J. Pharm. Pharmacol. 1988, 40: 758–762
Received December 9, 1987

© 1988 J. Pharm. Pharmacol.

Biotransformation of Primary Nicotine Metabolites:† Metabolism of *R*-(+)-[^{3}H-*N*′-CH$_3$; ^{14}C-*N*-CH$_3$] *N*-Methylnicotinium Acetate—The Use of Double Isotope Studies to Determine the In-vivo Stability of the *N*-Methyl Groups of *N*-Methylnicotinium Ion

WILLIAM F. POOL AND PETER A. CROOKS

Division of Medicinal Chemistry and the Graduate Center for Toxicology, University of Kentucky, Lexington, KY 40536–0053, USA

Abstract—The in-vivo metabolism of *R*-(+)-[^{3}H-*N*′-CH$_3$; ^{14}C-*N*-CH$_3$]-*N*-methylnicotinium acetate (NMN) was studied in the guinea-pig to determine the in-vivo stability of the *N*-methyl and *N*′-methyl groups of this primary nicotine metabolite. The results showed that *N*-demethylation does not occur. However, losses of 34 and 36%, respectively, of the ^{3}H label in the *N*′-CH$_3$ group of urinary NMN and the secondary metabolite, *N*-methyl-*N*′-oxonicotinium ion (NMNO), were observed. These results suggest that biotransformation of NMN may involve either an initial *N*′-demethylation step to *N*-methylnornicotinium ion (NMNor) followed by *N*′-methylation back to NMN, or the formation of an *N*′-methylene iminium species, which may be reductively converted back to NMN.

We have reported previously on the isolation of *N*-methyl-*N*′-oxonicotinium ion (NMNO), a urinary metabolite of both nicotine and *R*-(+)-[^{3}H-*N*′-CH$_3$]; [^{14}C-*N*-CH$_3$]-*N*-methylnicotinium acetate (NMN) in the guinea-pig (Pool et al 1986). NMNO was considered to be formed from NMN via direct *N*′-oxidation (Scheme 1a), however, NMN has been shown to be inactive as a substrate for the three major oxidation systems, cytochrome P-450 mixed function oxidase, flavin-peroxidase (cyclo-oxygenase), and flavin-containing mono-oxygenase (Damani et al 1988). The latter enzyme is able to catalyse the *N*′-oxidation of nicotine, but according to Ziegler (Ziegler 1985), it would be unlikely to accept NMN as a substrate due to the charge on the molecule. One possible route of formation of NMNO would be via an *N*-demethylation step to afford nicotine, followed by *N*′-oxidation to nicotine *N*′-oxide, and then *N*-methylation to NMNO (Scheme 1b). To examine this possibility we have prepared double labelled NMN containing a tritium label on the *N*′-methyl group, and a ^{14}C-label on the *N*-CH$_3$ group to detect any possible in-vivo *N*-demethylation. The results of the in-vivo metabolism of [^{3}H/^{14}C] NMN in the guinea-pig are presented herein.

Materials and Methods

Materials

R-(+)-[^{14}C-′*N*-tCH$_3$]-*N*-Methylnicotinium acetate (10 mCi m mol^{-1}) was synthesized via the previously reported procedure (Pool & Crooks 1985). *R*-(+)-[^{3}H-*N*CH$_3$]nicotine (76·5 Ci mmol^{-1}) was obtained from New England Nuclear, Boston, MA. *R*-(+)-Nicotine was prepared from (±)-nicotine via the procedure of Bowman et al (1982). All methylated nicotine derivatives were prepared as described previously (Cundy et al 1985). *R*-(+)-[^{3}H-*N*′-CH$_3$]-*N*-methylnicotinium acetate was synthesized as described below. Structure are shown in Fig. 1.

