Cytochrome P-450- and Flavin-containing Monoxygenase-catalyzed Formation of the Carcinogen N-Hydroxy-2-amino fluorene and Its Covalent Binding to Nuclear DNA

Clay B. Frederick, Joann B. Mays, Daniel M. Ziegler, F. Peter Guengerich, and Fred F. Kadlubar

National Center for Toxicological Research, Food and Drug Administration, Environmental Protection Agency, Jefferson, Arkansas 72079 [C. B. F., J. B. M., F. F. K.]; Clayton Foundation Biochemical Institute, University of Texas at Austin, Austin, Texas 78741 [D. M. Z.]; and Department of Biochemistry and Center in Environmental Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232 [F. P. G.]

ABSTRACT

The metabolic N-oxidation of the carcinogen 2-amino fluorene was examined in vitro using fortified hepatic microsomes from a variety of species. Rat, dog, human, and pig liver microsomes catalyzed the formation of N-hydroxy-2-amino fluorene (N-OH-AF) from AF at rates of 1.6, 1.0, 1.2, and 3.5 nmol/min/mg protein, respectively. The involvement of both cytochrome P-450 and the flavin-containing monooxygenase was demonstrated with hepatic microsomes and with purified enzymes by using specific enzyme inhibitors. 2-[(2,4-Dichloro-6-phenyl)phenoxy]ethylamine, a potent cytochrome P-450 inhibitor, decreased microsomal N-OH-AF formation by 96, 83, 70, and 46% in the rat, dog, human, and pig, respectively; and further addition of methimazole, a high-affinity flavin-containing monooxygenase substrate, abolished the residual N-hydroxylation activity. Using the purified porcine flavin-containing monooxygenase, metabolic formation of N-OH-AF occurred at a rate of 4.9 nmol/min/nmol flavin adenine dinucleotide and was insensitive to 2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine. In addition, purified rat liver cytochrome P-450 (isolated from 5,6-naphthoflavone-induced animals) N-hydrox ylated AF (1.1 nmol/min/nmol P-450) and was completely inhibited by 2-[(2,4-dichloro-6-phenyl)-phenoxy]ethylamine, but the reaction was insensitive to methimazole.

To determine whether or not the metabolic formation of N-OH-AF could lead directly to covalently bound adduct(s) with DNA under these incubation conditions (30 min, pH 7.5), the binding of synthetic and metabolically formed [3H]-N-OH-AF to added calf thymus DNA and to DNA in isolated rat liver nuclei was investigated. In all cases, the amount of DNA-bound carcinogen accounted for 0.8 to 0.15% of the N-OH-AF present in the incubation mixtures. These data, when compared to the levels of AF bound to hepatic nuclear DNA reported in vivo, suggest that the nonenzymatic reaction of N-OH-AF with nuclear DNA may be sufficient to account for a substantial portion of the observed in vivo binding of this carcinogen.

INTRODUCTION

Primary aromatic amines are a class of carcinogenic compounds to which humans may be exposed from industrial, environmental, and dietary sources (9, 22, 35, 54, 63). Numerous studies have suggested that N-hydroxylation of these compounds is a necessary step in their activation to electrophiles that covalently bind to DNA, induce mutations, and initiate carcinogenesis (9, 22, 35, 36, 42, 43, 46, 54, 63, 71). The enzymatic mechanism for the N-oxidation of primary aromatic amines has been examined in rodents and pigs, and the results have implicated both cytochrome P-450 and the FMO in this reaction (22, 23, 41, 46, 57, 66, 75, 76). Although this activation of certain N-hydroxyarylamines by cytosolic sulfotransferases (24) and seryltransferase (64, 74) has been demonstrated, several of these N-hydroxy metabolites have been shown to be electrophilic per se, especially under mildly acidic conditions (25-27, 33), and acid-catalyzed DNA binding has been proposed to be an essential step in arylamine-induced urinary bladder carcinogenesis (25, 54). The correlation between the covalent binding of carcinogens to nuclear DNA and their carcinogenicity in a variety of tissues has been discussed in several reviews (36, 38, 39, 47).

