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The ability of amine *N*-methyltransferases from rabbit liver to *N*-methylate azaheterocycles

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The substrate specificity of two homogeneous amine *N*-methyltransferases from rabbit liver has been demonstrated to extend to the azaheterocycles pyridine, *R*-(+)-nicotine and *S*-(−)-nicotine. Both enzymes methylate *R*-(+)-nicotine at the pyridyl nitrogen to afford the *N*-methylnicotinium salt, whereas *S*-(−)-nicotine does not act as a substrate for either enzyme. Surprisingly, *R*-(+)-nicotine is methylated at either the pyridyl nitrogen, or the pyrrolidine nitrogen, to afford the two isomeric monomethylate nicotinium ions when an enzymic preparation containing both methyl transferase activities was used. Under similar conditions *S*-(−)-nicotine was methylated only at the pyridyl nitrogen. The production of charged metabolites in-vivo, from the large number of pyridino-compounds that are used as drugs, or are present in the environment, may be of toxicological significance, in view of the reported toxicities of several such quaternary ammonium compounds.

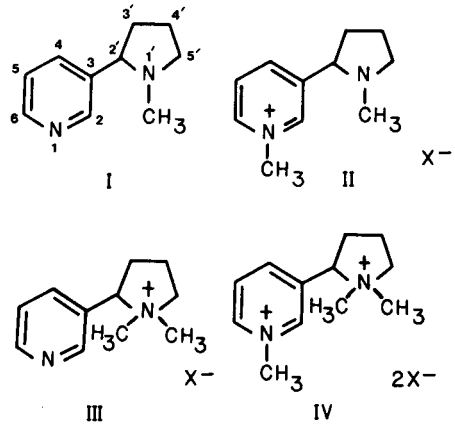
The broad substrate specificity attributed to the 'non-specific *N*-methyltransferases' from rabbit lung (Axelrod 1962a), other rabbit tissues (Axelrod 1962b; Saavedra et al 1973a) and tissues from other mammalian and non-mammalian species (Narasimhachari et al 1972; Wyatt et al 1973; Saavedra et al 1973a, b), suggests that these enzymes may play an important role in the methylation of xenobiotics. This prompted attempts at purification and characterization of the 'nonspecific *N*-methyltransferase' activity from rabbit liver cytosol (Lyon & Jakoby 1982) and has now resulted in a homogeneous preparation of an enzyme, amine *N*-methyltransferase A (Ansher & Jakoby 1986), that has a broad substrate specificity, transferring the methyl group of *S*-adenosyl-L-methionine (SAM) to the nitrogen of a number of primary and secondary amines. In addition, a second enzyme, amine *N*-methyltransferase B, has also been obtained and found to have overlapping specificity with transferase A (Ansher & Jakoby 1986). The availability of these two highly purified proteins offered an opportunity for testing the formation of *N*-methyl quaternary ammonium salts from tertiary amines in a system previously considered only in terms of methylating primary and secondary amines.

Since our recent studies had demonstrated the presence of 'azaheterocycle *N*-methyltransferase' activity in crude rabbit and guinea pig tissue preparations (Damani et al 1986; Cundy & Crooks 1985; Cundy et al 1985a, b), we investigated the capacity of the amine *N*-methyltransferases to catalyse the *N*-methylation of pyridine, *R*-(+)-nicotine, and *S*-(−)-nicotine. We have examined the reaction products by highly sensitive and specific high performance liquid radiochromatographic methods previously reported from our laboratories (Shaker et al 1982; Damani et al 1982; Cundy & Crooks 1984).

The data clearly demonstrate that these azaheterocycles are substrates for the homogeneous amine *N*-methyltransferases. The production of *N*-methylpyridinium ions as metabolites in-vivo of the scores of pyridino-compounds that are drugs or that are present in the environment, could be of toxicological significance, since several such quaternary compounds have been demonstrated to be highly toxic (see Lewin 1985).

