound. Therefore, it is possible that O-acetylation, similar to the acetyl-CoA-dependent activation of arylhydroxylamines in TA98 (18, 19), is one of the major pathways of activation of the unsubstituted hydroxamic acids in these cells. It was shown in our previous study (11) and those of others (3, 12, 13) that the presence of the N-H group of hydroxylamine moiety was essential for mutagenic activity. This observation led to the proposal (3, 12, 13) that a Lossen rearrangement (Ref. 20; Fig. 1), which transforms hydroxamic acids to reactive isocyanates, may be involved in the activation of hydroxamic acids and results in the carbamoylation of DNA. Our objective in this study was to explore the mechanisms by which NHA is activated in bacteria.

MATERIALS AND METHODS

Materials. The following were purchased from the sources indicated: 2-['ring-3H]naphthoic acid (Amersham Corporation, Inc., Arlington Heights, IL); 2-naphthyl['ring-C]oic acid (Chemsyn Science Laboratories, Lenexa, KS); 2-naphthoic acid, 2-naphthyl chloride, AN, and benzaldehyde (Aldrich Chemical Co., Inc., Milwaukee, WI); NpNCO (Adams Chemical Company, Round Lake, IL); Oxo2D nutrient broth (Oxoid USA, Inc., Columbia, MO); lysozyme, proteinase K, acetyl-CoA, HRP, 30% H2O2, NADH, NADPH, and PAPS (Sigma Chemical Co., St. Louis, MO); yeast tRNA (Calbiochem, La Jolla, CA); and RNase (Pharmacia Fine Chemicals, Piscatway, NJ). Other chemicals were of reagent grade.

S. typhimurium TA98 and TA98/1,8-DNP were obtained from Dr. B. N. Ames, University of California, Berkeley, CA, and Dr. E. C. McCoy, Case Western Reserve University, Cleveland, OH, respectively.

Instrumentation and General Procedures. Reverse-phase HPLC was carried out using a Waters Associates liquid chromatograph system equipped with a Model 440 absorbance detector and an automated gradient controller (Waters Associates, Inc., Milford, MA) interfaced with a radioactive flow detector (Flow-One HP; Radiomatic Instruments & Chemical Co., Inc., Tampa, FL) and an LKB 2140 rapid liquid chromatography spectrometer (LKB-Produkter AB, Bromma, Sweden) by an IBM computer using a data acquisition system (Series 760 interface; Nelson Instruments & Chemical Co., Inc., Tampa, FL) and an LKB 2140 rapid liquid chromatography spectrometer.
Torrance, CA), eluted with a linear gradient of methanol in 10 mM Tris-HCl (pH 7.4) at 1 ml/min from 50 to 100% methanol in 20 min and then at 100% methanol for 10 min. System II, a C18-Bondapak column (10 μm, 300 x 3.9 mm; Phenomenex, Torrance, CA), eluted at 1 ml/min linearly from 10 mM H3PO4 to methanol in 30 min and then 100% methanol for 5 min. System III, the same C18 column as above, eluted at 1 ml/min linearly from 40 to 80% methanol in 10 mM H3PO4 in 14 min, 80 to 100% methanol in 2 min, and then 100% methanol for 5 min. The Rf values of the compounds are listed in Table 1. TLC was carried out on silica gel plates with the following solvent systems: Solvent A, benzene:acetone (2:1); and Solvent C, benzene:methanol (9:1). Rf values were carried out on silica gel plates with the following solvent systems: Solvent A, benzene:acetone (2:1); and Solvent C, benzene:methanol (9:1). Rf values were listed in Table 1. Radioactivity was determined by liquid scintillation spectrophotometry. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Unless otherwise indicated, 0.1 M sodium phosphate buffer (pH 7.0) was used.

