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FORMATION OF QUATERNARY AMINES BY N-METHYLATION OF AZAHETEROCYCLES WITH HOMOGENEOUS AMINE *N*-METHYLTRANSFERASES*

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Abstract—Catalytic activities of two amine *N*-methyltransferases were documented for the following azaheterocycles: isomeric phenyl- and bispyridyls; 2-, 3- and 4-mono-substituted pyridines; and a miscellaneous group of azaheterocycles that included mono- and diazabenzenes and mono- and diazanaphthalenes. The broad substrate specificities of the two amine *N*-methyltransferases for primary and secondary amines are here extended to a large number of aromatic azaheterocycles in which N-methylation results in the formation of quaternary ammonium metabolites. Pyridine was the best substrate for both enzymes. Substitution in the ring at the 2-position sterically hindered methylation of the pyridyl nitrogen; 2-phenylpyridine and 2,2′-bispyridyl were not substrates.

Historically, N-methylation and quaternization of aromatic azaheterocycles were amongst the earliest recognized xenobiotic biotransformation pathways: in 1887, His [1] demonstrated that *N*-methylpyridinium ion is a urinary metabolite in dogs that had been administered pyridine. This metabolic step can be regarded as a detoxication reaction which, when effected, transforms a lipid-soluble substrate into a quaternary amine salt of somewhat greater water solubility. It is only in recent years that more detailed *in vivo* studies have been carried out on this route of metabolism of this azaheterocycle [2–5] and, hitherto, no studies have been carried at the level of the purified enzyme. The presence of *N*-methyltransferase activity with azaheterocycles has been demonstrated in crude, dialyzed rabbit and guinea pig tissue preparations [6–9]; these studies have also shown that the N-methylation of pyridino compounds is catalyzed by a cytosolic *S*-adenosyl-L-methionine (AdoMet§)-dependent *N*-methyltransferase which is present in high amounts in the lungs, and in significant amounts in the liver, kidney, spleen and brain [6–9].

The "nonspecific" *N*-methyltransferase activity in mammalian tissues has been attributed to an amine *N*-methyltransferase [10], indolethylamine *N*-methyltransferase [11, 12], and arylamine *N*-methyltransferase [13]. Most recently, purification of *N*-methyltransferase activity from rabbit liver cytosol resulted in the isolation of two homogeneous Ado-Met-dependent enzymes, amine *N*-methyltransferases A and B [14, 15]. These enzymes have broad and overlapping specificity for a large number of primary and secondary amines, and they appear to accomplish the reactions previously attributed to the above three *N*-methyltransferases. In preliminary studies, the homogeneous enzymes have also been shown to be capable of catalyzing the N-methylation of both pyridine and nicotine [6], and of metabolites of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [9]. These observations suggested that amine *N*-methyltransferases A and B may have truly broad substrate specificities and may, thereby, play a role in the metabolism of nitrogenous xenobiotics, particularly in the conversion of azaheterocylic compounds to quaternary ammonium ions. This report attempts to examine the breadth of substrate specificity that the individual purified rabbit amine *N*-methyltransferases exhibit toward a variety of aromatic azaheterocycles.

MATERIALS AND METHODS

Materials. *S*-Adenosyl-L-methionine (AdoMet) was obtained from the Sigma Chemical Co. (St. Louis, MO) and *S*-adenosyl-L-[*methyl*-^{3}H]methionine (sp. act. 11 Ci/mmol) from New England Nuclear (Boston, MA). HPLC grade methanol and Scintiverse LC scintillation fluid were obtained from Fisher Scientific (Cincinnati, OH). 2,2′-Bispyridyl was obtained from K & K Chemicals (Plainsview,

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§ Abbreviations: AdoMet, *S*-adenosylmethionine; MPP^+, *N*-methyl-4-phenylpyridinium ion; and MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

NY). *R*-(+)-Nicotine was prepared by the method of Bowman *et al.* [16], by resolution of (±)-nicotine with di-(*p*-toluoyl)-(+)-tartaric acid. *S*-(−)-Cotinine and (±)-cotinine were synthesized from *S*-(−)-nicotine and (±)-nicotine, respectively, according to published methods [17]. All other azaheterocycles were obtained from the Aldrich Chemical Co. (Milwaukee, WI). The N-methyl derivatives of mono-substituted pyridines were prepared by reaction of the appropriate substrate with iodomethane in methanol. The mono- and the di-N-methylated derivatives of symmetrical and unsymmetrical bispyridyls were prepared according to published methods [18–22]. The mono- and diazabenzenes and naphthalenes (Table 4) were reacted with iodomethane, and the mono- and di-N-methylated derivatives were isolated by chromatographic procedures and used as UV standards for the HPLC analyses. The N-methylated standards for nicotine were prepared essentially as described by Seeman and Whidby [23]. *S*-(−)-Cotinine and (±)-cotinine were both readily converted to the corresponding *N*-methylcotininium iodide by reaction with iodomethane. Under these conditions, metyrapone afforded two isomeric mono- and one di-N-methylated product(s); these were isolated by chromatographic procedures as UV standards for HPLC, but further structural assignment for the two isomeric mono-N-methylated products was not attempted.

