RAT LIVER CYTOCHROME P450 METABOLISM OF N-ACETYLBENZIDINE AND N,N'-DIACETYLBENZIDINE

VIJAYA M. LAKSHMI, TERRY V. ZENGER, AND BERNARD B. DAVIS

Veterans Administration Medical Center, and Department of Biochemistry and Division of Geriatric Medicine, St. Louis University School of Medicine

(Received September 24, 1996; accepted January 20, 1997)

ABSTRACT:

To provide the information necessary for assessing risk and preventing tumorigenesis, the metabolism of N-acetylbenzidine and N,N'-diacetylbenezidine was assessed with rat liver microsomes from control and β-naphthoflavone-treated rats. The oxidation of [3H]N-acetylbenzidine to [3H]N-hydroxy-N-acetylbenzidine (NHA), [3H]N-hydroxy-N-acetylbenzidine (NHA), and [3H]-ring oxidation products was assessed. For [3H]N,N'-diacetylbenezidine, the formation of [3H]N-hydroxy-N,N'-diacetylbenezidine (NHDA) and the [3H]-ring oxidation product was assessed. With β-naphthoflavone-treated microsomes, the rate of NHA formation was 8-fold more than observed with control. Although significant formation of ring-oxidation products was demonstrated, the formation of NHA was at the limit of detection. With control microsomes, NHA was a major metabolite with more NHA (49 ± 6 pmol/mg protein/min) produced than NHA (38 ± 5). Whereas the oxidation of N,N'-diacetylbenezidine was not observed with control microsomes, significant formation of NHDA (421 ± 49 pmol/mg protein/min) and ring-oxidation (182 ± 28) product was observed with β-naphthoflavone-treated microsomes. Metabolism of [3H]N-acetylbenzidine and [3H]N,N'-diacetylbenezidine by β-naphthoflavone-treated microsomes was completely inhibited by the specific cytochrome P4501A1/1A2 inhibitors α-naphthoflavone and ellipticine at 10 μM. Except for the <30% inhibition observed with the cytochrome P4502E1 inhibitor (disulfiram), inhibitors of cytochrome P4503A1/3A2 (troleandomycin) and P4502C6 (sulfinpyrazone) were not effective at 10 μM. NHA formation by control microsomes was not prevented by any of these inhibitors. Conditions that inhibit flavin-dependent monooxygenase metabolism, methimazole (1 mM), and heat treatment (37°C for 60 min) were also ineffective in preventing NHA formation. The nonspecific cytochrome P450 inhibitor SKF-525A (10 μM) exhibited a partial dose–response inhibition (maximum 41% of complete reaction mixture) of NHA formation, but did not alter NHA formation. In contrast, the nonspecific cytochrome P450 inhibitor, 2,4-dichloro-6-phenylphenoxyethylamine prevented formation of both NHA and NHA. β-Naphthoflavone treatment increased [3H]N-acetylbenzidine binding to DNA, but not [3H]N,N'-diacetylbenezidine. Binding of both compounds to DNA was inhibited by ellipticine. N'-([3-monophospho-deoxyguanosin-8-yl]-N-acetylbenzidine was detected by 32P-postlabeling in microsomal incubations with N-acetylbenzidine, but not N,N'-diacetylbenezidine. More adduct was detected with control than β-naphthoflavone-treated microsomes. Results are consistent with cytochrome P4501A1/1A2 playing the major role in N-acetylbenzidine and N,N'-diacetylbenezidine metabolism by liver microsomes from control and β-naphthoflavone-treated rats. The formation of NHA by control, but not by β-naphthoflavone-treated, rats and its insensitivity to inhibition by cytochrome P4501A1/1A2 inhibitors were unexpected.

Hepatic N- and ring-oxidations are important activation and inactivation steps in aromatic amine-induced toxicities. NADPH-dependent oxidation of N-acetylbenzidine occurs with rat and mouse liver microsomes (1). In mouse, the formation of NHA was 4 times greater than NHA. In rat, the formation of both products was about equal. Ring-oxidation products were considered minor and were not quantitated. The N-oxidation of N,N'-diacetylbenezidine to NHDA was much less than N-acetylbenzidine in both species. The specific oxidative pathways involved in the metabolism of these compounds were not determined. In both humans and rodents, benzidine is rapidly acetylated to N-acetylbenzidine and N,N'-diacetylbenezidine (2–4). Workers exposed to high levels of benzidine have as much as a 100-fold increased risk for bladder cancer (5). Benzidine causes bladder cancer in dogs and liver cancer in rats (6, 7).

