[CANCER RESEARCH 45, 3578–3585, August 1985]

# Metabolic Oxidation of Carcinogenic Arylamines by Rat, Dog, and Human Hepatic Microsomes and by Purified Flavin-containing and Cytochrome P-450 Monooxygenases[1]

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## ABSTRACT

Hepatic *N*-oxidation and aryl ring oxidation are generally regarded as critical activation and detoxification pathways for arylamine carcinogenesis. In this study, we examined the *in vitro* hepatic metabolism of the carcinogens, 2-aminofluorene (2-AF) and 2-naphthylamine (2-NA), and the suspected carcinogen, 1-naphthylamine (1-NA), using high-pressure liquid chromatography. Hepatic microsomes from rats, dogs, and humans were shown to catalyze the *N*-oxidation of 2-AF and of 2-NA, but not of 1-NA; and the rates of 2-AF *N*-oxidation were 2- to 3-fold greater than the rates of 2-NA *N*-oxidation. In each species, rates of 1-hydroxylation of 2-NA and 2-hydroxylation of 1-NA were comparable and were 2- to 5-fold greater than 6-hydroxylation of 2-NA or 5- and 7-hydroxylation of 2-AF. Purified rat hepatic monooxygenases, cytochromes P-450$_{UT-A}$, P-450$_{UT-H}$, P-450$_{PB-B}$, P-450$_{PB-D}$, P-450$_{BNF-B}$, and P-450$_{ISF/BNF-G}$ but not P-450$_{PB-C}$ or P-450$_{PB/PCN-E}$, catalyzed several ring oxidations as well as the *N*-oxidation of 2-AF. Cytochromes P-450$_{PB-B}$, P-450$_{BNF-B}$, and P-450$_{ISF/BNF-G}$ were most active; however, only cytochrome P-450$_{ISF/BNF-G}$, the isosafrole-induced isozyme, catalyzed the *N*-oxidation of 2-NA. The purified porcine hepatic flavin-containing monooxygenase, which was known to carry out the *N*-oxidation of 2-AF, was found to catalyze only ring oxidation of 1-NA and 2-NA. No activity for 1-NA *N*-oxidation was found with any of the purified enzymes. These data support the hypothesis that 1-NA is probably not carcinogenic. Furthermore, carcinogenic arylamines appear to be metabolized similarly in humans and experimental animals and perhaps selectively by a specific form of hepatic cytochrome P-450. Enzyme mechanisms accounting for the observed product distributions were evaluated by Hückel molecular orbital calculations on neutral, free radical, and cation intermediates. A reaction pathway is proposed that involves two consecutive one-electron oxidations to form a paired substrate cation-enzyme hydroxyl anion intermediate that collapses to ring and *N*-hydroxy products.

## INTRODUCTION

Primary arylamines were among the first chemicals to be identified as carcinogens in humans and experimental animals (1, 2). For example, 2-NA,[3] which has been used industrially and is present in cigarette smoke (3) and in synthetic fuels (4), induces primarily carcinomas of the urinary bladder in humans, dogs, and rats (1, 2); while 2-AF, also present in synthetic fuels (4), is carcinogenic to the liver, urinary bladder, and other tissues of a variety of species (1, 2, 5). In contrast, 1-NA has not been shown to be carcinogenic in animals (6); and, although epidemiological studies have implicated 1-NA as a human carcinogen, there was always concomitant exposure to 2-NA (1, 2, 6).

Since *N*-oxidation has long been considered an obligatory step in arylamine carcinogenesis (7), the identification and quantitation of N-OH arylamine and nitrosoarene metabolites have received considerable attention (8–15). From these studies, it has been postulated that differences in the rates of *N*-oxidation may account for compound and species specificity. In contrast, ring oxidation of arylamines has been proposed to be a critical detoxification process. The enzymatic mechanisms for the *N*- and ring oxidation of certain primary arylamines have been examined in several species, and the data suggest the involvement of hepatic cytochrome P-450 monooxygenases, the hepatic FMO, and PHS which is widely distributed in extrahepatic tissues (reviewed in Ref. 16). 2-AF, for example, has been shown to be *N*-oxidized by rat, dog, guinea pig, hamster, and human liver microsomes; by the purified porcine hepatic FMO; by purified rat hepatic cytochrome P-450$_{BNF-B}$; and by PHS from ram seminal vesicles (12, 13, 17, 18). In contrast, evidence for the *in vitro N*-oxidation of 2-NA is limited. However, the data have suggested the involvement of FMO (19), PHS (17), and hepatic (rat, pig) and bladder (bovine) microsomal monooxygenases (15, 20). Similar experiments with 1-NA have been inconclusive. However, the N-OH derivative of 1-NA is a potent direct-acting carcinogen and mutagen and is about 10 times more carcinogenic than is N-OH-2-NA (reviewed in Ref. 21).

In the present study, we have examined the oxidative metabolism of 2-NA and 1-NA by hepatic microsomal preparations from rats, from dogs, and from 10 individual human liver samples. To allow comparisons to be made with previous data, the *N*- and ring oxidation of 2-AF was also determined. In addition, the nature of these hydroxylating activities was assessed using the purified porcine liver FMO (10) and 8 different cytochrome P-450 isozymes purified from rat liver (22). The results demonstrate that *N*-oxidation of 2-AF and ring oxidation of 2-NA and 1-NA are catalyzed by several monooxygenases while *N*-oxidation of

Received 10/2/84; revised 4/24/85; accepted 4/30/85.

[1] This study was supported by an interagency agreement between the National Institute for Occupational Safety and Health (IA-81-71) and the National Center for Toxicological Research (224-82-0001) and by USPHS Grants ES01590 and CA30907.

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[3] The abbreviations used are: 2-AF, 2-aminofluorene; 2-NA, 2-naphthylamine; 1-NA, 1-naphthylamine; 2-OH-1-NA, 1-amino-2-naphthol; 1-OH-2-NA, 2-amino-1-naphthol; N-OH, N-hydroxy; P-450, hepatic microsomal cytochrome P-450; FMO, flavin-containing monooxygenase; PHS, prostaglandin H synthase; PB, phenobarbital; BNF, β-naphthoflavone; ISF, isosafrole; PCN, pregnenolone-16α-carbonitrile; UT, untreated; HPLC, high-pressure liquid chromatography; DPEA, 2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine. The nomenclature of individual forms of cytochrome P-450 and comparisons with preparations from other laboratories are described elsewhere (22).

2-NA is selectively carried out by the high-spin form of cytochrome P-450$_{ISF/BNF-G}$. *N*-Oxidation of 1-NA was not detected in any of these preparations, which supports the hypothesis that 1-NA is not carcinogenic because of its inability to form an N-OH metabolite.

## MATERIALS AND METHODS

**Chemicals.** [3-$^3$H]-1-NA (38 mCi/mmol), [5,6,7,8-$^3$H]-2-NA (47 mCi/mmol), and [5,6,7,8-$^3$H]-2-AF (55 mCi/mmol) were obtained from Dr. R. Roth, Midwest Research Institute, Kansas City, MO. The following materials were purchased: NADPH, dilauroyl-L-$\alpha$-phosphatidylcholine, and carboxylesterase (types I and II) from Sigma Chemical Co., St. Louis, MO; 1-OH-2-NA hydrochloride from Pfaltz and Bauer, Inc., Stamford, CT; 2-OH-1-NA, 1-amino-4-naphthol, and 2-amino-3-naphthol hydrochlorides, 1,2-naphthoquinone, and 2-amino-1,4-naphthoquinone from Aldrich Chemical Co., Milwaukee, WI; and desferrioxamine mesylate from Ciba Pharmaceuticals, Summit, NJ.