In this study, we investigated the relative contributions of cytochrome P-450 and FMO in the enzymatic N-oxidation of AF by fortified hepatic microsomes from a variety of species. Since N-hydroxyarylamines are subject to rapid decomposition (23, 37), it has often been necessary to measure total N-oxidation products when studying the in vivo and in vitro metabolism of aromatic amines (55, 65). However, we have recently developed an analytical procedure which permits estimation of N-hydroxyarylamine metabolites without derivatization (12). Using this method, hepatic microsomal N-OH-AF formation was estimated directly and has been shown to be sensitive to inhibitors of cytochrome P-450 and FMO. Purified porcine FMO and rat P-450 also catalyzed the N-oxidation of AF.

Several studies on the mutagenicity of the carcinogens AAF and N-OH-AAF in the Salmonella typhimurium test system (6, 7, 28, 60, 61) have indicated that N-OH-AF is the ultimate mutagen derived from these compounds. No further activation of N-OH-AF within the bacterium has been demonstrated, and the C-8 deoxyguanosine adduct produced in vitro (4, 31, 72) was also found in the bacterial DNA (6). The formation of this adduct was further demonstrated to have a high correlation with the observed mutagenicity (6). Based on the reactivity of
N-OH-AF in vitro (33) and the conclusion that N-OH-AF is an ultimate mutagen for bacteria, we have also reinvestigated the reaction of N-OH-AF with DNA under a variety of conditions. The covalent binding of synthetic [3H]-N-OH-AF to added calf thymus DNA at pH 7.5 was measured and compared to the level of DNA binding observed in isolated rat liver nuclei. Furthermore, to simulate the metabolic production and nuclear DNA binding of N-OH-AF, either microsomes or purified FMO were incubated with [3H]-AF, an NADPH-NADH generating system, and isolated nuclei. The nuclear DNA was then reisolated, and the efficiency of [3H]-AF binding was compared to each of the other in vitro studies. The results obtained from these reconstituted systems were compared to the levels of hepatic DNA binding observed in vivo with [3H]-AF (34, 62).

MATERIALS AND METHODS

Chemicals. [ring-3H]-N-OH-AF (80 mCi/mmol) and [ring-3H]AF (45 mCi/mmol) were obtained from Dr. R. Roth, Midwest Research Institute, Kansas City, Mo. DPEA was a gift of Eli Lilly and Co., Indianapolis, Ind., and SKF-525A was a gift of Smith, Kline, and French Co., Philadelphia, Pa. Glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides, calf thymus DNA (type I), and DNase (type I) were obtained from Sigma Chemical Co., St. Louis, Mo. Glucose-6-phosphate, NADP+, and NAD+ were obtained from P-L Biochemicals, Milwaukee, Wis. All other reagents were of analytical grade.

Animals and Tissues. Male Sprague-Dawley rats from our breeding colony weighing 300 to 500 g were used to prepare microsomes and nuclei. In some experiments, livers were obtained from rats pretreated with 3-methylcholanthrene in trioctanoin (50 mg/kg i.p. 72 and 48 hr before sacrifice and 25 mg/kg 24 hr before sacrifice). Mature male beagles were obtained from Marshall Research Animals, Inc. (North Rose, N. Y.), and were given free access to Purina dog chow and water for a minimum of 2 weeks prior to sacrifice. Normal human liver tissue was provided from biopsy samples which had been immediately frozen in liquid nitrogen and stored at —78° prior to microsomal preparation and analysis.