Chemical Synthesis. NHA, O-Ac-NHA, O-SO3H-NHA-Py, and di-Np urea were synthesized according to published procedures (11). For the preparation of 1-(2-naphthyl)-3-phenyl urea, a solution of 40 mg of NpNCO in 0.5 ml of DMSO was reacted with 0.1 ml of aniline at room temperature for 24 h. Benzene and water were added, and the organic phase was washed with 1 ml of 1 N HCl and then water (3×) to give crystals, m.p. 233–234°C (from methanol) [lit. m.p. 233–234°C (21)]. NpCONH2 was prepared by reaction of 2-naphthyl chloride with conc. NH4OH at room temperature, m.p. 194–195°C [lit. m.p. 195°C (22)]. O-CONp-NH2 was prepared by reaction of 2-naphthoyl chloride with conc. NH2OH (prepared by treating NH2OH-HCl with an equimolar quantity of NaOH. Evaporation of solvent and recrystallization from methanol gave a residue which was triturated with H2O, acidified to pH 2 with HCl, and recrystallized from acetone-ether to give [3H]NHA (70% to 75% yield) with a radiochemical purity greater than 98% and a specific activity of 43 to 53 mCi/mmol.

Radiochemical Synthesis. [3H]NHA was prepared by reaction of 2-[(ring)]H]naphthyl chloride (prepared by treating a benzene solution of the acid with 2.4 equivalents of SOCl2 in the presence of a catalytic amount of dimethylformamide at 40°C for 2 h) and 10 equivalents of NH2OH (prepared by treating NH2OH.HCl with an equimolar quantity of KOH in methanol, filtering off the solid KCl and evaporating methanol) in benzene at room temperature for 20 min. Evaporation of solvent gave a residue which was triturated with H2O, acidified to pH 2 with HCl, and recrystallized from acetone-ether to give [3H]NHA (70 to 75% yield) with a radiochemical purity greater than 98% based on TLC using Solvent C and a specific activity of 443 to 760 mCi/mmol.

Table 1 HPLC Rf, and TLC Rf values of compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Systems I</th>
<th>Systems II</th>
<th>Systems III</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHA</td>
<td>10.8</td>
<td>21.0</td>
<td>6.8</td>
</tr>
<tr>
<td>O-Ac-NHA</td>
<td>14.9</td>
<td>23.6</td>
<td>9.2</td>
</tr>
<tr>
<td>O-SO3H-NHA-Py</td>
<td>14.8</td>
<td>22.3</td>
<td>8.2</td>
</tr>
<tr>
<td>NpCONH2</td>
<td>21.3</td>
<td>16.3</td>
<td>5.0</td>
</tr>
<tr>
<td>NpCO2H</td>
<td>6.4</td>
<td>26.1</td>
<td>11.8</td>
</tr>
<tr>
<td>O-COP-NHA</td>
<td>31.7</td>
<td>15.9</td>
<td>0.61</td>
</tr>
<tr>
<td>di-Np urea</td>
<td>27.2</td>
<td>33.7</td>
<td>17.2</td>
</tr>
<tr>
<td>1-(2-naphthyl)-3-phenyl urea</td>
<td>23.2</td>
<td>4.34</td>
<td>0.60</td>
</tr>
</tbody>
</table>

* Rf values of the radioactive counterparts were 0.6 min longer than those listed in the table which were detected by a 254-nm detector.

The compounds were detected by viewing the TLC plates under 254-nm or 366-nm UV light.

For System I; a PRP-1 column was used and eluted with a linear gradient of methanol in 10 mM Tris-HCl (pH 7.4) at 1 ml/min from 50 to 100% methanol in 20 min and at 100% methanol for 10 min; for System II, a C18 column was used and eluted at 1 ml/min linearly from 10 mM H3PO4 to methanol in 30 min and then at methanol for 5 min; for System III, a C18 column was used and eluted at 1 ml/min linearly from 40 to 80% methanol in 10 mM H3PO4, in 14 min, 80 to 100% methanol in 2 min, and then 100% methanol for 5 min. The Rf values of the compounds were listed in Table 1.
then resuspended in 7 mg of the same buffer. The suspension was incubated with 1 mg of lysozyme at 37°C for 1 h, followed by freezing and thawing with liquid nitrogen (2×) and then centrifuging at 2000 rpm. The supernatant which contained 0.54 mg of protein/ml of preparation as determined by the published procedure (23) was stored at −70°C until use.

