N-METHYLATION AS A TOXICATION ROUTE FOR XENOBiotics.

II. In Vivo Formation of N,N'-Dimethyl-4,4'-bipyridyl ion (Paraquat) from 4,4'-Bipyridyl in the Guinea Pig

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(Received August 23, 1988; accepted September 16, 1988)

ABSTRACT:
The biotransformation of 4-phenylpyridine and 4,4'-bipyridyl to N-methylated quaternary ammonium metabolites in guinea pig and rabbit has been examined. Neither animal species excreted the neurotoxin N-methyl-4-phenylpyridinium ion as a urinary metabolite after ip administration of 4-phenylpyridine. However, treatment of rabbits with 4,4'-bipyridyl resulted in the formation of N-methyl-4,4'-bipyridinium ion in the urine (1.2% of the administered dose), and ip administration of 4,4'-bipyridyl to guinea pigs afforded both N-methyl-4,4'-bipyridinium ion and N,N'-dimethyl-4,4'-bipyridinium ion (paraquat) as urinary metabolites (0.8% and 2.9%, respectively, of the administered dose). The detection of the lung toxin paraquat as a urinary metabolite of 4,4'-bipyridyl is a significant finding, in that it represents the first documented report of the formation of a toxic metabolite via the N-methylation pathway.

Several studies have demonstrated the presence of 'azaheterocyclic N-methyltransferase' activity in animal tissues (1-5), and the in vivo biotransformation of aromatic azaheterocycles to N-methylated quaternary ammonium compounds has recently been investigated in our laboratories (6-10). In the rabbit, two homogeneous S-adenosylmethionine-dependent amine N-methyltransferases isolated from the soluble fraction of the liver have been demonstrated to have wide and overlapping substrate specificities for aliphatic, aralkyl, aromatic, and aromatic azaheterocyclic amines (11, 12). These observations indicate that the N-methylation of amino compounds by soluble nonspecific S-adenosylmethionine-dependent liver N-methyltransferases may constitute an important and general pathway for the biotransformation of a multitude of nitrogen-containing drugs and xenobiotics. In this respect, our interest has recently focused on the possibility that this metabolic route might be capable of generating toxicologically active compounds (13), because several N-methyl pyridinium salts are known to be highly toxic. The toxicity of paraquat (1) (N,N'-dimethyl-4,4'-bipyridyl) salt has long been known (14), and MPP+ (2) has recently been demonstrated to be a toxic metabolite of the Parkinsonism-inducing neurotoxin N-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (3) (15, 16). This study was, therefore, aimed at determining whether the administration of the potential 'protoxicants' 4,4'-bipyridyl (4) and 4-phenylpyridine (5) results in the in vivo formation of paraquat and MPP+, respectively, in guinea pig and rabbit.

Materials and Methods
Materials. Gold Label triethylamine, analytical grade sodium acetate, sodium borohydride, and 4,4'-bipyridyl were obtained from Aldrich.

For Part I, see C. S. Godin, P. A. Crooks, and L. A. Damani, Toxicol. Lett. 34, 217-222 (1986). This research was supported by a research grant from the Tobacco and Health Research Institute, Lexington, KY.

Abbreviations used are: MPP+, N-methyl-4-phenylpyridinium ion; MBP+, N-methyl-4,4'-bipyridinium ion; DMBP++, N,N'-dimethyl-4,4'-bipyridinium ion.

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in an manner identical to the procedure outlined above, and the resulting GLC-MS data were compared with those from the analysis of the authentic standards, in order to confirm the identity of the metabolites.

Quantitation of N-Methylated Metabolites of 4,4'-Bipyridyl. In order to quantify the urinary metabolites of 4,4'-bipyridyl obtained from the animal experiments, calibration curves of peak area vs. concentration were constructed for each authentic synthetic N-methylated metabolite. The analytical system used an analytical Partisil 10 SCX cation exchange column (25 x 0.46 cm) (Whatman). The isocratic mobile phase consisted of 0.3 M sodium acetate/methanol, 70:30, v/v, containing 3% triethylamine, adjusted with glacial acetic acid to a final pH of 4.5. Peak area integration was carried out on a Hewlett-Packard 3390 reporting integrator. Satisfactory calibration curves were generated by plotting peak area vs. the concentration of each potential metabolite. This was carried out on 3 consecutive days with freshly prepared samples. Concentration ranges utilized were 0.1 to 1.5 mg/ml for MBP* and 0.25 to 1.0 ml/ml for DMBP**. The calibration curves were used to determine the amount of each metabolite in the fractions from the preparative chromatography of the animal urine.

