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Inhibition of Acetylcholine Storage by Acetylcholine Analogs *In Vitro*

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SUMMARY

Forty-five acetylcholine (AcCh) analogs were chemically synthesized and characterized. They and two commercially available analogs were tested for the ability to inhibit active transport of AcCh by synaptic vesicles purified from the electric organ of *Torpedo californica*. A range of potencies greater than 4 orders of magnitude was found. A quaternary nitrogen and the presence of the carbonyl group are important to potency. The addition of hydrophobic groups to both ends of isonipecotic acid yielded the most potent analogs, which exhibited a nearly 1000-fold increase in potency relative to AcCh. The probable conformation of AcCh bound by the transporter has been deduced and confirmed by the synthesis of a potent rigid analog based on 2-amino-9-fluorenone. A potent analog was shown to be a competitive inhibitor with respect to AcCh, thus confirming that its site of action is the transporter active site. The structure-activity data clearly distinguish the binding site for AcCh from the site for vesamicol [(−)-(*trans*)-2-(4-phenylpiperidino)cyclohexanol], which is a noncompetitive inhibitor.

AcCh analogs that resemble AcCh sufficiently to be formed and released in a stimulation-dependent manner by intact cholinergic nerve terminals are termed false transmitters. A successful precursor (i.e., a choline analog) is transported by the sodium-dependent, high affinity, choline uptake system from the extracellular space into the cytoplasm, where it is acetylated by choline acetyltransferase (EC 2.3.1.6). Cytoplasmic false transmitter then is taken up by synaptic vesicles, from which it is released exocytotically upon stimulation.

Much has been learned regarding the specificities of sodium-dependent, high affinity, choline uptake and choline acetyltransferase through the study of choline analog metabolism in intact or semi-intact cholinergic preparations (1–6). In contrast, relatively little is known about the specificity of the AcCh transporter in synaptic vesicles. There are two reasons for this. First is a metabolic pathway-filter effect occurring in intact terminals. Only those choline analogs that successfully enter and become acetylated can test the specificity of the vesicle storage step. Second is the restriction to acetate esters that is imposed by the usual metabolism of intact terminals. Thus, relatively few structural variations in the two metabolic components of the AcCh molecule have been evaluated for their effects on the AcCh transporter. This greatly limits our ability to predict which compounds could be transported, which could be inhibitors, and which might be useful in affinity labeling and affinity purification applications.

An obvious solution to these limitations is to study AcCh analogs *in vitro*, using purified synaptic vesicles. With the recent development of the AcCh active transport assay using vesicles isolated from the *Torpedo* electric organ, this now is possible (7). In the current paper, we report the chemical synthesis of 45 AcCh analogs and derivatives, determination of their IC_{50} values (plus those of two commercially available compounds) as inhibitors of AcCh active transport, and a kinetic test of whether one of the most potent analogs acts as expected by binding to the AcCh transporter active site.

Materials and Methods

Melting points were determined in open capillaries on a Thomas Hoover capillary melting point apparatus and are uncorrected. ^1H NMR spectra were recorded on either a Varian EM360A, a Nicolet NT-300, or a General Electric GN-500 with tetramethylsilane as internal standard. UV spectra were recorded on a Perkin Elmer Lambda 3 spectrophotometer and IR spectra on a Perkin Elmer 1330 infrared spectrophotometer. Mass spectra were run on a VG Analytical 70-250 HF mass spectrometer in the FAB mode. TLC analyses were performed on Kodak 13181 silica gel plates and were visualized with UV light and I_2 vapor. Column chromatography was performed on Merck silica gel 60. AcCh (compound **1**) and acetyl β-methylcholine (**28**) were from commercial sources. All others were synthesized as described below, except compounds **46** and **47**, which were reported elsewhere (8). Precursor amines were purchased from Aldrich Chemical Co (Milwaukee, WI).

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ABBREVIATIONS: AcCh, acetylcholine; FABMS, fast atom bombardment mass spectrometry; TLC, thin layer chromatography; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Ac, acetyl; Me, methyl; Et, ethyl; Ph, phenyl; Bz, benzyl; GABA, γ-aminobutyric acid.

Quaternization of commercially available tertiary amines (Table 1; general synthetic method A). After the tertiary amine hydrochloride was dissolved in $CHCl_3$, the free base was liberated by neutralization with aqueous Na_2CO_3. The $CHCl_3$ solution was dried over Na_2SO_4 and filtered through K_2CO_3. Removal of the solvent on a rotary evaporator provided the amine, which was taken up in either CCl_4 or Et_2O and treated with either MeI (several-fold excess) or benzyl bromide (10% excess). Typically the product ammonium halide crystallized from solution within minutes or a few hours but for some hindered amines the reaction was incomplete even after several days. No attempt was made to optimize the yield.

General procedure for the preparation of quarternary ammonium esters and tertiary amino alcohols (Table 2; general synthetic method B). The appropriate amino alcohol (20–50 mmol) was dissolved in CH_2Cl_2 (15–50 ml) and cooled to 0°. An acid chloride or anhydride (1.3 equivalents) was added dropwise to the stirred solution, which was then allowed to come to room temperature slowly and stirred overnight. Solvent was removed on a rotary evaporator, the resulting oil or solid was dissolved in $CHCl_3$, and the solution was extracted several times with cold 10% Na_2CO_3. The $CHCl_3$ solution was dried over Na_2SO_4 and filtered through K_2CO_3. After removal of the $CHCl_3$ on a rotary evaporator, purity of the ester was assessed by ^1H NMR. When indicated, the compound was purified by column chromatography on silica gel. The amino ester was subsequently alkylated as in general synthetic method A above, except compound **2**, which was tested as the tertiary amine.