Incubation mixtures consisted of 135 μg of protein, 2.5 μmol of cofactor in 0.5 ml of pH 7.0 buffer, and 10 nmol of [3H]NHA (364 mCi/mmol) or O-Ac-[3H]NHA (760 mCi/mmol) in 10 μl of DMSO. Control experiments were carried out in the absence of cofactors or in the presence of heat-treated (100°C, 10 min) cell-free preparations. They were incubated at 37°C for 1 h and then mixed with 1 ml of methanol, and the extracts were analyzed by HPLC using System III. The cofactors used and the atmospheres under which the experiments were carried out are indicated in Table 2. In the case of PAPS, the buffer used was 0.1 m bicine, pH 8.0, containing 1.5 μmol of Mg(OAc)₂ and 0.5 μmol of dithiothreitol. The quantitation of the products formed was based on the percentage of the peak area after correction for appropriate control experiments. The limit of detection was 1% of the added substrate, i.e., 0.1 nmol.

In vitro Metabolism of NHA and O-Ac-NHA by TA98 Cell Suspensions. Cells from an overnight culture (8 ml) were washed twice with buffer (0.1 M sodium phosphate/0.9% NaCl solution, pH 7.2) and resuspended in 8 ml of the same buffer. To 0.5 ml of pH 7.0 buffer containing 10 nmol of [3H]NHA (364 mCi/mmol) or O-Ac-[3H]NHA (760 mCi/mmol) in 10 μl of DMSO was added 0.1 ml of the TA98 suspension or 0.1 ml of the same buffer as control. At 0 time and after incubation at 37°C for 2 or 4 h, 1 ml of methanol was added, and the extracts were analyzed by HPLC using System II. The quantitation of the products formed was based on the percentage of the peak area after correction for the 0 time control experiments. The limit of detection was 1% of the added substrate, i.e., 0.1 nmol.

Similar analyses of sonicates of TA98 or TA98/1,8-DNP cells that had been treated with [3H]NHA were also carried out.

Table 2 In vitro metabolism of NHA and O-Ac-NHA by a cell-free preparation of Salmonella typhimurium TA98

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cofactor</th>
<th>Atmosphere</th>
<th>Product formed (nmol)</th>
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</thead>
<tbody>
<tr>
<td>[3H]NHA</td>
<td>Acetyl-CoA</td>
<td>Air</td>
<td>O-Ac-NHA (1.2)</td>
</tr>
<tr>
<td></td>
<td>PAPS</td>
<td>Air</td>
<td>O-Ac-NHA (2.1)*</td>
</tr>
<tr>
<td>NADH</td>
<td>Argon</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>Argon</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>H₂O₂</td>
<td>H₂O₂</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>O-Ac-[3H]NHA</td>
<td>NADH</td>
<td>Air</td>
<td>NpCONH₂ (1.2)</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>Argon</td>
<td>NpCONH₂ (0.4)</td>
</tr>
<tr>
<td></td>
<td>Hypoxanthine</td>
<td>Argon</td>
<td>None</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>H₂O₂</td>
<td>Argon</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

* A heat-treated cell-free bacterial preparation was used.

Incubation was carried out in a pH 8.0 bicine buffer containing 1.5 μmol of Mg(OAc)₂ and 0.5 μmol of dithiothreitol.

