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THE CHEMISTRY AND PHARMACOLOGY OF
SOME ANALOGUES OF CHOLINE

A thesis submitted by
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for the degree of
DOCTOR OF PHILOSOPHY
in the
UNIVERSITY OF ASTON IN BIRMINGHAM

Department of Pharmacy
University of Aston in Birmingham

December, 1979.

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SUMMARY

The chemistry and pharmacology of choline analogues has been reviewed. Three series of choline analogues have been synthesised, namely (a) hemicholinium (HC-3) analogues, (b) long-chain choline analogues, and (c) cyclic hydroxypiperidinium analogues. All the analogues have been screened pharmacologically to investigate their action on (i) pre- and postjunctional sites at the neuromuscular junction, (ii) cholinesterase enzymes, (iii) choline acetyltransferase (ChAc) and (iv) transport of choline into synaptosomes. Conclusions have been made on the possible mechanisms of action of the analogues which result in a prejunctional block.

Structure/activity relationships have been discussed with regard to the acetylation *in vitro* of the analogues by ChAc. The optimum distance between the two quaternary nitrogen atoms in the HC-3 analogues for enzymatic acetylation *in vitro* was found to be 14Å. From a study of the hydroxypiperidinium analogues it has been concluded that the alkyl chain of the choline molecule may itself bind to the ChAc *in vitro*. The structure of acetylated HC-3 as synthesised by ChAc *in vitro* appears to be mostly in the cyclic form and it seems likely that this product is monoacetylated.

[¹⁴C]-HC-3 and 3-hydroxy-N-[¹⁴C]-methyl-N-methylpiperidinium have been synthesised chemically. [¹⁴C]-HC-3 has been shown to be transported into synaptosomes by a low affinity transport mechanism ($K_T=52.2\mu\text{M}$) but there was no evidence of enzymic acetylation of HC-3 by synaptosomes. 3-hydroxy-N-[¹⁴C]-methyl-N-methylpiperidinium has been shown to be transported into synaptosomes by both a high and low affinity transport mechanism ($K_{TH}=6.7\mu\text{M}$; $K_{TL}=192.9\mu\text{M}$) and was enzymatically acetylated *in situ* by synaptosomes.

3-hydroxy-N-[¹⁴C]-methyl-N-methylpiperidinium has also been shown to be transported into the phrenic nerve-endplate region of a mouse hemidiaphragm *in vitro* where it was enzymatically acetylated and the acetylated product released as a cholinergic false transmitter.

KEY WORDS: CHOLINE ACETYLTRANSFERASE:
CHOLINE ANALOGUES:
CHOLINERGIC FALSE TRANSMITTER:
HEMICHOLINIUM-3 (HC-3).

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CONTENTS

	<u>Page</u>
1. <u>Introduction</u>	1
1.1. Choline and the synthesis of acetylcholine in cholinergic neurons	2
1.2 Analogues of choline	9
1.2.1 Hemicholinium-3	9
1.2.2 Hemicholinium analogues	19
1.2.3 Triethylcholine	29
1.2.4 Monoethylcholine	51
1.2.5 Diethylcholine	54
1.2.6 Homocholine	55
1.2.7 Pyrrolcholine	59
1.2.8 Morpholinecholine	61
1.2.9 Polymethylene bis (hydroxyethyl) dimethylammonium salts	62
1.2.10 Other analogues of choline	66
1.2.11 <i>In vitro</i> acetylation of choline analogues by choline acetyltransferase	67
1.2.12 Inhibition of the transport of choline into synaptosomes by choline analogues	77
1.3 Inhibition of choline acetyltransferase	79
1.4 Possible sites of action for drugs that give a presynaptic block at cholinergic nerve terminals	88
1.5 Aims	89
1.6 Nomenclature	89

2.	<u>Materials</u>	90
2.1	Radiochemicals	91
2.2	Commercial enzyme preparations	91
2.3	Ion-exchangers	92
2.4	Materials used in liquid scintillation counting	92
2.5	Miscellaneous	92
3.	<u>Methods</u>	94
3.1	Rat phrenic nerve-hemidiaphragm	95
3.2	Frog rectus abdominis muscle	95
3.3	Guinea-pig ileum	95
3.4	Cholinesterase determinations	96
3.4.1.	Preparation of cholinesterase enzymes	96
3.4.2.	Determination of activity	96
3.4.2.1	Radiochemical procedure	96
3.4.2.1.1	Incubation system	96
3.4.2.1.2	Isolation and determination of the radioactive product of hydrolysis	97
3.4.2.2	Null-point titration method	97
3.5	Choline acetyltransferase (ChAc) determinations	98
3.5.1	Preparation of rat brain ChAc	98
3.5.2	Incubation system for the acetylation of choline and the choline analogues	99

3.5.3.	Incubation system for the measurement of the inhibition of ChAc by the choline analogues	99
3.5.4.	Isolation of the acetylated product	101
3.6	Transport studies with synaptosomes	101
3.6.1	Preparation of synaptosomes	101
3.6.2	Incubation of synaptosomes	102
3.6.3	Measurement of the transport of the radioactively labelled compound into synaptosomes	103
3.6.4	Synaptosomal metabolism of $[^{14}\text{C}]\text{-HC-3}$ and $[^{14}\text{C}]\text{-3-hydroxypiperidinium}$	103
3.7	Mouse phrenic nerve-diaphragm preparation	104
3.8	Paper electrophoresis	106
3.9	Liquid scintillation counting	107
3.9.1	Toluene-based scintillation fluor	107
3.9.2	Modified Bray's fluor	107
3.10	Determination of protein	107
3.11	Animals	107
3.12	Chemistry:- methods used to identify compounds synthesised	107
3.12.1	Melting points	
3.12.2	Infrared absorption spectra	107
3.12.3	Ultraviolet absorpition spectra	108
3.12.4	Nuclear magnetic resonance spectra	108
3.12.5	Microanalyses	108

4.	<u>Results Chapter I:- HC-3, some diphenylalkane analogues of HC-3 and a methyl analogue of HC-15</u>	110
4.1	Introduction	111
4.1.1	HC-3	111
4.1.1.1	Anticholinesterase activity	111
4.1.1.2	Action at the neuromuscular junction	112
4.1.1.3	Inhibition of choline transport	112
4.1.1.4	Inhibition of and acetylation by ChAc <i>in vitro</i>	113
4.1.1.5	Transport of HC-3 into cholinergic neurons and the false transmitter hypothesis	113
4.1.1.6	Structure of acetylated HC-3 as synthesised by ChAc	114
4.1.1.7	Aims	118
4.1.2	Some analogues of HC-3	118
4.1.2.1	General discussion	118
4.1.2.2	Analogues of HC-3 investigated and aims of this study	120
4.2	Chemistry	122
4.2.1	Synthesis of HC-3 and the diphenylalkane analogues	122
4.2.1.1	Step 1: Synthesis of 4,4'-bis(chloroacetyl)1,1'-biphenyl derivatives.	122
4.2.1.2	Step 2: Quaternisation reaction	125
4.2.1.3.	Step 3: Cyclisation	127
4.2.1.4	Recrystallisation	129
4.2.1.5	Confirmation of structures	129
4.2.2	Cyclisation study of HC-3 and the diphenylalkane analogues	131
4.2.3	Optical activity of HC-3 and the diphenylalkane analogues	135
4.2.4	Synthesis of tertiary-HC-3	135
4.2.5	Synthesis of methylmorpholinium	135

4.2.6	Attempted synthesis of cyclic acetyl-HC-3	137
4.2.7	[¹⁴ C]-HC-3	137
4.2.7.1	Synthesis	137
4.2.7.2	Specific activity and purity	137
4.2.7.2.1	Specific activity	137
4.2.7.2.2	Radiochemical purity	139
4.2.7.2.3	Chemical purity	139
4.2.8	Experimental	139
4.2.8.1	4,4'-bis(chloroacetyl)1,1'biphenyl derivatives	139
4.2.8.2	2,2' [1,1'-Biphenyl]-4,4'-diylbis [2-hydroxy-4,4-dimethylmorpholinium]dichloride analogues	142
4.2.8.3	2,2'-[1,1'Biphenyl]-4,4'-diylbis [2-hydroxy-4 methyl morpholinium]dihydrochloride (tertiary-HC-3)	146
4.2.8.4	4,4'-diethyl-2-hydroxy-2-methylmorpholinium chloride (methylmorpholinium)	147
4.2.8.5	Attempted synthesis of 2,2'-[1,1'biphenyl]-4,4'diylbis [2-acetyloxy-4,4-dimethylmorpholinium]dichloride (cyclic acetyl-HC-3)	147
4.2.8.5.1	Panormow modification of Schotten-Baumann reaction	147
4.2.8.5.2	Einhorn modification of Schotten-Baumann reaction	148
4.2.8.6	Synthesis of 2,2'-[1,1'-biphenyl]-4,4'diylbis [2-hydroxy-4 [¹⁴ C]methyl-4-methylmorpholinium]dichloride. ([¹⁴ C]-HC-3)	148
4.2.8.6.1	Reaction with non-radioactive methyl iodide	148
4.2.8.6.2	Reaction with radioactive [¹⁴ C]-methyl iodide	149
4.3	Pharmacology	152
4.3.1	Neuromuscular blocking action	152
4.3.1.1	Rat phrenic nerve-hemidiaphragm	152
4.3.1.2	Frog-rectus abdominis muscle	154
4.3.2	Anticholinesterase activity	154
4.3.3	<i>In vitro</i> acetylation by ChAc	157
4.3.4	<i>In vitro</i> inhibition of ChAc	165

4.3.5.	Determination of the structure of acetylated HC-3 as synthesised by ChAc <i>in vitro</i>	167
4.3.5.1	Theory of the experiment	167
4.3.5.2	Separation of open-chain Ac-seco-HC-3 from HC-3, ChAc and $[^{14}\text{C}]\text{-AcCoA}$	169
4.3.5.3	Isolation of $[^{14}\text{C}]\text{-acetylated HC-3}$ as synthesised by ChAc <i>in vitro</i>	172
4.3.5.4	Identification of $[^{14}\text{C}]\text{-acetylated HC-3}$ as synthesised by ChAc <i>in vitro</i>	173
4.3.5.5	Binding properties of HC-3 and acetylated HC-3 to the Amberlite ion-exchange resin.	179
4.3.6	Transport of $[^3\text{H}]\text{-choline}$ and $[^{14}\text{C}]\text{-HC-3}$ into synaptosomes	180
4.3.6.1	Inhibition of the high affinity choline transport by the hemicholinium compounds	180
4.3.6.2	Transport of $[^{14}\text{C}]\text{-HC-3}$ into synaptosomes	182
4.3.6.2.1	Verification of the modified incubation medium	185
4.3.6.2.2	Incubation of synaptosomes with $[^{14}\text{C}]\text{-HC-3}$	185
4.3.6.3	Identity of the radioactive materials accumulated by synaptosomes incubated with $[^{14}\text{C}]\text{-HC-3}$	192
4.4	Discussion	192
4.4.1	HC-3	192
4.4.1.1	Structure	192
4.4.1.2	Action at the neuromuscular junction	194
4.4.1.3	Anticholinesterase activity	194
4.4.1.4	Mechanism of action of HC-3 in causing a prejunctional block at the neuromuscular junction	195
4.4.1.4.1	Inhibition of the supply of choline for ACh synthesis	195
4.4.1.4.2	Inhibition of and acetylation by ChAc <i>in vitro</i>	196
4.4.1.4.3	False transmitter hypothesis	197
4.4.1.4.3.1	Transport of HC-3 into cholinergic neurons	197
4.4.1.4.3.2	Acetylation of HC-3 in cholinergic neurons	198
4.4.1.5	Summary of results	199

4.4.2	Some diphenylalkane analogues of HC-3 and a methyl analogue of HC-15	199
4.4.2.1	Structure	199
4.4.2.2	Action at the neuromuscular junction	200
4.4.2.3	Anticholinesterase activity	200
4.4.2.4	Mechanism of action of the analogues in causing a prejunctional block at the neuromuscular junction	201
4.4.2.4.1	Inhibition of the supply of choline for ACh synthesis	201
4.4.2.4.2	Inhibition of and acetylation by ChAc <i>in vitro</i>	202
4.4.2.4.3	Inhibition of and acetylation by ChAc <i>in vivo</i>	202
4.4.2.5	Structure/activity relationships	203
4.4.2.6.	Summary of results.	213
5.	<u>Results Chapter II:- Some long-chain choline analogues</u>	215
5.1	Introduction	216
5.1.1	Historical Introduction	216
5.1.2	Long-chain choline analogues investigated and aims of study	217
5.2	Chemistry	218
5.2.1	Synthesis of choline and 3C-choline	218
5.2.2	Synthesis of 4C-choline	218
5.2.3	Synthesis of 5C-choline	218
5.2.4	Experimental	220
5.2.4.1	2-hydroxy- <u>N,N,N</u> -trimethyl-ethanaminium iodide ([2-hydroxyethyl]-trimethylammonium iodide; choline as an iodide salt)	220
5.2.4.2	3-hydroxy- <u>N,N,N</u> -trimethyl-propanaminium iodide (3-trimethylamino-1-propanol iodide; homocholine as an iodide salt; 3C-choline)	220

5.2.4.3.	4-hydroxy-N,N,N-trimethyl-butanaminium iodide (3-trimethylamino-1-butanol iodide; 4C-choline)	220
5.2.4.4	5-hydroxy-N,N,N-trimethyl-pentanaminium iodide (5-trimethylamino-1-pentanol; 5C-choline)	221
5.3	Pharmacology	222
5.3.1	Neuromuscular blocking action	222
4.3.1.1	Rat phrenic nerve-hemidiaphragm	222
5.3.1.2	Frog rectus abdominis muscle	222
5.3.2.	Anticholinesterase activity	226
5.3.3	<i>In vitro</i> acetylation by ChAc	226
5.3.4	<i>In vitro</i> inhibition of ChAc	226
5.3.5	Inhibition of the high affinity transport of choline into synaptosomes by the long-chain choline analogues	229
5.4	Discussion	232
5.4.1	Action at the neuromuscular junction	232
5.4.2	Anticholinesterase activity	234
5.4.3	Mechanism of action of the long-chain choline analogues in causing a prejunctional block at the neuromuscular junction	234
5.4.3.1	Inhibition of the high affinity transport of choline into synaptosomes	234
5.4.3.2	Inhibition of and acetylation by ChAc <i>in vitro</i>	234
5.4.3.3.	False transmitter hypothesis	235
5.4.4.	Conclusions	235

6.	<u>Results Chapter III:- Some hydroxypiperidinium and hydroxypyridinium analogues of choline</u>	237
6.1	Introduction	238
6.2	Chemistry	241
6.2.1	Synthesis of the hydroxypiperidinium and hydroxypyridinium analogues	241
6.2.2	Synthesis of the acetoxypiperidinium analogues	241
6.2.3	[¹⁴ C]-3-hydroxypiperidinium	241
6.2.3.1.	Synthesis	241
6.2.3.2	Specific activity and purity	241
6.2.3.2:1	Specific activity	241
6.2.3.2.2	Radiochemical purity	242
6.2.3.2.3	Chemical purity	242
6.2.4	Experimental	242
6.2.4.1	3-hydroxy-N,N-dimethylpiperidinium iodide (3-hydroxypiperidinium)	242
6.2.4.2	2-hydroxymethyl-N,N-dimethylpiperidinium (2-hydroxymethylpiperidinium)	243
6.2.4.3	3-hydroxymethyl-N,N-dimethylpiperidinium (3-hydroxymethylpiperidinium)	243
6.2.4.4	4-hydroxy-N,N-dimethylpiperidinium iodide (4-hydroxypiperidinium)	243
6.2.4.5	5-hydroxy-2-methyl-N-methylpyridinium iodide (hydroxypyridinium)	244
6.2.4.6	3-acetoxy-N,N-dimethylpiperidinium iodide (3-acetoxypiperidinium)	244
6.2.4.7	3-methacetoxy-N,N-dimethylpiperidinium iodide (3-methacetoxy piperidinium)	245
6.2.4.8	4-acetoxy-N,N-dimethylpiperidinium iodide (4-acetoxypiperidinium)	245
6.2.4.9	Synthesis of 3-hydroxy-N-[¹⁴ C]methyl-N-methylpiperidinium iodide ([¹⁴ C]-3-hydroxypiperidinium)	246
6.2.4.9.1	Reaction with non radioactive methyl iodide	246
6.2.4.9.2	Reaction with radioactive [¹⁴ C]-methyl iodide	247

6.3	Pharmacology	248
6.3.1	Neuromuscular blocking action	248
6.3.1.1	Rat phrenic nerve-hemidiaphragm preparation	248
6.3.1.2	Frog rectus abdominis muscle	248
6.3.2	Anticholinesterase activity	251
6.3.3	<i>In vitro</i> acetylation by ChAc	251
6.3.4	<i>In vitro</i> inhibition of ChAc	256
6.3.5	Transport of [³ H]-choline and [¹⁴ C]-3-hydroxypiperidinium into synaptosomes	258
6.3.5.1	Inhibition of the high affinity transport of choline by the hydroxypiperidinium and hydroxypyridinium analogues	258
6.3.5.2	Transport of [¹⁴ C]-3-hydroxypiperidinium into synaptosomes	258
6.3.5.3	Identification of the radioactive materials accumulated by synaptosomes	267
6.3.6	Mouse phrenic nerve-hemidiaphragm preparation	267
6.3.6.1	Introduction	267
6.3.6.2	Verification of method	270
6.3.6.3	Incubation with [¹⁴ C]-3-hydroxypiperidinium	273
6.3.7	Pharmacological actions of the acetoxypiperidinium compounds	276
6.3.7.1	Guinea-pig ileum	276
6.3.7.2	Frog rectus abdominis muscle	280
6.3.7.3	Degradation of the acetoxypiperidinium compounds by cholinesterase enzymes	280
6.4	Discussion	280
6.4.1	Action at the neuromuscular junction	280
6.4.2	Anticholinesterase activity	281
6.4.3	Mechanism of action of the hydroxypiperidinium analogues in causing a prejunctional block at the neuromuscular junction	281

6.4.3.1.	Inhibition of the high affinity of transport of choline into synaptosomes	281
6.4.3.2	Inhibition of and acetylation by ChAc <i>in vitro</i>	282
6.4.3.3	False transmitter hypothesis	282
6.4.3.3.1	3-hydroxypiperidinium	282
6.4.3.3.1.1	Transport into synaptosomes	283
6.4.3.3.1.2	Metabolism by synaptosomes	284
6.4.3.3.1.3	Mouse phrenic nerve-diaphragm preparation	284
6.4.3.3.1.4	Conclusion	286
6.4.3.3.2	2-hydroxymethylpiperidinium, 3-hydroxymethylpiperidinium and 4-hydroxypiperidinium	286
6.4.4	Structure/activity relationships	286
6.4.5	Acetylated derivatives of the hydroxypiperidinium compounds	294
6.4.5.1	Guinea-pig ileum	294
6.4.5.2	Frog rectus abdominis muscle	298
6.4.5.3	Cholinesterase enzymes	298
6.4.6	Conclusions on the mechanism of action of the hydroxypiperidinium analogues in causing a prejunctional block at the neuromuscular junction	298
6.4.7	Summary of results	299
7.	<u>General discussion</u>	300
8.	<u>References</u>	303

TABLES

	Page
1.1	30
1.2	72
1.3	80
3.1	100
3.2	109
4.1	153
4.2	155
4.3	156
4.4	166
4.5	183
4.6	191
5.1	223
5.2	224
5.3	225
5.4	228
5.5	230
6.1	249
6.2	250
6.3	257
6.4	259
6.5	265
6.6	266
6.7	277
6.8	278
6.9	279
6.10	279

FIGURES

	Page
1.1	10
3.1	105
4.1	116
4.2	123
4.3	126
4.4	128
4.5	130
4.6	134
4.7	136
4.8	138
4.9	140
4.10	150
4.11	158
4.12	159
4.13	160
4.14	161
4.15	162
4.16	163
4.17	168
4.18	174
4.19	175
4.20	176
4.21	181
4.22	184
4.23	186
4.24	187
4.25	189

FIGURES (Cont.)

4.26	190
4.27	193
4.28	204
4.29	205
4.30	206
4.31	207-208
4.32	209
4.33	210
5.1	219
5.2	227
5.3	231
6.1	252
6.2	253
6.3	254
6.4	255
6.5	260
6.6	262
6.7	263
6.8	268
6.9	269
6.10	271
6.11	272
6.12	274
6.13	275
6.14	288
6.15	289
6.16	290
6.17	291
6.18	292

FIGURES (Cont.)

6.19

296

6.20

297

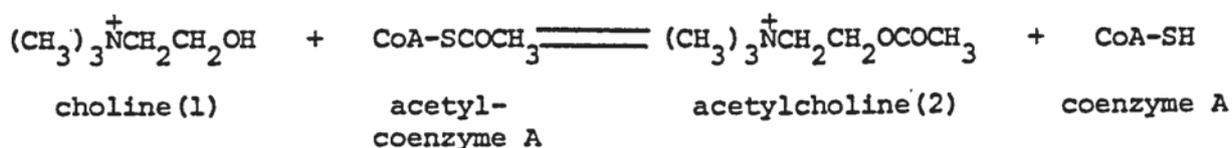
1. INTRODUCTION

1.

INTRODUCTION

1.1. CHOLINE AND THE SYNTHESIS OF ACETYLCHOLINE IN CHOLINERGIC NEURONS

Choline(1) is the trivial name for (2-hydroxyethyl)-trimethylammonium chloride. Chemical Abstracts, however, names this compound 2-hydroxy-N,N,N-trimethyl-ethanaminium. Choline(1) is an essential substrate for the synthesis of acetylcholine (ACh)(2) in neurons. The last stage of ACh(2) synthesis proceeds according to the equation:-



The synthesis is catalysed by the enzyme now known as choline acetyltransferase (ChAc, acetylcoenzyme A:choline-O-acetyltransferase, EC2.3.1.6.), Nachmansohn and Machado, (1943).

ACh serves as the transmitter of nerve impulses at cholinergic synapses (for a review see Kuhar, 1976). In man and homeothermic animals ACh effects transmission from motor nerves to skeletal muscle, from preganglionic parasympathetic and sympathetic fibres to neurons in the autonomic ganglia and adrenal medulla, from postganglionic parasympathetic fibres to the heart, and from postganglionic parasympathetic and some postganglionic sympathetic fibres to the glandular and smooth muscle cells. ACh is also the transmitter at some synapses in the central nervous system. A certain (but varying) quantity of ACh is released from the presynaptic nerve ending by each nerve impulse.

The released ACh diffuses into the synaptic gap and reacts with the cholinergic receptor protein, located in the postsynaptic membrane. The reaction with the receptor starts the chain of events resulting in the excitation or inhibition of the postganglionic cell.

Although choline is, chemically, a simple compound and is essential for the life of cells, it appears that the cells of nervous tissue are unable to synthesise it. The main source of choline in the animal body is the liver, (for a review of choline synthesis see Ansell and Spanner, 1975), where choline is synthesised not as a free compound, but as the choline moiety of phosphatidylcholine. Choline is released from phosphatidylcholine mainly via the steps lysophosphatidylcholine to glycerylphosphorylcholine to choline. Choline circulates in the blood and is supplied to the brain both as free choline and as choline bound in phospholipids. Additional free choline may be formed in the brain from phosphatidylcholine by the pathway described above. It is the free choline, found in the plasma bathing the nerve cells, which is utilised in the synthesis of ACh.

Most investigators have reported the plasma concentrations of unesterified choline in various animal species to be in the range 5-25 μ M (for a review see Tuček, 1978). However, Spanner, Hall and Ansell (1976) found that the choline concentrations in rat and human blood plasma are less than 1 μ M. The choline concentration in the cerebrospinal fluid of various animals has been found to be between 0.4 and 5.1 μ M (Tuček, 1978). The concentration of free choline in the rat brain has been found by Mann and Hebb (1977) to be 8.7nmol/g in the cerebrum and 14.9nmol/g in the cerebellum.

The problem now arises as to how the free choline, a positively charged molecule, present in the plasma bathing all cells gains access

to the site of ACh synthesis which takes place inside the nerve cells. It appears that most cells have the ability to transport choline. A specific carrier mediated transport system for the uptake of choline has been described in the giant axons of the squid (Hodgkin and Martin, 1965), brain slices (Schubeth, Sundwall, Sörbo and Lindell, 1966) and in nerve endings (Potter, 1968). Choline transport has also been reported in kidney slices (Sung and Johnstone, 1965), erythrocytes (Martin, 1968), heart (Buterbaugh, Figge and Spratt, 1968), small intestine (Sanford and Smyth, 1969), cervical ganglion (Collier and MacIntosh, 1969) and in the diaphragm (Chang and Lee, 1970 and Potter 1970). In general the transport systems show a varying requirement for sodium ions, exhibit Michaelis-Menten kinetics and are inhibited by drugs such as hemicholinium-3.

Yamamura and Snyder (1972 and 1973) and Haga and Noda (1973) showed that there are at least two transport systems for choline in the membranes of brain cells, differing in their affinity for the transported substance. This has been confirmed by many other workers (for a review see Kuhar and Murrin, 1978). The affinity of the carrier for the transported substance may be expressed by the value K_T (the apparent Michaelis constant for transport and corresponds to the concentration of substrate which provides one half of the maximal velocity of transport available). The high affinity transport of choline into brain synaptosomes has a $K_T < 10\mu\text{M}$ while the low affinity transport system has a K_T of $>10\mu\text{M}$ (usually between 40-100 μM). The high affinity carrier differs from the low affinity carrier by its greater dependence on the presence of sodium ions, by its greater sensitivity to

metabolic inhibitors, e.g. cyanide, dinitrophenol and ouabain (although results are conflicting) and by its greater sensitivity to the inhibitory action of hemicholinium-3.

Kuhar, Sethy, Roth and Aghajanian (1973) have shown in rat brain that the high affinity transport system is a feature of cholinergic nerve terminals only, as was originally suggested by Haga (1971). Chang and Lee (1970) made a similar observation in the rat phrenic nerve-hemidiaphragm. Therefore, it seems that the high affinity transport system for choline is specific for cholinergic nerve terminals whereas the low affinity transport system is probably present in all nerve cells and glial cells.

The low affinity transport of choline into synaptosomes most probably represents an example of facilitated diffusion, i.e. of carrier mediated transfer proceeding along the electrochemical gradient independent of an energy supply. The high affinity transport of choline could either be sodium dependent active transport (i.e. transport against the electrochemical gradient and dependent upon a supply of energy) or sodium dependent facilitated diffusion. None of the studies on choline transport to date have analysed this problem.

It has been suggested that for the synthesis of ACh cholinergic neurons can use both free choline, supplied by the extracellular plasma bathing the neuron and endogenous choline released inside the neuron from larger molecules (e.g. phospholipids), Collier, Poon and Salemoghaddam (1972). However Aquilonius, Flentge, Schuberth, Sparf and Sundwall (1973) administered IV radioactively labelled choline and found that the labelled atoms very rapidly appear in the brain ACh, so rapidly that it seemed as though free choline in the blood plasma is

used for the synthesis of ACh immediately it enters the cholinergic neurons. Collier and Katz, (1974) found that 50% of the choline formed as a product of cholinesterase enzyme action on neurally released ACh was re-utilized for the synthesis of new ACh. This evidence suggests that the high affinity carrier for choline in the membranes of the cholinergic nerve terminals plays an important if not an entirely specific role in ensuring the supply of choline for the synthesis of ACh.

Therefore it seems that free choline is transported into cholinergic nerve terminals by a low and high affinity transport system. Once inside the nerve terminals it can be acetylated by ChAc to ACh, the acetyl group being supplied by acetylcoenzyme A (AcCoA).

The properties of ChAc have been recently reviewed by Haubrich (1976), Bradshaw (1976) and Rossier (1977). ChAc is a water soluble globular protein and it has not yet been isolated in pure form. In some animal species ChAc is probably present in several molecular forms. ChAc is synthesised within cell bodies of cholinergic neurons and is subsequently transported along their axons to cholinergic nerve terminals, (Hebb and Waites, 1956), where it is highly concentrated along with the neurotransmitter (Hebb, Krnjević and Silver, 1964). Most studies indicate that the reaction catalysed by ChAc, that is the synthesis of ACh, follows the Theorell - Chance mechanism. AcCoA binds first followed by choline, the first product that is released is ACh followed by CoA. Evidence available indicates that one of the active groups at the active centre of the enzyme is imidazole. A thiol group is also present in the active centre, but it does not directly participate in the binding of reaction substrates or products.

The enzyme is not strictly specific for AcCoA and choline as a number of compounds with a similar structure can also serve as its substrates. The activity of ChAc is lower in media of low ionic strength. Most observations support the view that the presence of ChAc is specific for cholinergic neurons. However, extraneural ChAc is present in the placenta of some species, sperm, corneal epithelium and possibly also skeletal muscles (Tuček, 1973) and blood platelets.

AcCoA supplies the acetyl group for the synthesis of ACh. For a review see Barker (1976) and Browning (1976). Quantitatively the most important sources of AcCoA in the cells are oxidative decarboxylation of pyruvate and the β -oxidation of fatty acids; in both cases the AcCoA is formed in the mitochondria. It is not clear how the acetyl groups of the AcCoA pass out of the mitochondrial matrix into the cytoplasm where the synthesis of ACh is thought to occur.

Although some ACh is synthesised in all parts of the cholinergic neuron, most of it is produced in close proximity to the sites from which it is released, i.e. in most neurons, the terminal parts of the axon. ChAc is mainly dissolved in the cytoplasm of the nerve endings, but a proportion of it could be adsorbed to the cytoplasmic side of the surface membrane and of other membranes in the nerve terminals. ACh appears to be localised in the cytoplasm and some is membrane bound. Most features of ACh storage and release in synapses can be explained by the 'vesicular hypothesis' although there are objections to this hypothesis. For a review see Jones (1975). It should be noted here that Gray (1977) has proposed that synaptic vesicles are merely artifacts of the preparative procedure for electron microscope work.