Preparation of derivatives of isonipecotic acid and GABA (Table 3; general synthetic method C). The amino acid was dissolved in excess formic acid and treated with 2–3 equivalents of aqueous formaldehyde (Eschweiler-Clarke reaction) under gentle reflux overnight. Solvents were removed on a rotary evaporator, with the use of CH_3CN to aid in azeotropic distillation of residual water. For the synthesis of methyl esters, the formate salts of the tertiary amines were dissolved in MeOH and cooled to −5°. $SOCl_2$ (2.5 equivalents) was added slowly so that the solution temperature remained below 0°. After addition was complete, the solution was allowed to reach room temperature slowly and then was heated to 40° for several hours. For all other esters, the formate salt was converted with anhydrous HCl into the hydrochloride, which was crystallized. The tertiary amine hydrochloride and an excess of the appropriate alcohol were dissolved in CH_2Cl_2 and treated with 2 equivalents of $SOCl_2$, as for the methyl esters. The anilide derivative (**27**) was synthesized via the acid chloride, which was generated by heating the tertiary amine hydrochloride in excess $SOCl_2$ at 40° overnight. Alkylations of the tertiary amines were carried out as in general synthetic method A above.

Preparation of *N,N*-dimethyl-2-ketomorpholine iodide (33). *N,N*-Dimethylethanolamine (2.67 g, 30 mmol) and iodoacetic acid (5.58 g) were combined in 160 ml of $Et_2O/CHCl_3$ and placed in the dark overnight. Solvent was removed on a rotary evaporator, giving a yellow oil. Petroleum ether was layered over the oil to promote crystallization and was subsequently decanted. The mass of oil and crystals was treated with 50 ml of EtOH, which slowly dissolved the remaining oil and redeposited a pale yellow solid. The yield of the ammonium acid (IR and ^1H NMR) was 1.4 g (17%). Recrystallization from MeOH/EtOH gave material with m.p. = 227–228°. The ammonium acid (100 mg, 0.364 mmol) was dissolved in 1 ml of formic acid. A catalytic amount of toluene sulfonic acid was added to the solution, which was allowed to sit at room temperature overnight. Formic acid was removed on a rotary evaporator and the resulting solid was suspended in EtOH and collected by filtration. Yield of pale yellow solid was 82 mg (88%). Lactonization was confirmed by ^1H NMR in D_2O, which demonstrated significant hydrolysis occurring within 1 hr. Consequently, the stock solution and serial dilutions of this compound were prepared and used in the IC_{50} determination within 15 min. The effect of glycine (0.6 M in the titration buffer) on the stability of the lactone was not assessed.

Preparation of (±)-*O*-benzoyl-(*trans*)-2-(*N*-benzyl-*N,N*-dimethylammonium)cyclohexanol iodide (36). *N*-Benzylmethylamine (1.21 g, 10.0 mmol) and a 2-fold excess of cyclohexene oxide were dissolved in 25 ml of EtOH and refluxed for 2 days. TLC analysis of the reaction mixture indicated 90% completion. Solvent was removed on a rotary evaporator and the resulting oil was chromatographed on silica gel. Yield of amino alcohol was 1.95 g (89%).

(±)-(*trans*)-2-(*N*-Benzyl-*N*-methylamino)cyclohexanol (1.0 g, 4.6 mmol) and a 10% excess of benzoyl chloride were dissolved in 10 ml of CH_2Cl_2, with the addition of 50 mg of *p*-dimethylaminopyridine. TLC analysis indicated completion of the reaction after 4 days. The neutral amino ester was isolated by partitioning between Et_2O and aqueous base. ^1H NMR of the oil that was obtained after the ethereal solution was dried and evaporated revealed impurities. Therefore, the oil was applied to a silica gel column and eluted with 20% $CHCl_3/CCl_4$ to yield 700 mg (47%) of the pure ester (^1H NMR). This amino ester was treated with methyl iodide as in general synthetic method A above, to give the desired quaternary ammonium ester in 16% yield (FABMS shows base peak at *m/z* 338). The corresponding pentanol analog (**35**) was prepared in an analogous fashion.

Inhibition of AcCh active transport. VP_1 synaptic vesicles from *Torpedo californica* electric organ were isolated as described (9). After the final concentrative pelleting, vesicles were resuspended at 4° in buffer A, which contained 100 mM HEPES, 700 mM glycine, 1 mM EDTA, and 1 mM EGTA, adjusted to pH 7.9 with KOH. Protein concentration was determined by the method of Bradford (10), using bovine serum albumin as a standard. Vesicles were diluted to 0.25 mg of protein/ml and were treated with 150 μM paraoxon for 60 min at 23° to inhibit any AcCh esterase activity present. A MgATP/ATP-regenerating system was prepared in buffer B, which contained 100 mM HEPES, 240 mM glycine, 1 mM EDTA, 1 mM EGTA, 20 mM $MgCl_2$, 100 mM MgATP, 100 mM potassium phosphoenolpyruvate, and 10 μg of pyruvate kinase, adjusted to pH 7.8 with KOH. Inhibitors were dissolved either in buffer A or in aqueous/organic mixtures if necessary and were then serially diluted into buffer A. The final concentration of organic solvent (usually EtOH or Me_2SO) was ≤0.2% at the highest inhibitor concentration used. This concentration of organic solvent was shown not to inhibit AcCh active transport. Also, neither I^- nor Br^- inhibit transport, up to 10 mM (8). A 500 μM [^{3}H]AcCh solution (100 mCi/mmol; DuPont, Inc.) was prepared by evaporating an appropriate volume of an ethanolic solution to dryness, followed by dissolution in buffer B at 0°. This solution was always prepared immediately before use. Vesicles were preincubated with the AcCh analog for 15 min, after which uptake was initiated by addition of the AcCh/ATP solution. Final concentrations of protein, AcCh, and ATP were 0.20 mg/ml, 50 μM, and 10 mM, respectively, with variable analog concentrations. Transport was allowed to proceed for 30 min and was then quenched by vacuum-assisted filtration through prewetted (1 ml of buffer A, 0°) polyethylenimine-treated[1] glass-fiber filters (Whatman GF/F, 1.3 cm). Filters were immediately washed with three 1-ml volumes of ice-cold buffer A and were then placed in 6 ml of scintillation fluid. The scintillation vials were allowed to stand, with occasional shaking, for at least 6 hr before quantitation of [^{3}H]AcCh.