Hepatic N- and ring-oxidations are important activation and inactivation steps in aromatic amine-induced toxicities. NADPH-dependent oxidation of N-acetylbenzidine occurs with rat and mouse liver microsomes (1). In mouse, the formation of NHA was 4 times greater than NHA. In rat, the formation of both products was about equal. Ring-oxidation products were considered minor and were not quantitated. The N-oxidation of N,N'-diacetylbenezidine to NHDA was much less than N-acetylbenzidine in both species. The specific oxidative pathways involved in the metabolism of these compounds were not determined. In both humans and rodents, benzidine is rapidly acetylated to N-acetylbenzidine and N,N'-diacetylbenezidine (2–4). Workers exposed to high levels of benzidine have as much as a 100-fold increased risk for bladder cancer (5). Benzidine causes bladder cancer in dogs and liver cancer in rats (6, 7).

Rats, mice, or hamsters administered either benzidine or N-acetylbenzidine exhibit only a single adduct in liver, N'-[deoxyguanosin-8-yl]-N-acetylbenzidine (8, 9). This is also the major adduct detected in rat liver after administration of N,N'-diacetylbenezidine (9) and in exfoliated bladder cells from workers exposed to benzidine (10). One possible mechanism of N'-[deoxyguanosin-8-yl]-N-acetylbenzidine formation may involve cytochrome P450 oxidation of N-acetylbenzidine to NHA with subsequent O-acetylation to its unstable N-acetoxy ester that reacts with DNA to form the adduct. Alternatively, N,N'-diacetylbenezidine may be oxidized to NHDA and undergo intramolecular N,O-acetyltransfer to form this adduct (11, 12).

Understanding specific enzymes and the sequential steps involved in metabolism of chemicals are important for assessing chemical risk and preventing carcinogenicity. For example, by using specific cytochrome P450 inducers and inhibitors, aflatoxin B1 was demonstrated to undergo metabolic activation in rat by cytochrome P4503A2 and P4502C11 to aflatoxin-8,9-oxide (13, 14). Because pretreatment of rats with β-naphthoflavone, a specific P4501A1/1A2 inducer, dramat-
Reactions were linear with respect to protein concentration (0.5–1.5 mg/ml for concentration) and incubated (final volume of 0.1 ml) for 30 min at 37°C. 4-amino-4′-nitrobenzidine was synthesized from 4-trifluoroacetamido-4′-nitrobenzidine. Conversion of this product to N′-H' or NHDA was accomplished using a modification of a published procedure (17). N′-H' was synthesized from trifluorotoluamidom-4′-nitrobenzyl that was prepared from 4-amino-4′-nitrobiphenyl by trifluoracetylation. After conversion of the nitro group of 4-trifluorotoluamidom-4′-nitrobiphenyl to N′-hydroxy-N-acetamido, the latter compound was hydrolyzed to remove the trifluoracetyl group forming N′-H'. The identity and purity (>95%) of these synthetic compounds were established by TLC, NMR, and MS. Ring-oxidation products were prepared from 3-OH-benzidine, which was characterized by NMR and MS (18). To prepare 3-OH,N′,N,N′-diacetylbenzidine, 3-OH-benzidine was acetylated in pyridine/acetic anhydride, purified, and identified by MS (11). Ring-oxidation products of N-acetylbenzidine were prepared by treatment of 3-OH,N′-diacetylbenzidine with porcine liver carboxylesterase (Sigma Chemical Co.). Alternatively, ring-oxidation products of N-acetylbenzidine were prepared by direct chemical synthesis from 4-amino-4′-nitrobiphenyl after reaction with potassium persulfate. Male Fischer 344 rats (150–200 g) were purchased from Harlan Industries (Indianapolis, IN).