The following compounds were synthesized using published procedures: N-OH-2-NA and N-OH-1-NA (23); 2-nitrosonaphthalene and 1-nitrosonaphthalene (24); N-OH-2-AF and 2-nitrosofluorene (25); and 6-OH-2-NA (26). 1-OH, 3-OH-, 5-OH-, and 7-OH-2-AF were prepared from the corresponding ring hydroxy derivatives of 2-acetylaminofluorene (obtained from Illinois Institute of Technology Research Institute, Chicago, IL) by deacetylation with carboxylesterase (27). They were purified by ethyl acetate extraction and subsequent HPLC. Each of the synthetic compounds was analyzed for purity by HPLC and was routinely found to be >95%, and their identity was confirmed by electron impact mass spectrometry. All other reagents were of analytical grade.

**Tissues and Enzyme Preparations.** Human liver samples 21 (63-year-old male), 23 (24-year-old male), 25 (21-year-old male); 26 (24-year-old male), 27 (38-year-old female), 28 (13-year-old male), 29 (49-year-old male), 35 (28-year-old male), 36 (32-year-old male), and 37 (15-year-old female) were surgical samples obtained from organ donors immediately after death (28).

Mature male beagles were obtained from Marshall Research Animals, Inc., North Rose, NY. Male Sprague-Dawley rats (100 to 175 g) were purchased from the Charles River Breeding Laboratories, Inc., Wilmington, MA, or from Harlan Industries, Indianapolis, IN. Human (29), dog, and rat (22) liver microsomes were prepared as described previously and stored at −70°C. No difference in metabolic activity could be detected between fresh microsomes and those stored by this method. Protein concentrations were measured by the biuret reaction (30).

FMO, purified from porcine liver microsomes, was generously provided by Dr. D. M. Ziegler, University of Texas, Austin, TX. NADPH-cytochrome *c* (P-450) reductase was prepared from rat liver as described by Yasukochi and Masters (31) and modified elsewhere (22). Human hepatic cytochromes P-450 were prepared from 3 livers and designated P-450$_2$, P-450$_4$, and P-450$_8$ (28, 29). Dog liver P-450 fractions were prepared by column chromatography on *n*-octylamino-Sepharose 4B (22). The material eluted with buffer containing 0.06% Lubrol PX was pooled and designated Fraction 1, and the material subsequently eluted with buffer containing 0.3% Lubrol PX was pooled and designated Fraction 2. Both fractions were dialyzed extensively to remove sodium cholate, treated with Bio-Beads (Bio-Rad, Richmond, CA) to remove excess Lubrol PX, and concentrated using Amicon PM-30 ultrafiltration (Amicon Corp., Lexington, MS). Fractions 1 and 2 contained 11.6 and 9.8 nmol P-450/mg protein, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed the presence of several polypeptides (silver staining), but further attempts at purification were not made. The preparations were stored at −70°C in 10 mM Tris-acetate-1 mM EDTA-20% glycerol (pH 7.4). Eight distinct isozymes of rat hepatic cytochrome P-450, designated P-450$_{UT-A}$, P-450$_{UT-H}$, P-450$_{PB/PCN-E}$, P-450$_{PB-B}$, P-450$_{PB-C}$, P-450$_{PB-D}$, P-450$_{BNF-B}$, and P-450$_{ISF/BNF-G}$, were purified as described previously (22). Each of the purified cytochromes (0.25 to 2.0 nmol) were reconstituted immediately prior to enzyme assays by mixing with 0.5 to 4.0 nmol purified rat liver reductase (2-fold molar excess over P-450) and 30 $\mu$g dilauryl-L-$\alpha$-phosphatidylcholine in 10 mM sodium phosphate buffer (pH 7.4) for 3 min at 37°C. P-450 concentrations were estimated by the method of Omura and Sato (32).

**Enzyme Assays.** Incubation mixtures (1 ml) to determine the *N*- and ring oxidation of arylamines consisted of 10 mM sodium phosphate buffer (pH 7.4), 1 mM NADPH, and 0.1 mM $^3$H-labeled 2-AF, 2-NA, or 1-NA (added in 10 $\mu$l ethanol) and either (*a*) 0.5 to 1.5 mg hepatic microsomal protein, (*b*) 0.1 to 0.8 nmol purified porcine hepatic microsomal FMO, or (*c*) 0.25 to 2.0 nmol purified cytochrome P-450 reconstituted as described above. The assays were conducted in open 10-ml Erlenmeyer flasks and were preincubated at 37°C for 2 to 3 min. The reactions were then started by addition of NADPH. After incubation at 37°C for 3 to 5 min, the reactions were terminated by extraction with 0.5 ml of water-saturated ethyl acetate. Aliquots (50 $\mu$l) of the organic extracts were immediately analyzed by HPLC. These incubation conditions were selected because they were found to be optimal for the purified cytochrome P-450 monooxygenases and the rates could be directly compared with microsomal preparations. In the latter case, rates of 2-AF *N*-oxidation by hepatic microsomes and purified FMO were lower than previously reported (12) which was due to the use of NADPH (*versus* an NADPH-, NADH-generating system) and of lower substrate concentrations (0.1 *versus* 0.5 mM). However, for the purified monooxygenases and the microsomes of each species, reaction rates were linear with time and were directly proportional to the protein or enzyme concentrations used.

**Analytical Methods.** Instrumentation consisted of a Beckman/Altex Model 322 MP gradient HPLC system with a Radiomatic Flo-One Model HP radioactivity flow detector and a Hewlett-Packard HP 1040A high-speed spectrophotometric detector. Separation was achieved on an Altex ODS-3 column (4.6 mm x 25 cm) using a 20-min linear gradient of 20 to 100% methanol with a flow rate of 1 ml/min. The water contained 0.02% desferrioxamine mesylate to improve chromatographic properties and to minimize further oxidation of *N*- and ring-hydroxymetabolites (*cf.* Refs. 33 to 35).

The various metabolites were identified from both their UV spectra and their HPLC retention times (as compared with standards). Retention times (min) for the arylamine substrates and their *N*- and ring-oxidized metabolites are listed as follows: 2-NA (18.7); N-OH-2-NA (17.6); 2-nitrosonaphthalene (22.5); 1-OH-2-NA (14.9); 2-amino-1,4-naphthoquinone (16.8); 6-OH-2-NA (12.6); 1-NA (18.8); 2-OH-1-NA (16.8); 1,2-naphthoquinone (14.9); 2-AF (21.2); N-OH-2-AF (20.1); 2-nitrosofluorene (24.1); 5-OH-2-AF (17.5); and 7-OH-2-AF (16.0). 2-Amino-3-naphthol (16.8), N-OH-1-NA (17.7), 1-nitrosonaphthalene (22.7), 1-OH-2-AF (20.0), 3-OH-2-AF (18.4), and 1-amino-4-naphthol (13.4) were also analyzed by HPLC but were not detected as metabolites.

Quantitation of these metabolites was obtained directly using the radioactivity flow detector by measuring the percentage of total radioactivity eluting with each standard. Thus, rates were calculated as (% of total $^3$H in each peak ÷ 100) × (arylamine substrate concentration) ÷ (time) ÷ (protein concentration) and were expressed as pmol/min/mg. For the *N*-oxidation products of 2-AF and 2-NA, the relative proportions of N-OH-arylamine and nitrosoarene were variable; and greater amounts of the nitrosoarene product were observed with higher concentrations of products in the ethyl acetate extract and with increased use of the HPLC column. However, the rates of total *N*-oxidation, as calculated from the combined amounts of N-OH and nitroso products observed, were nearly constant for each enzyme preparation. Furthermore, recovery experiments showed that radiolabeled N-OH-AF, N-OH-2-NA, or N-OH-1-NA (10 to 50 $\mu$M) could be extracted from the microsomal incubations and analyzed by HPLC with 80 to 90% of the added radioactivity eluting with the N-OH and nitroso derivatives. Similar observations were made for (*a*) 1-OH-2-NA, a ring-oxidation product of 2-NA, which readily oxidized to 2-amino-1,4-naphthoquinone, and (*b*) 2-OH-1-NA, a ring oxidation product of 1-NA, which readily oxidized to 1,2-naphthoquinone. Therefore, total *N*-oxidation rates and ring oxidation rates are reported.

