were grown as monolayers in 25-cm^2 plastic culture flasks (Corning) in Waymouth's medium supplemented with 15% fetal calf serum. To produce chronic hypoxia, flasks were fitted with sterile rubber sleeve serum stoppers and exposed to an atmosphere of 95% nitrogen/5% CO_2 for 4 h at 37 °C before drug treatment. Parallel flasks were maintained in 95% air/5% CO_2. Drugs were added to each flask in 25 μL of acetone without breaking the hypoxia, and cells were exposed to each agent for 1 h under conditions of chronic hypoxia or normal aeration. At the end of this period, the medium was removed and the cells were washed with 3 mL of sterile phosphate-buffered saline, suspended by treatment with 0.05% trypsin, and plated and cultured in replicate dishes in Waymouth's medium plus 15% fetal calf serum under 95% air/5% CO_2; after 8 to 10 days, cell survival was measured by colony formation. No difference existed between the survival of untreated or vehicle only treated cells maintained under either the aerobic or hypoxic conditions employed; the plating efficiency for these control cultures was 65–80% depending upon the individual experiment. Cells exposed to hypoxic conditions appeared to continue traversing the cell cycle during the course of these experiments, since no decrease in the rate of [^{3}H]thymidine incorporation into acid-insoluble material or in mitotic index was observed after 4 h of incubation in the hypoxic atmosphere (data not shown). Each drug was tested in at least three separate experiments, and the bars on the figures represent the standard error of the mean. Using a two between group analysis of variance (i.e., factorial design: two factors), the aerobic/hypoxic differential exhibited by the *o*-nitrobenzyl compounds was significant at the $p < 0.001$ level.[27] The factors in this test were aerobic vs. hypoxic surviving cell fractions and drug concentration.

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Chemical Methods. All melting points were measured on a calibrated Thomas-Hoover capillary melting point apparatus. Analyses were performed by the Baron Consulting Co., Orange, CT. Analytical results were within ±0.4% of theoretical values. NMR data were obtained using a Varian T-60A spectrometer with Me_4Si as an internal standard. *o*-Nitrobenzyl and *p*-nitrobenzyl alcohols, bromides, and chlorides were obtained from commercial sources and recrystallized from ethanol prior to use in tissue culture.

General Procedure for the Preparation of N-Substituted Carbamates. *o*-Nitrobenzyl alcohol (7) or *p*-nitrobenzyl alcohol (1) (0.01 mol) was dissolved in a slight molar excess of methyl, phenyl, or chloroethyl isocyanate. Two or three drops of anhydrous pyridine or triethylamine were added to catalyze the reaction, and solutions were warmed for about 5 min. The reaction mixtures were then cooled in a beaker of ice to effect crystallization. All of the carbamates were purified for use in tissue culture by recrystallization from ethanol.

Polarography. First half-wave reduction potentials were measured in 0.05 M potassium phosphate buffer prepared in water–ethanol (1:1, v/v), pH 7.0, by differential pulse polarography (PAR 174A polarographic analyzer). The values were obtained in volts vs. a saturated calomel reference electrode using 0.1 M potassium chloride as the supporting electrolyte.

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Synthesis and Preliminary Binding Studies of 4,4-Ditritio-(−)-nicotine of High Specific Activity

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4,4-Ditritio-(−)-nicotine (**5**) of high specific activity (4.7 Ci/mmol) has been synthesized from (−)-nicotine via the readily prepared 4,4-dibromocotinine (**3**). Scatchard analysis of the binding of **5** to the crude mitochondrial fraction of whole rat brain revealed a K_a of 4.7×10^6 M^{-1} and 13 fmol of binding sites/mg of protein.

