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Inhibition of synaptosomal uptake of choline by various choline analogs

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Choline appears to be transported into cholinergic neurons by a high affinity mechanism [1–3] that is selectively localized to cholinergic nerve terminals [4]. Recent results indicate that this uptake mechanism plays a rate-limiting role in sustaining and regulating the synthesis of acetylcholine [5–7]. Thus, the ability to utilize and manipulate this uptake mechanism provides investigators with a very powerful tool for studying various aspects of acetylcholine synthesis, storage and release. To further a fundamental understanding of this uptake mechanism, we have examined the inhibition of synaptosomal choline uptake by choline analogs and some other compounds known to affect cholinergic systems.

dl-α-Methylcholine, *d*-β-methylcholine, 3-trimethylamino-propan-1-ol (homocholine) and *N*-hydroxyethyl pyrrolidinium (pyrrolidine choline) were prepared as iodide salts by reacting the corresponding tertiary amino alcohols with methyl iodide.

The reactions took place at room temperature overnight in the dark. The compounds were purified by recrystallization from acetone–methanol–ether mixtures. Homogeneity was ascertained by chromatography (*n*-butanol–methanol–acetic acid–water, 8:2:1:3) or electrophoresis at 1000 volts for 30 min (1·5 M acetic acid–0·75 M formic acid) on cellulose thin-layer plates (Chromagrams, Eastman Chemicals, Rochester, N.Y., lot Nos. 5102 and 5133).

We are grateful to Dr. C. Y. Chiou for his generous gift of diethylcholine, and to Dr. Darwin Cheney for his generous gift of 2-hydroxy-1,1,2,2-d_4-ethyl-trimethylammonium (D_4 choline) and 2-hydroxyethyl, d_9-trimethyl ammonium (D_9 choline). Triethylcholine, diethylethanolamine, *N*-methylethanolamine, hemicholinium-3, hemicholinium-15 and oxotremorine were purchased from Aldrich. Choline, 2-aminoethanol, carbamylcholine and phosphorylcholine were from Sigma, and *N*-hydroxyethyl-4-(1-naphthylvinyl) pyridinium (NVP), *d*-tubocurarine and physostigmine were purchased from Calbiochem. Acetylcholine was obtained from Calbiochem, Sigma and Aldrich, and thiocholine was supplied by Polysciences and K & K Labs. Tetramethylammonium and tetraethylammonium were from Baker and *t*-butylethanolamine was from K & K Labs. Other drugs were obtained as follows: BW-284C51 from Wellcome Research Labs; chlorpromazine from Smith Kline & French; haloperidol from McNeil; and neostigmine from Hoffman La-Roche. At least two IC_{50} determinations were made with each compound from each source.

Choline uptake was performed as previously described [4] with some modifications. These procedures are stated briefly as follows. Sprague–Dawley male rats (180–220 g) were killed by decapitation and the brains were quickly removed to a dish of chilled saline. The striatum was

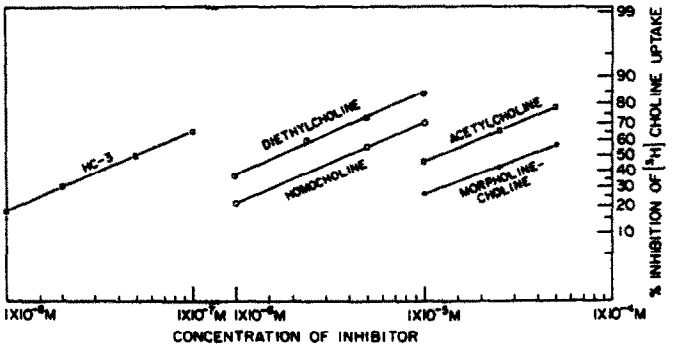
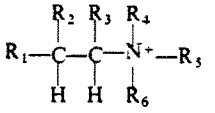


Fig. 1. Log-probit plot of some representative inhibitors of [^{3}H]choline uptake. Crude synaptosomal fractions were incubated for 4 min in the presence of 1 μM [^{3}H]choline and various concentrations of inhibitor. Results are the mean for at least two experiments which differed by less than 10 per cent and are expressed as per cent inhibition of uptake on a probability scale.