## RESULTS

### Rat, Dog, and Human Liver Microsomal-catalyzed Oxidation of 2-NA, 1-NA, and 2-AF

Since metabolic *N*- and ring oxidation are generally regarded as critical activation and detoxification pathways for arylamine carcinogenesis, the comparative metabolism of 2-NA, 1-NA, and 2-AF was assessed using rat, dog, and human liver microsomes. These data are summarized in Table 1. For the carcinogen 2-NA, the microsomal-catalyzed reactions involved primarily ring oxidation at C-1 (1-OH-2-NA and 2-amino-1,4-naphthoquinone formation) followed by *N*-oxidation (N-OH-2-NA and 2-nitrosonaphthalene formation). 6-OH-2-NA was also observed as a minor metabolite. These results are consistent with previous data on the *in vivo* metabolism of 2-NA in each of these species (reviewed in Ref. 26). In contrast, the oxidation of 1-NA appeared to proceed entirely through ring oxidation to form 2-OH-1-NA (and 1,2-naphthoquinone). Neither N-OH-1-NA nor 1-nitrosonaphthalene could be detected as a metabolite in rat, dog, or human liver microsomes. By comparison, 2-AF, a potent mutagen and carcinogen, was found to undergo *N*-oxidation almost exclusively, with ring oxidation at C-5 and C-7 occurring to only a minor extent. For dogs, the rates of arylamine *N*-oxidation and ring oxidation were about 2-fold higher than in rats and generally higher than most human liver samples.

The human liver microsomal preparations varied appreciably in their absolute activity toward the different arylamine substrates (Table 1). Since each human liver sample was obtained under similar conditions, the differences in metabolic activities may be the result of biochemical differences among individuals, perhaps as a result of induction of different monooxygenases through diet or life-style. For example, the rates of human microsomal 2-NA *N*-oxidation expressed per nmol P-450 varied at least 10-fold, while total cytochrome P-450 levels varied only 2 to 3-fold (Table 2). Furthermore, there was no correlation between total P-450 content and rate of 2-NA *N*-oxidation.

### *N*- and Ring Oxidation of 2-NA, 1-NA, and 2-AF by Purified Hepatic Monooxygenases

To assess the involvement of the different constitutive and inducible monooxygenases in arylamine oxidation, several purified enzyme preparations were examined.

**Involvement of the Hepatic FMO in the Ring Oxidation of 2-NA and 1-NA.** In our previous study (12), we reported that purified porcine hepatic FMO catalyzed the *N*-oxidation of 2-AF. Using DPEA, a specific P-450 inhibitor, and methimazole, a competitive inhibitor of FMO, we presented data which suggested that 2-AF *N*-oxidation involved both FMO and cytochrome P-450 in human and pig liver microsomes, but only cytochrome P-450 in rat or dog liver microsomes. We now report that addition of 0.5 mM DPEA completely inhibits the *N*-oxidation of 2-NA by rat, dog, and human liver microsomes (Table 1). In contrast, 1-hydroxylation of 2-NA and 2-hydroxylation of 1-NA was inhibited only 40 to 60% by DPEA, which suggests the partial involvement of FMO or other oxygenases in arylamine ring oxidation. This was investigated further using the purified porcine hepatic FMO (Table 3). Arylamine *N*-oxidation was detected only with 2-AF; however, the purified FMO catalyzed the ring oxidation of both 2-NA and 1-NA. These reactions were insensitive to inhibition by DPEA, catalase (1 mg/ml), or butylated hydroxytoluene (0.1 mM).

**Role of Hepatic Cytochrome P-450 Monooxygenases.** As mentioned above, evidence for the participation of cytochrome P-450 monooxygenases in 2-AF *N*-oxidation was previously obtained with rat, dog, and human liver microsomes; and purified rat hepatic P-450$_{BNF-B}$ was shown to catalyze this reaction at a rate comparable to that of purified porcine FMO (12). In the present study, we examined the metabolic oxidation of 2-NA, 1-NA, and 2-AF by 8 rat liver P-450s (22) as well as by several partially purified preparations from human and dog liver. As shown in Table 3, the rat cytochrome P-450$_{UT-A}$, a major constitutive form in untreated animals, had low activity toward ring oxidation of 2-NA and 1-NA and *N*-oxidation of 2-AF. Cytochrome P-450$_{UT-H}$, an additional major constitutive isozyme, did not oxidize 2-NA but had low activity toward ring oxidation of 1-NA,

Table 1

*Rates of metabolic oxidation of arylamines by rat, dog, and human liver microsomes*

Rates are shown as the mean ± SD or as individual determinations. Column headings (N-OH, 1-OH, etc.) signify total *N*- or ring oxidation rates as described in "Materials and Methods."

| Species/ individual | Rates (pmol/min/mg protein) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2-NA | | | 1-NA | | AF | | |
| | N-OH | 1-OH | 6-OH | N-OH | 2-OH | N-OH | 5-OH | 7-OH |
| Rat[a] | 116 ± 31[c] | 232 ± 36[d] | 28 ± 6[c] | <10 | 245 ± 46[e] | 340 ± 94[f] | 62 ± 16[c] | 37 ± 14[c] |
| Dog[b] | 242 ± 13[c] | 473 ± 48[d] | <10 | <10 | 383 ± 14[e] | 606 ± 77[f] | 50 ± 4[c] | <10 |
| Human-21 | <10 | <10 | 49 | <10 | 233 | <10 | <10 | <10 |
| Human-23 | 151[c] | 130[d] | 69[c] | <10 | 182[e] | 214[f] | 17[c] | 16[c] |
| Human-25 | 40 | 246 | 93 | <10 | 1194 | 256 | <10 | <10 |
| Human-26 | 44[c] | 129[d] | 33[c] | <10 | 358[e] | 213[f] | <10 | <10 |
| Human-27 | 63 | 111 | 16 | <10 | 144 | 49 | <10 | <10 |
| Human-28 | 352 | 308 | <10 | <10 | 281 | 519 | 19 | 14 |
| Human-29 | 161 | 131 | <10 | <10 | 113 | 234 | <10 | <10 |
| Human-35 | 109 | 146 | 22 | <10 | 160 | 184 | 35 | <10 |
| Human-36 | 138 | 110 | 34 | <10 | 104 | 319 | 26 | <10 |
| Human-37 | 151 | 99 | 20 | <10 | 170 | 150 | 37 | <10 |

[a] $n = 5$.
[b] $n = 3$.
[c] No activity was detectable (<10 pmol/min/mg) in the presence of 0.5 mM DPEA.
[d] Inhibited 30 to 50% by DPEA.
[e] Inhibited 60 to 70% by DPEA.
[f] Inhibited 70 to 90% by DPEA.

and ring- and *N*-oxidation of 2-AF. Two isozymes induced by PB ($P\text{-}450_{PB\text{-}C}$) and by PCN or PB ($P\text{-}450_{PB/PCN\text{-}E}$) had no detectable activity toward any of the arylamine substrates, while cytochromes $P\text{-}450_{PB\text{-}B}$ and $P\text{-}450_{PB\text{-}D}$ were quite effective in catalyzing the ring oxidation of 2-NA and 1-NA and had some activity for 2-AF *N*-oxidation. The most active monooxygenases for 2-AF *N*-oxidation and 2-NA and 1-NA ring oxidations were $P\text{-}450_{BNF\text{-}B}$ and $P\text{-}450_{ISF/BNF\text{-}G}$. Furthermore, the latter isozyme, which is a high-spin form of P-450 (22, 36, 37), was the only purified monooxygenase which catalyzed the *N*-oxidation of 2-NA.

