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 sufficient 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 carbonyl groups in the products, but NHA did not react. Enzymatic sulfation conjugation was not demonstrated. TA98 cells converted NHA to 2-aminoanthenylcarboxylic acid, 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 of the peroxidative product, 2-naphthoic acid. Both NHA and O-Ac-NHA or O-Ac-\( ^3 \)H, MC\(^ {14} \)NHA was obtained with H\(_2\)O\(_2\) 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 carbonyl of the naphthoyl moiety in the adduct(s). These results suggest that NHA may be activated in TA98 by esterification, and the resulting metabolites may amitate 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 acetohydroxamic acid (14) and NHA 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-\( ^{14} \)C]-naphthoic acid (Amersham Corporation, Inc., Arlington Heights, IL); 2-naphthyl\(^ {14} \)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); Oxoid No. 2 nutrient broth (Oxoid USA, Inc., Columbia, MO); lysozyme, proteinase K, acetyl-CoA, HRP, 30% H\(_2\)O\(_2\), NADH, NADPH, and PAPS (Sigma Chemical Co., St. Louis, MO); yeast tRNA (Calbiochem, La Jolla, CA); and RNase (Pharmacia Fine Chemicals, Piscataway, NJ). Other chemicals were of reagent grade.

S. typhimurium TA98 and TA98/1.8-DNP\(_6\) 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 absorption 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 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 system equipped with a Model 440 absorbance detector and an automated gradient controller (Waters Associates, Inc., Milford, MA). The following systems were used. System I, a PRP-1 column (10 µm, 150 x 4.1 mm) Phenomenex, * Unpublished observations.

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3 The abbreviations used are: NHA, 2-naphthohydroxamic acid; [\(^{14} \)H]NHA, 2-\( ^{14} \)C]-naphthohydroxamic acid; [\(^{3} \)H,NHA, 2-carboxyl-\( ^{14} \)C]naphthohydroxamic acid; [\(^{14} \)C]NHA, 2-carboxyl-\( ^{14} \)C]naphthohydroxamic acid; O-Ac-NHA, O-\(^ {14} \)C]-acetate of NHA; O-Ac-[\(^{3} \)H]NHA, the O-\(^ {14} \)C]-acetate of [\(^{3} \)H]NHA; O-Ac-[\(^{14} \)C]NHA, the O-\(^ {14} \)C]-acetate of [\(^{14} \)C]NHA; O-SO\(_3\)-NHA-Py, pyridinium salt of O-sulfonate of NHA; AN, 2-aminoanthenylcarboxylic acid; NpCO\(_2\)H, 2-naphthoic acid; NpCONH\(_2\), 2-naphthamide; O-CONP-NHA, O-\(^ {14} \)P]-phosphorylate of NHA; di-Np urea, 1,3-di-2-naphthyl urea; NpNP, 2-aminoanthenyl isocyanate; HRP, horseradish peroxidase; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; DMSO, dimethyl sulfoxide; FCT, pentachlorophenol; RCONO, nitrosoconlyl-alkanes or nitrosoconlyl-arenes; NpCONO, nitrosocarboxyl-2-naphthilamine.

4 C. Y. Wang, personal communication.

5 Unpublished observations.

6 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.
Fig. 1. Lossen rearrangement of O-acetate and O-sulfonate of NHA. R denotes a 2-naphthyl or phenyl group.

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:chloroform:methanol (9:1:1); Solvent B, benzene:acetone (2:1); and Solvent C, benzene:methanol (9:1). Rf values 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:chloroform:methanol (9:1:1); Solvent B, benzene:acetone (2:1); and Solvent C, benzene:methanol (9:1). Rf values are listed in Table 1. Radioactivity was determined by liquid scintillation spectroscopy. 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 18 h. It was extracted with 50 ml of ether, and the extract was washed with an equal volume of 1 N HCl and then 1 N NaOH. Evaporation of solvent and crystallization from methanol gave a solid which was analyzed by HPLC using System I and TLC using Solvent C for the detection of ureas. See Table 1 for the Rf and Rf of 1-(2-naphthyl)-3-phenyl urea and di-Np urea.

The potential for O-SO3H-NHA-Py to undergo Lossen rearrangement in pH 7.0 buffer at room temperature was established in our previous study (11). In this study, the half-life of this compound at room temperature was determined by measuring the decrease with time of the absorbance at 220 nm of a mixture of 1 μg/10 μl of ethanol in 1 ml of pH 7.0 buffer.