Bacterial DNA Binding with NHA and O-Ac-NHA. Exponentially growing cultures of TA98 were centrifuged, washed with a buffer of 0.85% NaCl/50 mM sodium phosphate, pH 7.2, and resuspended in the same buffer. Separate cultures were used for Experiments 1 and 2. For the reaction with [3H, 14C]NHA (150 mCi/mmol of H, 26.3 mCi/mmol of 14C), i.e., Experiment 1, 175 ml of a mixture containing 1.13 × 10¹³ cells and 1.5 μmol of compound in 75 μl of DMSO (0.4 mm in compound, 3 × 10⁶ cells/ml) were incubated at 37°C for up to 60 min. For the reaction with O-Ac-[3H, 14C]NHA (151 mCi/mmol of H, 26.3 mCi/mmol of 14C; 0.4 mm in compound and 4 × 10⁹ cells/ml), i.e., Experiment 2, incubation was for 60 min. Control samples received the test compound after a 60-min incubation just prior to work-up. Experiments were carried out in duplicate. After incubation, the cells were collected and washed 4 times with citrate-buffered saline, pH 7.2. Treatment of the cell pellets was as described by Beranek et al. (16). After the cells had been treated with sodium dodecyl sulfate at a final concentration of 0.5% at 37°C for 30 min (16), the solution was treated with protease K (0.25 mg/ml) for 1 h and then extracted with phenol/8-hydroxyquinoline/m-creosol/H₂O (500/50/70/55 by weight), chloroform/isooamyl alcohol (24/1), and ether. The solution was made 100 mM with sodium citrate to pH 7.0, and at 100% for 5 min. The limit of detection was 1% of the added substrate, i.e., 0.1 nmol.

RESULTS

Decomposition of Esters of NHA. The incubation of O-Ac-[3H]NHA at 37°C in pH 7.0 buffer resulted mainly in the formation of AN through a Lossen rearrangement. Hydrolysis of the acetate to NHA was a minor pathway. The 2-h incubation mixture showed the presence of both products. At the end of 24-h incubation, little of the ester remained, and AN was the major product as shown in the HPLC profiles (Fig. 2, A and B). The half-life was found to be 9.4 h as judged by the quantities of AN through a Lossen rearrangement. Hydrolysis of the acetate to NHA was a minor pathway. The 2-h incubation mixture showed the presence of both products. After the cells had been treated with sodium dodecyl sulfate at a final concentration of 0.5% at 37°C for 30 min (16), the solution was treated with protease K (0.25 mg/ml) for 1 h and then extracted with phenol/8-hydroxyquinoline/m-creosol/H₂O (500/50/70/55 by weight), chloroform/isooamyl alcohol (24/1), and ether. The solution was made 100 mM with sodium citrate to pH 7.0, and at 100% for 5 min. The limit of detection was 1% of the added substrate, i.e., 0.1 nmol.

O-SO₃H-NHA-Py was previously found to undergo Lossen rearrangement at pH 7.0 and room temperature within 30 min to yield AN and di-Np urea (11). We have now determined that the half-life of the sulfate ester in pH 7.0 buffer was 1.4 min based on the change in absorbance at 220 nm (Fig. 4). These results showed that these esters of NHA can undergo Lossen rearrangement to form NpNCO which can react with H₂O to yield AN or with amines to give ureas; the O-sulfonate is much more reactive than the O-acetate.

Oxidation of NHA and O-Ac-NHA with H₂O₂/HRP. Fig. 5 summarizes the results of the oxidation of these compounds.
Metabolic Activation of 2-Naphthohydroxamic Acid

Fig. 2. Reverse-phase HPLC profile of the incubation mixture of O-Ac-[\textsuperscript{3}H]-NHA at pH 7.0 and 37°C at 0 time (A) and 24 h (B). The mixture consisted of 10 nmol of O-Ac-[\textsuperscript{3}H]NHA (760 mCi/mmol)/10 \mu l of DMSO in 0.6 ml of pH 7.0 buffer. At 0 and 24 h, 50 \mu l were mixed with 0.1 ml of methanol, and the extracts were analyzed by HPLC using System II as described in Table 1.

Fig. 3. The half-life of O-Ac-NHA in pH 7.0 buffer at 37°C. The same incubation mixture and method of analysis as described for Fig. 2 were used to obtain the quantities of O-Ac-[\textsuperscript{3}H]NHA relative to the eluted label in the mixture at 0, 2, 4, and 24 h.