Rat and dog liver microsomes were prepared by the technique of Remmer et al. (58) and stored in liquid nitrogen. No difference in metabolic activity could be detected between fresh microsomes and those stored by this method. Female pig liver microsomes and purified porcine FMO (>900 units per mg protein; FAD (8 to 13 nmol per mg protein); 4,4'-dimethylaniline 4-oxidase). Although a specific inhibitor of another cytochrome P-450 activity (53). Accordingly, addition of 1.0 mM methimazole to the incubation mixtures, a range of inhibition was observed. Further addition of another cytochrome P-450 inhibitor, SKF-525A, did not augment this inhibition. The amount of N-hydroxylation that could not be inhibited appeared to correlate with the previously reported species differences (75) in hepatic FMO activity (N,N-dimethylamino-N-oxidase). Although a specific inhibitor of FMO has not been reported, methimazole has a very low Km for the enzyme (52) and can act as a competitive inhibitor of arylamine oxidation without significantly affecting cytochrome P-450 activity (53). Accordingly, addition of 1.0 mM methimazole and 0.5 mM DPEA completely inhibited N-hydroxylation of AF with the pig and human tissues.

Metabolic Formation of N-OH-AF by Cytochrome P-450

function oxidases. Porcine hepatic FMO (37 to 100 µg) was incubated with AF as described above for microsomes. Purified cytochrome P-450pur (2 µM) was reconstituted by admixture with 2 µM purified rat hepatic NADPH:cytochrome P-450 reductase (17) and dilauroyl lecithin (30 µg/ml) in 0.05 M Tris-HCl buffer (pH 7.5). AF and inhibitors were added as indicated above, and the reaction was initiated by the addition of NADPH (0.5 mM). Following incubation, the reaction mixtures were each extracted by adding 1 ml of water-saturated ethyl acetate and swirling vigorously under an argon stream. The resulting emulsion was then separated by centrifugation at 1500 rpm for 5 min in a clinical centrifuge, and a 100-µl aliquot of the organic layer was removed for high-pressure liquid chromatography analysis. A linear 20-min 0 to 100% water:methanol gradient with a flow rate of 2 ml/min was used with a Whatman Partisil 10 ODS-3 column, and N-OH-AF was specifically detected with a colorimetric post-column reactor as previously described (12).

Determination of Covalent Binding of N-OH-AF to Nuclear DNA. Nuclear DNA from the incubations described above was isolated by the method of Huberman and Sachs (18). The final DNA precipitate was dissolved in 5 mM bis(2-hydroxyethyl)aminomethane(hydroxy methyl)ethanolate-HCl, pH 7.1, containing 5 mM MgCl2. Aliquots were removed for DNA determinations (2) or were digested with DNase (0.1 mg/ml DNA) at 37° for 2 hr, mixed with scintillation fluid, and counted for radioactivity.

Calf thymus DNA that had been reacted with synthetic or metabolically formed N-OH-AF was extracted 3 times with ethyl acetate and 3 times with chloroform:isoamyl alcohol:phenol (48:2:50, v/v, v), precipitated with ethanol, triturated with ethanol (3 times), resuspended in water, precipitated with ethanol, triturated with ethanol and acetone, dried under an argon stream, and resuspended in 5 mM bis(2-hydroxyethyl)aminomethane(hydroxy methyl)ethanolate-HCl buffer (pH 7.1):5 mM MgCl2, and the specific radioactivity and DNA concentration were determined as noted above. To confirm that covalent binding was being measured, nuclear DNA was purified as described above and then enzymatically hydrolyzed to deoxyribonucleoside adducts by the technique of Beland et al. (4, 5).

Mutagenicity Assays. The procedure of Ames et al. (1), as modified by Pelroy and Gandolfi (50) was used to measure mutation induction in S. typhimurium strain TA98, except that 18 units of purified FMO were used per plate.

RESULTS

Metabolic Formation of N-OH-AF by Cytochrome P-450pur and FMO. Using a specific detection technique for N-hydroxyarylamines, the metabolism of AF to N-OH-AF by purified hepatic microsomes was determined for a variety of species (Table 1). When the specific and potent cytochrome P-450 inhibitor DPEA (23, 40, 76) was added to the incubation mixtures, a range of inhibition was observed. Further addition of another cytochrome P-450 inhibitor, SKF-525A, did not augment this inhibition. The amount of N-hydroxylation that could not be inhibited appeared to correlate with the previously reported species differences (75) in hepatic FMO activity (N,N-dimethylamino-N-oxidase). Although a specific inhibitor of FMO has not been reported, methimazole has a very low Km for the enzyme (52) and can act as a competitive inhibitor of arylamine oxidation without significantly affecting cytochrome P-450 activity (53). Accordingly, addition of 1.0 mM methimazole and 0.5 mM DPEA completely inhibited N-hydroxylation of AF with the pig and human tissues.