Oxidation of NHA and O-Ac-NHA with H2O2/HRP. Reaction mixtures consisted of 6 μmol of compound in 150 μl of DMSO in a total volume of 6 ml containing pH 7.0 buffer, 60 μmol of H2O2, and 30 units of HRP. Control incubations were carried out in the absence of HRP or H2O2/HRP. They were incubated at 37°C, and after 10 min, 1 h, and 24 h, 2-ml aliquots were extracted with ethyl acetate, and the organic phase was dried and redissolved in methanol for analysis of products by HPLC using System III.

Metabolism of NHA and O-Ac-NHA by Cell-free Preparations of S. typhimurium TA98. Cells from 7 ml of an overnight culture were washed twice with 7 ml of 0.1 M phosphate/0.9% NaCl buffer, pH 7.0, and

Table 1 HPLC Rf and TLC Rf values of compounds

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<th>Compound</th>
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<th>Solvents**</th>
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<td>di-Np urea</td>
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<td>33.7</td>
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<tr>
<td>1-(2-naphthyl)-3-phenyl urea</td>
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<td>0.43</td>
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</table>

* Rf 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 365-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 C8 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 at 100% methanol for 5 min.
A: Solvent A, benzene:chloroform:methanol (9:1:1); Solvent B, benzene:acetone (2:1); Solvent C, benzene:methanol (9:1).
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 (2x) 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 Vivo 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 7.0) 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 for product formation 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.

Reaction of NHA and O-Ac-NHA to tRNA. One mg of tRNA in 90 µl of 0.14 M NaCl/0.015 M citrate, pH 7, was incubated at 37°C for up to 4 h with 10 µl of DMSO containing 0.1 µmol of O-Ac-[3H]NHA (39 mCi/mmol of [3H]; 6.2 mCi/mmol of [14C]) or [3H, 14C]NHA (24.8 mCi/mmol of [3H]; 3.25 mCi/mmol of [14C]). The tRNA solution was organic solvents, recovered by precipitation with ethanol, and assayed for radioisotope content (24). The experiments were carried out in duplicate.

Reaction of NHA and O-Ac-NHA with tRNA in the Presence of H₂O₂/HRP. In order to examine the effect of peroxidation on the reactivity of these compounds toward tRNA, a mixture of 190 µl of pH 7.0 buffer, containing 1 mg of tRNA, 1 µmol of H₂O₂, and 5 units of HRP, was incubated at 37°C for 1 min with 0.1 µmol of [3H, 14C]NHA (18.5 mCi/mmol of [3H], 2.38 mCi/mmol of [14C]) or O-Ac-[3H, 14C]NHA (16.2 mCi/mmol of [3H], 2.28 mCi/mmol of [14C]) in 10 µl of DMSO. Control experiments received no H₂O₂. Experiments were carried out in duplicate, and the analysis of the extent of reaction with tRNA was as described (24).

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 [3H, 26.3 mCi/mmol of [14C]), i.e., Experiment 1, 75 ml of a mixture containing 1.13 x 10¹¹ cells and 1.5 µmol of compound in 75 µl of DMSO were added at 37°C for up to 60 min. For the reaction with O-Ac-[3H, 14C]NHA (151 mCi/mmol of [3H, 26.3 mCi/mmol of [14C]; 0.4 mm in compound and 4 x 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 triplicate. After incubation, the cells were collected and washed with a mixture of sodium dodecyl sulfate at a final concentration of 0.5% at 37°C for 30 min (16), the solution was treated with proteinase K (0.25 mg/ml for 1 h and then extracted with phenol/8-hydroxyquinoline/m-cresol/H₂O₂ (500/0.5/70/55 by weight), chloroform/isoamyl alcohol (24/1), and ether. The solution was made 0.3 M in sodium acetate, and the DNA was precipitated with 95% alcohol, washed successively with 70% alcohol, 95% alcohol, acetone, and ether, and then dissolved in 1 ml of 100 mM NaCl/10 mM Tris/10 mM EDTA, pH 7.2. Final purification of DNA included sequential incubations at 37°C for 30 min with heat-treated RNase (0.1 mg/ml) and proteinase K (0.25 mg/ml) before recovery by extraction and precipitation as described above. The DNA was dissolved in water for determination of A₂₆₀nm and tritium content (24). The estimation of DNA content assumed that a solution of 1 mg/ml would have an absorption of 20.