Haubrich and Chippendale (1977) have reviewed the regulation of ACh synthesis in nervous tissue. Continuous slow synthesis of ACh proceeds in the cholinergic neurons even at 'rest'. When the rate of ACh release is increased by nerve impulses the rate of synthesis is also increased so that the concentration of ACh is relatively constant under physiological conditions (Collier and MacIntosh, 1969). Recently synthesised ACh is preferentially released during synaptic activity (Collier, 1969; Potter, 1970). Several hypotheses have been proposed to explain the mechanism of the control of synthesis. These include control based on the law of mass action, i.e. feed back inhibition of the rate of synthesis by the products (ACh and/or AcCoA). It has also been suggested by Guyenet, Lesfresne, Rossier, Beaujouan and Glowinski (1975) and others, (see Haubrich and Chippendale, 1977) that there is a close functional link between the high affinity choline transport and the enzyme ChAc, and that the high affinity transport of choline plays an important role in the regulation of ACh synthesis. This view is in good agreement with experimental results showing the presence of membrane bound ChAc in the nerve terminal and results indicating the re-utilization of choline which has been formed by the action of cholinesterase enzymes on neurally released ACh. (Collier and MacIntosh, 1969; Potter, 1970; Collier and Katz, 1974).

Postsynaptically, choline has qualitatively similar pharmacological actions to ACh but is much less active (Goodman and Gilman, 1970).

In summary, choline plays an important role in the synthesis of ACh. Free choline is transported from the extracellular fluid into cholinergic neurons by a low affinity and a sodium dependent high affinity transport process. Once inside the nerve terminal it can be acetylated by the enzyme ChAc to ACh. The necessary acetyl group being

supplied by AcCoA. ACh so formed can then be released as the cholinergic transmitter. It is possible that a close functional link exists between the sodium dependent high affinity transport of choline and the synthesis of ACh.

1.2 ANALOGUES OF CHOLINE

1.2.1 Hemicholinium-3

Long and Schueler, (1954) and Schueler, (1955) reported an investigation into the anticholinesterase activity of a number of bis-quaternary ammonium salts. One of the compounds, originally called α, α' -dimethylethanolamino-4,4'-biacetophenone (now known as seco-HC-3(3)), had little anticholinesterase activity but it was found to exhibit an unusual type of delayed toxicity in laboratory animals. Seco-HC-3(3) contains two choline like moieties and Schueler, (1955) showed that it underwent spontaneous hemiacetal formation (Fig. 1.1) to give a compound (4) with four choline like moieties. Schueler then coined the name hemicholinium and the cyclic form of seco-HC-3(3) has since been known as hemicholinium-3 or HC-3(4). Chemical Abstracts, however, names this compound 2,2'-[1,1'-biphenyl] 4,4'-diylbis[2-hydroxy-4,4-dimethyl morpholinium] dibromide. (4).

The keto-hemiacetal (open chain-ring) tautomerism which gives the cyclic hemiacetal structure(4) is common in tertiary aminoalcohols (Cromwell and Tsou, 1949) and it has been reported for quaternary amines (Lutz and Jordan, 1949). Haarstad and Schueler (unpublished data, cited by DiAugustine and Haarstad, 1970) have provided direct evidence of the instability of seco-HC-3(3) and the persistence of HC-3(4) in the cyclic form, in aqueous and alcoholic solutions.

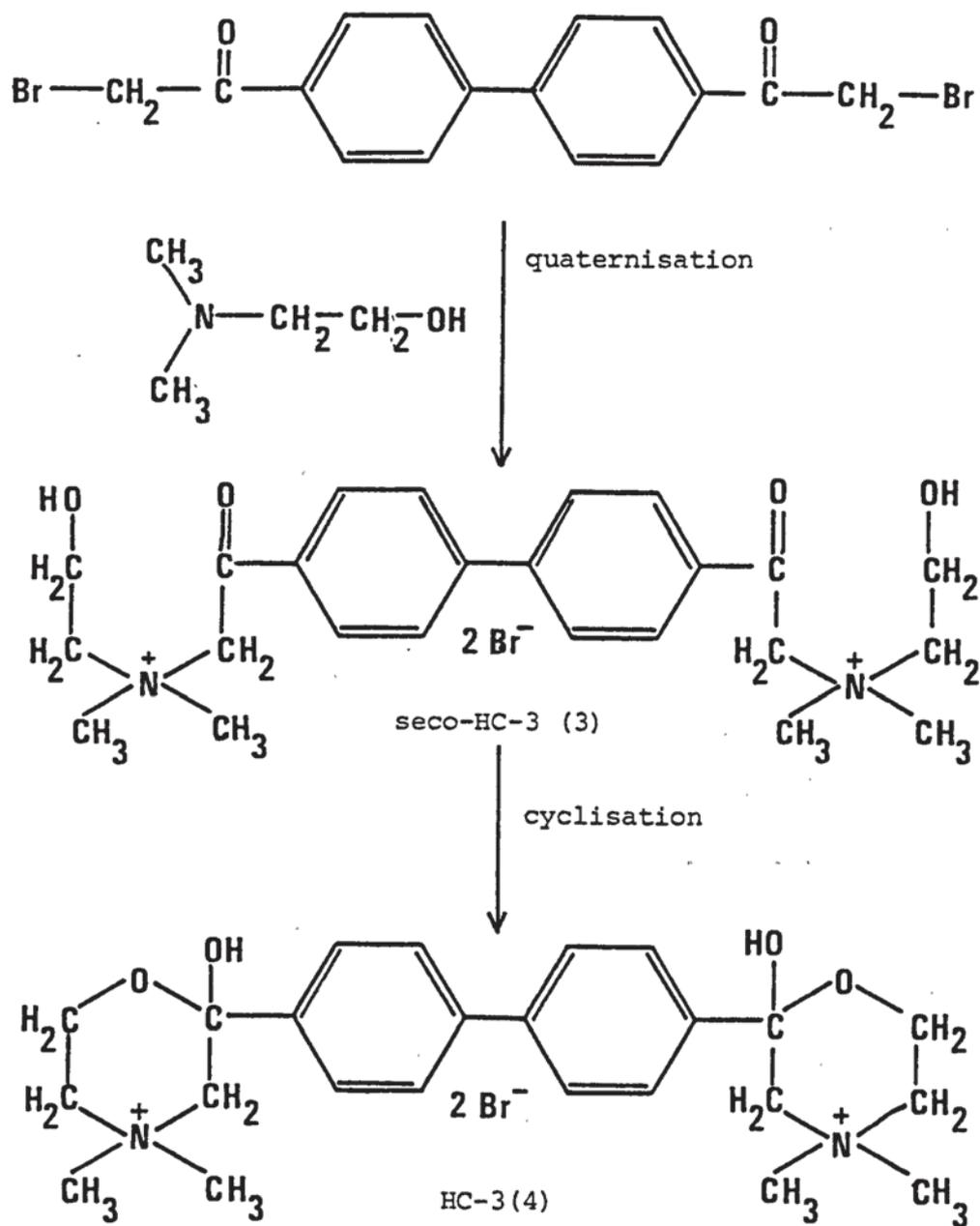


Fig. 1.1:- Cyclisation of hemicholinium-3

HC-3 was crystallised from ethanol/ether (Long and Schueler, 1954) giving colourless crystals with mp 179-180°. However, Long (1961) stated an alternative mp of 220° which he suggested was due to either a different isomer or a different crystalline form, although biological assay indicated no difference in the activity. HC-3, like most quaternary amines, was very hygroscopic, especially when first made and it has been found to crystallize in variable hydration states (unpublished data cited by Thampi, Domer, Haarstad and Schueler, 1966). The water of crystallization was very difficult to remove.

Most of the pharmacological studies on HC-3 have been carried out on whole animals, nerve-muscle preparations and cholinesterase enzymes. Very little biochemical work has been done. This is because the interest in HC-3 was at its highest in the 1950's to 1960's, and the techniques now available in the 1970's, especially the radiochemical techniques, were then unavailable.

In all species the onset of action of HC-3 is rather slow. With minimal lethal IV doses of HC-3, death results from a gradual failure of respiration. In general the larger the animal, the larger the minimal lethal dose on a body weight basis, and the longer the time to death (Schueler, 1955). This is an unusual observation, as with most drugs toxicity is greatest in larger animals because they metabolise drugs more slowly. Very large doses of HC-3 produce immediate respiratory paralysis which, like that produced by tubocurarine, is reversed by physostigmine (Schueler, 1955). A curare-like action of HC-3 might be expected from its structure, and the fact that large doses do produce this type of action has been confirmed using nerve-muscle preparations (Reitzel and Long, 1959b; Bowman and Rand, 1961; Marshall, 1969; Takagi,

Kojima, Nagata and Kuromi, 1970). However, the delayed respiratory paralysis produced by small doses is only slightly, or not at all reversed by anticholinesterase drugs (Schueler, 1955; Thies and Brooks, 1961), suggesting that it is due to a different mechanism from that giving rise to the abrupt paralysis with large doses. The delayed toxicity of HC-3 is however markedly antagonised by choline (Schueler, 1955; Giovinco, 1957; Thies and Brooks, 1961) but its abrupt curare-like effect is not.

Many studies (Schueler, 1955; Reitzel and Long, 1959b; Wilson and Long, 1959; Chang and Rand, 1960; Evans and Wilson, 1964; Gardiner and Sung, 1969; Marshall, 1969; Takagi, Kojima, Nagata and Kuromi, 1970; DiAugustine and Haarstad, 1970; Maggio-Cavaliere, 1976) have shown that small doses of HC-3 both *in vivo* and *in vitro* produce a slowly developing block of neuromuscular transmission in nerve-skeletal muscle preparations of guinea pigs, rats, rabbits, cats, dogs and chickens. The block is dependent on the frequency of stimulation of the motor nerve; in general small doses are ineffective at frequencies of stimulation below 1 Hz and effectiveness increases as the frequency is increased. The neuromuscular block is antagonised by choline but not by physostigmine. Similar effects of HC-3 and choline have been demonstrated at some autonomic cholinergic neuro-effector junctions (Rand and Ridehalgh, 1965; Everett, 1968; Appel and Vincenzi, 1970) and in sympathetic ganglia (Birks and MacIntosh, 1961; Bhatnagar, Lam and McColl, 1965). Direct bioassay of the ACh released into the fluid bathing isolated nerve-muscle preparations shows that HC-3 reduces the ACh output evoked by nerve stimulation (Matthews, 1966), indicating that small doses of HC-3 produce a presynaptic block at the neuromuscular junction.

Intracellular recording techniques at the neuromuscular junction have confirmed that HC-3 possesses both pre- and postjunctional actions. Initial studies showed that HC-3 in large doses blocks neuromuscular transmission and reduces the amplitudes of end plate potentials (e.p.p.s.) and miniature end plate potentials (m.e.p.p.s.). These effects were associated with a reduced sensitivity to applied ACh, showing that the site of action was postjunctional. The block was reversed by physostigmine but not by choline (Martin and Orkand, 1961; Thies and Brooks, 1961)

Elmqvist and Quastel (1965) performed similar experiments except that the preformed transmitter was rapidly released either by nerve stimulation or by potassium chloride. Under these conditions the amplitudes of the e.p.p.s. and m.e.p.p.s. were again reduced by HC-3 but by lower concentrations and in this case the changes occurred in the absence of any decrease in postjunctional chemosensitivity, showing that the effect was located in the nerve endings and was due to a reduction in size of the ACh quanta.

These results suggest that the respiratory paralysis produced by low doses of HC-3 is due to a presynaptic block of transmission at the neuromuscular junctions of the respiratory muscles, and there is strong evidence to support this (Longo, 1958, 1959). However some workers have maintained that HC-3 also depresses respiration through an action in the central nervous system (Kasé and Borison, 1958; Borison, 1961).

Intraventricular administration of HC-3 causes a reduction in brain ACh levels which is prevented by previous administration of choline (Rodriguez de Lores Arnaiz, Zieher and De Robertis, 1970). Gomez, Domino and Sellinger (1970) found that intraventricular HC-3 reduced the synthesis of ACh but enhanced choline conversion to phosphorylcholine and cytidine

diphosphocholine. This observation was supported by Spanner and Ansell, (1973) but Ansell and Spanner (1974) found that *in vitro* HC-3 inhibited choline kinase the enzyme responsible for the conversion of choline to phosphorylcholine, suggesting that *in vivo* the increase of synthesis of phosphorylcholine by HC-3 was not due to a direct action on the enzyme. However, it is possible that intraventricular HC-3 somehow enhances the synthesis of phosphorylcholine at the expense of ACh thereby causing a depletion in transmitter stores, resulting in a failure of transmission of cholinergic neurons which could lead to respiratory depression.

However, a central action of HC-3 after systemic administration, seems unlikely in view of the well known inability of quaternary ammonium compounds to penetrate the 'blood-brain barrier'. This is supported by the findings of Domer and Schueler (1960) who synthesized radioactively labelled [^{14}C]-HC-3 and found that tissue distribution studies failed to show any sites of localisation in various organs including the brain.

It is important to note here that Domer and Schueler (1960) using [^{14}C]-HC-3 conducted 24 hr. metabolism studies with rats and found that HC-3 is rapidly excreted in the urine and faeces. There appears to be no biotransformation.

The dependence of the transmission failure produced by HC-3 on the frequency of nerve stimulation suggests an explanation of the variation in toxicity among different species; toxicity is greatest and quickest in onset in the smaller animals, possibly because small animals breath at a faster rate than large animals. However, other factors probably also play a part and obvious possibilities are the activity of the animals and the circulating level of free choline which is an antagonist to the action of HC-3.

Several analogues of choline have been tested for their ability to reverse the neuromuscular transmission failure produced by small doses of HC-3 (Giovinco, 1957; Reitzel and Long, 1959a). All were ineffective except for N,N dimethyl,N,ethyl-aminoethan-2-ol (MEC) (27). ACh and some other esters of choline were also effective antagonists but only by virtue of their rapid hydrolysis with the production of choline.

The specific antagonistic action of choline suggested that HC-3 causes transmission failure at cholinergic junctions by interfering with choline metabolism in nervous tissue which leads to a decreased synthesis, and hence a decreased output of ACh. Choline, however, possesses a weak postjunctional depolarising action at the neuromuscular junction and it has been reported by Hutter, (1952) to have some action in increasing the release of preformed ACh. Both of these additional effects could contribute to restoration of the transmission failure. Therefore it is important to realize that reversal by choline is not by itself a reliable indication of a prejunctional block, although if the reversal is complete and long lasting it must be a strong indication that the transmission failure did arise from such a mechanism.

HC-3 was found by MacIntosh, Birks and Sastry, (1956) to inhibit the synthesis of ACh in the superior cervical ganglion of the cat and in minced brain tissue, but it had negligible direct inhibitory action on ChAc obtained from acetone-dried brain powders. MacIntosh, Birks and Sastry, (1958) postulated that HC-3 inhibited ACh synthesis in intact nervous tissue by competing with choline for the specific system necessary to transport extracellular choline to the intracellular sites of acetylation. Gardiner, (1961) supported this suggestion when he found that HC-3 inhibited ACh synthesis in minced guinea-pig brain and by

mitochondrial fractions of homogenates of the same tissue. This inhibition was antagonised by choline. However, ACh synthesis was unimpaired by HC-3 in homogenates which had been pretreated with ether. Ether is believed to disrupt membranes surrounding the synthesising enzyme, ChAc, (Hebb and Smallman, 1956), thus in the latter case ChAc is solubilized.

These observations have since been supported by several workers. HC-3 has been shown to inhibit ACh synthesis in minced mouse brain (Bhatnagar and MacIntosh, 1961; MacIntosh, 1961 and Bhatnagar, Lam and McColl, 1965) and in guinea-pig brain homogenates (Gardiner and Sung, 1969; DiAugustine and Haarstad, 1970 and Hemsworth, 1971a). There was no significant inhibition by HC-3 of solubilized ChAc from mouse brain (Bhatnagar, Lam and McColl, 1965; Domino, Mohrman, Wilson and Haarstad, 1973 and Hemsworth and Cholakis, 1978) and of ChAc solubilized from guinea-pig brain (Hemsworth, 1971a).

HC-3 has been found to inhibit the active secretion of choline by avian kidney tubules (MacIntosh, Birks and Sastry, 1958; MacIntosh, 1961; Trimble, Acara and Rennick, 1974) and to inhibit choline transport into erythrocytes (Martin, 1968, 1969), giant axon of the squid (*Loligo*) (Hodgkin and Martin, 1965), isolated perfused rabbit hearts (Buterbaugh, Figge and Spratt, 1968) and isolated synaptosomes (Marchbanks, 1968; Potter 1968; Diamond and Kennedy, 1969; Hemsworth and Bosmann, 1971; Hemsworth, Darmer and Bosmann, 1971; Diamond and Milfay, 1972; Guyenet, Lefresne, Rossier, Beaujouan and Glowinski, 1973a; Barker and Mittag, 1975; Simon, Mittag and Kuhar, 1975; Collier, Lovat, Ilson, Barker and Mittag, 1977; Hemsworth and Cholakis, 1978) and isolated synaptic vesicles (Hemsworth, Darmer and Bosmann, 1971).

These results support the original hypothesis of MacIntosh, Birks and Sastry (1958) that HC-3 inhibits ACh synthesis in intact nervous tissue by competing with choline for the choline transport system and thereby inhibiting the transport of choline into cholinergic nerve terminals.

Although it appears that *in vitro*, HC-3 does not inhibit soluble ChAc it was suggested that, like choline, HC-3 could itself act as a substrate for ChAc and be acetylated (Rodriguez de Lores Arnaiz, Zieher and De Robertis, 1970; Hemsworth, 1971a). This could be an alternative explanation of the presynaptic inhibitory action of HC-3 at the neuromuscular junction, as acetylated HC-3 could be released as a false transmitter by the cholinergic nerve.

However, Diamond and Milfay (1972) could find no evidence for the acetylation of HC-3 by ChAc *in vitro*. But Bradshaw and Hemsworth (1976) found that the method of isolation of the acetylated HC-3 used by Diamond and Milfay (1972) was at fault and that HC-3 is acetylated by soluble ChAc isolated from rat brain by about 27% compared to choline: 100%. Barker and Mittag (1975) and Mann and Hebb (1975) have also found that HC-3 is acetylated by ChAc, the latter workers found that HC-3 is a competitive substrate.

In order for HC-3 to be acetylated by ChAc *in vivo* it must, like choline, be transported from the extracellular medium into the cholinergic nerve terminal. Collier (1973) performed experiments with [^{14}C]-HC-3 on the cat superior cervical ganglion and failed to show the transport of HC-3 into the ganglion during nerve stimulation. He also found that compared to choline very little [^{14}C]-HC-3 is transported into mouse synaptosomes. However Guyenet, Lefresne, Rossier, Beaujouan and Glowinski

(1973b) showed that in rat synaptosomes HC-3 had an affinity for the carrier mechanism which was one hundred times greater than the affinity of choline itself. Sellinger, Domino, Haarstad and Mohrman, (1969) administered [¹⁴C]-HC-3 intraventricularly and they found that mitochondrial filled nerve endings, containing 30% of the acetylcholinesterase (AChE), had a high affinity for HC-3. Slater and Stonier, (1973) found that rat brain cortex slices accumulated HC-3 but it appeared to be by a different uptake mechanism compared to that of choline. These results seem to indicate that HC-3 can be transported into cholinergic neurons.

In conclusion the hypothesis proposed by MacIntosh, Birks and Sastry, (1958) is that the main action of HC-3 is to compete with choline for the transport mechanism in the nerve terminals with the result that ChAc is deprived of its substrate for ACh synthesis. Transmission failure becomes evident when the preformed stores of ACh have been partially exhausted by frequent nerve impulses and when synthesis is inhibited to the extent that it cannot keep up with demand. Excess choline then probably overcomes the transmission failure by competing more favourably with HC-3 for the transport mechanism and so restoring substrate to synthesising enzyme.

However, over the last decade it has been shown that HC-3 can itself act as a substrate for ChAc *in vitro*. If this occurs *in vivo*, the acetylated-HC-3 could be released as a false neurotransmitter and so attenuate the natural transmitter (ACh) released at the neuromuscular junction. An increase in the frequency of the nerve impulses would increase the rate of transport of HC-3 into the cholinergic nerve and the rate of acetylation of HC-3. Choline would antagonise this process. However, it has yet to be shown conclusively that HC-3 can be transported into intact

cholinergic nerve tissues. The false transmitter hypothesis provides an additional mechanism to that originally proposed by MacIntosh, Birks and Sastry, (1958).

1.2.2. Hemicholinium analogues

Several workers have studied structure-activity relationships in the hemicholinium series. Most of the studies have involved alterations at the cationic head, or in the biphenyl nucleus. For a summary of the hemicholinium analogues shown to have a presynaptic blocking action on cholinergic neurons see Table 1.1. Most of the pharmacological results obtained using the hemicholinium analogues have been from whole animals, isolated nerve muscle preparations and cholinesterase enzyme studies.

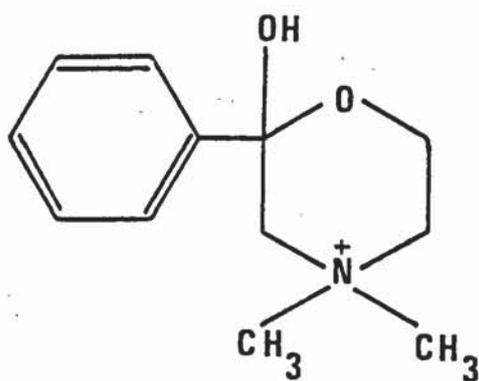
Schueler, (1955) showed that HC-15(5) which is exactly one half of the molecule of HC-3 exhibited no toxic activity in mice in doses up to 50mg/kg, (compared to LD₅₀ in mice of HC-3 of 0.02mg/kg). Bowman, Hemsworth and Rand, (1967) found that the primary action of HC-15 and other monoquaternary analogues of HC-3 on neuromuscular transmission is a postjunctional tubocurarine-like action. However, more recent studies have suggested that HC-15 may have a presynaptic action on cholinergic neurons. Hemsworth, (1971a) showed that HC-15 at low doses gives a presynaptic block in the rat phrenic nerve-hemidiaphragm when stimulated at 1Hz. He also showed that HC-15 inhibited ACh synthesis in guinea-pig brain homogenates but had no effect on synthesis where the membranes surrounding the ChAc are broken down (HC-3 had a similar action, Hemsworth, 1971a) Hemsworth, Darmer and Bosmann, (1971) showed that HC-15 is almost as active as HC-3 in inhibiting the uptake of [¹⁴C]-choline into synaptosomes. However, Diamond and Milfay, (1972); Barker and Mittag, (1975); Holden, Rossier, Beaujouan, Guyenet and Glowinski, (1975); and Simon,

Mittag and Kuhar, (1975) showed that HC-15 had a weaker inhibitory effect than HC-3, these results are probably more interesting as only the high affinity transport of choline was determined. HC-15 was found to be acetylated by soluble ChAc (Hemsworth, 1971a; Barker and Mittag, 1975) but at about half the rate of the acetylation of HC-3. In conclusion, HC-15 seems to have similar actions to HC-3 but is less potent.

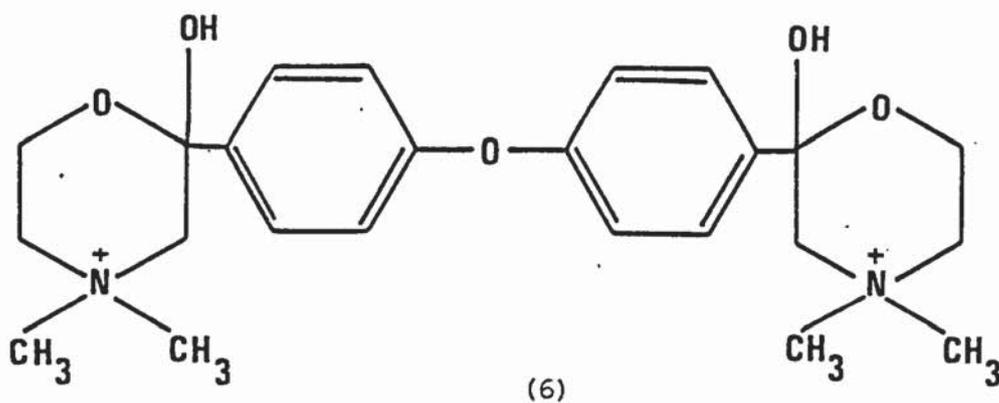
Marshall and Long, (1959) showed that the introduction of an ether (6) or methylene (7) linkage between the two phenyl rings, produced compounds which gave a presynaptic block but which were less potent than HC-3. Other studies have shown that the biphenyl moiety may be replaced by a hexamethylene chain (8) which approximates to the length of a biphenyl nucleus (Powers, Kruger and Schueler, 1962), one phenyl ring (norphenyl-HC-3) (9) (Thampi, Domer, Haarstad and Schueler, 1966) or three phenyl rings (terphenyl-HC-3) (10) (Gardiner and Sung, 1969) without appreciable loss of the characteristic HC-3 like activity in the nerve-muscle experiments conducted. Terphenyl-HC-3 (Gardiner and Sung, 1969) caused an HC-3 like blockade of neuromuscular transmission and inhibited ACh synthesis in guinea-pig brain mince (organised tissue). Barker and Mittag, (1975) showed that terphenyl-HC-3 was equipotent to HC-3 in inhibiting high affinity choline transport into synaptosomes.

However, the slight decrease in activity observed with most alterations of the biphenyl nucleus may indicate that in HC-3 the distance between the two quaternary nitrogens is optimal for maximal HC-3 like activity.

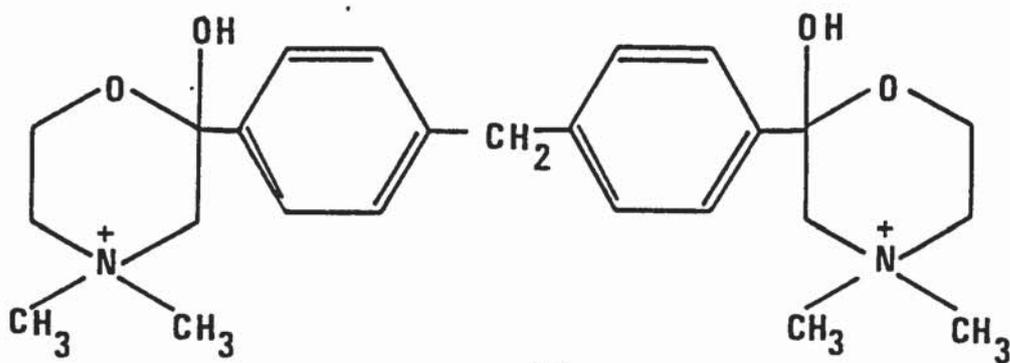
Schueler, (1955) in his original study found the characteristic HC-3 like activity, with regard to its toxic action in whole animals, only in those compounds containing an unmasked β -hydroxyl group with respect to the nitrogen centre. He concluded that the ethanolic group is necessary



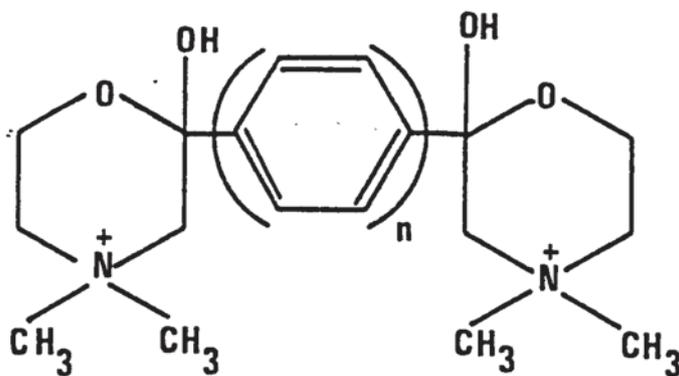
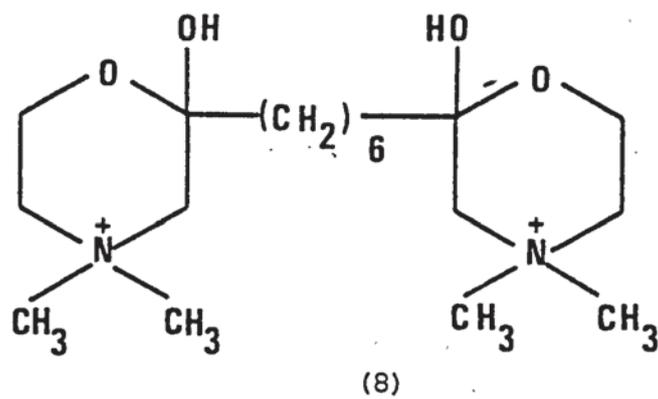
HC-15 (5)



(6)



(7)

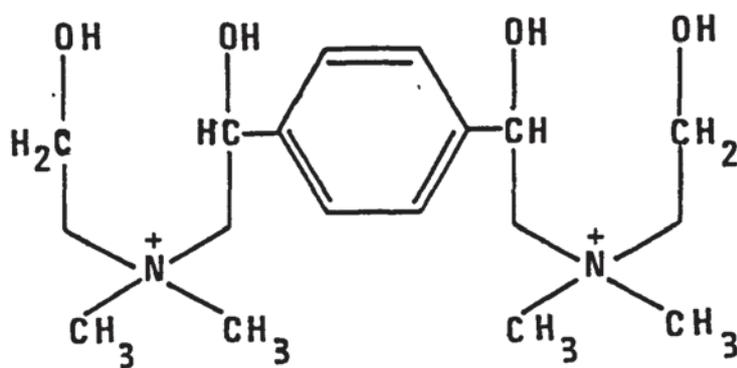
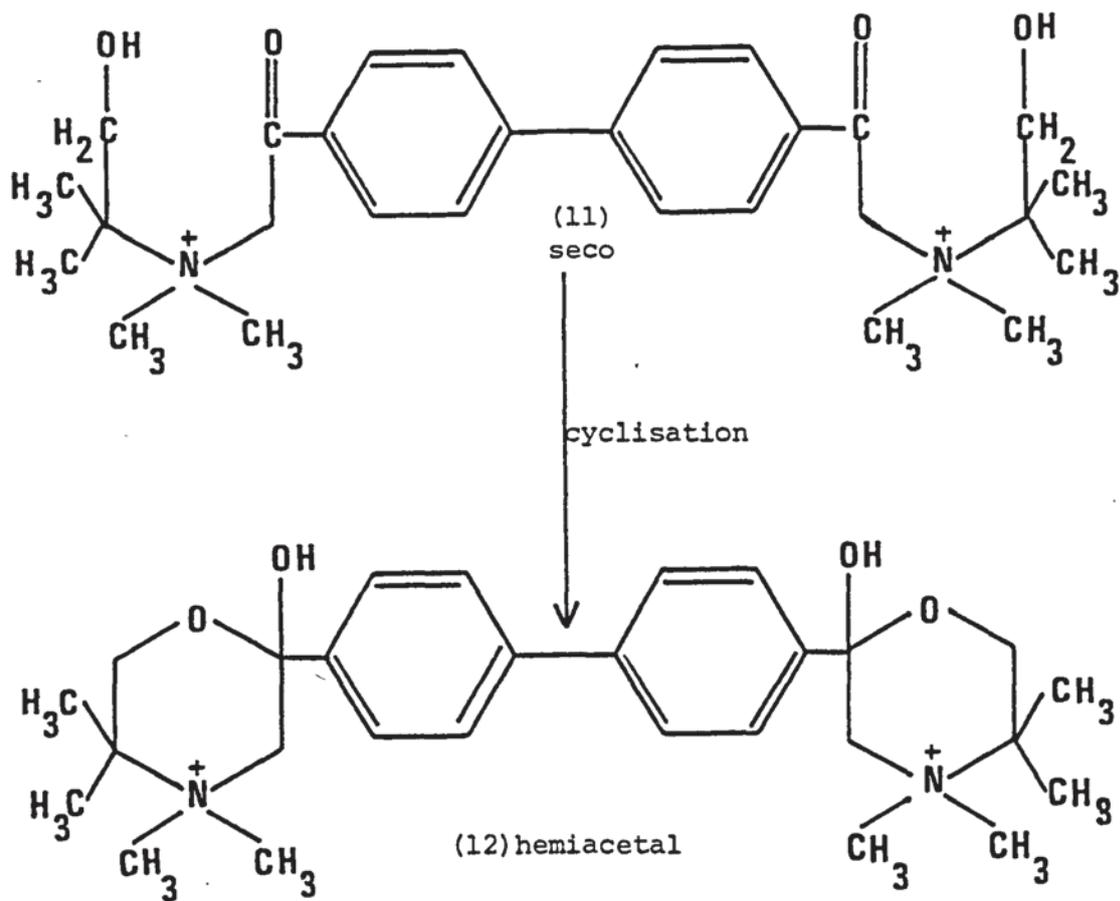


$n=1$ norphenyl-HC-3 (9)

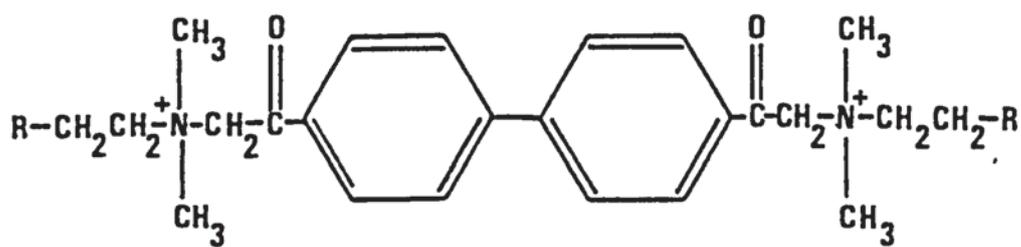
$n=3$ terphenyl-HC-3 (10)

but not sufficient and suggested that such compounds undergo hemiacetal formation (Fig. 1.1). Studies on α',α' , $\alpha\alpha$ -tetramethyl-HC-3 (Di Augustine and Haarstad, 1970) which can exist in both open chain (seco) (11) and cyclic hemiacetal forms (12), depending upon the experimental conditions, showed that the characteristic HC-3 like activity on nerve-muscle preparations is much more pronounced in the hemiacetal form (12). The open chain compound (11) inhibited ACh synthesis in minced mouse brain (organised tissue) to a lesser extent than the hemiacetal (12) though both had HC-3 like actions and both were less effective than HC-3. These workers concluded that the hemiacetal structure of this compound (12) is not essential for the inhibition of ACh biosynthesis but this structure appears to give a more potent HC-3 like compound on nerve muscle preparations and in inhibiting the synthesis of ACh.