Razzouk et al. (57) and Aune et al. (3) have previously suggested a role for cytochrome P-450 in AF N-oxygenation. To confirm these data and to validate our inhibitor studies, we
examine the N-hydroxylation of AF by purified cytochrome P-450BNF in a reconstituted system and compared the inhibitory effects of DPEA and methimazole. Using levels of P-450BNF comparable to those used in the microsomal studies, N-OH-AF was formed at a rate of 1.1 ± 0.2 (S.D.) nmol/min/nmol P-450. The accumulation of this product was linear for at least 10 min and was first order with regard to the concentration of enzyme used (2 to 4 μM). Addition of DPEA abolished N-oxidation completely, but addition of methimazole had no effect on product formation. In contrast, cytochrome P-450BNF was not observed to N-hydroxylate AF under the reaction conditions.

To establish the role of FMO in the N-oxidation of AF, the purified porcine liver enzyme was incubated with AF and the cofactor-generating system used in the microsomal studies. The accumulation of N-OH-AF was found to be linear for 30 min and to be first order with respect to enzyme concentration over the range used (0.5 to 1.5 μM). A representative chromatogram (Chart 1) indicated that N-OH-AF was the primary oxidative metabolite of the enzyme with significant amounts of presumed secondary oxidation products, 2-nitrosofluorene, 2-nitrofluorene, and 2,2'-bisazoxyfluorene, also being formed. Ring hydroxylation of AF was not detected with the purified enzyme. Although Rauckman et al. (56) have shown that FMO acts as a superoxide radical-generating system, the N-hydroxylation of AF was not significantly inhibited by superoxide dismutase (25 μg/ml), catalase (125 μg/ml), or 1.0 mM benzoate, indicating that neither superoxide, nor hydrogen peroxide, nor hydroxyl radicals are involved in this reaction. This N-hydroxylation was also insensitive to DPEA but was completely abolished with methimazole.

Using purified FMO, the mutagenic activation of AF was confirmed in the S. typhimurium mutagenicity assay as described previously by Pelroy and Gandolfi (50). In this system, 10, 25, and 50 μg AF per plate yielded 190, 700, and 1800 revertants per plate.

Cova lent Binding of Synthetic and Metabolically Formed N-OH-AF to DNA in Intact Nuclei. To determine whether or not the metabolic formation of N-OH-AF at neutral pH can result in appreciable DNA binding, we examined the level of reaction of [3H]-N-OH-AF with exogenous calf thymus DNA (Table 2) under these incubation conditions (pH 7.5, 30 min). The results show a small but significant level of binding of N-OH-AF (0.08 to 0.15%) to the DNA which increased with higher N-OH-AF concentration. The binding was not affected by the addition of Mg2+ or an NADPH-NADH-generating system (data not shown) but was somewhat decreased under aerobic conditions due to the decomposition of the N-OH-AF. However, the binding reaction was stimulated 36- to 65-fold when the incubation was conducted at pH 5.0.

Similar incubations were conducted with isolated rat liver nuclei instead of calf thymus DNA using a level of nuclear DNA and added N-OH-AF comparable to that of the previous experiment. As calculated from the data in Table 3, the amount of arylamine bound to the intact nuclear DNA in 30 min was found to be 0.09 ± 0.02% (n = 3) of the N-OH-AF in the medium.

