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Binding Characteristics of (−)- And (+)-Nicotine to the Rat Brain P_2 Fraction[1]

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SLOAN, J. W., W. R. MARTIN, J. HERNANDEZ AND R. HOOK. *Binding characteristics of (−)- and (+)-nicotine to the rat brain P_2 fraction*. PHARMACOL BIOCHEM BEHAV 23(6) 987–993, 1985.—Saturation studies employing (−)- and (+)-[^{3}H]nicotine indicate that the isomers bind to different very high and high affinity sites since the binding density for (−)-[^{3}H]nicotine is 10 times that for (+)-[^{3}H]nicotine. Both isomers also bind to a low affinity site (K_Ds = $\sim 10^{-5}$ to 10^{-4} M). Competition studies employing unlabelled (−)- and (+)-nicotine reveal greater complexities. The isomers also appear to bind to a separate site which enhances binding at the (−)- and (+)-nicotine high affinity sites. (+)-Nicotine is more effective in increasing the binding of (−)-[^{3}H]nicotine at its high affinity site than (−)-nicotine. Further, (+)-nicotine has a greater specificity for enhancing binding than (−)-nicotine in that it enhances (−)-[^{3}H]nicotine binding at lower concentrations and inhibits binding at higher concentrations than (−)-nicotine.

Nicotine Enhanced binding Very high affinity site High affinity site Nicotine stereospecificity (−)-and (+)-Nicotine Multiple nicotine binding sites

(−)-NICOTINE has been one of the primary drugs used to identify nicotine's cholinergic receptors and it has been presumed that these receptors are its primary site of action. Recent binding studies, however, indicate that (−)-nicotine may have more complex actions. Thus, both a high and a low affinity binding site have been identified using (±)-nicotine as the labelled ligand [6, 9, 14, 15]. Others, using (±)-[^{3}H]nicotine as the labelled ligand have obtained evidence for 5 nicotine binding sites, one of which enhances binding at other nicotine binding sites [11,13]. It has been assumed that (−)- and (+)-nicotine act at the same site with (−)-nicotine having the higher affinity. The present binding studies characterize the binding of (−)- and (+)-nicotine and indicate that nicotine binding may be more complex than previously assumed.

DRUGS AND CHEMICALS

Labelled and unlabelled ligands were checked periodically for purity using three TLC solvent systems (methanol:ammonium hydroxide, 99:1, silica gel; chloroform:methanol:diethylamine, 80:15:1, silica gel and methanol:acetic acid, 99:1, alumina).

Radiolabelled Drugs

(−)- And (+)-[^{3}H]nicotine (71.9 and 76.4 Ci/mMole respectively) were obtained from New England Nuclear Boston, MA. The purity of the labelled ligands was >99% as determined by HPLC using a Zorbax silica column and the following solvent system: hexane:isopropanol:triethylamine 60:40:0.1. The isotopes were diluted to 2 μM with a threefold molar excess of mercaptoacetic acid and stored at 4°C [9].

Non-Labelled Drugs

(−)-Nicotine was obtained from Research Plus (Bayonne, NJ) and (+)-nicotine was resolved by Dr. W. T. Smith and Miss Amy Howell (Chemistry Department, University of Kentucky). Smith and Howell employed a modification of a previously published method [5] for the racemization of (−)-nicotine. (1) Excess potassium hydride was used in place of sodium hydride. (2) Heating and refluxing was carried out under an atmosphere of argon instead of nitrogen. (3) Isopropanol was used for the decomposition of excess potassium hydride instead of ice and water. A specific rotation of 0.5° (C=5 in $CHCl_3$) indicated that racemization was essentially complete. (+)-Nicotine Di-(p-toluoyl)-(+)-tartrate prepared from (±)-nicotine [5] had a specific rotation of +98° (C=1 in methanol). (+)-Nicotine Di-(+)-tartrate was prepared [4] and recrystallized to a constant melting point of 137.5–139°C and a specific rotation of +10° (C=2.0 in H_2O). Confirmation of the product, (+)-nicotine Di-(+)-tartrate, was made by comparing its specific rotation to that of (−)-nicotine Di-(−)-tartrate [4] and purified to a melting point of 139–140°C. The specific rotation of the product was −8.8° (C=2 in H_2O) and therefore opposite in rotation to (+)-nicotine Di-(+)-tartrate.

