In summary, a method for selective measurement of [^{3}H]-DA uptake by the DA neuronal uptake mechanism in synaptosomal preparations of brain areas of mixed monoaminergic terminals was developed. Cortical, but not striatal or olfactory tubercle, synaptosomes showed substantial uptake of [^{3}H]-DA by transport mechanisms sensitive to 5-HT and NE re-uptake blockers. In the presence of DMI and fluoxetine, both amfonelic acid and AMPH isomers inhibited residual DA uptake in all areas. D-AMPH was about 5-fold more potent than L-AMPH in all three DA terminal areas. It now appears that D-AMPH is more potent than L-AMPH in (a) blocking DA re-uptake in terminals of VTA DA neurons, as well as (b) decreasing VTA DA impulse flow and (c) releasing dendritic DA in the VTA.

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Inhibition of high affinity choline transport by stereoisomers of some 3-quinuclidinol derivatives

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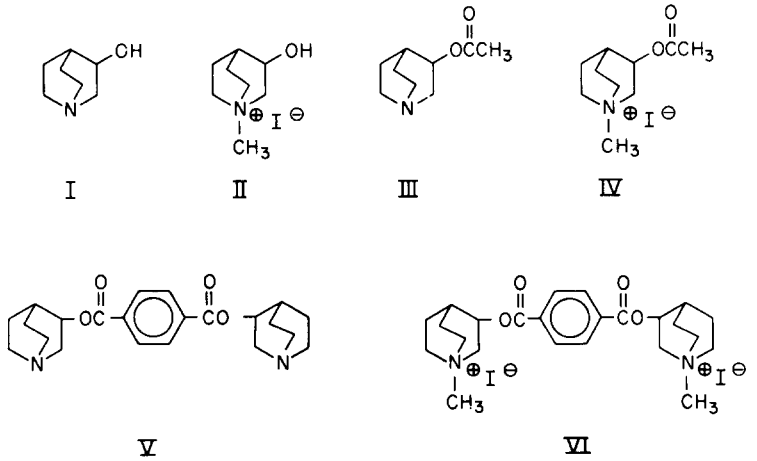
The transport of choline (Ch) into cholinergic nerve endings is mediated by a specific, high affinity ($K_T \sim 10^{-6}$ M), Na^+-dependent, hemicholinium-3 (HC-3) sensitive carrier system [1]. The structural demands on substrates for this high affinity Ch transport (HAChT) system are very strict [2]. Inhibitors, however, include both relatively simple analogues of Ch and more complex molecules most of which contain hydrophobic moieties [2, 3]. We have examined the inhibitory effects on HAChT in rat brain synaptosomes of stereochemical isomers of some tertiary amines and quaternary ammonium salts (I–VI, Fig. 1) structurally related to 3-quinuclidinol methiodide, a rigid Ch analogue. Our main interest was to compare the effects of optical isomers since the HAChT system has not been shown previously to display stereochemical selectivity [2]. We also examined the effects of Compounds I–VI on the isolated frog rectus abdominis muscle and on acetylcholinesterase (AChE) *in vitro*.

Materials and methods

Synthesis of new compounds. The enantiomers of 3-quinuclidinol (I), 3-acetoxyquinuclidine hydrochloride (III) and of 3-acetoxyquinuclidine methiodide (IV) were synthesized as previously described [4]. *R*-3-Quinuclidinol methiodide (*R*-II) was prepared by the addition of methyl iodide to a solution of *R*-I in methanol; m.p. 322–324° (from ethanol–ether), $[\alpha]_D^{23} - 32.2°$ (*c* 0.8, 95% ethanol). *S*-3-Quinuclidinol methiodide (*S*-II), prepared similarly, had m.p. 320–323° and $[\alpha]_D^{23} + 32.5°$ (*c* 0.8, 95% ethanol). Anal. Calc. for $C_8H_{16}INO$: C, 35.70; H, 5.99. Found: C, 35.82; H, 6.10.