Many open chain (seco) analogues of HC-3 (3) have been synthesised and some seem to have HC-3 actions. For example, Blase, Loomis, Collins and Sommer, (1974) found that an open chain analogue of norphenyl-HC-3 (13) still retained HC-3 like activity in the intact tibialis muscle preparation of the rat. Acetyl-seco-hemicholinium-3 (Ac-seco-HC-3) (14) is prevented from cyclising by the acetyl group which has replaced the hydrogen of the hydroxyl group on the ethanolic moiety, which is necessary for tautomerism to occur. Ac-seco-HC-3 has been studied by Domino, Mohrman, Wilson and Haarstad, (1973); Maggio-Cavaliere, (1976); Haarstad, Domer, Chihal, Rege and Charles, (1976); Domer, Chihal, Charles and Rege, (1977) and recently by Hemsworth and Cholakis, (1978). Ac-seco-HC-3 produced a toxicological picture similar to that of HC-3 and was first thought to be a ChAc inhibitor (Domino, Mohrman, Wilson and Haarstad, 1973). It depressed respiration, gave a slow progressive inhibition of neuromuscular transmission, which was reversible with choline



(13)



R = -OCOCH₃ Ac-seco-HC-3 (14)

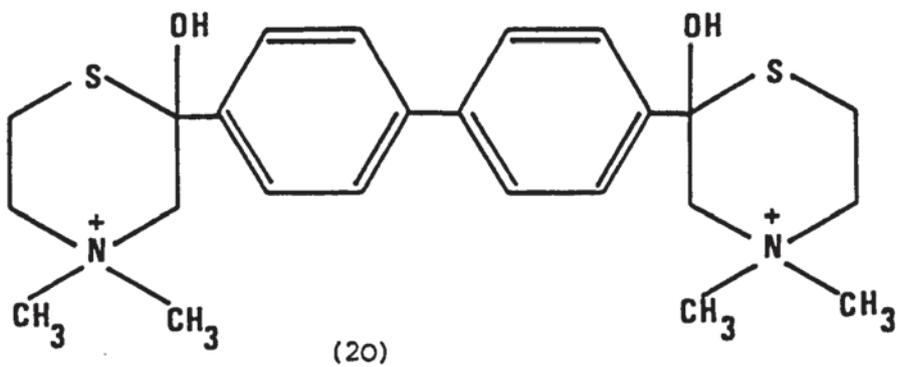
R = -OCH₂CH₃ (15)

R = -CH₂CO CH₃ (16)

R = -2CH₂CH₂CH₃ (17)

R = -S CO CH₃ thio-Ac-seco-HC₃ (18)

R = -SH thio-seco-HC₃ (19)



and inhibited the biosynthesis of ACh by minced mouse brain, all of which resemble the pharmacology of HC-3. Ac-seco-HC-3 has some characteristic actions of its own, (Maggio-Cavaliere, 1976). Hemsworth, and Cholakis, (1978) found that Ac-seco-HC-3 was de-esterified to a very small extent by cholinesterase (ChE) or acetylcholinesterase (AChE) and concluded that Ac-seco-HC-3 had an action of its own on neuromuscular transmission and that the pharmacological effects were not due to de-esterification of the compound to HC-3. This was supported by Haarstad, Domer, Chihal, Rege and Charles, (1976). Hemsworth and Cholakis, (1978) showed that Ac-seco-HC-3 was equipotent to HC-3 in inhibiting choline uptake into synaptosomes. They also showed that Ac-seco-HC-3 was equipotent to HC-3 in inhibiting synthesis of ACh in organised brain tissue and that both Ac-seco-HC-3 and HC-3 had no inhibitory action on ChAc. The different results with regard to ChAc inhibition compared to Domino, Mohrman, Wilson and Haarstad, (1973) was explained as being due to species difference and/or purity of ChAc. These results indicate that Ac-seco-HC-3 inhibits neuromuscular transmission by inhibiting the transport of choline into cholinergic nerve endings.

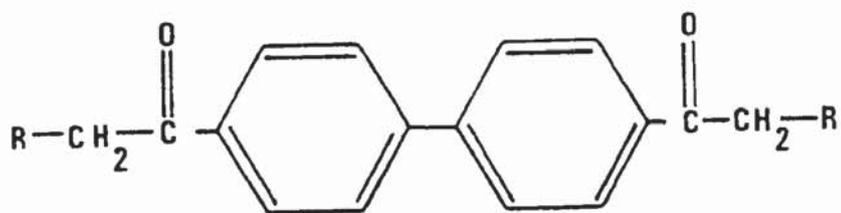
Other derivatives of HC-3 have been made (15, 16, 17, 18, 19,) by Haarstad, Domer, Chihal, Rege and Charles, (1976) and by Domer, Chihal, Charles and Rege, (1977) but little pharmacological study has been carried out. In toxicity studies all are much less toxic than HC-3 except for thio-Ac-seco-HC-3(18) which is equally toxic to HC-3 and Ac-seco-HC-3 and causes death by respiratory failure. It was found that thio-Ac-seco-HC-3(18) is broken down by ChE to a cyclic form of a sulphur analogue of HC-3(20). It was therefore suggested that thio-Ac-seco-HC-3(18) was active due to the action of its cyclic metabolite(20)

whereas thio-seco-HC-3(19) was less toxic as it was in an open chain form.

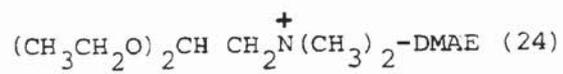
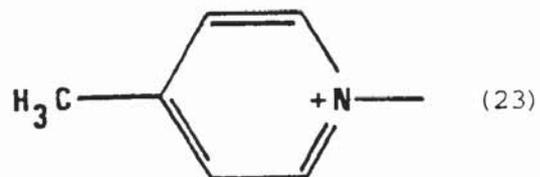
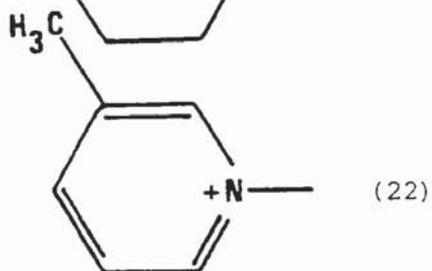
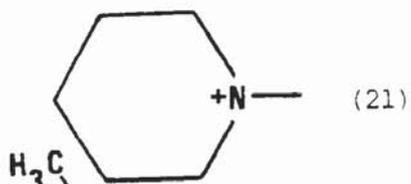
However, as open chain analogues of HC-3 have been found to inhibit ACh synthesis, it can be concluded that the cyclic hemiacetal form of the hemicholiniums is not essential for a presynaptic block at the cholinergic neuron, though HC-3(4) itself may act in this structural form.

In most studies, the effect of modifying the position of the β -hydroxyl groups and the alkyl substituents on the quaternary nitrogens of HC-3 produced marked changes in pharmacological activity (Schueler, 1955; Marshall and Long, 1959; Long, Evans and Wong, 1967). Long, Evans and Wong, (1967) were able to show different types of pharmacological activity by slightly varying the structure at the quaternary nitrogen. Long, Evans, and Wong, (1967) also noted that the N-methyl-piperidine analogue of HC-3 (21) exhibited HC-3-like activity on nerve-muscle preparations and toxicity studies. Subsequent studies (Benz and Long, 1969a, 1969b, 1970) have confirmed that various heterocyclic compounds lacking a β -hydroxyl substituent are active in this respect notably 3-methylpyridinium(22) and 4-methylpyridinium (23) derivatives. In addition Benz and Long, (1969b) have calculated that in the last three named compounds, the terminal methyl groups are separated from the quaternary nitrogen by a distance of 3.7 Å and have concluded that in the bisphenacyl quaternary salt an interonium distance of 14 Å is important for HC-3 like activity in neuromuscular preparations and toxicity studies and that this activity can be enhanced if the compound has a non-polar space-filling group 3.7 Å from the quaternary nitrogen.

Long, Evans and Wong, (1967) first synthesised α, α' -bis-(dimethyl-ammoniumacetaldehyde diethylacetal)-p,p'-diacetylbiphenyl bromide (DMAE) (24) and found that it resembled cocaine in that it potentiated



R =



catecholamines and sympathetic nerve stimulation. However, Chiou and Long, (1969) have shown that DMAE produces a biphasic block similar to that produced by tubocurarine followed by a secondary block of long duration which is reversible by choline. As the ED₅₀'s of the drugs are similar it was suggested that DMAE could be converted into HC-3 *in vivo*, with a concomitant inhibition of ACh synthesis. Volle, (1973) showed that DMAE reduced the quantal content released from a frog cholinergic nerve during rapid rates of stimulation and explained this in terms of a prejunctional action. However, he observed several differences between DMAE and HC-3 suggesting that DMAE has an action of its own on cholinergic neurons.

Many analogues of HC-3 have been synthesised and many have been shown to have a presynaptic blocking action of cholinergic neurons (see Table 1.1). In most cases insufficient research has been carried out to allow the precise site of action to be determined. The actual mechanism of action may vary from analogue to analogue, several analogues have been shown to inhibit choline transport, but some have a free hydroxyl group which could be acetylated by ChAc and the resultant acetylated product could then be released as a false transmitter. At the moment there is insufficient data to say that all the HC-3 analogues have an HC-3 like action, especially as there is some conflict over the precise mechanism of action of HC-3 itself.

1.2.3 Triethylcholine

Triethylcholine (TEC) (25) is the trivial name for N,N,N,-triethylethanolamine. Chemical Abstracts, however, names this compound, 2-hydroxy-N,N,N,-triethylethanaminium(25).

TABLE 1.1. PRESYNAPTIC ACTION OF ANALOGUES OF HC-3 AT THE CHOLINERGIC SYNAPSE



Analogue R =	Primary toxic action-respiratory depression	Presynaptic Block at Cholinergic nerve-muscle preparation (relative potency to HC-3)	Inhibition of ACh synthesis in brain homogenates	Inhibition of ACh synthesis by soluble ChAc	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes.
$\begin{array}{c} \text{CH}_3 \\ \\ +\text{N-CH}_2\text{CH}_2\text{OH}^1 \text{ (HC-3, (4))} \\ \\ \text{CH}_3 \end{array}$	Yes ³	1 ⁴	Yes ⁴	No ⁴	Yes ²¹	Yes ²²
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ +\text{N-CH}_2\text{CH}_2\text{OH}^1 \\ \\ \text{C}_2\text{H}_5 \end{array}$	Yes ³	0.81 ⁷				
$\begin{array}{c} \text{CH}_2\text{CH}_2\text{OH} \\ \\ +\text{N-CH}_2\text{CH}_2\text{OH}^1 \\ \\ \text{C}_2\text{H}_5 \end{array}$	Yes ³					
$\begin{array}{c} \text{CH}_3 \\ \\ +\text{N-CH}_2\text{CH}_2\text{OC(=O)CH}_3 \\ \\ \text{CH}_3 \end{array}$	Yes ³					

TABLE 1.1. (Continued)

Analogue R =	Primary toxic action-respiratory depression	Presynaptic Block at Cholinergic nerve-muscle preparation (relative potency to HC-3).	Inhibition of ACh synthesis in brain homogenates.	Inhibition of ACh synthesis by soluble ChAc.	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes.
$\begin{array}{c} \text{CH}_3 \\ \\ +\text{N}-\text{CH}-\text{CH}_2-\text{OH}^1 \\ \quad \\ \text{CH}_3 \quad \text{CH}_3 \end{array}$	Yes ^{7,8}	0.42 ^{7,8}				
$\begin{array}{c} \text{CH}_3 \quad \text{CH}_3 \\ \quad \\ +\text{N}-\text{CH}_2-\text{CH}-\text{OH}^1 \\ \quad \\ \text{CH}_3 \quad \text{CH}_3 \end{array}$	Yes ⁸	0.1 ⁸				
$\begin{array}{c} \text{CH}_3 \quad \text{CH}_3 \\ \quad \\ +\text{N}-\text{CH}_2-\text{C}-\text{OH}^1 \\ \quad \\ \text{CH}_3 \quad \text{CH}_3 \end{array}$	Yes ⁷	0.18 ⁷				
$\begin{array}{c} \text{CH}_3 \quad \text{CH}_3 \\ \quad \\ +\text{N}-\text{C}-\text{CH}_2-\text{OH}^2 \text{ (11,12)} \\ \quad \\ \text{CH}_3 \quad \text{CH}_3 \end{array}$	Yes ⁸	2.61 ⁷	Yes ⁸			
$\begin{array}{c} \text{CH}_3 \\ \\ +\text{N}-\text{CH}_2-\text{CH}_2-\text{OH}^1 \\ \\ \text{CH}_2-\text{CH}_2-\text{OH} \end{array}$	Yes ⁷	0.52 ⁷				

TABLE 1.1. (Continued)

Analogue R =	Primary toxic action-respiratory depression	Presynaptic Block at Cholinergic nerve-muscle preparation (relative potency to HC-3)	Inhibition of ACh synthesis in brain homogenates	Inhibition of ACh synthesis by soluble ChAc	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes.
$\begin{array}{c} \text{CH}_3 \\ \\ + \text{N}-(\text{CH}_2)_3\text{CH}_2\text{OH} \\ \\ \text{CH}_3 \end{array}$	Yes ⁷	0.05 ⁷				
-CH ₃		0.1 ⁹				
-CH ₂ CH ₃		0.33 ⁹				
-(CH ₂) ₂ -CH ₃		0.11 ⁹				
-(CH ₂) ₃ -CH ₃		0.05 ⁹				
-(CH ₂) ₄ -CH ₃		<0.03 ⁹				
-CH ₂ -CH=CH ₂		0.44 ⁹				

TABLE 1.1. (Continued)

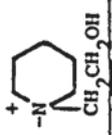
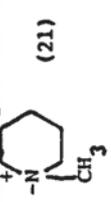
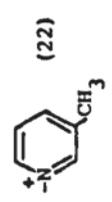
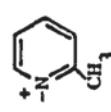
Analogue R =	Primary toxic action-respiratory depression	Presynaptic Block at cholinergic nerve-muscle preparation (relative potency to HC-3)	Inhibition of ACh synthesis in brain homogenates	Inhibition of ACh synthesis by soluble ChAc	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes
	Yes ⁷	0.52 ⁷				
 (21)	Yes ¹⁰	0.14 ^{7,10}				
	Yes ¹⁰	<0.03 ¹⁰				
 (22)	Yes ^{10,16}	0.87 ^{10,16}				
	Yes ¹⁰	<0.02 ¹⁰				

TABLE 1.1. (Continued)

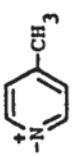
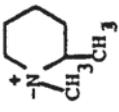
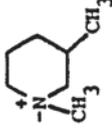
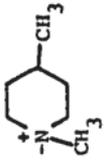
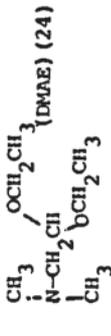
Analogue R =	Primary toxic action-respiratory depression	Presynaptic Block at cholinergic nerve-muscle preparation (relative potency to HC-3)	Inhibition of ACh synthesis in brain homogenates.	Inhibition of ACh synthesis by soluble ChAc	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes
 <p>(23)</p>	Yes ¹⁰	<0.02 ¹⁰				
	Yes ¹⁰	0.1 ¹⁰				
	Yes ¹⁰	0.09 ¹⁰				
	Yes ¹⁰	1.6 ¹⁰				
 <p>(DMAE) (24)</p>		Yes ^{11,23}				

TABLE 1.1. (Continued)

Analogue R =	Primary toxic action-respiratory depression	Presynaptic Block at cholinergic nerve-muscle preparation (relative potency to HC-3)	Inhibition of ACh synthesis in brain homogenates.	Inhibition of ACh synthesis by soluble ChAc	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes.
		0.22 ⁹				
		0.03 ⁹				
		0.03 ⁹				
		<0.03 ⁹				
		0.05 ⁹				

TABLE 1.1 (Continued)

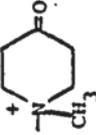
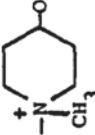
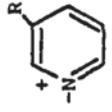
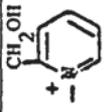
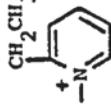
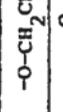
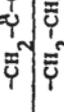
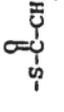
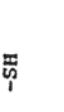
Analogue R =	Primary toxic action-respiratory depression	Presynaptic Block at cholinergic nerve-muscle preparation (relative potency to HC-3)	Inhibition of ACh synthesis in brain homogenates.	Inhibition of ACh synthesis by soluble ChAc	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes.
		0.09 ¹²				
		<0.03 ¹²				
 R' = CH ₂ OH		0.1 ¹²				
CHO		0.05 ¹²				
COOH		<0.03 ¹²				
Cl		0.19 ¹²				
Br		0.24 ¹²				
I		0.98 ¹²				
CH ₂ CH ₃		0.49 ¹²				
OH		<0.03 ¹²				

TABLE 1.1. (Continued)

Analogue R =	Primary toxic action-respiratory depression	Presynaptic Block at cholinergic nerve-muscle preparation (relative potency to HC-3)	Inhibition of ACh synthesis in brain homogenates.	Inhibition of ACh synthesis by soluble ChAc	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes.
		0.027 ¹³				
		<0.03 ¹²				
Analogue R* =						
 Ac-seco-HC-3 (14)	Yes ⁴	Yes ⁴		conflicting ⁴		Yes ²²
 (15)	Yes ⁵					
 (16)	Yes ⁵					
 (17)	Yes ⁵					
 thio-Ac-seco-HC-3 (18)	Yes ⁶					
 thio-seco-HC-3 (19)	Yes ⁶					

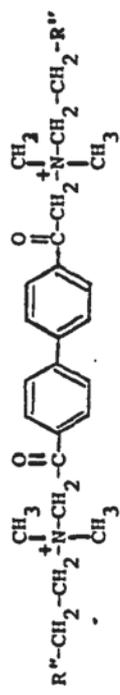


TABLE 1.1. (Continued)

Analogue	Primary toxic action-respiratory depression	Presynaptic Block at cholinergic nerve-muscle preparation (relative potency to HC-3)	Inhibition of ACh synthesis in brain homogenates.	Inhibition of ACh synthesis by soluble ChAc.	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes
	Yes ¹⁶	Yes ^{16,23}				
	Yes ¹⁶	Yes ^{16,23}				
		0.3 ¹³				
		0.09 ¹³				
		0.2 ¹³				
		< 0.01 ¹³				

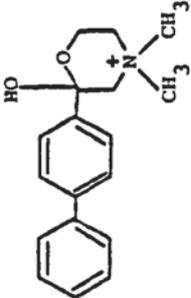
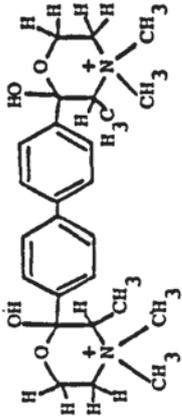
TABLE 1.1. (Continued)

Analogue	Primary toxic action-respiratory depression	Presynaptic Block at cholinergic nerve-muscle preparation (relative potency to HC-3)	Inhibition of ACh synthesis in brain homogenates.	Inhibition of ACh synthesis by soluble ChAc.	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes.
$\left(\begin{array}{c} \text{CH}_3 \\ \\ \text{HOCH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{C}(=\text{O})\text{C}_6\text{H}_4\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{CH}_3 \\ \\ \text{CH}_3 \end{array} \right)_2 \quad (7)$		0.15 ^{13,23}				
$\left(\begin{array}{c} \text{CH}_3 \\ \\ \text{HOCH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{C}(=\text{O})\text{N}(\text{H})\text{C}_6\text{H}_4\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{CH}_3 \\ \\ \text{CH}_3 \end{array} \right)_2$		0.01 ¹³				
$\left(\begin{array}{c} \text{CH}_3 \\ \\ \text{HOCH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{C}(=\text{O})\text{C}_6\text{H}_4\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{CH}_3 \\ \\ \text{CH}_3 \end{array} \right)_2 \quad (8)$	Yes ¹⁴	Yes ^{14,23}				
$\left(\begin{array}{c} \text{HO} \\ \\ \text{HOCH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{C}(=\text{O})\text{C}_6\text{H}_4\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{CH}_3 \\ \\ \text{CH}_3 \end{array} \right)_2$ <p style="text-align: center;">norphenyl-1-HC-3 (9)</p>	Yes ¹⁵	Yes ^{15,16,23}				
$\left(\begin{array}{c} \text{HO} \\ \\ \text{HOCH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{C}(=\text{O})\text{C}_6\text{H}_4\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{CH}_3 \\ \\ \text{CH}_3 \end{array} \right)_2$		Yes ^{16,23}				

TABLE 1.1.1. (Continued)

Analogue	Primary toxic action-respiratory depression	Presynaptic Block at cholinergic nerve-muscle preparation (relative potency to HC-3)	Inhibition of ACh synthesis in brain homogenates.	Inhibition of ACh synthesis by soluble ChAc	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes.													
$\left(\begin{array}{c} \text{CH}_3 \\ \\ \text{HOCH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{C} \begin{array}{l} \\ \text{OH} \\ \\ \text{C}_6\text{H}_5 \end{array} \\ \\ \text{CH}_3 \end{array} \right)_2 \quad (13)$		Yes ^{19,23}																	
$\begin{array}{c} \text{HO} \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{N}^+\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{CH}_2\text{CH}_3 \\ \\ \text{CH}_3 \end{array} \quad \text{terphenyl-1-HC-3 (10)}$		equipotent HC-3 ¹⁷	Yes ¹⁷		Yes ²¹	Yes ²²													
$\begin{array}{c} \text{OH} \\ \\ \text{C}_6\text{H}_5 \\ \\ \text{N}^+\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_2\text{CH}_2\text{R}' \\ \\ \text{CH}_3 \end{array}$ <table border="1" style="margin-left: auto; margin-right: auto;"> <tr> <td>R</td> <td>R'</td> </tr> <tr> <td>H</td> <td>H (HC-15)</td> </tr> <tr> <td colspan="2" style="text-align: center;">(5)</td> </tr> <tr> <td>CH₃</td> <td>H</td> </tr> <tr> <td>C₂H₅</td> <td>H</td> </tr> <tr> <td>(CH₂)₃CH</td> <td>H</td> </tr> <tr> <td>H</td> <td>CH₃</td> </tr> </table>	R	R'	H	H (HC-15)	(5)		CH ₃	H	C ₂ H ₅	H	(CH ₂) ₃ CH	H	H	CH ₃		Yes ^{18,23}	Yes ¹⁸	Yes ²¹	Yes ²²
R	R'																		
H	H (HC-15)																		
(5)																			
CH ₃	H																		
C ₂ H ₅	H																		
(CH ₂) ₃ CH	H																		
H	CH ₃																		
		Yes ²⁰				Yes ²⁰													
		Yes ²⁰				Yes ²⁰													
		Yes ²⁰				Yes ²⁰													
		Yes ²⁰				Yes ²⁰													

TABLE 1.1. (Continued)

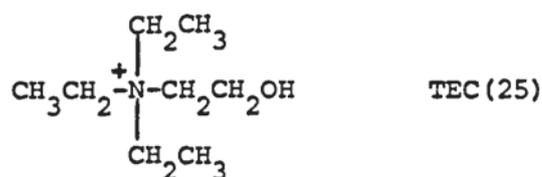
Analogue	Primary toxic action- respiratory depression	Presynaptic Block at cholinergic nerve muscle preparation (relative potency to HC-3)	Inhibition of ACh synthesis in brain homogenates.	Inhibition of ACh synthesis by soluble ChAc	Acetylation of analogues by ChAc.	Inhibition of choline uptake by synaptosomes.
	Yes ³					
	Yes ⁸	equipotent to HC-3 ⁸				

Notes

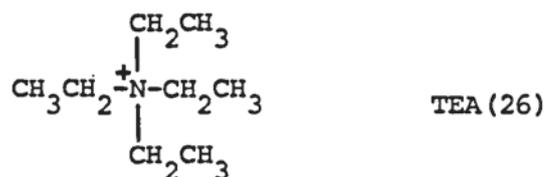
1. Analogue undergoes keto-hemiacetal tautomerism (not shown) and exists in aqueous solution in the cyclic hemiacetal form.
2. Depending upon the method of preparation analogue can exist in the cyclic hemiacetal form or in the open chain (seco) form.
3. Schueler, F.W., (1955).
4. See text.
5. Haarstad, Domer, Chihal, Rege and Charles, (1976).
6. Domer, Chihal, Charles and Rege, (1977).
7. Long, Evans and Wong, (1967).
8. Di Augustine and Haarstad, (1970).
9. Benz and Long, (1969b).
10. Benz and Long, (1969a).
11. Chlou and Long, (1969).
12. Benz and Long, (1970).
13. Marshall and Long, (1959)
14. Powers, Kruger and Schueler, (1962).

(continued . . .)

15. Thampi, Domer, Haarstad and Schueler, (1966)
16. Loomis, Sommer and Collins, (1974).
17. Gardiner, and Sung, (1969).
18. Hemsworth, (1971a).
19. Blase, Loomis, Collins and Sommer, (1974).
20. Hemsworth, (1972).
21. See Table 1.2 (Acetylation by ChAc).
22. See Table 1.3 (Inhibition of choline uptake)
23. No comparative study was made of HC-3.



Initially most of the work on TEC was concerned with its ability to replace choline in nutrition, (Jukes and Welch, 1942; Stekol and Weiss, 1950). TEC was found to inhibit the growth of rats, and this effect was reversible with choline. The first pharmacological investigations were carried out by Hunt and Taveau, (1909), who found that in sufficient amounts TEC is acutely toxic (LD_{50} in mice = 340 mg/kg). Keston and Wortis, (1946) showed that TEC gave rise to muscular weakness, convulsions and death and that protection against these symptoms is afforded by the injection of choline, given simultaneously with TEC. These workers suggested that TEC might interfere with ACh formation. Bowman and Rand, (1961) extensively investigated the actions of TEC, with regard to its general pharmacology. They found that in conscious animals, TEC caused a slowly developing muscular weakness which was more severe after exercise and which was reversed by an IV injection of choline. However, IV neostigmine did not reverse the muscular weakness. Toxicity tests in mice and rats showed that death was due to respiratory paralysis. The toxicity of TEC was antagonised by choline. TEC was also found to have a blocking action at ganglia (Rand and Ridehalgh, 1965) which is perhaps not surprising as it is structurally similar to tetraethylammonium (TEA) (26).