Synthesis of R-(+)-[^{3}H-N′-CH$_3$]-N-methylnicotinium acetate. Siliconization of all glassware utilized in this synthesis was necessary due to the high specific activity of the nicotine used (70 Ci mmol^{-1}), since it possesses high binding affinity

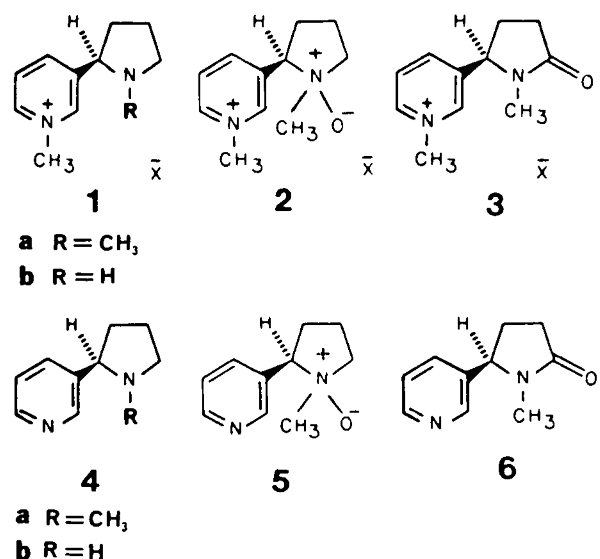
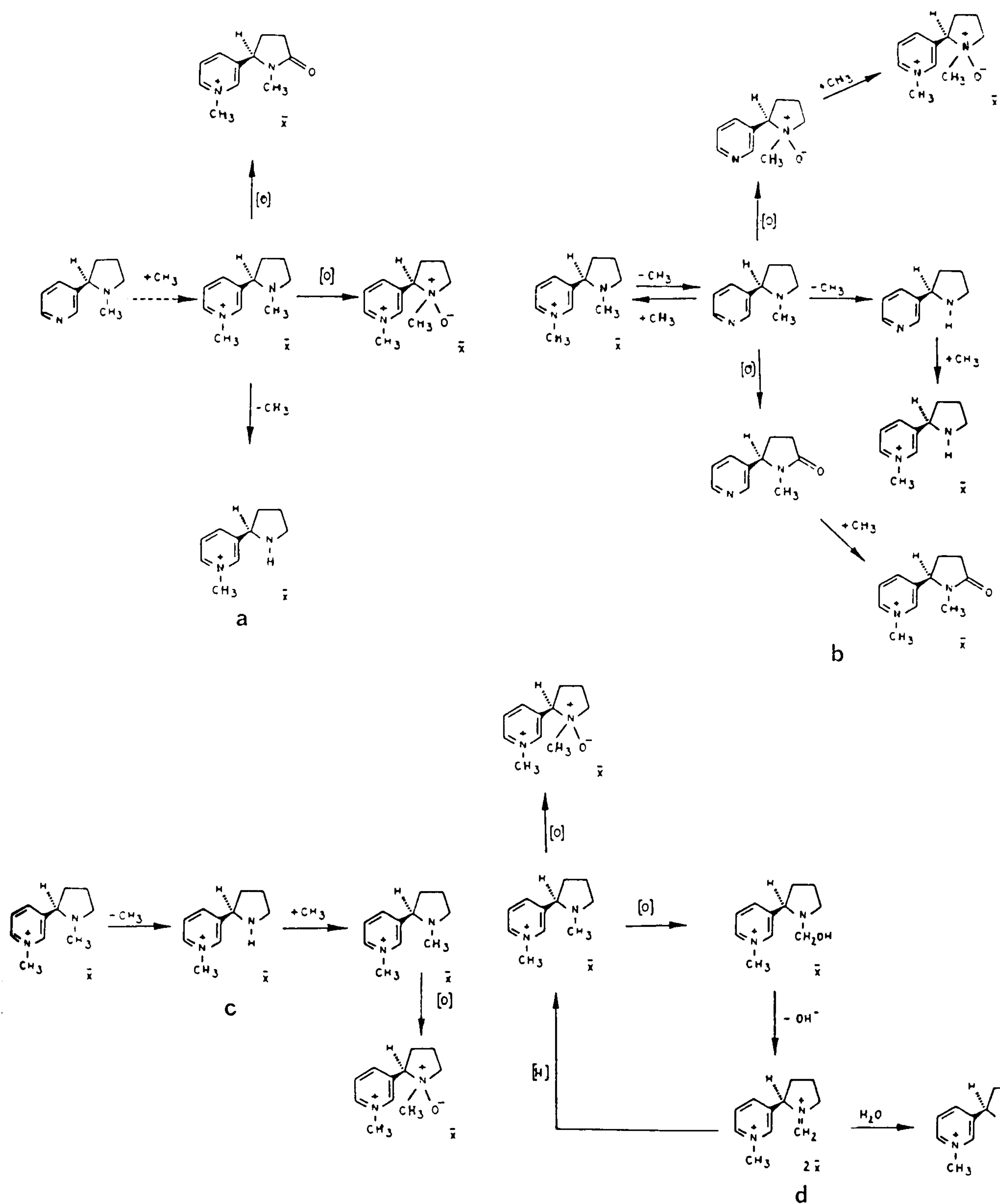