The ability of partially purified dog and purified human liver P-450s to oxidize the arylamine substrates was also examined. P-450 Fractions 1 and 2 from dog liver were able to *N*-hydroxylate 2-AF but only to ring oxidize 1-NA and 2-NA (Table 3). Human $P\text{-}450_2$ did not show activity toward any of the substrates, while human cytochromes $P\text{-}450_4$ and $P\text{-}450_8$ catalyzed only the ring oxidation of 1-NA. Additional studies will be required to determine if human or dog liver contains P-450 isozymes comparable to rat $P\text{-}450_{BNF\text{-}B}$ and $P\text{-}450_{ISF/BNF\text{-}G}$, since these enzymes may have been inactivated or lost during purification.

Table 2

*Human hepatic cytochrome P-450 levels and rates of 2-NA N-oxidation*

| Human liver microsomes | Cytochrome P-450 (nmol/mg protein) | 2-NA *N*-oxidation[a] (pmol/nmol P-450) |
|---|---|---|
| 21 | 0.36 | <30 |
| 37 | 0.36 | 419 |
| 26 | 0.38 | 116 |
| 35 | 0.38 | 287 |
| 23 | 0.40 | 378 |
| 29 | 0.41 | 393 |
| 28 | 0.41 | 859 |
| 36 | 0.42 | 329 |
| 27 | 0.44 | 143 |
| 25 | 0.92 | 43 |

[a] These values were calculated by dividing the rate data in Table 1 by the P-450 concentrations indicated above.

## DISCUSSION

Since the liver is usually considered to be the major site of drug and carcinogen biotransformation, comparative studies on metabolic activation and detoxification by hepatic microsomes of different species can provide important data on relative toxicity and carcinogenicity (reviewed in Ref. 38). For carcinogenic arylamines, microsomal *N*-oxidation results in the formation of highly reactive and mutagenic N-OH derivatives. Further oxidation yields nitrosoarenes, which can bind to proteins and glutathione but which can also be readily reduced back to N-OH-arylamines by reductases or ascorbic acid (reviewed in Ref. 39). In contrast, ring oxidation generally results in phenolic metabolites that are efficiently conjugated and excreted.

In previous studies on the metabolism of the carcinogen 2-NA, evidence for the *in vivo* formation of both *N*- and ring oxidation products has been obtained by analysis of urine from rats, dogs, monkeys, and humans (26, 40–42). With 1-NA, the results have been equivocal. Small quantities of N-OH-1-NA and 1-nitrosonaphthalene have been reported in the urine of some dogs given varying doses of 1-NA; however, only ring oxidation products appear to have been consistently detected (2, 6). Studies of the *in vitro* metabolism of 1-NA and 2-NA are also conflicting. Uehleke (43), Poulsen *et al.* (44), and Brill and Radomski (45) suggested that hepatic microsomes *N*-oxidized 2-NA but not 1-NA; however, more recently, Poupko *et al.* (15) did not detect *N*-oxidation of 2-NA and 1-NA by dog or bovine liver microsomes or by dog bladder epithelial microsomes, but activity for both substrates (2-NA > 1-NA) was seen with bovine bladder epithelial microsomes. Using electron spin resonance methods, Nakayama *et al.* (20) detected free radicals derived from N-OH-2-NA and 1-OH-2-NA in 2-NA incubations with rat liver microsomes; with 1-NA, a free radical tentatively identified as that derived from 2-OH-1-NA was also observed. In contrast, considerable evidence for the hepatic microsomal *N*-oxidation of 2-AF (but not ring

Table 3

*Rates of arylamine oxidation by purified monooxygenases*

Assay procedures are as described in "Materials and Methods." The values are an average of duplicate determinations which were within 15% of each other. The limit of detection in these assays was considered to be 0.1% of the total eluted radioactivity and was equivalent to 100 pmol/min/nmol enzyme for the pig FMO experiments and 50 pmol/min/nmol enzyme for the purified cytochromes P-450. Column headings signify total *N*- or ring oxidation products.

| | Rates (pmol/min/nmol enzyme) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2-NA | | | 1-NA | | AF | | |
| Purified monooxygenase | N-OH | 1-OH | 6-OH | N-OH | 2-OH | N-OH | 5-OH | 7-OH |
| Pig FMO[a] | <100 | 1505 | <100 | <100 | 1070 | 757 | <100 | <100 |
| Rat $P\text{-}450_{UT\text{-}A}$ | <50 | 152 | <50 | <50 | 274 | 543 | <50 | <50 |
| Rat $P\text{-}450_{UT\text{-}H}$ | <50 | <50 | <50 | <50 | 201 | 442 | 107 | 248 |
| Rat $P\text{-}450_{PB/PCN\text{-}E}$ | <50 | <50 | <50 | <50 | <50 | <50 | <50 | <50 |
| Rat $P\text{-}450_{PB\text{-}C}$ | <50 | <50 | <50 | <50 | <50 | <50 | <50 | <50 |
| Rat $P\text{-}450_{PB\text{-}B}$ | <50 | 773 | <50 | <50 | 2150 | 1062 | <50 | <50 |
| Rat $P\text{-}450_{PB\text{-}D}$ | <50 | 582 | <50 | <50 | 1661 | 456 | <50 | <50 |
| Rat $P\text{-}450_{BNF\text{-}B}$ | <50 | 2544 | 360 | <50 | 3910 | 5244 | 200 | 560 |
| Rat $P\text{-}450_{ISF/BNF\text{-}G}$ | 2517 | 2589 | 911 | <50 | 3851 | 7421 | 2318 | 520 |
| Dog P-450-I | <50 | 210 | <50 | <50 | 274 | 377 | <50 | <50 |
| Dog P-450-II | <50 | 175 | <50 | <50 | 327 | 756 | <50 | <50 |
| Human $P\text{-}450_2$ | <50 | <50 | <50 | <50 | <50 | <50 | <50 | <50 |
| Human $P\text{-}450_4$ | <50 | <50 | <50 | <50 | 160 | <50 | <50 | <50 |
| Human $P\text{-}450_8$ | <50 | <50 | <50 | <50 | 250 | <50 | <50 | <50 |

[a] Assays conducted at 0.5 mM substrate (*versus* 0.1 mM) gave initial rates which were 4- to 5-fold higher.

oxidation) has been obtained in several laboratories using a variety of species (12, 13, 46).

In the present report, the *N*- and ring oxidation of 2-NA, 1-NA, and 2-AF were determined by a direct HPLC method which simultaneously permitted quantification of these metabolites and their identification by spectral and chromatographic criteria. The results demonstrated that rat, dog, and human liver microsomes are able to ring oxidize all 3 arylamines and *N*-oxidize 2-NA and 2-AF but not 1-NA. In general, the rates of microsomal metabolism were in the order: 2-AF *N*-oxidation and 1-NA ring oxidation > 2-NA ring oxidation > 2-NA *N*-oxidation > 2-AF ring oxidation. Considering the importance of these pathways in the metabolic activation-detoxification for various species, the results are quite consistent with comparative mutagenicity data (47) for these arylamines in the S9-mediated (uninduced) *Salmonella typhimurium* assay; *i.e.*, 2-AF was a more potent mutagen than was 2-NA, and very little species differences were noted for rat, pig, mouse, and human hepatic preparations. 1-NA, on the other hand, has not been reported as a mutagen. Although the relative carcinogenicity of arylamines is influenced by numerous biological variables including metabolic conjugation reactions (reviewed in Refs. 26, 48, and 49), the data presented here are also consistent with the known carcinogenic activity of 2-NA and 2-AF and with the failure of 1-NA to induce tumors in experimental animals. One caveat, however, should be noted. PHS, which has recently been found to be present in the urinary bladder (16), can catalyze the *N*- and ring oxidation of 2-NA and the oxidation of 2-AF to DNA-bound products (17, 18). For 2-NA, this pathway is estimated to account for about 30% of the DNA binding in the dog urinary bladder, while the remainder can be attributed to hepatic *N*-oxidation and transport of proximate carcinogenic *N*-hydroxy-*N*-glucuronides (15, 26, 50). Thus, comparable studies with 1-NA and dog and human bladder PHS will be required to determine if 1-NA can be *N*-hydroxylated to an ultimate carcinogen by this metabolic pathway.