Enzyme purification. The two amine *N*-methyltransferases A and B were isolated as homogeneous products from the frozen livers of young (10–16 weeks) New Zealand white rabbits obtained from Pel-Freeze Biologicals (Little Rock, AR), as described previously [14].

Standard incubation conditions. The purified enzymes were tested for their substrate range by assaying, in duplicate, a total volume of 200 μl containing 60 μg of transferase A (or 100 μg of transferase B), 34 μM AdoMet (0.8 μCi), 50 mM Tris–HCl (pH 7.8), and 1 mM amine. The reaction was initiated by addition of enzyme and, after 60 min at 37°, was terminated by addition of 250 μl of 1.0 N HCl. Control incubations were performed in the absence of methyl group acceptor, as well as in the absence of enzyme, and treated in an identical manner.

High-performance radiochromatographic analysis. Analyses of enzyme-catalyzed reactions were carried out directly on samples that had been centrifuged at 10,000 *g* to remove precipitated protein. All chromatographic separations involved the use of a programmable HPLC system, consisting of two Altex model 110A solvent-metering pumps, an Altex model 420 system controller and an Altex model 153 analytical UV detector (254 nm) (Altex, Berkeley, CA). Chromatographic conditions varied depending on the N-methylated compound being analyzed. Most of the N-methyl quaternary ammonium metabolites were analyzed by cation-exchange chromatography (Systems A, D, F and G, Table 1), with appropriate mobile phases that effected efficient sep-

Table 1. Key to chromatographic systems used for the HPLC analysis of [^{3}H-CH_3]-N-methylated metabolites*

System	Chromatographic parameters (mobile phase composition, flow rate, and column)
A	Mobile phase: 0.3 M ammonium acetate buffer containing 0.17% triethylamine, pH adjusted to 3.7 with glacial acetic acid–methanol mixture (70:30, v/v). Flow rate: 2 ml/min. Column: Partisil-10 SCX cation exchange (25 × 0.46 cm).
B	Mobile phase: 0.3 M sodium acetate buffer, adjusted to pH 7.0 with glacial acetic acid. Flow rate: 2 ml/min. Column: Partisil-10 SAX anion exchange (25 × 0.46 cm).
C	Mobile phase: 0.3 M sodium acetate buffer, adjusted to pH 7.0 with glacial acetic acid–methanol mixture (70:30, v/v). Flow rate: 2 ml/min. Column: Partisil-10 ODS-2 reversed phase (25 × 0.46 cm).
D	Mobile phase: 0.15 M ammonium acetate buffer, containing 0.1% triethylamine, pH adjusted to 3.7 with glacial acetic acid–methanol mixture (70:30, v/v). Flow rate: 2 ml/min. Column: Partisil-10 SCX cation exchange (25 × 0.46 cm).
E	Mobile phase: 0.1 M sodium acetate buffer, containing 0.5% triethylamine, pH adjusted to 4.5 with glacial acetic acid–methanol mixture (60:40, v/v). Flow rate: 2 ml/min. Column: Partisil-10 ODS-2 reversed phase (25 × 0.46 cm).
F	Mobile phase: 0.15 M sodium acetate buffer, adjusted to pH 4.5 with glacial acetic acid–methanol mixture (70:30, v/v). Flow rate: 2.0 ml/min. Column: Partisil-10 SCX cation exchange (25 × 0.46 cm).
G	Mobile phase: 0.30 M sodium acetate buffer, adjusted to pH 4.5 with glacial acetic acid–methanol mixture (70:30, v/v). Flow rate: 2.0 ml/min. Column: Partisil-10 SCX cation exchange (25 × 0.46 cm).

* A 7 × 0.4 cm precolumn containing the appropriate pellicular packing material was incorporated into each system.

arations of the [^{3}H-CH$_3$]metabolite peak(s) from that of [^{3}H]AdoMet.

