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# Pharmacological actions of some cyclic analogues of choline

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**1** Two cyclic choline analogues (3-hydroxy-*N,N*-dimethylpiperidinium and 2-hydroxymethyl-*N,N*-dimethylpiperidinium) and two cyclic homocholine analogues (4-hydroxy-*N,N*-dimethylpiperidinium and 3-hydroxymethyl-*N,N*-dimethylpiperidinium) have been studied with regard to their actions at the cholinergic synapse.

**2** All the analogues had some direct depolarizing activity on the frog rectus abdominis muscle but they were less potent in this respect than acetylcholine. Compared to physostigmine, the analogues were weak inhibitors of cholinesterase enzymes.

**3** All the analogues were found to have a presynaptic blocking action on the rat phrenic nerve-hemidiaphragm preparation, which was reversed by choline. In addition, they all inhibited the high affinity transport of choline into synaptosomes but only the cyclic choline analogues were found to be acetylated by soluble choline acetyltransferase *in vitro*.

**4** We conclude that the hydroxypiperidinium analogues caused the presynaptic block seen at the neuromuscular junction by inhibiting acetylcholine synthesis.

## Introduction

A number of choline analogues have been shown to interact at cholinergic synapses (Burgen *et al.*, 1956; Bowman *et al.*, 1967; Barker & Mittag, 1975; Collier & Ilson, 1977). It has been demonstrated that many analogues inhibit the high affinity transport of choline into synaptosomes (Simon *et al.*, 1975) while some analogues are acetylated by choline acetyltransferase (ChAc) *in vitro* (Burgen *et al.*, 1956; Hemsworth, 1971a; Sollenberg *et al.*, 1979). Barker & Mittag (1975) showed that monoethylcholine and pyrrolcholine are transported into synaptosomes where they are acetylated by ChAc. Several choline analogues have been shown to be precursors of false transmitters. For example, homocholine (Collier *et al.*, 1977) and triethylcholine (Ilson *et al.*, 1977) are transported into the cat superior cervical ganglion where they are acetylated by ChAc *in vivo* and the acetylated products released as false cholinergic transmitters.

3-Hydroxy-*N,N*-dimethylpiperidinium (3-hydroxypiperidinium) and 2-hydroxymethyl-*N,N*-dimethylpiperidinium (2-hydroxymethylpiperidinium) are analogues in which the choline moiety is immobilized by being fixed in a ring (Figure 1). 4-Hydroxy-*N,N*-dimethylpiperidinium (4-hydroxypiperidinium) and 3-hydroxymethyl-*N,N*-dimethylpiperidinium (3-hydroxymethylpiperidinium) are analogues in which a homocholine moiety is incorporated into a ring structure (Figure 1).

It is interesting to observe that the choline moiety of 2,2′-[1,1-biphenyl]-4,4′diylbis (2-hydroxy-4,4-dimethylmorpholinium] (hemicholinium-3) is fixed in a morpholinium ring in a similar way to the choline moiety of the 3-hydroxypiperidinium molecule (Figure 1). Apart from the hemicholinium analogues, there has been very little work conducted on cyclic choline analogues and it was therefore thought of interest, particularly from the point of view of structure-activity relationships, to study these cyclic choline compounds and investigate their actions at the cholinergic synapse.