The effect of TEC on nerve-muscle preparations was also investigated by Bowman and Rand, (1961). TEC caused a slow reduction in muscle contraction in response to nerve stimulation of cat and rabbit tibialis, cat soleus and rat diaphragm muscles, although total block was not shown. This action was found to be selective for muscle contractions elicited by a high rate of nerve stimulation (1Hz). This slowly developing, frequency dependent neuromuscular block was antagonised by choline while anticholinesterases caused only a small increase in the twitch tension.

At these high rates of stimulation the first effect of IV TEC on the nerve-muscle preparations was to produce a small transient increase in twitch tension which was followed by the usual more slowly developing and longer lasting paralysis as previously described. Bowman, Hemsworth and Rand (1962) concluded that this first effect of TEC was not due to a depolarizing or an anticholinesterase action but due to an increase in the quantity of ACh released by each nerve impulse. TEA(26) is much more powerful than TEC in this respect. It is unlikely that this effect of TEC is of any significance in conscious animals.

Bowman and Rand, (1961) found that in the presence of TEC muscles responded normally to direct electrical stimulation which indicates that the block is not due to an effect on the contractile mechanism of the muscle. Conduction in the motor nerve trunk is also unaffected since nerve action potentials were unchanged in shape and size. Bartels, (1962) and Bowman, Hemsworth and Rand, (1962) showed that large doses of TEC can produce a postsynaptic neuromuscular blockade due to depressed sensitivity of the motor end plates, but the amounts of TEC required were approximately thirty times greater than those which selectively depressed twitches elicited by nerve impulses at a rate of 1Hz.

Bowman and Rand, (1961) compared TEC to HC-3. HC-3 was about twenty times more potent than TEC in giving a frequency dependent neuromuscular block which in both cases was antagonised by choline. HC-3 unlike TEC, did not give a transient increase in twitch tension.

Bowman and Rand, (1961) concluded that the "chemical similarity of TEC to choline, together with the fact that choline reverses the effects of TEC make it likely that TEC interferes with the metabolism of choline at the nerve endings, probably by interfering with the synthesis of ACh. The slow onset of the paralysis and its dependence upon high activity in the motor nerves support this view, since the preformed depots of ACh must be exhausted before transmission fails . . . The substance appears to resemble HC-3 in its mechanism of action . . . "

Bowman and Hemsworth, (1965b) studied the effects of TEC on the release of ACh from the isolated phrenic nerve-hemidiaphragm preparation. They found that the slight initial potentiation of the muscle twitches in the presence of TEC was accompanied by an increase in the amount of ACh released. This finding is similar to that previously reported by Bowman, Hemsworth and Rand, (1962) and discussed above. However, when the slowly developing neuromuscular block was at its peak, ACh output was depressed by 66-81%, but the release of ACh was not totally blocked. Addition of choline to the incubating medium restored ACh release to 72-90% of normal, whereas washing out the TEC restored ACh release to 82-100% of normal. These experiments indicate that TEC(25) has an inhibitory action on the synthesis of ACh from the cholinergic neuron.

This conclusion is supported by Matthews, (1966) who studied the effects of TEC on presynaptic ACh turnover in the perfused superior cervical ganglion of the cat. He showed that TEC inhibited ACh synthesis which was demonstrated by a declining output and a depletion of ganglionic stores

of ACh. The effect could be reversed by choline.

Elmqvist and Quastel, (1965) using intracellular recording techniques at the neuromuscular junction showed that in the presence of TEC the e.p.p.s. declined in amplitude this being due to a decrease in quantum size. This effect is similar to that shown for HC-3 by these workers and indicates that the amount of ACh released per impulse is decreased in the presence of these two choline analogues.

A further comparison between TEC and HC-3 was made on the frog rectus abdominis muscle (Bowman and Rand, 1961). HC-3 (150µg/ml) caused a 50% reduction in response to ACh, but TEC (5mg/ml) did not reduce responses to ACh.

It can be concluded from these pharmacological experiments that the primary action of TEC is a presynaptic block at the neuromuscular junction probably due to interference with the synthesis of ACh in the nerve terminals. It is also possible to conclude from these results that TEC has a weak, transient blocking action at the ganglia, similar to that produced by TEA(26), and appears to have little, if any, postsynaptic block at the neuromuscular junction.

Bull and Hemsworth, (1963) found that TEC inhibited the synthesis of ACh by subcellular fractions of rabbit brain homogenate. However, when the homogenate was treated with ether (a process which activates ChAc due to breakdown of the membranes enclosing the system) the inhibition was much smaller than that obtained with untreated particulates. In both cases the inhibition was antagonised by choline. These workers suggested that TEC acts by competing with choline for access to ChAc through a membrane. They also suggested that the results showed that TEC may possess a direct inhibiting action on ChAc. This latter effect of TEC was supported by Howard-Butcher, Cho and Schaeffer, (1974).

At high concentrations TEC has been found to inhibit choline and ACh transport across the renal tubule (Acara, Kowalski, Rennick and Hemsworth, 1975). TEC has also been found to inhibit choline transport into the rabbit heart (Buterbaugh, Figge and Spratt, 1968), the giant axon of the squid, (*Loligo*) (Hodgkin and Martin, 1965), brain synaptosomes (Potter, 1968; Hemsworth, Darmer and Bosmann, 1971; Howard-Butcher Cho and Schaeffer, 1974; Barker and Mittag, 1975; Holden, Rossier, Beaujouan, Guyenet and Glowinski, 1975; Simon, Mittag and Kuhar, 1975) and synaptic vesicles (Hemsworth, Darmer and Bosmann, 1971).

Hemsworth and Bosmann, (1971) also found that [^3H]-TEC was itself incorporated into synaptosomes and synaptic vesicles, but the experiments were performed under conditions favouring the low affinity transport system. Ilson, Collier and Boksa, (1977) found that minced rat cerebral cortex accumulated [^{14}C]-TEC. They also found that when the cat superior cervical ganglia were perfused with Krebs solution containing [^{14}C]-TEC the ganglia accumulated TEC. Electrical stimulation of the ganglia increased the accumulation of [^{14}C]-TEC. Collier and Ilson, (1977) found that this increased accumulation was not reduced by tubocurarine or by atropine, but it was blocked by choline and HC-3. When the calcium concentration was reduced the accumulation of [^{14}C]-TEC into resting ganglia was not altered but the increased accumulation into stimulated ganglia was reduced. However, changes in the magnesium concentration which depressed ACh release by amounts comparable to those induced by altered calcium concentrations did not alter the uptake of [^{14}C]-TEC. It was concluded that the transport of [^{14}C]-TEC is not regulated by transmitter release but that stimulation itself increases the uptake of [^{14}C]-TEC by a calcium dependent mechanism.

It can be concluded from these experiments that TEC(25) inhibits

both the low and high affinity transport of choline into synaptosomes. TEC is itself incorporated into synaptosomes by a low affinity process. Experiments to show a high affinity transport mechanism for TEC have not been conducted. TEC is also transported into the cat superior cervical ganglion. Stimulation of the ganglion increases the accumulation of TEC.

Burgen, Burke and Desbarats-Schonbaum, (1956) found that a partially purified extract of ChAc from rat brain acetylated TEC, *in vitro*, by 95% compared to choline 100%. However, Dauterman and Mehrotra, (1963) found no evidence of acetylation of TEC. Using partially purified, choline free ChAc from bovine caudate nucleus Hemsworth and Smith, (1970b) measured the rate of acetylation of a range of concentrations of TEC. They found that TEC was acetylated and at a constant concentration of AcCoA calculated the apparent Michaelis Menten constant (K_m) from a Lineweaver - Burke, (1934) plot. The K_m for TEC was 0.26mM, compared to the K_m for choline which was 0.19mM. Chiou, (1974) extracted ChAc from rat brain and found that TEC was acetylated 24% compared to choline 100%. Mann and Hebb, (1975) prepared ChAc from acetone dried powders of rat brain and removed endogenous choline. They found that TEC had a K_m of 50mM (The K_m for choline was 0.6mM). Barker and Mittag, (1975) prepared solubilized ChAc from rat forebrain and found that TEC had a K_m of 25mM. (The K_m for choline was 0.17mM). N.V.P.H. (40), a ChAc inhibitor, inhibited the acetylation of TEC by 70% compared to choline 95%. It is therefore possible that TEC is acetylated by another enzyme present in the extract other than ChAc (Bradshaw, 1976).

Ilson, Collier and Boksa, (1977) found that about 4% of the total [14 C]-TEC accumulated by resting superior cervical ganglia of the cat was acetylated to [14 C]-acetyltriethylcholine ([14 C]-AcTEC). Preganglionic nerve stimulation not only increased the uptake of [14 C]-TEC but also

increased the acetylation of [^{14}C]-TEC to 11% of the total [^{14}C]-TEC now accumulated. However, stimulated and unstimulated ganglia phosphorylated similar amounts of [^{14}C]-TEC. In their experiments with minced mouse brain they found that the [^{14}C]-TEC accumulated by this tissue was acetylated by 3% and phosphorylated by 9%. Howard-Butcher Cho and Schaeffer, (1974) found that TEC was transported into and acetylated in synaptosomes.

Ilson, Collier and Boksa, (1977) preloaded the cat superior cervical ganglion with [^{14}C]-TEC. After a 30 min. wash out period the preganglionic nerves were stimulated. Nerve stimulation was found to increase the efflux of radioactivity from ganglia. Radioactivity was released from non-stimulated ganglia and this was shown to be 95% [^{14}C]-TEC, whereas all of the extra radioactivity released by nerve stimulation was [^{14}C]-AcTEC. No [^{14}C]-AcTEC was detectable in the ganglia at the end of the experiments. Thus the total amount of [^{14}C]-AcTEC measured in these experiments as releasable [^{14}C]-AcTEC was similar to the total amount of [^{14}C]-AcTEC synthesised by ganglia. These workers also found that the rate of release of [^{14}C]-AcTEC was frequency dependent and calcium dependent.

Ilson, Collier and Boksa, (1977) preloaded minced rat brain with [^{14}C]-TEC and the preparation was stimulated by a high concentration of potassium ions. The efflux of radioactivity was increased. The radioactivity released from non stimulated tissue was almost all [^{14}C]-TEC whereas the extra radioactivity released on stimulation was [^{14}C]-AcTEC. The release of [^{14}C]-AcTEC was calcium dependent.

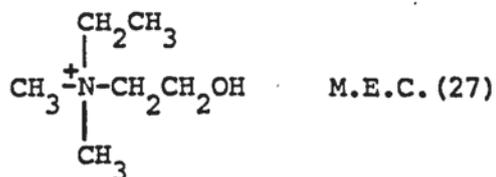
It can be concluded from these experiments that TEC is transported into the ganglia, is acetylated to AcTEC which is then released as a false transmitter. Experiments conducted on AcTEC (Holton and Ing, 1949;

Bowman, Hemsforth and Rand, 1962) indicate that this compound has a weak nicotinic action and is much less potent than ACh. AcTEC will therefore attenuate the quanta of transmitter released at the ganglia. Whether this is the main mechanism by which TEC affects cholinergic transmission is not clear.

Thus the presynaptic block of TEC on cholinergic neurons could result from a blockade of the transport of choline into nerve terminals and/or inhibition of acetylation of choline by ChAc and/or release of a false transmitter.

1.2.4. Monoethylcholine

Monoethylcholine (MEC) (27) is the trivial name for N,N,dimethyl-N-ethylethanolamine. Chemical Abstracts, however, names this compound 2-hydroxy-N,N dimethyl-N ethylethaniminium(27).



Bowman and Rand, (1962) investigated the action of MEC on the tibialis anterior muscle of the cat and the rat phrenic nerve-hemidiaphragm. MEC caused a paralysis which was rapid in onset and occurred in both rapidly and slowly stimulated muscles although the former was more usually affected. The block was shown to be due to a postsynaptic action on the motor end plates, since the response to close-arterially injected ACh was abolished. MEC showed the characteristics of a depolarising substance in that it caused contraction of the tibialis anterior muscle of the cat on close intrarterial injection, spastic paralysis of the chick and contracture of the frog rectus abdominis. These workers concluded that MEC gives a depolarising block at the neuromuscular junction.

Reitzel and Long, (1959a) found that MEC, unlike all the other choline analogues they tested, partially restored neuromuscular transmission which had been depressed by HC-3. Holton and Ing, (1949) found that the acetyl ester of this analogue (AcMEC) possessed weak ACh like activity and it is possible that MEC's limited action against HC-3 could be due to the formation of a false transmitter.

Burgen, Burke and Desbarats-Schonbaum, (1956) found that MEC was acetylated *in vitro* by ChAc extracted from rat brain 143% compared to choline 100%. MEC was also shown to inhibit the synthesis of ACh over and above that which can be accounted for as being due to its acetylation. Dauterman and Mehrotra, (1963) also found that MEC was acetylated *in vitro* by ChAc extracted from rat brain and determined a K_m of 3.3mM (choline in the same experiments had a K_m of 1.6mM). Using partially purified, choline free ChAc from the bovine caudate nucleus Hemsworth and Morris, (1964) found that MEC was acetylated 25-50% compared to choline 100%. Hemsworth and Smith, (1970b) later determined that MEC had a K_m of 0.5mM (choline had a K_m of 0.19mM). Barker and Mittag, (1975) prepared solubilised ChAc from rat forebrain. They determined that MEC had a K_m of 1.26mM (choline had a K_m of 0.16mM). NVPH (40), a ChAc inhibitor, inhibited acetylation by 95% indicating that MEC is acetylated by ChAc (compare with similar experiments with TEC (see page 49)).

Barker and Mittag, (1975) found that MEC competitively inhibited the high affinity transport of choline into synaptosomes isolated from rat forebrain. These workers also found that [3H]-MEC was itself transported into synaptosomes, having a K_{T_H} of 2.75 μ M (K_{T_H} is that concentration of substrate which provides one half the maximal velocity of uptake, with regard to the high affinity transport mechanism) and a V_{max} equal to that of choline. Barker and Mittag, (1975) found that choline and HC-3 inhibited this high

affinity transport. These workers also showed that about 45% of the total amount of [^3H]-MEC transported into synaptosomes is acetylated the maximal rate of acetylation of [^3H]-MEC being the same as for [^3H]-choline. There was no evidence of any other metabolite other than [^3H]-AcMEC.

When Collier, Barker and Mittag, (1976) perfused the cat superior cervical ganglion with Krebs solution containing [^3H]-MEC they found that the stimulated ganglion accumulated radioactivity. The spontaneous efflux from the preloaded ganglia was shown to be unchanged [^3H]-MEC, but nerve stimulation increased the amount of radioactivity released. All of this increased radioactivity was due to [^3H]-acetylmonoethylcholine ([^3H]-AcMEC). This release was calcium dependent. The sum of the amounts of the [^3H]-AcMEC released from and retained in the ganglia was about 63pmole, the sum of [^3H]-ACh was 75pmole, therefore, under the conditions of the experiment described by Collier, Barker and Mittag, (1976), choline and MEC are similar substrates for ChAc *in situ*. Thus the difference in the rates of acetylation of choline and MEC as seen *in vitro*, was less clear *in situ*. These workers also reported that ACh and AcMEC are equally available for release by nerve impulses.

These experiments show that MEC can be transported into cholinergic neurons, acetylated to AcMEC which can then be released on nerve stimulation as a false transmitter. AcMEC has been shown to behave as an agonist on nicotinic and muscarinic receptors, but is much less active compared to ACh. (Holton and Ing, 1949). Large and Rang, (1978 a,b) preloaded the phrenic nerve of the rat with MEC and using electrophysiological techniques they found that AcMEC was released as a false transmitter at the neuromuscular junction.

Although MEC has not been shown to have a presynaptic blocking action at the neuromuscular junction, it does appear to interfere with the synthesis of acetylcholine. Therefore it is possible that the presynaptic block of MEC has presumably been masked by its postsynaptic depolarising action.

1.2.5. Diethylcholine

Diethylcholine (28) is the trivial name for N,N,-diethyl-N-methylethanolamine, Chemical Abstracts names this compound 2-hydroxy-N,N-diethyl-N-methylethanaminium (28).



Bowman and Rand, (1962) showed that DEC had a presynaptic action at the neuromuscular junction. DEC caused a slowly developing paralysis of the more rapidly stimulated tibialis anterior muscle of the cat and diaphragm of the rat but was without effect on the maximal twitches of the slowly stimulated muscles. During the block in the cat, contractions of the muscle produced by close-arterially injected ACh were not depressed showing that the block was presynaptic. Chiou, (1974) showed that DEC had no effect on the dose response curve of ACh on the contraction of the guinea-pig ileum and frog rectus abdominis muscle. He also showed that acetyldiethylcholine (AcDEC) is a weak competitive antagonist on these tissues. Holton and Ing, (1949) showed that AcDEC was 300 times less potent than ACh on the frog rectus abdominis muscle.

Burgen, Burke and Desbarats-Schonbaum, (1956) showed that DEC was acetylated *in vitro* by ChAc extracted from rat brain 102% compared to

choline 100%. However, Dauterman and Mehrotra, (1963) also using ChAc extracted from rat brain could find no evidence for the acetylation of DEC *in vitro*. But, Hemsworth and Smith, (1970b) using partially purified choline free ChAc from bovine caudate nucleus found that DEC was acetylated, having a K_m of 7.5mM (compared to choline 0.19mM). Chiou, (1974) using partially purified ChAc from rat brain found that DEC was acetylated 67% compared to choline 100%. He also found that DEC is acetylated 61% by minced rat brain compared to choline 100%.

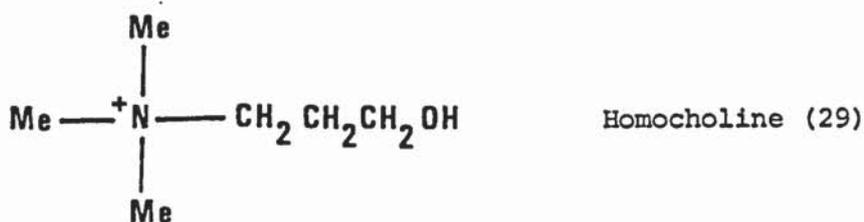
Barker and Mittag, (1975) found that DEC was acetylated by ChAc solubilised from rat forebrain having a K_m of 9.66mM (compared to choline 0.16mM). NYPH, a ChAc inhibitor, inhibited the acetylation by 92% (compared to TEC see page 49).

Barker and Mittag, (1975) and Simon, Mittag and Kuhar, (1975) showed that DEC inhibited the high affinity transport of choline into synaptosomes.

There has been no further work carried out on DEC to determine whether it is transported into the cholinergic neuron, therefore the precise mechanism of DEC's presynaptic block is unknown. However, it has been shown at least that it blocks the transport of choline into cholinergic nerve terminals and it is possible that DEC acts as a substrate for ChAc *in vivo*.

1.2.6. Homocholine

Homocholine(29) is the trivial name for 3-trimethylamino-1-propanol. Chemical Abstracts however names this compound 3-hydroxy-N,N,N-trimethylpropanaminium(29).



Bowman and Rand, (1962) showed that homocholine had a postsynaptic depolarising blocking action at the neuromuscular junction. It produced a paralysis in the cat tibialis anterior muscle and in the rat diaphragm which was rapid in onset and occurred in both rapidly and slowly stimulated muscles although the former was usually the more affected. The response to ACh injected close-arterially in the cat muscle at the height of the block due to homocholine was abolished. Homocholine caused a contraction of the tibialis anterior muscle of the cat on close-arterial injection, spastic paralysis of the chick and contracture of the frog rectus abdominis muscle. Homocholine has not been shown to possess a presynaptic blocking action at cholinergic neurons.

No evidence of any acetylation of homocholine *in vitro* by ChAc could be found by Burgen, Burke and Desbarats-Schonbaum, (1956); Dauterman and Mehrotra, (1963); Currier and Mautner, (1974); Barker and Mittag, (1975) and Collier, Lovat, Ilson, Barker and Mittag, (1977).

Simon, Mittag and Kuhar, (1975) and Collier, Lovat, Ilson, Barker and Mittag, (1977) showed that homocholine inhibits the high affinity transport of choline into synaptosomes.

Collier, Lovat, Ilson, Barker and Mittag, (1977) found that [³H]-homocholine was itself transported into synaptosomes. The uptake could be resolved into two components one had a high affinity ($K_{T_H} = 3\mu\text{M}$) and the other a low affinity ($K_{T_L} = 14.5\mu\text{M}$). [³H]-choline was shown to have a K_{T_H} of $2.8\mu\text{M}$ in this system. The total uptake of [³H]-homocholine is 60% that of [³H]-choline. HC-3 and choline reduced the accumulation of [³H]-homocholine. It seems as though homocholine is transported by the same mechanism as choline. Collier, Lovat, Ilson, Barker and Mittag, (1977) found that 32% of the [³H]-homocholine accumulated by synaptosomes was

acetylated compared to 50% of the [^3H]-choline, and 64% of [^3H]-homocholine which was accumulated by the high affinity transport system was acetylated, compared to 72% of the [^3H]-choline. These workers showed that acetylation was confined to the nerve terminals.

Collier and Ilson, (1977) perfused the cat superior cervical ganglion with Krebs containing [^3H]-homocholine. Electrical stimulation of the ganglia increased the amount of radioactivity accumulated by the ganglia. This increased accumulation was not reduced by tubocurarine, but it was completely blocked by choline and HC-3. When the calcium concentration was reduced the accumulation of [^3H]-homocholine into resting ganglia was not altered but the increased accumulation into stimulated ganglia was reduced. However, changes in the magnesium concentration which depressed ACh release by amounts comparable to those induced by altered calcium concentrations did not alter the uptake of [^3H]-homocholine. It was concluded that the transport of [^3H]-homocholine is not regulated by transmitter release but that nerve stimulation itself increases the uptake of [^3H]-homocholine by a calcium dependent mechanism.

Collier, Lovat, Ilson, Barker and Mittag, (1977) showed that in ganglia the proportion of accumulated [^3H]-homocholine converted to [^3H]-acetylhomocholine was much less than in synaptosomes; resting and stimulated ganglia acetylated 2.5% and 7.1% respectively (which takes into consideration the increase in the uptake of [^3H]-homocholine into the nerve terminals during electrical stimulation) of the accumulated [^3H]-homocholine. Stimulation did not increase the amount of [^3H]-phosphorylhomocholine formed. It is possible that uptake and acetylation are less closely coupled in ganglia than in synaptosomes.

Collier, Lovat, Ilson, Barker and Mittag, (1977) preloaded the cat superior cervical ganglion with [^3H]-homocholine. They found that [^3H]-homocholine was released from resting ganglia, however, on stimulation the

efflux of radioactivity was markedly increased. [^3H]-acetylhomocholine accounted for approximately 85% of the extra radioactivity released by electrical stimulation. These workers suggest that most of the other 15% consists of [^3H]-homocholine derived from hydrolysis of [^3H]-acetylhomocholine. These results show that acetylhomocholine is behaving as a false cholinergic transmitter.

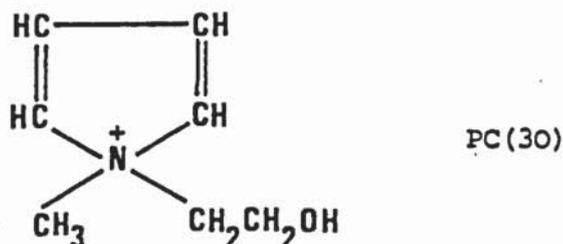
Barras, Brimblecombe, Rich and Taylor, (1970) found that acetylhomocholine is much less active than ACh on muscarinic receptors. A variety of actions has been reported for its nicotinic activity.

Collier, Lovat, Ilson, Barker and Mittag, (1977) found that the amount of homocholine acetylated by stimulated ganglia was not enough to replace all of the ACh released by nerve stimulation under their experimental conditions, probably indicating that the rate of acetylation was limiting, because stimulation enhanced the accumulation of unchanged homocholine. Their results showing the acetylation of homocholine by brain synaptosomes and sympathetic ganglia but not by ChAc *in vitro* suggest that either ChAc has different substrate specificity *in situ* compared to *in vitro* or there exists another enzyme which acetylates the choline analogues, but which does not survive isolation in an active form.

Although homocholine has not been shown to have a presynaptic blocking action at the neuromuscular junction it does appear to interfere with the synthesis of acetylcholine. Therefore, the presynaptic block of homocholine has presumably been masked by its postsynaptic depolarising action.

1.2.7. Pyrrolcholine

Pyrrolcholine (PC) (30) (Pyrrolidinecholine) is the trivial name for N-2-hydroxyethyl-N-methyl-pyrrolidinium. Chemical Abstracts however, names this compound 1-(2-hydroxyethyl)-1-methyl-pyrrolidinium(30).



Many agents influencing the actions and metabolism of ACh have been found to impair passive avoidance learning in rats. PC administered I.V. was found to do this (Glick, Craine, Barker and Mittag, 1975). This effect of PC was antagonised by choline and HC-3. These workers concluded that the results are consistent with the hypothesis that PC inhibits central cholinergic pathways by a presynaptic mechanism.

Von Schwarzenfeld and Whittaker, (1977) showed that PC has a direct neuromuscular blocking action on the rat gastrocnemius muscle.

PC was acetylated *in vitro* by ChAc extracted from rat forebrain by 53% compared to choline 100% (Barker and Mittag, 1975), squid optic lobe (Barker, Dowdall and Mittag, 1975) and electric organ of the *Torpedo* (Dowdall, unpublished data cited by Von Schwarzenfeld and Whittaker, 1977)

PC has been found to inhibit the high affinity transport of choline into synaptosomes from rat brain (Barker and Mittag, 1975; Simon, Mittag, and Kuhar, 1975) synaptosomes from the electric organ of the *Torpedo* (Dowdall, unpublished results cited by Von Schwarzenfeld and Whittaker, 1977), and synaptosomes from the squid optic lobe (Barker, Dowdall and Mittag, 1975).

[³H]-PC was itself shown to be transported into synaptosomes by Barker

and Mittag, (1975). The K_{T_H} was calculated as $5.5\mu\text{M}$ (compared to the K_{T_H} for choline $2.5\mu\text{M}$). HC-3 inhibited the high affinity uptake of $[^3\text{H}]\text{-PC}$. The V_{max} for $[^3\text{H}]\text{-PC}$ and $[^3\text{H}]\text{-choline}$ was the same.

Barker and Mittag, (1975) found that $[^3\text{H}]\text{-PC}$ is acetylated to $[^3\text{H}]\text{-acetylpyrrolcholine}$ ($[^3\text{H}]\text{-AcPC}$). About 45% of the total uptake of $[^3\text{H}]\text{-PC}$ transported by the high affinity transport process was acetylated (similar values were found for $[^3\text{H}]\text{-choline}$). The maximal rate of acetylation of $[^3\text{H}]\text{-PC}$ is the same as that for $[^3\text{H}]\text{-choline}$. There was no evidence of any other metabolite other than the acetyl ester. Barker, Dowdall and Mittag, (1975) showed that $[^3\text{H}]\text{-PC}$ was transported into squid (*Loligo*) optic lobe synaptosomes by a two component uptake system. The K_{T_L} for $[^3\text{H}]\text{-PC}$ was $35.7\mu\text{M}$ (compared to the K_{T_L} for $[^3\text{H}]\text{-choline}$ $37.5\mu\text{M}$), the K_{T_H} for $[^3\text{H}]\text{-PC}$ was $5.2\mu\text{M}$ (compared to the K_{T_H} for $[^3\text{H}]\text{-choline}$ of $2.7\mu\text{M}$) The V_{max} of $[^3\text{H}]\text{-PC}$ was the same as the V_{max} of $[^3\text{H}]\text{-choline}$. In squid synaptosomes only 30% of $[^3\text{H}]\text{-PC}$ taken up by the high affinity transport process was acetylated, this was less than the amount of choline which was acetylated after being transported by the high affinity process.

Collier, Barker and Mittag, (1976) showed that the electrically stimulated superior cervical ganglion accumulated $[^3\text{H}]\text{-PC}$. The spontaneous efflux of radioactivity was almost all due to unchanged $[^3\text{H}]\text{-PC}$, whereas nerve stimulation released $[^3\text{H}]\text{-AcPC}$. $[^3\text{H}]\text{-AcPC}$ accounted for about 99% of the extra radioactivity released on nerve stimulation. This release was calcium dependent. $[^3\text{H}]\text{-ACh}$ and $[^3\text{H}]\text{-AcPC}$ appear to be equally available for release by nerve impulses. The sum of the amount of $[^3\text{H}]\text{-AcPC}$ released from and retained in ganglia was 50pmoles (compared to 75pmoles of $[^3\text{H}]\text{-ACh}$). Thus $[^3\text{H}]\text{-choline}$ and $[^3\text{H}]\text{-PC}$ are similar substrates for ChAc, *in situ*.

Dowdall, Fox, Wachtler, Whittaker and Zimmerman, (1975) perfused a

single motor nerve trunk of *Torpedo* with [³H]-PC. They showed that [³H]-PC was transported into the nerve and that [³H]-AcPC was released on nervous stimulation.

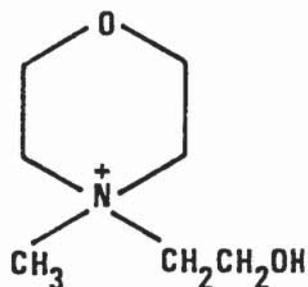
So it has been shown that AcPC can behave as a false transmitter at the ganglia of the cat. Collier, Barker and Mittag, (1976) showed that AcPC is about 29 times less active than ACh on the guinea-pig ileum. Cho, Jenden and Lamb, (1972) showed that AcPC is 11 times less active than ACh on the nicotinic receptor of the frog rectus abdominis muscle and is hydrolysed by rat brain cholinesterases 70% as rapidly as ACh is hydrolysed. Von Schwarzenfeld and Whittaker, (1977) showed that AcPC is a full cholinergic agonist with dose response curves parallel to that of ACh. AcPC is a more potent muscarinic drug than nicotinic. Equipotent Molar Ratio (ACh = 1) of AcPC to cause contracture of leech muscle is 28, frog rectus 15, and guinea-pig ileum 3.6.

However, Kilbinger, Wagner and Zerban, (1976) found that AcPC was only four times less potent on the frog rectus muscle and suggested that AcPC as a false transmitter does not modify transmission at the neuromuscular junction.