Materials and methods

Materials. Gold label pyridine, *S*-(−)-nicotine and triethylamine were purchased from Aldrich, Milwaukee, WI, USA. *R*-(+)-Nicotine was prepared by the method of Bowman et al (1982) by resolution of *RS*-(±)-nicotine (I) with di-(*p*-toluoyl)-(+)-tartaric acid. *N*-Methylnicotinium iodide (II) (NMN), *N'*-methylnicotinium iodide (III) (N'MN) and *N,N'*-dimethylnicotinium diodide (IV) (DMN) were prepared by the reaction of either *R*-(+)-, or *S*-(−)-nicotine with methyl iodide essentially as described by



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Seeman & Whidby (1976). *N*-Methylpyridinium iodide (NMP) was prepared by the reaction of pyridine with methyl iodide (Bergmann et al 1952). *S*-Adenosyl-L-[methyl-^{3}H]methionine (SAM) was obtained from New England Nuclear (Boston, MA, USA; specific activity 78 Ci mmol^{-1}). HPLC grade ammonium acetate and methanol were from Fisher Scientific Company (Fair Lawn, NJ, USA). The two amine *N*-methyltransferases, A and B, were homogeneous preparations from frozen New Zealand rabbit liver (Pel Freeze Biologicals) (Ansher & Jakoby 1986). Where indicated, a mixture of methyltransferases A and B was used; the mixture represents purification products eluted from hydroxyapetite, but before further purification and separation of the two transferases by HPLC ion exchange chromatography (Ansher & Jakoby 1986).

Incubation conditions. The incubation mixtures, in a total volume of 200 µl, contained 50 mM Tris HCl at pH 7·8, 34 µM [methyl-^{3}H]SAM, (final sp. act. 25 mCi mmol^{-1}) and either pyridine (2·0 mM), *R*-(+)-nicotine (1·0 or 2·0 mM), or *S*-(−)-nicotine (1·0 or 2 mM) (see Table 1). Reactions were initiated by the addition of an appropriate amount of enzyme, maintained at 37 °C for 60 min, and terminated by the addition of 250 µl 1·0 mM HCl. The quenched incubates were then lyophilized and stored in sealed vessels until analysed. Control incubations were performed in the absence of substrate, and others in the absence of enzyme, and treated in an identical manner to test incubates.

Table 1. *N*-Methylation of pyridine, *R*-(+)-nicotine and *S*-(−)-nicotine with amine *N*-methyltransferases A and B.

	Product formed (nmol mg protein^{-1} h^{-1})		
Substrate (mM)	*N*-Methyltransferase A	*N*-Methyltransferase B	A and B mixture
Pyridine (2·0)	157	60·8	109
R-(+)-Nicotine (1·0)	13·0[1]	7·5[1]	—[2]
R-(+)-Nicotine (2·0)	—[2]	—[2]	10·6[1], 5·0[3]
S-(−)-Nicotine (1·0)	0·0	0·0	—[2]
S-(−)-Nicotine (2·0)	—[2]	—[2]	13·6[4]

[1] Value for *R*-(+)-*N*-methylnicotinium ion formation.
[2] Not determined.
[3] Value for *R*-(+)-*N'*-methylnicotinium ion formation.
[4] Value for *S*-(−)-*N*-methylnicotinium ion formation.

Analysis of incubates. High performance liquid chromatographic analyses of the incubates were carried out by direct cation-exchange HPLC using a Partisil-10 SCX (Whatman) column (25 × 0·46 cm) to which was attached a pellicular cation-exchange guard column (CSK-1, Whatman) (7 × 0·4 cm). The lyophilized incubates were reconstituted in 100 µl of the mobile-phase, and aliquots co-injected onto the HPLC column together with an equal volume of methanol containing an authentic standard of the non-radiolabelled *N*-methylated product via a Rheodyne loop injector. Essential chromatographic operating conditions are to be found in the legends to Figs 1 and 2. Radioactivity in column effluents was determined using a radioactive flow-through detector (Radiomatic, model HS Flo-1) incorporating a stream splitter (Radiomatic model ES) which was linked in series with a UV flow-through detector (Altex, model 153) operating at 254 nm. In all analyses the scintillation cocktail used was Flo-Scint II, with a mixing ratio of 4 parts to 1 part by volume of a 50% split of effluent stream. The outputs from the UV and radioactive flow-through detectors were recorded simultaneously on the two channels of the dual channel recorder (Omniscribe, model 5000, Houston Instruments).