Analysis of 3-hydroxypyridine required an anion-exchange chromatographic system (System B), whereas other compounds were analyzed on a reversed-phase C_{18} column (Systems C and E). Details of the several chromatographic systems that were utilized are presented in Table 1. Samples of assay solutions, containing appropriate added standards, were co-injected onto the HPLC column using a loop injector (Rheodyne Inc., Cotati, CA) equipped with a 100-μl loop; output was recorded with an Omniscribe model 5000 dual channel recorder (Houston Instruments, Austin, TX). Standards in the column effluent were detected by UV absorption, and radioactivity in the column effluent was determined using a model HS Flo-1 flow-through radioactivity detector (Radiomatic, Tampa, FL), equipped with a Radiomatic model ES stream splitter. The scintillation fluid used was Scintiverse LC, with a mixing ratio of 4:1 (v/v) of a 50% split of effluent volume. The output of the radioactivity detector was recorded simultaneously on the second channel of the recorder. The data from these experiments are shown in Tables 2–4; each experiment represents the mean of two to four separate incubations with each of the *N*-methyltransferase enzymes; the individual values did not vary by more than 10%.

RESULTS AND DISCUSSION

The data, presented in Tables 2–4, provide evidence that the amine *N*-methyltransferases, each of which has an extraordinarily broad substrate range for primary and secondary amines [14], have a similarly broad spectrum of acceptance for tertiary

Table 2. N-Methylation of phenylpyridines and bispyridyls by amine *N*-methyltransferases A and B isolated from rabbit liver

Substrate	N-Methylated product [nmol·(mg protein)$^{-1}$·hr^{-1}]		Retention times of N-methylated compounds in min* (chromatographic system)
	Amine *N*-methyltransferase A	Amine *N*-methyltransferase B	
2-Phenylpyridine	5.1	ND†	10.6 (D)
3-Phenylpyridine	67	35	10.1 (D)
4-Phenylpyridine	59	17	10.6 (D)
2,2′-Bispyridyl	ND	ND	6.3, 29.1‡ (A)
3,3′-Bispyridyl	46§	11§	9.6, 45.2‡ (A)
4,4′-Bispyridyl	39§	13§	7.7, 33.5‡ (A)
2,4′-Bispyridyl	9.3§‖	3.1§‖	7.3, 25.0‡ (A)
2,3′-Bispyridyl	29§‖	8.1§‖	5.9, 43.6‡ (A)

* [^{3}H]AdoMet eluted at 4.1 min in System D and at 2.1 min in System A.
† Not detected.
‡ Retention time of the N,N-dimethylated authentic compounds.
§ Only the mono-N-methylated metabolite was detected.
‖ Identified as one or both of the two possible mono-N-methylated products.

Table 3. N-Methylation of some 2-, 3- and 4-substituted pyridines by amine *N*-methyltransferases A and B

Substrate	N-Methylated product [nmol·(mg protein)$^{-1}$·hr^{-1}]		Retention time of N-methylated compounds in min* (chromatographic system)
	Amine *N*-methyltransferase A	Amine *N*-methyltransferase B	
Pyridine	160	61	5.3 (A)
2-Methylpyridine	15	15	6.0 (A)
2,4,6-Trimethylpyridine	11	ND†	7.8 (A)
2-Chloropyridine	22	9.6	6.7 (A)
2-Bromopyridine	19	9.8	18.0 (D)
2-Aminopyridine	8.8	2.5	11.0‡ (F)
2-Vinylpyridine	7.7	6.0	6.3 (A)
2-Cyanopyridine	9.5	1.2	10.2 (G)
3-Bromopyridine	23	7.3	5.3 (A)
3-Aminopyridine	16	7.2	7.0‡ (D)
3-Cyanopyridine	1.8	ND	9.0 (G)
3-Hydroxypyridine	72	28	9.6‡ (B)
3-Benzylpyridine	60	31	4.0 (A)
4-Bromopyridine	22	5.9	17.3 (D)
4-Aminopyridine	2.0	ND	15.2‡ (F)
4-Vinylpyridine	10	6.4	5.6 (A)
4-Cyanopyridine	34	9.0	8.6 (G)

[^{3}H]AdoMet eluted at 2.1, 2.6, 2.1, 4.1, 2.0, 3.2, and 3.5 min in systems A, B, C, D, E, F, and G respectively.
† Not detected.
‡ Retention time for the N^1-methyl quaternary compound.

amines. Clearly, a variety of azaheterocycles are *N*-methylated to yield the respective quaternary ion. There is, however, a degree of structural selectivity that is exhibited, as is evident from examination of the activity with the phenylpyridine and bispyridyl series of compounds (Table 2). The presence of a bulky substituent at the pyridyl-2 carbon sterically hindered methylation of the pyridyl nitrogen (Table 3). Neither 2-phenylpyridine nor 2,2′-bispyridyl was an effective substrate despite very similar pK_a values [24] for all three bispyridyls. The effect of bulky substituents is also suggested by the observed decrease in N-methylation from pyridine to 2-methylpyridine and to 2,4,6-trimethylpyridine.

