

anol (by weight) by analyzing a series of known solutions of ethanol in gasoline. Integration was done by digital computer. Water was determined by Fischer titration (5).

Addition of HSPAN to the two-phase mixtures of gasoline, ethanol, and water resulted in selective absorption of water with nearly complete solution of the ethanol component into the gasoline phase (Table 1). Corn starch did not exhibit this selectivity. Because the HSPAN was not dry but contained 10 percent water, one should be able to reuse the polymer after air-drying it at room temperature and low relative humidity, and the energy required to regenerate HSPAN would thus be negligible [HSPAN air-dries readily (3), and the resulting polymer remains highly absorbent]. As expected, residual water increased with the final ethanol content of the gasohol. Water was removed more efficiently when gasoline-ethanol-water mixtures were passed through a column of HSPAN. For example, water contents of 0.24 and 0.28 percent (by weight) were found with ethanol contents of 10.4 and 10.9 percent (by weight), respectively, and these water contents are within the tolerance limit for a 90:10 ethanol-gasoline blend.

It apparently is not sufficient for HSPAN to merely absorb all of the lower aqueous ethanol phase; rather, enough HSPAN must be used so that the water present in the system is actually bound to the polymer by strong hydrogen bonds. The amount of polymer needed to dehydrate a particular system thus depends on the total water present and not on the degree of swelling or absorbency (6) of HSPAN in the ethanol-water mixture. Low-proof ethanol will require a large amount of HSPAN, even though much smaller amounts will absorb and solidify the aqueous ethanolic solution. Since the high-proof ethanol systems of Table 1 are essentially nonswelling media for HSPAN, addition of sufficient polymer to absorb all of the aqueous ethanol layer provides about the correct ratio of polymer to water to ensure its strong hydrogen bonding.

The presence of the gasoline phase as an extractant for ethanol, as water is being hydrogen-bonded to HSPAN, is essential to the success of our method, since high-proof ethanol in the absence of gasoline was not completely dehydrated by polymer. For example, when ethanol containing 23 percent (by weight) water was passed through 5 g of HSPAN loosely packed into a column 9 by 1.2 cm, the first 5 ml through the column still contained 15 percent water.

The water content of the effluent remained fairly constant until about the 15th milliliter and then began to increase, as the column reached its capacity for water.

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Stereospecific Nicotine Receptors on Rat Brain Membranes

Abstract. A stereospecific binding site for nicotine has been detected on rat brain membranes. Competition studies with cholinergic agonists suggest that this site is a nicotinic cholinergic receptor.

Nicotine is one of the most widely studied drugs. Its mechanism of action at the neuromuscular junction and at autonomic ganglia is well understood at both the molecular and the cellular levels (1). Nicotine also has many effects on the central nervous system: it is a primary reinforcer (2); it serves as a discriminative stimulus (3); it has anti-nociceptive properties (4); and it stimulates vasopressin release (5). Although it is usually assumed that these actions of nicotine are mediated by nicotinic cholinergic re-

ceptors on central neurons, there is no direct evidence for this assumption. Blockade of these effects by mecamylamine or pempidine is not a sufficient criterion, since these compounds are poor blockers at the neuromuscular junction, and their ganglionic blocking properties are largely noncompetitive and presynaptic (6, 7).

α -Bungarotoxin binds specifically to brain membranes, and its binding site shares ligand specificity and biochemical properties with the acetylcholine recep-