Current interest in nicotine receptors in the central nervous system has created a need for radiolabeled nicotine with high specific activity.[2-4] Commercially available (−)-nicotine-*t* is randomly labeled and varies in both quality and specific activity from batch to batch, while (−)-nicotine-^{14}C is of very low specific activity. We now report the synthesis and some preliminary binding data of 4,4-ditritio-(−)-nicotine (**5**). The distinct advantages of **5** synthesized by our method over commercially available radiolabeled (−)-nicotine are higher specific activity, good stability, and location of the tritium atoms at a specific carbon (4).

Chemistry. A prerequisite of the synthesis of (−)-nicotine (1) tritiated at a definite position was the accessibility of a stable intermediate which could be readily hydrogenated (tritiated). 4,4-Dibromocotinine (**3**), first reported by Pinner[5] in 1893 and isolated by Bowman and McKennis[6] as the crystalline $HBr{\cdot}Br_2$ complex **2** in an improved procedure, appeared to be such a compound. Conversion of **2** to **3** (Scheme I) in two stages, although more time-consuming than previously reported procedures,[7] was effected in superior yield (~90%).

In "pilot runs", hydrogenolysis of **3** to cotinine (5-oxonicotine)[7] and reduction of cotinine to (−)-nicotine (1) was accomplished with H_2 ($Pd–CaCO_3$) in EtOH and $LiAlH_4$, respectively. The resultant 1 could be isolated as the di-*l*-tartrate[8] and was identical with authentic 1. Furthermore, $LiAlH_4$, reduction of 4,4-dideuteriocotinine,[7] prepared from cotinine and D_2O as described by Glenn and

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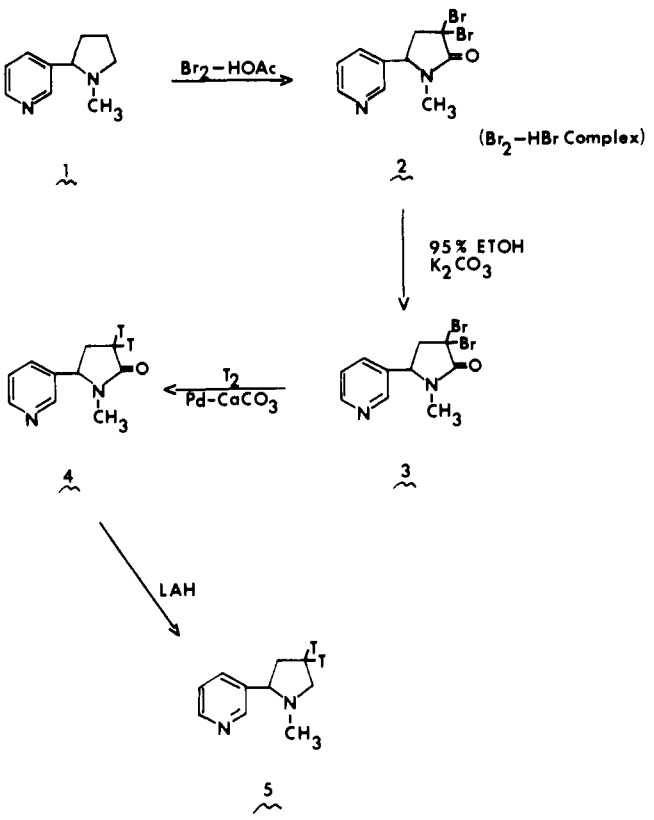
Scheme I



Edwards[9] for the 4-pyridyl isomer, gave 4,4-dideuterio-(−)-nicotine[7] with 96% incorporation of D_2.

Tritiation of **3** was carried out as described for hydrogenation above by Amersham-Searle (Arlington Heights, Ill.), who delivered the resultant **4** in PhMe. This **4** was reduced ($LiAlH_4$/THF) to **5**, which was stored in MeOH containing 0.025% *l*-tartaric acid for stability.[8] Using gas chromatography–mass spectrometry (GC–MS), the specific activity and the position of the tritium atoms were determined as described under Experimental Section.

