Prostaglandin H Synthase-catalyzed Metabolism and DNA Binding of 2-Naphthylamine

Jeff A. Boyd¹ and Thomas E. Eling²

Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

ABSTRACT

The oxidation of the bladder carcinogen 2-naphthylamine (2-NA) by prostaglandin H synthase (PHS) in vitro was examined. Oxygen uptake studies of 2-NA oxidation in the presence of glutathione, as well as extensive product analysis data, are consistent with a one-electron mechanism of 2-NA oxidation by PHS. The formation of 2-nitrosodiphenylamine is not observed under any condition. Metabolism studies with a purified PHS preparation confirm that 2-NA oxidation is dependent upon the peroxidase activity of the enzyme complex, and that a variety of organic hydroperoxides may support the reaction. Horseradish peroxidase oxidizes 2-NA to the same products but, depending on pH, in very different proportions from those obtained with PHS. Oxidation of 2-NA by a one-electron chemical oxidant results in a product profile similar to that obtained in the enzymatic systems. The above data are consistent with a one-electron mechanism of 2-NA oxidation by PHS. The metabolism data provide evidence for the formation of two types of potentially reactive electrophiles: 2-imino-1-naphthoxoquinone and a free radical product.

We further examine the time course of a product of PH2H2-NA to DNA in vitro, and compare this with the reaction of authentic 2H2-NA and phosphatase (2-A-1-N) products with DNA and protein. A significant amount of the PH2H2-catalyzed binding of 2-NA to DNA is derived from a short-lived intermediate; furthermore, the time course of binding is very rapid. Conversely, the binding to DNA of 2-NA and 2-A-1-N (presumably in the form of 2-A-1-naphthoxoquinone) occurs to a lower extent and is not time dependent under the conditions studied. 2-A-1-N binds to protein, however, at a rapid rate and to three orders of magnitude greater extent than to DNA. The PH2H2-catalyzed binding of 2-NA to DNA was studied under several conditions; binding was shown conclusively to result from the peroxidase activity of the PH2H2 complex. In addition, greater levels of binding were observed at pH 5.0 than at pH 7.6, and when catalyzed by horseradish peroxidase/H2O2 rather than PHS. These are conditions under which 2-A-1-N formation is negligible or nonexistent. These results demonstrate that in the PHS system, a reactive product(s) in addition to 2-A-1-N is generated which binds to DNA, and that this product is probably a free radical. This type of product should be considered in future studies of DNA adducts formed in vitro or in vivo by the PHS system.

INTRODUCTION

The metabolic activation of primary arylamine bladder carcinogens is considered an obligatory event in their initiation of carcinogenesis. Specifically, mutagenic alteration of macromolecules, primarily DNA, by the resulting electrophilic metabolites is currently thought to be the initiating event in chemical carcinogenesis (1). Arylamine metabolism studies have centered primarily on the role of the NADPH-dependent monooxygenase enzymes of the liver, including the cytochrome P-450 monooxygenase and flavin-containing monooxygenase (2-5). The mechanism frequently invoked for the bladder-specific carcinogenicity of arylamines requires that they undergo N-hydroxylation in the liver, followed by N-glucuronidation, transport to the bladder, and acid-catalyzed hydrolysis to yield the free N-hydroxyarylamine (6-9). Acid hydrolysis of the N-hydroxy compound would then yield a nitrenium ion, presumably the ultimate reactive electrophile involved in DNA binding and the initiation of carcinogenesis (8, 9). Cytochrome P-450-dependent N-hydroxylation may also occur in the bladder itself, as recently demonstrated using rabbit tissue (10).

The oxidation of xenobiotics by the peroxidase activity of PHS has been studied extensively during the past 10 years (reviewed in Refs. 11, 12). The hypothesis that PHS may serve as an alternative activating system in arylamine-induced bladder carcinogenesis has received considerable support from numerous studies in vitro (reviewed in Refs. 13-15). Furthermore, there is evidence of relatively high PHS activity in a target organ of arylamine-induced carcinogenesis, the dog bladder, which contains little or no detectable cytochrome P-450 activity (16). The PHS-catalyzed binding of several arylamines to nucleic acid in vitro (17, 18) serves further to prompt a complete investigation aimed toward discerning the potential relevance of this activating system in vivo.

Studies in our laboratory have focused on the metabolic pathway of PHS-catalyzed arylamine oxidation, in an effort to determine the reaction mechanism and the nature of the reactive products. Extensive studies with the model primary arylamine carcinogen 2-aminofluorene demonstrated that this compound is oxidized by PHS via a one-electron mechanism (19, 20). A comparison of PHS with other peroxidases indicated that 2-aminofluorene oxidation by HRP is very similar to that catalyzed by PHS, while chloroperoxidase catalyzes a dramatically different reaction (19, 20). Further studies suggested that a free radical or free radical-derived species is responsible for the DNA adducts formed in this system (21) as well as the PHS-catalyzed mutagenicity of 2-aminofluorene in a modified Ames test (22).