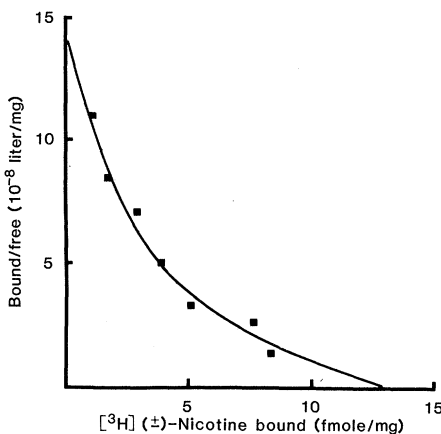


Fig. 1. Scatchard plot of (\pm) - $[^3\text{H}]$ nicotine binding to rat brain membranes. Male Simon-sen albino rats (Sprague-Dawley derived, Simonsen Laboratories, Gilroy, California), weighing 250 to 350 g were killed by decapitation and the brain was removed. Whole brain homogenate was prepared in ten volumes of ice-cold buffer (Hepes, 50 mM; NaCl, 118 mM; KCl, 4.8 mM; CaCl_2 , 2.5 mM; MgSO_4 , 1.2 mM; and NaOH to pH 7.40) and centrifuged at 17,500g for 30 minutes. The pellet was suspended in 20 volumes of ice-cold glass-distilled water, allowed to lyse for 60 minutes, and centrifuged as above. The membrane pellet was then suspended in buffer and centrifuged as above. The final pellet was suspended to a final concentration of 40 mg of original tissue per milliliter of buffer; 0.5 ml of the membrane suspension was added to 0.5 ml

of buffer containing (\pm) - $[^3\text{H}]$ nicotine at the appropriate concentration, in the absence or presence of unlabeled $(-)$ -nicotine at a 1.0 mM final concentration (each concentration in triplicate). Tubes were incubated for 40 minutes at 37°C, and then chilled on ice. After 20 minutes the mixture was diluted with 4.0 ml of ice-cold buffer, filtered through polylysine-soaked Whatman GFC filters, and washed with four 4.0-ml portions of cold buffer. To control for residual displaceable binding to filters, parallel tubes were incubated and chilled as above, but (\pm) - $[^3\text{H}]$ nicotine was not added until immediately before filtration. Radioactivity was determined by spectrometry in a dioxane-based liquid scintillation mixture at a counting efficiency of 43 percent. Binding not displaceable by 1.0 mM $(-)$ -nicotine, and displaceable binding to filters have been subtracted; plotted data represent only displaceable binding to brain membranes. Units for bound ligand are femtomoles per milligram of original tissue and for bound/free are liters per milligram of original tissue. This experiment yielded the following parameter estimates: high-affinity site $K_d = 28$ nM, density = 3.2 fmole per milligram of original tissue; low-affinity site $K_d = 0.46$ μM , density = 10.4 fmole per milligram of original tissue.

tor of electric organs and skeletal muscle (8). However, the inability of α -bungarotoxin to block cholinergic function in most neuronal systems examined has raised questions about the physiological relevance of its binding site (9).

To study the receptor through which nicotine exerts its central actions, we considered it essential to use nicotine itself as the primary ligand. A number of investigators have measured the binding of radiolabeled nicotine to brain tissue.

Some of these studies have lacked adequate controls for nonspecific binding (10). Recently, Abood *et al.* (11), unable to measure binding to a brain membrane preparation, detected a nicotine binding site on brain slices. On the basis of competition experiments they proposed that their site is a noncholinergic receptor for nicotine. Vincek *et al.* (12) described a centrifugal procedure for measuring binding of ^3H -labeled nicotine to brain membranes.

We now report the existence of a high-affinity, saturable, pharmacologically specific site for nicotine in rat brain membranes, detected with a filtration assay. $(\pm)\text{-}^3\text{H}$ nicotine of high specific radioactivity (23.6 Ci/mmol) was synthesized for us (New England Nuclear). The purity of the material was examined in three thin-layer chromatography systems (13) and two high-performance liquid chromatography systems (14). All the chromatographic methods revealed a single peak of radioactivity that migrated with authentic unlabeled nicotine.

Since nicotine binds avidly and specifically to glass-fiber filters (11), we initially separated bound from free radioactive nicotine by centrifugation. Although 15 to 20 percent of the bound ligand was displaceable by 1.0 mM unlabeled nicotine, Scatchard plots did not usually have negative slopes. This paradox implied that the displaceable binding was not saturable. One explanation may be the presence, at high density, of a number of moderate- and low-affinity sites for nicotine; the displaceable binding would then be a composite with these unusual properties. A separation technique that allows a thorough and rapid wash of the membranes, to remove non-specifically and loosely bound $(\pm)\text{-}^3\text{H}$ nicotine, was essential.

When Whatman GFC glass-fiber filters are presoaked in a 0.1 percent solution of polylysine (Sigma, type V), $(\pm)\text{-}^3\text{H}$ nicotine binding decreases by more than 85 percent. This reduction of background radioactivity was sufficient to permit detection of specific binding to brain membranes.