FIG. 1. Structures of *R*-(+)-*N*-methylnicotinium salt and related compounds.

1a, *N*-methylnicotinium salt (NMN); 1b, *N*-methylnornicotinium salt (NMNor); 2, *N*-methyl-*N*′-oxonicotinium salt (NMNO); 3, *N*-methylcotininium salt (NMC); 4a, nicotine; 4b, nornicotine; 5, *N*′-oxonicotine; 6, cotinine.

Correspondence to: P. A. Crooks, College of Pharmacy Division of Medicinal Chemistry, University of Kentucky, Lexington, KY 40536–0053, USA.

† Part III. For part II, see Cundy, K. C., & Crooks, P. A. (1987) Xenobiotica 17:785–792



Scheme 1. Hypothetical pathways for the formation of metabolites of *N*-methylnicotinium ion. a—direct oxidation pathway; b—*N*-demethylation pathway; c—*N'*-demethylation pathway; d—iminium ion pathway.

for glass surfaces. Glass disposable micro-sampling pipets (100 μL capacity) were sealed at one end under a bunsen flame and used as reaction vessels for this synthesis. An ethanolic solution of R-(+)-[^{3}H-N'-CH_3]nicotine (200 μL, 70Ci $mmol^{-1}$, 1μCi 1μL^{-1}) was added to a siliconized glass scintillation vial cooled in an ice-bath. Glacial acetic acid (14 μL) was then added and the resulting solution was gently agitated. The excess glacial acetic acid was required to ensure protonation of the pyrrolidine nitrogen. Methyl iodide (14 μL) delivered via a 100 μL Hamilton syringe was added to this mixture and the solution again gently agitated. Aliquots (approximately 80 μL) of the resulting mixture were transferred to the capillary reaction vessels, which were contained in an ice bath. The reaction vessels were then carefully sealed under a bunsen flame and placed in a water bath, which was heated to 100°C, and the reaction was allowed to proceed for 16 h. After this time, the reaction vessels were allowed to cool, scored at one end with a glass cutter, carefully opened, and the resulting crude R-(+)-[^{3}H-N'-CH_3]-N-methylnicotinium acetate solution subjected to high performance liquid radiochromatography. Analysis of the reaction mixture by cation-exchange chromatography using the procedure of Pool & Crooks (1985) indicated that 60% of the radiolabel co-migrated with an authentic UV-absorbing standard of NMN, 30% co-eluted with an authentic UV-absorbing standard of DMN and 10% of the radiolabel eluted in the void volume and was unidentified. No N'MN was detected in the reaction mixture. Purification of the crude reaction mixture utilized the previously reported preparative HPLC method for the preparation of R-(+)-[^{14}C-$N$$CH_3$]nicotinium acetate (Pool & Crooks 1985). Reanalysis of the purified product on the analytical system showed it to be greater than 99% radiochemically pure.

Metabolic experiments

Radiotracer experiments with R-(+)-[^{3}H-N'-CH_3]-N-methylnicotinium acetate (30 μCi, i.p.), R-(+)-[^{14}C-N-CH_3]-N-methylnicotinium acetate (10 μCi, i.p.) and R-(+)-[^{14}C-N-CH_3;, ^{3}H-N'-CH_3]-N-methylnicotinium acetate [5·5 μCi(^{14}C), 10 μCi(^{3}H)i.p.] were carried out on groups of four male Dunkin Hartley guinea-pigs (525 $\pm$ 24g) as described previously (Pool & Crooks 1985).