For the analysis of the potency of inhibition by each analog, a hyperbolic titration curve was fitted to a set of eight single data points (from 0 to high analog concentration) by nonlinear regression analysis, to determine an apparent inhibition constant, the IC_{50} value. These are listed in Tables 4–7. For the competition analysis, the linear competitive equations in hyperbolic form were fitted to the separate sets of velocities at different inhibitor concentrations, using simultaneous nonlinear regression analysis similar to the methods of Cleland (11). The best lines determined by this latter fit were graphed in the double-reciprocal format for presentation. Best fits are quoted ± 1 standard error.

[1] After soaking in 0.5% aqueous polyethylenimine for 3 hr, glass-fiber filters were washed three times with equal volumes of glass-distilled water and dried at 32°.

Results

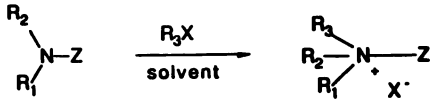
The specificity of AcCh transporter binding has been probed with 47 structurally related compounds, whose syntheses are summarized in Tables 1–3 and Materials and Methods. These compounds were tested for the ability to inhibit active transport of [^{3}H]AcCh by purified synaptic vesicles from *Torpedo* electric organ, and the results are summarized in Tables 4–7. Table 4 is composed of acyclic analogs of AcCh that preserve the β-amino alcohol structure of choline. Table 5 is composed of nitrogen heterocycles that also contain the same structural motif. Table 6 is composed of derivatives of isonipecotic acid and, therefore, are not amino alcohols but rather amino acids. Table 7 contains a variety of compounds that could not be accommodated in Tables 4–7 without introducing schematic confusion.

Acyclic β-amino alcohols. From the entries in Table 4, it is obvious that the transporter will bind AcCh analogs with any or all of the *N*-methyl groups replaced with bulkier ethyl groups (**3** and **4**) or with five methylene groups forming the piperidinyl ring, as in compound **5**. Removal of one methyl group from AcCh, as in compound **2**, to produce the tertiary amine is deleterious to potency by a factor of 4. Substitution of the more hydrophobic benzyl group for an *N*-methyl (**6**) demonstrates that a phenyl ring here makes favorable contacts with the transporter, increasing potency by 5-fold. Steric bulk in the *ortho*-position of the benzylic ring is well-tolerated (**7**), but a carboxylate in the *para*-position is not (**8**). The diminished potency seen for compound **8** could be due to charge repulsion between the carboxylate and a negatively charged group in the receptor, which almost certainly exists to ion-pair with the positive ammonium ion of AcCh, to a high hydration requirement, or to a steric effect. On the other end of AcCh, replacement of the acetate with propionate or benzoate (**9** and **10**) promotes greater potency in both instances. However, replacement with the even more hydrophobic benzilate (**12**), which contains two aromatic rings, increases potency no further.

Relative importance of the ester oxygens. Another acyclic analog of note is tetramethyl-GABA (**31**). This "reverse ester" binds to the transporter with an affinity nearly equal to that of AcCh. Thus, the position (indeed, as discussed below, even the presence) of the alcohol oxygen in AcCh is not important to binding affinity. The carbonyl oxygen, in contrast, is critical. Several compounds (**34**, **44**, and **45**) are aldehydes or ketones and have IC_{50} values 2.6-, 22-, or 350-fold lower than that of AcCh, respectively. Compound **27** is an amide with an IC_{50} value 150-fold lower than that for AcCh. Additionally, the IC_{50} value for choline is about 100-fold higher than that for AcCh (**12**), which again emphasizes the importance of the carbonyl group to binding. However, the distance of the carbonyl group from the ammonium ion is not highly critical, because acetylhomocholine (**29**) and acetyldiethylhomocholine (**30**) exhibit potencies within 2-fold of AcCh itself. The 9-fold lower IC_{50} value obtained by substituting a benzyl (**32**) for a methyl (**31**), as in the GABA derivatives, compared with a 5-fold lower value in the analogous benzylated AcCh derivative (**6** versus **1**) is noteworthy and implies a slightly different positioning of the ammonium ion of the GABA family in the transporter active site.

