N-Methylation: Potential mechanism for metabolic activation of carcinogenic primary arylamines

DANIEL M. ZIEGLER*, SHERRY S. ANSHER[†‡], TOSHIYUKI NAGATA*, FRED F. KADLUBAR[§], AND WILLIAM B. JAKOBY[†]

*Clayton Foundation Biochemical Institute, Department of Chemistry, The University of Texas, Austin, TX 78712; [†]Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD 20892; and [§]National Center for Toxicological Research, Jefferson, AR 72079

ABSTRACT   Two amine *N*-methyltransferases isolated from rabbit liver catalyze *S*-adenosylmethionine-dependent N-methylation of benzidine and 4-aminobiphenyl but not of 4-aminoazobenzene or 2-aminobiphenyl. The enzymatic reaction products were analyzed and found to be identical to synthetic *N*-methylbenzidine and *N*-methyl-4-aminobiphenyl. N-Methylation may be a critical step in the metabolic activation of primary arylamines because *N*-methylarylamines, unlike primary arylamines, are readily N-oxygenated by the NADPH- and oxygen-dependent microsomal flavin-containing monooxygenase. Kinetic studies carried out with the purified porcine liver monooxygenase demonstrate that, while activity with primary arylamines could not be detected, *N*-methyl derivatives of benzidine, 4-aminoazobenzene, and 4-aminobiphenyl are substrates. Products formed from *N*-methyl-4-aminobiphenyl had the properties of the hydroxylamine and/or nitrone in that the enzyme- and time-dependent incubation product(s) reduced Fe^{3+} to Fe^{2+}, and formaldehyde was formed during the course of the reaction. These data suggest that *N*-methyl-4-aminobiphenyl is oxidized to *N*-hydroxy-*N*-methyl-4-aminobiphenyl, which can undergo further oxidation to a nitrone that hydrolyzes to formaldehyde and *N*-hydroxy-4-aminobiphenyl.

Benzidine, 4-aminobiphenyl, and several other primary arylamines are known to produce tumors at sites removed from their point of application. The reactions responsible for the activation and transport of carcinogenic metabolites have been extensively investigated (see refs. 1–3 for recent reviews). Several routes for activation of arylamines have been described in different species or tissues with virtually all studies suggesting that oxidation at nitrogen is an early obligatory event. In a few extrahepatic tissues, there is sequential one-electron peroxidation leading to the formation of reactive imines or iminoquinones; an example is that catalyzed by prostaglandin H synthase without formation of N-oxygenated intermediates (4). In the liver, however, acetylation followed by N-oxygenation, or direct N-oxygenation of the amine, is usually considered essential for bioactivation of primary arylamine carcinogens (1, 2). For arylamides, oxidation to hydroxamic acids catalyzed by P-450 monooxygenases is a reasonable possibility, whereas an equally chemically sound mechanism for enzymatic N-oxidation of primary arylamines remains to be described.

Most studies indicate that P-450 oxygenases catalyze oxidation of amines by a radical mechanism (5); charge delocalization into the ring upon abstraction of the first electron at nitrogen should lead to oxygen rebound on carbon as well as nitrogen. Therefore, formation of ring hydroxylated products would limit production of N-

oxygenated metabolites from the amine radical cation. An alternate mechanism, in which the arylamine is oxidized to a cation species by stepwise abstraction of two electrons from nitrogen before oxygen addition, has been proposed (6). Although oxidation of arylamines by this mechanism would yield primarily N-oxygenated products, it differs considerably from the generally accepted mechanism for P-450-catalyzed oxidation of the closely related *N*-alkylarylamines (5, 7). With the latter amines, oxygen rebound occurs exclusively on the neutral side-chain carbon-centered radical after proton loss and rearrangement of the amine radical cation. The nature of products formed is consistent with this interpretation, and it is generally accepted that P-450 catalyzes N-dealkylation of secondary and tertiary amines without formation of N-oxygenated intermediates (8).