NADPH-Dependent Oxidation. Microsomes were prepared from control or β-naphthoflavone-treated rats (19). Treated rats received 40 mg/kg β-naphthoflavone in corn oil ip, once a day for three consecutive days (20). Control rats received only corn oil. Animals were euthanized by CO2 asphyxiation and thioflavine in corn oil ip, once a day for three consecutive days (20). Control animals were treated with RNase followed by sodium dodecyl sulfate and proteinase K. After extraction with phenol and then chloroform:isoamyl alcohol (24:1) (21), the aqueous fraction was quickly adjusted to pH 7.4 with 0.08 ml of 1.0 M NaOH, and extracted twice with 1.6 ml of CHCl3 saturated with 0.1 M NaOH. The aqueous fraction was adjusted to a final concentration of 0.25 M NaCl, cold ethanol added, and nucleic acid precipitated at 20°C overnight. Pellets were washed with 70% ethanol and dissolved in H2O. Purity of DNA was determined by absorbance at 260 and 280 nm, with a ratio of A260/A280 of 1.7 achieved for each sample. The radioactivity bound to DNA was determined. To detect adducts by 32P-postlabeling, the DNA was digested with micrococcal nuclease and spleen phosphodiesterase to 3′-monophosphate deoxynucleotides, and analyzed as previously described (22). Samples were enriched by n-butanol extraction and separated on PEI-cellulose sheets (23). Chromatographic conditions were selected to detect N(3′)-monophosphate deoxyguanosin-8-yl)-benzidine which was recently characterized (24).

β-Naphthoflavone treatment resulted in a substantial increase in [3H]-N-acetylbenzidine metabolism (table 1). The rate of NHA formation increased 8-fold, and the formation of ring oxidation products was demonstrated. In contrast, N′-H' formation was at the limit of detection (<1% of total HPLC recovered radioactivity) with

### Materials and Methods

[3H]Benzidine (189 mCi/mmol) was purchased from Chemsys (Lenexa, KS). Benzidine free base and hydrochloride salt, NADPH, β-naphthoflavone, diethylenetriaminepentacetic acid, ascorbic acid, and EDTA were bought from Sigma Chemical Co. (St. Louis, MO). DPEA was a gift from Eli Lilly Laboratories (Indianapolis, IN). Furafylline was purchased from Gentest Corp. (Woburn, MA), whereas the other cytochrome P450 inhibitors were bought from Sigma Chemical Co. N-Acetylbenzidine was synthesized by acetylation of benzidine in glacial acetic acid and N,N′-diacetylbenzidine was synthesized by acetylation of benzidine with acetic anhydride as previously described (16). N′-H', NHA, and NHDA were synthesized by Dr. Shu Wen Li (Department of Biochemistry, St. Louis University Medical School, St. Louis, MO). 4-Amino-4′-nitrophenyl (TCl America, Portland, OR) was acetylated to 4-acetamidom-4′-nitrophenyl. Conversion of this product to N′-H' or NHDA was accomplished using a modification of a published procedure (17). N′-H' was synthesized from trifluorotoluamidom-4′-nitrobenzyl that was prepared from 4-amino-4′-nitrobiphenyl by trifluoracetylation. After conversion of the nitro group of 4-trifluorotoluamidom-4′-nitrobiphenyl to N′-hydroxy-N-acetamido, the latter compound was hydrolyzed to remove the trifluoracetyl group forming N′-H'. The identity and purity (>95%) of these synthetic compounds were established by TLC, NMR, and MS. Ring-oxidation products were prepared from 3-OH-benzidine, which was characterized by NMR and MS (18). To prepare 3-OH,N′,N,N′-diacetylbenzidine, 3-OH-benzidine was acetylated in pyridine/acetic anhydride, purified, and identified by MS (11). Ring-oxidation products of N-acetylbenzidine were prepared by treatment of 3-OH,N′-diacetylbenzidine with porcine liver carboxylesterase (Sigma Chemical Co.). Alternatively, ring-oxidation products of N-acetylbenzidine were prepared by direct chemical synthesis from 4-amino-4′-nitrobiphenyl after reaction with potassium persulfate. Male Fischer 344 rats (150–200 g) were purchased from Harlan Industries (Indianapolis, IN).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>β-Naphthoflavone</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]-N-Acetylbenzidine</td>
<td>49 ± 6</td>
<td>ND</td>
</tr>
<tr>
<td>NHDA</td>
<td>39 ± 5</td>
<td>309 ± 56</td>
</tr>
<tr>
<td>Ring-oxidation</td>
<td>ND</td>
<td>87 ± 14</td>
</tr>
<tr>
<td>[3H]-N,N′-Diacetylbenzidine</td>
<td>421 ± 49</td>
<td></td>
</tr>
<tr>
<td>NHDA</td>
<td>ND</td>
<td>182 ± 28</td>
</tr>
</tbody>
</table>