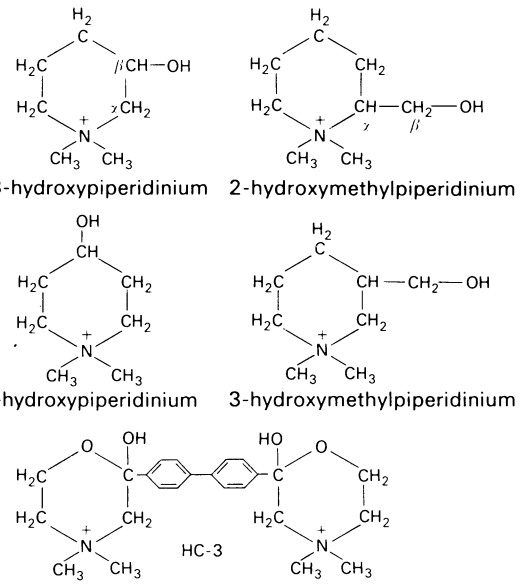


**Figure 1** Chemical structures of the hydroxypiperidinium analogues studied and hemicholinium-3 (HC-3).

## Methods

### *Materials*

[$^3$H-methyl]-choline chloride (6.4 Ci mmol$^{-1}$), [$^{14}$C-acetyl]-coenzyme A (AcCoA) (59.5 mCi mmol$^{-1}$), [$^{14}$C]-methyliodide (58 mCi mmol$^{-1}$), [$^{14}$C]-acetyl choline iodide (ACh) (10.2 mCi mmol$^{-1}$ diluted to 2.7 mCi mmol$^{-1}$ with non-labelled ACh) were purchased from the Radiochemical Centre, Amersham. [$^{14}$C]-acetyl-β-methylcholine iodide (Ac-β-MeCh) (3.2 mCi mmol$^{-1}$ diluted to 0.07 mCi mmol$^{-1}$ with non-labelled AC-β-MeCh) and [$^{14}$C]-butyrylcholine iodide (BuCh) (4.8 mCi mmol$^{-1}$ diluted to 0.12 mCi mmol$^{-1}$ with non-labelled BuCh) were purchased from New England Nuclear, Winchester. Acetylcholinesterase (AChE) (ACh hydrolase E.C.3.1.1.7. obtained from bovine erythrocytes, 2.6 units mg$^{-1}$ of solid), cholinesterase (ChE) (type IV obtained from horse serum, 21.3 units mg$^{-1}$ of solid) and bovine serum albumin were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Physostigmine sulphate and disodium edetate (EDTA) were obtained from BDH Chemicals, Ltd, Poole. Choline chloride and Triton X-100 were purchased from Hopkin & Williams, Essex. The hydroxypiperidinium compounds were synthesized as iodide salts by reacting the appropriate hydroxy-*N*-methylpiperidine (Aldrich Chemical Co., London) with methyliodide (BDH Chemicals, Ltd, Poole). The products were recrystallized from ethanol. The structure and purity of the compounds were confirmed by the determination of melting points, i.r. spectra and microanalyses.

### *Frog rectus abdominis muscle*

The frog rectus abdominis muscle was suspended in Krebs Ringer solution (composition in mM: NaCl 111, KCl 1.9, $CaCl_2$ 1.1, $NaH_2PO_4$ 0.1, $NaHCO_3$ 4.8; glucose 11) which was equilibrated with air at room temperature (20°C). ACh or another agonist (see Results) were added at 4 min intervals and left in contact with the muscle for 30 s. The preparation was then washed twice with fresh Krebs Ringer solution and allowed to relax to its baseline position. In experiments investigating the inhibition or potentiation of the ACh-induced submaximal muscle contraction, the analogue was added to the bath 1 min before the next dose of ACh, which was given in the presence of the analogue. Contractions were recorded isometrically by means of a force displacement transducer with a Washington recorder.

### *Cholinesterase determination*

The rates of hydrolysis of $^{14}$C-labelled substrates (ACh, Ac-β-MeCh and BuCh) in the presence of the analogues were estimated by incubation with cholinesterase enzyme and by quantitative determination of the radioactive products of hydrolysis by the method described by Siakotos *et al.* (1969). AChE and ChE were obtained commercially and were made up in distilled water to a concentration of 1 mg protein ml$^{-1}$. A brain homogenate was prepared by homogenization of rat brains on ice in 0.1% Triton X-100 containing 200 mM KCl (10 ml g$^{-1}$ wet weight), using a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 20,000 *g* for 20 min and the supernatant containing the enzymes was used immediately or stored at −20°C.

The incubation mixture was placed in a 10 ml graduated test tube and contained 100 μl of enzyme, 100 μl of $^{14}$C-labelled substrate (3 mM, giving a final concentration of 1 mM), 30 μl of the analogue at the appropriate concentration and 70 μl of the buffer-salt-detergent mixture (composition in mM: NaCl 300, $NaH_2PO_4$ 20, $Na_2HPO_4$ 80 and 10 ml Triton X-100, all made up to one litre with distilled water and adjusted to pH 7.4). The mixture was incubated in a shaking water bath at 37°C for 10 min, over which time the reaction followed a linear time course. Controls contained 30 μl of distilled water in place of the analogue. Blank values were determined in which the enzyme was replaced with distilled water.