Thus the presynaptic blocking action of PC on the cholinergic neuron could be due to the inhibition of the transport of choline and/or the release of a false transmitter.

1.2.8. Morpholinecholine

Morpholinecholine (MC) is the trivial name for N-2-hydroxyethyl-N-methyl-morpholinium. Chemical Abstracts names this compound 4-(2-hydroxyethyl)-4-methyl morpholinium (31).



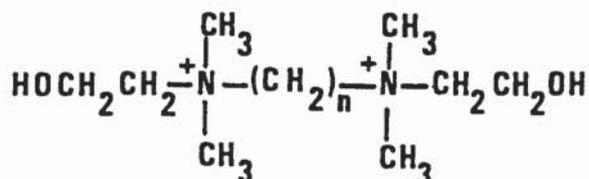
MC(31)

Very little work has been carried out on this analogue. M.J. Dowdall, unpublished results cited by Von Schwarzenfeld and Whittaker, (1977), found that MC(31) was acetylated 16% compared to choline 100% by ChAc extracted from the electric organ of the *Torpedo*.

MC has been shown to inhibit the high affinity transport of choline into synaptosomes isolated from rat brain (Simon, Mittag and Kuhar, 1975), synaptosomes isolated from squid (*Loligo*) optic lobe (Barker, Dowdall and Mittag, 1975) and synaptosomes isolated from the electric organ of the *Torpedo* (Dowdall, unpublished results cited by Von Schwarzenfeld and Whittaker, 1977).

Acetylmorpholinecholine (AcMC) was shown to be a full cholinergic agonist by Von Schwarzenfeld and Whittaker, (1977) but was far less potent.

1.2.9. Polymethylene bis (hydroxyethyl) dimethylammonium salts.



$n = 3 (C_3, 33), 4 (C_4, 34); 5 (C_5, 35); 6 (C_6, 36); 7 (C_7, 37);$

$n = 8 (C_8, 38); 9 (C_9, 39); 10 (C_{10}, 32).$

Bowman and Hemsworth, (1965a) studied several of these analogues, C_3 (33), C_4 (34), C_5 (35), C_6 (36) and C_{10} (32), of which C_{10} (32) was the most potent. C_{10} (32) showed evidence of having a depolarising action at the neuromuscular junction as it produced a contraction of the non stimulated-tibialis anterior muscle of the cat on close-arterial injection, spastic paralysis of the conscious chick on I.V. injection and contracture of the isolated frog rectus abdominis muscle. These results support the findings of Barlow and Zoller, (1962).

However, C_{10} (32) was shown to have a two phase blocking action in the cat, (Bowman and Hemsworth, 1965a). On I.V. injection of C_{10} (32) there was a decrease in the amplitude of the maximal indirectly elicited twitches which rapidly followed an initial small potentiation. This first phase of block occurred in both rapidly (1Hz) and slowly (0.1Hz) stimulated tibialis anterior muscles and exhibited characteristics of block by depolarisation as produced by decamethonium in this muscle. Close arterially injected ACh during this first phase of block had no effect, confirming C_{10} (32) has a postjunctional block.

In the muscle stimulated at 0.1Hz recovery was usually complete within 15-20 minutes after injection of C_{10} (32). However, with the more rapidly stimulated muscle the recovery was interrupted by a second phase of block. Recovery from this second phase of block was prolonged and occurred 1-2 hrs after injection. Choline slightly enhanced the first phase of block which occurred in both muscles but antagonised the second phase of block which occurred selectively in the more rapidly stimulated muscles, (Bowman and Hemsworth, 1965a).

The first effect of C_{10} (32) after I.V. injection into conscious chicks was to cause a spastic paralysis (like that produced by depolarising

blocking drugs). On recovery the chick stood up and looked normal, however, if the chick was placed on its back to elicit its righting reflex a pronounced muscular weakness developed, (Bowman and Hemsworth, 1965a)

These results suggest that the first phase of block produced by C_{10} (32) at the neuromuscular junction was due to depolarisation of the motor end plates while the second phase, occurring during higher frequencies of stimulation and reversible by choline appeared to be a presynaptic block.

Bowman and Hemsworth, (1965a) investigated the action of C_{10} (32) on the isolated phrenic nerve-diaphragm preparation of the rat. This preparation is much less sensitive to a block by depolarisation than the cat tibialis anterior muscle and consequently the first phase of block was less obvious in this tissue. C_{10} (32) produced a slight potentiation of the twitches followed by a slowly developing depression which occurred selectively in the diaphragm stimulated at 1 shock/sec. These workers showed that the amount of ACh was reduced during this block. Twitches were restored by the addition of choline, and at the same time ACh output was restored to 65 to 90% of the control output. These results support the hypothesis that C_{10} (32) has a presynaptic blocking action at the neuromuscular junction.

The derivatives C_3 (33), C_4 (34), C_5 (35), and C_6 (36) did not produce effects characteristic of a depolarising drug in the cat and the chick as described above. In the chick C_4 (34), C_5 (35) and C_6 (36) produced an immediate flaccid paralysis.

All of the dicholine compounds (32 to 39) were found to block the effect of ACh on the nicotinic receptors of the frog rectus abdominis muscle. C_5 (35) was the most potent analogue in this respect, (Hemsworth, 1976).

Hemsworth, (1976) also showed that all the dicholine compounds were more effective as inhibitors of acetylcholinesterase than cholinesterase. However, in both cases a decrease in chain length decreased activity. This is in agreement with the findings of Barlow, (1955).

In the isolated rat diaphragm C_5 (35), C_6 (36), C_7 (37), C_8 (38) and C_9 (39) had similar effects to C_{10} (32). They produced a presynaptic block which was frequency dependent and reversible with choline. C_4 (34) on this preparation caused a slight depression in the more rapidly stimulated muscle which was reversible with choline and C_3 (33) was found to have no effect in the concentrations used. (Bowman and Hemsworth, 1965a; Hemsworth, 1976).

Hemsworth, Darmer and Bosmann, (1971) showed that the dicholine compounds (32-36) inhibited choline transport into the synaptosomes and synaptic vesicles. The result for C_{10} (32) was supported by Holden, Rossier, Beaujouan, Guyenet and Glowinski, (1975). Bowman and Hemsworth, (1965a) showed that C_{10} (32) inhibited the synthesis of ACh by the mitochondrial fraction of the rabbit brain. Hemsworth, (1971b) showed that the inhibition only occurred in intact synaptosomes and that C_{10} (32) had no effect on the synthesis of ACh when the membranes surrounding the ChAc were broken down.

Burgen, Burke and Desbarats-Schonbaum, (1956) could find no evidence that C_3 (33) and C_5 (35) were acetylated *in vitro* by ChAc extracted from rat brain. However, they showed that C_{10} (32) was acetylated. Hemsworth, (1971b) showed that C_3 (33), C_4 (34) and C_5 (35) were acetylated *in vitro* by ChAc by negligible amounts, whereas C_6 (36), and C_{10} (32) were acetylated by large amounts. C_{10} (32) was acetylated 100% compared to choline 100%. Hemsworth, (1976) showed that C_7 (37), C_8 (38) and C_9 (39) were also acetylated by very high amounts.

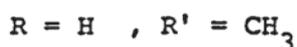
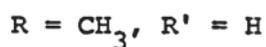
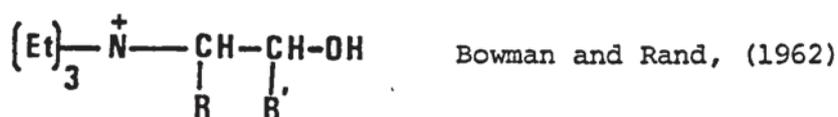
Hemsworth, (1976) noted that the rate of acetylation of these dicholine

compounds (32-39) paralleled the activity of these analogues at the prejunctional site at the neuromuscular junction, suggesting that part of the pharmacological activity of these compounds may be due to their incorporation into cholinergic nerve endings, where they could be acetylated and released as false transmitters.

Barlow and Zoller, (1962) investigated the postjunctional activity of some of the acetylated derivatives. They were weaker than ACh on the tissues used.

1.2.10. Other analogues of choline

Several other analogues of choline have been investigated, but only limited pharmacological testing has been carried out. Some of these analogues have been shown to have a presynaptic blocking action on nerve-muscle preparations:



Other choline analogues have been shown to be substrates for ChAc, *in vitro*, and are acetylated (Table 1.2) and/or inhibit the high affinity transport of choline into synaptosomes (Table 1.3).

1.2.11. *In vitro* acetylation of choline analogues by choline acetyltransferase

Table 1.2. is a review of the choline analogues that have been investigated as possible substrates for ChAc *in vitro*, (see references for experimental details). Choline is the natural substrate for ChAc.

From Table 1.2. it can be seen that an increase in the length of the methylene chain separating the quaternary nitrogen from the hydroxyl group in the choline molecule results in a decrease in the rate of acetylation by ChAc *in vitro*. Homocholine (29) was acetylated by less than 5% in five studies and the four carbon analogue was acetylated by less than 5% in two studies, although in one study it was acetylated by 29% compared to choline 100%. This suggests that the ethyl chain in the choline molecule is essential for its high rate of acetylation by ChAc *in vitro*.

Methyl substitution on the α carbon of the ethyl chain, however, gives an analogue which if in the DL form can be acetylated by ChAc *in vitro*, although it has a low affinity for the enzyme. A certain amount of stereospecificity exists since the D α isomer is a better substrate than the L α isomer. Methyl substitution on the β carbon of the ethyl chain resulted in analogues which in one study were acetylated by less than 5% compared to choline 100%. However, in another study the D- β methyl substituted choline analogue was found to be acetylated although it had a low affinity for the enzyme.



A cyclic choline moiety in a pyridine ring was acetylated by ChAc *in vitro* at less than 5% the rate of choline. However, HC-3(4) is acetylated by about 27% and HC-15(5) by about 15% compared to choline 100%. It therefore appears as though *in vitro* ChAc can acetylate a cyclic form of choline as found in the latter two analogues but it cannot acetylate the analogue in which the choline skeleton is part of an aromatic ring. It is not known which of the choline moieties of HC-3 and HC-15 are acetylated. Alkyl substitution on the morpholine ring of HC-15 resulted in analogues which were acetylated at a lower rate compared to HC-15. The substitutions were made on the ether choline moiety possibly indicating that ChAc combines with this moiety. Barker and Mittag, (1975) suggested that the cyclic forms of HC-3 and HC-15 being hemi-acetals might open up to the straight chain (seco) tautomer on the enzyme surface so rendering the now terminal hydroxyl group free to be acetylated. However, the cyclic forms of HC-3 and HC-15 are β -substituted choline analogues and it is interesting to observe that in one study a D- β -substituted analogue of choline, discussed above, was acetylated but it had a low affinity for ChAc. It is possible that the cyclic form of the D- β -substituted choline moiety as found in the HC-3 and HC-15 molecules could confer some favourable rigidity to this moiety.

The rate of the *in vitro* acetylation by ChAc of the dicholine compounds (32-39) increased with an increase in the length of the methylene chain separating the two quaternary nitrogen atoms. It appears as though the distance between the two quaternary nitrogen atoms is important in determining the rate of acetylation of these analogues.

Replacement of one, two or all three of the N-methyl groups in choline by an hydroxy ethyl chain reduced substrate activity by 21%, 37% and 84% respectively compared to the acetylation of choline. However, if two hydroxy ethyl groups are attached to a quaternary nitrogen atom

which is either in a piperidine ring or in a morpholine ring no acetylation of these analogues by ChAc occurs.

The quaternary nitrogen in the choline molecule can be replaced by a positively charge sulphur ion (having three groups attached to it unlike the quaternary nitrogen atom which has four) giving a choline analogue, thiocholine, which was acetylated by the incubation medium used in the *in vitro* experiments, but its acetylation was not inhibited by NVPH (40), a ChAc inhibitor, suggesting that it represented non-enzymic acyl exchange between AcCoA and thiocholine.

Replacement of the quaternary nitrogen in the choline molecule for a tertiary nitrogen resulted in a compound which was acetylated by ChAc by 100% in one study, but only by 6% in another study, compared to choline 100%. However, it does seem that as the number of groups attached to the nitrogen was decreased from four (quaternary amine; choline) to one (primary amino derivative of choline) there was a decrease in the rates of acetylation.

Substitution of one of the N-methyl groups in the choline molecule by an ethyl group resulted in an analogue, monoethylcholine(27), which can be acetylated *in vitro* by ChAc at a rate at least equal to that of choline. When one of the N-methyl groups was replaced by a longer n-alkyl chain then provided the number of carbons in the chain did not exceed ten, the resultant analogues were acetylated having V_{\max} 's equal to that of choline although their affinity for ChAc was about five times less than that of choline. When the number of carbons in the n-alkyl chain increased from eleven to sixteen the rates of acetylation of these analogues drastically decreased.

Substitution of one of the N-methyl groups in the choline molecule by an isopropyl group resulted in an analogue which according to two studies was a poor substrate for *in vitro* ChAc. Similarly, the introduction of more bulky branched alkyl chains on the nitrogen atom led to analogues which were poorly acetylated.

Replacement of one of the N-methyl groups in the choline molecule by a phenyl group resulted in an analogue with a very low rate of acetylation (V_{\max} approximately equal to 4% that of choline). The introduction of an alkyl chain between the phenyl group and the quaternary nitrogen gave analogues which were still poor substrates for ChAc *in vitro*. When one of the N-methyl groups was substituted for a pyridinium group the resultant analogue had a low rate of acetylation. However, the introduction of an alkyl chain between the quaternary nitrogen of the pyridinium group and the quaternary nitrogen of the choline moiety of these analogues led to analogues which were better substrates for ChAc. The dicholine compounds (32-39), discussed above, also followed this trend, in that as the distance between the two quaternary nitrogens increased so the rates of acetylation increased. Sollenberg, Stensiö and Sörbo, (1979) concluded that the improved substrate properties obtained with these two classes of compounds by elongation of the polymethylene chain is due to electrostatic binding of the second cationic head in the substrate to an anionic group located outside the active site of the enzyme.

Progressive substitution of the N-methyl groups in the choline molecule with ethyl groups resulted in analogues which were acetylated by ChAc *in vitro* at progressively decreasing rates. However, even when all three of the N-methyl groups were replaced by N-ethyl groups the

analogue, triethylcholine(25), was still acetylated. When one, two or all three of the N-ethyl groups in the triethylcholine molecule(25) were replaced with propyl or butyl groups the resultant analogues, according to one study were not substrates for the enzyme ChAc.

If the quaternary nitrogen of the choline moiety is part of a pyrrole (pyrrolcholine, 30) or morpholine (morpholinecholine, 31) ring, the analogues were poorer substrates than choline but were acetylated to some extent *in vitro* by ChAc.

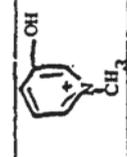
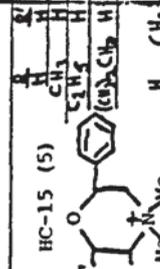
One study has shown the importance of the hydroxyl group in the choline molecule. Replacement of this group with an -SH group resulted in an analogue which was not acetylated *in vitro* by ChAc. However, replacement of this hydroxyl group with an -NH₂ group resulted in an analogue which was acetylated at about 50% the rate of choline. Presumably the acetyl group (from AcCoA) has a greater affinity for the oxygen atom due to its greater electronegativity, compared to sulphur or nitrogen. The -NH₂ derivative is still capable of being acetylated to some extent as nitrogen is fairly electronegative, more so than sulphur.

Any incongruities in the results from the different studies could well be due to methodology which includes the use of different sources of ChAc enzyme, different enzyme purification techniques, different concentrations of AcCoA, differences in the ionic strengths of the incubation media and different methods of isolating and quantitatively measuring the acetylated products.

It is apparent from a number of studies (Barker and Mittag, 1975; Collier, Barker and Mittag, 1976; Collier, Lovat, Ilson, Barker and Mittag, 1977) that the rate of acetylation of the choline analogue *in vivo*

Table 1.2 In vitro ACETYLATION OF CHOLINE ANALOGUES BY CHOLINE ACETYLTRANSFERASE

(see notes for references)

Choline Analogue	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}-\text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{CH}_3 \end{array}$ (1)	100%	$K_m=1.6\text{mM}$ ($V_{max}=52\mu\text{M/g/hr}$)	$K_m=0.166\text{mM}$ ($V_{max}=100\%$)	4nmole/ K_m of pr-0.17mM product/ V_{max} per 30 mins/ml of enzyme (/hr)	100%	100%	100%	$K_m=0.29\text{mM}$	100%	100%	$K_m=0.135\text{mM}$ ($V_{max}=100\%$)	100%	$K_m=0.99\text{mM}$ ($V_{max}=4.1\mu\text{M/g/hr}$)	100%	100%	$K_m=0.19\text{mM}$ (V_{max} of enzyme /hr=18.83)	$K_m=1.8\text{mM}$ ($V_{rel}=100\%$)	100%	$K_m=0.1$ ($V_{max}=775\mu\text{M/g/hr.}$)		
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{CH}_3 \end{array}$ (29)	0%	(0%)	(less than 5%)	0.05nm	less than 5%																
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{CH}_3 \end{array}$ (72)	29%	(0%)	(less than 5%)																		
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}-\text{CH}-\text{CH}_2-\text{OH} \\ \\ \text{CH}_3 \end{array}$ (D)					8.9mM (17)																
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}-\text{CH}-\text{CH}_2-\text{OH} \\ \\ \text{CH}_3 \end{array}$ (L)					39mM (9.88)																
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}-\text{CH}-\text{CH}_2-\text{OH} \\ \\ \text{CH}_3 \end{array}$ (DL)	0%		18.3mM (60%)																		
$\begin{array}{c} \text{CH}_3 \\ \\ \text{C}-\text{H}_2-\text{CH}_2-\text{CH}-\text{OH} \\ \\ \text{CH}_3 \end{array}$ D			(less than 5%)		16mM (7.62)																
$\begin{array}{c} \text{CH}_3 \\ \\ \text{C}-\text{H}_2-\text{CH}_2-\text{CH}-\text{OH} \\ \\ \text{CH}_3 \end{array}$ L			(less than 5%)		0																
	DL	0%	(less than 5%)																		
HC-3 (4)								27%													
HC-15 (5)			$K_m=1.88\text{mM}$ (65%)					$K_m=2.9$ (18%)													20%
			$K_m=20\text{mM}$ (15%)																		7%
																					5%
																					15%
																					12%

Choline Analogue	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
$\begin{array}{c} \text{CH}_3 \\ \\ \text{OH}-\text{CH}_2-\text{N}^+ \\ \quad \\ \text{CH}_2 \quad \text{CH}_3 \\ \\ (\text{CH}_2)_n \end{array}$ $\begin{array}{c} \text{CH}_3 \\ \\ \text{HO}-\text{CH}_2-\text{N}^+ \\ \quad \\ \text{CH}_2 \quad \text{CH}_3 \\ \\ (\text{CH}_2)_n \end{array}$ (32-39)	n=3 0% n=5 0% n=10 63%									n=3 (2%) n=4 (5%) n=5 (8%) n=6 ((78%)) n=10 (100%) when n=10 Km= 0.24mM	n=7 91% n=8 90% n=9 85%										
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}^+-\text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{CH}_2-\text{CH}_2-\text{OH} \end{array}$	79%																				
$\begin{array}{c} \text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{CH}_3-\text{N}^+-\text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{CH}_2-\text{CH}_2-\text{OH} \end{array}$	63%																				
$\begin{array}{c} \text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{HOCH}_2-\text{N}^+-\text{CH}_2-\text{CH}_2-\text{OH} \\ \quad \\ \text{CH}_2-\text{CH}_2-\text{OH} \quad \text{CH}_2-\text{CH}_2-\text{OH} \end{array}$	16%																				
	0%																				
	0%																				
$\begin{array}{c} \text{CH}_3-\text{S}-\text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{CH}_3 \end{array}$			NVPB did not inhibit the acetylation of this compound																		
$\begin{array}{c} \text{CH}_3-\text{N}^+-\text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{CH}_3 \end{array}$															100% 6%						
$\begin{array}{c} \text{CH}_3-\text{N}^+-\text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{H} \end{array}$	15%														0% 2%						
$\begin{array}{c} \text{H}-\text{N}^+-\text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{H} \end{array}$	15%																				

Notes

1. Burgen, Burke and Desbarats-Schonbaum, (1956)
2. DaUterman and Mehrotra, (1963)
3. Barker and Mittag, (1975)
4. Currier and Mautner, (1974)
5. Hemsworth and Smith, (1970a)
6. Collier, Lovat, Ilson, Barker and Mittag, (1977)
7. Bradshaw and Hemsworth, (1976)
8. Mann and Hebb, (1975)
9. Rodriguez de Lorez Arnaiz, Zieher and De Robertis, (1970)
10. Hemsworth, (1971a)
11. Hemsworth, (1971b)
12. Hemsworth, (1976)
13. Frakenber Heimburger, Nilsson and Sörbo, (1973)
14. Korey, Braganza and Nachmansohn, (1951)
15. Reibsberg, (1957)
16. Hemsworth and Smith, (1970b)
17. Sollenberg, Stensiö and Sörbo (1979)
18. Chlou, (1974)
19. Barker, Dowdall and Mittag, (1975)
20. Dowdall, cited by Von Schwarzenfeld and Whittaker, (1977)
21. Hemsworth, (1972)

may be much different to that found *in vitro*. This is possibly due to a coupling of the transport and acetylation processes and/or due to differences in the ionic strengths of the acetylating environment *in vivo* as compared to *in vitro* (Mann and Hebb, 1975; Hersh, Barker and Rush, 1978) or it could be that the enzyme purification procedures in some way change the behaviour of ChAc.

1.2.12. Inhibition of transport of choline into synaptosomes by choline analogues.

Table 1.3. is a review of the choline analogues that have been shown to inhibit the high affinity transport (except where noted otherwise in the table) of choline into synaptosomes, (see references for experimental details). Choline is the natural substrate for the transport system and in most studies the concentration of choline was $1\mu\text{M}$.

From Table 1.3. it can be seen that an increase in the length of the alkyl chain separating the quaternary nitrogen from the hydroxyl group in the choline molecule by one methylene group gave an analogue, homocholine (29), which inhibited the high affinity uptake of choline. Homocholine had a K_i (reciprocal of enzyme-inhibitor affinity) equal to the K_{T_H} (substrate concentration which provides one half of the maximal velocity of transport of the substrate into synaptosomes with regard to the high affinity process) of choline. Methyl substitution of the alkyl chain in the α or β position gave analogues which had some inhibitory activity against the high affinity choline transport system. The D- β -methylcholine analogue was a weak inhibitor ($IC_{50} = 240\mu\text{M}$), whereas the DL- α -methylcholine analogue was a much stronger inhibitor ($IC_{50} = 6-18\mu\text{M}$). This is a similar trend to that seen with the *in vitro* acetylation of

these analogues by ChAc, where the α analogue was acetylated at a greater rate than the β analogue.

HC-3(4) is a very powerful inhibitor of the high affinity choline transport system ($IC_{50} = 50$ to $100nM$). Terphenyl-HC-3(10) seems just as potent but HC-15(5) ($IC_{50} = 8$ to $15\mu M$) is much less potent.

An increase in the length of the alkyl chain separating the two quaternary nitrogen atoms in the dicholine compounds (32-36) did not seem to alter the inhibitory potency of these compounds on the transport of choline although in this case only the low affinity transport of choline was determined (see Table 1.3.). This is a different situation to that seen when the *in vitro* acetylation of these analogues was determined since an increase in the polymethylene chain separating the two nitrogens resulted in analogues which were acetylated at a correspondingly increasing rate.

When the quaternary nitrogen of the choline molecule was part of a pyrrole ring then the analogue, pyrrolcholine(30), was quite a potent inhibitor of the high affinity choline transport system (IC_{50} approximately equals $5\mu M$). However, the potency was decreased by about ten times when the quaternary nitrogen was part of a morpholine ring (morpholinecholine, 31). Tertiary choline analogues were very weak inhibitors of choline transport (IC_{50} greater than $1mM$).

Progressive substitution of the N-methyl groups in the choline molecule by ethyl groups (mono di- and triethylcholine 27, 28, 25) did not affect the potency of these analogues too much as inhibitors of high affinity choline transport system. They were all potent inhibitors (IC_{50} approximately equal to 2 to $5\mu M$).

Replacement of the hydroxyl group in the choline molecule with an -SH group resulted in a marked reduction in the inhibitory potency of the analogue ($IC_{50} = 75\mu M$). Esterification of the hydroxyl group also reduced the inhibitory potency of the choline analogue against the high affinity choline transport system although ACh(2) had some inhibitory activity ($IC_{50} = 12\mu M$).

Choline analogues, some bis quaternary, others monoquaternary with no hydroxyl group have been shown to be potent inhibitors of the high affinity choline transport system (see Table 1.3.). The bis quaternary analogues showed an increase in inhibitory potency as the n-alkyl chain separating the two quaternary nitrogens increased. A similar trend is seen with the *in vitro* acetylation of bis quaternary choline analogues by ChAc. As the distance between the two nitrogens increases, the rates of acetylation increase. Many other compounds have been shown to inhibit choline transport to some extent.

In conclusion it appears that there is very little structural specificity for the inhibitors of the high affinity choline transport system. This is a different situation to that found with the choline analogues which act as substrates for ChAc *in vitro*, since in this case the structure is very important. (See Table 1.2.). The most powerful inhibitors of choline transport that have been studied are HC-3 and terphenyl-HC-3.

1.3. INHIBITION OF CHOLINE ACETYLTRANSFERASE

The ability of styrylpyridine analogues to inhibit ChAc was first demonstrated in *in vitro* experiments by Smith, Cavallito and Foldes, (1967). Quaternary pyridinium analogues such as N-hydroxyethyl-4-(1-naphthylvinyl)

TABLE 1.3. INHIBITION OF THE HIGH AFFINITY TRANSPORT (EXCEPT WHERE STATED OTHERWISE) OF CHOLINE INTO SYNAPTOSOMES BY CHOLINE ANALOGUES.

(Values, unless otherwise noted are IC₅₀'s) (See notes for references)

choline analogue	1	2	3	4	5	6	7	8	9	10	11	12	13	14
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}-\text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{CH}_3 \end{array}$ (1)	1.6μM													
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{OH} \\ \\ \text{CH}_3 \end{array}$ (29)	4μM		K _i =3.6μM											
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}-\text{CH}-\text{CH}_2-\text{OH} \\ \quad \\ \text{CH}_3 \quad \text{CH}_3 \end{array}$ [DL]	18μM	6μM												
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}-\text{CH}_2-\text{CH}-\text{OH} \\ \quad \\ \text{CH}_3 \quad \text{CH}_3 \end{array}$ [D]	240μM													
HC-3 (4)	50nM	80nM	0.1μM	30% inhibition at 100μM.	K _i =25nM 60nM	K _i =25nM	80% inhibition at 10μM	0.1mM	K _i =40μM	K _i =21μM	K _i =19μM			
HC-15 (5)	15μM	8μM				7.7μM		0.1mM		K _i =0.17mM				
Terphenyl-HC-3 (10)		80nM												

Table 1.3 (cont.)

choline analogue	1	2	3	4	5	6 (40% inhibition)	7	8 (100% inhibition)	9	10	11	12	13	14
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{C}-\text{N}-\text{CH}_2\text{CH}_2\text{OH} \\ \quad \\ \text{H} \quad \text{CH}_3 \end{array}$	>1mM													
$\text{CH}_3-\text{NH}-\text{CH}_2\text{CH}_2\text{OH}$	>1mM													
$\text{NH}_2\text{CH}_2\text{CH}_2\text{OH}$	>1mM													
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{CH}_3-\text{N}-\text{CH}_2\text{CH}_2\text{OH} \\ \\ \text{CH}_3 \end{array} \quad (27)$		$K_1 = 2.98 \mu\text{M}$ $\cdot 2 \mu\text{M}$												
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{CH}_3-\text{N}-\text{CH}_2\text{CH}_2\text{OH} \\ \\ \text{C}_2\text{H}_5 \end{array} \quad (28)$	1.6 μM	2.5 μM												
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{C}_2\text{H}_5-\text{N}-\text{CH}_2\text{CH}_2-\text{OH} \\ \\ \text{C}_2\text{H}_5 \end{array} \quad (25)$	3.8 μM	5 μM				15 μM		0.1mM			$K_1 = 1\text{mM}$			1mM to 1 μM inhibit choline uptake

Table 1.3 (cont)

choline analogue	1	2	3	4	5	6	7	8	9	10	11	12	13	14
						(40% inhibition)		(100% inhibition)						
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}^+-\text{CH}_2-\text{CH}_2-\text{SH} \\ \\ \text{CH}_3 \end{array}$	75 μM													
$\begin{array}{c} \text{CH}_2 \\ \\ \text{CH}_3-\text{N}^+-\text{CH}_2-\text{CH}_2-\text{COCH}_3 \\ \\ \text{CH}_3 \end{array}$ (2)	12 μM													
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}^+-\text{CH}_2-\text{CH}_2-\text{CONH}_2 \\ \\ \text{CH}_3 \end{array}$	350 μM													
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{N}^+-\text{CH}_2-\text{CH}_2-\text{OPO}_3\text{H}_2 \\ \\ \text{CH}_3 \end{array}$	1mM													
Troxypyrrolium tosylate														
BW 284C51	0.24 μM										0.17 μM			
CT 5263											0.41 μM			
AC-seco-HC-3 (14)											5.2 μM			
														10mM gives 83% inhibition

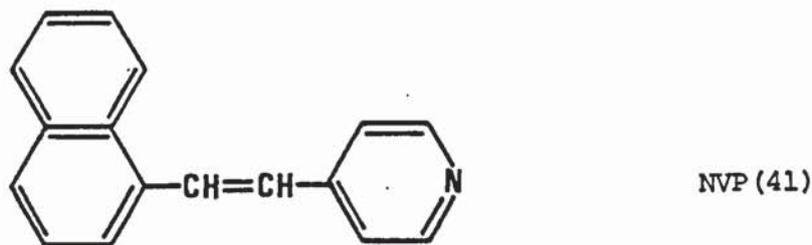
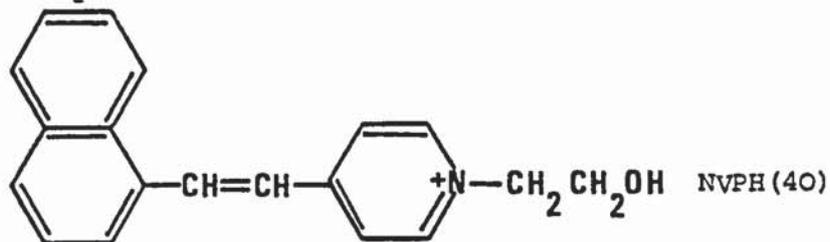
Table 1.3 (cont.)

inhibitor	1	2	3	4	5	(40% inhibition)	7	(100% inhibition)	9	10	11	12	13	14
decamethonium									8mM					
edrophonium									9mM					
physostigmine	>100µM													

Notes

1. Simon, Mittag and Kuhar (1975)
2. Barker and Mittag (1975)
3. Collier, Lovat, Ilson, Barker and Mittag (1977)
4. Marchbanks (1968) (low affinity transport)
5. Guyenet, Lesfresne, Rossier, Beaujolan and Glowinski (1973^{a, b})
6. Holden, Rossier, Beaujolan, Guyenet and Glowinski (1975)
7. Hemsworth and Cholakis (1978)
8. Hemsworth, Dørner and Bossman (1971) (low affinity transport)
9. Diamond and Kennedy (1969) (low affinity transport)
10. Diamond and Milfay (1972) (low affinity transport)
11. Potter (1968) (low affinity transport)
12. Dowdall cited by Von Schwarzenfeld and Whittaker (1977)
13. Barker, Dowdall and Mittag (1975)
14. Howard-Butcher, Cho and Schaeffer (1974)

pyridinium (NVPH) (40) are the most potent of the styrylpyridine inhibitors, however, they are also potent inhibitors of AchE. The tertiary nitrogen analogues such as 4-(1-naphthylvinyl)-pyridine (NVP) (41) and certain halogenated styrylpyridines (e.g. 4-chlorostilbazole, CS) (Baker and Gibson, 1971;1972), are more selective in their ability to inhibit ChAc.

