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SHORT COMMUNICATIONS

Stereospecific *in vitro* N-methylation of nicotine in guinea pig tissues by an *S*-adenosylmethionine-dependent *N*-methyltransferase

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The biotransformation of nicotine into quaternary *N*-methyl pyridinium metabolites was first demonstrated in the dog, and in man, by McKennis *et al.* [1], and was shown to be a significant route in the *in vivo* metabolism of nicotine. Since that initial report, no new studies on the qualitative and quantitative aspects of nicotine methylation appear to have been carried out, and the site of methylation, the nature of the enzyme(s) involved and the substrate requirements for the enzymic reaction are still not known. The guinea pig has been identified previously as an extensive *in vivo* N-methylator of the heterocycle pyridine [2, 3] and will also methylate nicotine *in vivo* [4]. We have, therefore, undertaken a preliminary investigation of the *in vitro* N-methylation of nicotine by guinea pig tissues. These studies have determined the co-factor requirements, stereochemical specificity, and tissue distribution of an azaheterocycle *N*-methyltransferase enzyme involved in the N-methylation of nicotine.

Materials and methods

Materials. [^{14}C-2′]-*RS*-Nicotine (0.25 mCi, sp. act. 57.6 mCi/mmole) was obtained from New England Nuclear (Boston, MA); [^{14}C-NCH$_3$]-*RS*-nicotine-(+)-bitartrate (0.25 mCi, sp. act. 57 mCi/mmole) was obtained from the Radiochemical Centre, Amersham (Great Britain). [^{3}H-NCH$_3$]-*S*-Nicotine (1 mCi, sp. act. 68.6 Ci/mmole) and [^{3}H-NCH$_3$]-*R*-nicotine (1 mCi, sp. act. 76.5 Ci/mmole) were gifts from New England Nuclear. *S*-Adenosyl-L-[*methyl*-^{14}C]-methionine (0.05 mCi, sp. act. 45 mCi/mmole) was obtained from R.P.I. (Mount Prospect, IL). The radiochemical purity of the nicotine substrates was determined by cation-exchange high performance liquid chromatography (HPLC) and, in all cases was better than 95%.

S-Nicotine was obtained from the Aldrich Chemical Co. (Milwaukee, WI) and *R*-nicotine was a gift from Drs. E. R. Bowman and E. L. May, Medical College of Virginia, Virginia Commonwealth University (Richmond, VA). *R*-*N*-Methylnicotinium iodide was synthesized from *R*-nicotine by the method of Seeman and Whidby [5].

Preparation of tissue homogenates. Four male Hartley guinea pigs (630 ± 15 g) were deprived of food and water for 24 hr and killed by nembutal overdose. The liver, lung, spleen, and brain from each animal were removed, weighed and homogenized in 3 vol. of ice-cold 1.15% KCl solution using an Ultra-Turax homogenizer (Janke & Kunkle, Kh.). The lung and liver were both perfused with ice-cold 1.15% KCl solution prior to removal. The crude homogenates were centrifuged at 40,000 *g* for 20 min in a model J2–21 refrigerated ultracentrifuge (Beckman Instruments) to remove nuclei and cell debris, and the volume of the supernatant fraction was adjusted with 1.15% KCl solution, such that 1 ml was equivalent to 250 mg of original tissue. One-half of each tissue homogenate was dispensed into dialysis bags (Spectrapor 2, molecular weight cut-off 12,000–14,000, Spectrum Medical Industries) and dialyzed at 4° against 2 × 1 liter of 1 mM phosphate buffer, pH 7.9, over 24 hr. Protein contents of both dialyzed and undialyzed homogenates were determined by the method of Lowry *et al.* [6]. All homogenates were maintained at −20° prior to use.

In vitro *incubations.* All incubations of both dialyzed and undialyzed tissue homogenates were performed in 6 × 50 mm disposable glass tubes sealed with rubber septa. In initial experiments, the incubation mixture (250 μl) contained homogenate (125 μl), *S*-nicotine (20 μg/250 μl), [^{14}C-2′]-*RS*-nicotine (0.25 μCi/250 μl), *S*-adenosyl-L-methionine (SAM; 12.5 μl, 10 mM in 1 mM hydrochloric acid) and 0.1 M phosphate buffer, pH 7.9 (to 250 μl). In later experiments, the nature and position of the radiolabeled atom and the enantiomeric purity of the radiolabeled nicotine were varied. In all experiments, the final concentration of SAM used was 10 mM. Control incubations were performed in which the cofactor SAM or the homogenate was omitted, or in which the homogenate was boiled for 5 min prior to use. All homogenates were preincubated with co-factor for 10 min at 37°, prior to addition of nicotine. Incubations were then carried out at 37° for 1 hr unless otherwise stated. After incubation, homogenates were immediately frozen and stored at −20° until analyzed.