*Rat phrenic nerve-hemidiaphragm*

In experiments on the isolated phrenic nerve-hemidiaphragm of the rat (Bülbring, 1946), both hemidiaphragms were mounted in the same bath, in Krebs solution (composition in mM: NaCl 117, KCl 5.4, $CaCl_2$ 2.6, $MgSO_4 7H_2O$ 0.6, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, glucose 11) which was equilibrated with 5% $CO_2$ plus 95% $O_2$ at 37°C. The muscles were excited by supramaximal rectangular pulses of 0.1 ms duration applied to the phrenic nerves. One nerve was stimulated at 1 Hz and the other at 0.1 Hz. The muscle contractions were recorded isometrically by means of a force displacement transducer with a Washington recorder.

*Inhibition of the high affinity transport of [$^3$H]-choline into synaptosomes*

A crude preparation of synaptosomes ($P_2$ fraction of Gray & Whittaker, 1962) was prepared from a rat forebrain. Synaptosomes from 30 mg of tissue were suspended in 1.0 ml of Krebs Ringer Phosphate (KRP) buffer (composition in mM: NaCl 175, KCl 4, glucose 10, $CaCl_2$ 1, $MgCl_2$ $6H_2O$ 2, sodium phosphate buffer (pH 7.4) 10) containing [$^3$H]-choline (1 μM, 0.55 μCi nmol$^{-1}$) and analogue at the required concentration. All additions were made at 4°C. The synaptosomes were incubated at 37°C (blank values were obtained by incubating at 4°C), after which uptake was terminated by cooling the suspension in ice and subsequent centrifugation (2,000 *g* for 10 min). The supernatant was discarded and the resulting pellet was washed with KRP solution (5 ml) at room temperature. After a second centrifugation process, 200 μl of NaOH (1 M) was added to the pellet, which was then pipetted onto glass fibre papers. After drying at room temperature, any radioactivity on these papers was detected using a toluene scintillation fluor and counting in a liquid scintillation spectrometer.

*Choline acetyltransferase (ChAc) activity*

Freshly dissected rat brains were placed in an ice-cold mortar and acetone, precooled to − 20°C, was added in a large excess. The tissue was disintegrated in the mortar, using a pestle, and was then filtered under suction in a Buchner funnel. After washing with cold acetone, the powder was left on the filter for 5 min with suction, was then transferred to a dessicator and dried over $P_2O_5$ at 4°C for 4 h. When dry, the powder was extracted and ChAc was purified by the method described by Mann & Hebb (1975).

The rate of acetylation of choline and other substrates was estimated by incubation with [$^{14}$C]-AcCoA and ChAc and by a quantitative determination of the amount of the labelled product formed. The incubation mixture was placed in a plastic microfuge tube (Beckman) and contained 5 μl ChAc, 10 μl choline or analogue at the required concentration and 10 μl of a buffer (initial composition in mM: NaCl 30, $MgSO_4$ 5, disodium EDTA 0.1, potassium phosphate (pH 7.7) 15, physostigmine sulphate 0.2, [$^{14}$C]-AcCoA (59.5 mCi mmol$^{-1}$) 0.045 and bovine serum albumin (0.05%). Blank values were determined by replacing the substrate with distilled water. All additions were made at 4°C and, when complete, the tubes were shaken, transferred to a shaking waterbath at 37°C and incubated for 10 min. The reaction was stopped by transferring the tubes to an ice bath.

The inhibition of ChAc by the choline analogues was measured by a quantitative determination of the amount of [$^{14}$C]-ACh formed by a reaction of [$^{14}$C]-AcCoA and choline in the presence of the analogue compared to control. The incubation mixture was placed in a plastic microfuge tube (Beckman) and contained 5 μl ChAc, 10 μl choline (14 mM), 10 μl of analogue (14 mM) and 10 μl of the buffer containing [$^{14}$C]-AcCoA (as described above). Control values were determined by replacing the analogue with distilled water. Blank values were determined by replacing both choline and the analogue with distilled water. Additions and incubations were effected, as described above.

The [$^{14}$C]-acetylated product was routinely extracted into the toluene-based scintillation fluor, as described by Fonnum (1975).

*Protein determination*

This was effected by following the method of Lowry *et al.* (1951).