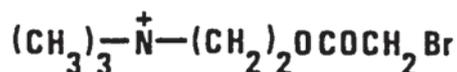


Studies have been effected on the binding of these inhibitors to ChAc *in vitro* which have led to suggestions for the chemical structure of the active site of this enzyme. Baker and Gibson, (1972) suggested that the inhibition of ChAc by these compounds involves an interaction between a nucleophilic residue on the enzyme and the positive partial charge on the vinyl bridge, White and Cavallito, (1970) suggested that this nucleophilic residue is a catalytic imidazole group.

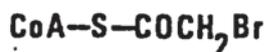
Biochemical studies suggest that NVP and CS inhibit brain ChAc *in vivo* (Krell and Goldberg, 1975). NVP has been shown to cause a reduction in the rate of ACh synthesis in the brain (Saelens, Simke, Schuman and Allen, 1974), but neither NVP nor CS cause a reduction in the concentration of ACh in the brain *in vivo*.

NVP, *in vivo*, produces a depression of the central nervous system, skeletal muscle weakness and death, however, the toxicity of NVP is different to that of HC-3 (Hemsworth and Foldes, 1970). NVP does, however, possess weak, anticholinergic activity (Hemsworth and Foldes, 1970). These workers showed that the neuromuscular block induced by NVP might be related to the inhibition of ChAc, or a postsynaptic block of the cholinergic receptors.

Haloacetyl derivatives of either ACh (Morris and Grewaal, 1969) (42) or AcCoA (Roskowski, 1974) (43) are potent inhibitors of ChAc *in vitro*.

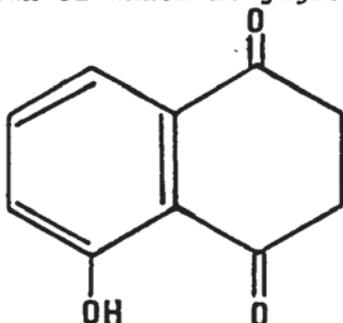


Bromoacetylcholine (42)



Bromoacetyl-CoA (43)

Some quinone derivatives are known to inhibit ChAc *in vitro*; the most well known of which is juglone (Häubrich and Wang, 1976) (44)



Juglone (44)

Several other compounds have been shown to have an inhibitory action on ChAc *in vitro*, for example, halogenated aldehyde analogues of ACh (Persson, Larsson, Schuberth and Sörbo, 1967) and both products of the forward reaction of ChAc; CoA is about 1,000 times more potent than is ACh (Kaita and Goldberg, 1969).

1.4. POSSIBLE SITES OF ACTION FOR DRUGS THAT GIVE A PRESYNAPTIC
BLOCK AT CHOLINERGIC NERVE TERMINALS.

Since many choline analogues have been shown to give a presynaptic block at cholinergic nerve terminals it is interesting to speculate as to the possible sites of action of these compounds.

A presynaptic block, produced by a drug, at a cholinergic synapse can be the result of one or more of the following actions:-

1. Interference in the electrical conduction of the neurons.
2. Inhibition of the synthesis of the transmitter, ACh.

(a) interference with the supply of choline for ACh synthesis by (i) an inhibition of the transport of choline from extracellular sites into the neuron or (ii) by enhancement of an alternative metabolic pathway at the expense of the pathway involving the synthesis of ACh.

(b) interference with the supply of AcCoA by preventing its synthesis in the neuron.

(c) impairment of ChAc by (i) preventing its synthesis or (ii) by direct inhibition of the enzyme by the drug which may or may not be acetylated to a derivative which could possibly act as a false transmitter.

(d) interference in the subcellular localisation of choline, AcCoA or ChAc.

(e) interference with the control mechanism of ACh synthesis.

3. Interference with the subcellular localisation of ACh.
4. Impairment of the release of ACh by the action potential.
5. Release of a false transmitter which has a weaker postsynaptic action than ACh and therefore attenuates the quanta of transmitter released.

1.5 AIMS

Thirteen choline analogues have been studied which have been classified into three series, that is, the hemicholinium analogues, the long-chain choline analogues and the hydroxypiperidinium and hydroxypyridinium analogues. Most of the analogues are commercially unobtainable and therefore have had to be synthesised and their chemical structure and purity has had to be confirmed.

The aim of the present study was to investigate the action of these analogues at the cholinergic synapse especially with regard to their presynaptic action. To this end a variety of chemical, pharmacological and biochemical techniques have been employed. In addition structure/activity relationships have been studied and are discussed.

1.6 NOMENCLATURE

Where appropriate the choline analogues have been initially referred to by their trivial name, for example choline; their common chemical name as found in chemical catalogues such as "Aldrich", for example (2-hydroxyethyl) trimethylammonium and their chemical name as found in "Chemical Abstracts", for example 2-hydroxy-N,N,N-trimethyl-ethanaminium. They are thereafter referred to by their trivial name only except that in the chemistry sections all three names will be used when appropriate. In general common chemical names have been quoted for other reagents such as starting materials. Most of the choline analogues and, where appropriate, some other compounds have been numbered, the numbers referring to a chemical structure which has been drawn out. In general these numbers are only used initially although they have sometimes been used later for the sake of clarity.

2. MATERIALS

2.

MATERIALS

2.1. RADIOCHEMICALS

[1-¹⁴C]-Acetyl-Coenzyme A (59.5mCi/mmole). The Radiochemical Centre, Amersham, England.

[1-¹⁴C]-Acetyl-β-methylcholine iodide (3.2mCi/mmole diluted to 0.07mCi/mmole with non-labelled Ac-β-MeCh). New England Nuclear, Winchester, Hants.

[1-¹⁴C]-Butyrylcholine iodide (4.8mCi/mmole diluted to 0.12mCi/mmole with non-labelled BuCh). New England Nuclear, Winchester, Hants.

[1-¹⁴C]-Acetylcholine iodide (10.2mCi/mmole diluted to 2.7mCi/mmole with non-labelled ACh). The Radiochemical Centre, Amersham, England.

[Methyl-³H]-Choline chloride (6.4Ci/mmole). The Radiochemical Centre, Amersham, England.

[¹⁴C]-Methyl iodide (58mCi/mmole). The Radiochemical Centre, Amersham, England.

2.2. COMMERCIAL ENZYME PREPARATIONS

Acetylcholinesterase; Acetylcholine hydrolase E.C.3.1.1.7. from bovine erythrocytes (2.6units/mg of solid). Sigma Chemical Co., St.Louis, Mo., U.S.A.

Cholinesterase; from horse serum. Type IV. (21.3units/mg of solid) Sigma Chemical Co., St.Louis, Mo., U.S.A.

2.3. ION-EXCHANGERS

Amberlite CG-50; 100-200 mesh. ($\text{RCOO}^{\ominus}\text{Na}^{\oplus}$) Chromatographic grade.

Mallinkrodt Chemical Works, St.Louis, Mo., U.S.A.

Amberlite CG-120; 200-400 mesh. ($\text{RSO}_3^{\ominus}\text{Na}^{\oplus}$) Chromatographic grade.

Mallinkrodt Chemical Works, St.Louis, Mo., U.S.A.

Sephadex G-25; CM-Sephadex C-50, SP-Sephadex C-25. Sigma Chemical

Co., St.Louis, Mo., U.S.A.

Kalignost; (sodium tetraphenylboron) B.D.H. Chemicals Ltd.,

Poole, England.

Ammonium Feineckate; ammonium tetrathiocyanodiammonochromate.

Schuchardt Chemical Co., Munich, Germany.

2.4. MATERIALS USED IN LIQUID SCINTILLATION COUNTING

PPO; 2,5 diphenyloxazole. Hopkin and Williams, Chadwell Heath,

Essex, England.

POPOP; 1,4 di (2-[5-phenyloxazolyl]) benzene. Hopkin and Williams,

Chadwell Heath, Essex, England.

Naphthalene; (scintillation grade). B.D.H. Chemicals Ltd.,

Poole, England.

NE260; (Micellar Scintillator), Nuclear Enterprises Ltd.,

Sighthill, Edinburgh.

Ready-Solv, Solution VI; Beckman, RIIC Ltd., Fife, Scotland.

Glass Microfibre Paper; (2.5cm) GF/A, Whatman, Maidstone, Kent, England.

2.5 MISCELLANEOUS

Bovine Serum Albumin; Sigma Chemical Co., St.Louis, Mo., U.S.A.

EDTA; disodium ethylenediaminetetra-acetic acid. B.D.H. Chemicals Ltd.,

Poole, England.

DTT; dithiothreitol, Sigma Chemical Co., St. Louis, Mo., U.S.A.

Tris buffer; (Tris [hydroxymethyl]aminomethane) Sigma Chemical Co.,
St. Louis, Mo., U.S.A.

Triton X-100; (scintillator grade). Hopkin and Williams, Chadwell
Heath, Essex, England.

Choline chloride; Hopkin and Williams, Chadwell heath, Essex, England.

Acetylcholine chloride; Sigma Chemical Co., St. Louis, Mo., U.S.A.

Hemicholinium-3; Aldrich Chemical Co., London, England.

Acetyl-seco-hemicholinium-3; Eastman Organic Chemicals, Rochester,
New York, U.S.A.

3. METHODS

3.

METHODS

3.1. RAT PHRENIC NERVE-HEMIDIAPHRAGM

In experiments on the isolated phrenic nerve-diaphragm preparation of the rat (Bülbring, 1946) both hemidiaphragms were mounted in the same bath in Krebs solution (Table 3.2.) at 37°. The muscles were excited by supramaximal rectangular pulses of 0.1msec duration applied to the phrenic nerves. One nerve was stimulated at 1Hz and the other at 0.1Hz. The muscle contractions were recorded isometrically by means of a force displacement transducer on a Washington recorder.

3.2. FROG RECTUS ABDOMINIS MUSCLE

The frog rectus abdominis muscle was suspended in frog-Ringer solution (Table 3.2.) at room temperature (20°). The frog-Ringer was aerated with air. ACh (or other agonists) were added at 4 min. intervals and left in contact with the muscle for 30 sec. The preparation was then washed twice with fresh frog-Ringer and allowed to relax to its baseline position. In experiments investigating the inhibition of the ACh induced submaximal muscle contraction the choline 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 and a Washington recorder.

3.3. GUINEA-PIG ILEUM

The terminal portion of the ileum, 2-4cms in length, was removed and suspended in Tyrode solution (Table 3.2.) at 37°. The Tyrode was aerated with air. One of the acetylated hydroxypiperidinium compounds as

synthesised in this study (Section 6.2.) was added to the bath at 2 min. intervals and left in contact with the tissue for 30 sec. The preparation was then washed twice with fresh Tyrode. Contractions were recorded isototonically using a Washington recorder.

3.4. CHOLINESTERASE DETERMINATIONS

3.4.1. Preparation of cholinesterase enzymes

Fresh rat brains were homogenised on ice in 0.1% Triton X-100 containing 200mM KCl (10ml/g wet weight) using a glass homogeniser with a Teflon pestle. The homogenate was centrifuged at 20,000g for 20 min. and the supernatant containing the enzyme was used immediately or stored at -20°.

Commercial preparations of acetylcholinesterase (AChE) and pseudo-cholinesterase (ChE) were made up in distilled water to a concentration of 1mg of protein/ml.

3.4.2. Determination of activity

3.4.2.1. Radiochemical procedure

The method used was the radiochemical procedure described by Siakotos, Filbert and Hester, (1969).

3.4.2.1.1. Incubation system

The rates of hydrolysis of [¹⁴C]-labelled substrates (ACh, acetyl-β-methylcholine and butyryl choline) in the presence of the choline analogues were estimated by incubation with the cholinesterase preparations obtained as described above and by quantitative determination of the radioactive product of hydrolysis.

The incubation mixture was placed in a 10ml graduated test-tube and contained 100µl of enzyme, 100µl of [^{14}C]-labelled substrate (3mM, giving a final concentration of 1mM), 30µl of the choline analogue at the appropriate concentration and 70µl of the buffer - salt - detergent mixture (containing NaCl 300mM, NaH_2PO_4 20mM, Na_2HPO_4 80mM and 10mls 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 waterbath at 37° for 10 min. over which time the reaction followed a linear time course. Controls contained 30µl of distilled water in place of the choline analogue. Blank values were determined in which the enzyme was replaced with distilled water.

3.4.2.1.2. Isolation and determination of the radioactive product of hydrolysis

After incubation the reaction was stopped by the addition of 5ml of a resin-dioxan mixture (20g washed Amberlite CG-120, 200-400 mesh in 100ml dioxan). Dioxan was then added to the test-tube up to a volume of 10mls. The contents of the tube were mixed. After centrifugation 5ml of the supernatant was added to 5mls of modified Brays (1960) scintillation fluor and any radioactivity was detected by liquid scintillation spectrometry.

3.4.2.2. Null-point titration method

The de-esterification of the acetylated-piperidinols, synthesised in this thesis (Section 6.2.), by AChE and ChE were estimated *in vitro* by a null-point titration method using a Radiometer (Copenhagen) pH-Stat unit (Burette unit ABU12; Titration assembly TTA60; Tritrator TTT2; pH Meter PHM22 and Recorder SBR3) with a 25ml water jacketed reaction

chamber for automatic titration of N/200 NaOH from the 25ml burette. The temperature was maintained at 37°.

Solutions of AChE and ChE (commercially obtained) were made up in distilled water and 0.2ml of this solution was added to 6mls of a modified Ringer solution (containing NaCl 153.7, KCl 4.02, CaCl₂ 3.31mM). Sufficient water was then added so that the final incubation volume (after the addition of the substrate) was 10ml. The incubation system was left for 5 min. to equilibrate to 37° and the pH was then adjusted to pH 7.4. The substrate was then added and the hydrolysis of the compound was followed for 5 min. Various substrate concentrations were used and blank values were obtained using denatured enzymes.

3.5 CHOLINE ACETYLTRANSFERASE (ChAc) DETERMINATIONS

3.5.1. Preparation of rat brain ChAc

Acetone-dried powders were prepared from rat brains by a similar procedure to that used by Hemsworth and Morris, (1964). Freshly dissected rat brains were placed in an ice-cold mortar and acetone, pre-cooled to -20°, was added in 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 and was then transferred to a dessicator and dried over P₂O₅ at 4° for 4 hr.

When dry the powder was extracted following the method described by Mann and Hebb, (1975). The powder was extracted with 0.1M sodium phosphate buffer (pH 6.5) containing 1mM disodium EDTA and 1mM DTT, the concentration of the powder being 50mg/ml. The powder and buffer mixture was frozen (-20°) and 12 hr later thawed and centrifuged (0°)

at 80,000g for 1hr. The supernatant was run through a Sephadex G-25 column to remove the endogenous choline; before use the column was equilibrated with the extracting medium. The eluate containing the ChAc contained little, if any, free choline and when stored frozen at -20° for two months did not form further amounts of free choline.

3.5.2. Incubation system for the acetylation of choline and the choline analogues

The rates of acetylation of choline and other substrates were estimated by incubation with $[^{14}\text{C}]$ -AcCoA and ChAc and by a quantitative determination of the amount of labelled product formed.

The incubation mixture was placed in a plastic microfuge tube (Beckman) and contained 5 μ l ChAc, 10 μ l choline or other substrate at the required concentration and 10 μ l of a buffer containing $[^{14}\text{C}]$ -AcCoA and other constituents (Table 3.1.). Blank values were determined by replacing the substrate with distilled water.

All additions were made at 0° and when complete the tubes were shaken and then transferred to a shaking waterbath at 37° and incubated for 10min. The reaction was stopped by transferring the tubes to an ice-bath.

3.5.3. Incubation system for the measurement of the inhibition of ChAc by the choline analogues

The inhibition of ChAc by the choline analogues was measured by a quantitative determination of the amount of $[^{14}\text{C}]$ -ACh formed by a reaction of $[^{14}\text{C}]$ -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 μ l ChAc, 10 μ l choline (14mM), 10 μ l of choline analogue (14mM) and 10 μ l of a buffer containing $[^{14}\text{C}]$ -AcCoA and other

Substance Added	Initial Concentration, mM	Final Concentration in incubation medium, mM	
		acetylation experiments	inhibition experiments
NaCl	30	12	8.6
MgSO ₄	5	2	1.4
disodium EDTA	0.1	0.04	0.03
potassium phosphate (pH7.7)	15	6	4.3
bovine serum albumin	(0.05%)	(0.02%)	(0.014%)
physostigmine sulphate	0.2	0.08	0.06
[¹⁴ C]-AcCoA (59.5mCi/mmole)	0.045	0.018	0.013

TABLE 3.1: Buffer used in ChAc determinations

constituents (Table 3.1). Control values were determined by replacing the choline analogue with distilled water. Blank values were determined by replacing both the substrate and choline analogue with distilled water.

Additions and incubation were effected as described above (Section 3.5.2.).

3.5.4. Isolation of the acetylated product

The radioactive $[^{14}\text{C}]$ -acetyl-labelled product was routinely extracted into the toluene based scintillation fluor as described by Fonnum, (1975). Using this procedure the microfuge tube, after incubation, was placed in the scintillation vial and washed out with 5ml of sodium phosphate buffer (10mM, pH 7.4) using a syringe. To the vial was then added 2ml of acetonitrile containing 10mg of Kalignost (sodium tetraphenylboron). The vial was shaken and 10ml of toluene scintillation fluor was then added. The vial was then shaken lightly for 1 min., and the $[^{14}\text{C}]$ -acetyl-labelled product was thus extracted into the toluene phase whereas the unreacted $[^{14}\text{C}]$ -AcCoA was left in the aqueous phase. The amount of radioactive acetylated product in the scintillation fluor was determined by liquid scintillation spectrometry. The efficiency of the extraction procedure and the counting efficiency were determined by adding a known amount of $[^{14}\text{C}]$ -ACh of known specific activity to the incubation medium in the absence of a choline analogue and extracted as described. The total efficiency of both the extraction procedure and the counting procedure was 85%.

3.6 TRANSPORT STUDIES WITH SYNAPTOSOMES

3.6.1. Preparation of synaptosomes

A crude synaptosomal fraction (P_2) was prepared from rat fore-brain by a method similar to that described by Gray and Whittaker, (1962).

A freshly dissected rat fore-brain was homogenised (30 up and down strokes) in ice-cold 0.32M sucrose (10%w/v). The homogenate was then centrifuged (0°) at 1000g for 10 min. The pellet (P_1) was discarded and the supernatant (S_1) was centrifuged again (0°) at 10,000g for 20 min. The supernatant (S_2) was discarded whereas the pellet consisting of the crude synaptosomes was routinely resuspended in 4.5mls of ice cold 'Krebs Ringer Phosphate', buffer (KRP), (NaCl 175mM; KCl 4mM; glucose 10mM; $CaCl_2$ 1mM; $MgCl_2 \cdot 6H_2O$ 2mM; sodium phosphate buffer 10mM, pH 7.4) which gave 30mg of original wet brain weight per 150 μ l. In some experiments the synaptosomal fraction was resuspended with (a) KRP with NaCl replaced by LiCl on a molar basis or (b) Tris-Krebs buffer (KCl 4mM, glucose 10mM; $CaCl_2$ 1mM; $MgCl_2 \cdot 6H_2O$ 2mM; Tris buffer 50mM; pH 7.4). The synaptosomes were used within 1 hr of being prepared.

3.6.2. Incubation of synaptosomes

(a) 150 μ l of the synaptosome suspension in KRP buffer was added to a 10ml test-tube containing [3H]-choline (1 μ M, 0.55 μ Ci/nmole) and KRP buffer such that the final volume was 1ml. In inhibition experiments the choline analogue was added at the appropriate concentration. In some experiments [3H]-choline was replaced with [^{14}C]-3-hydroxypiperidinium (11-509 μ M, 0.55 μ Ci) and in some experiments using this compound metabolic inhibitors were added to the KRP buffer and in other experiments the KRP buffer was modified such that the NaCl was replaced with LiCl on a molar basis. In the latter case the synaptosomes were resuspended in the appropriate buffer (Section 3.6.1.).

(b) 150 μ l of the synaptosome suspension in Tris-Krebs buffer was added to a 10ml test-tube containing, either [3H]-choline (1-100 μ M; 0.55 μ Ci) and 500 μ l of NaCl (1M), or [^{14}C]-HC-3 (0.87-200.67 μ M, 0.160 μ Ci) in NaCl (1M) (as discussed in Section 4.3.6.2.) and Tris-Krebs buffer such that the

final volume was 2ml. In some experiments metabolic inhibitors were added to the incubation medium.

All additions were made at 0°. The synaptosomes were always added last. The tubes were gently shaken by hand and incubated in a shaking waterbath at 37°. Blank values were obtained by incubating at 0°.

3.6.3. Measurement of the transport of the radioactively labelled compounds into synaptosomes

After incubation the tubes were immediately placed in ice. They were then centrifuged at 2,000g using a bench centrifuge. The supernatant was discarded and the pellets were washed once with the appropriate KRP solution (5ml) at room temperature. The tubes were centrifuged once again and the supernatant was discarded as before. 200µl of NaOH (1N) was added to the remaining pellets and the tubes were mixed with a 'Whirlimixer'. The contents of the tubes were pipetted onto glass fibre papers. The papers were allowed to dry at room temperature and any radioactivity on them was detected using a toluene scintillation fluor and counting in a liquid scintillation spectrometer. Counting efficiencies were determined by counting a known amount of [¹⁴C]-choline of known specific activity on a glass fibre filter as described above. The counting efficiency was 90%.

3.6.4 Synaptosomal metabolism of [¹⁴C]-HC-3 and [¹⁴C]-3-hydroxy-piperdinium

Synaptosomes were incubated as previously described (Section 3.6.2) in the absence of any metabolic inhibitors. After incubation the transport was terminated by centrifugation and the pellets were washed as described in Section 3.6.3. except that the KRP solution contained physostigmine sulphate (0.5mM). After the final centrifugation the Krebs-physostigmine solution was discarded and each pellet was extracted

with 1ml of a 1:10 dilution of electrophoresis buffer (see Section 3.8.) containing physostigmine sulphate (0.5mM). The extract was freeze dried and dissolved in 20 μ l of methanol. The radioactively labelled compounds present in the extract were separated and identified by electrophoresis (Section 3.8).

3.7. MOUSE PHRENIC NERVE-DIAPHRAGM PREPARATION

Experiments with this preparation were based on the methods of Potter, (1970).

The phrenic nerve endplate region was dissected out from a mouse hemidiaphragm (Fig. 3.1.) and was placed in a 10ml glass beaker containing 5ml of a mixture of Krebs solution (Table 3.2) and physostigmine sulphate (30 μ M). This solution was vigorously bubbled with 5% CO₂ in O₂ delivered from a fine glass pipette. The incubation medium was maintained at 37^o by mounting the beaker in a water bath. [³H]-choline (30 μ M; 10 μ Ci/0.15 μ mole) or [¹⁴C]-3-hydroxypiperidinium (36 μ M; 10 μ Ci/0.18 μ mole) was added to the beaker. Due to the purification procedure the latter compound is in a solution of 300mM NaCl (Section 6.2.4.9.) and therefore in this case the Krebs-physostigmine solution was modified so that the correct concentration of NaCl was maintained. The phrenic nerve was held just out of the incubation medium by a pair of fine platinum electrodes and the nerve was kept moist by the splattering of fluid induced by the bubbler. Rectangular pulses of 0.1msec duration were applied to the phrenic nerve at a voltage initially required for vigorous muscle twitching (before dissection). The nerve was stimulated at 1Hz for 30 min in the presence of the radioactively labelled compounds.

The preparation was then washed three times with fresh Krebs-physostigmine solution and the tissue was finally incubated with 5 ml of this solution. The tissue was left at rest for 10 min and then stimulated

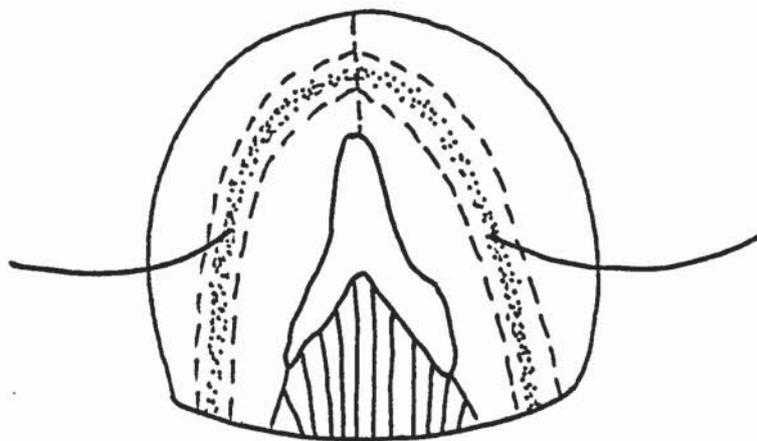


Fig. 3.1:- A stretched whole diaphragm from a mouse is shown diagrammatically. Dots indicate the position of neuromuscular end-plates seen with the aid of a light beneath the tissue. Dashed lines show where the diaphragm was cut.

as described above for 30 min. The tissue was then removed from the bath and extracted with a 1:10 dilution of electrophoresis buffer (Section 3.8) containing physostigmine sulphate (0.5mM). This extract was freeze dried. The incubation medium was also freeze dried. Sufficient methanol was added to the residues and the radiolabelled compounds present in the extract were separated and identified by paper electrophoresis.

Each experiment was conducted in duplicate using both hemidiaphragms from a mouse.

3.8 PAPER ELECTROPHORESIS

Paper electrophoresis was used to identify and confirm the purity of the radiolabelled choline analogues synthesised in the present study (Sections 4.2.8.6 and 6.2.4.9) it was also used to isolate and identify radiolabelled metabolites synthesised by synaptosomes (Section 3.6.4.) and by the mouse phrenic nerve-diaphragm preparation (Section 3.7).

Non-radioactively labelled compounds were added to the solutions containing the radiolabelled compounds, to be separated and identified. Samples of such solutions were applied to the mid-line of the electrophoresis paper and allowed to dry. The compounds were then subjected to electrophoresis at 500 volts for 2hr in a V-type Durrum cell (Beckman) using 1.5M acetic acid/0.75M formic acid buffer as described by Potter and Murphy, (1967). After electrophoresis, the paper was stained in iodine vapour whilst barely damp and the position of any stained band was recorded. The iodine was allowed to evaporate off and after drying the electrophoresis paper was cut into 0.5cm or 1cm strips. The strips were placed in a scintillation vial containing toluene scintillation fluor and radioactivity on the strips was detected and estimated by liquid scintillation spectrometry. The counting efficiency of the paper strips was determined by performing electrophoresis on a known amount of [^{14}C]-ACh of known specific activity and then cutting the electrophoresis paper into strips and detecting the radioactivity as described above. The counting efficiency was 45%.

3.9 LIQUID SCINTILLATION COUNTING

Radioactive measurements were made using a Beckman LS-230 liquid scintillation spectrometer.

3.9.1 Toluene-based scintillation fluor

This fluor contained PPO (5g/l) and POPOP (0.3g/l) in toluene.

3.9.2 Modified Bray's fluor

This fluor was based on that described by Bray, (1960) and contained naphthalene (100g/l), methanol (100ml/l), PPO (4g/l) and POPOP (0.2g/l) in 1,4 dioxan.

3.10 DETERMINATION OF PROTEIN

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall, (1951). Bovine serum albumin was used as a standard.

3.11 ANIMALS

All the animals used, that is, rats (Wistar), mice (BKW), guinea-pigs (Dunkin Hartley) and frogs (*Rana temporaria*), were male and they were killed by decapitation.

3.12 CHEMISTRY:- Methods used to identify compounds synthesised

3.12.1 Melting points

Melting points were determined using an Electrothermal melting point apparatus, melting points are uncorrected.

3.12.2 Infrared absorption spectra

Infrared spectra were recorded using a Unicam S.P. 200 spectrophotometer. The samples were run as mulls in liquid paraffin, as potassium

bromide discs and as solutions in water (using IRTRAM cells).

3.12.3 Ultraviolet absorption spectra

Ultraviolet spectra were recorded using a Unicam SP 8,000 spectrophotometer. The samples were run as solutions in water unless otherwise indicated.

3.12.4 Nuclear magnetic resonance spectra

Nuclear magnetic resonance spectra were determined in deuterium oxide on a Varian A60 spectrometer using tetramethyl silane as an internal standard. All the peaks were assigned in τ values.

3.12.5 Microanalyses

Microanalyses were carried out by B.M.A.C., the Butterworth Microanalytical Consultancy Ltd., Middlesex, U.K.