It would appear that the basicity of the nitrogen is not a principal limitation for N-methylation as catalyzed by these two transferases, a result that was predicted on the basis of the behavior of both enzymes with primary and secondary amines [14]. The symmetrical 3,3′- and 4,4′-bispyridyls were better substrates than the 2,3′- and 2,4′-bispyridyls despite similar pK_a values. Although the insertion of an additional nitrogen atom into the phenyl ring of the phenylpyridines, to form the appropriate bispyridyl, would decrease the basicity of the original pyridyl nitrogen, the 3,3′- and 4,4′-bispyridyls were not significantly less active than the corresponding phenylpyridines.

Table 4 contains other examples of this phenomenon. In comparing two weak bases, pyridazine and phthalazine, only the former was N-methylated. Quinoline and isoquinoline, similar in structure and in pK_a values, differed appreciably in their performance as substrates.

Thus, it would appear that structural features predominate in governing the effectiveness of compounds that could serve as substrates. Consistent with this view is the stereoselectivity of both enzymes for only the *R*(+)-isomer of nicotine [7]. However, such stereoselectivity was not observed with cotinine, a metabolite of nicotine; the (−)-isomer was effective as a substrate (Table 4).

Interpretation of the data presented here for product formation is subject to the caution that only a single concentration of substrate was used. Although that concentration, 1 mM, is relatively high, there is no assurance that the data were obtained at substrate saturation, thereby setting aside any definitive conclusion as to mechanism. Complicating the situation is the observation that both 4-phenylpyridine and 4,4-bispyridyl were inhibitory in this concentration range. K_m and V_{max} values, therefore, cannot be determined readily in this system because of substrate inhibition, an observation made previously for primary and secondary amines with both amine *N*-methyltransferases [14]. Nevertheless, the wide variety of azaheterocyclic compounds that can be methylated to form quaternary ions does support a role for these enzymes in the metabolism of xenobiotics [25]. That role could lead to toxicity, as in the formation of the *N*-methyl-4-phenylpyridinium ion (MPP), or it could have a positive pharmacological effect, as in the methylation in brain of a prodrug to produce a more slowly effluxing quaternary ion [9].

Table 4. N-Methylation of some aromatic mono- and diazaheterocycles by amine *N*-methyltransferases A and B

Substrate	N-methylated product [nmol·(mg protein)$^{-1}$·hr^{-1}]		Retention times of N-methylated compounds in min* (chromatographic system)
	Amine *N*-methyltransferase A	Amine *N*-methyltransferase B	
Imidazole	9.4†	8.7†	–
Pyrazole	20	5.4	90‡ (C)
Indole	ND§	ND	19.6 (C)
7-Azaindole	45	21	7.5‖ (D)
Pyridazine	34	19	4.6¶ (A)
Purine	9.1	ND	17** (E)
Quinoline	19	30	12.6 (D)
Isoquinoline	ND	8.5	13.5 (D)
Phthalazine	9.7	ND	5.6†† (A)
Quinoxaline	16	4.2	6.8‡‡ (A)
S-(−)-Cotinine	3.4	0.68	6.0 (A)
R,S-(±)-Cotinine	3.3	0.22	6.0 (A)
Metyrapone	4.2, 11§§	4.9, 1.1§§	6.4‖‖, 9.0‖‖, 26.1¶¶ (A)

* [^{3}H]AdoMet eluted at 2.1, 2.1, 4.1 and 2.0 min, respectively, in systems A, C, D, and E.
† Data taken from Ansher and Jakoby [14].
‡ Retention time for N^2-methylpyrazolium ion.
§ Not detected.
‖ Retention time for N^7-methylazaindolinium ion.
¶ Retention time for *N*-methylpyridazinium ion.
** Retention time for *N*-methylpurine ion.
†† Retention time for *N*-methylphthalazinium ion.
‡‡ Retention time for *N*-methylquinoxalinium ion.
§§ Data for the two isomeric mono-N-methylated metabolites of metyrapone [2-methyl-1,2-*bis*(3-pyridyl)-1-propanone].
‖‖ Retention time for each of the two mono-*N*-methylated metyrapone derivatives.
¶¶ Retention time for the *N,N*′-dimethylated metyrapone derivative.

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