Guanyl Oα-Arylation and O6-Arylation of DNA by the Carcinogen N-Hydroxy-1-naphthylamine

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ABSTRACT

The carcinogen N-hydroxy-1-naphthylamine reacted with nucleic acids and protein under slightly acidic conditions (pH 5) to form covalently bound derivatives with 3 to 20 naphthyl residues/1000 monomer units. The level of binding was in the following order: DNA > polyguanylic acid > denatured DNA and ribosomal RNA > serum albumin > transfer RNA > polyadenylic acid. Reactions with nucleosides and nucleotides were not detected, and the binding of N-hydroxy-1-naphthylamine to DNA was not inhibited by the addition of nucleosides, nucleotides, methionine, or glutathione. The reaction rates were first order with respect to both DNA and N-hydroxy-1-naphthylamine concentrations. Enzymatic hydrolysis of the DNA containing naphthyl residues yielded 3 nucleoside-arylamine adducts. The major adduct was identified by chemical, ultraviolet, nuclear magnetic resonance, and mass spectrometric analyses as N-(deoxyguanosin-Oα-yl)-1-naphthylamine. The other two adducts were identified as 2-(deoxyguanosin-Oα-yl)-1-naphthylamine and its decomposition product. Direct evidence for acid-dependent arylnitrenium ion formation was obtained by isotope exchange upon solvolysis of N-hydroxy-1-naphthylamine in acidic H318O, and carboxylation formation was indicated by the formation of the solvolysis products, 1-amino-2-naphthol and 1-amino-4-naphthol.

These studies demonstrated the conversion of a carcinogenic N-hydroxy arylamine to electrophilic arylnitrenium ion and carboxylation species that display high selectivity toward macromolecules. The roles of these electrophiles and their macromolecular adducts in the initiation of urinary bladder carcinogenesis through formation of promutagenic lesions in DNA are suggested.

INTRODUCTION

A number of arylamines and arylamides are carcinogenic for the liver, urinary bladder, and other tissues of humans and experimental animals (reviewed in Ref. 10). The initial step in the metabolic activation of these amines and amides is the formation of N-hydroxy metabolites in the hepatic endoplasmic reticulum (10, 21, 30, 38). These N-hydroxy derivatives are generally more carcinogenic than are the parent amines and arylamides when administered p.o., at sites of s.c. or i.p. injection, or on implantation into the lumen of the urinary bladder (7, 9, 10, 30, 39, 40, 47, 48).

It has been suggested that the carcinogenicity of certain arylamines for the urinary bladder may depend on the conjugation of their N-hydroxy metabolites in the liver and the subsequent transport of the conjugates to the urinary bladder (18, 22, 49). A number of N-hydroxy amines, including the N-hydroxy derivatives of 1- and 2-naphthylamine and 4-aminobiphenyl, are rapidly N-glucuronidated in vitro by uridine 5′-diphosphoglucuronic acid-fortified liver microsomes (22), and Radomski et al. (Ref. 49; J. L. Radomski, personal communication) have isolated N-glucuronides of N-hydroxy-4-aminobiphenyl and N-hydroxy-2-naphthylamine from the urine of dogs given the parent amines. While N-hydroxy arylamine N-glucuronides are relatively stable near neutral pH (22), they are hydrolyzed rapidly to yield the free N-hydroxy amines at the acidic pH (5.0 to 6.0) of most human and dog urines (22, 49). These N-hydroxy derivatives may then be subject to further metabolic activation in the urinary bladder epithelium, or they may undergo an acid-dependent conversion in the bladder lumen to reactive derivatives capable of altering critical cellular macromolecules. The covalent binding of N-HO-1-NA,2 N-hydroxy-2-naphthylamine, and N-hydroxy-4-aminobiphenyl to DNA and RNA at slightly acidic pH has been reported (22, 26, 27). Under these conditions the greater reactivity of N-HO-1-NA, as compared to the activities of N-hydroxy-4-aminobiphenyl and N-hydroxy-2-naphthylamine, is consistent with the much greater carcinogenic potency of N-HO-1-NA at sites of direct application (Refs. 5, 8, and 48; E. C. Miller, F. F. Kadlubar, J. D. Scribner, and J. A. Miller, unpublished data).

This report describes the isolation and characterization of two Oα-substituted deoxyguanosine adducts formed by reaction of the carcinogen N-HO-1-NA with DNA at pH 5. Evidence is presented that the reaction proceeds via an intermediate hydrated arylnitrenium ion that is in resonance with hydrated carbocations. The possible role of the guan-Oα-yl naphthylamine adducts as promutagenic lesions involved in the initiation of carcinogenesis by arylamines is also discussed.