Results and discussion

The identities of *N*-methylpyridinium, *S*-(−)- and *R*-(+)-*N*-methylnicotinium ions and the *R*-(+)-*N'*-methylnicotinium ion, as in-vitro metabolites of their corresponding azaheterocycles when incubated with amine *N*-methyltransferase and [methyl-^{3}H]SAM, were established by co-elution of radioactivity in quenched incubates with authentic standards from the cation-exchange HPLC column (Figs 1, 2). In no case was any methylated product formed in control incubations, i.e. in the absence of enzyme, or of substrate (e.g. see Fig. 1, panel A). However, in complete test incubations, both amine *N*-methyltransferase A and B readily catalysed the transfer of the [methyl-^{3}H] group from SAM to both pyridine and *R*-(+)-nicotine, but not to

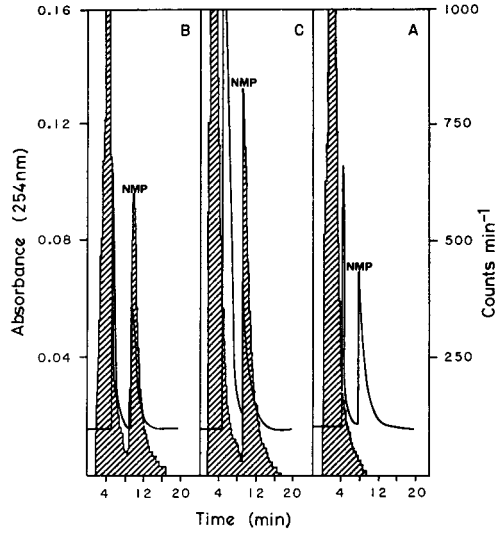

FIG. 1. HPLC-radiochromatograms from analysis of incubations containing amine *N*-methyltransferase enzymes A and B, pyridine and *S*-adenosyl-L-[methyl-^{3}H]methionine. A = control incubation without enzyme, B = test incubation with 0·2 mM pyridine. HPLC mobile phase: isocratic 0·1 M sodium acetate + 0·5% triethylamine adjusted to pH 4·2 with glacial acetic acid, flow rate 1·5 ml min^{-1}. Cross-hatching refers to counts min^{-1} in eluate, superimposed on the UV chromatogram. NMP = *N*-methylpyridinium ion peak.