Chemicals

The following chemicals were analytical reagent grade unless otherwise specified. Poly-1-lysine (type V), N-

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2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), Tris-HCl, DFP, mercaptoacetic acid (grade V), sucrose and PPO (scintillation grade), Sigma Chemical Co. (St. Louis, MO); methanol and chloroform (distilled in glass), Bodman Chemicals (Doraville, GA); NaCl, $MgSO_4$, Mallinckrodt (St. Louis, MO); KCl, $CaCl_2$, acetic acid, naphthalene and p-dioxane (scintillation grade) Fisher Scientific (Louisville, KY); xylene (scintillation grade) Research Products International Corp. (Elk Grove Village, IL) and dimethyl POPOP (scintillation grade) New England Nuclear (Boston, MA).

METHOD

Binding Assay

The procedures for the preparation of the P_2 fraction from the whole brain of female rats and the binding method have been described previously [13]. Binding was determined in triplicate for each drug concentration. Inhibition studies were repeated 4 times using different homogenate preparations. Each tube contained 0.5 ml of the P_2 suspension (~2 mg protein), 0.25 ml of either Hepes (total binding) or the appropriate concentration of the competing drug followed by 0.25 ml of either (−)- or (+)-[^{3}H]nicotine (1.2 and 4.0×10^{-8} M respectively). The concentration of (−)-[^{3}H]nicotine was chosen so that it would be comparable to the concentration of (−)-[^{3}H]nicotine in the racemic mix, (±)-[^{3}H]nicotine, 2.4×10^{-8} M, used in previous studies [13]. The concentration of the labelled ligand in those and the present studies was chosen in order to label both high and low affinity sites in a single experiment. Further, the concentration of (±)-[^{3}H]nicotine was similar to that used by others [6,9]. The concentration of (+)-[^{3}H]nicotine was chosen since saturation studies indicated that the K_D for the (+)-[^{3}H]nicotine high affinity site was 3.7 times higher than that obtained for (−)-[^{3}H]nicotine. Nonspecific binding for (−)- or (+)-[^{3}H]nicotine was determined in the presence of 10^{-2} unlabelled (−)- or (+)-nicotine respectively. This concentration was chosen because lower concentrations inhibited [^{3}H]nicotine to a lesser extent whereas higher concentrations produced no further increase in inhibition. When saturation studies were employed each sample contained 0.5 ml of tissue suspension, 0.25 ml of either (−)- or (+)-[^{3}H]nicotine and either 0.25 ml of Hepes (total binding) or unlabelled (−)- or (+)-nicotine, 10^{-2} M (nonspecific binding). Each sample was incubated at 4°C for exactly 1 hour in a shaking ice bath [13]. After incubation each sample was diluted with 3.5 ml of ice cold Hepes and filtered at a reduced pressure (460–510 mm Hg) using a filter apparatus (Hoeffer Scientific Instruments, San Francisco, CA) and Whatman GF/C glass fiber filters previously soaked in poly-1-lysine, 0.1%. The filters were washed 4 times with 3.5 ml cold Hepes and after 20 seconds of suction were counted by liquid scintillation.

Protein Determination

Protein was determined in 5 μl of the P_2 preparation with bovine serum albumin as the standard [7]. The reading obtained with 5 μl of Hepes buffer was substracted from each tissue sample since it produced significant interference.

Data Analysis: Curve Fitting Procedures

The data were analyzed several ways. Two iterative nonlinear curve fitting procedures were employed for the estimation of K_Ds (dissociation constants) and binding site densities: LIGAND [8] and ANOVA I and ANOVA II [13]. The IC_{50} is the concentration of the unlabelled drug which inhibited the binding of the labelled ligand by 50% and was determined graphically from the inhibition curve.