The critical involvement of hepatic monooxygenases in carcinogen metabolism and the possibility of genetic polymorphism in humans has stimulated considerable research interest in identification of the specific enzymes that catalyze activation and detoxification reactions (12, 14, 28, 51–53). In this study, high rates of 2-AF *N*-oxidation and ring oxidation of 1-NA and 2-NA were observed with purified P-450$_{ISF/BNF-G}$ and P-450$_{BNF-B}$, followed by P-450$_{PB-B}$ and P-450$_{PB-D}$. Also effective for these pathways was the purified pig hepatic FMO, although substrate concentrations were nonsaturating. However, the only hepatic monooxygenase that was active for 2-NA *N*-oxidation was the ISF-induced, high-spin form of cytochrome P-450$_{ISF/BNF-G}$. The high selectivity of this isozyme for arylamine *N*-oxidation has been noted previously for 4-aminobiphenyl and for several mutagenic heterocyclic arylamines, although an isozyme corresponding to P-450$_{BNF-B}$ was also active (54). These findings are consistent with preliminary studies in our laboratory on the effects of enzyme inducers on 2-NA, 1-NA, and 2-AF metabolism by rat liver microsomes. Accordingly, 2-AF *N*-oxidation and ring oxidation of 1-NA and 2-NA were increased by 2- to 5-fold by pretreatment with PB, BNF, or ISF, but not PCN; while 2-NA *N*-oxidation is increased only by ISF (10-fold) and BNF (3-fold).[4] Furthermore, the catalytic selectivities of the different rat monooxygenases were consistent with the hypothesis that the differences in oxidative metabolism with the human liver samples, even when expressed per nmol P-450 (Table 2), may be a function of enzyme induction and/or genetic polymorphism.

The mechanisms of enzymatic oxidation of arylamines by P-450s, FMO, and PHS pose an interesting and important problem. All 3 enzyme systems are known to catalyze the *N*-oxidation of 2-AF and ring oxidation of 2-NA and 1-NA, while 2-NA *N*-oxidation is mediated only by cytochrome P-450$_{ISF/BNF-G}$ and by extrahepatic PHS. For each of these enzymes, numerous studies have demonstrated that an activated oxygen species functions at the catalytic site to oxidize a variety of substrates (55–57). For nitrogen and carbon hydroxylation, 3 general mechanisms (Chart 1) have received the most attention: Mechanism a, an "addition-rearrangement" (58–61); Mechanism b, a "one-electron transfer" (57–59, 62–64); and Mechanism c, a 2-electron transfer" (61, 62, 65). In addition, the catalytic efficiency of substrate oxidation is also considered to reflect the conformational constraints imposed by enzyme-substrate active sites, the oxidation potential of the substrate (57, 58, 66) or enzyme (67), and the thermodynamic stabilities of intermediates or products (61). However, the relative distribution of *N*-hydroxylated or ring-hydroxylated products should be dictated by the type of intermediate formed (Chart 1) just prior to substrate-oxygen bond formation and by the electronic charge distribution of the nitrogen or carbon atom being hydroxylated. For an "addition-rearrangement" mechanism, the product distribution would reflect the electron density (nucleophilicity) at the nitrogen and ring-carbon atoms of the parent arylamine; while in the "one- and 2-electron transfer" mechanisms, the product distribution would reflect the positive charge (electrophilicity) localization at nitrogen and ring-carbon atoms in the neutral radical and cation intermediates, respectively.

The charge distribution in each of these species can be estimated from Hückel molecular orbital calculations (68, 69), the results of which are shown in Table 4. In the parent arylamines, the greatest positive charge resides on the amine nitrogens, with a relative ordering of 1-NA > 2-AF > 2-NA. Since the "addition-rearrangement" mechanism (Chart 1*a*) involves an activated oxygen attacking an electron-rich center, these results predict that substitution would not occur at the nitrogens, but rather only on the ring carbons, which does not correspond to our experimental observations for 2-NA and 2-AF (Tables 1 and 3). In the "one-electron transfer" model (Chart 1*b*), an activated oxygen will react preferentially with electron-deficient atoms. Since the Hückel calculations indicate that the nitrogens in the radical intermediates are electron rich (Table 4), this mechanism is also inconsistent with any *N*-oxidation of these arylamines. In the "2-electron transfer" mechanism (Chart 1*c*), a hydroxide anion will combine with the most electron-deficient centers of the nitrenium-carbenium cation intermediates. As shown in Table 4, the charge densities for the substrate cations obtained from the Hückel calculations correspond reasonably well with the experimental observation; *i.e.*, the amine nitrogens of the cations of 2-NA and 2-AF are more electrophilic than the nitrogen in the 1-NA cation which agrees with the finding that *N*-oxidation occurs with 2-NA and 2-AF but not with 1-NA. With the cation of 2-AF, the most electron-deficient centers are carbon 4a and the nitrogen; thus, oxidation should occur at both these positions. Oxidation products were not detected at carbon 4a, but this is

[4] Y. Yamazoe, G. J. Hammons, and F. F. Kadlubar, unpublished data.