We have undertaken the present study to characterize the peroxidase-catalyzed oxidation of the human bladder carcinogen 2-NA. Previous studies have shown that the PHS of dog bladder epithelium supports the metabolism of 2-NA to products which bind to tRNA and protein (16). The PHS present in ram seminal vesicle microsomes also catalyzes the metabolism of 2-NA to a mutagenic metabolite in the Ames test (23) and the binding of 2-NA to DNA in vitro (17). In this paper, we present a product profile and other data that is consistent with the hypothesis that 2-NA oxidation by PHS occurs via a one-electron mechanism. HRP oxidized 2-NA to the same products as PHS, but in markedly different proportions, depending on the reaction conditions. Unlike 2-aminofluorene, however, two distinctly different types of 2-NA metabolites are generated by PHS which could potentially react with macromolecules. These metabolites include the C-oxygenated product.

¹ To whom requests for reprints should be addressed, at Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709.

² The abbreviations used are: PHS, prostaglandin H synthase; ADN, 2-amino-1-naphthylamine; 2-A-1-N, 2-A-1-naphthol; AQI, 2-amino-1,4-naphthoquinone-NH2; 2-A-1-naphthylamine; CIP, chloroform/isoamyl alcohol/phenol; DBP, di-benz(o, a)phenazine; GSH, glutathione; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; 15-HPETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 2-NA, 2-naphthylamine.
2-NA OXIDATION AND DNA BINDING

2-A-1-N, and a free radical species. While this work was in progress, the PHS-dependent formation of a 2-imino-1-naphthoquinone/DNA adduct was demonstrated by Yamazoe et al. (24). Additional DNA adducts isolated in that study were attributed to N-hydroxy-2-NA. However, as shown in this paper, extensive metabolism studies fail to demonstrate the formation of N-hydroxy-2-NA or 2-nitrosophenanthrene in the PHS system. Thus, we believe that these additional adducts are more likely derived from some other reactive intermediate. The second aim of this study is therefore to compare the reactivity with DNA of the 2-imino-1-naphthoquinone (the oxidized form of 2-A-1-N) metabolite with that of other metabolic intermediates, presumably free radicals. The PHS-catalyzed binding of 2-NA to DNA is also characterized under a variety of conditions, to further assess the relative contribution of various reactive metabolites formed in this system. Finally, evidence is provided that the binding of 2-NA to DNA is dependent upon the peroxidase activity of PHS.

MATERIALS AND METHODS

Materials. [ring-3H]2-NA (196 mCi/mmol) was obtained from Midwest Research Institute, Kansas City, MO. The [3H]2-NA was consistently >98% pure as determined by HPLC analysis. 2-Amino-1-naphthyl HCl was obtained from Pfaltz and Bauer, Inc., Stanford, CT. Unlabeled 2-NA, potassium superoxide, phenylbutazone, glutathione, indomethacin, and horseradish peroxidase (type VI), calf thymus DNA (type I), ribonuclease T, (grade IV), ribonuclease-A (type I-A), and protease (type XIV) were from Sigma Chemical Co. Potassium nitrosodisulfonate, potassium persulfate, 2-nitrophenylalanine, palladium on carbon (5%), hydrazine hydrate, diethylazodicarboxylate, and potassium ferricyanide were products of Aldrich Chemical Co. Arachidonic acid was from the Nu-Chek-Prep, Inc., Elysian, MN, and 2,2'-azobis(2-methylpropionitrile) was obtained from Alfa Products, Danvers, MA. Hydrogen peroxide (30%), HPLC-grade methanol, and HPLC-grade water were products of Fisher Scientific Co. All other materials were of reagent grade and purchased from standard commercial suppliers.

Preparation of Standard Compounds and Substrates. [5,6,7,8-3H]2-A-1-N (14.6 mCi/mmol) was prepared by first synthesizing the 2-amino-1-naphthyl sulfate by oxidation of [3H]2-NA with potassium persulfate (25). This compound was then converted to [3H]2-A-1-N as described (26). 2-Nitroso-phenanthrene was synthesized from N-hydroxy-2-NA by diethylazodicarboxylate-catalyzed oxidation, as described (27). N-Hydroxy-2-NA was prepared by reduction of 2-nitroso-phenanthrene with palladium on carbon and hydrazine hydrate as described by Westra (28). DBP was synthesized from 2-NA using potassium superoxide (29). AFO was prepared by potassium nitrosodisulfonate-catalyzed oxidation of 2-NA (30). ADN was synthesized by potassium ferricyanide-catalyzed oxidation of 2-NA, as described below. The identity of all standards was confirmed by mass spectrometry and UV-visible spectrophotometry. 15-HPETE was prepared by soybean lipoygenase-catalyzed oxidation of arachidonic acid as described (31).

Prostaglandin H Synthase Preparation. Microsomes from rat seminal vesicles were prepared as described previously (19), with the exception that the final resuspension was in 100 mM potassium phosphate buffer, pH 7.6. Purified PHS (67,000 units/mg protein) was purchased from Oxford Biomedical Research, Inc., Oxford, MI. Both PHS preparations were stored at −70°C until use. Enzyme activity was assured by the addition of either 100 μM arachidonic acid or 100 μM H2O2. Reactions were carried out in 100 mM sodium acetate buffer, pH 5.0, and initiated with 100 μM H2O2. Reaction mixtures were determined by measuring UV absorbance at 240 nm, ε = 43.6 (33). All incubations were run for 10 min at 37°C. Reactions were terminated and the products extracted with two volumes of buffered saturated ethyl acetate:diethyl ether, 1:1. Two such organic extracts from each reaction mixture were pooled and evaporated to dryness under reduced pressure at 37°C. The residues were resuspended in ethanol and analyzed by HPLC as described below. Radioactivity remaining in the aqueous fraction was quantitated by counting an aliquot using Atomlight scintillation cocktail (New England Nuclear, Boston, MA), and a Packard model 460C liquid scintillation spectrometer, with quench correction. Reaction mixtures containing boiled microsomal protein with and without arachidonic acid or H2O2 served as nonenzymatic controls.