Effect of dialysis. Both dialyzed and undialyzed homogenates of each of the four tissues were examined with appropriate controls, as described above. Experiments were repeated for each of three animals to provide statistically valid results.

The thawed, centrifuged incubates were analyzed directly by cation-exchange high performance liquid chromatography (see Fig. 1) using a procedure previously developed in our laboratory [4], which allows the analysis of seven potential metabolites of nicotine including four N-methylated derivatives. In each analysis, the extent of nicotine methylation in each homogenate sample was assessed as a percentage of the recovered radioactivity. The values were adjusted, based upon mg of protein/ml, to give a value of percent methylation of nicotine per mg of protein.

Determination of stereospecific N-methylation of nicotine. To ascertain the stereochemical requirements for nicotine methylation, incubations of dialyzed lung homogenate were performed using the pure enantiomers of ^{3}H-labeled nicotine as substrate. These were then compared with incubations in which racemic nicotine, labeled in one of two positions with ^{14}C, was used as the substrate. Thus, incubations were carried out using [^{3}H-NCH$_3$]-*S*-nicotine (0.5 μCi/incubate), [^{3}H-NCH$_3$]-*R*-nicotine (0.5 μCi/incubate), [^{14}C-2′]-*RS*-nicotine (0.25 μCi/incubate) or [^{14}C-NCH$_3$]-*RS*-nicotine-(+)-bitartrate (0.25 μCi/incubate). Appropriate controls were also performed in each case, as previously described. The mixtures were then incubated at 37° for 4 hr. Analysis of nicotine N-methylation for each sample was achieved by HPLC as described above.

Source of methyl group. To establish unequivocally that SAM was the source of the methyl group in the methylated metabolite, a double-label experiment was performed. ^{3}H-Labeled nicotine of either enantiomeric form was used as the substrate, and SAM labeled with ^{14}C in the methyl group was used as the cofactor. Thus, one set of incubations contained [^{3}H-NCH$_3$]-*S*-nicotine (0.5 μCi/incubate) as substrate and one set contained [^{3}H-NCH$_3$]-*R*-nicotine (0.5 μCi/incubate) as substrate. In both cases, *S*-adenosyl-L-[*methyl*-^{14}C]-methionine (0.25 μCi/incubate) was added along with unlabeled SAM. Appropriate controls were