TABLE 1

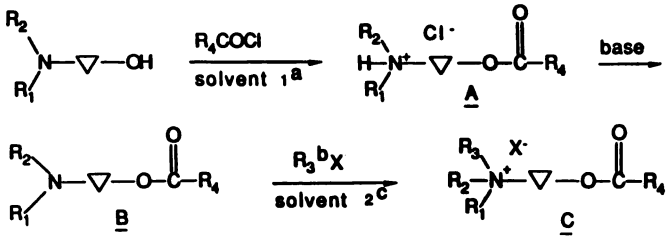
General synthetic method A



Compound	Solvent	R_3X	Yield	Melting point	Characterization
			%		
12	Et_2O	MeI	26	140.5–142°	FABMS
34	Neat	MeI	30	150–151°	FABMS
3	Et_2O	MeI	58	172–173°	FABMS
41	Et_2O/CH_2Cl_2	BzBr	95	207–208°	^{1}H NMR; FABMS
42	Et_2O/CH_2Cl_2	BzBr	74	125–126°	^{1}H NMR; FABMS
43	CH_3CN	BzBr	39	199–200.5°	FABMS
44	Et_2O/CH_2Cl_2	BzBr	45	203–204°	FABMS
45	CH_3CN/C_6H_6	BzBr	16	≥85° (dec)	FABMS

TABLE 2

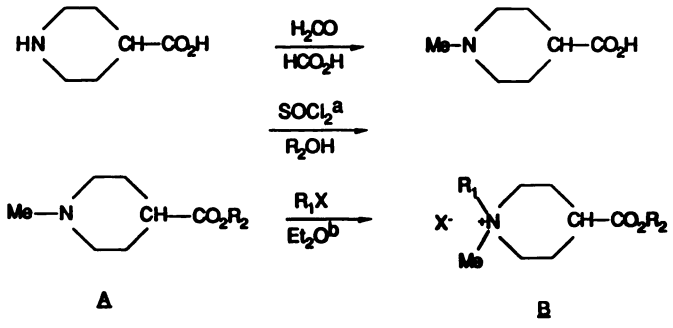
General synthetic method B



Compound	X	Yield[d]	Melting point	Method of purification[e]	Characterization
		%			
3	I	17	84–88°	I	^{1}H NMR (*B*); FABMS (*C*)
4	I	19	120.5–121°	II	^{1}H NMR, FABMS (*C*)
5	I	49	108–110°	I	^{1}H NMR (*B*); FABMS (*C*)
6	Br	32	127.5–128.5°	III	^{1}H NMR (*A*, *C*); FABMS (*C*)
7	Br	[f]	[f]	I	^{1}H NMR (*A*); FABMS (*C*)
8		[f]	184–187°	IV	^{1}H NMR (*A*, *C*); FABMS, IR, UV (*C*)
9	I	30	133°	I	^{1}H NMR (*B*)
10	I	80	243–245°	I	^{1}H NMR (*B*); FABMS (*C*)
11	Br	71	113.5–114.5°	V	^{1}H NMR (*A*); FABMS (*C*)
13	I	41	173–177°	I	^{1}H NMR (*B*); FABMS (*C*)
14	Br	[f]	[g]	I	^{1}H NMR (*B*)
15	I	38	[f]	I	^{1}H NMR (*B*, *C*); FABMS (*C*)
16	Br	13	[f]	III	^{1}H NMR (*B*, *C*); FABMS (*C*)
17	Br	30	[f]	III	^{1}H NMR (*B*, *C*); FABMS (*C*)
18	Br	[f]	[f]	III	^{1}H NMR (*B*, *C*); FABMS (*C*)
19	I	58	169–171°	I	^{1}H NMR (*B*); FABMS (*C*)
20	Br	31	[g]	I	^{1}H NMR (*B*); FABMS (*C*)
29	I	61	93–103°	I	^{1}H NMR (*B*); FABMS (*C*)
30	I	62	110.5–111°	VI	^{1}H NMR (*B*, *C*); FABMS (*C*)
37	Br	87[h]	219–222°	III	^{1}H NMR (*B*); FABMS (*C*)
38	Br	87[h]	204–207°	III	^{1}H NMR (*B*); FABMS (*C*)
40	I	42	163–164°	VII	^{1}H NMR (*B*); FABMS (*C*)

[a] $CHCl_3$ except compounds **18**, **37**, and **38** (CH_2Cl_2).
[b] See R_3 of Table 4 or R_1 of Tables 5 or 6, as appropriate. For compounds **30**, **37**, **38**, and **40**, R_3 = Me, Bz, Bz, or Me, respectively.
[c] CCl_4 (**3–7**, **10**, **11**, **29**, **30**, and **40**); $CCl_4/CHCl_3$ (**8**); $CHCl_3$ (**9**); Et_2O (**13–17**, **19**, and **20**); CH_2Cl_2 (**18**); $Et_2O/CCl_4/CH_2Cl_2$ (**37** and **38**).
[d] Overall yields.
[e] I, Crystallized from solvent 2; II, crystallized from CH_3CN/Et_2O; III, chromatographed on silica gel; IV, crystallized from MeOH/EtOH; V, crystallized from CH_3CN/C_6H_6; VI, crystallized from EtOH/Et_2O; VII, crystallized from CH_3CN.
[f] Not determined.
[g] Product is an *E*,*Z* mixture.
[h] Before separation into *E*- and *Z*-isomers.