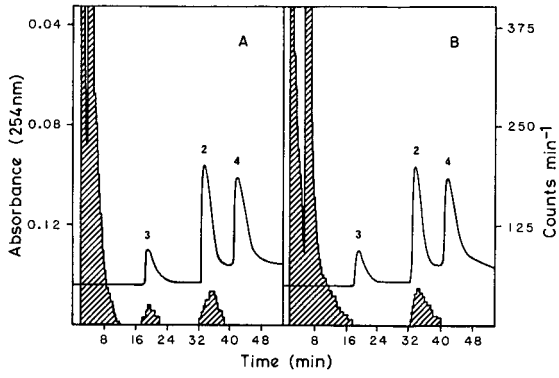

FIG. 2. HPLC-radiochromatograms from analysis of incubations containing amine *N*-methyltransferase enzymes A and B and *S*-adenosyl-L-[methyl-^{3}H]methionine, with either *R*-(+)-nicotine (2·0 mM, Panel A), or *S*-(−)-nicotine (2·0 mM, Panel B) as substrate. HPLC mobile phase: buffer (X) 0·3 M sodium acetate-methanol, 70:30, pH 4·5; buffer (Y) 0·3 M sodium acetate-methanol, 70:30, +1% triethylamine, final pH 4·5. Gradient: 100% X until 12 min, rising to 100% Y over 10 min, flow rate 2·0 ml min^{-1}. Crosshatching refers to counts min^{-1} in eluate, superimposed on the UV chromatogram. Key: 2 = *N*-methyl nicotinium ion; 3 = *N'*-methylnicotinium ion; 4 = *N,N'*-dimethyl nicotinium ion.

S-(−)-nicotine. The total amount of radioactivity (d min^{-1}) associated with each metabolite peak was converted into nmol product (mg protein)$^{-1}$ h^{-1} (Table 1). Of the three substrates tested, pyridine was the best substrate, with a turnover rate about ten-fold greater than that for the two isomers of nicotine.

Interestingly, when a mixture of methyl transferases A and B was used, *R*-(+)-nicotine was not only methylated at the pyridyl nitrogen to give *R*-(+)-*N*-methylnicotinium ion, but also underwent *N*-methylation at the pyrrolidine nitrogen to afford the isomeric *R*-(+)-*N'*-methylnicotinium ion (Fig. 2, panel A). This demonstrates the first reported *N'*-methylation of the more basic *N'*-methyl pyrrolidine nitrogen of nicotine. *S*-(−)-Nicotine was methylated exclusively at the pyridyl nitrogen to afford *S*-(−)-*N*-methylnicotinium ion (Fig. 2, panel B). This in itself is interesting, since *S*-(−)-nicotine is not *N*-methylated by either methyltransferase A or B in-vivo in the guinea-pig, nor is it a substrate in-vitro for crude guinea-pig lung cytosolic *N*-methyltransferases. Thus, in the guinea-pig, as with rabbit liver methyl transferases A and B, nicotine exhibits both regiospecific and stereospecific biotransformation; *R*-(+)-nicotine only affording *R*-(+)-*N*-methylnicotinium ion and none of the isomeric *R*-(+)-*N'*-methylnicotinium ion (Cundy et al 1985a, b; Cundy & Crooks 1985). In fact, the *S*-(−)-isomer of nicotine is a potent competitive inhibitor of the *N*-methylation of its optical antipode by guinea-pig lung *N*-methyltransferase (Cundy et al 1985b).

By use of pyridine as a model substrate, this *N*-methyltransferase activity has been demonstrated in crude cytosolic preparations from rabbit lung, liver and kidney, but not in those from the brain (Damani et al 1986)

With *R*-(+)-nicotine as the model substrate, 'azaheterocycle *N*-methyltransferase' activity was demonstrated in crude cytosolic preparations from guinea-pig liver, lung, spleen and brain (Cundy et al 1985a). This present study has clearly demonstrated that the substrate specificity of the rabbit liver amine *N*-methyltransferases does extend to tertiary heterocyclic amines.

The presence in mammalian tissues, particularly in CNS tissues, of 'azaheterocycle *N*-methyltransferase' activity may contribute to the observed toxicity of azaheterocycles, in view of the reported toxicity of several methyl quaternary ammonium compounds, e.g. paraquat (Haley 1979) and 1-methyl-4-phenylpyridinium ion (MPP+) (Javitch et al 1985). In this respect, it is important to establish whether compounds such as bis-pyridyls and phenylpyridines, some of which are either used industrially or are present as environmental pollutants, could act as 'protoxicants', i.e. xenobiotics that undergo *N*-methylation in-vivo to form toxic *N*-methyl quaternary ammonium metabolites in target tissues.