	Krebs	Frog-Ringer	Tyrode
NaCl	6.87	6.50	8.00
KCl	0.40	0.14	0.20
MgCl ₂	-	-	0.10
MgSO ₄ ·7H ₂ O	0.14	-	-
NaH ₂ PO ₄	-	0.01	0.05
KH ₂ PO ₄	0.16	-	-
Glucose	2.00	2.00	1.00
NaHCO ₃	2.10	0.40	1.00
CaCl ₂	0.28	0.12	0.20
aerating gas	5% CO ₂ in O ₂	air	air

TABLE 3.2: Physiological salt solutions. Composition in g/l of distilled water.

4. RESULTS CHAPTER I:- HC-3, SOME DIPHENYLALKANE ANALOGUES

OF HC-3 AND A METHYL ANALOGUE OF HC-15

4. RESULTS CHAPTER I:- HC-3, SOME DIPHENYLALKANE ANALOGUES OF HC-3
AND A METHYL ANALOGUE OF HC-15

4.1 INTRODUCTION

4.1.1 HC-3

Long and Schueler (1954) and Schueler (1955), were the first to synthesise α,α' -dimethylethanolamino,4,4'biacetophenone (3) which was shown by Schueler (1955) to undergo spontaneous hemiacetal formation in aqueous solutions as shown in Fig. 1.1, to give a compound which has since been known as hemicholinium-3 or HC-3(4). Chemical Abstracts however, names this compound 2,2'-[1,1'-biphenyl] 4,4'diylbis [2-hydroxy-4,4-dimethyl morpholinium] dibromide.

4.1.1.1 Anticholinesterase activity

HC-3 has been reported to have weak anticholinesterase activity. Long and Schueler (1954), reported that HC-3 inhibits AChE (obtained from bovine erythrocytes) *in vitro* at concentrations greater than 10^{-5} M when acetyl- β -methylcholine (2×10^{-2} M) was used as the substrate. Marshall and Long (1959) showed that HC-3 (10^{-3} M) inhibited by 25% the breakdown of ACh (10^{-3} M) by AChE. Domino, Shellenberger and Frappier (1968), also demonstrated that HC-3 has some anticholinesterase action. These workers showed that HC-3 had an IC_{50} of 10^{-4} M in inhibiting the breakdown of acetyl- β -methylcholine (10^{-3} M) by cholinesterases in rat brain homogenate. HC-3 inhibition was found to be reversible and competitive. Hemsworth (1971a), reported that the concentration of HC-3 which inhibits by 50% the breakdown of ACh (3×10^{-2} M) *in vitro* by AChE is 9×10^{-3} M whereas a concentration of 7×10^{-4} M of HC-3 inhibits by 50% the breakdown of ACh *in vitro* by ChE.

4.1.1.2 Action at the neuromuscular junction

Large doses of HC-3 have been shown to produce a postjunctional curare-like blocking action at nerve-muscle preparations (Reitzel and Long, 1959b; Bowman and Rand, 1961; Marshall, 1969; Takagi, Kojima, Nagata and Kuromi, 1970), which was reversed by anticholinesterase drugs such as physostigmine. However, many studies (Schueler, 1955; Reitzel and Long, 1959b; Wilson and Long, 1959; Chang and Rand, 1960; Evans and Wilson, 1964; Gardiner and Sung, 1969; Marshall, 1969; Takagi, Kojima, Nagata and Kuromi, 1970; Di Augustine and Haarstad, 1970; Maggio-Cavaliere, 1976), have shown that small doses of HC-3 both *in vivo* and *in vitro* produce a slowly developing frequency dependent block of neuromuscular transmission, which is reversible by choline but not by anticholinesterases. It was concluded that this block is prejunctional.

4.1.1.3 Inhibition of choline transport

The specific antagonistic action of choline against the HC-3 induced block of nerve transmission suggested that HC-3 causes a presynaptic block at cholinergic junctions by interfering with the metabolism of choline in nerve tissue leading to a decreased synthesis and hence decreased output of ACh. MacIntosh, Birks and Sastry (1958), postulated that HC-3 inhibited ACh synthesis in intact nervous tissue by competing with choline for the specific transport system which is necessary to transport extracellular choline to the intracellular sites of acetylation and in this way reducing the amount of available substrate necessary for the synthesis of ACh.

This hypothesis is supported by many studies which have shown that HC-3 inhibits the high affinity choline transport system in synaptosomes (Guyenet, Lefresne, Rossier, Beaujouan and Glowinski, 1973a; Barker and Mittag, 1975; Simon, Mittag and Kuhar, 1975; Collier, Lovat, Ilson, Barker and Mittag, 1977; Hemsworth and Cholakis, 1978). HC-3 has also been reported

to inhibit choline transport into synaptosomes by Marchbanks, (1968); Potter, (1968); Diamond and Kennedy, (1969); Hemsworth and Bosmann, (1971); Hemsworth, Darmer, and Bosmann, (1971); Diamond and Milfay; (1972). (Table 1.3).

4.1.1.4 Inhibition of and acetylation by ChAc *in vitro*

HC-3 appears not to inhibit ChAc directly (Bhatnager, Lam and McColl, 1965; Hemsworth, 1971a; Domino, Mohrman, Wilson and Haarstad, 1973; Hemsworth and Cholakis, 1978). However, it has been shown that HC-3, like choline, can itself act as a substrate for ChAc *in vitro* (Rodriguez de Lores Arnaiz, Zieher and De Robertis, 1970; Hemsworth, 1971a; Barker and Mittag, 1975; Mann and Hebb, 1975; Bradshaw and Hemsworth, 1976), (Table 1.2).

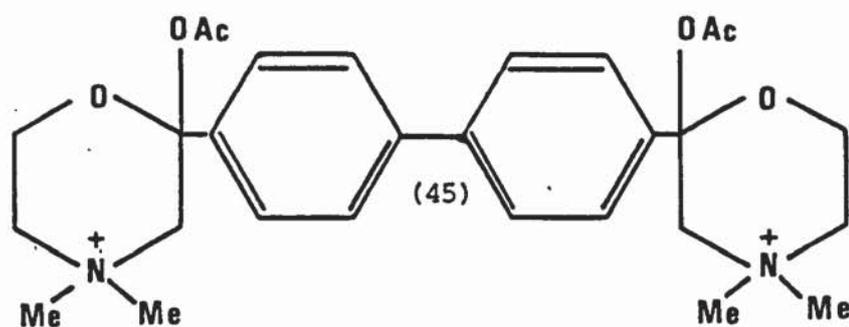
4.1.1.5 Transport of HC-3 into cholinergic neurons and the false transmitter hypothesis

It has been suggested that the failure in nerve transmission could be caused by the release of acetylated HC-3, as a false transmitter, in place of the natural transmitter ACh. However, in order for HC-3 to be acetylated by ChAc *in vivo* it must, like choline, be transported from the extracellular medium into the cholinergic neuron. Collier (1973), failed to show that HC-3 is transported into the cat superior cervical ganglion specifically during nerve stimulation unlike choline, although a little was shown to be transported into the ganglion at rest. He also found that compared to choline very little HC-3 is transported into mouse synaptosomes. However, it has been shown (Table 1.3), that HC-3 has a greater affinity for the high affinity choline transport system, than choline itself. Sellinger, Domino, Haarstad and Mohrman (1969), after administering HC-3 intraventricularly, found that mitochondrial filled nerve endings had a high affinity for HC-3. Slater and Stonier (1973) found that rat brain cortex slices accumulate

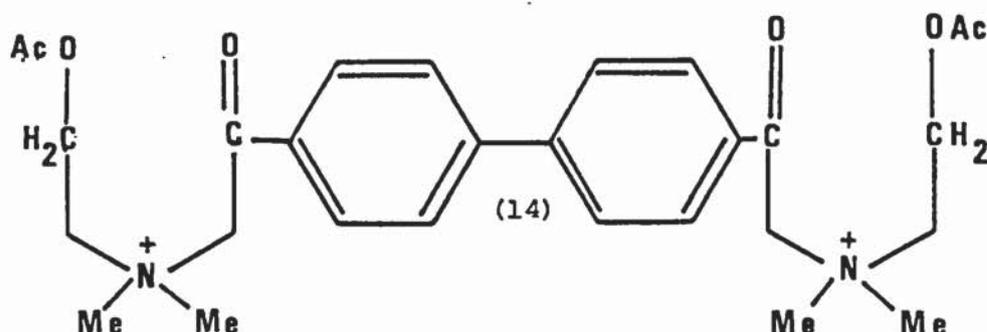
HC-3, but by a different uptake mechanism compared to that of choline. Therefore some work does support the theory that HC-3 can be transported into cholinergic neurons, where it could be acetylated by ChAc to acetylated HC-3 which could then be released as a false transmitter. An increase in the frequency of nerve impulses would increase the rate of transport of HC-3 into the cholinergic nerve and the rate of acetylation. Choline would antagonise this process. However, it has yet to be shown conclusively that HC-3 can be transported into cholinergic nerve terminals.

4.1.1.6 Structure of the acetylated HC-3 as synthesised by ChAc

The false transmitter hypothesis provides an additional mechanism of action of HC-3 to that originally proposed by MacIntosh, Birks and Sastry, (1958), which could result in the presynaptic block seen at cholinergic synapses. The structure of this possible false transmitter is therefore of interest. It is not known whether the acetylated HC-3 is in the cyclic form, acetyl-HC-3 (45), or in the open chain form, Ac-seco-HC-3 (14), or whether it is monoacetylated or biacetylated.



biacetylated



Schueler (1955), found that the characteristic HC-3 like activity causing respiratory paralysis was present only in those compounds containing an unmasked β -hydroxyl group with respect to the nitrogen centre. He showed that such compounds, including HC-3 undergo hemiacetal formation (Fig. 1.1). Therefore this cyclic structure must be the most stable tautomer and HC-3 (4) might be expected to act biochemically in this cyclic structural form at the cholinergic neuron. This hypothesis is supported by Di Augustine and Haarstad, (1970), who on studying both the open-chain tautomer (11) and the cyclic hemiacetal tautomer (12) of $\alpha, \alpha', \alpha, \alpha$ -tetramethyl-HC-3, showed that the characteristic HC-3 like activity of causing a prejunctional block at nerve-muscle preparations is much more pronounced in the cyclic hemiacetal form (12). This suggests that HC-3 itself is biologically more active in the cyclic hemiacetal form. Therefore if acetylated HC-3 is synthesised *in vivo* by ChAc then according to these experimental results acetylated HC-3 is likely to be in the cyclic form. This hypothesis is further supported by investigating the action of ChAc *in vitro*. The 'hydroxylcholine moiety' of the cyclic form of HC-3 (Fig. 4.1) is a β substituted choline analogue and it is interesting to observe that in one study (Hemsworth and Smith, 1970a) a D- β -substituted analogue of choline was acetylated *in vitro* by ChAc to a small extent, (8% compared to choline 100%). It is possible that the cyclic form of a D- β substituted choline moiety as found in the HC-3 molecule could confer some favourable rigidity on the moiety since HC-3 is acetylated *in vitro* by ChAc by about 27% compared to choline 100%.

However, many other choline analogues, including choline itself, possess straight chain choline moieties and are acetylated *in vitro* by ChAc (Table 1.2). Barker and Mittag (1975), suggested that the cyclic form of HC-3 (4) could undergo ring opening to give the straight chain form of the analogue (3), which lacks a β substituent, so rendering the now terminal hydroxyl group free to be acetylated by ChAc to Ac-seco-HC-3 (4).

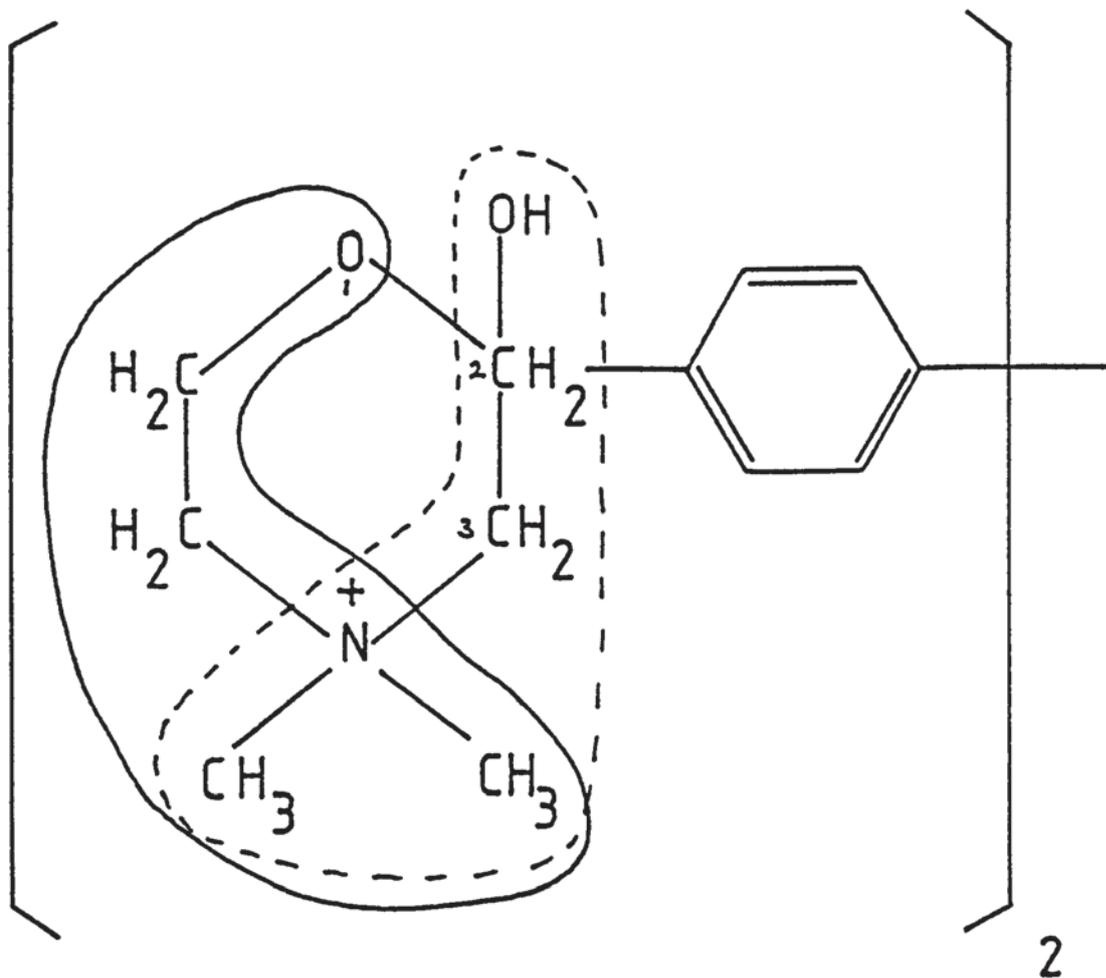


Fig. 4.1:- HC-3(4) :--- hydroxylcholine moiety
 —— ether choline moiety

Di Augustine and Haarstad (1970), after studying some methyl derivatives of HC-3 concluded that in general methyl substitution on the 'ether moiety' of choline resulted in compounds which were less potent than HC-3 with regard to toxicity, anticholinesterase activity and blocking action at nerve-muscle preparations, whereas methyl substitution on the 3 position of the 'hydroxyl moiety' resulted in a compound which was equipotent to HC-3. This work suggests that the unaltered ether moiety is biologically more important than the hydroxyl moiety of HC-3 implying that if the false transmitter hypothesis is relevant for the presynaptic action of HC-3 *in vivo*, it is the ether moiety which is acetylated by ChAc. However, this hypothesis is not supported by the study made by Di Augustine and Haarstad (1970) on the $\alpha, \alpha', \alpha, \alpha$ -tetramethyl-HC-3 analogue, discussed above. Hemsworth (1972), showed that alkyl substitution of the ether moiety of HC-15 resulted in analogues which were acetylated *in vitro* by ChAc but at a reduced rate compared HC-15 itself, although a comparative study, with alkyl substituents on the hydroxyl moiety was not conducted. These two studies by Di Augustine and Haarstad (1970), and Hemsworth (1972) suggest that it is the 'ether moiety' of the morpholine ring that is acetylated by ChAc. Since it is unlikely that an ether oxygen can be acetylated, due to its relatively unreactive nature these two studies support the possibility that cyclic HC-3 undergoes enol-keto (ring-chain) tautomerism on the surface of the enzyme to yield the more easily recognisable choline moiety with a free hydroxyl group which is then acetylated to the open chain Ac-seco-HC-3 (14).

The experimental evidence for the structural form of the acetylated HC-3 as synthesised by ChAc is conflicting. Neither is it known if both or just one of the morpholine rings of the HC-3 molecule are acetylated. This only has any relevance to the action of HC-3, if HC-3 is acetylated by ChAc *in vivo* as well as *in vitro*.

4.1.1.7 Aims

The present study involved an investigation of the action of HC-3 at cholinergic synapses and in particular the neuromuscular junction. Therefore this study sought to confirm or deny:-

- a. the cyclic structure of HC-3
- b. the prejunctional and postjunctional action of HC-3 at the neuromuscular junction
- c. the weak anticholinesterase activity of HC-3
- d. the *in vitro* acetylation of HC-3 by ChAc
- e. the lack of inhibitory activity of HC-3 on ChAc *in vitro*
- f. the inhibitory action of HC-3 on the transport of choline into synaptosomes.

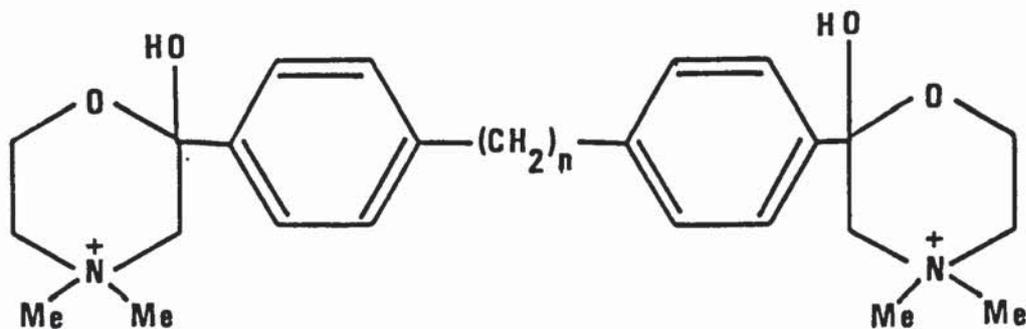
Since HC-3 was found to be acetylated by 28% compared to choline 100%, the false transmitter hypothesis was also investigated. Hence, the possible transport and acetylation of HC-3 in synaptosomes and the structure of the acetylated HC-3 as synthesised by ChAc *in vitro* were studied. In order to study the transport of HC-3 into synaptosomes radioactively labelled HC-3 has to be used. Unfortunately this is commercially unavailable unless the compound is "custom made" at a very high cost. It was therefore decided to synthesise $[^{14}\text{C}]$ -HC-3 (70) from tertiary-HC-3 and $[^{14}\text{C}]$ -methyl iodide.

4.1.2. Some analogues of HC-3

4.1.2.1 General discussion

Many analogues of HC-3 have been synthesised (Table 1.1). Several workers have altered the biphenyl nucleus of HC-3. HC-15(5) is one half of the HC-3 molecule and Hemsworth (1971a), showed that it has HC-3 like actions in that it gives a frequency dependent prejunctional block at the neuromuscular junction, inhibits the transport of choline into syna-

ptosomes and is acetylated by ChAc *in vitro*. Marshall and Long, (1959), showed that the introduction of an ether (6) or methylene(7) linkage between the two phenyl rings of HC-3 produced compounds which gave a prejunctional block in nerve-tissue experiments but with a reduced potency when compared to HC-3. These workers showed that the methylene analogue (7) at a concentration 1.5×10^{-5} M inhibited by 50% the break down of acetyl- β -methylcholine (2×10^{-2} M) by AChE. Subsequent studies have shown that the biphenyl moiety of HC-3 may be replaced by a hexamethylene chain (8), which approximates to the length of a biphenyl nucleus (Powers, Kruger and Schueler, 1962), one phenyl ring (9) (Thampi, Domer, Haarstad and Schueler, 1966) or three phenyl rings (10) (Gardiner and Sung, 1969), without appreciable loss of the characteristic HC-3 like activity in giving a prejunctional block in the nerve-muscle experiments conducted. Some of these findings contradict the suggestion made by Benz and Long (1969b), that a distance of 14\AA separating the two quaternary nitrogens of analogues of HC-3 is important for HC-3 like activity with regard to their presynaptic blocking action on cholinergic neurons.

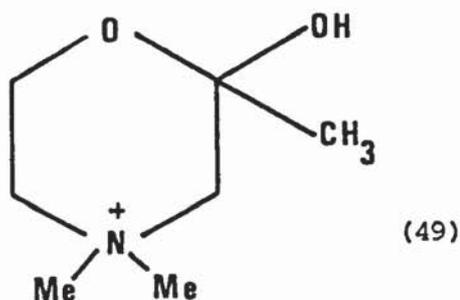


n=1 1CHC (7)
 n=2 2CHC (46)
 n=3 3CHC (47)
 n=4 4CHC (48)

4.1.2.2 Analogues of HC-3 investigated and aims of this study

Since it has been shown that certain alterations of the biphenyl nucleus gave compounds with HC-3 like activity, in that they gave a prejunctional block on nerve-muscle preparations, it was thought of interest to study a series of hemicholinium analogues which have an increasing number of methyl groups between the two phenyl rings, that is 2,2'-[1,1'-diphenylethane]-4,4'-diylbis-[2-hydroxy-4,4-dimethylmorpholinium](1CHC) (7), 2,2'-[1,1'-diphenylethane]-4,4'-diylbis-[2-hydroxy-4,4-dimethylmorpholinium] (2CHC) (46) 2,2'-[1,1'-diphenylpropane]-4,4'-diylbis[2-hydroxy-4,4-dimethylmorpholinium] (3CHC) (47) and 2,2'-[1,1'-diphenylbutane]-4,4'-diylbis[2-hydroxy-4,4-dimethylmorpholinium] (4CHC) (48).

To investigate the importance of the phenyl group on the action of HC-3 at the neuromuscular junction an analogue of HC-15(5) was synthesised in which the phenyl group was replaced with a methyl group to give the compound called 4,4-dimethyl-2-hydroxy-2-methyl-morpholinium (methylmorpholinium)(49)



In the present study these five hemicholinium analogues were synthesised and their chemical structures confirmed. An investigation was conducted into their action at cholinergic synapses and in particular at the neuromuscular junction. Therefore this study investigated these compounds with regard to their

- a. prejunctional and postjunctional activity at the neuromuscular junction.

- b. action on cholinesterase enzymes *in vitro*.
- c. action on ChAc *in vitro*.
- d. action on the transport of choline into synaptosomes.

In each case the activities of the analogues are compared to that of the parent compound, HC-3. Structure/activity relationships are discussed particularly with regard to the interatomic distance between the two quaternary nitrogen atoms in the molecules.

4.2. CHEMISTRY

4.2.1. Synthesis of HC-3 and the diphenylalkane analogues

The syntheses of HC-3(4) and the diphenylalkane analogues (7, 46, 47, 48) were based on the method described by Long and Schueler, (1954) with a number of modifications which improved the yields. The synthetic reaction can be divided into three steps.

4.2.1.1. Step 1:

Synthesis of 4,4'-bis (chloroacetyl) 1,1'-biphenyl derivatives

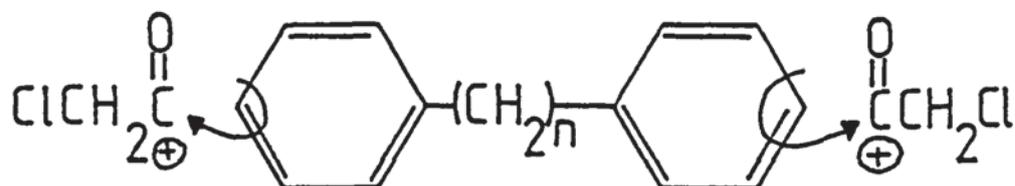
The first step was a Friedel-Crafts acylation which resulted in the synthesis of 4,4'-bis (chloroacetyl) 1,1'-biphenyl derivatives (58-62). The most likely mechanism for this reaction is now described (Fig.4.2.). Chloroacetyl chloride reacts with aluminium chloride to yield an acylium ion (50). The acylium ion (50) is a very strong electrophile due to the presence of two electronegative atoms, namely chlorine and oxygen, which pull electrons away from the positive centre. The diphenyl compounds (51-55) being electron rich undergo, electrophilic attack by the acylium ion(50) yielding ionic unstable intermediates(56) which immediately lose two protons to give the 4,4'-bis (chloroacetyl) 1,1'-biphenyl derivatives (58 -62).

As a ketone will complex with aluminium chloride, both chloroacetyl chloride and the products 4,4'-bis (chloroacetyl) 1,1'-biphenyl derivatives (58-62) will combine with the catalyst as shown (57, 63) (Fig.4.2.). Therefore excess aluminium chloride must be used, and the 4,4'-bis (chloroacetyl) 1,1'-biphenyl derivative - aluminium chloride complex(57) must be broken up by adding it to a mixture of hydrochloric acid, methanol and iced water to yield the 4,4'-bis (chloroacetyl) 1,1'-biphenyl derivatives (58-62).

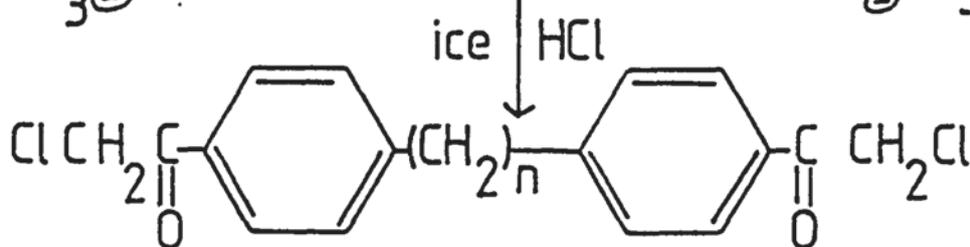
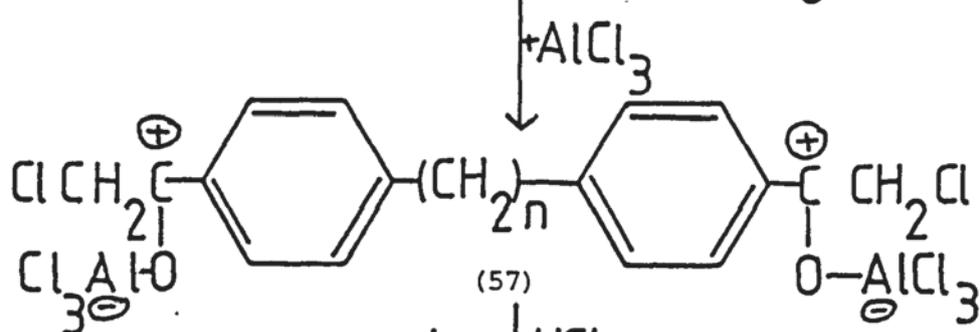
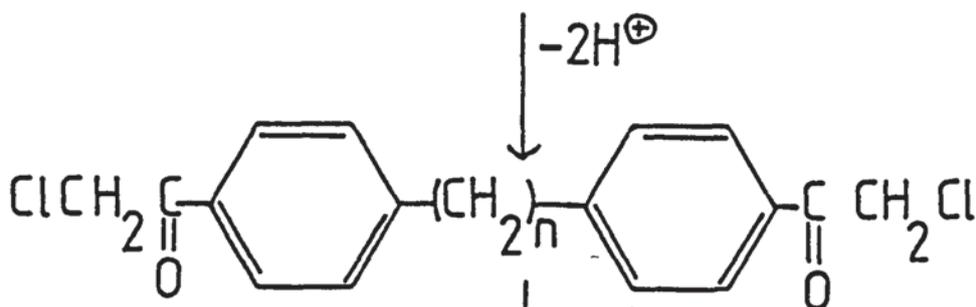
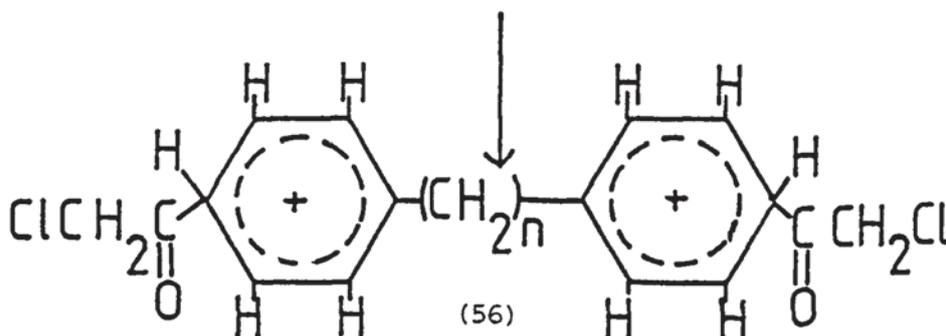


Lewis acid
(Catalyst)

acylium ion (50)



$n = 0$ (51); $n = 1$ (52); $n = 2$ (53);
 $n = 3$ (54); $n = 4$ (55) electrophilic attack



$n=0$ (58); $n=1$ (59); $n=2$ (60)
 $n=3$ (61); $n=4$ (62)

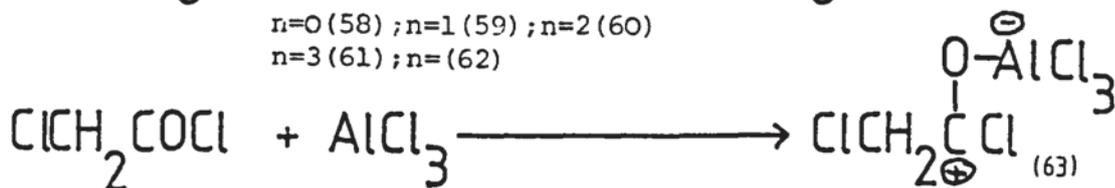


Fig. 4.2:- Synthesis of the hemicholinium analogues. Step 1; Friedel Craft acylation of diphenyl analogues

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Long and Schueler, (1954) used carbon disulphide ($\mu = 0.06$ at 20°) as the solvent for this synthetic reaction, but it was thought that a more polar solvent would stabilise the ionic intermediate(56) formed during the reaction, so encouraging the reaction to go to completion and so giving higher yields. The solvents tried were ether ($\mu = 0.28$ at 20°) chloroform ($\mu = 1.15$ at 25°), acetone ($\mu = 2.69$ at 20°) and nitrobenzene ($\mu = 4.03$ at 25°) but the yields were equal to those obtained when carbon disulphide was used as the solvent.