Formation of significant amounts of hydroxylamines could also occur by direct two-electron oxidation at nitrogen without formation of intermediate radical cations. At present, the flavin-containing monooxygenases (7, 9) are the only known candidates for catalysis of amines by this mechanism. These enzymes, whether purified from hog liver (10) or from rabit lung microsomes (11), are inactive toward most primary arylamines. Nevertheless, they readily N-oxygenate secondary and tertiary arylamines; one group (12) has indicated that the flavin-containing monooxygenase is the primary catalyst for bioactivation of the carcinogen *N*-methyl-4-aminoazobenzene in rat liver. The formation of phenylhydroxylamine from *N*-methylaniline by sequential N-oxidations, followed by hydrolysis of the nitrone in reactions catalyzed by this enzyme, also has been described (13). Therefore, enzymatic N-methylation followed by N-oxygenation provides a mechanism for the generation of substantial quantities of *N*-hydroxy metabolites from primary arylamines capable of undergoing rapid N-methylation by liver enzymes.

The studies described in this report demonstrate that carcinogenic primary arylamines are excellent substrates for rabbit liver amine *N*-methyltransferases and that the resulting secondary amines are readily N-oxygenated by the flavin-containing monooxygenase.

MATERIALS AND METHODS

The components of the enzyme assay were purchased at the highest grades available from Sigma. Benzidine and 4-aminoazobenzene were obtained from Merck and ICN, respectively. All other arylamines and the reagents used for the synthesis of the *N*-methyl derivatives were from Aldrich.

N-Methyl-4-aminobiphenyl, *N*,*N*'-dimethylbenzidine, and *N*-methylbenzidine were synthesized by reduction of the corresponding carbethoxyamino derivatives with lithium

[‡]Present address: Laboratory of Bacterial Toxins, Food and Drug Administration, Bethesda, MD 20892.

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aluminum hydride (14). 4-Carbethoxylaminobiphenyl and N,N'-biscarbethoxybenzidine were prepared by dropwise addition of ethylchloroformate (1.3 and 2.2 mol/mol of 4-aminobiphenyl and benzidine, respectively) to 30 mmol of the parent arylamine dissolved in 500 ml of chloroform containing 60 mM pyridine. The product, which forms almost immediately as an insoluble precipitate upon addition of ethylchloroformate, was collected by filtration. The product remaining in the filtrate was recovered by washing the filtrate with water (three or four times to remove pyridine) before removing chloroform under reduced pressure. The combined product fractions were washed with water, dried under reduced pressure, and crystallized from 95% ethanol. The yield was 90–92% of theoretical for both derivatives.

N-Carbethoxybenzidine was synthesized by essentially the same procedure except that the reaction was carried out in benzene and the molar ratio of ethylchloroformate to benzidine was reduced to 1:2. After 15 min at room temperature, an equal volume of 0.1 M potassium carbonate in water was added to the reaction mixture. The organic phase was collected, and the aqueous phase was extracted three times with benzene. The combined benzene extracts were washed several times with water and dried with sodium sulfate, and the solvent was removed under reduced pressure. The residue was dissolved in chloroform/methanol, 10:1 (vol/vol), and N-carbethoxybenzidine was separated from unreacted benzidine and N,N'-biscarbethoxybenzidine by chromatography on a column of silica gel developed with 10:1 chloroform/methanol. The desired product, which was eluted between the two major contaminants, was concentrated under reduced pressure and crystallized from 95% ethanol (yield = 20% of theoretical).