MATERIALS AND METHODS

Materials. Calf thymus DNA (type I), tRNA (type III), polyadenylic acid, polyguanylic acid, polycytidylic acid, polyyuridylic acid, human serum albumin (fraction V), phosphodiesterase I (type II), alkaline phosphatase (type III-S),

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1 The abbreviations used are: N-HO-1-NA, N-hydroxy-1-naphthylamine; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography.

2 To whom requests for reprints should be addressed.

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protease (type V), deoxyadenosine, deoxyguanosine, deoxyctydine, dAMP, dGMP, L-methionine, reduced glutathione, Sephadex G-15, bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)methane base, and bis(trimethylsilyl)trifluoroacetamide were obtained from the Sigma Chemical Co. (St. Louis, Mo.). Benzytrithiyammonium chloride, tetrabutylammonium chloride, 4-(p-nitrobenzyl)pyridine, 1-amino-2-naphthol hydrochloride, 1-amino-4-naphthol hydrochloride, and deuterated NMR solvents were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). DNase I (code naphthol hydrochloride, 1-amino-4-naphthol hydrochloride, and polyuridylic acid were estimated spectrophotometrically; and protein was measured by the biuret reaction (15). Aliquots of the reisolated macromolecules were analyzed for bound 3H by solution in Scintisol (Isolab, Inc., Akron, Ohio) and by subsequent scintillation spectrometry. DNA or protein samples were treated with DNase (22) or protease (0.2 mg/ml, at 37° for 30 min) to effect complete solution in Scintisol.

Analyses for reaction products of N-HO-1-NA with deoxyribonucleosides and deoxyribonucleotides were carried out by means of thin-layer chromatography in Solvents A to D (Table 1) of neutralized aliquots containing 0.01 μCi of 14C or 0.03 μCi of 3H, removal of appropriate chromatogram sections, and estimation of radioactivity. Radioactivity that accumulated in areas with Rf's greater or less than that of the radiolabeled reactant and that was dependent on incubation (at pH 5, but not at pH 7) of N-HO-1-NA with the nucleophile provided an estimate of adduct formation. Solvents A and D were most useful for detection of 14C-nucleoside or 14C-nucleotide adducts, and Solvents B and C were most satisfactory for separation of [3H]-N-HO-1-NA adducts (Table 1).

The reactivity of N-HO-1-NA with 4-(p-nitrobenzyl)pyridine was assayed in a 40% ethanol solution that contained 10 mM potassium citrate buffer (pH 5 or 7), 0.1 mM EDTA, 100 mM 4-(p-nitrobenzyl)pyridine, and 0.05 mM N-HO-1-NA at 37° under argon for 30 min or 4 hr. An equal volume of 0.1 M Na2CO3 that contained 80% acetone was added to stop the reaction and, after centrifugation, the resulting supernatant was analyzed by UV spectrometry at 600 nm (13). N-Benzoyloxy-N-methyl-4-aminoazobenzene (62) was used as a positive control.

Preparation, Isolation, and Characterization of DNA-DNA Adducts. DNA (5 mg/ml) was incubated as described in the previous section with 1 mM [3H]-N-HO-1-NA (11.5 μCi/mmol) at pH 5 for 4 hr under argon (total volume, 30 ml). The DNA was then reisolated (21, 22) and dissolved (1 mg DNA per ml) in 5 mM bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)methane-HCl buffer (pH 7).5 mM MgCl2 (total volume, 150 ml). Enzymatic hydrolysis of the DNA solution was carried out by addition of DNase (15 ml of 10 mg protein per ml in 0.15M NaCl) and subsequent incubation at 37° for 2 hr under argon. The incubation mixture was then cooled to 5°, and 15 ml of 1 M Tris base, 150 units of alkaline phosphatase, and 6 units of venom phosphodiesterase were added (final pH, 8.8 to 9.2). The mixture was filtered with argon and incubated at 37° for 6 hr. At this point the hydrolysate could be stored at −20° for up to 1 wk. Ammonium sulfate (2 g) was added, and the (N-HO-1-NA)-nucleoside adducts were extracted twice with equal volumes of water-saturated n-butyl alcohol (redistilled). The n-butyl alcohol proportion of DNA was determined by optical density measurements of neutralized aliquots at 260 nm.