In the presence of H\textsubscript{2}O\textsubscript{2}/HRP, NHA was almost completely converted in 10 min at 37°C, pH 7.0, to the 2 major products, NpCO\textsubscript{2}H and O-CONp-NHA, and 3 minor unknown products as evidenced from the HPLC profile using System III (data not shown). At the end of 1 h (Fig. 6), the product distribution remained similar to that of the 10-min reaction mixture. At the end of 24 h, only the 2 major products remained; there were approximately 5 times more of the acid (data not shown).

Control experiments were carried out in the presence of H\textsubscript{2}O\textsubscript{2} alone or in the absence of H\textsubscript{2}O\textsubscript{2}/HRP. At the end of a 24-h incubation at 37°C, no NpCO\textsubscript{2}H was detected, and NHA was recovered unchanged from these control experiments (data not shown). Presumably, as proposed for the oxidation of hydroxamic acids (25), in the presence of H\textsubscript{2}O\textsubscript{2}/HRP, the reactive intermediate, NpCONO, was generated which reacted with H\textsubscript{2}O or NHA to give NpCO\textsubscript{2}H and O-CONp-NHA, respectively (Fig. 5).

At short intervals, e.g., 10 min, the peroxidation of O-Ac-NHA gave only a small amount of NpCO\textsubscript{2}H (Fig. 1A). Subsequently, hydrolysis of the acetate to NHA became the major pathway. At the end of a 24-h incubation, NHA became the major component. Very little AN was produced (Fig. 1B). Control incubations of O-Ac-NHA and H\textsubscript{2}O\textsubscript{2} underwent mainly hydrolysis of the acetate to NHA; very little Lossen rearrangement occurred. At the end of 24 h, the major product was NHA with very little AN or O-Ac-NHA present (<1%). These results indicate that only in the presence of H\textsubscript{2}O\textsubscript{2}/HRP did oxidation of O-Ac-NHA take place to form NpCO\textsubscript{2}H. H\textsubscript{2}O\textsubscript{2} suppressed the Lossen rearrangement and facilitated hydrolysis to form NHA. Thus, the formation of NpCO\textsubscript{2}H is an indication of peroxidation of NHA or O-Ac-NHA.

Metabolism of NHA and O-Ac-NHA by Cell-free Preparations of TA98. In order to better understand the activation...
using System III as described in Table 1. and 1.95 ml of pH 7.0 buffer, containing 20 μmol of H2O2 and 10 units of HRP. After 1 h incubation at 37°C, its ethyl acetate extract was analyzed by HPLC at 1 h. The reaction mixture consisted of 2 μmol of compound/50 μl of DMSO with these cofactors. As in the case of NHA, the TA98 preparations cannot oxidize NHA and TA98 can not sulfonate, reduce, or oxidize NHA, since the oxidation product, NpCO2H, was not detected when it was treated with H2O2 and cell-free preparation. On the other hand, TA98 reduced O-Ac-NHA to NpCONH2 in the presence of NADH or NADPH; the reduction was more efficient using NADH and was sensitive to oxygen. However, hypoxanthine and benzaldehyde could not serve as electron donors in the reduction of O-Ac-NHA.

**In Vivo Metabolism of NHA and O-Ac-NHA by Intact TA98 Cells.** The activation mechanisms of NHA and O-Ac-NHA by TA98 were further studied using intact cells. The metabolism by intact cells converted NHA to AN and 2-naphthamide in a time-dependent fashion (Table 3), presumably through the formation of a conjugate. The conversion of NHA to O-Ac-NHA was detected only in experiments in which the cells were ruptured by sonication prior to extraction with methanol. Furthermore, a similar experiment using TA98/1,8-DNP6, the N-arylhydroxylamine O-acetyltransferase-deficient strain of Salmonella (26, 27), also showed the formation of O-Ac-NHA. These results suggest that the conjugation of NHA does not require the O-acetyltransferase that can utilize N-arylhydroxylamines as substrates. Small amounts of NpCO2H were detected at each time point from NHA (Table 3); however, the formation was not dependent on the presence of bacteria. Reduction of O-Ac-NHA to the amide, NpCONH2, occurred in the presence of bacteria. In contrast, formation of AN and NHA from O-Ac-NHA did not require bacteria. No production of NpCO2H was observed from O-Ac-NHA in the presence or absence of bacteria; thus, no peroxidation was detected.