Table 1

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Ratea</th>
<th>Range of inhibition by DPEA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver microsomes</td>
<td>1.6 ± 0.9b (8)c</td>
<td>93-100</td>
</tr>
<tr>
<td>Dog liver microsomes</td>
<td>1.0 ± 0.2 (4)</td>
<td>75-90</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>1.2 ± 0.6 (3)</td>
<td>69-71</td>
</tr>
<tr>
<td>Pig liver microsomes</td>
<td>3.5 ± 1.9 (4)</td>
<td>45-48</td>
</tr>
<tr>
<td>Purified pig liver FMO</td>
<td>4.9 ± 0.5 (3)</td>
<td>0</td>
</tr>
<tr>
<td>Purified rat liver P-450BNF</td>
<td>1.1 ± 0.2 (3)</td>
<td>100</td>
</tr>
<tr>
<td>Purified rat liver P-450BF</td>
<td>&lt;0.1 (3)</td>
<td>-</td>
</tr>
</tbody>
</table>

a Rates are expressed as nmol/min/mg protein for microsomes and nmol/min/nmol enzyme for purified FMO and P-450.
b Mean ± S.D.
c Numbers in parentheses, number of individual determinations.

Additional to 1 mM methimazole to DPEA (0.5 mM)-containing incubation mixtures resulted in >99% inhibition of N-OH-AF formation.

Some stimulation of enzyme activity (30 to 40%) was observed in the presence of DPEA.

**Enzymatic Formation of N-OH-AF**

**Chart 1.** A, elution profile from an ethyl acetate extract of a FMO incubation mixture containing AF (see “Materials and Methods”). Detection is by UV absorbance. B, the same elution profile after the column effluent has been mixed with a Fe3+-bathophenanthroline reagent to give an intense visible absorption characteristic of N-hydroxyarylamines. AF gives a very slight positive response with the reagent.
This value is quite similar to that observed above after reaction of \([^{3}H]\)-N-OH-AF with isolated calf thymus DNA. To confirm that the binding measured in these reactions represented the same covalent AF:DNA adduct observed from the acid-catalyzed reaction of N-OH-AF with DNA (4), the modified nuclear DNA was enzymatically hydrolyzed and chromatographed (Chart 2) with synthetic N-(deoxyguanosin-8-yl)-2-aminofluorene (4). Most of the radioactivity (73%) cochromatographed with the synthetic standard, with the majority of the remaining radioactivity (22%), presumed to be incompletely hydrolyzed AF:DNA, eluting at the column void volume.

To simulate the in vivo metabolic production of N-OH-AF, isolated rat liver nuclei and \([^{3}H]\)AF were coincubated with rat liver microsomes or purified FMO under conditions in which the total production of \([^{3}H]\)-N-OH-AF would be comparable to that in the preceding experiments. The results as summarized in Table 3 indicate that the nuclei alone had a low but significant ability to activate AF to an electrophilic species. However, addition of either rat liver microsomes or purified FMO increased this covalent binding 2- to 3-fold. Furthermore, DPEA inhibited the rat liver microsomal-mediated DNA-binding reaction, but not that supported by FMO.

Table 2

<table>
<thead>
<tr>
<th>pH</th>
<th>N-OH-AF (( \mu \text{M} ))</th>
<th>N-OH-AF bound (pmol/mg DNA)</th>
<th>% of N-OH-AF bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>7.5</td>
<td>10</td>
<td>16.8 ± 3.7(a)</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>10</td>
<td>995 ± 49</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>7.5</td>
<td>5</td>
<td>9.6 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>10</td>
<td>630 ± 80</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>10</td>
<td>28.3 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>10</td>
<td>1032 ± 45</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>25</td>
<td>39.0 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>25</td>
<td>1958 ± 10</td>
</tr>
</tbody>
</table>

\(a\) Mean ± S.D. of 3 determinations.

\(b\) Mean of 3 determinations.