DNA-(Guan-O*yl)-1-naphthylamine Adducts

Reactivity of N-HO-1-NA with Nucleic Acids, Proteins, Nucleosides, Nucleotides, and Other Nucleophiles. For determination of the covalent binding of [3H]-N-HO-1-NA to cellular nucleophiles, incubations were carried out under argon at 37° in 10 mM potassium citrate buffer (pH 5 or 7) containing 0.1 mM EDTA, 0.5 mM [3H]-N-HO-1-NA (11.5 μCi/mmol) (unless otherwise specified), and either 5 mg of nucleic acid per ml (equivalent to 15 μmol of total nucleoside residues or 3 μmol of deoxyguanosine), or 5 mg of protein per ml, or 2 mM deoxyribonucleoside or deoxyribonucleotide. For determination of the reaction products of N-HO-1-NA with 14C-labeled deoxyribonucleosides or deoxyribonucleotides, the incubations were similar, except that 10 mM N-HO-1-NA and 1 mM [14C]deoxyribonucleoside or [14C]deoxyribonucleotide (2 μCi/ml) were used. Stock solutions of 25 mM N-HO-1-NA were prepared in absolute ethanol and stored at 5° for up to 3 hr.

Nucleic acids and proteins were isolated from the reaction mixtures by multiple solvent extractions and multiple precipitations as previously described (21, 22) and were dissolved in 150 mM NaCl:1.5 mM sodium citrate buffer (pH 7). DNA was estimated by the diphenylamine reaction (2); rRNA, tRNA, polyadenylic acid, polyguanylic acid, polyctydilic acid, and polyuridylic acid were estimated spectrophotometrically; and protein was measured by the biuret reaction (15). Aliquots of the reisolated macromolecules were analyzed for bound 3H by solution in Scintisol (Isolab, Inc., Akron, Ohio) and by subsequent scintillation spectrometry. DNA or protein samples were treated with DNase (22) or protease (0.2 mg/ml, at 37° for 30 min) to effect complete solution in Scintisol.

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extracts, which contained 85 to 90% of the DNA-bound $^3$H, were evaporated at 35° under reduced pressure. The residue was suspended in 5 to 10 ml of 10 mM potassium phosphate buffer (pH 12) containing 35% methanol, applied to a Sephadex G-15 column (2.5 x 28 cm), and developed with the same solvent.

Table 1: Separation of nucleosides, N-HO-1-NA, and N-HO-1-NA nucleoside adducts by thin-layer chromatography

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Deoxyadenosine</th>
<th>Deoxyguanosine</th>
<th>Deoxythymidine</th>
<th>dAMP</th>
<th>dGMP</th>
<th>N-HO-1-NA</th>
<th>Adducts I, II, and III</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Isopropyl alcohol: NH$_4$OH:H$_2$O (6:3:1)$^a$</td>
<td>0.68</td>
<td>0.56</td>
<td>0.75</td>
<td>0.81</td>
<td>0.35</td>
<td>0.16</td>
<td>0.90-0.98</td>
</tr>
<tr>
<td>B. Aqueous phase of n-butyl alcohol:n-propyl alcohol: H$_2$O (4:1:5)$^a$</td>
<td>0.65</td>
<td>0.72</td>
<td>0.89</td>
<td>0.93</td>
<td>0.85-0.95</td>
<td>0.95</td>
<td>0-0.30</td>
</tr>
<tr>
<td>C. Solvent B: NH$_4$OH (100:1)$^a$</td>
<td>0.65</td>
<td>0.77</td>
<td>0.85</td>
<td>0.92</td>
<td>0.91</td>
<td>0.96</td>
<td>0.05-0.20</td>
</tr>
<tr>
<td>D. Benzene: NH$_4$OH (7:3)$^a$</td>
<td>0.66</td>
<td>0.33</td>
<td>0.20</td>
<td>0.45</td>
<td>0</td>
<td>0</td>
<td>0.90-0.98</td>
</tr>
</tbody>
</table>

$^a$ The adsorbant was cellulose (Brinkman MN300).

RESULTS

Reactivity of N-HO-1-NA with Nucleic Acids and Protein at Acidic pH. As reported previously, N-HO-1-NA reacted at acidic pH with nucleic acids, presumably through the formation of an electrophilic arylnitrenium ion, to yield high levels of bound naphthylamine residues that were not removed by repeated solvent extractions or multiple precipitations (22). Additional data on the reactivity of [16O]-N-HO-1-NA with macromolecules are presented in Table 2. The extent of reaction with nucleic acids was in the order: native DNA > polyguanylic acid > denatured DNA, rRNA > tRNA, polyadenylic acid > polyctydlylic acid and polyuridylic acid.
At pH 5, about 30% of the [3H]-N-HO-1-NA added to the incubation mixture reacted with the DNA and yielded approximately 1 bound arylamine residue/100 nucleotides. At pH 7 the reaction with native DNA was only about 2%, and marked reductions in the extents of reaction were also observed for each of the other polynucleotides when the reactions were carried out at neutrality. The reaction with serum albumin was about one-third of that observed with native DNA at pH 5, but at pH 7 the reaction with serum albumin was about 3 times that with native DNA. The reaction with protein, particularly at pH 7, may reflect in part the slow oxidative decomposition of N-HO-1-NA to 1-nitrosonaphthalene, which would then be expected to react with sulfhydryl groups in the protein (3, 16, 36).