The metabolism of 2-NA by purified PHS was examined in reaction mixtures containing 100 mM potassium phosphate buffer, pH 7.6, 36.5 μg (2.44 × 10^3 units) of enzyme, and 50 μM [3H]2-NA in a total volume of 2 ml. Reactions were initiated with either 100 μM arachidonic acid, 30 μM 15-HPETE, or 100 μM H2O2. Some arachidonic acid-containing reaction mixtures also contained 100 μM indomethacin. Incubation mixtures with only arachidonic acid or H2O2 and 2-NA served as nonenzymatic controls. Other reaction conditions and product analysis procedures were identical to those described above for microsomal reactions. Reaction conditions for HRP-dependent 2-NA metabolism were identical to those described for PHS, except that 1 μg/ml of HRP was used, and all reactions were initiated with H2O2. Product analysis procedures were identical to those described above. Structural identification of products from all three enzyme systems was carried out as described below.

High-Performance Liquid Chromatography of Products. The HPLC method described by Kadlubar et al. was used without modification (34). The Waters (Milford, MA) HPLC hardware consisted of a μBondapak C18 column, 3.9 mm x 30 cm, a Model U6K injector, two Model 6000A solvent pumps, and a Model 721 programmable system controller. Absorbance at 280 nm was monitored with a Perkin-Elmer LC-55 spectrophotometer (Coleman Instruments Division, Oak Park, IL). Radioactivity was monitored with a Flo-One radioactive flow detector programmed for quench correction (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL) interfaced with a MicroMate computer system (Personal Micro Computers, Inc., Mt. View, CA) for peak integration and plotting. All chromatograms were plotted as functions of both UV absorbance and radioactivity. For preparative scale HPLC (see below), a Waters μBondapak C18 column, 7.8 mm x 30 cm, was used with a flow rate of 3 ml/min. All other conditions were identical to the analytical method described above.

Chemical Oxidation of 2-NA. The potassium ferricyanide-catalyzed oxidation of 2-NA was carried out in a 50-ml solution consisting of 25 ml of water adjusted to pH 10.0 with NaOH, 25 ml of acetone, 5 mM potassium ferricyanide, and 5 mM unlabeled 2-NA. The reaction was allowed to proceed for 20 min at 23°C; the products were extracted and analyzed as described above for the enzymatic reactions.

Structural Identification of Products. All products were initially characterized by cochromatography on HPLC with authentic standards and comparison of UV-visible absorbance spectra. The absorbance spectra were measured with a Hewlett-Packard Model 8450A diode array spectrophotometer, and plotted on a Hewlett-Packard Model 7225B pen plotter. For absorbance and mass spectral analysis, unknown product eluants were collected from the HPLC, evaporated to dryness under reduced pressure at 37°C, and the residue resuspended in a small volume of absolute ethanol. Products were then purified by a second injection and collection from the HPLC. The preparative scale HPLC
system and 50-ml reaction mixtures were used for unknown product generation.

Mass spectra were obtained by direct probe, 70-eV electron impact ionization using a VG 7070E mass spectrometer equipped with a VG 2050 data system. Samples were scanned at 1000 resolving power from m/e 600 to 50 at a scan speed of 1.5 s/decade of mass, with perfluorokerosene as an internal standard. The probe temperature ranged from 180 to 350°C.

DNA Binding Reaction Conditions. Incubation mixtures for study of the PHS-dependent binding of 2-NA to DNA consisted of the following: 100 mM potassium phosphate buffer, pH 7.6, or 100 mM sodium acetate buffer, pH 5.0, 200 µg/ml of solubilized ram seminal vesical microsomal PHS, 50 µM [3H]2-NA, 100 µM arachidonic acid, or 100 µM H2O2, and 1 mg/ml of calf thymus DNA in a total volume of 10 ml. Reactions were initiated by the addition of arachidonic acid or H2O2 and allowed to proceed for 15 min at 37°C, then terminated by the addition of an equal volume of CIP, 24:1:25. Some reaction mixtures were preincubated with indomethacin (100 µM) for 2 min prior to initiation. Control reaction mixtures to correct for nonenzymatic binding contained heat-denatured PHS, obtained by heating at 55°C for 10 min. HRP-dependent reaction mixtures were identical, except that the enzyme concentration was 1 µg/ml. Reactions were initiated by the addition of 100 µM H2O2; control reaction mixtures contained no H2O2. All reactions were carried out in triplicate.

For studies monitoring the time course of DNA binding, reactions were terminated at the appropriate time point by the addition of CIP. For reactions monitoring the disappearance of reactive products, DNA was added at the appropriate time point so that final concentrations of reaction components were as described above. The reaction was then allowed to proceed for an additional 5 min before termination with CIP.