## Results

*Frog rectus abdominis muscle*

3-Hydroxypiperidinium, 2-hydroxymethylpiperidinium and 4-hydroxypiperidinium (0.4 to 1.0 μmol ml$^{-1}$) slightly enhanced the ACh-induced contraction of the frog rectus abdominis muscle. However, 3-hydroxymethylpiperidinium was shown to have a direct depolarizing action on the muscle at a dose of 0.7 μmole ml$^{-1}$ and above. The equipotent molar ratio of this compound, compared to ACh, was 887 : 1.

*Anticholinesterase activity*

All of the hydroxypiperidinium analogues were shown to inhibit both ChE and AChE; however, they

**Table 1** $IC_{50}$ (molar) values for the inhibition of cholinesterase activity by the hydroxypiperidinium analogues

| *Analogue* | *Enzyme* | | | *Substrate* |
|---|---|---|---|---|
| | *Bovine erythrocyte (AChE)* | *Horse serum (ChE)* | *Rat brain Homogenate* | |
| 3-Hydroxypiperidinium | $1.7 \times 10^{-2}$ | $5.7 \times 10^{-2}$ | $5.6 \times 10^{-3}$ | ACh |
| | | | $2.1 \times 10^{-3}$ | Ac-β-MeCh |
| | | | $8.9 \times 10^{-2}$ | BuCh |
| 2-Hydroxymethylpiperidinium | $1.6 \times 10^{-2}$ | $9.6 \times 10^{-3}$ | $1.8 \times 10^{-3}$ | ACh |
| | | | $1.7 \times 10^{-3}$ | Ac-β-MeCh |
| | | | $5.3 \times 10^{-3}$ | BuCh |
| 4-Hydroxypiperidinium | | | $1.6 \times 10^{-2}$ | ACh |
| 3-Hydroxymethylpiperidinium | | | $1.7 \times 10^{-2}$ | ACh |
| Physostigmine | $1.3 \times 10^{-6}$ | $3.0 \times 10^{-6}$ | | |

$IC_{50}$ values were calculated from Dixon (1953) plots. For each plot, a regression line with a correlation coefficient of 0.98 to 0.99 was drawn through at least five points. Each point was the mean of at least three determinations ± s.e.mean of 10% or less.

were much weaker in this respect than physostigmine. Their $IC_{50}$ values (concentration of inhibitor which inhibits the hydrolysis of the various substrates by 50%) are given in Table 1. Because of their weak anticholinesterase activity, 4-hydroxypiperidinium and 3-hydroxymethylpiperidinium were only tested with rat brain homogenate using ACh as the substrate.

*Rat phrenic nerve-hemidiaphragm*

All the hydroxypiperidinium analogues were found to cause a slowly developing block of the rapidly stimulated muscle (1 Hz) but had no effect on the more slowly stimulated muscle (0.1 Hz) at the doses employed. When choline was added to the incubating medium, in the presence of one of the analogues, or if the analogue was washed out of the bath, then the muscle contractions of the rapidly stimulated muscle returned to the control height of contraction. It was concluded that all these analogues gave a frequency-dependent prejunctional block at the doses employed (Table 2) and, in this respect, 4-hydroxypiperidinium was found to be the most potent.

**Table 2** Effect of hydroxypiperidinium analogues on nerve transmission in the rat phrenic nerve-hemidiaphragm preparation

| *Analogue* | *Minimum effective dose giving a prejunctional block* ($\mu$mol ml$^{-1}$) |
|---|---|
| 3-Hydroxypiperidinium | 2.7 |
| 2-Hydroxymethylpiperidinium | 1.8 |
| 4-Hydroxypiperidinium | 1.2 |
| 3-Hydroxymethylpiperidinium | 2.6 |

The prejunctional block was reversed by choline (0.2 to 0.14 $\mu$mol ml$^{-1}$). Each experiment was repeated twice; ± s.e.mean was 10% or less.

*Inhibition of the high affinity transport of [$^3$H]-choline into synaptosomes*

A crude preparation of synaptosomes from mammalian brain tissue accumulates choline from the extracellular medium by two distinct transport mechanisms; one has a high affinity for choline and is sodium-dependent and the other has a low affinity for choline (Yamamura & Snyder, 1973).

In the present experiments, the transport of [$^3$H]-choline (1 $\mu$M, 0.55 $\mu$Ci nmol$^{-1}$) into synaptosomes at 37°C was linear for 7 min. The amount of choline accumulated by synaptosomes at 37°C was 4–6 times greater than that accumulated at 4°C for 7 min. The concentration of the analogues inhibiting the transport of choline into synaptosomes by 50% (Table 3) was determined graphically from a Dixon (1953) plot. 3-Hydroxymethylpiperidinium was the most potent inhibitor of the high affinity choline transport system.