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Evidence against leukotriene-mediation of propranolol-induced airway hyperreactivity to acetylcholine

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In unanaesthetized guinea-pigs, propranolol treatment (0·1 mg kg^{-1} i.v.) substantially increased reactivity to intravenous acetylcholine infusion or aerosolized histamine to a comparable degree. Neither BW755c (5 mg kg^{-1} i.v.), FPL 55712 (1 mg kg^{-1} i.v.), nor piriprost (5 mg kg^{-1} i.v.) pretreatment influenced propranolol's effect on muscarinic reactivity although BW755c abolished histaminic hyperreactivity. This suggests that propranolol-induced muscarinic hyperreactivity in the guinea-pig is not mediated by leukotrienes whereas histaminic hyperreactivity may be.

Propranolol may increase airway reactivity to different bronchoconstricting agents in a variety of species, including man (Zaid & Beall 1966; Douglas et al 1973; MacLagan & Ney 1979; Mue et al 1980). The mechanism of this effect is unclear. Several years ago, MacLagan & Ney (1979) found no correlation, and thus, no causal relationship, between the bronchospasm and the pulmonary β-adrenoceptor blockade produced by propranolol treatment. Ney (1983) found that propranolol-induced hyperreactivity to histamine or 5-hydroxytryptamine (5-HT) was inhibited by either BW755c, an antagonist of both the lipoxygenase and cyclooxygenase pathways of arachidonic acid metabolism, or FPL 55712, an antagonist of slow reacting substance of anaphylaxis. This evidence suggests that the bronchial hyperreactivity produced by propranolol to some agonists may be leukotriene-mediated. Stimulation of the airways by muscarinic agonists (Miller et al 1976) differs in several respects from that by 5-HT and histamine (Gold et al 1972). To investigate whether propranolol-induced bronchial hyperreactivity to acetylcholine (ACh) or histamine occurred in unanaesthetized guinea-pigs and whether leukotrienes were involved, we studied muscarinic or histaminic bronchomotor tone before and after propranolol treatment in guinea-pigs pretreated with BW755c. In those challenged with ACh, the effect of pretreatment with FPL 55712, or piriprost, a pyrroloprostacyclin antagonist of leukotriene C/D biosynthesis (Bach et al 1983), was also assessed.

Methods

Ten male, English short-haired guinea-pigs (550–750 g) were used. Both baseline specific airway resistance (SRaw (ml × cm H_2O/(ml s^{-1})) and bronchial reactivity to either intravenous ACh or aerosolized histamine were determined before (on 3 separate occasions) and after drug treatment. Treatment consisted of propanolol (0·5 mg kg^{-1} i.v. via an indwelling jugular venous cannula (Roum & Murlas 1984)) given 15 min before ACh or histamine. On another occasion in each animal, propranolol was followed by BW755c (5 mg kg^{-1} i.v.) administered 1 min before ACh or histamine challenges. In those animals tested with ACh, the effect of FPL 55712 (1 mg kg^{-1} i.v.) or piriprost (5 mg kg^{-1} i.v.), administered 1 min before ACh infusion, was also evaluated.

SRaw in unanaesthetized guinea-pigs was measured using a constant volume body plethysmograph. The techniques were those of Roum & Murlas (1984). Briefly, each animal was positioned in a two-compartment chamber designed to isolate the head (with mouth closed) from the body and the plethysmograph. Flow at the snout was measured using a pneumotachograph (No. 0; Fleish Instruments, Pres Laussane, Switzerland) connected to a differential pressure transducer (Model MP45-1; Validyne, Northridge, CA). Airflow and box pressure signals were displayed simultaneously on an x-y oscilloscope so that the angle described during the rapid inspiratory phase of the animal's breathing could be measured, and SRaw calculated from it.

Bronchial reactivity was assessed by measuring SRaw as a function of the rate of ACh infused or concentration of histamine inhaled. For ACh testing, if no change occurred in baseline SRaw during normal saline infusion, 1 μg kg^{-1} min^{-1} ACh was delivered, and mean SRaw for 3 consecutive breaths was recorded after steady state had been reached. At the end of 90 s, the rate of infusion was approximately doubled, and the