Another technical advance was necessary before $(\pm)\text{-}^3\text{H}$ nicotine binding assays could be performed routinely. $(\pm)\text{-}^3\text{H}$ nicotine of high specific radioactivity is subject to radiolysis, and storage conditions that hinder this process had to be found. The severity of the problem is best illustrated by example (Table 1). $(\pm)\text{-}^3\text{H}$ nicotine was stored as described in the legend to Table 1. After 105 days, the extent of radiochemical breakdown was assessed chromatographically, and the ability of the different preparations to bind specifically to rat brain membranes was determined. The results show that although the amount of specific binding was comparable among all the storage forms, nonspecific binding varied over a four-fold range. Storage with mercaptoacetic acid provided the most protection against radiolytic breakdown and the lowest nonspecific binding. Accordingly, this condition was adopted for all sub-

Table 1. Effect of storage condition on ^3H -labeled (\pm) -nicotine breakdown and binding to rat brain membranes. $(\pm)\text{-}^3\text{H}$ nicotine was kept frozen in aqueous solution (2 μM) with no additives or with a threefold molar excess of taurine, tartaric acid, sulfuric acid, or mercaptoacetic acid. After 105 days of storage, the purity was examined by thin-layer chromatography (TLC) (MeOH and NH_4OH , 99:1, silica plates) and high-performance liquid chromatography (HPLC) [conditions as in (14) except solvent CHCl_3 , 91 percent, MeOH and NH_4OH , 99:1, 9 percent]. Data represent percentage of recovered counts not migrating with nicotine. Binding assay was performed as in legend to Fig. 2, except that the final concentration of $(\pm)\text{-}^3\text{H}$ nicotine was 18.5 nM. Binding data are means \pm standard deviations of quadruplicate determinations.

Storage form	Percent impurity		Bound (count/min)			
	TLC	HPLC	Total	Non-specific	Specific	Specific (%)
No addition	4	5	1531 \pm 40	1155 \pm 21	376	25
Taurine	6	7	1416 \pm 22	1006 \pm 19	410	29
Tartaric acid	4	6	979 \pm 32	555 \pm 19	424	43
Sulfuric acid	4	5	1006 \pm 25	568 \pm 25	438	44
Mercaptoacetic acid	2	2	707 \pm 22	275 \pm 22	432	61