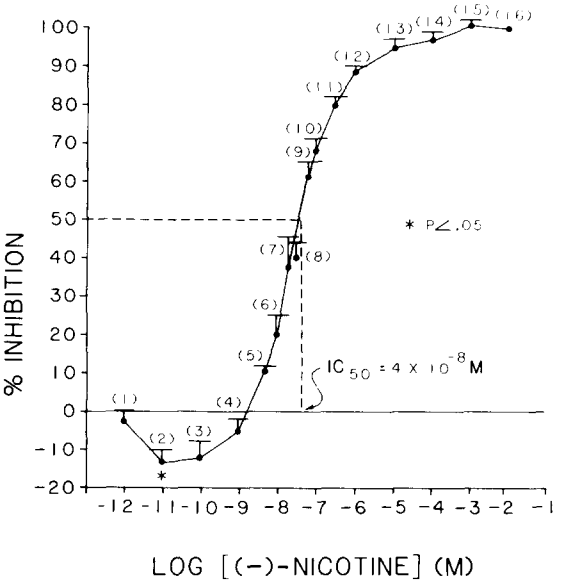

FIG. 1. The inhibition of (−)-[^{3}H]nicotine (1.2×10^{-8} M) by graded concentrations of (−)-nicotine. The inhibition produced by (−)-nicotine is shown as a percentage of the maximum displacement achieved by 10^{-2} M (−)-nicotine. Each point is the mean of 4 experiments with its standard error.

The LIGAND program, available in basic language through Vanderbilt University (Biomedical Computing Technology Information Center (BCTIC), R-1302 Vanderbilt Medical Center, Nashville, TN 37232) uses the total ligand concentration (labelled and unlabelled) and provides a weighted least squares estimate of affinity constants, binding capacity and nonspecific binding.

ANOVA I and ANOVA II also provide a least squares best fit of the binding curves but uses non-weighted data. ANOVA I segregates the variance of the bound/free (B/F) values into between homogenates and between concentrations variance compartments. The residual variance is used as an error term. The between concentration variance is further analyzed by determining the variance accounted for by the regression of the B/F values against the appropriate mean B values. ANOVA II involves iterative fitting of multiple regression lines of B and B/F Scatchard data to achieve the least mean squares. The line or set of lines which reduced the variance the most was chosen [13].

Data Analysis: Enhancement of Binding

Enhanced binding is defined as a statistically significant ($p<0.05$) increase in the amount of [^{3}H]nicotine bound to the rat brain P_2 preparation in the presence of a given concentration of a drug relative to the amount of [^{3}H]nicotine bound in the absence of the drug. Figure 1 illustrates this phenomenon for the binding of (−)-[^{3}H]nicotine in the presence of unlabelled (−)-nicotine in concentrations ranging from 10^{-12} M to 10^{-9} M. Enhanced binding is represented graphically by the values which fall below "0" percent inhibition. Drugs which enhance [^{3}H]nicotine binding produce this effect consistently

TABLE 1

BINDING PARAMETERS* OF NICOTINE IN COMPETITION STUDIES EMPLOYING (±)- OR (−)-[^{3}H]NICOTINE AS LABELLED LIGANDS AND (−)- AND (+)-NICOTINE AS THE UNLABELLED LIGANDS

Unlabelled Ligand	(±)-[^{3}H]nicotine (2.4×10^{-8} M) K_D	Site Density	IC_{50}	(−)-[^{3}H]nicotine (1.2×10^{-8} M) K_D	Site Density	IC_{50}
(−)-nicotine	2.2×10^{-11}	0.070				
	5.2×10^{-9}	1.2	3.0×10^{-8}	2.5×10^{-8}	2.8	4×10^{-8}
	4.5×10^{-5}	801.0		1.5×10^{-5}	129.0	
(+)-nicotine	4.9×10^{-13}	0.008				
	4.3×10^{-7}	3.1	5.3×10^{-6}	6.7×10^{-7}	60.0	0.8×10^{-6}
	1.1×10^{-5}	287.0		1.5×10^{-4}	1478.0	

*K_Ds and IC_{50}s are expressed in molar units and site densities are expressed in fm/mg of tissue.

over certain concentrations; it is not a random effect. Both the amount of enhancement and the dose range producing it varies with different compounds [12,13]. The level of enhancement also varies between homogenates. In order to test the statistical significance of this enhancement a variety of procedures were used. The data obtained by concentrations of the unlabelled ligand which led to apparent enhancement of [^{3}H]nicotine binding were analyzed by a two-way analysis of variance (ANOVA) which partitioned the variance into between homogenates and between doses. The between doses variance was then partitioned into the variance due to linear and quadratic regression components and the significance of these components assessed. If the between doses variance obtained by the two-way ANOVA was not statistically significant then each concentration of the drug for each homogenate was considered to be an independent determination, the data were pooled and the significance of the mean determined using a *t*-test. A *t*-test was also used to assess the significance of the mean enhancement of binding for individual concentrations of the ligand.