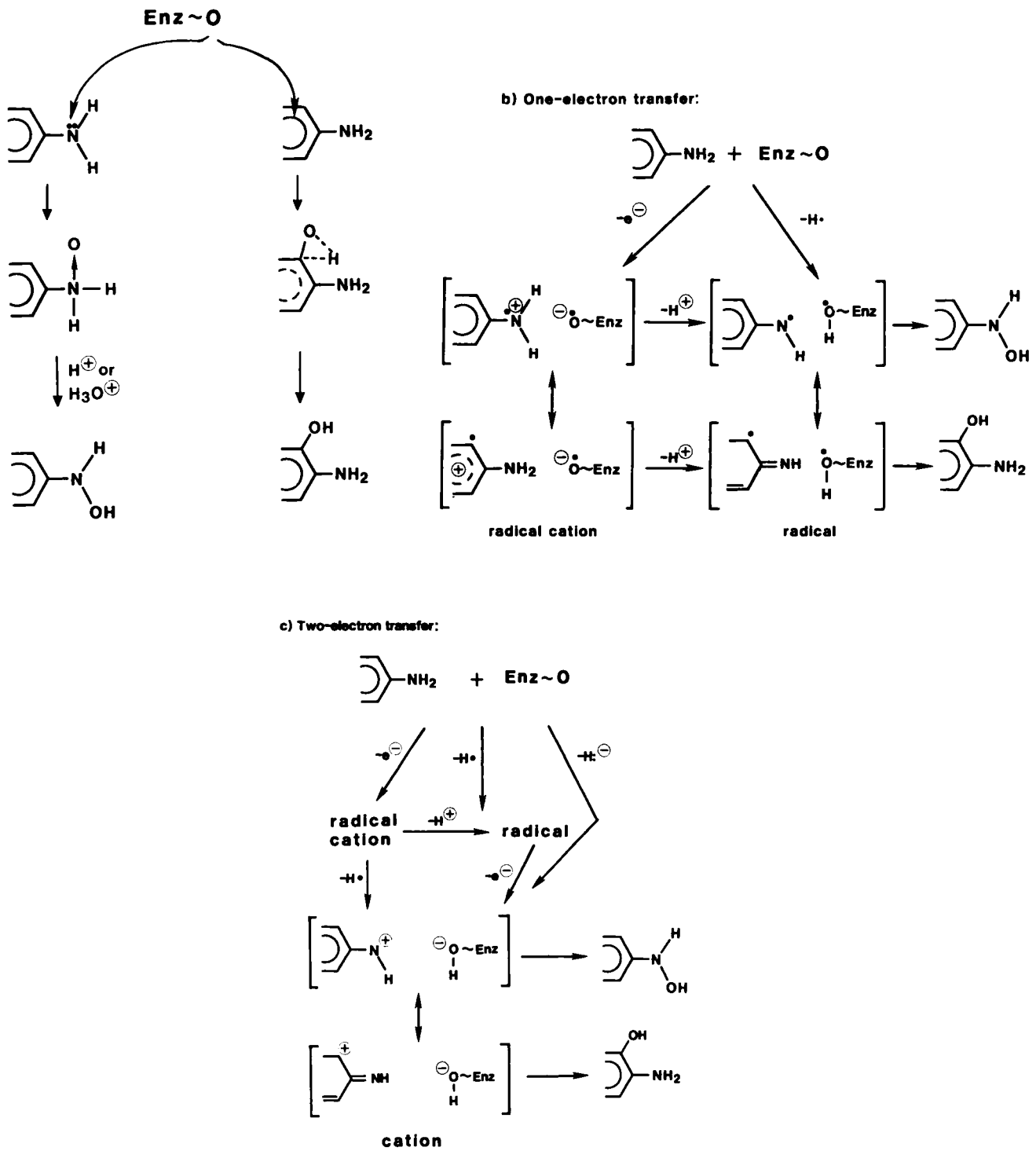



Chart 1. Proposed mechanisms for enzymatic oxidation of arylamines: addition-rearrangement (*a*); one-electron transfer (*b*); and 2-electron transfer (*c*). *Enz~O* signifies enzyme-activated oxygen complex.

presumably due to thermodynamic control of the transition state which would be highly unfavorable due to loss of aromaticity. In this regard, a 4-hydroxy product is observed upon solvolysis of *N*-acetoxy-2-acetylaminofluorene which proceeds by way of a kinetically controlled nitrenium-carbenium ion transition state (70, 71). The most electron-deficient centers in the 1-NA cation are at C-2 and C-4. Since oxidation occurred only at C-2, this may indicate the existence of steric constraints in the enzyme-substrate complex. For the 2-NA cation, C-1 was the most electron-deficient atom; and with the exception of rat P-$450_{ISF/BNF-G}$, the only site of oxidation occurred at this position. It is possible, however, that this isozyme could form an additional ligand with

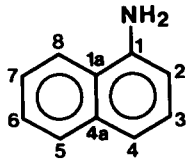
Table 4

*Charge distribution[a] in the parent arylamines and their neutral radical and cation derivatives*

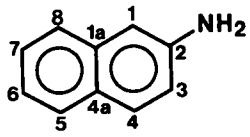
| | Arylamine | | | Neutral radical | | | Cation | | |
|---|---|---|---|---|---|---|---|---|---|
| Atom[b] | 1-NA | 2-NA | 2-AF[c] | 1-NA | 2-NA | 2-AF[c] | 1-NA | 2-NA | 2-AF[c] |
| N | 0.141 | 0.129 | 0.131 | −0.296 | −0.264 | −0.263 | 0.081 | 0.187 | 0.180 |
| 1 | 0.084 | −0.104 | −0.070 | 0.090 | 0.080 | 0.020 | 0.121 | 0.332 | 0.146 |
| 1a | −0.036 | 0.003 | 0.006 | −0.005 | 0.004 | 0.012 | 0.039 | 0.005 | 0.019 |
| 2 | −0.102 | 0.070 | 0.072 | 0.037 | 0.076 | 0.078 | 0.233 | 0.103 | 0.107 |
| 3 | 0.007 | −0.045 | −0.070 | 0.013 | −0.014 | 0.020 | 0.020 | 0.031 | 0.146 |
| 4 | −0.077 | 0.007 | 0.006 | 0.073 | 0.020 | 0.012 | 0.285 | 0.033 | 0.019 |
| 4a | 0.001 | −0.026 | −0.049 | 0.001 | 0.014 | 0.052 | 0.001 | 0.072 | 0.192 |
| 5 | −0.014 | 0.003 | −0.010 | 0.032 | 0.012 | 0.018 | 0.094 | 0.021 | 0.057 |
| 5a | | | 0.003 | | | 0.008 | | | 0.013 |
| 6 | 0.003 | −0.019 | 0.000 | 0.008 | 0.033 | 0.001 | 0.012 | 0.103 | 0.002 |
| 7 | −0.018 | 0.000 | −0.009 | 0.021 | 0.001 | 0.022 | 0.076 | 0.002 | 0.063 |
| 8 | 0.011 | −0.018 | 0.000 | 0.024 | 0.038 | 0.001 | 0.038 | 0.111 | 0.002 |
| 8a | | | −0.010 | | | 0.018 | | | 0.057 |

[a] Calculations were performed using the simple Hückel method. The parameters for $n$ of the diagonal elements (68), $\alpha + n\beta$, were: C(H) = 0; N(H) = 0.5; N($H_2$) = 1.5. The parameters for $m$ of the nondiagonal elements (68), $m\beta$, were: C—C = 1.0; C—N = 1.0.

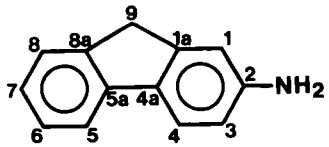
[b] The numbering system used is:




1-NA




2-NA




2-AF

[c] In these approximations, C-9 of 2-AF is not explicitly considered, but rather the calculations were performed on a 4-aminobiphenyl $\pi$ system. We have shown previously (69) that this approximation gives reasonable values for the reactivity of analogous compounds.

the substrate nitrogen or otherwise result in an altered electron distribution (increased positive charge on the nitrogen) with a concomitant altering of the ratio of oxidized products.

From these data and theoretical considerations, we suggest an overall "2-electron transfer" mechanism for the metabolic oxidation of these arylamines to *N*-hydroxylated and ring-hydroxylated products. For cytochrome P-450 and PHS, this pathway may involve consecutive "one-electron transfers" prior to the oxygen rebound.