*ND, not detected.*

Identification of Metabolites. The characteristic response of each of these compounds to pH was used in solvent extraction purification and in their identification by HPLC. To a 0.8 ml incubation mixture, cold standards (0.125–0.25 mM) and 0.08 ml of 1.0 N NaOH were added, and immediately extracted twice with 1.6 ml of CHCl3, saturated with 0.1 N NaOH. The aqueous fraction was quickly adjusted to pH 7.4 with 0.08 ml of 1.0 N HCl, and extracted twice with 1.6 ml of ethyl acetate saturated with pH 7.4, 100 mM phosphate buffer. N′-H' was extracted into CHCl3, whereas NHDA, NHA, and ring-oxidation products were extracted into ethyl acetate. Recovery of each compound was judged to be at least 75% by analysis of the UV peak of synthetic standard on HPLC. For N-acetylbenzidine, further HPLC analysis was performed using the following solvent system: mobile phase contained 32% methanol:68% of 50 mM citrate/phosphate buffer (pH 4.0), 0–9 min; 32–34%, 9–11 min; 34–60%, 14–19 min; and flow rate, 1 ml/min. Elution times of the ring-oxidation products, N′-H', N-acetylbenzidine, and NHA, were 6.8 and 7.4, 8.8, 10.5, and 12.7 min, respectively. For N,N′-diacetylbenzidine, further HPLC analysis was performed using the following solvent system: mobile phase contained 20% methanol:80% of 20 mM phosphate buffer (pH 8.2), 0–2 min; 20–38%, 2–15 min; 38–80%, 25–32 min; and flow rate, 1 ml/min. Elution times of the ring-oxidation products, N′-H', N-acetylbenzidine, and NHA, were 20, 23.7, and 24.6 min, respectively. Because the UV peaks of the cold synthetic standards corresponded to the radioactive peaks of the proposed metabolites on at least two different HPLC systems, the radiolabeled microsomal incubation products are assumed to be the assigned compounds.

Analysis of Metabolite Binding to DNA and DNA Adducts. Samples were treated with RNase followed by sodium dodecyl sulfate and proteinase K. After extraction with phenol and then chloroform/isooamyl alcohol (24:1) (21), the aqueous fraction was adjusted to a final concentration of 0.25 M NaCl, cold ethanol added, and nucleic acid precipitated at −20°C overnight. Pellets were washed with 70% ethanol and dissolved in H2O. Purity of DNA was determined by absorbance at 260 and 280 nm, with a ratio of A260/A280 of 1.7 achieved for each sample. The radioactivity bound to DNA was determined. To detect adducts by 32P-postlabeling, the DNA was digested with micrococcal nuclease and spleen phosphodiesterase to 3′-monophosphate deoxynucleotides, and analyzed as previously described (22). Samples were enriched by n-butanol extraction and separated on PEI-cellulose sheets (23). Chromatographic conditions were selected to detect N(3′)-monophosphate deoxyguanosin-8-yl)-benzidine which was recently characterized (24).

### Results

- β-Naphthoflavone treatment resulted in a substantial increase in [3H]-N-acetylbenzidine metabolism (table 1). The rate of NHA formation increased 8-fold, and the formation of ring oxidation products was demonstrated. In contrast, N′-H' formation was at the limit of detection (<1% of total HPLC recovered radioactivity) with
Oxidation of N-Acetylbenzidine and N,N'-Diacetylbenzidine

Methods

Purified material from reaction mixtures (see details in Materials and Methods) was verified by comparison to synthetic standards after partial purification from the reaction mixture and HPLC on at least two different solvent systems (see details in Materials and Methods).

Although the metabolism of [3H]N,N'-diacetylbenzidine was not detected with control microsomes, β-naphthoflavone treatment resulted in significant metabolism (table 1). The formation of NHA was more than twice that observed for the ring-oxidation product. The HPLC profile observed with β-naphthoflavone-treated microsomes is illustrated in fig. 2A. Blank incubations without NADPH yielded no products of metabolism (fig. 2B). Cold NHA (~10 nmol of each) was co-injected on the HPLC to allow recovery of the radioactive NHA and NHA peaks. The UV peak derived from the cold synthetic standards corresponded to the radioactive peaks. The identity of these metabolites was verified by comparison with synthetic standards after partial purification from the reaction mixture and HPLC on at least two different solvent systems (see details in Materials and Methods).