RR-Bis(3-quinuclidinyl)terephthalate dihydrochloride (*RR*-V) was synthesized from *R*-I [4.0 g, $[\alpha]_D^{23} - 44.6°$ (*c* 2.0, 1 N HCl)], 1.7 M butyl lithium in hexane (25 ml) and 3.35 g of terephthaloyl chloride [5] according to a previously described method [6]. The yield was 4.5 g (74%)



I II III IV

V VI

Fig. 1. Chemical structures of Compounds I–VI.

of the free base of *RR*-V. This base (0.5 g) was dissolved in 20 ml of 2 N NaOH, and the solution was kept at 60° for 3 hr. Extraction with chloroform and evaporation gave a residue which was recrystallized from benzene yielding 0.21 g (64%) of *R*-I which had $[\alpha]_D^{23} - 44.5°$ (*c* 1.8, 1 N HCl), showing that no racemization had occurred during the transformation of *R*-I to *RR*-V.

A solution of *RR*-V (1.7 g) in dichloromethane (25 ml) was added dropwise to a saturated solution of HCl in dichloromethane. The precipitate was washed thoroughly with dichloromethane followed by acetone and left in vacuum (1 mm Hg) for 2 days. The yield of *RR*-V dihydrochloride was 1.8 g (89%); m.p. > 330°, $[\alpha]_D^{23} - 46.1°$ (*c* 1.1, 50% ethanol). Anal. Calc. for $C_{22}H_{28}N_2O_4 \cdot 2HCl$: C, 57.77; H, 6.61; N, 6.12; Cl, 15.50. Found: C, 57.60; H, 6.63; N, 6.07; Cl, 15.64.

RR-Bis(3-quinuclidinyl)terephthalate dimethiodide (*RR*-VI) was prepared by the addition of a solution of *RR*-V (0.9 g) in 30 ml of acetone–chloroform (1 : 1) to a solution of methyl iodide (0.77 g) in methanol (10 ml); yield 0.97 g (62%), m.p. > 330° (from 50% ethanol–acetone), $[\alpha]_D^{23} - 39.2°$ (*c* 1.1, 50% ethanol). Anal. Calc. for $C_{24}H_{34}I_2N_2O_4$: C, 43.13; H, 5.13; N, 4.19. Found: C, 43.32; H, 5.25; N, 4.12.

SS-Bis(3-quinuclidinyl)terephthalate dihydrochloride (*SS*-V) was prepared from *S*-I as described above for *RR*-V. Yield 84%, m.p. > 330°, $[\alpha]_D^{23} + 47.2°$ (*c* 1.1, 50% ethanol). Found: C, 57.56; H, 6.73; N, 5.90.

SS-Bis(3-quinuclidinyl)terephthalate dimethiodide (*SS*-VI) was prepared from *SS*-V as described above for *RR*-VI. Yield 71%, m.p. > 330°, $[\alpha]_D^{23} + 41.6°$ (*c* 1.1, 50% ethanol). Found: C, 42.96; H, 5,24; N, 3.92.

Inhibition of high affinity choline transport. The crude synaptosomal fraction (P_2) was prepared from whole rat brain (minus cerebellum, pons and medulla) and incubated as previously described [7]. Paraoxon (40 μM) was always present to inhibit AChE. HAChT was measured as the difference between the transport of [$^2H_4$]Ch (2 μM) at 37° and 0° during a 4-min incubation. Inhibitors were present during the measurement of Ch transport. ACh and Ch were extracted from the synaptosomal pellets and supernatant fractions using [$^2H_9$]ACh and [$^2H_9$]Ch as internal standards [8]. The concentrations of endogenous ([$^2H_0$]) and tracer ([$^2H_9$]) variants of ACh and Ch were determined by gas chromatography mass spectrometry [9]. Duplicate or triplicate samples were used.

Frog rectus abdominis. Stimulant activity on the frog rectus abdominis muscle was measured as previously described [10]. Compounds that showed no stimulatory actions were assayed as antagonists to carbachol. Schild plots were used to test for competitive antagonism [11].

Acetylcholinesterase inhibition. The ability of the compounds to inhibit electric eel AChE (Sigma Chemical Co., St. Louis, MO) was measured with a microprocessor-controlled constant pH titration system devised in this laboratory. Assays were carried out at 37° under N_2. IC_{50} Values were obtained by plotting percent inhibition versus log inhibitor concentration.