INHIBITION EXPERIMENTS EMPLOYING (−)-[^{3}H]NICOTINE AS THE LABELLED LIGAND

(−)-Nicotine

Figure 1 shows the results obtained in a study of (−)-nicotine's ability to compete with the binding of (−)-[^{3}H]nicotine. As can be seen, low concentrations of (−)-nicotine enhanced the binding of (−)-[^{3}H]nicotine and the enhancement is maximal and significant at 10^{-11} M. Higher concentrations first produced a lesser enhancement and then inhibited binding. Scatchard analysis of these data show that up-regulation is not maximal until a concentration of 10^{-8} M of (−)-nicotine is obtained even though inhibition of binding occurs with concentrations as low as 10^{-10} M. LIGAND analysis of data for concentrations of 10^{-8} to 10^{-2} M showed that there were two binding sites (Table 1).

(+)-Nicotine

Figure 2 shows the inhibition of (−)-[^{3}H]nicotine binding by graded concentrations of (+)-nicotine. Small but significant enhancement of (−)-[^{3}H]nicotine binding occurred over a wide concentration range, 10^{-14} M to 10^{-8} M, whereas higher concentrations inhibited binding. A Scatchard analysis of the data showed that maximum enhancement of binding occurred at 10^{-9} M. A LIGAND analysis of data obtained with concentrations of 10^{-8} to 10^{-2} M showed that two sites best fit the data (Table 1). The K_D for (+)-nicotine for the high affinity site was approximately one-tenth that of (−)-nicotine, however, B_{max} was 20 times larger.

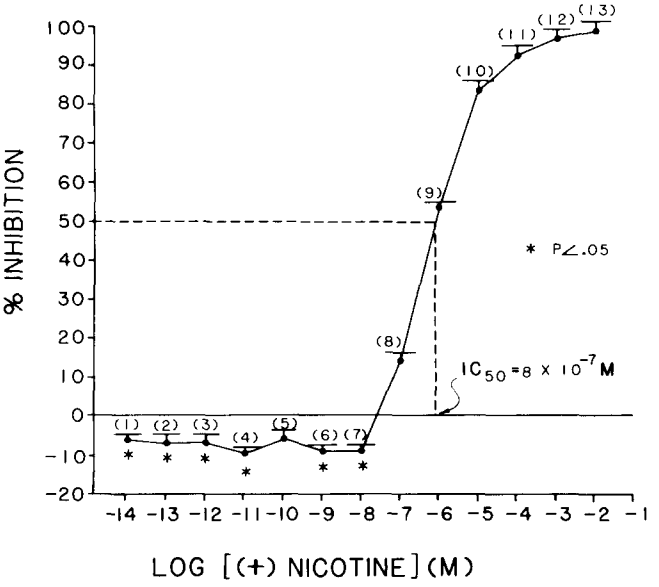

FIG. 2. The inhibition of (−)-[^{3}H]nicotine (1.2×10^{-8} M) binding by graded concentrations of (+)-nicotine. Each point is the mean of 4 experiments shown with its standard error.

SATURATION EXPERIMENTS WITH (−)-[^{3}H]NICOTINE

The effect of increasing concentrations of (−)-[^{3}H]nicotine on its specific binding to the rat brain P_2 preparation is shown in Fig. 3. Of the total counts added, about 12% were bound at the lowest concentrations, whereas about 0.3% were bound at the highest concentration. Of the total counts bound about 70% represented specific binding at the lower concentrations and about 30% at the higher concentrations. Figure 3A shows that the Scatchard plot of the sat-