Failure to Detect Reaction of N-HO-1-NA with Nucleosides, Nucleotides, or Other Low-Molecular-Weight Nucleophiles. Under the conditions that resulted in acid-dependent binding of N-HO-1-NA to nucleic acids, no adduct formation was detected with a variety of low-molecular-weight nucleophiles. With incubations of 0.5 mM [3H]-N-HO-1-NA and 2 mM deoxyadenosine, deoxyguanosine, deoxycytidine, thymidine, dAMP, or dGMP, no tritiated arylamine-nucleoside or nucleotide adducts were detected by means of thin-layer chromatography (cf. "Materials and Methods"). Because of some instability of [3H]-N-HO-1-NA during the thin-layer chromatography, reaction of less than 5% of the [3H]-N-OH-1-NA was not detectable. Similar incubations of 10 mM N-HO-1-NA and 1 mM 14C-labeled deoxyribonucleosides, [3H]dAMP, or [3H]dGMP likewise yielded no detectable adducts; conversion of 1% of the [14C]-labeled nucleosides or nucleotides to adducts would have been detected. Similar negative results were obtained when the reaction mixtures were mixed vigorously with an organic phase (equal volumes of n-hexane) or with a phase-transfer catalyst (10 mM benzyltriethylammonium chloride or 5 mM tetrabutylammonium chloride) during the entire incubation. Even when N-HO-1-NA was incubated with the strong nucleophile 4-(p-nitrobenzyl)pyridine, no reaction was detected; the chromophore obtained was less than 2% of that formed on incubation with an equimolar amount of N-benzyloxy-N-methyl-4-aminoazobenzene. Furthermore, the addition of 5 mM deoxyadenosine, deoxyguanosine, deoxycytidine, thymidine, methionine, or glutathione to reaction mixtures containing N-HO-1-NA and DNA (described in Table 2) resulted in no significant diminution (<10%) of arylamine-adduct formation; the addition of serum albumin (5 mg/ml) reduced the yield of DNA-bound arylamine by 30%.

Isolation of (N-HO-1-NA)-Nucleoside Adducts from DNA. The apparent selectivity of the N-HO-1-NA binding reaction for macromolecules, particularly DNA, prompted an investigation of the nature of the arylamine-DNA adducts. DNA that had been incubated with 1 mM [3H]-N-HO-1-NA at pH 5 for 4 hr in an argon atmosphere was resolated by solvent extractions and precipitations. Treatment of the arylamine-substituted DNA, which contained 40 to 60 nmol of tritiated adduct per mg DNA, with DNase, phosphodiesterase, and alkaline phosphatase (cf. "Materials and Methods"), converted 85 to 90% of the tritiated products to n-butyl alcohol-, but not diethyl ether-, extractable derivatives. All 3 enzymes were required for the release of extractable tritium.

Chromatography of the n-butyl alcohol extract of the DNA-arylamine hydrolysate on Sephadex G-15 separated 3 putative arylamine-nucleoside adducts, which together accounted for 85 to 95% of the 3H applied to the column (Chart 1). Adducts I and II were recognized both as 254 nm-absorbing and as 3H-containing peaks. The concentrations of Adduct III in the eluates were too dilute to be detected by 254 nm absorbance, but this adduct was visualized as a slowly eluting purple band. In addition, the 3H in Adduct III was labile and was slowly exchanged with the solvent.

The eluants containing each adduct were pooled, concentrated, and extracted with n-butyl alcohol, and each n-butyl alcohol fraction was then analyzed by HPLC. The Adduct I fraction, which contained 5 to 15% of the tritium eluted from the Sephadex column, contained 2 components...
F. F. Kadlubar et al.

The peak that eluted at 5 min was identified as Adduct I. The more rapidly eluting peak was eventually found to be identical with Adduct III, which was slowly formed from Adduct I. Therefore, the yields of these 2 adducts from either Sephadex or HPLC columns were variable and were inversely related. The Adduct II fraction, which accounted for 50 to 60% of the tritium eluted from the Sephadex column, exhibited 1 major UV-absorbing and 3H-containing component on HPLC analysis (Chart 2B). Adduct III, chromatographed with a different solvent program, yielded a single major UV/absorbing component that was identical in its UV spectrum with the presumed Adduct III from the Adduct I fraction. Preparations of Adduct III differed considerably in their specific radioactivities; further purifications eventually resulted in complete loss of tritium from this adduct. On the assumption that Adduct III has an $E_{\text{max}}$ (at its $\lambda_{\text{max}}$) similar to those of Adducts I and II, this decomposition product accounts for 20 to 30% of the total adducts eluted from the Sephadex column. If all of Adduct III is formed from Adduct I, the N-HO-1-NA-reacted DNA contains Adducts I and II in a ratio of about 1:2.