Toxicity in mice. LD_{50} Values were determined by intravenous injection using the "up and down" method for small samples [12]. Each animal was observed for 1 hr.

Results and discussion

High affinity choline transport. The HAChT was calculated as the sum of the synaptosomal [$^2H_4$]Ch and [$^2H_4$]ACh plus the released [$^2H_4$]ACh after a 4-min incubation at 37° minus those concentrations obtained at 0°. All of the tested compounds inhibited each of these components equally, i.e. none altered the percent of the transported [$^2H_4$]Ch that was acetylated or altered the release of [$^2H_4$]ACh differently from its effect on HAChT. Therefore, the primary effect of the inhibitors was at the site of Ch transport.

3-Quinuclidinol methiodide (II) may be regarded as a rigid analogue of Ch. The distance between the quaternary nitrogen and the oxygen in Compound II and in Ch is similar [3]. The 12-fold higher inhibitory potency of *S*-II as compared to *R*-II (Table 1) is the first demonstration that the HAChT system exhibits stereoselectivity. It shows that not only the distance between the ammonium group and the hydroxyl group [3] but also the spatial orientation of the two groups are important for interaction with the HAChT system. 3-Quinuclidinol (I), which has a pK_a value of 9.88 [14], is fully ionized at physiological pH. The low potency of *S*-I and *R*-I in inhibiting HAChT (Table 1), therefore, indicates that, in these simple analogues of Ch, a protonated nitrogen cannot replace the N-methyl quaternary ammonium group without dramatic loss of affinity. Acetylation of the hydroxyl group of Compounds I and II appeared to eliminate stereoselectivity and potency difference between tertiary amines and quaternary ammonium compounds since the four stereoisomers of 3-acetoxyquinuclidine (III) and its methiodide (IV) were almost equipotent. The finding that the enantiomers of the semi-rigid ACh analogue IV were less effective inhibitors of Ch transport than *S*-II is in agreement with the observation that ACh has a lower affinity for the Ch carrier than Ch itself [15].

Inspection of molecular models reveals that Compound

Table 1. Effects of Compounds I–VI on high affinity choline transport (HAChT) in rat brain synaptosomes, on the frog rectus abdominis muscle, and on acetylcholinesterase (AChE) *in vitro**

Compound	HAChT inhibition IC_{50}† (μM)	Frog rectus abdominis EC_{50} or K_B (μM)	AChE inhibition IC_{50}‡ (μM)	LD_{50} (μmoles/kg)
S-I	400	2580 ± 580 (4)	>1000§ (2)	
R-I	640	3660 ± 990 (4)	>1000§ (2)	
S-II	6.4	>5000 (2)	584 ± 43 (3)	153 ± 14
R-II	76	>5000 (3)	343 ± 101 (3)	367 ± 32
S-III	>10‖	668 ± 13 (4)	Substrate [13]	
R-III	>10‖	697 ± 47 (4)	Substrate [13]	
S-IV	>10‖	278 ± 21 (4)	Substrate [13]	
R-IV	>10‖	1810 ± 340 (4)	Substrate [13]	
SS-V	2.4	ND¶	4.2 ± 0.8 (4)	63 ± 4
RR-V	6.2	ND¶	6.0 ± 0.3 (4)	49 ± 5
SS-VI	1.0	12.4 ± 1.0** (12)	2.4 ± 0.4 (4)	2.6 ± 0.2
RR-VI	3.2	8.0 ± 0.6** (9)	3.1 ± 0.6 (4)	4.5 ± 0.5
HC-3	0.012			0.14 ± 0.02
d-Tubocurarine		0.57 ± 0.05** (9)		

* Values are means ± S.E. Number of estimates is given in parentheses.
† Concentration of compound required to produce 50% inhibition of choline transport.
‡ Concentration of compound required to inhibit the initial velocity of acetylcholine hydrolysis by 50%.
§ *S*-I and *R*-I caused 30–35% inhibition when present at 10^{-3} M.
‖ These compounds caused 21–27% inhibition when present at 10 μM.
¶ Not determined; see text.
** Antagonist, K_B value.