Reaction conditions for 2-A-1-N binding were similar to those for 2-NA, except that substrate concentration was 15 µM. Protein binding was studied by substituting bovine albumin for DNA at a concentration of 1 mg/ml. DNA reactions were terminated by the addition of CIP, while protein reactions were terminated by the addition of an equal volume of 10% trichloroacetic acid.

Quantification of Binding. After termination of reactions, the DNA was purified by an extraction technique adapted from Gupta (35). Following the addition of CIP to reaction tubes, the mixture was centrifuged at 10,000 x g for 10 min, and the aqueous layer was carefully removed. This procedure was repeated once more. The aqueous fraction was then subjected to three extractions with one volume of buffer-saturated ethyl acetate:diethyl ether, 1:1. Any remaining organic solvent was then removed by treating the aqueous layer with a stream of nitrogen for several minutes. The solution was then incubated with RNase A (100 µg/ml) and RNase T1 (50 U/ml) for 45 min at 37°C, followed with Pronase (500 µg/ml) for 30 min at 37°C. The aqueous solution was then subjected to one additional CIP extraction followed by an ethyl acetate:ether extraction. The solution was made 0.1 M in sodium chloride, and the DNA was precipitated by the addition of two volumes of ice-cold ethanol. The DNA was pelleted by centrifugation at 1,500 rpm for 10 min, the supernatant was removed, and the DNA redissolved in water at a concentration of approximately 1 mg/ml for analysis.

DNA was quantitated by measuring absorbance at 260 nm in a Hewlett-Packard model 8450A diode array spectrophotometer. The amount of hydrogen-3 binding was determined by counting an aliquot in a Packard model 4600 liquid scintillation spectrophotometer, programmed for quench correction. The cocktail was Atomlight Scintillation Fluid (New England Nuclear). The purity of the isolated DNA was determined by measuring A260/A280; values were consistently greater than 1.9, indicating negligible protein contamination. In early experiments, DNA was also purified by hydroxyapatite chromatography. The extraction technique described above was found to yield DNA of an equal or greater purity as the chromatography technique; the extraction procedure was therefore used throughout this study.

The determination of 2-A-1-N binding to protein was carried out as previously described in detail (36).

**RESULTS**

Glutathione/Oxygen Uptake Assay. As shown in Fig. 1, the peroxidase-dependent oxidation of 2-NA in the presence of glutathione results in the rapid and extensive utilization of molecular oxygen. In both enzymatic systems, the extent of oxygen uptake was greater at pH 7.6 than at pH 5.0. Little or no oxygen utilization under any condition was measurable when either 2-NA or glutathione was omitted from the reaction mixture.

Isolation and Identification of Metabolites. The metabolites of 2-NA were separated and isolated by preparative HPLC. Representative chromatograms from reactions catalyzed by PHS and HRP, under various conditions, are shown in Fig. 2. The product profile obtained from the PHS-dependent oxidation of 2-NA at pH 7.6 (initiated by either arachidonic acid or H2O2) indicated the presence of at least five metabolites above background levels, with three major products observed (Fig. 2A). The product profile from PHS/H2O2-dependent 2-NA oxidation at pH 5.0 was different from that observed at pH 7.6. An obvious metabolite shift occurred with different major metabolites observed. HRP/H2O2-dependent 2-NA oxidation at either pH 5.0 or 7.6 gave a very similar product profile to that observed with PHS at pH 5.0. Although significantly greater metabolism was observed under these conditions than in the PHS system, other experiments with decreased HRP concentration and/or increased O2 concentration did not alter the product profile. An HPLC chromatogram of this product profile is shown in Fig. 2B.

All products were isolated and initially characterized by cochromatography with synthetic standards. A standard for 2,2'-azobis(1,1'-dihydroxynaphthalene) (the hypothesized structure of unknown II) was not available, since an unambiguous synthetic procedure for this compound could not be found in the literature. The UV-visible absorbance spectra of the metabolites were then recorded. The spectra of DBP (37) and AQI (30) were identical to those published. Characteristic chromophores in the DBP spectrum were observed at 414, 405, 372, 297, 291, 262, and 242 nm. Characteristic chromophores in the AQI spectrum were observed at 458, 334, and 289 nm. The UV-visible spectrum of ADN contained chromophores at 342, 290, 280, 261, and 242 nm. The UV-visible spectrum of unknown I contained chromophores at 345, 287, 273, and 242 nm, while...
that of unknown II contained chromophores at 370, 349, 330, 300, 275, 238, 223, and 210 nm.

The mass spectra of the three major enzymatic products, AQI, DBF, and ADN contained molecular ions and major fragments consistent with the proposed structures. For AQI, the spectrum is identical to that of its standard synthesized as in reference 30. The molecular ion and base peak is observed at m/e 298. The peak at m/e 183 results from cleavage of the C'1—C'2 and C'2—C'3 bonds with charge retention of the larger fragment. The peak at m/e 115 results from the same reaction with charge retention on the smaller fragment. The peak at m/e 127 results from cleavage of the N—C'1 bond with charge retention on the naphthalene system. The peak at m/e 143 results from the loss of CO from the larger fragment of the previous reaction. For ADN, a molecular ion and base peak is observed at m/e 284. A fragment at m/e 267 results from loss of NH3. Loss of the naphthalene ring system from this fragment, with charge retention on the naphthalene function, results in a peak at m/e 127. The same reaction, with charge retention or the nitrogen-containing fragment, results in a peak at m/e 140. For DBP, the molecular ion and base peak corresponds to m/e 280. A peak at m/e 140 results from symmetrical cleavage of both N—C'1 bonds, with charge retention on either fragment. A peak at m/e 126 results from cleavage of the N—C'1 and N—C'2 bonds, with charge retention on the naphthalene ring system.