## REFERENCES


1. Kriek, E. Aromatic amines and related compounds as carcinogenic hazards to man. *In:* P. Emmelot and E. Kriek (eds.), Environmental Carcinogenesis, pp. 143–164. Amsterdam: Elsevier/North-Holland Biomedical Press, 1979.
2. Radomski, J. L. The primary aromatic amines, their biological properties and structure-activity relationships. Annu. Rev. Pharmacol. Toxicol., *19:* 129–157, 1979.
3. Patrianakos, C., and Hoffmann, D. Chemical studies on tobacco smoke. LXIV. On the analysis of aromatic amines in cigarette smoke. J. Anal. Toxicol., *3:* 150–154, 1979.
4. Later, D. W., Andros, T. G., and Lee, M. L. Isolation and identification of amino polycyclic aromatic hydrocarbons from coal-derived products. Anal. Chem., *55:* 2126–2132, 1983.
5. Morris, H. P. Hepatocarcinogenesis by 2-acetylaminofluorene and related compounds including comments on dietary and other influences. J. Natl. Cancer Inst., *15:* 1535–1545, 1955.
6. Radomski, J. L., Deichmann, W. B., Altman, N. H., and Radomski, T. Failure of pure 1-naphthylamine to induce bladder tumors in dogs. Cancer Res., *40:* 3537–3539, 1980.
7. Miller, J. A., and Miller, E. C. The metabolic activation of carcinogenic aromatic amines and amides. Prog. Exp. Tumor Res., *11:* 273–301, 1969.
8. Uehleke, H. The role of cytochrome P-450 in the *N*-oxidation of individual amines. Drug Metab. Dispos., *1:* 299–313, 1973.
9. Kadlubar, F. F., Miller, J. A., and Miller, E. C. Microsomal *N*-oxidation of the hepatocarcinogen *N*-methyl-4-aminoazobenzene and the reactivity of *N*-hydroxy-*N*-methyl-4-aminoazobenzene. Cancer Res., *36:* 1196–1206, 1976.
10. Ziegler, D. M. Microsomal oxidases. *In:* S. Fleischer, Y. Hatefi, D. H. MacLennan, and A. Tzagoloff (eds.), The Molecular Biology of Membranes, pp. 193–204. New York: Plenum Publishing Corp., 1978.
11. McMahon, R. E., Turner, J. C., and Whitaker, G. W. The *N*-hydroxylation and ring-hydroxylation of 4-aminobiphenyl *in vitro* by hepatic microoxygenases from rat, mouse, hamster, rabbit and guinea pig. Xenobiotica, *10:* 469–481, 1980.
12. Frederick, C. B., Mays, J. B., Ziegler, D. M., Guengerich, F. P., and Kadlubar, F. F. Cytochrome P-450- and flavin-containing monooxygenase-catalyzed formation of the carcinogen *N*-hydroxy-2-aminofluorene and its covalent binding to nuclear DNA. Cancer Res., *42:* 2671–2677, 1982.
13. Razzouk, C., and Roberfroid, M. B. Species differences in the biochemical properties of liver microsomal arylamine and arylamide *N*-hydroxylases. Chem.-Biol. Interact. *41:* 251–264, 1982.
14. Kato, R., Kamataki, T., and Yamazoe, Y. *N*-Hydroxylation of carcinogenic and mutagenic aromatic amines. Environ. Health Perspect., *49:* 21–25, 1983.
15. Poupko, J. M., Radomski, T., Santella, R. M., and Radomski, J. L. Organ, species and compound specificity in the metabolic activation of primary aromatic amines. J. Natl. Cancer Inst., *70:* 1077–1080, 1983.
16. Wise, R. W., Zenser, T. V., Kadlubar, F. F., and Davis, B. B. Metabolic activation of carcinogenic aromatic amines by dog bladder and kidney prostaglandin H synthase. Cancer Res., *44:* 1893–1897, 1984.
17. Kadlubar, F. F., Frederick, C. B., Weis, C. C., and Zenser, T. V. Prostaglandin endoperoxide synthetase-mediated metabolism of carcinogenic aromatic amines and their binding to DNA and protein. Biochem. Biophys. Res. Commun., *108:* 253–258, 1982.
18. Boyd, J. A., Harvan, D. J., and Eling, T. E. The oxidation of 2-aminofluorene