Binding Studies. Binding of **5** to the CM fraction of whole rat brain was found to be dependent upon several conditions of incubation. The specific binding of **5** [total binding minus that in the presence of excess unlabeled (−)-nicotine (1)] increased as protein concentration was increased, as the pH was increased, and as the temperature was decreased. It decreased as preincubation time was increased (presumably due to degradation of binding sites) and when Krebs–Henseleit (K–H) buffer was replaced with isotonic sucrose. Nicotine-*t* binding (5×10^{-10} to 10^{-7} M) was then analyzed in vitro at steady state using the following conditions: 5 mg of protein, 5-min incubation, 2 °C, pH 8.4, K–H buffer. Binding of **5** was displaceable in this range, with (+)-[8] and (−)-nicotine being equally effective. A Scatchard plot of these data revealed a $K_a = 4.7 \times 10^6$ M^{-1} and a binding capacity of 13 fmol/mg of protein.

When **5** was analyzed in the concentration range of 5×10^{-10} to 10^{-3} M, a second binding component was revealed of $K_a = 4.4 \times 10^3$ M^{-1} and with a binding capacity of 7 nmol/mg of protein. Binding to the crude nuclear fraction of whole brain was studied also and **5** binding was similar to that of the CM fraction, quantitively and qualitatively.

Lack of stereospecific binding to brain tissue could be interpreted as nonspecific nicotine binding. On the other hand, (−)- and (+)-nicotine could have equal binding affinity for the receptor but different efficacies (intrinsic activities). The latter suggestion is consistent with the fact that both (−)- and (+)-nicotine produce similar pharmacologic effects, the primary difference being that the (+) isomer is less potent.[8-11] Further experiments are in progress.

Experimental Section

Melting points (capillary, uncorrected) were taken in a Thomas-Hoover apparatus. IR and NMR data (nontritiated materials) were in agreement with literature assignments.[7] The THF was freshly distilled from $LiAlH_4$ into flame-dried glassware; all solvents were reagent grade. (−)-Nicotine (98%) and *l*-tartaric acid were purchased from Aldrich Chemical Co. TLC's were performed on Analtech, silica gel GHLF 250 μm, 4 × 10 cm, detection with UV or potassium iodoplatinate.

$LiAlH_4$ Reduction of Cotinine to (−)-Nicotine (1). To a flask charged with $LiAlH_4$ (0.065 g, 1.7 mmol) in 10 mL of THF was added (−)-cotinine[6] (0.3 g, 1.7 mmol) in 2.0 mL of Et_2O. The mixture was refluxed for 1 h and treated cautiously with 0.1 mL of H_2O, 0.1 mL of 15% NaOH, and then 0.3 mL of H_2O. The precipitate was filtered and washed with 5 mL of Et_2O, and the combined filtrates were dried (K_2CO_3). Removal of solvents in vacuo gave 0.26 g (93%) of (−)-1 (pale yellow oil): TLC pure, R_f 0.53 (1:4 Me_2CO–Et_2O).

Similarly, 4,4-dideuterio-(−)-cotinine,[7] prepared from cotinine and D_2O as described for the 4-pyridyl isomer,[9] gave 4,4-dideuterio-(−)-nicotine with 96% incorporation of D_2.

Conversion of 2 to 3. A solution of **2** (mp 139–146 °C; 0.5 g, 1.1 mmol) in 10 mL of 95% EtOH was boiled for about 5 min (color change from red-orange to yellow) and cooled to 25 °C and then to 5 °C. The crystals were filtered and dissolved in 5 mL of H_2O. K_2CO_3 was added to pH 10–11 and the free base extracted with $CHCl_3$ (3 × 15 mL). The dried (K_2CO_3) $CHCl_3$ was evaporated in vacuo, giving 0.33 g (90%) of **3**, mp 122–123 °C (lit.[7] mp 124 °C).

