NOTES

Separation of d-(+)-Nicotine from a Racemic Mixture by Stereospecific Degradation of the L-(-) Isomer with Pseudomonas putida

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Incubation of racemic mixtures of DL-(±)-nicotine with Pseudomonas putida resulted in a complete stereoselective degradation of the L-(-) isomer. Unnatural d-(+)-nicotine, which is of pharmacological interest for stereochemical studies of various nicotine-responsive systems, was not affected by the bacterium and was recovered by extraction.

Nicotine is known to exert specific pharmacological effects on cholinergic receptors located in ganglionic synapses, neuromuscular end plates, and Renshaw cells (3, 4, 6) and may exert specific noncholinergic receptor-mediated effects (1) as well. Elucidation of the binding requirements of nicotine-specific receptors has progressed mainly by studying the effects of structurally related drugs, but little can be learned of the stereochemical specificity of these interactions until unnatural stereoisomers become available.

The present work describes a technique which can be used to efficiently recover optically pure unnatural d-(+)-nicotine from racemic DL-(±)-nicotine by selectively degrading the natural L-(-) isomer with Pseudomonas putida. Existing methods for isolating this material are considerably more laborious, relying upon treatment of DL-(±)-nicotine with d-tartaric acid, followed by di-p-toluyl-l-tartaric acid, and fractional recrystallization of the resulting diastereomeric salts (2).

The nicotine-degrading abilities of P. putida have been the subject of various patents in the tobacco industry relating to the production of milder smoking tobaccos (L. E. Gravely, V. L. Geiss, and R. P. Newton, U.S. patent 4,011,141, 8 March 1977; V. L. Geiss, C. F. Gregory, R. P. Newton, and L. E. Gravely, U.S. patent 4,038,993, 2 August 1977). As discussed in these patents, the organisms degrade nicotine by an oxidative metabolism, resulting first in the production of 3-succinopyrroidine (International Union of Pure and Applied Chemistry [IUPAC] designation, γ-o xo-3-pyridinebutanoic acid) and then in the production of 6-hydroxy-3-succinopyrroidine (IUPAC designation, 6-hydroxy-γ-oxo-3-pyridinebutanoic acid) and other metabolites. Before this work, the stereospecificity of nicotine degradation by P. putida had not been reported.

DL-(±)-Nicotine was a gift from H. McKennis, Jr., and E. R. Bowman, Medical College of Virginia, Richmond. Methods for the preparation of racemic nicotine have appeared in the literature (5). P. putida NRRL B-8061 was obtained from T. G. Pridham, Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Ill. Bacteria were grown to stationary phase in aerobic liquid culture at 30°C, using 1% nutrient broth (no. 11479; BBL Microbiology Systems, Cockeysville, Md.) in buffer consisting of 0.5% NaCl and 0.4% KH2PO4, pH 6.5. Cells were harvested by centrifugation at 8,000 × g for 10 min at 4°C and were washed twice with pH 6.5 buffer (minus nutrient broth).

Experiments for testing the stereoselectivity of the organisms toward nicotine degradation were performed by comparing absorption spectra for the metabolic conversion of natural L-(-)-nicotine versus racemic DL-(±)-nicotine to succinopyrroidine derivatives. P. putida organisms (0.50 g, wet weight) were suspended in a minimum volume of pH 6.5 buffer and placed in dialysis sacks (Spectrapor 2 dialysis tubing; 12,000- to 14,000-molecular-weight cutoff; Spectrum Medical Industries, Los Angeles, Calif.). Identical sacks were immersed in 100 ml of pH 6.5 buffer containing 3.1 mM L-(-)-nicotine or racemic DL-(±)-nicotine and incubated at 30°C with constant agitation. At designated times, 1-ml samples were withdrawn from each solution and frozen for subsequent ultraviolet and circular dichroic spectral analyses.

The resultant time course of degradation of...
natural L-(-)-nicotine is shown by the ultraviolet spectral scans in Fig. 1A. The nicotine absorption (maximum at 259 nm) shown at time zero was first replaced by a 3-succinoylpyridine absorption (maximum at 252 nm) shown after 8 h of incubation and subsequently by an absorption characteristic of 6-hydroxy-3-succinoylpyridine (maximum at 275 nm) shown after 24 h. Further metabolic conversion resulted in a loss of the aromatic chromophore, as evidenced by little ultraviolet absorption after 36 h of incubation. In contrast, the time course for metabolic degradation of racemic DL-(±)-nicotine (Fig. 1B) resulted in a loss of only one half of the original nicotine chromophore. Thus, unnatural D-(+)-nicotine is apparently neither a substrate nor an inhibitor of nicotine-metabolizing enzymes in P. putida.

Circular dichroic spectra corroborated this hypothesis. During the production of 3-succinoylpyridine, the first step in P. putida nicotine metabolism, asymmetry at the beta carbon of the pyrrole ring is lost. Thus, no optical activity was observed in the ensuing succinoylpyridine derivatives. Circular dichroic spectra monitoring the time course of optical activity during L-(-)-nicotine degradation (Fig. 2A), therefore, showed decreased molecular ellipticity as the substrate decreased to a zero concentration. By contrast, when racemic DL-(±)-nicotine was used as a substrate (Fig. 2B), spectra showed little initial net molecular ellipticity (+ and - rotations canceled) but revealed a mirror-image spectrum as the natural isomer was degraded. The absolute value of the resulting ellipticity was half that of the natural isomer (Fig. 2A),

**Fig. 1.**(A) Ultraviolet spectra representing the time course of L-(-)-nicotine metabolism by P. putida after 0, 8, 24, and 36 h of incubation; (B) spectra for the time course of racemic DL-(±)-nicotine metabolism. All spectra were recorded on a Cary 210 spectrophotometer at a concentration equivalent to 0.15 mM of the original nicotine substrate.

**Fig. 2.**(A) Circular dichroic spectra representing the time course of changes in molecular ellipticity during the degradation of L-(-)-nicotine by P. putida. Curves representing 0, 8, and 24 h of incubation demonstrate a loss in optical activity as achiral metabolites were formed. (B) Spectra for the time course of reaction with racemic DL-(±)-nicotine as the substrate. After 24 h, D-(+)-nicotine emerged as the sole chiral product observable. All spectra were recorded on a Cary 60 spectropolarimeter at a concentration equivalent to 0.41 mM of the original nicotine substrate, using a 1-cm cell path.
because only half of the original racemic mixture was D- (+)-nicotine. [A slight excess of the D- (+) isomer was present in the racemic modification used for these studies and was visible in the zero-hour circular dichroic spectrum.]

Efficient recovery of D- (+)-nicotine on a preparative scale was attained by increasing the initial concentration of racemic nicotine up to 0.1 M or by increasing the entire incubation volume or both. When spectra indicated that no L- (-) isomer remained in the reaction mixture, it was adjusted to pH 11 with sodium hydroxide and extracted repeatedly with diethyl ether. Polar metabolites remained in the aqueous phase. Ether extracts were combined, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. D- (+)-Nicotine was recovered quantitatively by this extraction procedure. Further purifications can be performed by preparative chromatography on silica gel, using a chloroform-ethanol-ammonium hydroxide (85:14:1) solvent system (nicotine Rf = 0.35), or by vacuum distillation.

In conclusion, this work demonstrates that nicotine-metabolizing enzymes in P. putida are stereoselective and can be utilized to resolve unnatural D- (+)-nicotine from racemic mixtures.

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LITERATURE CITED