To assess the specific oxidative pathways metabolizing [3H]N-acetylbenzidine and [3H]N,N'-diacetylbenzidine in β-naphthoflavone-treated microsomes, specific cytochrome P450 inhibitors were used (25, 26) (table 2). Because β-naphthoflavone is known to induce rat hepatic cytochrome P4501A1/1A2, a specific inhibitor of this family was examined for possible dose–response inhibition. Ellipticine elicited a dose–response inhibition of NHA and ring-oxidation product formation with an IC50 of ~0.3 μM and complete inhibition at 1 μM (fig. 3). A similar dose–response inhibition was observed with ellipticine for [3H]N,N'-diacetylbenzidine metabolism. For this reason, all inhibitors were tested at 10 μM (table 2). At this concentration, both α-naphthoflavone and ellipticine, specific cytochrome P4501A1/1A2 inhibitors, elicited complete inhibition of metabolism of [3H]N-acetylbenzidine and [3H]N,N'-diacetylbenzidine. A small amount of inhibition might have been detected with the cytochrome P4502E1 inhibitor, disulfiram (26). Inhibitors of cytochrome P4503A1/3A2 (troleandomycin) and P4502C6 (sulfonpyrazone) did not alter metabolism (26). In addition, yohimbine and ajmaline, inhibitors of human cytochrome P4502D6, did not alter metabolism.

Additional test agents and conditions were used to evaluate [3H]N-acetylbenzidine metabolism by control microsomes (table 3). Consistent with metabolism by β-naphthoflavone-treated microsomes, α-naphthoflavone and ellipticine, specific cytochrome P4501A1/1A2 inhibitors (25, 26), completely prevented formation of NHA. In contrast, NHA formation was not prevented by these compounds or any of the other cytochrome P450 inhibitors tested in table 2. Furafylline, a specific inhibitor of cytochrome P4501A2 (27), was also tested and reduced NHA formation to 53% of the complete reaction mixture, with the rat liver microsomal NADPH-dependent oxidation of [3H]N-acetylbenzidine (ABZ). (A–C) [3H]N-acetylbenzidine metabolism by β-naphthoflavone-treated microsomes, control microsomes, and β-naphthoflavone-treated microsomes without NADPH, respectively. Ring Ox., N-acetylbenzidine ring-oxidation products.
Discussion

This is the first study to assess the cytochrome P450s that oxidize N-acetylbenzidine and N,N'-diacetylbenzidine in rat control and \( \beta \)-naphthoflavone-treated liver microsomes. A previous study had assessed metabolism of these compounds by control Sprague-Dawley rat liver microsomes (1). In that study, the rates of NHA and NHA formation were 0.13 ± 0.03 and 0.11 ± 0.01 nmol/mg/min, respectively, with NHDa formation over 20-fold less (0.005 ± 0.001). These previous values for NHA and NHA are about 2–3 times more than that reported for control microsomes in our study (table 1). In both studies, the relative rates of formation are NHA > NHA > NHDa, with ring-oxidation at the limit of detection. Thus, the results were qualitatively similar in both studies, with quantitative differences attributed to strain differences in the rats used.

The cytochrome P4501A family has been shown to oxidize a variety of carcinogenic aromatic and heterocyclic amines, including 4-aminobiphenyl (29–31). \( \beta \)-Naphthoflavone is a specific inducer for this family (20) and increased the oxidation of both N-acetylbenzidine and N,N'-diametylbenzidine (table 1). In addition, both \( \alpha \)-naphthoflavone and ellipticine, specific cytochrome P4501A family inhibitors (25, 26), elicited complete inhibition of oxidation of these acetylated benzidine analogs. Thus, the P4501A family seems to be responsible for oxidation by \( \beta \)-naphthoflavone-treated liver microsomes. Although \( \alpha \)-naphthoflavone and ellipticine were also effective in preventing NHA formation by control microsomes, neither compound inhibited formation of NHA. Although these chemicals inhibit both cytochrome P4501A1 and P4501A2, furafylline, a relatively selective, irreversible/mechanism-based inhibitor of P4501A2 (26, 27, 32), was also tested for inhibition of NHA formation. Furafylline was ineffective in inhibiting NHA at 10 \( \mu \)M and only caused a partial inhibition of NHA formation by control microsomes, thus suggesting greater involvement of cytochrome P4501A1 than P4501A2 in NHA formation. The nonspecific cytochrome P450 inhibitors SKF-525A and DPEA have been previously shown to prevent aromatic amine oxidation (28, 29, 33) and elicited partial inhibition of NHA formation by control microsomes at 10 \( \mu \)M (table 1). At 100 \( \mu \)M, DPEA completely inhibited NHA formation. In control microsomes, different cytochrome P450s seem to be eliciting the formation of NHA and NHA. Conditions that inhibit flavin-dependent monoxygenase metabolism did not seem to alter significantly NHA or NHA formation. Therefore, whereas the P4501A family contributes to the oxidation of NHA by control microsomes, the cytochrome P450 responsible for NHA formation by control microsomes was not determined.