Due to the lack of resolvable fragments in the mass spectra of the two minor products (unknowns I and II), it is impossible to identify them with certainty. The spectrum of unknown I contains a molecular ion at m/e 314, which corresponds to the structure of 2,2'-azobis(1,1'-dihydroxy-naphthalene). Another possible 2-NA oxidation product, 2-amino-1,4-naphthoquinone-N'-(1-hydroxy-2-naphthyl)imine, would also have a molecular ion of m/e 314, but the published UV-visible spectrum of this compound (38) is distinctly different from that of unknown II.

**Quantitation of [3H]2-NA Metabolism.** The quantitation of 2-NA metabolism by PHS and HRP was carried out using [3H]-2-NA, and results are presented in Table 1. Under the conditions employed in this study, total metabolism by PHS at pH 7.6 was approximately 34% when initiated with arachidonic acid. Substitution of arachidonic acid with equimolar H2O2 resulted in only half as much metabolism. At pH 5.0, H2O2 supported approximately 36% total metabolism. At pH 7.6, the same three products were isolated from the PHS system, whether the reaction was initiated with arachidonic acid or H2O2. The major organic-extractable product at pH 7.6 was AQI. Minor products were DBP and unknown product II. A significant amount of organic-extractable polymeric/oligomeric products was also formed. This material migrated as dark, colored bands near the origin of thin layer chromatography plates, and could not be volatilized from the mass spectrometer probe. These products are quantitated collectively as "organic-extractable polymer." The majority of 2-NA products in all cases was nonorganic extractable. No attempt was made in this study to differentiate between water soluble products and metabolites covalently bound to microsomal macromolecules. At pH 5.0, the product profile was significantly different. The major organic-extractable metabolites were two nitrogen to carbon dimers, ADN and DBP. Products formed to a lesser extent included unknown products I and II. Only trace amounts of AQI, the major product at pH 7.6, were formed at pH 5.0. Again, the majority of 2-NA products were nonorganic extractable. 2-Nitrosonaphthalene, the standard of which is clearly separable from the other metabolites, including unknowns (Fig. 2.4), was not detected as a product, in any amount under any condition. Likewise, no 2-nitronaphthalene was detected under any condition. When enzyme concentration was limited such that total metabolism was approximately 10%, the product profiles remained qualitatively similar to those described above.

**Table 1 Quantitation of enzymatic [3H]2-NA metabolism**

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>Total percentage of metabolism</th>
<th>Total nmol of metabolites formed</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ADN</td>
<td>AQI</td>
</tr>
<tr>
<td>PHS, pH 7.6, AA</td>
<td>34.1</td>
<td>14.8</td>
</tr>
<tr>
<td>PHS, pH 7.6, H2O2</td>
<td>16.9</td>
<td>8.0</td>
</tr>
<tr>
<td>PHS, pH 5.0, H2O2</td>
<td>36.4</td>
<td>16.9</td>
</tr>
<tr>
<td>PHS, pH 7.6, H2O2</td>
<td>78.3</td>
<td>39.9</td>
</tr>
<tr>
<td>HRP, pH 5.0, H2O2</td>
<td>98.7</td>
<td>22.2</td>
</tr>
<tr>
<td>Purified PHS, pH</td>
<td>18.5</td>
<td>10.6</td>
</tr>
<tr>
<td>7.6, AA</td>
<td>15.9</td>
<td>6.8</td>
</tr>
<tr>
<td>Purified PHS, pH</td>
<td>15.9</td>
<td>6.8</td>
</tr>
<tr>
<td>7.6, 15-HPETE</td>
<td>8.6</td>
<td>2.7</td>
</tr>
<tr>
<td>Purified PHS, pH</td>
<td>14.6</td>
<td>8.1</td>
</tr>
</tbody>
</table>

* Reaction conditions are described in detail in "Materials and Methods." Notation indicates the enzyme system used, the pH at which the reaction was studied, and the substrate used to initiate the peroxidase activity.
* Reaction conditions are described as detailed in "Materials and Methods."
Identical product profiles were also obtained when a solubilized PHS preparation (20) was used for metabolism studies (data not shown).

Total HRP-dependent 2-NA metabolism results are also shown in Table 1, and as with prostaglandin H synthase, H2O2 supported greater metabolism at pH 5.0 than pH 7.6. The product profiles of HRP-dependent 2-NA oxidation were very similar at both pH values studied. The major organic-extractable products were DBP and ADN. Lesser amounts of unknown products I and II were also isolated. Only trace amounts of AQI were detected as product in the HRP system at either pH value. Lowering the HRP concentration to give approximately 10% metabolism, or conducting experiments under a 100% oxygen atmosphere did not significantly alter the product profile.