Table 1. Arylamine substrates for rabbit liver N-methyltransferases

| | Kinetic constants* | | | |
| | Transferase A | | Transferase B | |
Arylamine	$K_m \times 10^3$	k_{cat}	$K_m \times 10^3$	k_{cat}
Benzidine	1.7	26	0.45	18
N-Methylbenzidine	0.45	24	2.8	43
N,N'-Dimethylbenzidine	1.1	21	0.52	31
4-Aminobiphenyl	1.6	30	0.56	29
N-Methyl-4-aminobiphenyl	0.29	63	0.56	24
2-Aminobiphenyl	0.28	0.1	0.37	0.2
4-Aminoazobenzene	—	0†	—	0†

*Kinetic constants were calculated from velocities determined at 37°C in medium containing Tris (pH 7.8), 34 μM [*methyl*-^{3}H]-AdoMet, and either 0.45 mg of amine N-methyltransferase A or 0.9 mg of amine N-methyltransferase B per ml.
†The value of 0 refers to the inability to detect activity at the highest concentrations tested.

The N-methylarylamines, prepared by reduction of the N-carbethoxyamine derivatives with lithium aluminum hydride in ether, were converted to the hydrochloride salts and crystallized from 95% ethanol. The crystals were thoroughly dried under reduced pressure and stored in sealed containers in the dark. The structure of each compound was verified by proton NMR spectroscopy, and purity was assessed by chromatography. Only one spot could be detected for each of the derivatives on plates developed in several solvent systems.

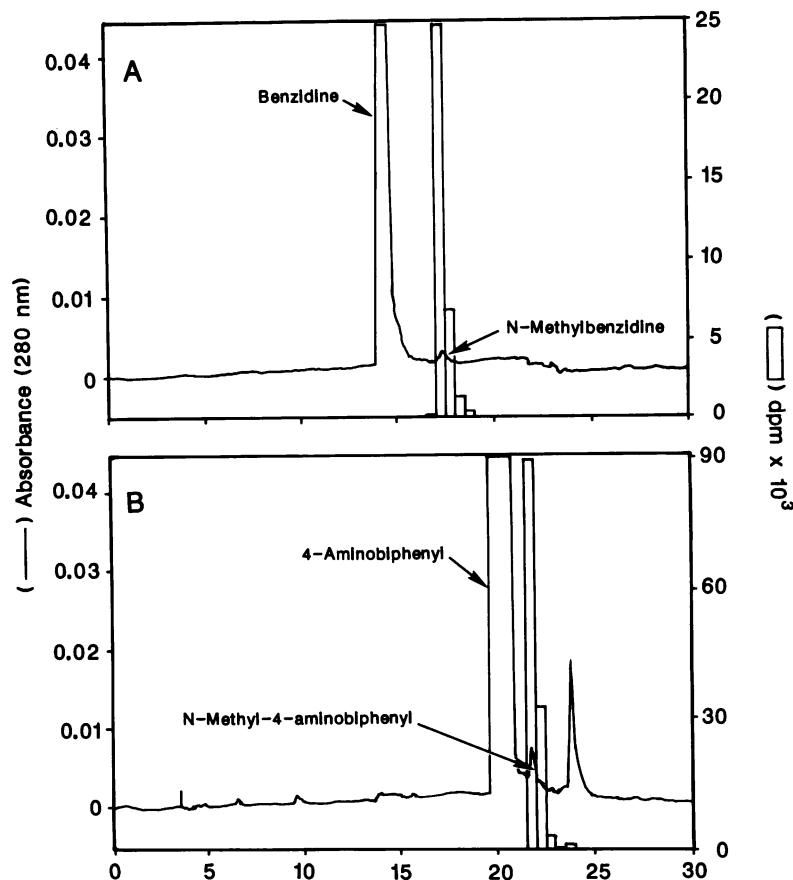The porcine flavin-containing monooxygenase (10) and rabbit liver amine N-methyltransferases (15) were isolated

Fig. 1. HPLC elution profiles of ethyl acetate extracts of incubation mixtures containing N-methyltransferase B, [*methyl*-^{3}H]AdoMet, and benzidine (A) or 4-aminobiphenyl (B). The solid line shows the absorbance at 280 nm, and the histogram shows the eluted radioactivity. Ultraviolet absorption spectra of the metabolites were obtained during elution and were identical to the synthetic N-methyl derivatives. Similar results were obtained on analyses of incubations containing N-methyltransferase A.