In vitro *acetylation by ChAc*

The relative rates of *in vitro* acetylation of the hydroxypiperidinium analogues were determined and compared to the acetylation of choline (Figure 2). Additional studies demonstrated that the rates of acetylation of the analogues were linear for at least 10 min at the concentrations employed and that there was no substrate inhibition up to a concentration of 200 mM. As a relatively high rate of acetylation was seen, the acetylation of 3-hydroxypiperidinium by ChAc was further investigated. At a constant concentration of AcCoA (18 $\mu$M), the plot of velocity of

**Table 3** Inhibition of high-affinity choline transport into synaptosomes

| *Inhibitor* | $IC_{50}$ (μM) |
|---|---|
| 3-Hydroxypiperidinium | 31 |
| 2-Hydroxymethylpiperidinium | 14 |
| 4-Hydroxypiperidinium | 33 |
| 3-Hydroxymethylpiperidinium | 8 |

$IC_{50}$ values were calculated from Dixon (1953) plots. For each plot a regression line with a correlation coefficient of 0.98 was drawn through at least six points. Each point was the mean of at least five determinations ± s.e.mean of 10% or less.

acetylation against substrate concentration showed a typically shaped curve for an enzyme-one substrate reaction. However, at a substrate concentration of 3-hydroxypiperidinium of 400 mM, substrate inhibition was seen. The apparent Michaelis-Menten constants ($K_m$) were derived from plots according to Lineweaver & Burk (1934). 3-Hydroxypiperidinium had a $K_m$ (concentration of substrate providing one-half of the maximal rate of acetylation) of 0.88 mM, which is about three times that of choline (0.29 mM).

In vitro *inhibition of ChAc*

A partially purified extract of ChAc and AcCoA were incubated with equimolar concentrations of choline and one of the hydroxypiperidinium analogues and the amount of acetylated product synthesized was assayed. These incubations were compared with controls containing the same concentration of choline, but distilled water instead of the analogue. The method of isolation of the acetylated

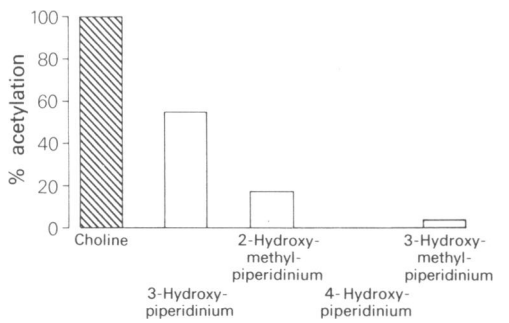


**Figure 2** Rates of acetylation of choline and the hydroxypiperidinium analogues by choline acetyltransferase (ChAc) extracted from rat brain. The incubation was carried out for 10 min at 37°C. The final substrate concentration was 20 mM. Each experiment was performed in duplicate and repeated six times; ± s.e.mean were less than 5%.

product does not distinguish between ACh and any acetylated hydroxypiperidinium analogue. This need only be taken into consideration in the case of 3-hydroxypiperidinium and, to a lesser extent, 2-hydroxymethylpiperidinium, which have been shown to be acetylated by ChAc. However, it should be remembered that in these experiments choline, the natural substrate for ChAc, is present in relatively high concentrations and therefore, will presumably favourably compete with these choline analogues for the enzyme. All the analogues were found to be weak inhibitors of ChAc (Table 4).

## Discussion

Bowman *et al.* (1967) proposed a number of pharmacological tests for examining the pre- and postjunctional blocking action of a drug at the neuromuscular junction. The frog rectus abdominis muscle is one such preparation and it is useful for determining the competitive or the depolarizing postjunctional activity of a drug. None of the hydroxypiperidinium compounds antagonized the ACh elicited contracture of this muscle. However, 3-hydroxypiperidinium showed depolarizing activity of its own, although its activity was slight compared to ACh. The other analogues did not elicit a contracture of the muscle on their own, but in low doses they did slightly potentiate the responses to ACh. It is unlikely that this potentiation is due to any anticholinesterase activity since all the hydroxypiperidinium compounds were weak inhibitors of these enzymes.