Conversion of 3 to (−)-Nicotine (1). To a suspension of 10 mg of 5% Pd/$CaCO_3$ and 20 mg of $CaCO_3$ in 1.0 mL of 100% EtOH (in a microvial with a Teflon-lined septum and containing a stirring bar) was added (after evacuating and flushing with 20 mL of H_2 four times) **3** (35 mg, 0.1 mmol) in 0.5 mL of 100% EtOH by syringe. Upon addition, vigorous stirring was begun. During 2.5 h 5.0 mL of H_2 (theory, 4.4 mL) was consumed. The suspension was filtered through a cotton pad and the EtOH removed (without heat) under reduced pressure. The residue (R_f 0.19, 5:20:0.02 Me_2CO–Et_2O–NH_4OH) was dissolved in 4 mL of concentrated NH_4OH and extracted with Et_2O (3 × 5 mL). The combined Et_2O extracts were dried (K_2CO_3) and evaporated in vacuo. The residue in 5 mL of THF was added dropwise to a flask containing 10 mg (0.3 mmol) of $LiAlH_4$ in 10 mL of THF. The mixture was heated at reflux for 15 min and cooled to 25 °C. Et_2O (15 mL) and then $Na_2SO_4 \cdot 10H_2O$ were added. The solvents were dried (K_2CO_3) and evaporated in vacuo, giving a pale-yellow oil (R_f 0.53; 1:4 Me_2CO–Et_2O) identical with authentic (−)-nicotine. The (unrecrystallized) di-*l*-tartrate melted at 135–137 °C alone or when mixed with authentic di-*l*-tartrate.[8]

4,4-Ditritio-(−)-nicotine (5). Compound **3** was tritiated by Amersham-Searle (code Tr. 3) as follows: To 5% Pd/$CaCO_3$ (10 mg) and 20 mg of $CaCO_3$ in 1 mL of 95% EtOH under an atmosphere of tritium (10 Ci) was added 35 mg of **3** (0.1 mmol) with stirring. After 3 h, excess T_2 was removed and the mixture was filtered through a cotton pad. The EtOH was removed in vacuo to yield a gummy residue, to which 4 mL of concentrated NH_4OH was added. The basic solution was shaken with Et_2O (5 × 5 mL) and the combined extracts[12] were evaporated in vacuo. The oily residue (4) was dissolved in PhMe and sent to us.

The PhMe solution of 4 was concentrated (under vacuum, without heat) to ~50 μL. This material was streaked onto a silica gel plate (Analtech, GHLF, 250 μm, 4 × 10 cm) and developed

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in Me_2CO–Et_2O–NH_4OH (2:20:0.02). The band corresponding to cotinine[6] (R_f 0.15) was removed and eluted with ~10 mL of MeOH. The MeOH was removed in vacuo and the clear oil was dissolved in 2 mL of THF. This (4) was added to 17 mg (0.5 mmol) of $LiAlH_4$ in 15 mL of THF (by syringe) under an atmosphere of Ar. The mixture was refluxed for 20 min and cooled to 25 °C. Et_2O (20 mL) was added and then (cautiously) $Na_2SO_4 \cdot 10H_2O$ to destroy excess $LiAlH_4$. The precipitate was filtered and THF–Et_2O was removed in vacuo. The residue 5 (oil, 0.17 Ci, 3.6 mmol) was dissolved in MeOH containing 0.025% *l*-tartaric acid.

Determination of Specific Activity of 5. The specific activity of 5 was determined by liquid-scintillation counting and GS/MS. A Finnigan 4000 GC/MS was equipped with a 1.83 m × 2 mm glass column packed with 3% SP 2250 on Supelcoport 80–100 mesh. Mass spectra were obtained under the following conditions: column temperature, 120 °C; carrier gas (He) flow, 30 mL/min; electron energy, 70 eV. Single-ion monitoring was used to construct a standard calibration curve for 1 (0.1–1000 ng). The ions monitored for both the standard curve and samples were *m/e* 84, 86, and 88, which corresponded to the base peaks of nicotine, nicotine-*t* and nicotine-t_2, respectively. The nicotine standards did not contain detectable levels of *m/e* 86 and 88. A known quantity of radiolabeled 1 (5) (1 μCi as detemined by liquid-scintillation counting) was injected into the GC/MS, and the quantity of total nicotine (labeled and unlabeled) was determined from the calibration curve. The specific activity was found to be 4.7 Ci/mmol. Since the base peak of 1 corresponds to the pyrrolidine ring fragment,[7] the presence of *m/e* 86 and 88 established that the tritium atoms were in this ring.