**Reaction of NHA and O-Ac-NHA with tRNA.** The reactivities of these compounds toward macromolecules were studied with a tRNA binding assay (24) using both ring-3H- and carbonyl-14C-labeled materials. The results are shown in Fig. 8. O-Ac-NHA bound to tRNA, and the adduct retained both ring and carbonyl labels. On the other hand, NHA had very little reactivity toward tRNA. These results suggest that NHA needs activation in order to react with macromolecules and that adduct formation occurs with retention of both the ring and naphthyl carbonyl moieties.

**Reaction of NHA and O-Ac-NHA with DNA in TA98.** Binding of these compounds to DNA in bacteria was studied using both ring-3H- and carbonyl-14C-labeled compounds. The results

![Graph](https://via.placeholder.com/150)

**Fig. 6.** Reverse-phase HPLC profile of products from peroxidation of NHA at 1 h. The reaction mixture consisted of 2 μmol of compound/50 μl of DMSO and 1.95 ml of pH 7.0 buffer, containing 20 μmol of H2O2 and 10 units of HRP. After 1 h incubation at 37°C, its ethyl acetate extract was analyzed by HPLC using System III as described in Table 1.
METABOLIC ACTIVATION OF 2-NAPHTHYDROXAMIC ACID

Table 3: Metabolism of NHA and O-Ac-NHA by intact Salmonella typhimurium TA98 cells

Mixtures of 10 nmol of [3H]NHA or O-Ac-[3H]NHA/10 μl of DMSO in 0.5 ml of pH 7.0 buffer were incubated at 37°C with or without 0.1 ml of an overnight culture. After incubation, 1 ml of methanol was added, and the extracts were analyzed by HPLC using a C18 column, eluted at 1 ml/min linearly from 10 mM H3PO4 to methanol in 30 min. The limit of detection was 1% of the added substrate, i.e., 0.1 nmol.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Substrate</th>
<th>Metabolites formed (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[3H]NHA</td>
<td>NHA</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>−</td>
<td>0.1</td>
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<tr>
<td></td>
<td>+</td>
<td>2.3</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>O-Ac-[3H]NHA</td>
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</tr>
<tr>
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<td>−</td>
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<tr>
<td></td>
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<td>1.2</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* ND, not detected.

Table 4: Reactions of NHA and O-Ac-NHA with tRNA in the presence of H₂O₂/HRP at 37°C for 1 min

The incubation mixtures consisted of 0.1 μmol of compound in 10 μl of DMSO and 190 μl of pH 7 buffer, containing 1 mg of tRNA, 1 μmol of H₂O₂ and 5 units of HRP. The procedure for quantitation of adducts was as described (24). The results are from duplicate experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>H₂O₂</th>
<th>Net binding (nmol/mg of tRNA)</th>
<th>Ring/carbonyl ratio bound to tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H, 14C]NHA</td>
<td>+</td>
<td>Binding level (nmol/mg of tRNA)</td>
<td>Net binding (nmol/mg of tRNA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ring</td>
<td>Carbonyl</td>
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<tr>
<td>[3H, 14C]NHA</td>
<td>+</td>
<td>1.00</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>−</td>
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<td>0.04</td>
</tr>
<tr>
<td>O-Ac-[3H, 14C]NHA</td>
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<td>0.10</td>
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</tr>
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</table>