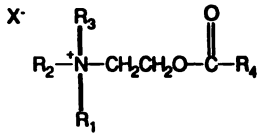
TABLE 3
General synthetic method C



Compound	X	Yield[c]	Melting point	Method of purification[d]	Characterization
21	I	37	151–152°	I	^{1}H NMR (*A*); FABMS (*B*)
22	Br	29	[e]	I	^{1}H NMR (*A*); FABMS (*B*)
23	Br	15	195–197°	II	^{1}H NMR; FABMS (*B*)
24	Br	9	[f]	II	^{1}H NMR; FABMS (*B*)
25	Br	42	[e]	III	^{1}H NMR (*B*)
26	Br	40	[e]	III	^{1}H NMR (*A*, *B*); FABMS (*B*)

[a] Dicyclohexylcarbodiimide was used for compound **26**.
[b] Except compounds **25** (CH_2Cl_2) and **26** (Et_2O/CH_2Cl_2).
[c] Overall yield.
[d] Crystallized from the reaction solvent; II, chromatographed on silica gel; III, crystallized from EtOH/Et_2O.
[e] Mixture of *E*,*Z*-isomers.
[f] Isolated as a glass.

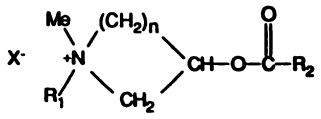
TABLE 4
Derivatives of ethanolamine

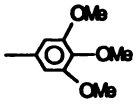


Compound	R_1	R_2	R_3	R_4	IC_{50}
					μM
1	Me	Me	Me	Me	290 ± 20
2	Me	Me	H	Me	1200 ± 500
3	Me	Me	Et	Me	550 ± 100
4	Et	Et	Et	Me	310 ± 10
5	[C_5H_{10}]		Me	Me	270 ± 20
6	Me	Me	Bz	Me	60 ± 10
7	Me	Me	CH_2—C6H4—CH_2Br	Me	150 ± 30
8	Me	Me	CH_2—C6H4—CO_2^-	Me	1700 ± 700
9	Me	Me	Me	Et	150 ± 50
10	Me	Me	Me	Ph	54 ± 18
11	Me	Me	Bz	Et	63 ± 9
12	Et	Et	Me	$C(Ph)_2OH$	54 ± 9

Derivatives of 3- and 4-hydroxycycloamines. Table 5 is composed of six derivatives of 3-pyrrolidinol and two derivatives of 3-piperidinol. Going from the simplest member of this family of heterocycles (**13**), which has a potency essentially equal to that of AcCh, to the *N*-benzyl derivative (**14**) shows the same 9-fold lowering of the IC_{50} value as seen in the GABA derivatives. Likewise, replacement of the acetyl of compound **13** with benzoyl (**15**) increases potency by 8-fold. Somewhat to our surprise, the combination of these two aromatic-for-alkyl changes was fully additive and produced a compound (the

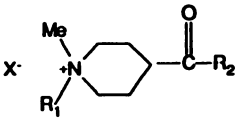
TABLE 5
Derivatives of 3-hydroxycycloamines



Compound	Isomer	R_1	R_2	*n*	IC_{50}
					μM
13	(±)	Me	Me	2	270 ± 40
14	(±)-*E*,*Z*	Bz	Me	2	30 ± 10
15	(±)	Me	Ph	2	33 ± 3
16[a]	(±)-*Z*	Bz	Ph	2	2.5 ± 0.4
17[a]	(±)-*E*	Bz	Ph	2	4.3 ± 0.8
18[a]	(±)-*Z*	Bz		2	3.7 ± 0.6
19	(±)	Me	Me	3	4700 ± 500
20	(±)-*E*,*Z*	Bz	Me	3	1600 ± 900

[a] The assignment of structures of geometrical isomers was made using ^{1}H NMR based on the phenomenon of deshielding by steric compression (Ref. 22 and references therein).

TABLE 6
Derivatives of isonipecotic acid



Compound	Isomer	R_1	R_2[a]	IC_{50}
				μM
21		Me	OMe	140 ± 15
22	*E*,*Z*	Bz	OMe	11 ± 2
23[b]	*Z*	Bz	OBz	1.7 ± 0.4
24[b]	*E*	Bz	OBz	1.6 ± 0.2
25	*E*,*Z*	Bz	OCH_2—C6H5	0.58 ± 0.16
26	*E*,*Z*	Bz	OCH_2—C6H5	0.63 ± 0.03
27	*E*,*Z*	Bz	NH—C6H3(F)—NO_2	2.0 ± 0.7

[a] The R_2 notation includes the heteroatom bonded to the carbonyl, whereas it does not in Table 3.
[b] The assignment of structures of geometrical isomers was made using ^{1}H NMR based on the phenomenon of deshielding by steric compression (Ref. 22 and references therein).

Z-isomer, **16**) which is over 100-fold more potent than the parent (**13**) or AcCh. Also of interest is the finding that the *E*/*Z* pair (**16** and **17**) are within a factor of two of each other in potency. In two other *E*/*Z* pairs (**23** and **24** as well as **37** and **38**) where the isomers were separated and tested individually, this near identity of potencies was observed also. A tentative conclusion from these observations is that there are two hydrophobic sites in the AcCh transporter near the ammonium ion binding site. Perhaps a compound bearing two aromatic or other hydrophobic groups on the nitrogen could take advantage of binding to both sites to increase potency even further. We have used the approximate equipotency of these three pairs of *E*/*Z*-isomers as justification for quantitating the potencies of