The metabolism of 2-NA by purified PHS was also studied, and a quantitative summary of metabolism is presented in Table 1. The organic-extractable product profiles were qualitatively identical to those obtained with the microsomal enzyme system. Under the conditions of this study, total metabolism in the purified system was approximately half that of the microsomal system. Arachidonic acid supported the greatest amount of 2-NA metabolism, with 15-HPETE and H2O2 supporting approximately 86 and 46%, respectively, of the total metabolism obtained with arachidonic acid (at pH 7.6). Addition of indomethacin, a cyclooxygenase inhibitor, to arachidonic acid supported reactions resulted in a 70% inhibition of total metabolism. Indomethacin did not inhibit metabolism initiated with 15-HPETE or H2O2.

Concerning the relative rates of 2-NA oxidation, the results closely parallel those obtained with 2-aminofluorene (19, 20). Briefly, rates are slightly greater with H2O2 than with arachidonic acid, greater with HRP than with PHS, and greater at pH 5.0 than at pH 7.0. These rate data are reflected in Figs. 1, 3, and 4.

Chemical Oxidation of 2-NA. The oxidation of 2-NA with potassium ferricyanide resulted in the formation of organic-extractable products identical to those formed in the enzymatic systems. A qualitative analysis indicated that the major products were DBP and ADN, but detectable amounts of AQI and unknown II were also formed. A very small amount of unknown I was formed. Total product formation was approximately 10% of the starting material (data not shown).

DNA Binding Time Course. The time course of PHS-catalyzed binding of 2-NA to DNA is shown in Fig. 3. The time course was measured at pH 7.6, using arachidonic acid to initiate the reaction. Binding increases rapidly at time points up to 1 min, and then appears to level off from 2 to 5 min. The time course of 2-A-1-N binding to DNA, in the presence of enzyme, is also shown in Fig. 3. The reaction conditions were identical to those for 2-NA binding, except that 2-A-1-N concentration was 15 μM. This concentration was in excess of the maximal possible concentration of 2-A-1-N formed by 2-NA oxidation, based on the quantitative metabolism studies. As shown, 2-A-1-N binding did not increase at time points up to 15 min, and binding levels were near the lower limit of detection (5–8 pmol/mg). At a time point of 2 h (data not shown) binding had increased slightly, to approximately 10 pmol/mg.

The effect of adding DNA to PHS/2-NA reaction mixtures at various times of incubation is shown in Fig. 4. Maximal binding is obtained only when DNA is present from the beginning of the reaction. Binding levels rapidly decrease such that only half maximal binding is seen by 45 s. After 5 min, the addition of DNA to reaction mixtures results in binding levels near the lower limit of detection, or approximately 10% of that seen when DNA is present at time 0.

Time Course of 2-A-1-N Binding to Protein. The time course of 2-A-1-N binding to protein was also studied, under conditions identical to those used for the DNA binding experiment (in which the appropriate macromolecule is present from the beginning of the reaction). As shown in Fig. 5, binding to protein is rapid, reaching maximal levels after 5–10 min. Binding levels are also three orders of magnitude greater than observed with DNA.

Binding of 2-NA to DNA under Various Conditions. The data presented in Table 2 show the quantitative nature of the bulk binding of 2-NA to DNA under several different conditions. As

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**Fig. 3.** Time course of PHS-catalyzed binding of 2-NA (•) and 2-A-1-N (○) to DNA. Reaction conditions are described in "Materials and Methods." Points, mean of three determinations; arrow, limit of detection.

**Fig. 4.** Time course of decay of DNA-binding species generated during PHS-catalyzed 2-NA oxidation. Reaction protocol is described in "Materials and Methods." Points, mean of three determinations.

**Fig. 5.** Time course of 2-A-1-N binding to protein in PHS system. Reaction protocol is described in "Materials and Methods." Points, mean of three determinations.

**Table 2 Binding of [3H]2-NA to DNA under various enzymatic conditions**

<table>
<thead>
<tr>
<th>Incubation conditions*</th>
<th>pmol [3H]2-NA/mg DNA</th>
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<tbody>
<tr>
<td>PHS, pH 7.6, arachidonic acid</td>
<td>53.7 ± 2.5</td>
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<tr>
<td>PHS, pH 7.6, arachidonic acid, indomethacin</td>
<td>5.5 ± 2.4</td>
</tr>
<tr>
<td>PHS, pH 7.6, H2O2</td>
<td>49.8 ± 12.5</td>
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<tr>
<td>PHS, pH 7.6, H2O2, indomethacin</td>
<td>71.6 ± 2.9</td>
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<tr>
<td>PHS, pH 5.0, H2O2</td>
<td>100.8 ± 6.4</td>
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<tr>
<td>HRP, pH 7.6, H2O2</td>
<td>681.9 ± 123.0</td>
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<tr>
<td>HRP, pH 5.0, H2O2</td>
<td>3337.0 ± 225.1</td>
</tr>
</tbody>
</table>

*Incubations were carried out as described in "Materials and Methods" and results are expressed as mean ± SD, n = 3.
previously reported (17, 24), arachidonic acid supports the
PHS-catalyzed binding of 2-NA to DNA; the PHS cyclooxygenase inhibitor indomethacin inhibits this binding by 90%.
Here, we provide additional data demonstrating that H₂O₂ also
initiates a similar level of binding, that is not inhibited by
indomethacin.