Numerous studies have suggested that activation of aromatic amines to bind DNA involves a number of metabolic and distribution steps (34, 35). After hepatic N-oxidation, the N-OH analogs are thought to undergo N-glucuronidation, excretion, and accumulation in the lumen of the bladder. The acid lability of these N-glucuronides results in their hydrolysis to the corresponding N-OH arylamine that can form DNA adducts in the bladder resulting in tumor formation (35, 36). Because the amount of N-OH products formed in liver may have a direct impact on bladder carcinogenicity, the proportion of ring vs. N-oxidized products is an important oxidative parameter. For control microsomes, only N-oxidation products are formed with N-acetylbenzidine. For \( \beta \)-naphthoflavone-treated liver microsomes, N-oxidation is 3.6-fold and 2.3-fold greater than ring-oxidation for N-acetylbenzidine and N,N'-diacetylbenzidine, respectively. Thus, under all conditions, N-oxidation exceeds ring-oxidation. Similar results were observed for 4-aminobiphenyl with 10-fold more N-oxidation than ring-oxidation products (37). In contrast, ring-oxidation of 2-amino-\( \alpha \)-carboline exceeds N-oxidation by 5.7-fold (38).
Effects of different cytochrome P450 inhibitors on NADPH-dependent oxidation of N-acetylbenzidine and N,N'-diacetylbenzidine by β-naphthoflavone-treated rat liver microsomes

<table>
<thead>
<tr>
<th>Inhibitor (10 μM)</th>
<th>Type of Inhibition</th>
<th>NHA</th>
<th>ABZ-ROX</th>
<th>NHDA</th>
<th>DABZ-ROX</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Naphthoflavone</td>
<td>1A1/1A2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>3A1/3A2</td>
<td>97</td>
<td>94</td>
<td>94</td>
<td>94</td>
</tr>
<tr>
<td>Sulfipyrazone</td>
<td>2C6</td>
<td>100</td>
<td>100</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>2E1</td>
<td>81</td>
<td>73</td>
<td>94</td>
<td>94</td>
</tr>
<tr>
<td>Ellipticine</td>
<td>1A1/1A2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

All inhibitors were tested at 10 μM. ABZ-ROX, N-acetylbenzidine ring-oxidation products; DABZ-ROX, N,N'-diacetylbenzidine ring-oxidation product.

Effects of different inhibitors on NADPH-dependent oxidation of N-acetylbenzidine by control rat liver microsomes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Type of Inhibition</th>
<th>NHA %</th>
<th>NHDA %</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Naphthoflavone</td>
<td>1A1/1A2</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>3A1/3A2</td>
<td>89</td>
<td>86</td>
</tr>
<tr>
<td>Sulfipyrazone</td>
<td>2C6</td>
<td>96</td>
<td>100</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>2E1</td>
<td>86</td>
<td>86</td>
</tr>
<tr>
<td>Ellipticine</td>
<td>1A1/1A2</td>
<td>88</td>
<td>0</td>
</tr>
<tr>
<td>Furafylline</td>
<td>1A2</td>
<td>94</td>
<td>53</td>
</tr>
<tr>
<td>SKF-525A</td>
<td>Nonspecific</td>
<td>41</td>
<td>100</td>
</tr>
<tr>
<td>DPEA</td>
<td>Nonspecific</td>
<td>49</td>
<td>29</td>
</tr>
<tr>
<td>Methimazole</td>
<td>FMO</td>
<td>96</td>
<td>70</td>
</tr>
<tr>
<td>Heated microsomes</td>
<td>FMO</td>
<td>79</td>
<td>82</td>
</tr>
</tbody>
</table>

DNA binding was used as an index of reactivity of microsomal oxidation products. Although β-naphthoflavone treatment increased N-acetylbenzidine binding to DNA, no increase was detected for N,N'-diacetylbenzidine (table 4). Binding was attributed to the N-OH products of metabolism (34, 39). Binding data are consistent with the greater N-oxidation, compared with ring-oxidation observed for N-acetylbenzidine than N,N'-diacetylbenzidine, and with the greater stability of the N-OH analog of the latter compared with the former. That is, N-acetoxy-N,N'-diacetylbenzidine reacts only slowly with 2'-deoxyguanosine at pH 7.0, requiring 6 hr for significant adduct formation (24).