Table 1. Inhibitory potency of choline and three groups of choline analogs*



Choline analogs

Group I: $R_1 = OH$; $R_2 = R_3 = H$

	R_4	R_5	R_6	IC_{50} (μM)
Choline	CH_3	CH_3	CH_3	1·6
Diethylcholine	CH_3	C_2H_5	C_2H_5	1·6
Pyrrolidine choline	CH_3			3·7
Triethylcholine	C_2H_5	C_2H_5	C_2H_5	3·8
Morpholine choline	CH_3			37
Diethylethanolamine	C_2H_5	C_2H_5		75
2-Aminoethanol	H	H		>1000
N-methylethanolamine	CH_3	H		>1000
t-Butylethanolamine	$C(CH_3)_3$	H		>1000

Group II: $R_2 = R_3 = H$; $R_4 = R_5 = R_6 = CH_3$

	R_1	IC_{50} (μM)
Acetylcholine	O_2CCH_3	12
Thiocholine	SH	75
Carbamylcholine	O_2CNH_2	350
Phosphorylcholine	OPO_3H_2	>1000

Group III: $R_4 = R_5 = R_6 = CH_3$

	R_1	R_2	R_3	IC_{50} (μM)
Homocholine	CH_2OH	H	H	4·0
dl-α-Methylcholine	OH	H	CH_3	18
d-β-Methylcholine	OH	CH_3	H	240

* See Methods for experimental details. The IC_{50} values represent the concentration of inhibitor required to produce a 50 per cent inhibition of [^{3}H]choline uptake and were determined graphically as shown in Fig. 1. In experiments with acetylcholine, paraoxon (20 μM), which by itself had no effect on uptake, was included in the incubation media. Results are means of at least two experiments which differed by less than 10 per cent.

dissected and homogenized in 20 vol. of ice-cold 0·32 M sucrose in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 1000 *g* for 10 min to remove nuclei and large cellular debris. The resulting supernatant fluid was thoroughly stirred and aliquots were utilized for the uptake procedures. One hundred μl of the supernatant fluid was added to 0·9 ml of Krebs–Ringer buffer (pH 7·4) in Corex centrifuge tubes, containing 1 μM concentrations of tritiated choline (final sp. act., 0·25 Ci/mmole). After thorough mixing, the tubes were transferred to an incubation bath at 30°, and blank values were obtained by maintaining parallel duplicate samples at 0°. At the end of the 4-min incubation period, the tubes were transferred to an ice-water bath and centrifuged at 5000 *g* for 15 min to collect the particulate matter in a pellet. After discarding the supernatant fluid, the pellets were surface-washed with 2 ml of ice-cold 0·9% saline, dissolved with Protosol (New England Nuclear Corp.) and counted in a Packard scintillation spectrometer. Under these conditions, choline uptake was linear with time and tissue concentration [2, 4].

For uptake inhibition experiments, various drugs were added in various concentrations to the Krebs–Ringer media immediately prior to the addition of tissue. The IC_{50} values are expressed as the molar concentration of the drug that inhibited 50 per cent of the tritiated choline uptake as determined graphically from a logarithmic/probability plot. The plots of some potent uptake inhibitors are shown in Fig. 1.

Choline analogs were divided into groups I, II and III, those with alterations of the nitrogen atom, of the hydroxyl group, and of the carbon chain respectively (Table 1). In order to obtain a valid comparison of the various analogs with choline, we examined the inhibitory potency of non-radioactive choline on the uptake of [^{3}H]choline. The necessity of this experiment becomes apparent when one realizes that the amount of choline present in the media from endogenous tissue sources may be significant compared to the amount of added, exogenous radiolabeled choline. While the process that generates the endogenous choline is not understood, it is known that in brain tissue there occurs a rapid post-mortem increase in choline [8]. We found an IC_{50} value of 1·6 μM for choline and compared other results to this value. Of the eight analogs tested in group I, diethylcholine appeared to be the best inhibitor with an IC_{50} value of 1·6 μM. Pyrrolidine choline and triethylcholine, also good inhibitors, were approximately half as potent as diethylcholine. The remaining compounds were much less potent inhibitors. These included morpholine choline as well as those which were

Table 2. Inhibitory potency of other compounds known to affect choline uptake*

Compound†	IC_{50} (μM)
Hemicholinium-3	0·05
BW-284 C51	0·24
D_4 choline	1·9
D_9 choline	2·2
Hemicholinium-15	15
Tetramethylammonium	18
NVP	20
Chlorpromazine	22
Tetraethylammonium	37
Haloperidol	60
d-Tubocurarine	70
Neostigmine	100
Physostigmine	>100
Oxotremorine	>100
QNB	>100

* See Methods for experimental detail. The IC_{50} values represent the concentration of inhibitor required to produce a 50 per cent inhibition of [^{3}H]choline uptake, and were determined graphically as shown in Fig. 1. Results are means of at least two experiments which differed by less than 10 per cent.