Results and Discussion

The isolation of quaternary ammonium compounds from biological fluids is a formidable challenge in metabolic studies because such compounds are polar, water soluble, and not usually extractable into organic solvents. The use of preparative cation exchange liquid chromatography was used in the isolation of the N-methylated urinary metabolites of 4-phenylpyridine and 4,4'-bipyridyl. This procedure afforded a satisfactory methodology for the desalting of urine samples and the fractionation of the quaternary ammonium metabolites. The structural identification of the metabolites required a sensitive and unambiguous technique, because relatively low amounts of the N-methylated metabolites in the urine were expected. The procedure used involved initial reductive derivatization of the N-methyl quaternary ammonium metabolites to more volatile products, followed by GLC-electron impact mass spectroscopic analysis. The reduction of the cationic N-methyl-pyrindinium rings in 6 and 1 with hydride ion was expected to afford the corresponding N-methylhydropyridine derivatives 7 and 9, and 8 and 10, respectively, which should possess greater volatility and lipophilicity as a result of the reductive destruction of the positively charged quaternary ammonium center. Similarly, reduction of 2 was expected to afford 3.

Fig. 1, B and C illustrate the preparative high performance cation exchange liquid chromatograms obtained from the analysis of 24-hr urine samples from rabbit and guinea pig, respectively, which had each been injected ip with 50 mg/kg 4,4'-bipyridyl. A chromatogram obtained from the analysis of guinea pig urine spiked with both MBP* and DMBP** authentic standards is shown in fig. 1A. The results clearly demonstrated that a UV-absorbing peak (not present in the control urine) could be detected in the urine from the 4,4'-bipyridyl-treated animal, which eluted with a retention time identical to that of the authentic metabolic standard MBP*. The column eluent containing this peak (indicated by the shaded area) was collected together with the fraction in which any DMBP** might be expected to elute (note, DMBP** was not observable by UV detection at 254 nm in the 24-hr rabbit urine analysis). Both fractions were evaporated and the solvent-free samples were subjected to reductive derivatization followed by GLC-MS analysis. Analysis of fraction 1 (fig. 1B) confirmed the presence of the tetrahydroborohydride reduction product, 7, of the mono-N-methylated metabolite of 4,4'-bipyridyl (see fig. 2). The results
were identical to those obtained from similar analysis of control urine spiked with synthetic MBP\(^+\) after the same fractionation procedure. No reduction product(s) of DMBP\(^+\) in fraction II (Fig. 1B) could be detected by GLC-MS analysis. Thus, DMBP\(^+\) does not appear to be an in vivo metabolite of 4,4'\'-bipyridyl in the rabbit.

The results from the analysis of 24-hr urine from guinea pigs that had been treated with 4,4'\'-bipyridyl were significantly different from those obtained in the rabbit (see fig. 1C). In guinea pig urine fractions, in addition to MBP\(^+\), a UV-absorbing peak that had a retention time on cation exchange HPLC similar to that of authentic DMBP\(^+\) was observed. Results from the GLC-MS analysis of the reduction products of these metabolites showed conclusively that both MBP\(^+\) and DMBP\(^+\) were urinary metabolites of 4,4'\'-bipyridyl in the guinea pig (figs. 2 and 3).

The borohydride reduction of both authentic and metabolically formed MBP\(^+\) and DMBP\(^+\), in each case, afforded two compounds, a major and a minor reduction product, with GLC R\(_T\) of 14.7 min (minor) and 15.9 min (major), and 14.2 min (minor) and 17.2 (major), respectively. The minor product was shown by MS analysis to be the hexahydro derivative (9) in the case of MBP\(^+\), and the decahydro derivative (10) in the case of DMBP\(^+\). The electron impact mass spectra and the proposed fragmentation pathways for the major borohydride reduction products 7 and 8 of MBP\(^+\) and DMBP\(^+\), respectively, are given in figs. 2 and 3.