Binding of 5 to Brain Tissue. Male, Sprague–Dawley rats (200–250 g) were decapitated and their brains removed for homogenization in 4 volumes of 0.32 M sucrose. The homogenates were centrifuged at 1000*g* for 10 min at 0 °C. The resulting supernatants were recentrifuged at 10000*g* for 20 min at 0 °C to provide crude mitochondrial (CM) pellets. The pellets were suspended in Krebs–Henseleit (K–H) buffer (2–4 mg of protein/mL) and pipetted into microfuge tubes. Compound 5, in 50 μL of K–H buffer, was added to the tubes and optimum incubation conditions were established. For displacement experiments, unlabeled drugs were incubated 5 min prior to the addition of 5. After incubation, the tubes were spun on a Beckman Microfuge, Model B, for 3 min and the supernatants were pipetted into scintillation vials containing 10 mL of scintillation fluid [2 parts of PhMe containing 0.4% of diphenyloxazole, 0.01% of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, and 1 part of Triton X-100 (TPP-TX)]. The tips of the centrifuge tubes containing the bound 5 were cut off and counted in 10 mL of TPP-TX. Radioactivity was quantitited by liquid-scintillation spectrometry and quench corrected by external standardization.

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Thymidine Phosphorylase. Substrate Specificity for 5-Substituted 2′-Deoxyuridines

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A continuous spectrophotometric assay for thymidine phosphorylase has been developed and used to analyze the substrate properties of a number of 5-substituted 2′-deoxyuridines. A QSAR was found which relates the catalytic efficiency for various substrates to a single variable, the inductive field constant $\mathcal{F}$.

Thymidine (dThd) phosphorylase (EC 2.4.2.4) catalyzes the reversible conversion of dThd and phosphate to thymine (Thy) and 2-deoxyribose 1-phosphate. The enzyme also catalyzes transfer of the deoxyribosyl moiety of dThd to appropriate pyrimidine bases. In addition to its role as a salvage enzyme in pyrimidine metabolism, the enzyme is responsible for the catabolism of a number of 5-substituted pyrimidine deoxyribonucleoside analogues with real or potential chemotherapeutic usefulness. In this report we describe the behavior of a number of 5-substituted pyrimidine nucleosides as substrates of mammalian dThd phosphorylase, as well as an improved continuous spectrophotometric assay for phosphorolysis and transferase activities of this enzyme.

Results and Discussion

Continuous Spectrophotometric Assay. dThd phosphorylase activity is usually monitored by use of radiolabeled substrates and separation and quantitation of reactants and products,[3,4] or by quenching reaction mixtures and observing differences in the UV spectra of nucleoside and pyrimidine base in strongly alkaline solutions.[4-6] Both methods suffer from the tedium and inaccuracies of single point assays. A continuous spectrophotometric assay has been reported for Urd phosphorylase which monitors the decrease in absorbance at the 282 nm which accompanies conversion of Urd to uracil;[7] while this assay is convenient, the change in absorbance at 282 nm is small ($\Delta\epsilon$ = 1370), and it is less sensitive than either of the aforementioned single point assays. The assay described here is a modification of the reported continuous assay in which the change in absorbance is monitored at the wavelength ($\Delta\lambda_{max}$) where the *maximal* difference in absorbance ($\Delta\epsilon_{max}$) exists between the nucleoside and base

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