Table 3

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>DPEA</th>
<th>DNA binding (pmol AF bound/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver nuclei only</td>
<td>–</td>
<td>8.2 ± 2.5(a)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>15.2 ± 1.6</td>
</tr>
<tr>
<td>Rat liver nuclei + microsomes</td>
<td>–</td>
<td>22.0 ± 2.6(a)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10.4 ± 3.7(b)</td>
</tr>
<tr>
<td>Rat liver nuclei + porcine FMO</td>
<td>–</td>
<td>12.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>17.6 ± 2.6(c)</td>
</tr>
<tr>
<td>Rat liver nuclei + AF + N-OH-AF</td>
<td>–</td>
<td>16.9 ± 4.6(d)</td>
</tr>
</tbody>
</table>

\(a\) Incubations were conducted aerobically for 30 min at pH 7.5 as described in Table 1 using 0.5 mm \([^{3}H]\)AF and 2 mg nuclear protein per ml. Where indicated, 0.5 mm DPEA, 3-methylcholanthrene-induced rat liver microsomes (0.5 mg/ml), FMO (74 units/ml), or 20.6 mm \([^{3}H]\)-N-OH-AF was added.

\(b\) Mean ± S.D. of 3 determinations.

\(c\) The increase in DNA binding, cf. to “rat liver nuclei only,” was statistically significant (\(p < 0.05\)).

\(d\) The extent of DPEA inhibition was statistically significant (\(p < 0.025\)).

DISCUSSION

The metabolic N-oxidation of primary arylamines in certain species is believed to be catalyzed by microsomal mixed-function oxidase systems involving cytochrome P-450 (23, 41, 46, 57, 67). In this study, the hepatic microsomal N-oxidation of AF to N-OH-AF was shown to occur for rats, dogs, pigs, and humans (Table 1). DPEA, a specific inhibitor of cytochrome P-450-catalyzed reactions, was strongly inhibitory to N-OH-AF formation in the rat and dog, while complete inhibition in human and pig liver fractions required addition of methimazole, a competitive inhibitor of FMO. These results are consistent with previous determinations of high FMO activity (N,N-dimethylaniline N-oxidase) in the latter 2 species (75) and indicate a dual mode of activation for AF as indicated in Chart 3. The ability of FMO to catalyze the N-oxidation of AF was then established by in vitro metabolism studies with the purified enzyme from porcine liver. Similarly, a purified rat cytochrome P-450\(_{SKN}\) preparation N-hydroxylated AF in a reconstituted system at a rate (Table 1) comparable to that of normal rat liver microsomes [based on a total cytochrome P-450 content of 0.8 nmol/mg protein (16)]. In a reconstituted system with the major form of cytochrome P-450 from phenobarbital-induced rats (17), N-hydroxylation of AF was not observed, which suggests that only certain forms of the native cytochrome P-450s may be involved in primary arylamine N-oxidation.

In comparison, purified porcine MFO has also been shown to catalyze the N-oxidation of the secondary arylamine hepatocarcinogen, N-methyl-4-aminobenzene; and, using DPEA as a selective P-450 inhibitor, the involvement of FMO was demonstrated in several species (23). However, with this substrate, FMO activity was highest in the rat. Thus, the substrate specificity of FMO for arylamines may differ significantly between various species, making extrapolation from animal models to humans rather difficult. Furthermore, recent studies have indicated that mouse and rat hepatic MFO are under hormonal control and that both are decreased by inducers of
hepatic microsomal cytochrome P-450 (10, 48, 73). Therefore, both normal and induced rodent liver S-9 fractions, which are used extensively for mutagenicity screening, may not provide a representative model of human arylamine metabolism to mutagenic products.

In this report, the purified FMO was used in a S. typhimurium assay to activate AF to a mutagen (cf. Ref. 50), presumably by forming N-OH-AF. This is consistent with several studies using AAF and N-OH-AAF, which have concluded that the deacetylated metabolite, N-OH-AF, is the ultimate mutagen derived from these carcinogens (6, 7, 28, 60, 61). Further metabolic activation of N-OH-AF within the bacterial cell has also been suggested (59, 68); however, no evidence for such conversions has been reported. In summary, the published data suggest that the production of N-OH-AF and its transport into the bacterial cell may be sufficient for DNA binding and mutations, presumably under physiological conditions and neutral pH.