by methods described earlier. The activity of the latter was determined by measuring the formation of N-[methyl-^{3}H]methylarylamine after incubation at 37°C for 30 min with enzyme, 34 μM [methyl-^{3}H]AdoMet (375,000 cpm), and the arylamine (15). Product identification was carried out by HPLC and ultraviolet spectral analysis using a Waters Associates model 510 HPLC system equipped with a μBondapak C_{18} "semi-prep" column and a Hewlett–Packard model 1040A high-speed spectrophotometric detector. The solvents were 20 mM diethylamine acetate (pH 6.5) (solvent A) and methanol (solvent B), and the elution program (30 min) was 20–100% solvent B from 0 to 20 min and 100% solvent B from 20 to 30 min; the flow rate was 3 ml/min. The activity of the flavin-containing monooxygenase was determined by measuring substrate-dependent oxygen uptake at 37°C and pH 7.4 in 0.1 M potassium phosphate containing 0.15 mM NADPH and a glucose-6-phosphate dehydrogenase-linked NADPH-generating system (9). Kinetic constants were calculated from double reciprocal plots of velocity as a function of substrate at concentrations above and below K_m.

The formation of N-hydroxy products was measured as described (11) and of formaldehyde by the method of Nash (16).

RESULTS

Benzidine, N-methylbenzidine, and N,N'-dimethylbenzidine are substrates for both amine N-methyltransferases A and B that were purified to homogeneity from rabbit liver cytosol (Table 1). The number of methyl substituents primarily affected K_m with transferase A, whereas both K_m and V_{max} were affected in reactions catalyzed by transferase B. 4-Aminobiphenyl and its N-methyl derivatives were also substrates for both amine N-methyltransferases. In contrast, N-methylation of 2-aminobiphenyl was barely detectable (Table 1). Whereas the K_m of 2-aminobiphenyl was less than that of 4-aminobiphenyl for each methyltransferase, k_{cat} was at least 2 orders of magnitude less than that of the latter with either enzyme.

The formation of N-methylbenzidine and N-methyl-4-aminobiphenyl from their parent arylamines was confirmed by HPLC and ultraviolet spectral analyses of ethyl acetate extracts of incubations containing N-methyltransferase A or B and [methyl-^{3}H]AdoMet (Fig. 1). The analyses showed an enzyme-dependent conversion of both arylamine substrates to a single radioactive component that was chromatographically identical to the corresponding N-methyl derivative. In

addition, the metabolites exhibited ultraviolet absorption spectra that were identical to the synthetic N-methylbenzidine (λ_{max} = 287 nm) and N-methyl-4-aminobiphenyl (λ_{max} = 285 nm), respectively.

N-Methyltransferase activity with 4-aminoazobenzene could not be detected with either enzyme. Since this azo dye did not inhibit activity with other substrates, it does not appear to interact with the methyltransferases.

Kinetic constants calculated from measurements of activity of these arylamines with the purified hog liver flavin-containing monooxygenase (Table 2) show that only the N-methyl derivatives are substrates. Oxidation of benzidine, 4-aminobiphenyl, and 4-aminoazobenzene could not be detected even at concentrations approaching their solubility in the assay medium. Benzidine and 4-aminobiphenyl also do not inhibit the oxidation of methimazole (data not shown), suggesting that they do not bind at or near the catalytic site. However, the N-methyl derivatives of these arylamines are substrates, although there was an unexpected difference in V_{max} (Table 2) between N-methylbenzidine and N,N'-dimethylbenzidine. The turnover of the latter is close to that obtained with a saturating concentration of N,N'-dimethylaniline, but V_{max} for N-methylbenzidine is only half of this rate. The reason for this finding is not known, but it appears that the latter N-methylarylamine may affect more than one step in the catalytic cycle (17, 18).