Analysis of urinary metabolites

Animals were maintained in custom-built glass metabolism cages (Crown Glass Co., Sommerville, New Jersey) for 24 h, which incorporated an efficient unit for the collection of urine, faeces and expired air. Urine and faeces were collected at the completion of the study and were stored at -20°C until analysed. At the end of the studies, animals were killed with sodium pentobarbitone (approximately 100 mg kg^{-1} body weight). Blood was removed via cardiac puncture and gastric samples were taken from the stomach by hypodermic syringe. Tissues were excised, weighed, and immediately stored at -20°C until analysed for total radio-activity.

Expired carbon dioxide was trapped in 75 mL of a mixture of 2-methoxyethanol–ethanolamine (2:1, v/v) in those experiments involving the collection of ^{14}C-labelled carbon dioxide. Air was pulled through the metabolism cages at 400 mL min^{-1} by a vacuum pump. [^{14}C]Carbon dioxide collection was carried out over 24h during which time the trapping solution was changed three times. Aliquots (1 mL) from the [^{14}C]-CO_2 trapping solution were added to 15 mL of Scintiverse II scintillation cocktail. Distilled water (2 mL) was then added and the samples analysed by scintillation spectrometry. Addition of water to the cocktail is necessary to prevent the carbonate salt of ethanolamine from precipitating out of solution, and to achieve a homogeneous distribution.

Urine samples were analysed for total radioactivity by accurately measuring the volume of the 24 h urine collected, and adding a 100 μL aliquot to RPI 3a70b scintillation cocktail (10 mL) and analysing on a Packard Model 3255 liquid scintillation spectrometer. This procedure was performed in triplicate. Cage rinses were analysed in an identical manner, and total radioactivity was determined from these values.

Samples containing both ^{3}H and ^{14}C radioisotopes from the double labelled studies, which required ratio analysis, were quantitated in a Packard Model 3375 liquid scintillation spectrometer in which the ^{3}H and ^{14}C windows had been adjusted to correct for ^{14}C spillover into the ^{3}H channel. Sample quench was compensated for by the use of quench curves obtained by utilizing automatic external standardization. Quench curves were calculated every three months for each instrument. Scintiverse II was utilized as the scintillation cocktail in these experiments.

Total 24 h faeces were pulverized using a glass mortar and pestle. Aliquots of approximately 500 mg were placed in a Cumbusto cone (Packard Instruments, Downers Grove, Illinois) and oxidized in a Packard Model 306B sample oxidizer using the appropriate scintillation cocktails. The oxidized samples were then counted for radioactivity as described previously.

Blood samples were separated into blood clot and blood serum which were each oxidized separately in amounts of 500 mg or less along with the bile and gastric samples; all oxidized samples were then counted for radioactivity as described previously.

Amounts of 500 mg or less of all tissue samples were oxidized and counted for radioactivity. Some tissues including the brain, spleen, pancreas, and adrenals were oxidized entirely. The liver was homogenized in phosphate buffer, pH 7·4, and 0·5 mL amounts were oxidized.

Total 24 h urine samples from animals that had been administered radio-labelled NMN were centrifuged at 250 g for 5 min before chromatographic analysis to pellet any particulates. Aliquots (0.1 mL) were then injected directly onto the appropriate chromatographic column.

Initial analysis of quaternary ammonium metabolites utilized a Partisil 10/25 SCX (25 $\times$ 0·46 cm) cation exchange column (Whatman) 10 μm particle size, 37 000 AV plates per metre, and a mobile phase of 0·3M sodium acetate–methanol (70:30) containing 1% triethylamine (v/v) adjusted to pH 4·5 with glacial acetic acid, and then to pH 5·5 with ammonium hydroxide. The flow rate was 2 mL min^{-1}. Urine samples were also analysed on a second HPLC system which afforded a different order of elution of the expected quaternary ammonium metabolites: this system comprised a Partisil cation exchange column and a mobile phase of 0·3 ammonium acetate–methanol (70:30) containing 1% triethylamine (v/v) adjusted to pH 3·7 with glacial acetic acid.

Oxidative urinary metabolites were determined using the previously reported HPLC procedure (Pool & Crooks 1985). Identification of all metabolites was accomplished by co-chromatography with authentic standards utilizing UV detection at 254 nm on the above chromatographic systems. Quantitation of the radiolabelled metabolite was achieved by collecting 1 min fractions and analyzing them by scintillation spectrometry.