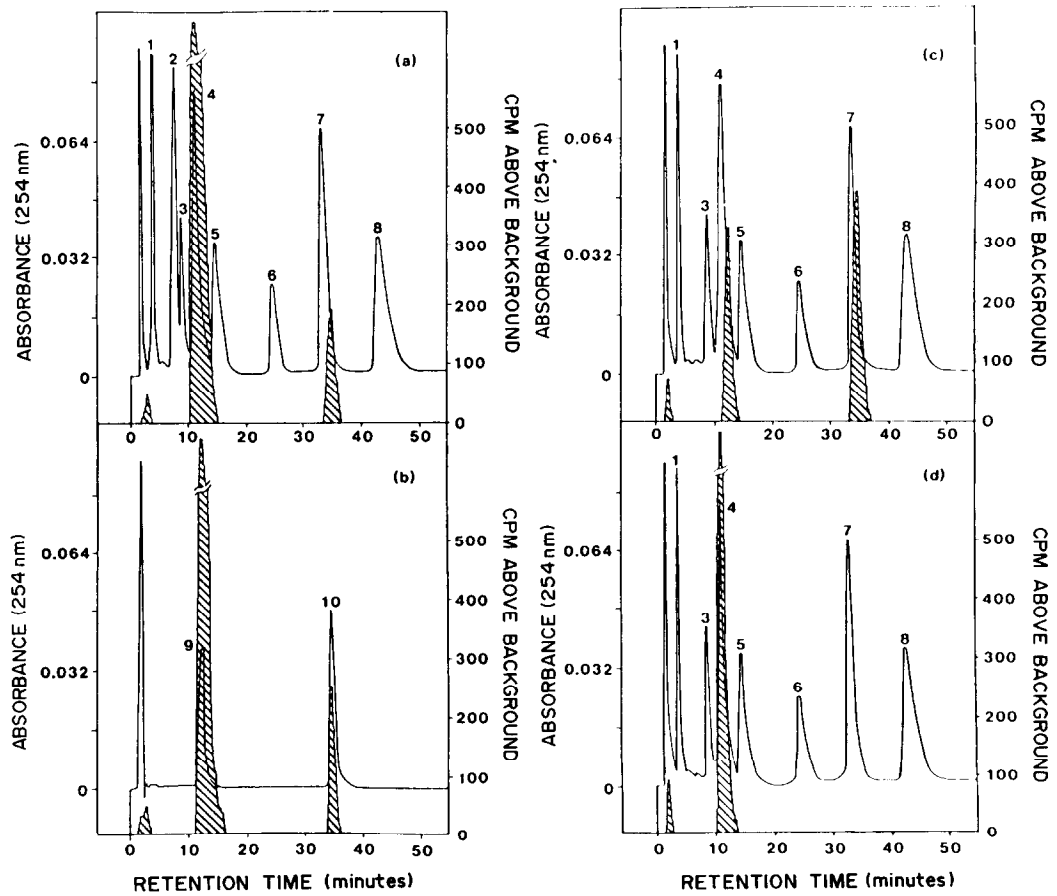

Fig. 1. Radiochromatograms from analysis of *in vitro* homogenates by HPLC. Methylation of [^{14}C-2′]-*RS*-nicotine by dialyzed spleen coinjected with (a) *S*-*N*-methylnicotinium standard, and (b) *R*-*N*-methylnicotinium standard. Stereospecific methylation of nicotine by dialyzed lung using (c) [^{3}H-NCH$_3$]-*R*-nicotine and (d) [^{3}H-NCH$_3$]-*S*-nicotine. The standards used are: (1) *S*-cotinine, (2) *S*-nornicotine, (3) *S*-nicotine-1′-oxide, (4) *S*-nicotine, (5) *S*-*N*-methylcotininium iodide, (6) *S*-*N*′-methylnicotinium iodide, (7) *S*-*N*-methylnicotinium iodide, (8) *S*-*N*,*N*′-dimethylnicotinium diiodide, (9) *R*-nicotine, and (10) *R*-*N*-methylnicotinium iodide. The HPLC system is described elsewhere [4].

prepared, and all samples were incubated at 37° for 1 hr. Analysis of incubations was performed, after centrifugation, by a modified HPLC system described in Fig. 2. However, duplicate analyses were performed to determine the distribution of both ^{3}H and ^{14}C among the metabolites eluting from the column. This was achieved by selecting the appropriate channel of the radioactive flow-through detector for the desired radioisotope. In this way, the presence of both the nicotine moiety and the original methyl group of SAM, in the radiolabeled metabolite, could be established.

Results and discussion

Identity of the methylated metabolite. In all four tissues examined, racemic [^{14}C-2′]-nicotine gave rise to a single cationic metabolite, with a retention on the HPLC system similar to that of the *S*-*N*-methylnicotinium standard. However, in all cases this radioactive metabolite did not exactly co-elute with the unlabeled standard, but had a longer retention time (Fig. 1a). We have observed previously a similar phenomenon in the analysis of radiolabeled nicotine, on the same HPLC system [7]. Neither pure enantiomer of radiolabeled nicotine co-elutes with an unlabeled nicotine standard of the opposite enantiomeric composition when coinjected on the above HPLC system. The phenomenon responsible for this behaviour has been studied in detail by us elsewhere [7], and can be explained by some form of differential association between like and unlike enantiomers of nicotine, resulting in the formation of *homo*- and *hetero*-dimers of nicotine that exhibit slightly different retention times on the above HPLC system. It therefore appeared that the same phenomenon may be responsible for the separation of the radiolabeled metabolite and the *N*-methylnicotinium standard observed in the present study. For this reason, analysis of the homogenate was repeated with *R*-*N*-methylnicotinium iodide as the unlabeled standard. As is demonstrated in Fig. 1b, the radioactive metabolite and unlabeled *R*-*N*-methylnicotinium standard had identical retention on the same HPLC system. This result suggested that only one optical