The inherent instability of the oxidation products (hydroxylamines and/or nitrones) of N-methylbenzidine and N-methyl-4-aminobiphenyl prevented their isolation and positive identification. Nevertheless, as shown in Fig. 2, the
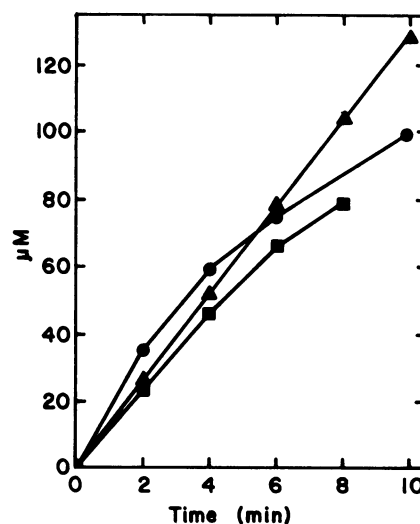

FIG. 2. Rates of N-methyl-4-aminobiphenyl-dependent oxygen uptake and product formation as a function of reaction time: ▲, Oxygen uptake; ●, hydroxylamine (Fe^{3+} reducing equivalents = 2); ■, formaldehyde. The reactions were carried out in an Oxygraph vessel at 37°C in medium containing 0.1 M potassium phosphate (pH 7.4), 0.2 mM $NADP^+$, 1.5 mM glucose 6-phosphate, *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase, and 0.45 μM flavin-containing monooxygenase. The reaction was started by adding N-methyl-4-aminobiphenyl (2 mM). An aliquot of 0.1 ml was withdrawn at zero time for hydroxylamine estimation; the reaction vessel was then closed, and oxygen uptake was recorded for 6 min. Aliquots for hydroxylamine estimation were withdrawn at 2, 4, 6, and 10 min through the capillary access port. The rate of formaldehyde formation was measured in separate experiments under identical conditions with 0.5-ml aliquots withdrawn at 2-min intervals. Samples were deproteinized with 0.3 M trichloroacetic acid and warmed to 60°C for 5 min to ensure complete hydrolysis of the nitrone before estimating formaldehyde. Oxygen consumption, measured repeatedly in separate experiments, was linear for at least 15 min and reproducible within ±2%.

Table 2. Substrate activity of primary and secondary arylamines for the flavin-containing monooxygenase

Arylamine	Kinetic constants*	
	$K_m \times 10^3$	k_{cat}†
Benzidine	—	0‡
N-Methylbenzidine	0.56	13
N,N'-Dimethylbenzidine	0.18	29
4-Aminobiphenyl	—	0‡
N-Methyl-4-aminobiphenyl	0.36	26
4-Aminoazobenzene	—	0‡
N,N'-Dimethyl-4-aminoazobenzene	0.13	30

*Kinetic constants were calculated from substrate-dependent oxygen uptake at 37°C in medium at pH 7.8 containing 0.1 M potassium phosphate, 0.1 mM NADPH, 5 mM glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase per ml, 0.45 μM monooxygenase (FAD), and arylamine substrate.
†Values are mol of substrate oxidized per mol of enzyme flavin at infinite substrate concentration.
‡The value of 0 refers to the inability to detect activity at the highest concentrations tested.

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$$\text{ArNH}_2 \xrightarrow{(1)} \text{ArNHCH}_3 \xrightarrow{(2)} \overset{\text{OH}}{\underset{|}{\text{ArNCH}_3}} \xrightarrow{(3)} \overset{\text{O}}{\underset{\uparrow}{\text{ArN=CH}_2}} \xrightarrow{(4)} \text{ArNHOH} + \text{CH}_2\text{O}$$
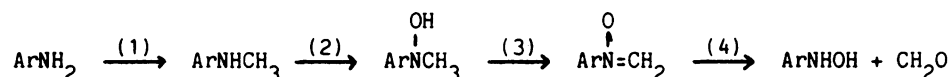
FIG. 3. Reaction sequence for N-oxygenation of 4-aminobiphenyl and benzidine. Reaction 1 is catalyzed by cytosolic *S*-adenosylmethionine-dependent amine *N*-methyltransferases. Reactions 2 and 3 require NADPH and oxygen and are catalyzed by the microsomal flavin-containing monooxygenase. Nonenzymatic hydrolysis of the nitrones, reaction 4, is quite rapid at 37°C.