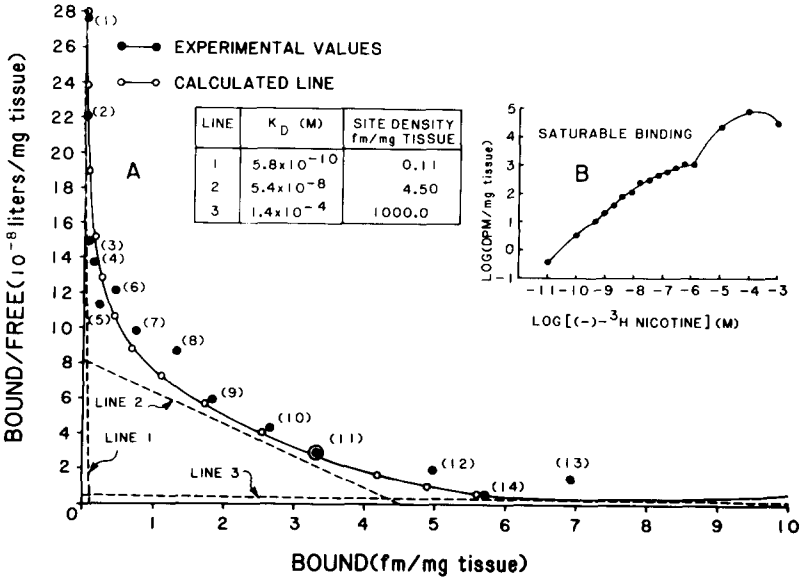

FIG. 3. Scatchard plot of (−)-[^{3}H]nicotine saturation binding. Concentrations of (−)-[^{3}H]nicotine ranging from 10^{-11} M (point 1 in A) to 10^{-3} M were employed. The B and B/F values associated with concentrations of 10^{-5}, 10^{-4} and 10^{-3} M (points 15 to 17) could not be shown on the scale of this graph but were used in calculating the K_D for line 3. Inset (B) shows the saturation curve (corrected for non-saturable binding). A Scatchard conversion of these data is shown in (A) as solid circles. The dashed lines represent the best fit to the data obtained by using the ANOVA I and ANOVA II program. K_Ds (in molar units (M)) and site densities (in femtomoles (fm)/per mg of tissue) calculated from these lines are shown in the box. The open circles represent the calculated values for the line [10].

uration curve (3B) is curvilinear, suggesting three binding sites. Further, the best fit lines to these data as obtained by ANOVA I and ANOVA II suggest the presence of three binding sites which are indicated by the dashed lines in Fig. 3A. The K_Ds and site densities for these lines are shown in the box in Fig. 3A. The theoretical values calculated from these lines [10] provide a good fit of the experimental data.

INHIBITION EXPERIMENTS EMPLOYING (+)-[^{3}H]NICOTINE AS THE LABELLED LIGAND

(−)-Nicotine

Figure 4 shows that (−)-nicotine produced a dose-related enhancement of the binding of (+)-[^{3}H]nicotine over a concentration range of 10^{-14} M to 10^{-10} M and doubled the amount of (+)-[^{3}H]nicotine bound at a concentration of 10^{-10} M. Higher concentrations inhibited binding. A two-way ANOVA of the data between the concentrations of 10^{-13}M and 10^{-6} M showed that there was a significant between homogenates variance. Analysis further revealed that the curve had a significant quadratic component and that the slope of the dose-response line over the concentration range of 10^{-14}–10^{-10} M (line 1) had a highly significant negative slope. Further, there was a significant dose-related inhibition of binding over the concentration range of 10^{-10} M to 10^{-3} M. Because of the marked enhancement of binding produced by (+)-nicotine and the small amounts of (+)-[^{3}H]nicotine specifically bound LIGAND and Scatchard analysis of the data yielded ambiguous estimates of K_Ds and binding site densities.