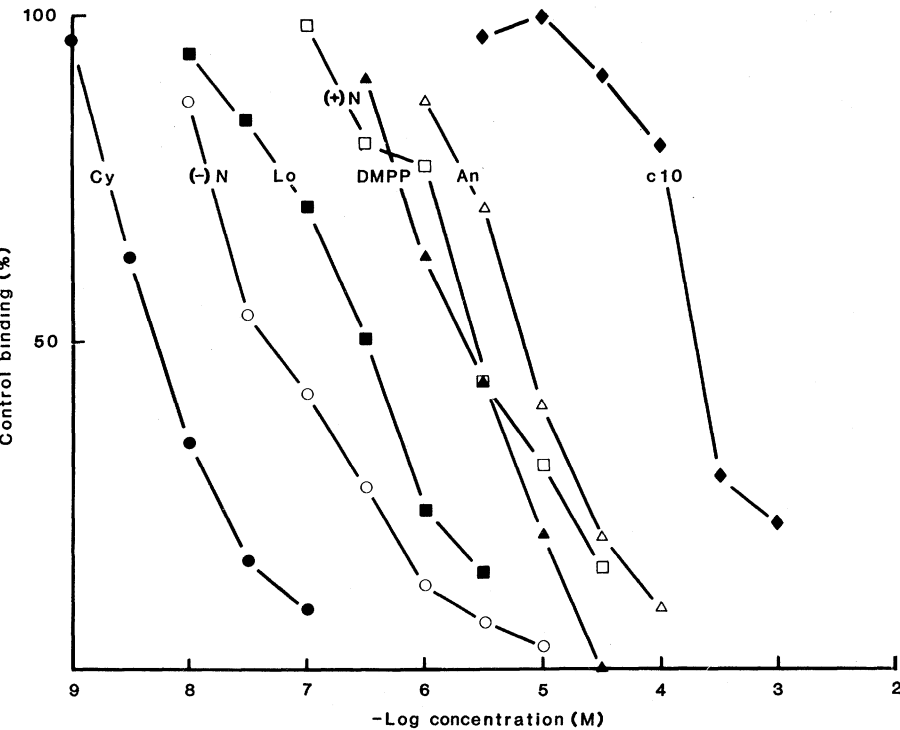


Figure 2. Competition of various compounds for the binding site (19). Homogenate was prepared as described in the legend to Fig. 1. Each milliliter of final incubation mixture contained membranes from 15 mg of original tissue; incubation time was 60 minutes; $(\pm)\text{-}^3\text{H}$ nicotine concentration was 40 nM. Binding reaction was started by addition of membranes to buffer containing $(\pm)\text{-}^3\text{H}$ nicotine and inhibitor. Nonspecific binding was determined in the presence of 10 μM $(-)\text{-}^3\text{H}$ nicotine. At this concentration of $(\pm)\text{-}^3\text{H}$ nicotine, displaceable filter binding was negligible. Abbreviations: Cy, cytisine; $(-)\text{N}$, $(-)$ -nicotine; Lo, lobeline hydrochloride; DMPP, dimethylphenylpiperazinium iodide; $(+)\text{N}$, $(+)$ -nicotine; An, anabasine; and c10, decamethonium bromide.

sequent experiments. (Some of the experiments reported here used the taurine salt at a time in its storage prior to extensive breakdown.)

A typical Scatchard plot is shown in Fig. 1. Free ligand concentration ranged from 10 to 640 nM. Specific, displaceable binding to brain membranes was 65 to 80 percent of total binding at the lowest concentration and usually above 30 percent at the highest. The plots were always curvilinear, suggesting the presence of multiple sites. Computer fitting (15) to a two-site Scatchard equation yielded the following estimates for the high-affinity site: K_d (dissociation constant) = 43 nM (range 20 to 89), site density = 4.4 fmole per milligram of tissue (range 2.7 to 6.1) in six experiments. Parameter estimates for the low-affinity site were widely disparate from experiment to experiment.

Competition studies characterized the high-affinity site pharmacologically (Fig. 2); results from all compounds tested are given in Table 2. The stereospecificity is noteworthy; (+)-nicotine is only 1/63 as potent as (–)-nicotine. Since the radio-labeled ligand is racemic, the affinity is approximately twice that determined from the Scatchard analysis. The most effective competing compounds are potent ganglionic stimulants known to interact with the nicotinic cholinergic receptor present on autonomic ganglion cells.

None of the nicotinic cholinergic antagonists tested were potent competitors. In particular, α -bungarotoxin, in concentrations up to 5 μ g/ml, did not substantially reduce binding. The efficacy of this preparation (Boehringer Mannheim, lot 1449506) was tested by bioassay on the rat phrenic nerve-hemi-diaphragm preparation (16). At 4 μ g/ml, neuromuscular transmission was completely blocked in 20 minutes, as predicted for authentic, efficacious α -bungarotoxin (17). It would be tempting to conclude that the toxin's ineffectiveness in neuronal preparations is due to a difference in the neuronal receptor as compared with the skeletal muscle receptor. However, the ineffectiveness of hexamethonium, pentolinium, tetraethylammonium, and trimethaphan, all competitive antagonists at ganglia (7), implies a general impotence of antagonists at this site.

The high potency of nicotinic cholinergic agonists in displacing (\pm)-[³H]nicotine binding suggests that this site is indeed similar to, or identical with, the ganglionic nicotinic receptor and not a unique, noncholinergic nicotine recep-

tor. Two anomalies demand explanation. (i) Why are antagonists ineffective? (ii) Why is the dissociation constant for nicotine in the low nanomolar range, whereas micromolar concentrations are required to elicit ganglionic stimulation (6, 7) and behavioral effects (3)? One hy-

pothesis is that the prolonged incubation used in these in vitro studies causes an agonist-induced shift of the receptor to a high-affinity, agonist-selective state—perhaps analogous to the desensitization seen in vivo (18). This would explain both anomalies, but proof requires further experimentation.