enzymatic product formed from *N*-methyl-4-aminobiphenyl has properties characteristic of an *N*-hydroxy or nitrone derivative. The product reduces Fe^{3+} to Fe^{2+} and, upon hydrolysis, yields formaldehyde. Although the rate of *N*-methyl-4-aminobiphenyl-dependent oxygen uptake is linear for at least 10 min, the formation of the products that will reduce Fe^{3+} or yield formaldehyde appears to decrease during the course of the reaction. This suggests that *N*-hydroxy-*N*-methyl-4-aminobiphenyl, as with other secondary hydroxylamines, does not accumulate but is further oxidized to the nitrone. Without exception, the concentration of secondary *N*-hydroxyarylamines required to half-saturate flavin-containing monooxygenases is considerably less than that of the parent secondary amine (11).

Direct measurements with the authentic *N*-hydroxy derivative of *N*-methyl-4-aminobiphenyl would be necessary to avoid ambiguity. Unfortunately, synthesis of this derivative has not been described, and attempts to prepare it were unsuccessful.

Similar measurements of product formation could not be carried out with *N*-methylbenzidine. This substrate reduces Fe^{3+} directly; the enzyme-catalyzed oxidation product, presumably the *N*-hydroxyamine derivative, decomposes to colored products that interfere with the estimation of formaldehyde.

DISCUSSION

The early studies of Miller and Miller (19) demonstrated the importance of *N*-methyl substituents for the hepatocarcinogenicity of 4-aminoazobenzene in rats: the primary arylamine is not carcinogenic in the rat, but *N*-methyl derivatives of this amine produce tumors in virtually all animals tested. *N*-Methyl-4-aminoazobenzene, administered directly or formed metabolically by demethylation of *N,N*-dimethylaminoazobenzene, is oxidized to the secondary *N*-hydroxy product by the microsomal flavin-containing monooxygenase (12). N-Oxygenation, apparently catalyzed largely by this monooxygenase, is a critical step in the formation of the ultimate carcinogen through the sulfation-dependent formation of *N*-sulfonyloxy-*N*-methyl-4-aminoazobenzene. As with most other primary amines, 4-aminoazobenzene is not N-oxygenated by the flavin-containing monooxygenase (Table 2) nor is it methylated by liver amine *N*-methyltransferases (Table 1). The lack of carcinogenicity of 4-aminoazobenzene in the rat may be related to its inability to serve as substrate for the amine *N*-methyltransferases and, consequently, for the monooxygenase.

In contrast to 4-aminoazobenzene, benzidine and 4-aminobiphenyl are carcinogenic in several species; as described here (Table 1), both undergo N-methylation catalyzed by the liver *N*-methyltransferases. The secondary *N*-methylarylamines are then readily N-oxygenated (Table 2) to products with the properties of *N*-hydroxy derivatives (Fig. 2). On the other hand, the noncarcinogenic 2-aminobiphenyl is neither N-oxygenated directly by the mo-

nooxygenase (Table 2) nor is it N-methylated at significant rates by amine *N*-methyltransferases (Table 1).

These data suggest that N-methylation followed by N-oxygenation of the secondary amine, as illustrated in Fig. 3, may be a significant pathway of metabolic activation for at least one group of primary arylamines. Sequential N-oxidation of the secondary amine produces a reactive nitrone which, upon hydrolysis, yields the primary hydroxylamine that in turn is converted to the ultimate carcinogen. Alternatively, the nitrone may react directly with cellular macromolecules without additional activation (12). The studies described in this report provide a chemically sound metabolic pathway for the N-oxygenation of primary arylamines that can serve as substrates for the hepatic amine *N*-methyltransferases. These enzymes may well catalyze the critical first step in the metabolic activation of some primary arylamines.

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