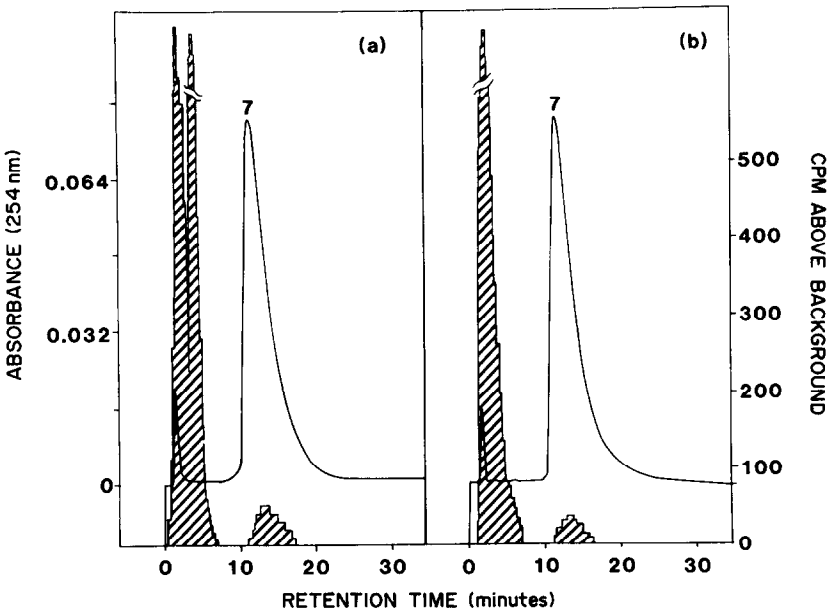

Fig. 2. Radiochromatograms from a double-label experiment using [^{3}H-NCH$_3$]-*R*-nicotine substrate and *S*-adenosyl-[^{14}C-*methyl*]-L-methionine cofactor. Key: (a) distribution of ^{3}H-activity in effluent, and (b) distribution of ^{14}C-activity in effluent. HPLC system: isocratic 0.3 M sodium acetate/methanol (70:30) + 1% triethylamine, pH 4.5, 2.0 ml/min. For identity of standards, see legend of Fig. 1.

isomer of the racemic ^{14}C-labeled nicotine was being N-methylated by the homogenates. There was no evidence of the formation of any metabolites arising from N-methylation of the more basic pyrrolidine nitrogen of nicotine. The values for the percent N-methylation of nicotine per mg protein are shown in Table 1 for dialyzed and undialyzed homogenates of each tissue. Spleen appears to be the most active tissue, although levels of the methylase in brain are also high and perhaps, because of this location, more significant. The dialyzed homogenates invariably showed a significant increase in methylase activity over undialyzed material for each tissue examined ($P < 0.05$). Comparison with the protein content of the original homogenates suggests this may be due to removal on dialysis of a small molecular weight endogenous compound capable of inhibiting the N-methylation of nicotine. Such an inhibitor molecule has been shown to exist for other methylase enzymes [8, 9].

Stereospecificity of N-methylation. The results from comparison of different enantiomers of radiolabeled nicotine as substrate are summarized in Table 2. From this data, it appears that the methyltransferase enzyme exhibits specificity for the *R*-enantiomer of nicotine, and thus the observed metabolite is *R*-*N*-methylnicotinium ion (Fig. 1c). No methylation was observed when radiolabeled *S*-nicotine was used as the substrate (Fig. 1d). This result is consistent with the observation that the radioactive metabolite formed from racemic radiolabeled nicotine only coelutes with unlabeled *R*-*N*-methylnicotinium standard.

Both racemic ^{14}C-2′- and racemic ^{14}C-NCH$_3$-labeled nicotines showed similar levels of methylation. However, these levels were less than 50% of that observed for pure radiolabeled *R*-nicotine under the same conditions. This suggests that, although *S*-nicotine is not a substrate for the methylase enzyme, its presence may perturb the N-methylation of the *R*-enantiomer. This effect is currently

Table 1. Methylation of [^{14}C-2′]-*RS*-nicotine by guinea pig tissue homogenates

	[^{14}C]Nicotine activity* (% methylated/mg protein)	
Tissue	Undialyzed	Dialyzed
Liver	0.82 ± 0.37	2.60 ± 1.12†
Lung	1.82 ± 0.47	4.08 ± 0.55†
Spleen	3.62 ± 0.69	8.42 ± 2.14†
Brain	2.84 ± 0.25	5.21 ± 0.55†

* Values are expressed as a percentage of total ^{14}C-activity in injection sample per mg protein and are the mean of four determinations ± S.E.

† Significantly different from undialyzed, $P < 0.05$ (paired Student's *t*-test).

Table 2. Stereospecificity of N-methylation of nicotine by guinea pig tissue*

Substrate	[^{14}C]Nicotine activity† (% methylated/mg protein)
[^{3}H-NCH$_3$]-*R*-nicotine	46.2
[^{3}H-NCH$_3$]-*S*-nicotine	None detected
[^{14}C-2′]-*RS*-nicotine	11.7
[^{14}C-NCH$_3$]-*RS*-nicotine (+)-bitartrate	12.0

* Using dialyzed lung homogenate and incubating for 1 hr at 37°.