The data shown in the lower half of Table 2 also demonstrate
that the PHS/H₂O₂ system catalyzes 2-NA binding to DNA at
pH 5.0, to twice as great an extent as at pH 7.6. In addition,
the horseradish peroxidase/H₂O₂ system supports a relatively
large amount of binding, again greater at pH 5.0 than at pH
7.6. As shown by the metabolism studies above, these are
conditions under which little or no 2-A-1-N is formed. The
increased DNA binding catalyzed by both enzymes systems at
pH 5.0, when compared to that at pH 7.6, is consistent with
the greater 2-NA metabolism observed at the lower pH.

**DISCUSSION**

The metabolism of 2-NA by PHS appears to be mediated by
the peroxidase component of PHS. Substitution of the hydro-
peroxides 15-HPETE and H₂O₂ for arachidonic acid supports
the oxidation of 2-NA by purified PHS. The oxidation of 2-
NA by the peroxidase activity of PHS occurs through a free
radical mechanism, as observed for other aromatic amines. As
with other primary amines, the extreme instability of a
radical species prevents detection of the initial 2-NA free radical
intermediate by electron spin resonance spectroscopy.

However, indirect evidence for the formation of a radical
intermediate in the enzymatic systems was obtained with the
glutathione-dependent oxygen uptake assay described by Mol-
deus et al. The free radical intermediates generated by peroxi-
dase-catalyzed oxidation of xenobiotics are reduced by gluta-
thonine in this assay to produce a thiol radical, which then reacts
with molecular oxygen (32, 39, 40). The reaction of thiol radicals with molecular oxygen is well documented (41, 42).
These observations are consistent with the reaction scheme presented:

\[
\text{Ar—NH}_2 \quad \text{e}^- \quad \text{Ar—NH} \quad \text{(A)}
\]

\[
\text{Ar—NH} + \text{GSH} \rightarrow \text{Ar—NH}_2 + \text{GS}^- \quad \text{(B)}
\]

\[
\text{GS}^- + \text{O}_2 \rightarrow \text{GSOO}^- \quad \text{(C)}
\]

Using this assay, rapid and extensive oxygen uptake was ob-
served during 2-NA oxidation by either PHS or HRP, at either
pH 5.0 or 7.6, in the presence of glutathione suggesting the
formation of a 2-NA free radical.

Additional evidence for the formation of free radical inter-
mediates in the peroxidase-dependent oxidation of 2-NA was
obtained by an analysis of the stable organic-extractable prod-
ucts. The formation of nitrogen to carbon dimers, DBP and
ADN, is indicative of radical coupling, probably involving
nitrogen-centered and carbon-centered species. HRP-catalyzed
reactions occur through a one-electron oxidation of donor
molecules (43); arylamine oxidation in particular has been
documented in great detail (44). The isolation of the same prod-
ucts from the HRP, PHS, and one-electron chemical oxidation
systems provides additional evidence for the one-electron oxida-
tion of 2-NA by PHS. Furthermore, a recent report provides
evidence that the peroxidase reaction of PHS involves a cycle
of native enzyme, compound I, and compound II, typical of
heme-containing peroxidases such as HRP (45). While it is
doUBtful that coupling products such as these would be formed
in vivo, we believe that their isolation in vitro offers valuable
insight into the mechanism of 2-NA oxidation, as well as to the
nature of the initial oxidation product, e.g., a free radical.

The formation of ring-oxygenated 2-NA products by PHS
predominated at pH 7.6, and is also consistent with a one-
electron oxidation pathway (Fig. 6). Due to the highly reactive
nature of the one-position of the naphthalene ring system, a 2-
NA free radical would be expected to result in a resonance
hybrid consisting of a nitrogen centered and carbon-1-centered
species (Fig. 6). This mechanism was implied in a previous
study showing DBP as the major product of HRP-catalyzed 2-
NA oxidation (37). We propose that the carbon centered form
of the initial 2-NA free radical reacts with molecular oxygen to
form a peroxy radical. Insufficient metabolite formation, how-
ever, prevents detection of O₂ uptake by 2-NA. A series of
reduction steps would then ultimately result in the formation
of 2-amino-1-naphthol. Reactions of this type have been de-
scribed for polycyclic aromatic hydrocarbons (46, 47). Air oxida-
tion or further enzymatic oxidation of 2-amino-1-naphthol
to 2-imino-1-naphthoquinone, followed by spontaneous cou-
pling with unchanged 2-NA, would yield the product we have
isolated, AQI. This series of reactions is also well documented
(38, 48, 49). The tentative structures that we have assigned to
unknown products I and II are also consistent with this mech-
anism, and are shown in brackets in Fig. 6. We could not detect
the formation of 2-nitrosonaphthalene in any amount by either
the microsomal, solubilized, or purified forms of PHS, or HRP,
at either pH. This is in agreement with our previous studies on
2-aminofluorene metabolism (19, 20), but in contrast with
another communication (17), where products were tentatively
identified by chromatographic properties only.

Finally, the product profiles obtained using the purified PHS
were identical to those obtained from the microsomal system;
lowering the total amount of 2-NA turnover (to approximately
5-15%, as compared to 15–35% in the microsomal system) did
not alter the qualitative product profile.