TABLE 7
Other analogs

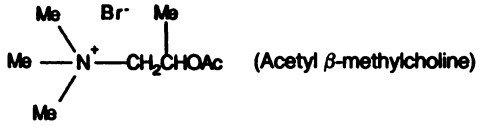
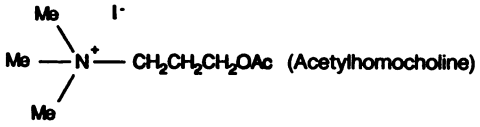
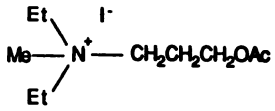
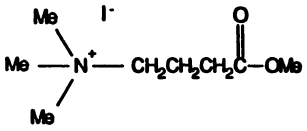
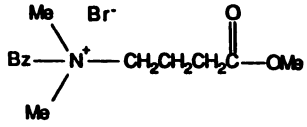
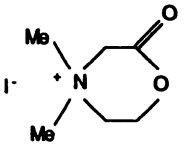
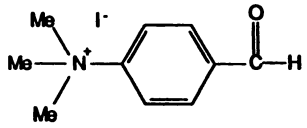
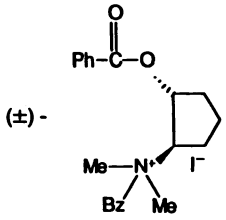
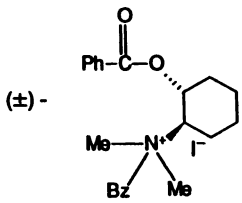
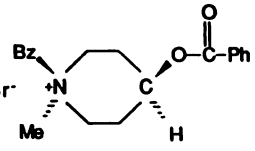
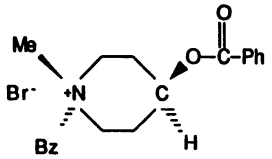
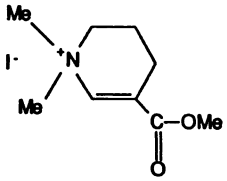
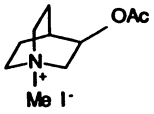
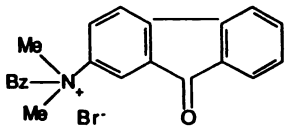
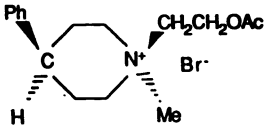
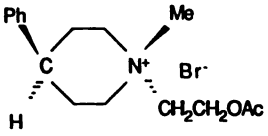
Compound	Structure (Name)	IC_{50}
		μM
28	(±) -  (Acetyl β-methylcholine)	750 ± 150
29	 (Acetylhomocholine)	400 ± 100
30		600 ± 200
31		410 ± 108
32		45 ± 14
33		7000 ± 1400
34		110 ± 3000
35	(±) - 	4.7 ± 1.5
36	(±) - 	6.4 ± 2.4
37	(Z) - Br⁻ 	4.5 ± 0.6

TABLE 7-Continued

Compound	Structure (Name)	IC_{50}
38	(*E*) - 	3.9 ± 0.8
39		1700 ± 700
40	(±)- 	400 ± 300
41	(exo)-*N*-Benzylatropine bromide	1.9 ± 0.5
42	(exo)-(−)-*N*-Benzylscopolamine bromide	9 ± 3
43	*N*-Benzylsecurinine bromide	8.5 ± 3
44	(±)-*N*-Benzylmethadone bromide	13 ± 4
45		0.83 ± 0.11
46[a]	(*Z*) - 	5.5 ± 2.0
47[a]	(*E*) - 	3.8 ± 0.5

[a] The assignment of structures of geometrical isomers was made using ^1H NMR based on the phenomenon of deshielding of steric compression (Ref. 22 and references therein).

TABLE 8

Pharmacological comparisons of AcCh analogs

Ratios for AcCh transporter values are derived from IC_{50} values from Tables 4 and 7. All others are from Ref. 6. AcChT, AcCh transporter; ChAT, choline acetyltransferase; AcChE, AcCh esterase; mAcChR, muscarinic AcCh receptor; nAcChR, nicotinic AcCh receptor.

Compound	Equipotent molar ratio (AcCh = 1)				
	AcChT	ChAT[a]	AcChE[b]	mAcChR	nAcChR
Acetylhomocholine (29)	1.3	–[c]	0.04	40–50	5–8
Acetylmonoethylcholine (3)	1.8	2–7	1.0	2–3	5
Acetyltriethylcholine (4)	1.0	150–170	0.94	1700–2000	5000

[a] Ratios of K_m values for acetylation of the respective alchols.
[b] These values are ratios of rates rather than molar ratios.
[c] Not observed in vitro (Ref. 6 and references cited therein).

some compounds without separating them into their individual components (**14**, **22**, **25**, **26** and **27**).

Earlier studies on the high affinity choline uptake system revealed that binding of some esters could be greatly enhanced by use of 3,4,5-trimethoxybenzoyl group (13). In order to examine this possibility in the AcCh transport system, compound **18** was synthesized. Compared with the relevant control (**16**), it shows no enhancement of binding due to the methoxy substituents.

The remaining two derivatives in Table 5 are the 3-piperi-

dinol derivatives (**19** and **20**). Although quite low in potency (**19** is a 16-fold lower than AcCh), these derivatives are significant because they likely define a conformation of AcCh that does not closely fit the active site of the transporter. If one contrasts compound **40** (it also contains the 3-piperidinol structure) with compound **19**, then it is obvious that the steric bulk of the extra methylene group (compared with **13**) is not the reason for the lowered binding affinity. Rather, the reason must reside in the conformational differences between 5- and 6-membered rings. Another 3-substituted piperidinyl compound (**39**, *N*-methylarecoline) also is a low potency drug. In contrast, the two derivatives of 4-piperidinol (**37** and **38**) exhibit reasonably good potencies, about as good as the similarly substituted derivatives of 3-pyrrolidinol (**16** and **17**).