by prostaglandin endoperoxide synthetase. J. Biol. Chem., *258:* 8246–8254, 1983.
19. Ziegler, D. M., McKee, E. M., and Poulson, L. L. Microsomal flavoprotein-catalyzed *N*-oxidation of arylamines. Drug. Metab. Dispos., *1:* 314–321, 1973.
20. Nakayama, T., Kodama, M., Nagata, C. Electron spin resonance study in the metabolism of 2-naphthylamine and 1-naphthylamine in rat liver microsomes. Gann, *73:* 382–390, 1982.
21. Dooley, K. L., Beland, F. A., Bucci, T. J., and Kadlubar, F. F. Local carcinogenicity, rates of absorption, extent and persistence of macromolecular binding, and acute histopathological effects of *N*-hydroxy-1-naphthylamine and *N*-hydroxy-2-naphthylamine. Cancer Res., *44:* 1172–1177, 1984.
22. Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., and Kaminsky, L. S. Purification and characterization of liver microsomal cytochromes P-450; electrophoretic, spectra, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. Biochemistry, *21:* 6019–6030, 1982.
23. Willstätter, R., and Kubli, H. Uber die Reduction van Nitroverbindungen nach der Methode von Zinin. Chem. Ber., *41:* 1936–1940, 1908.
24. Brill, E. Diethyl azodicarboxylate oxidation of some carcinogenic arylhydroxylamines to nitroso derivatives. Experientia (Basel), *25:* 680, 1961.
25. Lotlikar, P. D., Miller, E. C., Miller, J. A., and Margreth, A. The enzymatic reduction of the *N*-hydroxy derivatives of 2-acetylaminofluorene and related carcinogens by tissue preparations. Cancer Res., *25:* 1743–1752, 1965.
26. Kadlubar, F. F., Unruh, L. E., Flammang, T. J., Sparks, D., Mitchum, R. K., and Mulder, G. J. Alteration of urinary levels of the carcinogen, *N*-hydroxy-2-naphthylamine, and its *N*-glucuronide in the rat by control of urinary pH, inhibition of metabolic sulfation, and changes in biliary excretion. Chem.-Biol. Interact., *33:* 129–147, 1981.
27. Martin, C. N., Beland, F. A., Roth, R. W., and Kadlubar, F. F. Covalent binding of benzidine and *N*-acetylbenzidine to DNA at the C-8 atom of deoxyguanosine *in vivo* and *in vitro*. Cancer Res., *42:* 2678–2696, 1982.
28. Wang, P. D., Beaune, P., Kaminsky, L. S., Dannan, G. A., Kadlubar, F. F., Larrey, D., and Guengerich, F. P. Purification and characterization of six cytochrome P-450 isozymes from human liver microsomes. Biochemistry, *22:* 5375–5383, 1983.
29. Wang, P., Mason, P. S., and Guengerich, F. P. Purification of human liver cytochrome P-450 and comparison to the enzyme involved from rat liver. Arch. Biochem. Biophys., *199:* 206–219, 1980.
30. Gornall, A. G., Bardawill, C. J., and David, M. M. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., *177:* 751–766, 1949.
31. Yasukochi, Y., and Masters, B. S. S. Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. J. Biol. Chem., *251:* 5337–5344, 1976.
32. Omura, T., and Sato, R. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem., *239:* 2370–2378, 1964.
33. Corbett, M. D., and Chipko, B. R. Quantitative determination of *N*-arylaceto- and *N*-arylglycohydroxamic acids in biochemical reactions. Anal. Biochem., *98:* 169–177, 1979.
34. Frederick, C. B., Mays, J. B., and Kadlubar, F. F. A chromatographic technique for the analysis of oxidized metabolites: application to carcinogenic *N*-hydroxyarylamines in urine. Anal. Biochem., *118:* 120–125, 1981.
35. Smith, C. L., and Thorgeirsson, S. S. An improved high pressure liquid chromatography assay for 2-acetylaminofluorene and eight of its metabolites. Anal. Biochem., *113:* 62–67, 1981.
36. Fisher, G. J., Fukushima, H., and Gaylor, J. L. Isolation, purification, and properties of a unique form of cytochrome P-450 in microsomes of isosafrole-treated rats. J. Biol. Chem., *256:* 4388–4394, 1981.
37. Larrey, D., Distlerath, L. M., Dannan, G. A., Wilkinson, G. R., and Guengerich, F. P. Purification and characterization of rat liver microsomal cytochrome P-450 involved in the 4-hydroxylation of debrisoquine, a prototype for genetic variation in oxidative drug metabolism. Biochemistry, *23:* 2787–2795, 1984.
38. Kato, R. Characteristics and differences in the hepatic mixed function oxidases of different species. Pharmacol. Ther., *6:* 41–98, 1979.
39. Mulder, G. J., Unruh, L. E., Evans, F. E., Ketterer, B., and Kadlubar, F. F. Formation and identification of glutathione conjugates from 2-nitrosofluorene and *N*-hydroxy-2-aminofluorene. Chem.-Biol. Interact., *39:* 111–127, 1982.
40. Troll, W., and Nelson, N. *N*-Hydroxy-2-naphthylamine, a urinary metabolite of 2-naphthylamine in man. Fed. Proc., *20:* 41, 1961.
41. Boyland, E., and Manson, D. The biochemistry of aromatic amines: the metabolism of 2-naphthylamine and 2-naphthylhydroxylamine derivatives. Biochem. J., *101:* 84–102, 1963.
42. Radomski, J. L., Conzelman, G. M., Jr., Rey, A. A., and Brill, E. *N*-Oxidation of certain aromatic urinary acetamides and nitro compounds by monkeys and dogs. J. Natl. Cancer Inst., *50:* 989–995, 1973.
43. Uehleke, H. *N*-Hydroxylation of carcinogenic amines *in vivo* and *in vitro* with liver microsomes. Biochem. Pharmacol., *12:* 219–221, 1962.
44. Poulsen, L. L., Masters, B. S. S., and Ziegler, D. M. Mechanism of 2-naphthylamine oxidation catalyzed by pig liver microsomes. Xenobiotica, *6:* 481–498, 1976.
45. Brill, E., and Radomski, J. L. Comparision of the *in vitro* and *in vivo* *N*-oxidation of carcinogenic aromatic amines. Xenobiotica, *1:* 347–348, 1971.
46. Kiese, M., Renner, G., and Wiedemann, I. *N*-Hydroxylation of 2-aminofluorene in the guinea pig and by guinea pig liver microsomes *in vitro*. Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol., *252:* 418–423, 1966.
47. Phillipson, C. E., and Ioannides, C. Activation of aromatic amines to mutagens by various species including man. Mutat. Res., *124:* 325–336, 1983.
48. Mulder, G. J., and Meerman, J. H. N. Sulfation and glucuronidation as competing pathways in the metabolism of hydroxamic acids: the role of *N,O*-sulfonation in chemical carcinogenesis. Environ. Health Perspect., *49:* 27–32, 1983.
49. Ketterer, B., Coles, B., and Meyer, D. J. The role of glutathione in detoxification. Environ. Health Perspect., *49:* 59–69, 1983.
50. Yamazoe, Y., Miller, D. W., Gupta, R. C., Zenser, T. V., Weis, C. C., and Kadlubar, F. F. DNA adducts formed by prostaglandin H synthase-mediated activation of carcinogenic arylamines. Proc. Am. Assoc. Cancer Res., *25:* 356, 1984.
51. Masson, H. A., Ioannides, C., Gorrod, J. W., and Gibson, C. G. The role of highly purified cytochrome P-450 isozymes in the activation of 4-aminobiphenyl to mutagenic products in the Ames test. Carcinogenesis (Lond.), *4:* 1583–1586, 1983.
52. Lotlikar, P. D., Pandey, R. N., Clearfield, M. S., and Paik, S. M. Oxidative activation and inactivation of the carcinogen 2-acetylaminofluorene by various purified cytochromes P-450 from 3-methylcholanthrene-pretreated rats. Toxicol. Lett., *21:* 111–118, 1984.
53. Prough, R. A., Brown, M. I., Dannan, G. A., and Guengerich, F. P. Major isozymes of rat liver microsomal cytochrome P-450 involved in the *N*-oxidation of *N*-isopropyl-2-(2-methylazo)-*p*-toluamide, the azo derivative of procarbazine. Cancer Res., *44:* 543–548, 1984.
54. Kamataki, T., Maeda, K., Yamazoe, Y., Matsuda, N., Ishii, K., and Kato, R. A high spin form of cytochrome P-450 highly purified from PCB-treated rats: catalytic characterization and immunological quantitation in liver microsomes. Mol. Pharmacol., *24:* 146–155, 1983.
55. Poulsen, L. L., and Ziegler, D. M. The liver microsomal FAD-containing monooxygenase. Spectral characterization and kinetics studies. J. Biol. Chem., *254:* 6449–6455, 1979.
56. Samuelsson, B. Prostaglandins, thromboxanes and leukotrienes. *In:* T. J. Powles, R. S. Bockman, K. V. Honn, and P. Ramwell (eds.), Prostaglandin and Cancer: First International Conference, pp. 1–19. New York: Alan R. Liss, Inc., 1982.
57. Guengerich, F. P., and MacDonald, T. L. Chemical mechanisms of catalysis by cytochromes P-450: a unified view. Accts. Chem. Res., *17:* 9–16, 1984.
58. Gorrod, J. W. The current status of the $pK_a$ concept in the differentiation of enzyme *N*-oxidation. *In:* J. W. Gorrod (ed.), Biological Oxidation of Nitrogen, pp. 201–210. Amsterdam: Elsevier Publishing Co., 1978.
59. Shea, J. P., Nelson, S. D., and Ford, G. P. MNDO calculations of kinetic isotope effects in model cytochrome P-450 oxidations. J. Am. Chem. Soc., *105:* 5451–5454, 1983.
60. Hanzlik, R. P., Hogberg, K., and Judson, C. M. Microsomal hydroxylation of specifically deuterated monosubstituted benzenes. Evidence for direct aromatic hydroxylation. Biochemistry, *23:* 3048–3055, 1984.
61. Pack, G. R., and Lowe, G. H. Semiempirical studies of the mechanism of models for the *N*-hydroxylation of amines by cytochrome P-450. Int. J. Quantum Chem. Symp., *6:* 381–390, 1979.
62. Miwa, G. T., Walsh, J. S., Kedderis, G. L., and Hollenberg, P. The use of intramolecular isotope effects to distinguish between deprotonation and hydrogen atom abstraction mechanisms in cytochrome P-450- and peroxidase-catalyzed *N*-demethylation reactions. J. Biol. Chem., *258:* 1445–1449, 1983.
63. Groves, J. T., and Subramanian, D. V. Hydroxylation by cytochrome P-450 and metalloporphyrin models. Evidence for allylic rearrangement. J. Am. Chem. Soc., *106:* 2177–2181, 1984.
64. Wagner, W. R., Spero, D. M., and Rastetter, W. H. Oxygen transfer from oxaziridines: a chemical model for flavin-dependent monooxygenases. J. Am. Chem. Soc., *106:* 1476–1480, 1984.
65. Nguyen, T.-L., Gruenke, L. D., and Castagnoli, N., Jr. Metabolic oxidation of nicotine to chemically reactive intermediates. J. Med. Chem., *22:* 259–263, 1979.
66. Cavalieri, E., Rogan, E., and Roth, R. Multiple mechanisms of activation in aromatic hydrocarbon carcinogenesis. *In:* R. A. Floyd (ed.), Free Radicals and Cancer, pp. 117–158. New York: Marcel Dekker, Inc., 1982.
67. Sligar, S. G. Coupling of spin, substrate and redox equilibria in cytochrome P-450. Biochemistry, *15:* 5399–5406, 1976.
68. Streitwieser, A., Jr. Molecular Orbital Theory for Organic Chemists. New York: John Wiley and Son, Inc., 1961.
69. Fu, P. P., Harvey, R. H., and Beland, F. A. Molecular orbital theoretical prediction of the isomeric products formed from reactions of arene oxides and related metabolites of polycyclic aromatic hydrocarbons. Tetrahedron, *34:* 857–866, 1978.
70. Scribner, J. D. Conversion of the carcinogen *N*-acetoxy-2-acetamidofluorene to 4-hydroxy-2-acetamidofluorene. J. Am. Chem. Soc., *99:* 7384–7395, 1977.
71. Underwood, G. R., and Kirsch, R. B. The solvolysis of *N*-acetoxy-2-acetylaminofluorene and *N*-acetoxy-4-acetylaminobiphenyl: delicate balance between nitrenium ion formation and hydrolysis. J. Chem. Soc. Chem. Commun., 136–138, 1985.