Determination of deuterium exchange of the N′-CH_3 *protons of NMNO and NMN acetate*

Both NMNO and NMN acetate were dissolved in either acidic or alkaline D_2O solutions, and the resulting mixtures analyzed by ^{1}H-NMR spectroscopy. An initial spectrum of NMNO was recorded at 300 MHz in deuterium oxide (pH 6.5), and integrals of the *N′*CH_3 and N^+-CH_3 signals noted. The NMNO sample was then split into two samples; one in which the final pH was adjusted to pH 4·0 with 0·01 M aqueous deuterium chloride while the second was adjusted to a final pH of 9·0 with a 0·01 M solution of sodium deuteroxide. Spectra of the pH-modified NMNO samples were then recorded and integrals of the *N′*-CH_3 and N^+-CH_3 signals noted. Analysis of the ratio of *N′*-CH_3-integral to its corresponding N^+-CH_3-integral for each of the three samples was then carried out to determine whether any exchange of the *N′*-CH_3 protons with deuterium had occurred at these pH extremes. Experiments using NMN acetate in place of NMNO were similarly made.

Results and Discussion

Double channel analysis of the $^{14}C/^{3}H$ labelled NMN used in the metabolism studies afforded a value of 1·87:1·0 (^{3}H: ^{14}C). Percent recovery of each radio nuclide over 24 h after i.p. administration of doubled labelled NMN to guinea-pigs is given in Table 1. As can be seen, slightly lower recovery of the ^{3}H-label compared with the ^{14}C-label was observed in the urine, however, this was not statistically significant. Very little of either nuclide was excreted in the faeces, and 24 h tissue label retention was low (1·3 and 1·0% of the administered dose, respectively, for ^{3}H and ^{14}C) for both nuclei. No $^{14}CO_2$ was detected in the expired air of guinea-pigs that had been administered [^{3}H/^{14}C]NMN.

A more detailed analysis of the tissue radiolabel is shown in Table 2. The adrenal glands and caudate of the epididymis contained the greatest percentage of both ^{3}H- and ^{14}C-label in the tissues on a per gram basis. Other tissues containing significant levels of both radiolabelled forms of NMN on a per gram basis, included the stomach contents, small intestine, kidney, heart, spleen, and liver. While a small percentage of the ^{14}C-radiolabel per gram tissue is associated with the brain, the level of ^{3}H-label in the brain is greater than ten times that of the ^{14}C-label. Other tissues containing a disproportionally high level of ^{3}H on a per gram basis included blood clot, blood serum, perirenal adipose tissue, and the lung. Analysis of the ^{3}H: ^{14}C (d min^{-1}:d min^{-1}) data in Table 2 demonstrated that all tissues assayed at a greater ^{3}H:^{14}C ratio than that in the administered NMN. The most striking tissue ratios were found in the brain (48·51:1·0), blood clot (14·79:1·0), blood serum (10·17:1·0), perirenal adipose tissue (9·12:1·0), and lung tissue (5·89:1·0).

Analysis of the urinary metabolites from the double label study are given in Table 3. Most of the radiolabel was associated with NMN, however, the ^{3}H:^{14}C ratio analysis of urinary NMN was significantly lower than the ^{3}H:^{14}C ratio of the administered NMN. Approximately 25% of urinary radiolabel was in the form of *N*-methylcotininium ion, which had a ^{3}H:^{14}C ratio similar to that of administered NMN. However, the minor metabolite NMNO, afforded a ^{3}H:^{14}C ratio of 1·19, which is almost identical to that observed for urinary NMN. As expected, the *N′*-demethylated metabolite, NMNor contained only the ^{14}C-label.

These data indicate that 34 and 36% loss of ^{3}H has occurred, respectively, in urinary NMN and NMNO, compared with the original ^{3}H:^{14}C ratio of the administered *R*-(+)-NMN. No loss of either nuclide was observed in the urinary NMC.