Derivatives of isonipecotate. Table 6 contains seven derivatives of isonipecotic acid. Benzyl for methyl substitution on the nitrogen (compare **21** with **22**) provided a nearly 13-fold increase in potency, compared with 5- to 9-fold in the previous examples. Encouraged by this greater effect, we prepared *N*-benzyl derivatives with tree different hydrophobic alcohols replacing the methyl ester of compound **22** (**23**, **24**, **25**, and **26**). These four derivatives are among the five most potent compounds presented here. The cyclopentylmethyl ester is at least 500-fold more potent than AcCh and is our most potent derivative. The good potency of amide **27** should be contrasted to the very low potency previously observed for carbachol (12).

Other analogs. Other quaternary derivatives of heterocyclic drugs with known cholinergic effects are found in Table 7. *N*-Benzylatropine (**41**) binds to the transporter with 150-fold greater apparent affinity than does AcCh. The epoxide derivative, *N*-benzylscopolamine (**42**), also was prepared and found to bind nearly 5 times more weakly than the benzylated atropine derivative. Because the epoxide potentially could react with a binding site nucleophile, AcCh transport partially inhibited by *N*-benzylscopolamine was assayed over a 2-hr period, in order to assess whether there was progressive inhibition with time, but none was observed. Thus, there is no evidence for covalent labeling of the AcCh transporter by this agent. *N*-Benzylsecurinine is interesting because it has reasonable potency (over 30-fold greater than AcCh) and is a lactone. As discussed below, it is much more potent than anticipated.

Competitive versus noncompetitive inhibition. Increasing the hydrophobicity of the AcCh analogs has a generally favorable effect on the potency of transport inhibition. However, it is possible that inhibition is the result of occupancy of a site different from the AcCh binding site in the transport system. To assess this possibility, we examined the competitive or noncompetitive nature of the inhibition of [^{3}H]AcCh transport by our most potent isomerically pure compound (**23**). Not only was it isolable in pure *E* and *Z* forms, but the compound also is achiral, meaning that truly only one structure was present. In Fig. 1 are the results of that test, displayed in a double-reciprocal format. Initial fits of hyperbolic equations to the separate data files obtained at the indicated inhibitor concentrations resulted in V_{max} values that were within errors of each other. Because this result is consistent with a competitive interaction, the constrained linear competitive model was used to evaluate the K_m value for AcCh (300 ± 30 μM) and the K_i value for the drug (350 ± 50 nM) by simultaneous nonlinear regression analysis. The K_i is lower than the IC_{50} value because

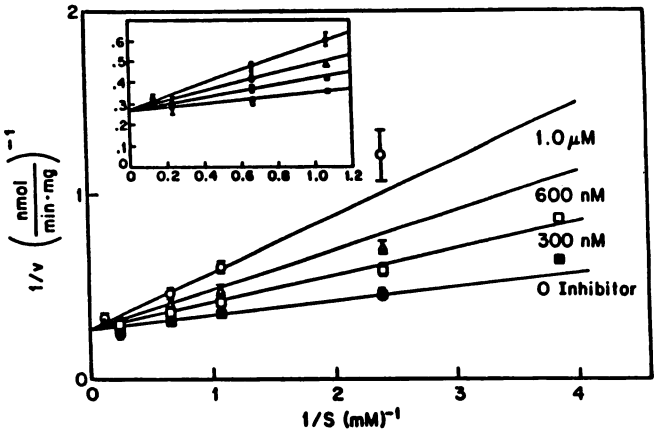


Fig. 1. Inhibition of [^{3}H]AcCh active transport by compound **23**. Data are presented as the reciprocal [^{3}H]AcCh transport velocity versus reciprocal [^{3}H]AcCh concentration at the changing fixed concentrations of compound **23** and are the mean values of triplicate observations. *Error bars* are 1 SE. Inhibitor concentrations were 0 nM (■), 300 nM (□), 600 nM (△), and 1.0 μM (○). Vesicle concentration was 0.20 mg of protein/ml. A constrained linear competitive model was used to evaluate the K_m value for AcCh (300 ± 30 μM), the V_{max} value (3.69 ± 0.08 nmol min^{-1} mg^{-1}), and the K_i value for compound **23** (350 ± 50 nM).

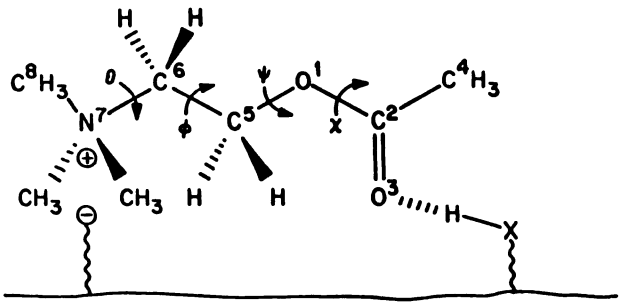


Fig. 2. Probable conformation of AcCh bound by the transporter active site, as deduced from structure-activity relationships.

of the competition present in the IC_{50} measurement and perhaps because of variability in the properties of different preparations of the vesicles (14, 15). From this analysis, we conclude that the isonipecotate derivatives, and likely our other compounds, are binding to the AcCh binding site in the transporter.

Discussion

The structure-activity data can be summarized as follows. Quaternization of the nitrogen and the presence of the carbonyl are important to potency. The addition of hydrophobic groups to both ends of the AcCh structure enhances the ability of the molecule to inhibit uptake of [^{3}H]AcCh by synaptic vesicles. Additionally, the conformation of isonipecotate derivatives allows better contacts to be made between the hydrophobic groups and their binding sites.