Identification of the Major Adduct (II) as N-(Deoxyguanosin-O$^\text{6}$-yl)-1-naphthylamine. Mass spectral analyses of Adduct II indicated that it was a deoxyguanosinyl-naphthylamine derivative. The molecular ion ($m/e$ 444) obtained by field desorption mass spectrometry corresponds to a deoxyguanosinyl-naphthylamine dihydrate, and a fragment at $m/e$ 302 corresponds to the loss of naphthylamine minus one proton. Electron impact mass spectrometry of the silylated product (cf. "Materials and Methods") yielded a molecular ion at $m/e$ 624, which was consistent with a tris(trimethylsilyl)deoxyguanosinyl-naphthylamine adduct.

The partition coefficients of Adduct II between aqueous buffers and n-butyl alcohol:ethyl ether, calculated according to the method of Moore and Koreeda (41), were identical from pH 6 to pH 10 (Chart 3). This finding showed that the adduct did not ionize in alkali and suggested that substitution occurred on the N$^\text{7}$ or O$^\text{6}$ atoms of guanine residues. Identical UV spectra at pH 7 and pH 13 also indicated the lack of a basic pK$_\alpha$ (Chart 4). The absorption maxima at 243 nm (pH 7 and pH 13) and 290 nm (pH 1) were similar to those of O$^\text{6}$-alkylguanosines (54) and were thus also consistent with substitution at the O$^\text{6}$ atom of guanine. Furthermore, the spectrum of an equimolar mixture of N-HO-1-NA and deoxyguanosine in 0.1 N NaOH was almost identical with the spectra of Adduct II at pH 7 and pH 13. The IR spectrum also indicated that the adduct was an O$^\text{6}$-substituted guanine derivative (Chart 5). The presence of the N$^\text{7}$-H bend (1610 cm$^{-1}$) and the absence of a C$^\text{6}$=O stretch (1733 cm$^{-1}$) and of a N$^\text{1}$—H bend (1538 cm$^{-1}$) clearly indicated an aromatized guanosine derivative (1).

Final structural identity was obtained from 90-MHz Fourier transform-NMR spectroscopy, and the assignments were made after comparison of the spectrum with 90-MHz spectra of 1-naphthylamine, N-HO-1-NA, and deoxyguanosine and with previously reported spectra and assignments (12, 14). The spectral data (summarized in Chart 6A) showed the absence of the guanine N$^\text{1}$ proton (11 to 11.5 ppm; Ref. 14)
and an upfield shift of the N² protons from 6.9 to 5.8 ppm, both of which are consistent with an aromatic guanosine derivative. The spectrum also showed the presence of a single naphthylamine N—H proton at 9.5 ppm and all 7 aromatic protons of the naphthylamine ring. All of the NMR and other spectral data are uniquely consistent with O⁶ substitution of the guanine base through the amino group of the naphthylamine residue.

In contrast to the acid lability of the O⁶-alkylguanosines that have been studied (33, 54, 61), this guanosine derivative, in which the O⁶ was substituted by the nitrogen of 1-naphthylamine, was stable for 4 hr at 37° at pH 1, 7, and 13.

| Chart 4. UV spectra of Adduct II (25 μM) in 10% methanol that contained 0.1 N HCl (-----), 10 mM potassium citrate (--), pH 7, and 0.1 N NaOH (----). |
| Chart 5. IR spectrum of Adduct II (0.6 mg) in a 15-mm KBr (100-mg) pellet. |

Chart 6. Proton NMR data for Adducts II (A) and I (B). Adduct II (0.8 mg) and Adduct I (0.07 mg) were each dissolved in 0.3 ml of deuterated dimethyl sulfoxide. Because of the small sample size only a partial spectrum of Adduct I could be obtained. The undeuterated dimethyl sulfoxide and water signals at 2.5 and 3.3 ppm (downfield from tetramethylsilane), respectively, were suppressed by irradiation, but the residual peaks were still of such an intensity that the sample signals could be detected above signal noise only at 6 to 11 ppm.