All the hydroxypiperidinium compounds were shown to have a prejunctional blocking action on the rat phrenic nerve-diaphragm preparation, which was reversed by choline. This reversal indicates that the analogues caused a prejunctional block by inhibiting the synthesis of ACh. Indeed, many choline analogues which have exhibited a prejunctional block on the rat diaphragm have also been shown to interfere with neuronal ACh synthesis (Bowman & Rand, 1962; Bowman & Hemsworth, 1965;

**Table 4** Percentage inhibition of the acetylation of choline by ChAc extracted from rat brain

| *Inhibitor* | *% Inhibition* |
|---|---|
| 3-Hydroxypiperidinium | 5 |
| 2-Hydroxymethylpiperidinium | 5 |
| 4-Hydroxypiperidinium | 0 |
| 3-Hydroxymethylpiperidinium | 0 |

Both choline and hydroxypiperidinium analogues were at a concentration of 14 mM. Each value is the average of 4 determinations ± s.e.mean of 10% or less.

DiAugustine & Haarstad, 1970; Hemsworth, 1971b; Barker & Mittag, 1975; Simon *et al*, 1975). Therefore, a further study of the hydroxypiperidinium compounds was made, using biochemical techniques, to try to determine how they might inhibit ACh synthesis.

Choline is transported into rat brain synaptosomes by both high and low affinity transport mechanisms (Yamamura & Snyder, 1972; 1973; Haga & Noda, 1973). Low affinity choline uptake occurs in most cells, but the high affinity transport of choline is localized specifically in cholinergic neurones (Kuhar *et al.*, 1973). There is some controversy over whether the high affinity system is coupled directly to ACh synthesis (Barker & Mittag, 1975; Marchbanks & Kessler, 1982), but Ach synthesis will be impaired if the high affinity uptake of choline is antagonized (for review, see Kuhar & Murrin, 1978). Many choline analogues have been shown to be inhibitors of the high affinity uptake system (Simon *et al.*, 1975; Batzold *et al.*, 1980) and therefore of ACh synthesis and it was thought possible that the hydroxypiperidinium compounds could cause the prejunctional block seen in the rat diaphragm preparation by acting in this manner.

All the hydroxypiperidinium compounds were found to inhibit the high affinity transport of choline into synaptosomes. The analogues having a choline moiety fixed in a piperidinium ring, namely 3-hydroxypiperidinium ($IC_{50} = 31\,\mu M$) and 2-hydroxymethylpiperidinium ($IC_{50} = 14\,\mu M$), were less potent than choline itself ($IC_{50} = 2\,\mu M$; Simon *et al.*, 1975)). Similarly, hemicholinium-15 (HC-15), which has a choline moiety fixed in a morpholinum ring structure and which is exactly one-half of the hemicholinium-3 molecule, has been found to have an $IC_{50}$ of $15\,\mu M$ (Simon *et al.*, 1975). Thus, in monoquaternary compounds, immobilizing part of the choline moiety in a ring structure appears to decrease its potency as an inhibitor of the high affinity uptake system. However, when the cyclic choline moieties are part of the bisquaternary molecule, hemicholinium-3, their inhibitory potencies exceed that of choline (Barker & Mittag, 1975; Hemsworth *et al.*, 1979).

Increasing the carbon chain length between the quaternary nitrogen and the hydroxyl group in the choline molecule from two to three methylene groups gives the compound homocholine. This molecule inhibits the high affinity uptake of choline, having an $IC_{50}$ of $2-4\,\mu M$ and, in this respect, it is just as potent as choline (Simon *et al.*, 1975; Batzold *et al.*, 1980). The homocholine analogue 3-hydroxymethylpiperidinium has two of its methylene groups fixed in a ring structure and, with an $IC_{50}$ of $8\,\mu M$, was only slightly less potent than the parent compound. Therefore, unlike the choline moiety, the homocholine moiety does not lose its potency when part of its carbon chain is fixed in a ring. However, the potency of the homocholine moiety is markedly reduced when all three of the methylene groups are in a ring structure. Thus, 4-hydroxypiperidinium was found to have an $IC_{50}$ of $33\,\mu M$. It is interesting to observe that, in this study, the two least potent inhibitors of the high affinity transport system are 3-hydroxypiperidinium and 4-hydroxypiperidinium. It appears that the potency of monoquaternary choline analogues is reduced when the hydroxyl group is attached to a methylene group which is immobilized in a ring structure.