† NVP = *N*-hydroxyethyl-4-(1-naphthylvinyl)pyridinium; QNB = quinuclidynil benzilate.

not quaternary nitrogen derivatives: diethylethanolamine, 2-aminoethanol, *N*-methylethanolamine and *t*-butylethanolamine.

The compounds in group II, those with alterations on the hydroxl group, were generally poor inhibitors. Acetylcholine was the best inhibitor in this group, while thiocholine, carbamylcholine and phosphorylcholine showed IC_{50} values of 75, 350 and greater than 1000 μM respectively.

The compounds in group III showed a wide range of inhibitory potency. Homocholine had the lowest IC_{50} value in this group, exhibiting an inhibitory potency approximately 60 times greater than that observed for *d*-β-methylcholine. *dl*-α-Methylcholine displayed an intermediate level of potency.

We examined the kinetics of inhibition of choline uptake by triethylcholine and thiocholine. Lineweaver–Burk analysis revealed competitive inhibition as expected (data not shown). Since all of the log-probit lines from experiments with compounds in Table 1 were parallel and since the compounds are structurally alike, similar competitive kinetics would be expected of all of the compounds.

We examined a group of other compounds known to affect cholinergic systems and again found a very wide range of inhibitory potency (Table 2). Hemicholinium-3 was an extremely potent inhibitor of choline uptake, while physostigime, oxotremorine and quinuclidinyl benzilate (QNB) were the least potent. After hemicholiuium-3, the most potent inhibitor was BW-284 C51. This compound, a cholinesterase inhibitor, while displaying a high level of inhibitory potency, was only one-fifth as potent as hemicholinium-3. D_4 choline and D_9 choline, deuterated analogs of choline, exhibited inhibitory potencies similar to that of choline itself. Hemicholinium-15, tetramethylammonium, *N*-hydroxyethyl-4-(1-naphthylvinyl)pyridinium (NVP), chlorpromazine and tetraethylammonium exhibited intermediate IC_{50} values of between 15 and 37 μM.

In the first group of uptake inhibitors listed in Table I, diethylcholine and triethylcholine were very potent inhibitors, indicating that substitution of methyl groups with ethyl groups does not greatly impair inhibitory potency. Similar results have been obtained by other investigators [6, 9, 10]. However, complete removal of methyl groups results in a very large loss of inhibitory potency, as evidenced by the high IC_{50} values of the last four compounds in this group. Of the two compounds which had ring substituents on the nitrogen, pyrrolidine choline was 10 times more potent than morpholine choline. This difference may be due to differences in the size of the ring and resulting conformational differences, or may arise from differences in polarity and lipid solubility of the ring substituents.

None of the compounds in group II (alterations on the hydroxyl group) was a highly potent inhibitor of choline uptake. Acetylcholine showed the lowest IC_{50} value, while carbamylcholine was much less potent. This difference may be due to the charged amide nitrogen on carbamylcholine. Thiocholine was a surprisingly poor inhibitor, displaying an IC_{50} value of 75 μM. Since the mercapto group of thiocholine is more acidic, a fraction of the thiocholine molecules will be ionized. This may account, in part, for its poor inhibitory potency. It seems physiologically significant that choline analogs with alterations of the hydroxyl group have a much reduced affinity for the transport system. Recent experiments indicate that acetylcholine, the best inhibitor in this group, is not selectively transported by the choline uptake mechanism [11]. It is tempting to speculate that a portion of the choline uptake mechanism evolved in such a way as to become highly discriminatory for free hydroxyl groups; one could imagine that this discrimination has functional importance in that the uptake system at the synaptic cleft carefully excludes acetylcholine but recycles choline. This notion fits in nicely with the recent evidence that choline uptake is coupled to choline acetylation in nerve terminals [5–7].

In group III, the analogs with the lengthened carbon chain, homocholine was the best inhibitor of uptake, displaying an IC_{50} value of 4 μM. Substituting a methylene hydrogen with a methyl group resulted in a loss of potency, especially in the case of *d*-β-methylcholine. *dl*-α-Methylcholine appeared to be about 4·5 times less effective than homocholine, but since we utilized the racemic mixture of α-methylcholine, it is possible that one of the isomers would display a greater inhibitory potency.

From these data we can summarize some features characterizing analogs that would be potent choline uptake inhibitors: (1) with regard to the substituents on the nitrogen atom, methyl groups are not essential, but quaternary nitrogen compounds appear to be very effective; (2) a free hydroxyl group appears to be necessary; and (3) it may be possible to alter the carbon chain slightly, either by lengthening it or perhaps by substituting a methylene hydrogen.