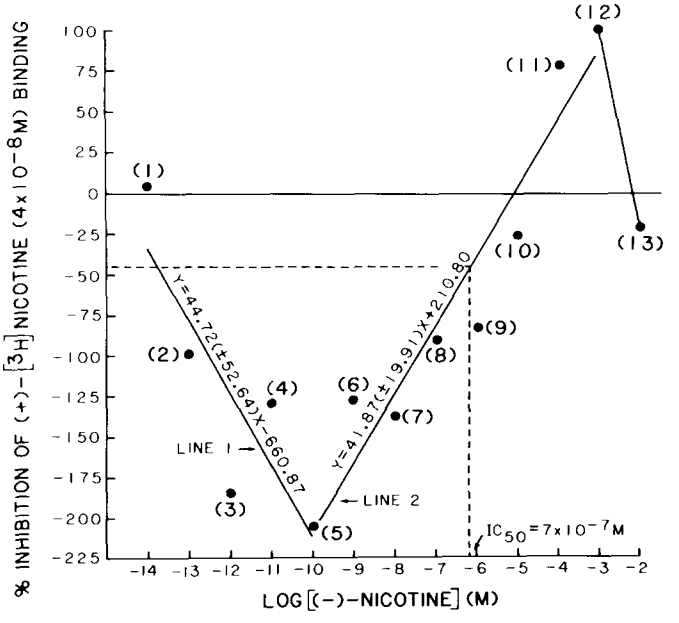

FIG. 4. The inhibition of (+)-[^{3}H]nicotine (4×10^{-8} M) binding by graded concentrations of unlabelled (−)-nicotine. Each point is the mean of 4 experiments. The least squares fit for lines 1 (points 1–5) and 2 (points 5–12) are shown with the equation for the fit. The slope for each line (± its 95% confidence limits) was calculated by regressing the % inhibition against the log of the molar concentration of (−)-nicotine.

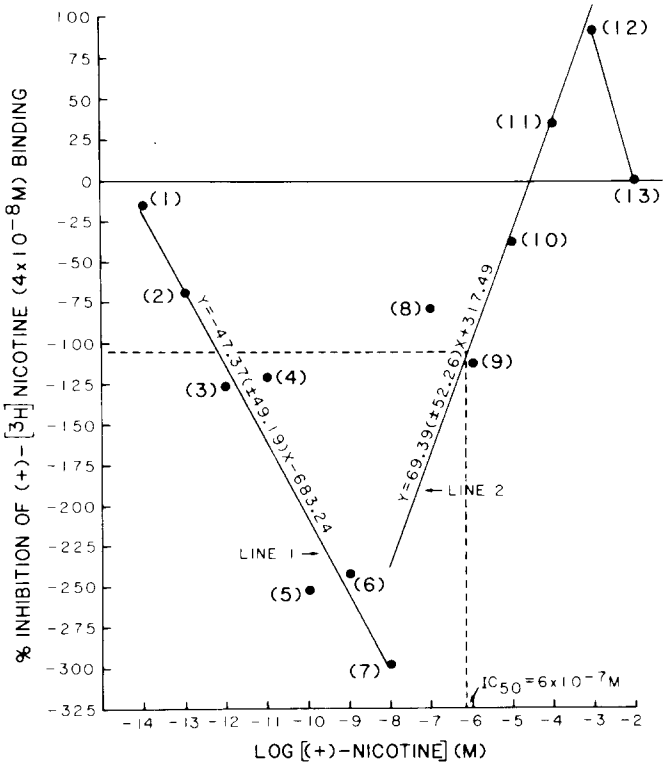

FIG. 5. The inhibition of (+)-[^{3}H]nicotine (4×10^{-8} M) binding by graded concentrations of unlabelled (+)-nicotine. Each point is the mean of 4 experiments. The least squares fit for lines 1 (point 1–7) and 2 (points 7–12) are shown with the equation for the fit. The slope for each line (± its 95% confidence limits) was calculated by regressing the % inhibition against the log of the molar concentration of (+)-nicotine.