Several choline analogues which are choline uptake inhibitors have also been shown to be substrates for the high affinity uptake system and, in addition, were acetylated by ChAc within the nerve terminal (Dowdell, 1975; Simon *et al.*, 1975; Collier *et al.*, 1977; Ilson *et al.*, 1977). 3-Hydroxypiperidinium is one such compound since it has been found to be transported into the nerve terminal where it is acetylated *in situ* by ChAc and the acetylated product is released as a false cholinergic transmitter (Hemsworth *et al.*, 1984). When compared to ACh, 3-acetoxypiperidinium is a weak agonist at the neuromuscular junction. (Borkhataria *et al.*, 1979). Thus, the presynaptic blocking action of 3-hydroxypiperidinium is probably due, at least in part, to the synthesis of a cholinergic false transmitter which is released from the nerve in place of ACh. It appears that in order to be a precursor of a false transmitter the choline analogues have to be acetylated by ChAc. Therefore, in order to test whether all the hydroxypiperidinium compounds could cause presynaptic inhibition by acting as precursors of false transmitters, the activities of these compounds were determined as substrates for a soluble preparation of ChAc extracted from rat brain. Only the analogues with a choline moiety in their structure were shown to be significantly acetylated. 3-Hydroxypiperidinium was acetylated by 55% and 2-hydroxymethylpiperidinium by 18%, compared to 100% acetylation of choline.

Collier *et al.* (1977) showed that homocholine was acetylated within the cholinergic neurone and although homocholine has been shown to be acetylated *in vitro* by membrane-bound ChAc, it has not been found to be a substrate for soluble ChAc (Benishin & Carroll, 1981; Luqmani & Richardson, 1982). This indicates that it might be membrane bound ChAc and not soluble ChAc that is responsible for acetylation *in situ*. However, Hersh *et al.* (1978) showed that homocholine was acetylated by soluble ChAc *in vitro* provided the reaction was run in a medium of low ionic strength (10 mM compared to 300 mM). Thus, these experiments suggest that soluble ChAc could, after all, be responsible for acetylation *in situ*. In our

experiments using soluble ChAc, a medium with an ionic strength of about 40 mM was used and no significant acetylation of homocholine was detected (Hemsworth, Shreeve & Veitch, unpublished observation). Thus, it is possible that although 4-hydroxypiperidinium and 3-hydroxymethylpiperidinium, the two homocholine analogues, were apparently not acetylated under the experimental conditions recounted here, acetylation may have been detected if a membrane bound ChAc and/or a medium of a lower ionic strength had been used. Interestingly, Hersh *et al.* (1978) observed that although the absolute rates of acetylation of certain choline analogues were dependent upon the ionic strength of the medium, the relative rates of acetylation of the analogues remained the same. Therefore, it seems reasonable to conclude from our studies that 3-hydroxypiperidinium is a better substrate for soluble ChAc than 2-hydroxymethylpiperidinium and that they are both better substrates than the cyclic homocholine analogues.

Thus, soluble ChAc is able to acetylate a choline moiety which is fixed in a piperidinium ring. In contrast to the structural requirements of the high affinity choline transport system, soluble ChAc favours the analogue in which the hydroxyl group is attached to a methylene group which is part of the cyclic ring structure. Hemicholinium-3 has a cyclic choline moiety which is structurally similar to 3-hydroxypiperidinium except that it is fixed in a morpholinium ring. Hemicholinium-3 is also acetylated by soluble ChAc (Hemsworth, 1971b), but it is not known for certain whether it is acetylated to a cyclic form of acetylhemicholinium-3 or whether it undergoes ring opening on the enzyme surface, to give open-chain acetyl-seco-hemicholinium-3 (DiAugustine & Haarstad, 1970; Barker & Mittag, 1975). Due to the stability of the piperidinium ring, it seems unlikely that 3-hydroxypiperidinium will undergo ring opening upon enzymatic acetylation. Thus, the data presented in this study show that it is at least possible for cyclic choline analogues like hemicholinium-3 to be acetylated in the ring conformation.

In conclusion, it has been shown that all the cyclic choline analogues investigated in this study can inhibit ACh synthesis. This will result in the prejunctional block observed at the neuromuscular junction.

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