Adduct II: N-(Deoxyguanosin-0'-yl)-1-naphthylamine

<table>
<thead>
<tr>
<th>Signal (Protons, Multiplicity)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5 (1,5)</td>
<td>NH (Naphthyl)</td>
</tr>
<tr>
<td>8.5 (1,5)</td>
<td>H-8 (Deoxyguanosine)</td>
</tr>
<tr>
<td>8.1-8.3 (2, M)</td>
<td>H-5,H-8 (Naphthyl)</td>
</tr>
<tr>
<td>7.2-7.9 (4, M)</td>
<td>H-3,H-4,H-6,H-7 (Naphthyl)</td>
</tr>
<tr>
<td>6.9-7.0 (1, D)</td>
<td>H-2 (Naphthyl)</td>
</tr>
<tr>
<td>6.6-6.8 (1, T)</td>
<td>H-1' (Deoxyguanosine)</td>
</tr>
<tr>
<td>5.8 (2, S)</td>
<td>NH₂ (Deoxyguanosine)</td>
</tr>
<tr>
<td>4.5 (1, 5)</td>
<td>H-3' (Deoxyguanosine)</td>
</tr>
<tr>
<td>3.0-4.0 (6)</td>
<td>OH-3',OH-5',H-4',H-2',H-5' (Deoxyguanosine)</td>
</tr>
</tbody>
</table>

Adduct I: 2-(Deoxyguanosin-0'-yl)-1-naphthylamine

<table>
<thead>
<tr>
<th>Signal (Protons, Multiplicity)</th>
<th>Tentative Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3 (1, 5)</td>
<td>H-8 (Deoxyguanosine)</td>
</tr>
<tr>
<td>8.1-8.2 (1, D)</td>
<td>H-3 or H-4 (Naphthyl)</td>
</tr>
<tr>
<td>7.6-7.7 (1, M)</td>
<td>H-5 or H-8 (Naphthyl)</td>
</tr>
<tr>
<td>7.3-7.5 (2, M)</td>
<td>H-6 and H-7 (Naphthyl)</td>
</tr>
<tr>
<td>7.1-7.2 (1, M)</td>
<td>H-8 or H-5 (Naphthyl)</td>
</tr>
<tr>
<td>6.6-6.75 (1, Q)</td>
<td>H-1' (Deoxyguanosine)</td>
</tr>
<tr>
<td>6.2 (2, S)</td>
<td>NH₂ (Deoxyguanosine or Naphthyl)</td>
</tr>
</tbody>
</table>

* The solvent was d₂-dimethylsulfoxide. 
* Expressed as ppm from tetramethylsilane; S=singlet, D=doublet, T=triplet, Q=quartet, M=multiplet. 
* Exchangeable with D₂O. 
* Not resolved due to residual H₂O signal. 
* Due to adduct lability, a spectrum in D₂O-d₆-DMSO was not obtained. 
* i=8.0 
* j=8.6
as assayed by HPLC. The stability of this adduct, an O-substituted N-hydroxy arylamine, is consistent with the stabilities of other O-substituted N-hydroxy arylamines (Ref. 56, p. 5).

Identification of Adduct I as 2-(Deoxyguanosin-O6-yl)-1-naphthylamine. Adduct I was isolated by repetitive HPLC and analyzed in a manner similar to that of Adduct II, but these studies were limited by the smaller amounts available. Electron impact mass spectrometry of silylated Adduct I yielded a molecular ion at m/e 696, which corresponds to a tetrakis(trimethylsilyl)-deoxyguanosinyl-naphthylamine adduct. As in the case of Adduct II, Adduct I showed an identical partition coefficient between aqueous buffers and n-butyl alcohol-ethyl ether at neutral and alkaline pH (Chart 3); this finding was consistent with substitution at the O6 or N1 atom of the guanine.

The UV spectra of Adduct I at pH's 7 and 13 (Chart 7) were also identical and were very similar to those of Adduct II under these conditions (Chart 4). The absorbance of Adduct I at pH 1 was lower at 240 nm and higher at 290 nm, compared to the absorbances of this adduct at pH 7 or 13. These spectral data are characteristic of O6-guanine substitutions and therefore suggest that Adduct I is substituted at this site.

Only a partial 360-MHz NMR spectrum was possible with the small quantities of Adduct I (70 µg) that were available (Chart 6B). On comparison of the NMR spectra of Adduct II, 1-naphthylamine, and 2-methoxy-1-naphthalene (13, 44) with that of Adduct I, the presence of 2 slightly downfield aromatic proton doublets (7.6 and 7.7 ppm; 8.1 and 8.2 ppm) with identical ortho coupling constants in the spectrum of Adduct I strongly suggested substitution at C-2 of the 1-naphthylamine residue. The data for Adduct I clearly support its characterization as 2-(deoxyguanosin-O6-yl)-1-naphthylamine.