To support this scheme, the binding of [3H]-N-OH-AF to purified calf thymus DNA, although substantially higher at pH 5 (cf. Table 2), readily occurred at pH 7.5 under in vitro conditions used to measure AF metabolism, accounting for 0.08 to 0.15% of the added N-OH-AF. Similar experiments using isolated rat liver nuclei at pH 7.5 (Table 3) resulted in a comparable trapping efficiency (0.09%). When this reaction was conducted with S. typhimurium, a virtually identical trapping efficiency (0.11%) was observed (6). These values are still 2 orders of magnitude higher than the levels calculated from data reported by Kriek (34) and Stout et al. (62) for the binding of AF to rat liver DNA in vivo after an i.p. injection (approximately 0.001 and 0.002%, respectively, of the dose bound to liver DNA). This difference is reasonable if some allowance is made for a significant amount of metabolic detoxification of AF in vivo. The similar N-OH-AF-trapping efficiencies observed between calf thymus DNA at neutrality, the DNA of isolated rat liver nuclei, and bacterial DNA infer that the nonenzymatic reaction of N-OH-AF with nuclear DNA under physiological conditions could be sufficient to explain a substantial portion of the low level of binding to hepatocyte DNA observed in vivo [as earlier proposed by King and Phillips (30) and Weisburger et al. (70)]. Nevertheless, the existence of some undefined nuclear activation of N-OH-AF cannot be excluded.

A critical variable in the above comparisons is the rate of reaction of N-OH-AF with DNA in the nucleus. However, since reactions of N-hydroxyarylamines with DNA are first order with respect to DNA concentrations (26) and since the concentration of DNA within rat liver nuclei is very high (about 50 mg/ml), the binding of N-OH-AF to nuclear DNA may be 100-fold faster than was observed with calf thymus DNA in solution (cf. Table 2, where DNA was 0.5 mg/ml). This would allow for the concomitant binding of N-OH-AF to protein while still resulting in a high level of binding to nuclear DNA in vitro (cf. Table 3) and in vivo.

This study has focused on the metabolism of the primary arylamine, AF, and its N-hydroxylated metabolite. However, both AF and N-OH-AF can be acetylated in vivo in certain species (44, 70), and these N-acetylated derivatives can further undergo a variety of metabolic conversions which may ultimately lead to macromolecular binding and carcinogenicity (Chart 3; reviewed in Refs. 9, 36, 42, and 71). In addition, N-OH-AAF, a proximate carcinogenic metabolite of AAF (42), can be deacetylated to yield N-OH-AF (15, 19), which may react directly with nuclear DNA as shown by this study. Two other metabolic routes involving N-OH-AAF have also been suggested to produce the deacetylated AF:DNA adducts which predominate in vivo (21, 34). In the first, N-O-acetyltransferase can mediate the formation of a highly reactive O-acetyl ester of the hydroxylamine (reviewed in Ref. 29). In the second route, O-glucuronidation of N-OH-AAF in the liver produces a stable metabolite that is excreted in the urine of rats dosed with AAF (20, 45). Subsequently, Cardona and King (8) found that this compound can be deacetylated to a highly reactive O-glucuronide of N-OH-AAF which will bind to tissue macromolecules. Therefore, we propose that the direct reaction of N-OH-AF with nuclear DNA may provide a basal level of AF:DNA binding, which can be significantly augmented in certain tissues by the enzymatic activation modes indicated above. However, the direct binding mechanism could be predominant in dogs (51) and certain humans (13) deficient in arylamine N-acetylation activity and in those species which lack N-O-acetyltransferase activity (29) or deacetylase activity toward the O-glucuronide of N-OH-AAF (8).

ACKNOWLEDGMENTS

The contribution of Dr. Robert Heffich and Gail White in conducting the mutagenicity assay is gratefully acknowledged.

REFERENCES

Enzymatic Formation of N-OH-AF


Cytochrome P-450- and Flavin-containing Monooxygenase-catalyzed Formation of the Carcinogen \( N \)-Hydroxy-2-aminofluorene and Its Covalent Binding to Nuclear DNA

Clay B. Frederick, Joann B. Mays, Daniel M. Ziegler, et al.


Updated version  Access the most recent version of this article at:  
http://cancerres.aacrjournals.org/content/42/7/2671

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.