DISCUSSION

NHA is stable in neutral aqueous medium, and its O-acetate and O-sulfonate undergo Lossen rearrangements to form a reactive isocyanate which reacts with H₂O to give AN. At high concentrations, the isocyanate can react with AN to form di-Np urea (Fig. 1). Thus, the production of AN is an indication of the presence of some type of NHA ester. Both NHA and O-Ac-NHA can be peroxidized to form NpCO₂H, although to a much lesser extent with the acetate (Figs. 6 and 7, A and B), presumably through the reaction of H₂O₂ with the reactive intermediate, NpCONO., (Fig. 5). The formation of RCONO₂⁻ (R = 2-naphthyl in the present study), which has been proposed as a strong acylating agent (25), from the oxidation of hydroxamic acids is further confirmed by the formation of O-CONp-NHA in the peroxidation mixture of NHA. Thus, the formation of NpCO₂H from these compounds is an indication of peroxidation.

In order to better understand the activation pathways of NHA in TA98, in vitro and in vivo metabolism of NHA and O-Ac-NHA with TA98 was investigated. Since there was no evidence of formation of NpCO₂H from NHA or O-Ac-NHA in the presence of H₂O₂/TA98 cell-free preparations (Table 2) or in the presence of bacteria themselves (Table 3), it is unlikely that TA98 can catalyze the oxidation of these compounds, although a peroxidative system (H₂O₂/HRP) increased the reactivity of NHA and O-Ac-NHA toward tRNA (Table 4).

O-Sulfonation of NHA by PAPS in the presence of TA98 cell-free preparations was not demonstrated (Table 2); otherwise the Lossen rearrangement product, AN, would have been detected. A previous study also failed to detect the formation of O-sulfonates from N-arylhydroxylamines by Salmonella (28), although mammalian liver cytosols can O-sulfonate N-hydroxy-2-acetylaminofluorene (29-31) as well as N-hydroxy-2-amino-fluorene (31).

TA98 cell-free preparations can reduce O-Ac-NHA but not NHA to NpCONH₂ in the presence of NADH or NADPH, and the reduction is sensitive to oxygen (Table 2). On the other hand, TA98 cells can reduce NHA to its amide, thus providing additional support for the conclusion that NHA was activated to the O-acetate or other conjugate(s) which can be similarly reduced as is the O-acetate. It has been reported that liver aldehyde oxidase can reduce hydroxamic acids to amides (32), and that NADH, NADPH, or hypoxanthine is not a cofactor in this reaction. It is also known that N-hydroxy-2-acylamino-fluorene reductase from rabbit liver cytosol required NADH or NADPH as an electron donor and that the reduction is not...
affected by oxygen (33). Thus, the TA98 enzyme which catalyzes the reduction of O-Ac-NHA has characteristics that are different from those of the mammalian enzymes.

In neutral solution, O-Ac-NHA reacted with tRNA, but NHA had very limited reactivity (Fig. 8). However, incubation of NHA or O-Ac-NHA with TA98 cell suspensions resulted in their binding to the bacterial DNA (Table 5) with the retention of both naphthyl and carbonyl (of naphthoyl) moieties. These experiments were carried out with different bacterial cultures and under slightly different conditions and cannot, therefore, provide for quantitative comparison of the binding of these two compounds. However, these data do distinguish qualitatively between adduct formation from the activated AN as compared with reactions with NpNCO or metabolites that are capable of naphthoylation or naphthamidation. The activation of NHA through the formation of reactive metabolites is further supported by the demonstration that PCP inhibits the mutagenicity of NHA, but not that of O-Ac-NHA, in TA98 in a dose-dependent fashion. Thus, the use of PCP as an inhibitor of metabolic activation may facilitate the search for the enzymatic pathway(s) involved in the activation.

In conclusion, our present results show that NHA requires metabolic activation by TA98 to bind to its DNA and that the metabolites responsible for adduct formation may be the O-acetate and/or other metabolites with similar reactivity. These data further suggest that DNA modification pathways may involve naphthamidation or carbamoylation. The characterization of the structure of this adduct(s) is in progress.

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