Fig. 4A illustrates the preparative high performance liquid chromatogram obtained from an injection of urine spiked with authentic MPP\(^+\), whereas fig. 4, B and C are representative chromatograms obtained from the injection of 24-hr urine samples from rabbit and guinea pig, respectively, which had each been injected ip with 50 mg/kg 4-phenylpyridine. The results clearly illustrate that no observable UV-absorbing peak representing metabolically formed MPP\(^+\) is present in the urine of either animal. Nevertheless, the fraction eluting with the same retention time as authentic MPP\(^+\) was collected from both urine analyses, the solvent was removed, and the sample was submitted to borohydride reduction using the more sensitive analytical GLC-MS procedure described previously for the 4,4'\'-bipyridyl metabolites. The results indicated that no detectable amounts of MPP\(^+\) were present in either of the animal urine samples.

Results from the quantitative analysis of the N-methylated urinary metabolites of 4,4\'-bipyridyl recovered from preparative chromatography showed that these metabolites constitute only a minor fraction of the administered dose. In the rabbit, the MPP\(^+\) isolated from the urine represented 1.28% and 1.14% of the administered dose of 4,4\'-bipyridyl in the two animals examined, whereas in the guinea pig MPP\(^+\) was 0.92% and 0.68%, and DMBP\(^+\) was 2.98% and 2.78% of the administered dose in each of the two animals studied.

In conclusion, although the neurotoxin MPP\(^+\) could not be demonstrated as a urinary metabolite in either rabbits or guinea pigs that had been treated with 4-phenylpyridine, both animals formed and excreted MBP\(^+\) following administration of 50 mg/kg 4,4\'-bipyridyl. Although no data on the toxicity of MBP\(^+\) exist, there is a great deal of structural similarity between MBP\(^+\) and MPP\(^+\). Both structures are 4-substituted pyridinium ions and it is, therefore, possible that MBP\(^+\) may exhibit some of the neurotoxicity manifested by MPP\(^+\). Studies are currently underway to examine the possible neurotoxicity of MBP\(^+\) following intracerebral administration of this compound. Although lower amounts of MBP\(^+\) were excreted by the guinea pig compared with the rabbit, significantly higher amounts of DMBP\(^+\) were excreted in the urine of the guinea pig. However, in the rabbit, DMBP\(^+\) could not be detected as a urinary metabolite of 4,4\'-bipyridyl. The presence of DMBP\(^+\) in the urine of guinea pigs
FIG. 2. Mass spectral analysis of the major borohydride reduction product of metabolically formed MBP* in the rabbit (A), metabolically formed MBP* in the guinea pig (B), and an authentic sample of MBP* (C).

The scheme illustrates the proposed electron impact mass spectral fragmentation pathway for the reduced MBP*.
that had been treated with 4,4'-bipyridyl is significant, in that it is the first documented report wherein an *in vivo* *N*-methylation pathway has been shown to biotransform a xenobiotic into a known toxic metabolite. Thus, the reported detection of 4,4'-bipyridyl in cigarette smoke (17) may be of possible toxicological importance. Although 4,4'-bipyridyl is present in only trace amounts in cigarette smoke, the *N*-methylation of this compound to either MBP* or DBP* by cells in the lung may result in a cumulative toxic response, because DMBP* is concentrated by certain cells, especially type II alveolar cells (18). It is interesting to note that, although previous *in vitro* data in the rabbit (13) have shown that 4,4'-bipyridyl is avidly *N*-methylated to MBP* by lung soluble fractions, no *N*,*N'*-dimethylation to DBP* could be demonstrated in this study. This suggests that MBP* is probably not a substrate for the azaheterocycle *N*-methyltransferase enzymes in this animal species.

**References**

A. Preparative HPLC analysis of synthetic MPP* in urine by cation exchange chromatography (for chromatographic conditions see Materials and Methods). B. Analysis of 24-hr urine obtained from rabbits that had been administered 50 mg/kg 4-phenylpyridine. C. Analysis of 24-hr urine obtained from guinea pigs that had been administered 50 mg/kg 4-phenylpyridine. The shaded areas indicate fractions of column effluent collected for further analysis.