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Table 2. Values of IC_{50} (concentration necessary to reduce specific binding of (\pm)-[³H]nicotine by 50 percent) for various compounds as competitors (19). Assays were performed as described in legends to Figs. 1 and 2. Values of IC_{50} were determined by a least-squares regression line drawn through points providing 15 to 85 percent reduction of specific binding. Each drug was examined in at least two experiments, and the value given is the average. Abbreviations: DMPP, dimethylphenylpiperazinium, iodide; TMA, tetramethylammonium chloride; TEA, tetraethylammonium chloride; cyclic AMP, adenosine 3',5'-monophosphate; DADLE, [D-Ala²,D-Leu⁵]enkephalin; GABA, γ -aminobutyric acid; PGE₁, prostaglandin E₁; α -MSH, α -melanocyte-stimulating hormone; and α -BTX, α -bungarotoxin.

Drug	IC_{50} , μ M
<i>IC₅₀ values less than 1.0 mM</i>	
Cytisine	0.014
(–)-Nicotine	0.062
Lobeline	0.30
DMPP	1.8
Carbachol	2.4
(+)-Nicotine	3.9
Anabasine	4.6
N-Benzyl normicotine	13
TMA	40
d-Tubocurarine	150
Decamethonium	200
Piperidine	280
Neostigmine	320
<i>IC₅₀ values approximately 1.0 mM*</i>	
Oxotremorine	
Atropine	
N-Benzyl piperidine	
Diazepam	
Pentolinium	
<i>IC₅₀ values greater than 1.0 mM†</i>	
Hexamethonium	Haloperidol
TEA	Epinephrine‡
Phenylpropyl-diethylamine	Norepinephrine‡
Mecamylamine	Dopamine‡
Chlorisondamine	Serotonin‡
Pempidine	Histamine‡
Gallamine	Ouabain
Trimethaphan	GABA
D,L-Muscarine	Glycine
Adenosine	Glutamic acid
Cyclic AMP	Aminophylline
Levorphanol	Nicotinic acid
Naloxone	Nicotinamide
DADLE	Substance P‡
Isoproterenol	PGE ₁
D,L-Propranolol	Somatostatin§
Clonidine	α -MSH§
Ascorbic acid	α -BTX§

*Drugs in this group caused 40 to 60 percent inhibition when tested at 1.0 mM. †Drugs in this group gave less than 40 percent inhibition at 1.0 mM, except as noted below. ‡Ascorbic acid (1.0 mM) was present as preservative. §For somatostatin, α -MSH, and α -BTX, no inhibition was observed at the highest concentrations tested, 150, 15, and 0.6 μ M, respectively.

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biochem; ascorbic acid, Baker; GABA, California Corp. for Biochemical Research; glutamic acid, Pfanstiehl; piperidine, Fisher; trimethaphan camphorsulfonate, diazepam, and levorphanol tartrate, Hoffmann-La Roche; pentolinium tartrate, May and Baker; chlorisondamine chloride, Ciba; haloperidol, McNeil Laboratories; naloxone hydrochloride, Endo Laboratories; (+)-nicotine (> 96 percent optically pure), T. Kisaki; *N*-benzyl nornicotine and *N*-benzyl piperidine, L. G. Abood; phenylpropanediethylamine hydrobromide, R. B. Barlow;

and clonidine hydrochloride, Boehringer-Ingelheim. All other drugs were obtained from Sigma.

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Carbon-13 Nuclear Magnetic Resonance Study of Osmoregulation in a Blue-Green Alga

Abstract. *The process of osmoregulation in a unicellular blue-green alga, Synechococcus sp., has been studied by natural-abundance carbon-13 nuclear magnetic resonance spectroscopy of intact cells and cell extracts. 2-O- α -D-Glucopyranosylglycerol was identified as the major organic osmoregulatory solute. This demonstrates the presence of a major osmoregulatory solute in a blue-green alga and is also an example of an osmoregulatory role for glucosylglycerol.*