Comparing molecular models of the various conformationally constrained molecules that have apparent binding affinities equal to or greater than that of AcCh allows a relatively unambiguous determination of the AcCh conformation complementing the transporter active site. The hypothesized bound conformation is depicted in Fig. 2. The dihedral angles defined by C^8-N^7-C^6-C^5 (θ), N^7-C^6-C^5-O^1 (ϕ), and C^6-C^5-O^1-C^2 (ψ) are 180°, whereas that defined by C^5-O^1-C^2-O^3 (χ) is 0°. This conformation corresponds to the global energy minimum as

determined by an *ab initio* procedure (16). The carbonyl and nitrogen of the isonipecotate derivatives can be aligned with the illustrated AcCh model, with excellent correspondence. The other analogs with some conformational restraint imposed by various ring systems (such as the 3-pyrrolidinols, *N*-benzylatropine, and compounds **35** and **36**) all give reasonably good fits to the proposed conformation (*N*-benzylsecurinine is an exception, perhaps indicating that it binds at a different site). The weakly inhibitory 3-piperidinol derivatives (**19** and **20**) definitely do not produce a good fit. In order to more critically test this conformational hypothesis, we synthesized a rigid structure (**45**) based upon 2-amino-9-fluorenone, which would provide the ammonium ion and carbonyl contacts at the distance required in Fig. 2. As predicted, the compound is a potent inhibitor of AcCh active transport, exhibiting a 350-fold greater affinity than AcCh (Table 7). The result strongly supports the claim that AcCh is bound in a confirmation closely resembling the one shown.

These structure-activity results can be compared with the physiological behavior of choline analogs observed in intact or semi-intact nerve terminal preparations (1–6). Such a comparison is valid because no differences in the utilization of false transmitters by different species have been found, which suggests that the AcCh transporter is highly conserved. More detailed information, of course, might result in discovery of specificity differences among species or even possibly among the different metabolic forms of synaptic vesicles found within a single terminal (1). Acetylhomocholine (**29**), acetylmonoethylcholine (**3**), and acetyltriethylcholine (**4**) are formed and serve as false transmitters in tissues like *Torpedo* electric organ, mammalian brain, superior cervical ganglion, myenteric plexus, and neuromuscular junction (1–6). This is consistent with the observation that these analogs are bound to the AcCh transporter with affinities similar to that of AcCh, and this suggests strongly that they are actively transported. In agreement with the weak binding observed here, acetyl 3-hydroxy-*N*,*N*-dimethylpiperidinium (**19**) is a relatively poor false transmitter in brain and phrenic nerve (17). The analogs of AcCh substituting antimony or sulfur for the nitrogen, an amino group for one of the *N*-methyl groups, or a carbamyl group for the acetyl also serve as false transmitters, but no analogs incorporating these chemical features were tested here.

The structure-activity profile for the AcCh transporter is markedly different from those of muscarinic and nicotinic receptors and AcCh esterase (6). Thus, compared with AcCh, acetylhomocholine (**29**), acetylmonoethylcholine (**3**), and acetyltriethylcholine (**4**) are 40–50-, 2–3-, and 1700–2000-fold less potent as muscarinic ligands, and 5–8-, 5-, and 5000-fold less potent as nicotinic ligands, respectively (Table 8). Acetylhomocholine (**29**) is a much poorer substrate for the esterase than AcCh (25-fold). The close similarity of the three analogs as false transmitters suggests that the AcCh transporter is less discriminating than the other AcCh-binding proteins. Perhaps this is because the transporter is intracellular, where the number of different types of molecules presented to it is restricted by the nerve terminal metabolism, whereas the extracellular receptors and esterase must confront and distinguish among a greater diversity of structures. Where specificity is not required, it likely will not evolve.

The only well-documented example of an abortive false transmitter failing at the synaptic vesicle storage step in intact preparations is that of acetyldiethylhomocholine (**30**) (18, 19). This compound was only slightly less potent than AcCh in the current study. Overall, the physiological data suggest that analogs incorporating a substantial increase in the size of the substituents on the quaternary nitrogen *or* an increased distance between the acetate and the quaternary nitrogen are transported successfully by synaptic vesicles *in vivo*. However, simultaneous changes in these regions of the analog, as occurs in acetyldiethylhomocholine, are not accepted by the transport mechanism. As demonstrated here, such compounds still can bind to the transporter active site and inhibit AcCh transport. Thus, we would predict that, when radiolabeled versions of the new analogs reported here are available and tests of transport can be carried out, a compound like **21** might be transported even though it is a reverse ester. Because little information about the transport of analogs containing altered acyl groups, like **9** and **10**, is available from previous false transmitter work, it will be of unique interest to test this feature in an *in vitro* transport assay. Conflicting evidence exists regarding **9**, with one worker suggesting that it is synthesized and utilized by *Torpedo* electric organ (20) and another group disputing this claim (21).

These structure-activity results also can be compared with those obtained for vesamicol [(−)-(*trans*)-2-(4-phenylpiperidino)cyclohexanol, also known as AH5183], which is a noncompetitive inhibitor of the AcCh active transport system (15). The relationship elucidated in the current study is opposite to the behavior of the vesamicol family (8), where quaternization of the tertiary nitrogen or esterification or removal of the alcohol group greatly decreases the binding affinity. Compounds **46** and **47** are hybrid analogs of AcCh and vesamicol, but they probably belong in the AcCh family for this reason. Even though a β-amino alcohol core is common to both AcCh and vesamicol, the structure-activity relationships for them support the conclusion that AcCh and vesamicol bind to different loci in the synaptic vesicle membrane.

Acknowledgments

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