Adduct I was relatively labile, particularly at acid or neutral pH, and yielded Adduct III. This purple decomposition product could be decolorized by addition of a reducing agent (sodium dithionite), was considerably more polar than was Adduct I, readily lost 3H from the naphthylamine ring, and had UV absorption spectra (Chart 8) that were consistent with characterization as 2-(deoxyguanosin-O6-yl)-4-naphthoquinone-1-imine. Further studies on the identity of Adduct III were not carried out.

Mechanisms of Reaction of N-HO-1-NA with DNA. The high reactivity of N-HO-1-NA toward macromolecules and the high selectivity toward substitution at the O6 atom of guanine in the DNA prompted an investigation of the reaction mechanisms involved. The reaction rate was first order with respect to the concentrations of both N-HO-1-NA and DNA (Chart 9); thus, an apparent overall second-order reaction occurred.

Direct evidence for the formation of an electrophilic arylnitrenium ion was obtained from solvolysis experiments in 18O-enriched H2O. Incubation of N-HO-1-NA in 50% 18O-enriched H2O at 37° for 2 hr in the absence of DNA resulted in a 42% incorporation (84% exchange) of 18O into N-HO-1-NA at pH 5 and a 1% incorporation (2% exchange) at pH 7. These data were obtained after neutralization of the pH 5 incubation mixture, recovery of N-HO-1-NA by extraction into ethyl acetate, and subsequent analysis by electron
impact mass spectrometry (cf. "Materials and Methods").

Evidence for carboxylation formation was obtained by incubation of N-HO-1-NA at pH 1, 5, and 7 in the absence of DNA followed by determination of the yields of the probable carboxylation solvolysis products, 1-amino-2-naphthol and 1-amino-4-naphthol, by high performance thin-layer chromatography (cf. "Materials and Methods"). A slow conversion to products that cochromatographed with the amiono-2-naphthol was obtained during a 4-hr incubation (Table 3). The accumulation of these carboxylation solvolysis products was markedly acid-dependent, and the formation of 1-amino-2-naphthol was preferred over that of 1-amino-4-naphthol. These results were consistent with the formation of the 2- rather than the 4-substituted naphthyl carboxylation adduct. The lower yield of the carboxylation solvolysis products as compared to the nitrenium ion product, [18O]-N-HO-1-NA, was consistent with the preferred formation of the nitrenium adduct, N-(deoxyguanosin-O6-yl)-1-naphthylamine, over that of the carboxylation adduct, 2-(deoxyguanosin-O6-yl)-1-naphthylamine.

Table 3
Conversion of N-HO-1-NA to 1-amino-2-naphthol and 1-amino-4-naphthol

<table>
<thead>
<tr>
<th>pH</th>
<th>1-Amino-2-naphthol</th>
<th>1-Amino-4-naphthol</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

The reaction mixtures contained either 0.1 M HCl (pH 1) or 10 mM potassium citrate buffer (pH 5 or pH 7), 0.1 mM EDTA, and 1 mM [18H]-N-HO-1-NA. The incubations were carried out under argon for 4 hr. 1-Amino-2-naphthol and 1-amino-4-naphthol were separated by high-performance thin-layer chromatography and determined radiochemically as described in "Materials and Methods."

DISCUSSION

Evidence supporting the somatic mutation theory of cancer induction has come from studies on the reactions with DNA of structurally simple carcinogenic alkylating agents, N-nitrosoamines, and N-nitrosamides. A considerable amount of data suggests that substitution of certain oxygen atoms in the DNA bases may be critical to the initiation of neoplasia (11, 25, 33, 43, 50, 55). No similar generalization has yet emerged for the reactions of the structurally more complex chemical carcinogens. Previous studies in vivo and in vitro have identified C-8 and N2-substituted guanine, N8-substituted adenine and N4-substituted cytosine adducts as products of the reactions of ultimate carcinogenic derivatives of arylamines and arylamides with nucleic acids (23, 28, 29, 31, 34, 35, 40, 52, 53, 59). In addition, this study has demonstrated that the O6-atom of guanine in DNA is substituted by N-HO-1-NA under the mildly acidic conditions frequently found in human and dog urines.