All cells that grow in a solution must "osmoregulate" to prevent net water movement across their plasma membranes and consequent cell damage (1). Species of eukaryotic algae (2-5) and marine bacteria (6) achieve this by accumulating specific, low-molecular-weight, neutral organic solutes in their cytoplasm in direct response to the salt concentration of their growth media. By contrast, prokaryotic algae (blue-green algae or cyanobacteria) have been reported to accumulate only inorganic ions (7), particularly KCl (8), in response to high-saline environments. Up to now extensive accumulation of organic solutes

in the cytoplasm has not been demonstrated in this division (Cyanophyta). Therefore, we have investigated osmoregulation in intact cells of a unicellular blue-green alga with the aid of natural-abundance ^{13}C nuclear magnetic resonance (NMR) spectroscopy. This technique is a useful new means of studying the process of osmoregulation in marine and halophilic organisms because it enables the identification and quantitation of all major organic solutes in both living cells and cell extracts (9-11). The results reported here show that 2-O- α -D-glucopyranosylglycerol is the major organic osmoregulatory solute.

This demonstrates the presence in a blue-green alga of a major organic osmoregulatory solute and is an example of an osmoregulatory role for glucosylglycerol.

The unicellular blue-green alga, *Synechococcus* sp. N \ddot{a} geli (Chroococcales, Cyanophyta) (strain RRIMP N 100), isolated from rock surfaces in the marine intertidal zone in Sydney, has a broad salt tolerance in culture, growing in basal medium (12) with no added NaCl (that is, 0.03M total salts) and in media with NaCl additions up to 1.69M total salts (13). Figure 1a shows a natural-abundance ^{13}C NMR spectrum of intact *Synechococcus* cells grown in medium with 1.04M salts. Except for a prominent resonance from tris buffer present in the growth medium, the only observable resonances come from the glycoside 2-O- α -D-glucopyranosylglycerol. A ^{13}C NMR spectrum of the extracellular supernatant recorded after accumulation (Fig. 1a) was complete showed only the tris resonance, which indicates that (i) all the glucosylglycerol was intracellular and (ii) it did not leak out of the cells during spectral accumulation. Figure 1b shows the spectrum of an aqueous extract of completely broken cells. Quantitative comparison of spectra from intact cell suspensions and cell extracts indicates that essentially all of the glucosylglycerol is visible in the former. Therefore, most of the glycoside must be freely mobile in intact cells.

The compound was identified princi-

Fig. 1. Natural abundance ^{13}C NMR spectra of (a) intact *Synechococcus* cells (0.13 g, wet weight, per milliliter) grown in medium with 1.04M total salts and harvested in the late exponential phase (culture details are given in the legend of Fig. 2) and (b) an aqueous extract of cells (0.4 g, wet weight, per milliliter) grown under the same conditions as in (a). Cells were broken by three freeze-thaw treatments followed by French pressing twice at 18,000 pounds per square inch, the debris was removed by centrifugation, and the resulting supernatant was diluted with D_2O (to 12 percent by volume) to provide a lock signal. Spectra were obtained on a JEOL spectrometer (model FX-60) operating in the pulsed Fourier transform mode at 15.04 MHz and incorporating a 4000-Hz band-pass crystal filter. Sample tubes, 10 mm in outside diameter, were used. For (a) 19,000 scans with a recycle time of 1.0 second (total time, 5.3 hours) were accumulated in 4096 time-domain addresses, a further 4096 addresses with zero value were added before Fourier transformation, and exponential broadening was 1.46 Hz. Sweep width was 2500 Hz, 90° radio-frequency pulses (pulse width, 18 μsec) were employed, and the sample tube was spun at 10 to 15 Hz. Conditions for (b) were as for (a) except that 12,000 scans with a recycle time of 4.0 seconds (total time, 13.3 hours) were accumulated in 8192 time-domain addresses, and exponential broadening was 1.0 Hz. Temperature of the supernatants varied between 26° and 31°C and pH between 5.5 and 6.5, depending on composition. The 90° pulse width varied by only 1 to 2 μsec over the range of salt concentrations examined. The resonance at 60.7 parts per million (ppm) arises from the three methylene carbons of tris buffer in the medium. The corresponding quaternary carbon resonance (at 62.7 ppm) is partially saturated under our accumulation conditions. Repeated washing of cells prior to breakage reduced the height of these peaks in extracts below the level indicated in (b). Me_4Si is tetramethylsilane.