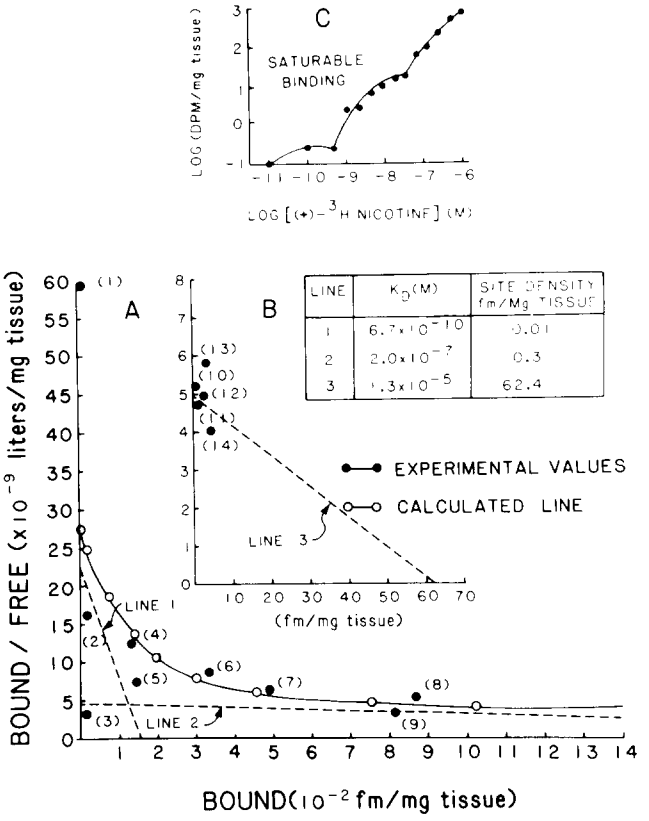

FIG. 6. Scatchard plot of (+)-[^{3}H]nicotine saturation binding data. Concentrations of (+)-[^{3}H]nicotine ranging from 10^{-11} M (point 1 A) to 10^{-6} M (point 14) were employed. Inset (C) shows the saturation curve (corrected for non-saturable binding). A Scatchard conversion of this data is shown in (A) and (B) as solid circles. The dashed lines represent the best fit to these data obtained by using the ANOVA I and ANOVA II program. K_Ds and site densities calculated from these lines are shown in the box. The open circles represent the calculated values for the line [10].

(+)-Nicotine

Figure 5 shows that (+)-nicotine also produced a significant concentration-related enhancement of binding (Fig. 5, line 1) over a concentration range of 10^{-14} M to 10^{-8} M. The line had a significant negative slope. A two-way ANOVA of the data through concentrations ranging from 10^{-14} M to 10^{-3} M showed that there was a significant between homogenates variance and a highly significant concentration related inhibition of binding from 10^{-8} M to 10^{-3} M (Fig. 5, line 2).

Saturation Experiments

Figure 6A and 6B show the Scatchard plot of the triphasic saturation binding curve (Fig. 6C). About 0.4 percent of the total counts added were bound at the lowest concentrations and about 0.2 percent at the highest concentrations. Specific binding represented about 10 percent of the total binding and in contrast to (−)-[^{3}H]nicotine specific binding remained relatively constant across concentrations. The best fit lines were obtained using ANOVA I and ANOVA II and produced three binding sites which are indicated by the dashed lines in Fig. 6A and 6B. The K_Ds and the site densities estimated from these lines are shown in the box in Fig. 6B. The calculated values derived from the best fit lines for points 1–9 are shown in Fig. 6A. Although these lines resulted in the greatest reduction of variance it is apparent that the resulting theoretical line [10] obtained provides only a moderately good fit so that there are ambiguities in interpreting the data.

DISCUSSION

The present studies provide additional evidence of the complexities of the binding of nicotine to rat synaptosomal preparations. Saturation studies employing (−)- and (+)-[^{3}H]nicotine confirm the existence of a very high affinity binding site ($K_D \sim 10^{-10}$ M) previously identified [13] in inhibition studies employing (±)-[^{3}H]nicotine. This site was not seen in inhibition studies using (−)- or (+)-[^{3}H]nicotine. However, it could have been obscured by the enhancement of binding produced by low concentrations of the unlabelled ligands. Others have also identified a very high and high affinity site using (−)-[^{3}H]nicotine [1].

A perplexing aspect of these studies was that less than

1/10 as much (+)-nicotine bound specifically to the P_2 fraction as (−)-nicotine. This was seen in saturation studies (Figs. 3 and 6). These data suggest that (+)-nicotine is either binding to a separate population of binding sites or to a subpopulation of the sites to which (−)-nicotine binds.