† Values are expressed as a percentage of the total radioactivity in the injection sample per mg protein.

being investigated in more detail in our laboratory.

Source of the methyl group. The results of the double-label experiment designed to confirm the origin of the methyl group in the methylated metabolite are shown in Fig. 2, using [^{3}H-NCH$_3$]-*R*-nicotine as the substrate. Approximately 7.4% of the ^{3}H-activity and 1.1% of the ^{14}C-activity recovered in the column effluent were associated with the *N*-methylated metabolite, indicating transfer of the labeled methyl group from SAM to nicotine. The absence of methylation in the controls proved unequivocally that this process is enzymic.

Again no methylation was observed when [^{3}H-NCH$_3$]-*S*-nicotine was used as the substrate, and hence no incorporation of the ^{14}C-labeled methyl group was seen.

These preliminary studies describe the presence of a methyltransferase system which is widely distributed in guinea pig tissues and which is capable of methylating nicotine to *N*-methylnicotinium ion. The enzyme utilized *S*-adenosyl-L-methionine as co-factor and exhibited specificity for the *R*-isomer of nicotine. Although this type of metabolic reaction was noted many years ago by His [10], and subsequently by others [11–14], it would appear that the *in vivo* methylation of aromatic tertiary amines to water-soluble quaternary metabolites may have been overlooked as a significant route in the biotransformation of heterocyclic drug molecules. We are currently involved in the isolation, purification, and substrate kinetics of the above *N*-methyltransferase enzyme.

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* Author to whom all correspondence should be addressed.

Division of Medicinal Chemistry and Pharmacognosy
University of Kentucky
Lexington, KY 40502, U.S.A.

KENNETH C. CUNDY
C. STEVEN GODIN
PETER A. CROOKS*

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Dopamine microinjection into the nucleus accumbens: correlation between metabolism and behavior

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Many studies have demonstrated that microinjection of dopamine (DA) into the nucleus accumbens of rodents produces an increase in locomotor activity [1–3]. The doses required to produce this effect are in the range of 20–100 nmoles (3–15 μg) DA/accumbens. In contrast, the entire nucleus accumbens contains approximately 0.3 to 0.6 nmole DA [4]. This discrepancy between the dose of exogenous DA required to elicit a behavioral response and the endogenous level of DA has been problematic in interpreting these pharmacological responses in terms of a physiological function for DA in the nucleus accumbens. It is generally assumed that the rapid elimination of injected DA from the synaptic cleft by DA uptake into the presynaptic terminal, degradation to biologically inactive metabolites or diffusion necessitates the use of seemingly non-physiological concentrations of DA.

In this study, we examined the metabolism of [^{3}H]DA which was microinjected into the nucelus accumbens. Further, we evaluated the temporal relationship between the degradation of DA and DA-induced motor activity.

Materials and methods

Male Sprague–Dawley rats were housed individually with food and water made available *ad lib*. When the rats attained a weight of 300–350 g, they were anesthetized with ketamine (80 mg/kg, i.p.) and pentobarbital (10 mg/kg, i.p.), and were stereotaxically implanted with chronic bilateral injection cannulae (26 gauge stainless steel) 1 mm over the nucleus accumbens according to the atlas of Pelligrino *et al.* [5]. The stereotaxic coordinates employed were A/P 9.2, M/L 1.7 and, D/V-0.7 (relative to the interaural line). The range of variation for the nucleus accumbens implantation is described in detail elsewhere [6]. The cannulae were secured in position with set screws and dental acrylic. The rats were then divided into two groups: one group to be used for behavioral measurements (N = 10) and one group for neurochemical analysis (N = 10).

Behavioral measurements were obtained with a photocell apparatus. Each cage, 25 cm × 50 cm × 35 cm high, was equipped with two photocells located 4 cm above the cage floor to estimate locomotor activity. Rats were adapted to the cage for 90 min and then microinjected with either 0.9% (w/v) sterile saline or DA (10 μg/side). Simultaneous bilateral microinjections were made in the unrestrained rat in a volume of 0.5 μl/side over 60 sec with a 33 gauge needle connected via PE-10 tubing to a 1 μl syringe. Immediately after microinjection, the rat was placed in the photocell cage and motor activity was monitored for 90 min. After each rat received one saline and one DA injection in random order with a 72-hr intertrial interval, the rat was killed with ether and the brain was perfused with saline followed by 4% (w/v) paraformaldehyde. The brain was