The selectivity of the reaction of N-HO-1-NA with polynucleotides as compared to low-molecular-weight nucleophiles and the dependence of the reaction rate on the concentrations of both DNA and N-hydroxy arylamine suggest a mechanism by which the activated carcinogen is inserted into the DNA at the O6 atom of the guanine base (Chart 10). A H+-dependent formation of an electrophilic arylnitrenium ion has been suggested for several N-hydroxyarylamines (3, 17, 22, 26, 27, 32, 64), and direct evidence for the formation of a 1-naphthyl nitrenium ion is provided in this study by the relatively slow H+-dependent incorporation of 18O from H218O into N-HO-1-NA. The existence of the 2- and 4-naphthyl carboxylations as contributing resonance forms was indicated by the accumulation of their solvolysis products, 1-amino-2-naphthol and 1-amino-4-naphthol. The lower yield of Adduct I (plus Adduct III), compared to that of Adduct II on reaction of N-HO-1-NA with DNA, may reflect the relative contribution of each resonance form. Simple Hückel molecular orbital calculations also indicate that the charge should be localized preferentially at the N-naphthyl rather than the 2- and 4-naphthyl positions (N. R. Drinkwater, unpublished studies). Alternatively, the DNA may assist in adduct formation at the N-naphthyl and 2-naphthyl positions through steric interactions such as intercalation, hydrogen bonding, or kinking. Other mechanistic studies on the conversion of N-hydroxy arylamines to aminophenols (32, 57) support the proposed intermolecular conversion of N-HO-1-NA to aminonaphthols via an intermediate arylnitrenium ion-carbocation electrophile. The proposed mechanism also depicts the solvolyses of the carbocations as proceeding irreversibly through oxonium ions to the aminonaphthols; this is consistent with the lack of reactivity of aminonaphthols with nucleic acids (24).

The suggested existence of the electrophile (with contributing arylnitrenium ion and carbocation resonance structures) as a hydrated species is consistent with its lack of reaction with mononucleosides; thus, a macromolecular structure may assist the desolvation of these electrophiles and facilitate adduct formation. The existence of a hydrated naphthylammonium ion (51) and of solvated carbocations (46) has previously been suggested, and desolvation of the cation by the nucleophile was suggested as a rate-determi-
ing step. Other ultimate carcinogens [e.g., aflatoxin-B1, 2,3-
dichloride (58), N,N-diethylnyl-2-chloroethylamine (45), and
7,8-dihydridiol-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)py-
rene (42)] have also shown high selectivity toward macro-
molecules, and their reaction mechanisms may also involve
hydrated electrophilic species.

The N-HO-1-NA-DNA reaction kinetics, which was first
order with respect to both reactants, indicate that the
mechanism may be either $S_{1}$ with formation of an inter-
mediate involved in a rate-determining step or $S_{2}$ (6). An
$S_{1}$ mechanism seems more likely, since it has been shown
with the simple alkylating agents that $S_{2}$ reactivity leads to
appreciable substitution at the O$_{6}$ atom of guanine and to
greater carcinogenicity (reviewed in Ref. 33). The $S_{1}$ or
$S_{1}^{'}$ intermediate could be the hydrated arylnitrenium ion-
carbocation electrophile. In addition, the reaction may
proceed through the intermediate formation of an intimate
ion pair (6, 46) between the desolvated electrophile and the
O$_{6}$ of the guanine just prior to adducci formation. The DNA
may also stereochemically assist in directing the electro-
philic region of the carcinogen to the O$_{6}$-guanine atom
and thus promote the selectivity of the reaction.

This reaction mechanism has several biological implica-
tions for arylamine-induced urinary bladder carcinogenesis.
If the hydrolysis of the N-hydroxy arylamine N-glucuronide
and the acid-dependent conversion of the free N-hydroxy
arylamine to reactive derivatives in the urine are significant
in bladder carcinogenesis, the electrophilics must be able
to survive in the presence of urinary nucleophiles, enter the
bladder epithelium, and induce a heritable lesion in a
cellular constituent. N-HO-1-NA would appear to be capable
of fulfilling these requirements. Under the acidic conditions
normally found in the bladder lumen, N-HO-1-NA and its
reactive derivatives are quite stable and unreactive with
low-molecular-weight nucleophiles (cf. "Results"). As lipo-
philic cations the reactive naphthyl derivatives may have
detergent properties that facilitate their transport across
the epithelial cell membrane to react with intracellular
nucleic acids and proteins. If reaction with DNA is a critical
event in arylamine carcinogenesis, the formation of the O$_{6}$-
guanine-substituted adducts, which would interfere with
normal Watson-Crick base pairing, could provide the initial
step toward neoplasia. Computer-generated graphic
models of the N-(deoxyguanosin-06-yl)-1-naphthylamine
adduct in the DNA double helix indicate that, like the N$^{2}$-
DNA-(Guan-O6-yl)-1-naphthylamine Adducts


F. F. Kadlubar et al.


Guanyl O⁶-Arylation and O⁶-Arylation of DNA by the Carcinogen N-Hydroxy-1-naphthylamine

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