**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 relative to the eluted label (Fig. 3).

At higher concentrations, the NpNCO intermediate could be trapped by reaction with aniline. At the end of an 18-h incubation, both 1-(2-naphthyl)-3-phenyl urea and di-Np urea (Fig. 1) were present. Their structures were established from their HPLC Rₘ, TLC Rₙ values (Table 1, Solvent C), and UV spectra which were all identical with those of authentic samples.

O-SO₂H-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 H₂O₂ 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

In the presence of H$_2$O$_2$/HRP, NHA was almost completely converted in 10 min at 37°C, pH 7.0, to the 2 major products, NpCO$_2$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$_2$O$_2$ alone or in the absence of H$_2$O$_2$/HRP. At the end of a 24-h incubation at 37°C, no NpCO$_2$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$_2$O$_2$/HRP, the reactive intermediate, NpCONO, was generated which reacted with H$_2$O or NHA to give NpCO$_2$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$_2$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$_2$O$_2$ 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$_2$O$_2$/HRP did oxidation of O-Ac-NHA take place to form NpCO$_2$H. H$_2$O$_2$ suppressed the Lossen rearrangement and facilitated hydrolysis to form NHA. Thus, the formation of NpCO$_2$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...
mechanisms of NHA and its O-acetate with TA98, these compounds were treated with cell-free bacterial preparations and various cofactors to determine the potentials for conjugations, oxidation, or reduction. The results are summarized in Table 2. NHA was acetylated to O-Ac-NHA by acetyl-CoA in the presence of either a freshly prepared cell-free protein preparation or one that had been heat treated. In the presence of PAPS, NADH, hypoxanthine, benzoaldehyde, or H2O2, no product was formed from NHA and cell-free preparations. Thus, under these conditions, TA98 can not sulfonate, reduce, or oxidize NHA with these cofactors. As in the case of NHA, the TA98 preparations cannot oxidize O-Ac-NHA, since the oxidation product, NpCO2H, was not detected when it was treated with H2O2 and a 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 benzoaldehyde 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 tRNA in the Presence of H2O2/HRP. The effect of peroxidation of these compounds on the reactivities towards macromolecules was studied with a tRNA binding assay (24) using both ring-3H- and carbonyl-14C-labeled materials. The results are shown in Table 4. Adduct formation was greatly enhanced in the presence of the peroxidation system. NHA was more reactive than O-Ac-NHA, and the adducts retained both labels, presumably through naphthoylation by the reactive intermediate NpCONO (Fig. 5) as proposed by others for the oxidation of hydroxamic acids (25). The binding levels of these compounds (Table 4) reflect the extent of formation of NpCO2H from them in the presence of H2O2/HRP as mentioned above in the section of oxidation of NHA and O-Ac-NHA with H2O2/HRP.

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...
METABOLIC ACTIVATION OF 2-NAPHTOHYDROXAMIC 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.

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<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not detected.

Incubation

Substrate time (h) [3H]NHA O-Ac-[3H]NHA
2 1.4 0.1
4 2.3 0.2
0.6 0.4
1.2 1.1
ND ND

Fig. 8. Binding of [3H, 14C]NHA and O-Ac-[3H, 14C]NHA to tRNA at pH 7, 37°C. Incubation mixture consisted of 0.1 μmol of compounds in 10 μl of DMSO and 1 mg of tRNA in 90 μl of 0.14 M NaCl/0.015 M citrate buffer. The assay procedure was as described by King (24). Points, means; bars, SD from duplicate experiments.

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 μg 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>Binding level (nmol/mg of tRNA)</th>
<th>Net binding level (nmol/mg of tRNA)</th>
<th>Ring/carbonyl ratio bound to tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ring</td>
<td>Carbonyl</td>
<td>Ring</td>
</tr>
<tr>
<td>[3H, 14C]NHA</td>
<td>+</td>
<td>1.00</td>
<td>0.99</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.03</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>O-Ac-[3H, 14C]NHA</td>
<td>+</td>
<td>0.10</td>
<td>0.08</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.00</td>
<td>0.00</td>
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</tr>
</tbody>
</table>

showed that the reaction of NHA with TA98 DNA was time dependent and that binding levels increased linearly up to 60 min (graph not shown). The adducts obtained from both compounds (60-min incubation) retained both labels (Table 5). These results suggest that NHA might be activated by forming a conjugate, such as O-Ac-NHA, and the DNA was modified by NpCONH2 or the Lossen rearrangement product, NpNCO. AN, which could be formed from reaction of NpNCO with water, was not involved in adduct formation; otherwise, it would have lost the carbonyl label.

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 RCONO (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-aminofluorene (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-acylaminofluorene 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 naphthyl) 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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REFERENCES


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

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