Among the other compounds tested, hemicholinium-3 was the most potent, and this great potency has been observed by others [1, 2, 5–7]. It was, in fact, 30 times more potent than the most potent choline analog tested in Table 1, diethylcholine. Surprisingly, hemicholinium-15, which is one-half of the symmetrical molecule of hemicholinium-3, was only 1/300 as potent as hemicholinium-3. There is no obvious reason for this difference or for the great potency of hemicholinium-3. Of the acetylcholinesterase inhibitors that were tested, BW-284 C51 was more potent than any of the choline analogs tested, while neostigmine and physostigmine were weak inhibitors. The two receptor blockers tested, *d*-tubocurarine (nicotinic) and quinuclidinyl benzilate (muscarinic), were poor inhibitors; *d*-tubocurarine exhibited an IC_{50} value of 70 μM and was the more potent. NVP, a cholineacetyltransferase inhibitor, was also found to be a reasonably potent uptake inhibitor, not much weaker than hemicholinium-15. D_4 choline and D_9 choline were very potent inhibitors, but seemed to be consistently less potent than choline itself, suggesting that increasing deuterium content may result in a slight "isotope" effect.

A surprising finding was that tetramethylammonium and tetraethylammonium were fairly potent inhibitors, consi-

dering that they are quaternary nitrogen compounds with no alcohol-containing side chains at all. This finding indicates that quaternary ammonium compounds have an affinity for the uptake mechanism, and supports our suggestion above that a quaternary ammonium recognition site is a major feature of the transport system. Chlorpromazine and haloperidol, drugs that are known to block dopamine receptors and to have effects on cholinergic systems *in vivo* [12], showed IC_{50} values of 22 and 60 μM respectively. Thus it appears that a relatively wide variety of drugs and compounds can affect choline uptake. While this does not detract from the usefulness of the three guidelines presented above, it implicates uptake inhibition as some part of the mechanism of action of these various drugs.

While these studies point out some potent uptake inhibitors, we cannot establish, on the basis of these data alone, whether they are transported by the choline system or acetylated. However, acetylation of these compounds by choline acetyltransferase can be examined directly [6, 7], and there are two types of experiments that one can perform to test whether a compound is transported. One approach is to study the kinetics of transport; the similarity of the transport constant (K_t) of a compound and its inhibition constant (K_i) for choline transport indicates that the same transport system may be involved. Another approach is to show a reduction of uptake of the compound in question in synaptosomal preparations after cholinergic nerve terminals have degenerated [4]. By utilizing these procedures, it has recently been shown that monoethylcholine and pyrrolidine choline are transported by this system [6, 7], but acetylcholine is not [11]. Additional studies have shown that monoethylcholine, diethylcholine, triethylcholine and pyrrolidine choline (potent uptake inhibitors) are transported, acetylated, and in some cases form false neurotransmitters [6, 7, 9, 10]. Thus, the data presented here provide one with a framework for designing compounds which may be potent inhibitors of choline uptake, or compounds that would be transported by cholinergic nerve terminals and perhaps form false neurotransmitters.

A preliminary report of this study has been presented [13].

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Effect of androgenic steroid nitrates on rat liver lysosomes *in vitro*

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It is generally accepted that anti-inflammatory steroids stabilize the lysosomal membrane. This effect might be one of the mechanisms of their anti-inflammatory action as shown by studies *in vitro* of Weissman *et al.* [1–3] and by experiments *in vivo* [4, 5]. However, Tanaka and Iizuka [6] showed that the purified "light" lysosomal fraction, unlike the crude lysosomal fraction, is not stabilized by anti-inflammatory drugs. Malbica [7] and Malbica and Hart [8] working with a purified lysosomal fraction at pH 7·4 could not find any stabilizing effect of hydrocortisone and cloroquine.

In previous experiments we found that some androgenic steroid nitrates (4-chloro-testosterone nitrate and androstanolone nitrate) have anti-inflammatory activity against exudative experimental inflammation (formaline, kaolin and carrageenin edema and cotton pellet inflammation), whereas another compound from the same class, testosterone nitrate, has pro-inflammatory activity. Thus, it seems worthwhile to study their effect on lysosomes, attempting to find a correlation with their action on the experimental inflammation.

The effect of incubating the particulate fraction containing mitochondria and lysosomes (ML) isolated from rat liver [9] with steroid nitrates, synthesized according to published procedures [10] and with one of the parent compounds, androstanolone, for 90 min at 37° and pH