VI may adopt conformations in which the interquaternary distance is the same as in HC-3, i.e. 14 Å [16]. In spite of this, the more potent *SS*-enantiomer of Compound VI was almost 100 times less effective than HC-3 as a Ch transport inhibitor. *SS*-VI was only 2.4-fold more potent than its corresponding tertiary amine (*SS*-V), in sharp contrast to the large potency difference between *S*-I and *S*-II (62-fold).

We examined the kinetics of HAChT in the presence of 10^{-6} M of *SS*-VI (Fig. 2). There was only a slight reduction in the V_{max} whereas the apparent K_T was increased from 0.53 to 2.2 μM, indicating competitive inhibition of Ch transport.

Frog rectus abdominis. The observed effects of the enantiomers of Compounds I–IV on the frog rectus (Table 1) were in general agreement with the results reported for the racemic compounds [17]. The contractile responses to all compounds were prevented or abolished by *d*-tubocurarine (10^{-5} M). The 6-fold potency difference observed between *S*-IV and *R*-IV must be considered in view of the fact that *R*-IV is a much better substrate for AChE than *S*-IV [13].

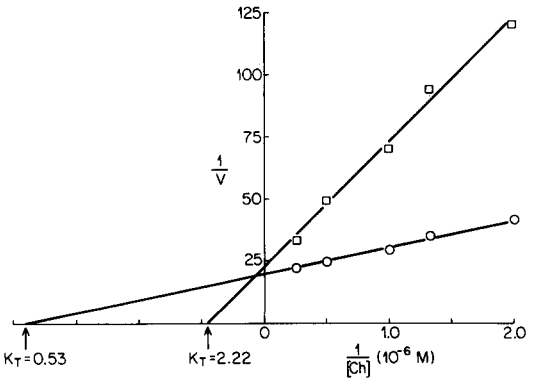


Fig. 2. Inhibition of high affinity choline transport in rat brain synaptosomes by *SS*-VI. Synaptosomes were incubated with [$^2H_4$]Ch (0.50, 0.75, 1.0, 2.0 and 3.0 μM) without inhibitor (○) and with 10^{-6} M of *SS*-VI (□) at 37° and 0° for 4 min.

The enantiomers of Compound VI blocked competitively the actions of carbachol on the frog rectus. The increased sensitivity of the muscle to ACh caused by *S*-VI (2- to 4-fold potentiation at 10^{-5}–10^{-4} M) most probably was due to its ability to inhibit AChE (Table 1). *R*-VI which was a somewhat weaker AChE inhibitor and a more potent blocker of post-junctional nicotinic receptors than *S*-VI did not potentiate ACh responses. It is interesting to note that, whereas the terephthalic acid ester VI had curare-like actions, the corresponding ester of Ch is a depolarizing neuromuscular blocking agent which contracts the frog rectus [18]. A concentration of 2×10^{-5} M of *RR*-V and *SS*-V had no effect on carbachol-induced contractions of the frog rectus. At higher concentrations (10^{-4} M), the compounds caused a slowly progressing elevation of the baseline which was not influenced by washing or by *d*-tubocurarine (10^{-4} M). This phenomenon precluded determination of dissociation constants.

Toxicity in mice. The LD_{50} values of the compounds found to be the most potent inhibitors of HAChT are summarized in Table 1. No correlation could be made between ability to inhibit HAChT *in vitro* and toxicity in mice. Also, there was no clear correlation between degree of AChE inhibition *in vitro* and toxicity. The absence of muscarinic effects, frequently seen after high doses of AChE inhibitors, also appeared to exclude AChE inhibition as a primary cause of death. These observations and the short time to death (<3 min) suggest that the relatively high toxicity of *SS*-VI and *RR*-VI was due mainly to curare-like actions. *S*-II produced toxic effects of slower onset similar to those seen after administration of HC-3.

In summary, *S*-3-quinuclidinol methiodide (*S*-II) was considerably more potent than the *R*-enantiomer as an inhibitor of Ch transport. In contrast to the Ch transport inhibitors V and VI, whose enantiomers were rather potent neuromuscular blocking agents and AChE inhibitors, *S*-II appeared to be a specific inhibitor of the HAChT system.

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