The results rule out *N*-demethylation as the initial step in the metabolism of NMN (Scheme 1b) and suggest a direct rapid oxidation of NMN to NMC due to the similar ratios of the administered NMN, and urinary NMC. However, the significant loss of ^{3}H seen in both the NMNO and NMN recovered from the urine is unexpected and could arise from an additional and more complex metabolic pathway for the formation of the former compound. The double labelled experiment, as was observed in similar experiments with ^{14}C-labelled NMN, clearly demonstrated the *N′*-demethylation of NMN to NMNor. Now, if NMNor is a substrate for an azaheterocycle *N*-methyltransferse system present in guinea-pig tissues, this metabolite could be *N′*-methylated back to NMN, some of which could be subsequently oxidized to NMNO. Both could then be excreted along with unmetabolized NMN, and its oxidized product NMNO (Scheme 1c). The combination of the above biotransformations would result in a reduction in the ^{3}H-label in both the urinary NMNO and urinary NMN, as was observed in the double label study.

Another explanation for the loss of ^{3}H label at the *N′*-CH_3 group of NMN and NMNO, could be that some of the NMN is channelled into the oxidation pathway illustrated in Scheme ld, undergoing initial hydroxylation of the *N′*-CH_3 group, formation an iminimum salt intermediate, followed by either hydrolysis to NMNor, or reduction back to NMN and *N′*-oxidation. This pathway would result in loss of tritium at the *N′*-CH_3 if the NMN is recycled. As far as we are aware, there is no precedent for the reductive step in this hypothetical pathway.

An alternative chemical mechanism to explain the observed ^{3}H loss could be that the hydrogens of the methyl group attached to the quaternary, positively charged pyrroli-

Table 1. Percent radioactivity recovered after i.p. administration of [^{14}C-*N*-CH_3; ^{3}H-*N′*-CH_3]-*N*-methylnicotinium acetate in the guinea-pig.

	% Administered dose over 24 h	
Radiolabel	[^{14}C]NMN	[^{3}H]NMN
Urine	69·8±9·8$_a$	57·4±7·5
Faeces	1·7±0·7	2·0±0·8
Tissue	1·0±0·2	1·3±0·1
Total	72·4±9·6	60·7±7·6

a data expressed as Mean ± sem, n = 4.

Table 2. Tissue retention of radiolabel 24 h after i.p. administration of R-(+)-[^{14}C-N-CH_3; ^{3}H-N'-CH_3; ^{3}H-N'-CH_3]-N-methylnicotinium acetate in the guinea-pig.

Radiolabel	% Administered dose/g × 10^{-3} ^{3}H	^{14}C	^{3}H:^{14}C ($dmin^{-1}$:$dmin^{-1}$)[c]
Liver	32·6 ± 3·6[a]	17·6 ± 3·1	3·56 ± 0·23
Lung	13·1 ± 1·3	4·4 ± 1·1	5·89 ± 0·7
Kidney	37·7 ± 4·0	22·4 ± 3·5	3·22 ± 0·19
Spleen	32·2 ± 1·8	18·5 ± 1·4	3·3 ± 0·2
Bladder	29·8 ± 6·5	16·2 ± 6·3	4·01 ± 0·79
Gall Bladder	17·5 ± 0·8	7·7[b]	4·26 ± 0·06
Bile	19·8 ± 1·9	8·6 ± 1·0	4·35 ± 0·24
Serum	10·6 ± 0·7	2·2 ± 0·6	10·17 ± 2·06
Clot	5·7 ± 0·3	0·8[b]	14·79 ± 1·21
Adrenals	74·1 ± 16·5	60·8 ± 15·5	2·34 ± 0·09
Heart	38·4 ± 7·0	23·6 ± 5·4	3·13 ± 0·24
Pancreas	28·8 ± 2·9	20·0 ± 2·2	2·72 ± 0·05
Brain	7·3 ± 0·1	0·4 ± 0·1	48·51 ± 6·00
Peri-renal adipose	4·4 ± 0·5	1·9 ± 1·1	9·12 ± 4·84
Stomach contents	66·6 ± 1·7	44·6 ± 5·8	2·92 ± 0·43
Sm. Int. contents	48·5 ± 9·6	33·6 ± 4·5	2·73 ± 0·12
Testis	22·8 ± 8·3	12·0 ± 6·5	4·29 ± 0·69
Epididymis (Cauda)	72·5 ± 43·3	51·2 ± 34·4	3·07 ± 0·33

[a] data are expressed as Mean ± sem; n = 3
[b] n = 2, values did not vary by ± 10%
[c] the administered [^{3}H/^{14}C]NMN had an isotope ratio of 1·87

Table 3. Urinary metabolite quantitation after i.p. administration of R-(+)-[^{14}C-N-CH_3]; [^{3}H-N'-CH_3]N-methylnicotinium acetate in the guinea-pig.