Although previous studies employing (±)-[^{3}H]nicotine had suggested that both (+)- and (−)-nicotine could enhance the binding of the labelled ligand, the use of (−)- and (+)-[^{3}H]nicotine have not only established this action but have reconciled several unexplained observations. (−)-Nicotine produced an enhancement of (−)-[^{3}H]nicotine binding over a concentration range of 10^{-12} to 10^{-8} M probably at the high affinity site. However, the degree of enhancement may have been masked by a significant inhibition of binding by concentrations of 10^{-10} M and greater. The radioligand (1.2×10^{-8} M) itself may also have produced almost maximal enhancement such that additional unlabelled ligand produced only a modest additional increase. The enhancement of the binding of (−)-[^{3}H]nicotine by (+)-nicotine was of the same order of magnitude but was seen with concentrations as low as 10^{-14} M. Increasing the concentration did not markedly change the amount of enhancement of binding until concentrations of 10^{-7} were achieved which inhibited binding. The difference in configuration of inhibition curves of (−)- and (+)-nicotine competing with (−)-[^{3}H]nicotine binding can be explained by assuming that (+)-nicotine has a higher affinity for the (−)-nicotine enhancement site than (−)-nicotine but a lower affinity for the high affinity binding site. Previous studies also suggested that (+)-nicotine was both more potent than (−)-nicotine in enhancing the binding of (±)-[^{3}H]nicotine [13] and that it had a lesser affinity for the high affinity site than (−)-nicotine [2, 3, 6, 9, 12, 13]. Further, it is important to note that the binding density for the high affinity (−)-nicotine site was about the same in saturation studies and in inhibition studies employing either (±)-[^{3}H]nicotine or (−)-[^{3}H]nicotine (1.0–4.5 fm/mg of tissue). However, (+)-nicotine increased the high affinity (−)-nicotine binding site to 60 fm/mg of tissue indicating that not only does it have a very high affinity for the nicotine enhancement site but has a greater ability to enhance binding at this site than (−)-nicotine. Further, these data indicate that the enhancement site must be able to bind both (+)- and (−)-nicotine. Evidence that other drugs also differ in efficacy in producing enhancement of nicotine binding has been obtained and the enhancement is viewed as an agonistic action ([13] and in preparation).

The binding of (+)-[^{3}H]nicotine is enhanced in a dose-related manner by both (−)- and (+)-nicotine and they are probably about equipotent in this regard since their dose response lines have about the same slopes and intercepts (Figs. 4 and 5). The fact that the degree of enhancement of binding of (+)-[^{3}H]nicotine is greater than that seen when (−)-[^{3}H]nicotine was used as a ligand may be explained in two ways. (1) It may be that the K_D of (+)-nicotine is greater than the K_D of (−)-nicotine for the enhancement site, hence the concentrations of (+)-[^{3}H]nicotine employed in the inhibition studies may produce a lesser degree of enhancement in their own right. As indicated, the enhancement site may have a greater ability to bind (+)-nicotine. (2) (−)- and (+)-nicotine have lower affinities for the (+)nicotine binding sites than for the (−)-nicotine binding sites, hence inhibition of binding does not obscure the enhancement of binding to the same degree at the (+)-nicotine binding site as it does the (−)-nicotine binding. site.

Characterization of the low affinity site, or sites, has not been as complete as for the other sites, primarily because of the large amounts of (−)- and (+)-[^{3}H]nicotine which must be employed to investigate this site. As can be seen from Table 1 and Figs. 3 and 6, the estimates of the K_Ds of the low affinity site ranged from 10^{-5} to 10^{-4} M. A low affinity site was not identified with (+)-[^{3}H]nicotine, however, this may have been a consequence of the relatively low concentration of the radioligand employed.

The binding of both (+)- and (−)-nicotine to the P_2 fraction of the rat brain is very complicated and the two isomers differ in their binding characteristics. Most evidence indicates that (−)-nicotine has a greater affinity for the high affinity site than (+)-nicotine. The high affinity site is probably comprised of existing sites and recently enhanced sites. Whether the binding characteristics of existing and recently enhanced sites are the same is not known. Some data presented would suggest that recently enhanced high affinity sites may have a greater affinity for (+)-nicotine than existing high affinity binding. (+)-Nicotine has a greater affinity for the enhancement site than (−)nicotine and has greater activity in producing enhancement. Clearly the (+) and (−) isomers interact with each other. There are no existing mathematical models for these complex interactions and hence no way to compare predicted outcomes with observed data. The physiologic importance of these different binding and enhancement sites is unknown.

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