The generation of two electrophilic metabolites by the PHS-
dependent oxidation of 2-NA has potentially important biolog-
ical consequences. A free radical species is a potentially reactive
metabolite. We have previously provided evidence that 2-ami-
nofluorene is oxidized by PHS to a free radical species (20)
which is capable of causing mutagenesis in the Ames Salmo-
rella system (22) and binding irreversibly to DNA in vitro (21).
Extensive studies of the metabolism of p-phenetidine by per-
oxidase also suggest that the product responsible for the ob-
served DNA binding is a radical species (50, 51). In addition,
several reports provide evidence that a variety of free radical
species are capable of reacting with DNA (52–54).

The second potentially reactive metabolite, which appears to
be unique to 2-NA oxidation by PHS, is 2-A-1-N. Oxidation of

![Fig. 6. Proposed pathway for the oxidation of 2-NA by PHS and HRP. A detailed description of this scheme is presented in the "Discussion." Structures in brackets, only tentative.](10.2307/20771277)

4012
this very unstable compound occurs readily, and results in the formation of 2-imino-1-naphthoquinone. According to one report, an oxidation product of 2-A-l-N (presumably 2-imino-1-naphthoquinone) binds to DNA (55), but a subsequent report contradicts this data, concluding instead that the oxidation product of the orfAo-aminophenol reacts with protein, but not nucleic acids (56).

The binding of 2-NA to DNA is dependent upon the peroxidase activity of PHS. The relative levels of 2-NA/DNA binding under various conditions correlate well with the amount of 2-NA metabolized. 2-A-1-N formation is negligible in the PHS system at pH 5.0, and in the horseradish peroxidase system at either pH 5.0 or 7.6. Yet, the relatively high levels of binding under these conditions clearly suggest that a reactive metabolite other than 2-A-1-N (or its oxidation product) is formed which is capable of binding to DNA.

Maximal levels of DNA binding resulting from 2-NA metabolism are reached very rapidly (1–2 min) during the course of an incubation and closely parallel the time course of 2-NA metabolism under these conditions. However, 2-A-1-N binding levels are only about 10–20% of those obtained from the reaction of the parent 2-NA and show little or no increase at time points of 2 and 4 h (~10 pmol/mg DNA). These data support the hypothesis that a reactive species other than a metabolite of 2-A-1-N is responsible for a significant amount of the binding to DNA observed during 2-NA metabolism under these conditions. This other reactive product(s) appears to be very unstable and short lived, since binding rapidly decreased when DNA was added after initiation of 2-NA oxidation, such that after 5 min, only about 20% of the maximal binding level was observed. The apparent discrepancy between the data presented here and by Yamazoe et al. (24), in regard to the amount of 2-A-1-N which binds to DNA, may relate to different experimental conditions. Our data were derived from reaction mixtures containing lower amounts of 2-A-1-N and proceeding for shorter time periods, in order to more closely approximate the actual conditions that exist during 2-NA metabolism by PHS, as explained in “Results.”

The reactivity of 2-A-1-N is significantly greater with protein than with DNA. A reactive oxidation product of 2-A-1-N, probably the iminoquinone, therefore appears to have a much greater affinity for protein than for DNA, based on the more rapid and dramatically larger extent of binding. This is consistent with the previously observed reactivity of 2-imino-1-naphthoquinone with protein, but not with nucleic acids (56). At the very least, there clearly appears to be a PHS/2-NA oxidation product in addition to 2-A-1-N which is capable of covalent binding to DNA.

In the recent publication by Yamazoe et al. (24), 2-NA/DNA adducts formed by PHS were isolated and characterized. Three unique 2-NA/DNA adducts were identified as corresponding to those formed by a reaction of 2-A-1-N with DNA. Additional, less well-characterized adducts were attributed to the reaction of N-hydroxy-2-NA with DNA; there was also a significant amount of uncharacterized adduct material. Our data presented here do not contradict their conclusion regarding the binding of 2-A-1-N with DNA, but suggest that additional adducts are formed from free radical intermediates rather than N-hydroxy-2-NA. Their supposition that N-hydroxy-2-NA is formed in the PHS reaction appears to be based solely on the cochromatography of a 2-NA metabolite with a 2-nitrosomonaphtalene standard (17). Rigorous metabolism studies carried out in our laboratory indicate that 2-nitrosomonaphtalene is not formed under any condition by PHS oxidation. The absence of 2-nitrosomonaphtalene is consistent with our proposed mechanism, our previous studies of 2-aminofluorene metabolism, and known HRP (43) and PHS (49) chemistry.

In summary, we propose a one-electron mechanism for 2-NA oxidation, resulting in free radical metabolites in addition to the C-oxygenated metabolites, that is consistent with our metabolism and DNA binding data, as well as the DNA adduct data presented elsewhere (24). The C-oxygenated 2-NA product (2-A-1-N) clearly reacts with DNA to form adducts, while it is likely that the DNA adducts attributed to N-hydroxy-2-NA (24), as well as other uncharacterized adducts, may instead result from a free radical product. We feel that the available data suggest that DNA adducts produced from radical metabolites should be considered in future studies, in vitro and in vivo, and that additional studies are required to identify all of the 2-NA/DNA adducts formed from PHS-catalyzed oxidation.

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REFERENCES

Prostaglandin H Synthase-catalyzed Metabolism and DNA Binding of 2-Naphthylamine

Jeff A. Boyd and Thomas E. Eling