Radiolabel	[^{14}C]NMN	[^{3}H]NMN	^{3}H:^{14}C ($dmin^{-1}$:$dmin^{-1}$)
Void	1·6 ± 0·1[a]		
NMC	25·8 ± 2·0	23·7 ± 0·6	1·88
NMN	56·2 ± 2·3	66·6 ± 0·5	1·24
NMNO	6·6 ± 0·5	8·4 ± 0·9	1·19
NMNor	7·5 ± 0·6	—[b]	—[c]

[a] data expressed as Mean ± sem, n = 4
[b] not detecteddue to loss of label
[c] contained only ^{14}C label

dine nitrogen in the NMNO structure may be acidic enough to be removed by a nucleophilic moiety or by solvent exchange, thus resulting in a change in the specific activity of the tritium label. Subsequent N'-deoxygenation would give rise to NMN also with an altered specific activity of tritium at the N'-methyl group. Studies were therefore conducted to determine this, using ^{1}H-NMR spectroscopy. Both NMNO and NMN acetate were subjected to both acidic and basic conditions in D_2O solution to facilitate deuterium exchange of the protons on the N'-CH_3 group of the pyrrolidine ring. No exchange was observed, indicating that the tritium label in both NMNO and NMN is stable and does not dissociate in-vivo.

In summary, the results from this double label study indicate that in-vivo N-demethylation of NMN to form nicotine is an unlikely metabolic step, since none of the urinary metabolites isolated showed any loss of ^{14}C-label. Thus the metabolic pathway illustrated in Scheme 1b would appear not to be operating in the formation of metabolites of NMN. However, unexpected loss of ^{3}H-label in urinary NMN, and NMNO suggests that an additional pathway to the direct N'-oxidation pathway may be operating in the formation of NMNO. This pathway may involve an initial N'-demethylation to NMNor followed by N'-methylation back to NMN and N'-oxidation to NMNO (Scheme 1c), or it may be due to the formation of an iminium species, which may be reductively converted back to NMN followed by N'-oxidation (Scheme 1d). Clearly in-vitro studies involving either radiolabelled NMNor or SAM would be of value in distinguishing between the above mechanisms, as would the determination of the fate of radiolabelled NMNor in-vivo.

Acknowledgement
This study was supported by a research grant (to PAC) from the Tobacco and Health Research Institute, Lexington, Kentucky (Project number 4C018).

References

Bowman, E. E., McKennis, H., Jr., Martin, B. (1982) A convenient method for the preparation of racemic nicotine. Synthetic Commun. 12: 871–879
Cundy, K. C., Sato, M., Crooks. P. A. (1985) Stereospecific in vivo N-methylation of nicotine in the guinea-pig. Drug Metab. Disposit. 13: 175–185.
Damani, L. A., Pool, W. F., Crooks, P. A., Kaderlik, R. K., Ziegler, D. M. (1988) Stereoselectivity in the N′-oxidation of nicotine isomers by flavin-containing monooxygenase. Molec. Pharmacol. 33: 702–705
Pool, W. F., Crooks, P. A. (1985) Biotransformation of primary nicotine metabolites. 1. In vivo metabolism of R-(+)-[^{14}C-NCH_3]-N-methylnicotinium ion in the guinea-pig. Drug Metab. Disposit. 13: 578–581
Pool, W. F., Houdi, A. A., Damani, L. A., Layton, W. J., Crooks, P. A. (1986) Isolation and characterization of N-methyl-N′-oxonicotinium ion, a new urinary metabolite of R-(+)-nicotine in the guinea-pig. Ibid. 14: 574–579
Ziegler, D. M. (1985) Molecular basis for N-oxygenation of sec- and tert-amines. In Biological Oxidation of Nitrogen in Organic Molecules—Chemistry, Toxicology and Pharmacology, edit. Gorrod, J. W. Damani, L. A., Ellis-Norwood, Chichester, UK, p. 43-52