N-acetylbenzidine is observed whereas metabolism of N,N'-diacetylbenzidine by control microsomes, and N H A can react with DNA to form this adduct directly. Using the same incubation conditions, neither N H A nor NHDA would be expected to form this adduct.

N-Acetylation of aromatic amines makes them harder to oxidize and is considered a detoxification step (40). For the diamine benzidine, N-acetylbenzidine is considered a more active metabolite of benzidine than N,N'-diacetylbenzidine, which has been considered a more detoxified metabolite (8, 9, 41). Results with control microsomes are consistent with this hypothesis (table 1). That is, substantial metabolism of N-acetylbenzidine is observed whereas metabolism of N,N'-diacetylbenzidine is not detected. In addition, the N H A produced by control microsomes (table 1) is probably responsible for the N-acetylbenzidine to DNA adduct. However, because control animals administered benzidine exhibit rapid acetylation to N,N'-diacetylbenzidine with little accumulation of benzidine or N-acetylbenzidine (2–4, 24), N-acetylation would seem to result in detoxification in control animals. After β-naphthoflavone treatment, N H A is not formed, and the metabolism of N,N'-diacetylbenzidine exceeds N-acetylbenzidine (table 1). N,N'-Diacetylbenzidine seems to be a better substrate than N-acetylbenzidine for the oxidizing enzyme(s) induced by β-naphthoflavone treatment. In these induced rats, N-acetylation may not be a detoxification step.
The product of cytochrome P450 oxidation of benzidine (18). The rate of 3-OH-benzidine formation by control microsomes (68 ± 16 pmol/mg/min) is increased 12.3-fold after β-naphthoflavone treatment (835 ± 81). Similar rates were reported for N-acetylbenzidine (table 1). Inhibitor studies demonstrated that cytochrome P4501A1/1A2 was responsible for benzidine metabolism. Thus, the oxidation of benzidine by rat liver microsomes is comparable with that reported herein for N-acetylbenzidine and N,N'-diacetylbenzidine.

The constitutive level of cytochrome P4501A2 in control rat liver microsomes is quite low and that for cytochrome P4501A1 even lower (42, 43). β-Naphthoflavone treatment can increase the activity of rat liver cytochrome P4501A1 and P4501A2 by 88-fold and 11-fold, respectively (44). This would be consistent with the low level of metabolism observed for benzidine and its acetylated analogs by control microsomes and the increased metabolism with β-naphthoflavone treatment. Treatment by β-naphthoflavone dramatically lowers hepatic cytochrome P4502C11 content (45). The latter effect on this or another cytochrome P450 could explain the lack of formation of N9HA by control microsomes was not determined.

In conclusion, the cytochrome P4501A family is responsible for oxidative metabolism of benzidine and its N-acetylated analogs in control and β-naphthoflavone-treated rat liver microsomes. Relative rates of N-acetylbenzidine and N,N'-diacetylbenzidine oxidation favored activation (N-OH) rather than detoxification (ring-oxidation). A summary of structures and putative pathways is illustrated in fig. 4. Included in this summary are recent results demonstrating benzidine oxidation by cytochrome P4501A1/1A2 to 3-OH-benzidine (18), N9HA, a potential carcinogenic metabolite, formation was observed with control, but not β-naphthoflavone-treated microsomes. The cytochrome P450(s) producing N9HA by control microsomes was not determined.

Acknowledgments. MS was performed at the Center of Mass Spectrometry Resource at Washington University School of Medicine (St. Louis, MO) through National Institutes of Health Grants RR-00954 and AM-20579. We thank Dr. F. F. Hsu for mass spectral analysis, and Cindee Rettke and Priscilla DeHaven for excellent technical assistance.

References
Oxidation of N-Acetylbenzidine and N,N′-Diacetylbenzidine