However, by using a large excess of the catalyst, aluminium chloride, (three times the amount used by Long and Schueler, 1954) and by using butan-1-ol as the recrystallising solvent (instead of toluene) the yields were doubled when compared to those obtained by Long and Schueler, (1954).

All the reactants and apparatus used in the reaction were dry as aluminium chloride reacts violently with water.

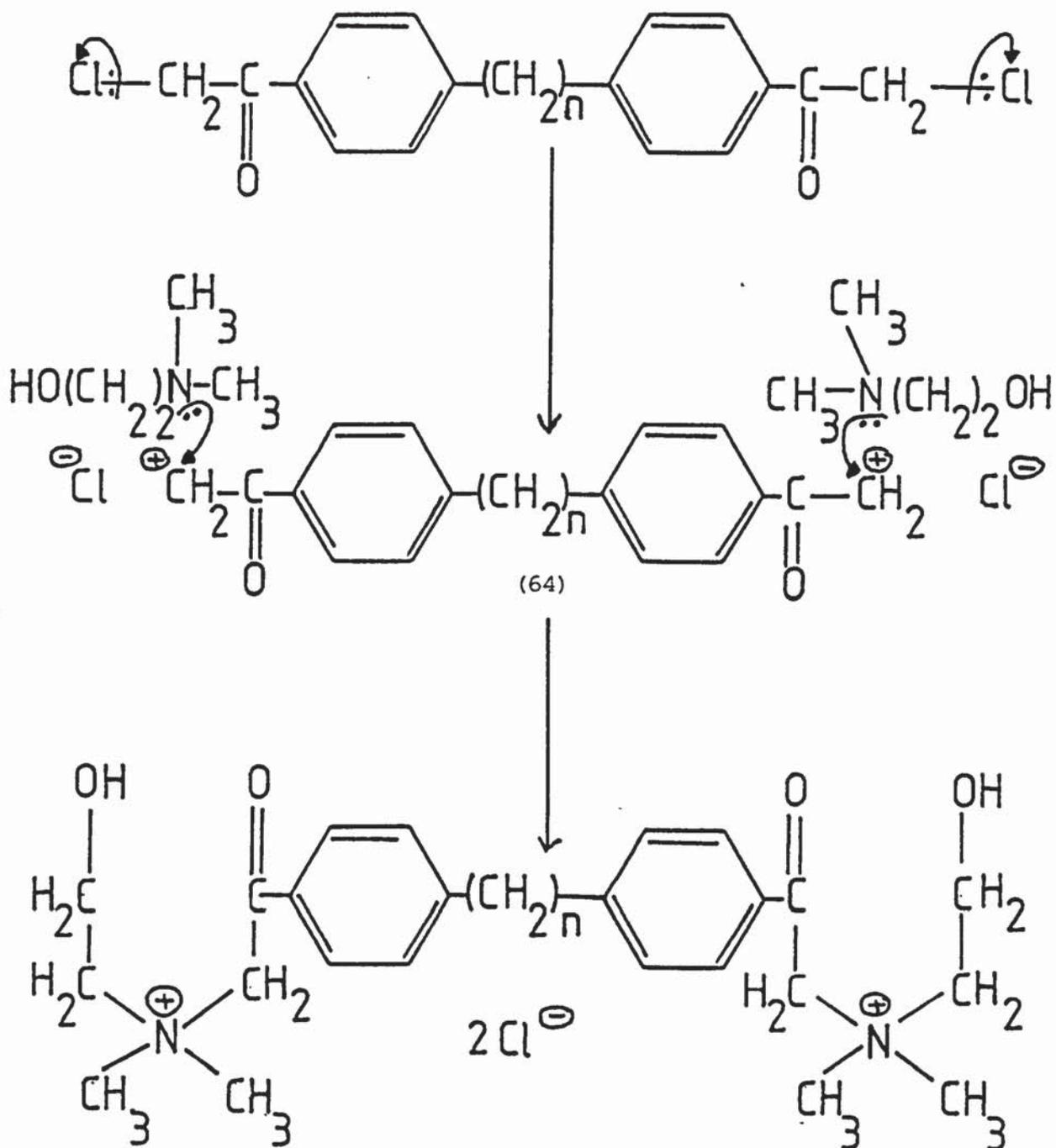
The structures of the products were confirmed by ir spectrometry. The 4,4'-bis (chloroacetyl) 1,1'-biphenyl derivatives (58-62) showed absorption indicating that the compounds are para substituted aromatic ketones containing a carbon-chloride bond.

4.2.1.2. Step 2:

Quaternisation Reaction

The second step of the reaction is a quaternisation process (Fig.4.3.). 2-Dimethylaminoethanol was reacted with the 4,4'-bis (chloroacetyl) 1,1'-biphenyl derivatives (58-62) and the reaction proceeds via a nucleophilic substitution to yield the open-chain (seco) form of the hemicholinium derivatives (3, 65-68).

It was thought that steric effects would encourage this reaction to proceed via an S_N1 mechanism as opposed to an S_N2 mechanism. This is



$n=0$ (3) ; $n=1$ (65) ; $n=2$ (66) ; $n=3$ (67) ; $n=4$ (68)

Fig. 4.3:- Synthesis of the hemicholinium analogues. Step 2;
Quaternisation process involving a nucleophilic substitution.

because the S_N2 reaction involves a transition state where the nucleophile becomes partially bonded to the reacting carbon atom, before the incipient chlorine ion has become wholly detached from it, and it was thought that the bulky groups surrounding the reacting carbon atom of the 4,4'-bis (chloroacetyl) 1,1'-biphenyl derivatives (58-62) will make it unlikely for this transition state to occur.

If this quaternisation step is proceeding via an S_N1 mechanism then polar solvents will stabilise the ionic intermediates (64) and possibly result in higher yields. Therefore, a variety of solvents were tried, that is ether, nitrobenzene and acetone. Acetone ($\mu = 2.69$ at 20°) was found to be the most suitable solvent almost doubling the yield compared to that obtained with 1,4-dioxan ($\mu = 0$ at 20°) which was used as the solvent by Long and Schueler, (1954).

It was concluded that, particularly in acetone, the Step 2 reaction proceeds via an S_N1 mechanism (Fig.4.3.). Thus the chlorine atom, due to its size, readily leaves the 4,4'-bis (chloroacetyl) 1,1'-biphenyl derivatives (58-62) which results in their becoming carbonium ions (64). The lone pair of electrons on the nitrogen atom of 2-dimethylaminoethanol makes this molecule a nucleophile and the nitrogen centre reacts with the carbonium ion (64) to yield the highly unstable straight chain form of the hemicholinium analogues (3, 65-68).

4.2.1.3. Step 3:

Cyclisation

In the solvents used the open-chain (seco) form of the hemicholinium analogues (3, 65-68) underwent a spontaneous internal keto-enol (open-chain -cyclic) tautomerism to give the cyclic hemiacetal structure of the hemicholinium derivatives (HC-3(4), ICHC (7), 2CHC (46), 3CHC (47), 4CHC (48)) (Fig.4.4.)

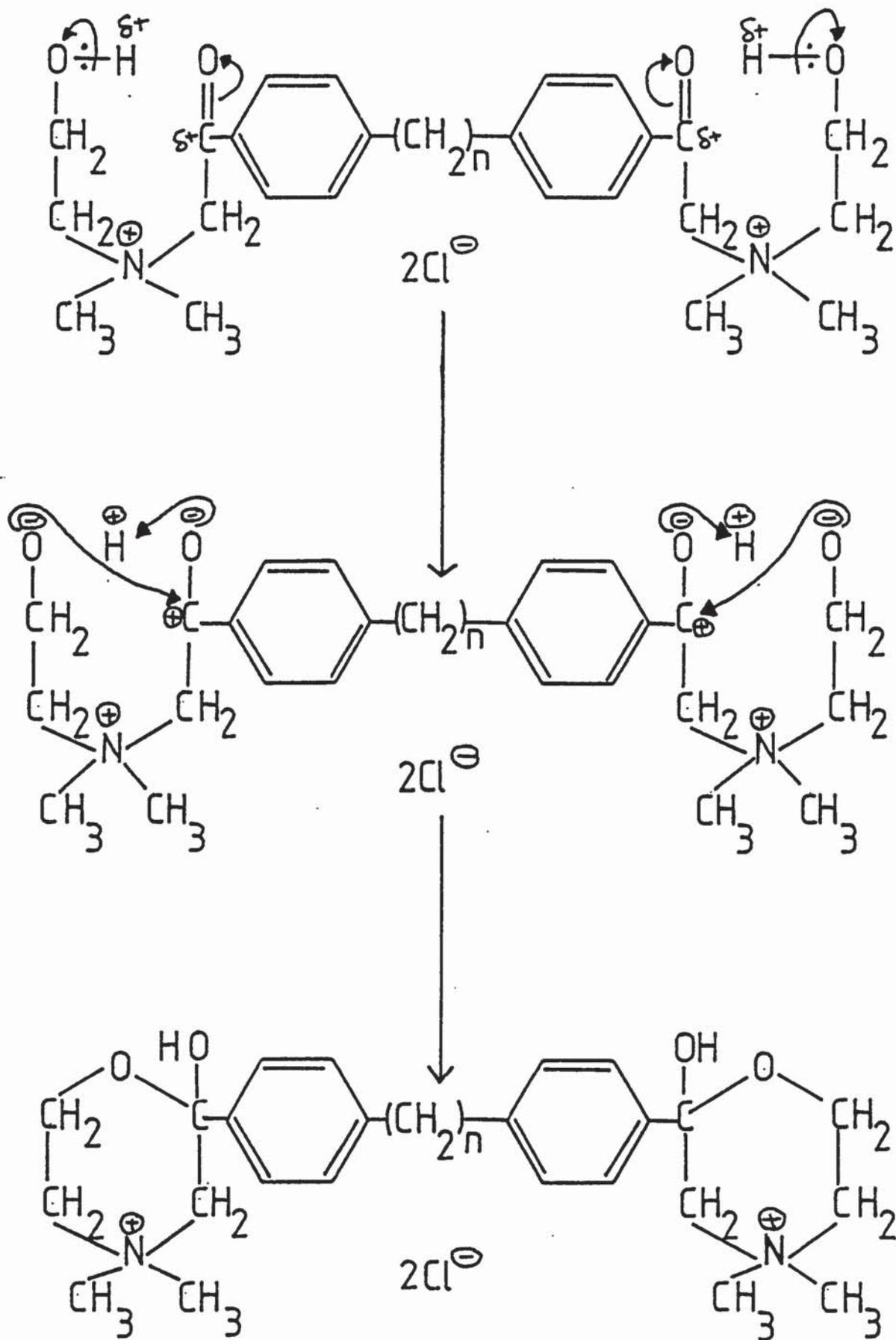


Fig. 4.4:- Synthesis of the hemicholinium analogues. Step 3;
 spontaneous cyclisation from the seco form to the
 cyclic hemiacetal

Synthesis of 4CHC(48) was effected using 1,4-dioxan as solvent as 4,4'-bis(chloroacetyl)1,1'-diphenylbutane, the starting material for Step 2 of the synthesis of this analogue, was insoluble in acetone.

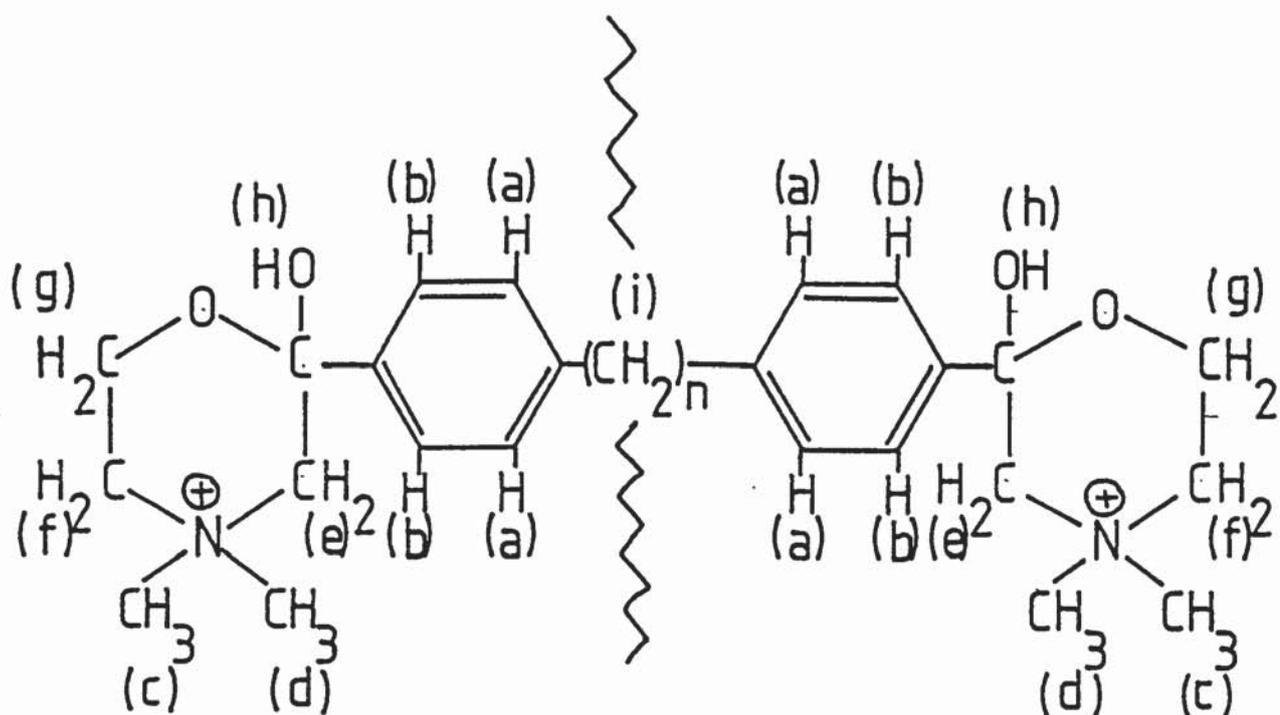
4.2.1.4. Recrystallisation

The hemicholinium analogues (4,7,46-48) were recrystallised from ethanol/ether. Like most quaternary amines they are very hygroscopic especially when first made and it is important to keep them under vacuum until they are completely dry. Because these analogues are so moisture sensitive all the reactants and apparatus used in the Step 2 reaction must be dry.

4.2.1.5. Confirmation of Structures

The structures of the hemicholinium analogues (4,7,46-48) were confirmed by ir, uv and nmr spectra. Each analogue showed strong absorptions between 3,600 to 3,100 cm^{-1} indicating the presence of an -OH group, 840 cm^{-1} indicating the presence of an aromatic (para substituted) group, and 257 - 260nm indicating the presence of a diphenyl group.

Further confirmation of structure was obtained from nmr spectra. The analogues are symmetrical, (Fig.4.5.). The nmr spectrum for HC-3(4) shows a doublet at 2.32 τ which is due to the eight protons on the diphenyl rings, ((a) and (b) in this compound are equivalent). An absorption at 5.15 τ is due to the presence of water. The methyl protons (c) and (d) absorb at 6.5 τ and 6.72 τ . Protons (c) have a different chemical shift to protons (d) are affected by the aromatic nucleus whereas protons (c) are less affected. Despite the fact that the solvent is D_2O , one hydroxyl proton (h) absorbs at 6.98 τ . It might be expected that this proton would not show, as hydroxyl protons are labile and easily deuterated, however, in this case the exchange rate must be very slow, with only one of these protons deuterated at any one time. The



when $n=0$ there are no (i) protons HC-3(4)

$n=1$	-	(i) CH ₂	1CHC (7)
$n=2$	-	(i) (i) CH ₂ - CH ₂	2CHC (46)
$n=3$	-	(i) (i) CH ₂ - CH ₂ - CH ₂	3CHC (47)
$n=4$	-	(i) (i) (i) (i) CH ₂ - CH ₂ - CH ₂ - CH ₂	4CHC (48)

Fig. 4.5:- Diagram indicating the symmetry of the hemicholinium analogues showing the equivalent protons indicated by the letters in parentheses. When $n=1$ and 3 the molecule is symmetrical about the central carbon atom, when $n=0, 2$ and 4 the molecule is symmetrical about the central bond.

methylene protons (e), (f) and (g) are very difficult to identify from this spectrum. They each must have different shifts as their electronic environments are so different. In all the absorptions discussed integration confirmed the assignments.

nmr spectra for the hemicholinium analogues ICHC(7), 2CHC(46), 3CHC(47), 4CHC(48), are similar to the nmr spectrum for HC-3(4) the differences being due to the methylene groups separating the two phenyl rings. Two doublets, instead of one doublet seen with HC-3(4), are seen at around 2.5 τ and 2.7 τ which are due to the eight protons on the aromatic ring. The methylene groups separating the two phenyl rings results in their being two types of protons (a) and (b) on the phenyl rings which give the two doublets described. Additional absorptions are seen due to the (i) protons.

4.2.2. Cyclisation study of HC-3 and the diphenylalkane analogues

The keto-hemiacetal tautomerism which gives the cyclic hemiacetal structure of HC-3(4) is common in tertiary amino alcohols (Cromwell and Tsou, 1949) and it has been reported for quaternary amines (Lutz and Jordan, 1949). Schueler, (1955) was the first to show that the open-chain (seco) form of HC-3(3) underwent this tautomerism. Haarstad and Schueler, (unpublished data, cited by DiAugustine and Haarstad, 1970) have provided direct evidence of the instability of seco-HC-3 and the persistence of the cyclic HC-3 in aqueous and alcoholic solutions. They found that the cyclic hemiacetal form of HC-3 in aqueous solutions was extremely stable, maintaining the same uv spectrum and the same activity in mice for over two years. Highly basic or acidic solutions failed to convert cyclic HC-3 to its seco form. They have also shown that HC-3 can be prepared in a manner such that it can exist, prior to recrystallisation,

as the seco form, provided that the compound remains in a relatively anhydrous environment.

Due to the lack of published data and the fact that it is the choline moieties which are involved in this tautomerism it was thought of interest to study the structures of HC-3 and the analogues synthesised here. Their structures in aqueous solutions are of special interest, as this is the environment they are in when they act on biological preparations. It was thought possible, particularly in an aqueous solution, that an equilibrium might exist between the open chain, seco forms (3,65-68) and the cyclic hemiacetal forms (4,7,46-48) of HC-3 and the analogues.

A study of the ir absorption spectra of HC-3 and the analogues (1CHC, 2CHC, 3CHC, 4CHC) as a mull of the solid in liquid paraffin indicates that there is no sharp absorption between 1650 to 1750cm^{-1} (i.e. no carbonyl absorption). Absorption would be expected at this wavelength if any portion (provided it is in a sufficient amount to be detected by ir) of HC-3 or the analogues was in the open chain, seco form. Therefore from these ir studies it appears as though solid HC-3 and the analogues are totally in the cyclic hemiacetal form (4,7,46-48).

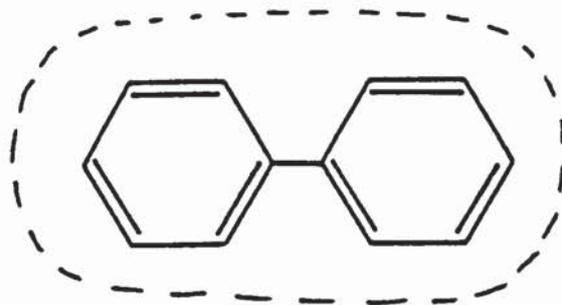
The ir absorption spectra of aqueous solutions of HC-3 and the analogues (1CHC, 2CHC, 3CHC, 4CHC) also showed no carbonyl absorption. This indicates that HC-3 and the analogues are totally in the cyclic hemiacetal form (4,7,46-48) in an aqueous environment.

The uv absorption spectra of HC-3 and the analogues (1CHC, 2CHC, 3CHC, 4CHC) as aqueous solutions show maximal absorptions below 260nm . 1,1'-Biphenyl also has a maximal absorption below 260nm , this absorption is unaffected by the addition of acid or base to the solvent. However, Ac-seco-HC-3(14), an open chain analogue of HC-3 has a maximal absorption

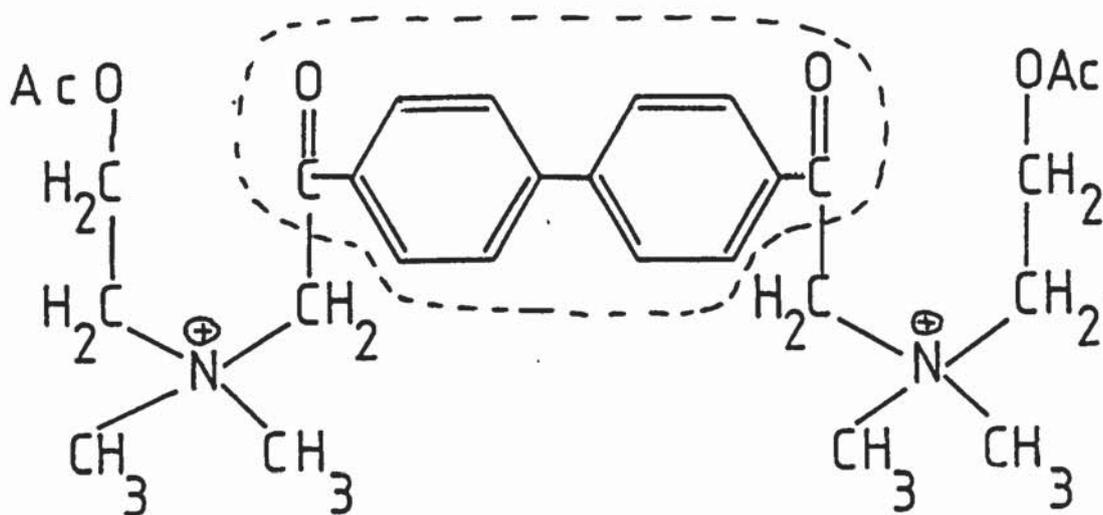
at 304nm at pH 7.0. In this molecule the chromophore includes the diphenyl moiety and the two carbonyl groups as shown (Fig.4.6.). In this chromophore the π electron orbitals of the biphenyl nucleus combine with the π electron orbitals of the carbonyl groups which results in a new orbital which is of higher energy and will therefore absorb uv light of a lower energy (longer wavelength) as compared to 1,1'biphenyl. The addition of base (i.e. sodium hydroxide) to the aqueous solution of Ac-seco-HC-3 shifts the absorption to 278nm. This is because the base stabilises the electrons of the carbonyl groups due to hydrogen bonding.

The open-chain forms of HC-3(3) and the analogues(3,65-68) will have the same chromophore as Ac-seco-HC-3. Therefore if any portion of HC-3 or the analogues (1CHC, 2CHC, 3CHC, 4CHC) was in the open-chain form, a maximal absorption of uv light would occur at around 304nm, and on the addition of sodium hydroxide it should move to a shorter wavelength. However, HC-3 and the analogues in aqueous solutions, do not absorb uv light at longer wavelengths than 260nm and the addition of NaOH or HCl has no effect on their maximal absorptions. The results of this uv study indicate that HC-3 and the analogues are totally in the cyclic hemiacetal form (4,7,46-48) in aqueous solutions.

A study of the nmr spectra run in D_2O of HC-3 and the analogues (1CHC, 2CHC, 3CHC, 4CHC) shows small doublets at 2.1 τ and 2.53 τ (HC-3) 2.3 τ and 2.6 τ (1CHC), 2.2 τ and 2.75 τ (2CHC), 2.25 τ and 2.75 τ (3CHC) and 2.2 τ and 2.72 τ (4CHC) which could indicate the presence of small amounts of open-chain compound (3,65-68 respectively) as the protons of the diphenyl nucleus would be deshielded by the adjacent carbonyl groups and subsequently give the peaks indicated. However, these peaks could



1,1 Biphenyl $\lambda_{\max} \text{CHCl}_3 = 252\text{nm}$



Ac-seco-HC-3 (14) $\lambda_{\max} (\text{H}_2\text{O}) = 304\text{nm}$

$\lambda_{\max} (\text{H}_2\text{O}) = 278\text{nm}$
(NaOH)

Fig. 4.6:- Diagram showing chromophores (in dotted circles) giving absorptions indicated of 1,1'-biphenyl and Ac-seco-HC-3.

also be due to impurity i.e. the 4,4'-bis(chloroacetyl)1,1'-biphenyl derivatives (58-62).

As the peaks in the nmr are so small and as the uv does not detect any carbonyl groups adjacent to the biphenyl nucleus it is concluded that in aqueous solutions HC-3 and the analogues described here, (1CHC, 2CHC, 3CHC, 4CHC) are totally in the cyclic hemiacetal form (4,7,46-48).

4.2.3. Optical activity of HC-3 and the diphenylalkane analogue

Each molecule of HC-3(4) and the analogues described here (1CHC, 2CHC, 3CHC, 4CHC) has two asymmetric carbon atoms. Polarimeter experiments indicate that the compounds are optically inactive and are therefore present in aqueous solution as a racemic mixture.

4.2.4. Synthesis of tertiary-HC-3

Tertiary-HC-3 (69) was prepared by the method of Domer and Schueler, (1960). This reaction involves a nucleophilic substitution similar to that described above (Fig.4.3.). The structure was confirmed by ir and uv.

4.2.5. Synthesis of methylmorpholinium

Methylmorpholinium(49) was synthesised by a nucleophilic substitution of chloroacetone by 2-dimethylaminoethanol. Due to the small size of the neighbouring groups around the reacting carbon atom it is possible that the reaction proceeds via an S_N2 mechanism, but there is no factual evidence for this. The product spontaneously cyclises in a similar way to the hemicholinium analogues (Fig.4.7.). The structure was confirmed by ir.

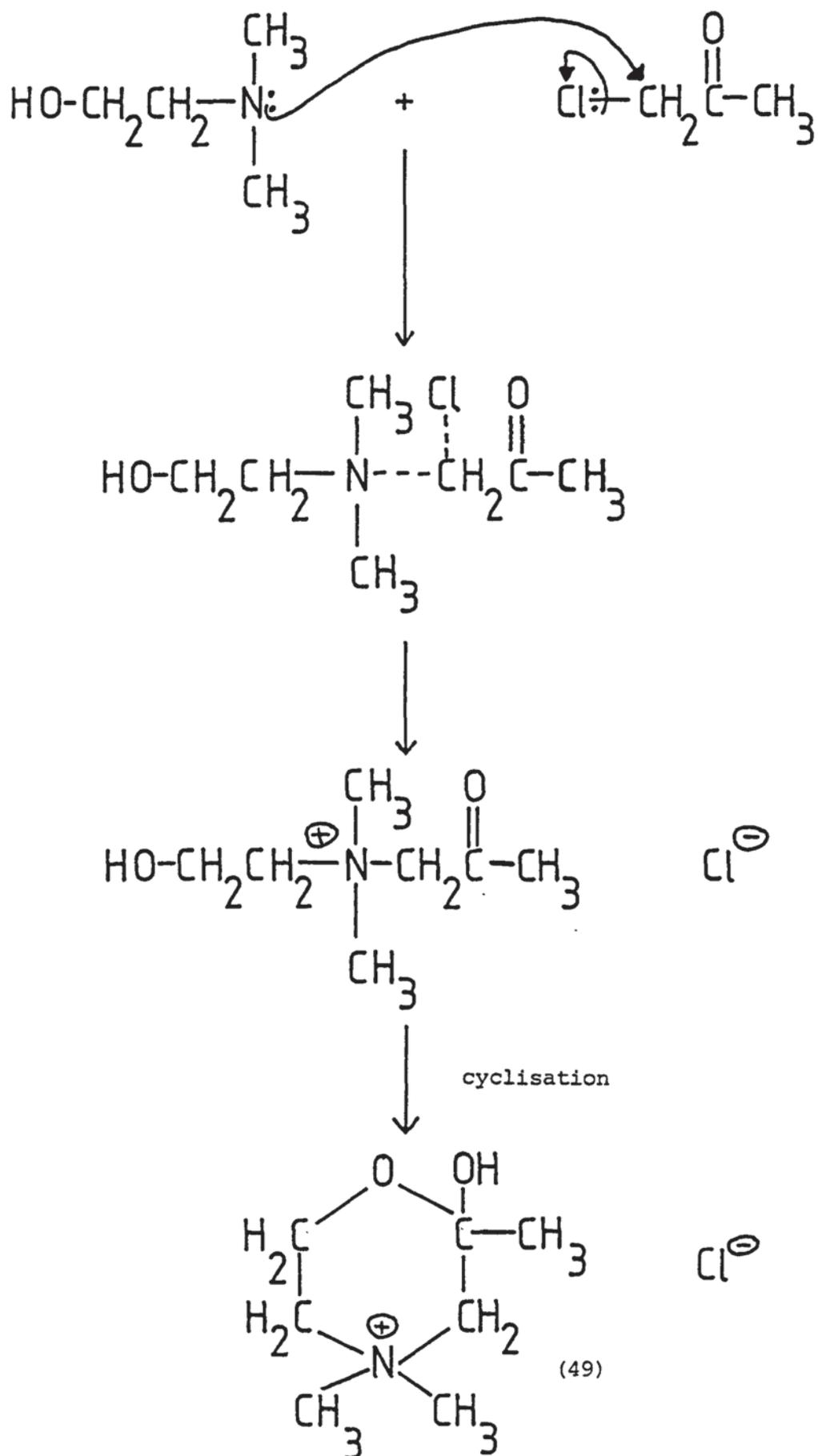


Fig. 4.7:- Synthesis of methylmorpholinium chloride

4.2.6. Attempted synthesis of cyclic acetyl-HC-3

Tertiary alcohols are very difficult to acetylate due to steric hindrance and this is probably why the attempted synthesis of cyclic acetyl-HC-3(45) failed (Fig.4.8.).

The phenyl group, and the -o- and -CH₂- groups in the morpholine ring must hinder the approach of the bulky acetyl group towards the hydroxyl group.

4.2.7. [¹⁴C]-HC-3

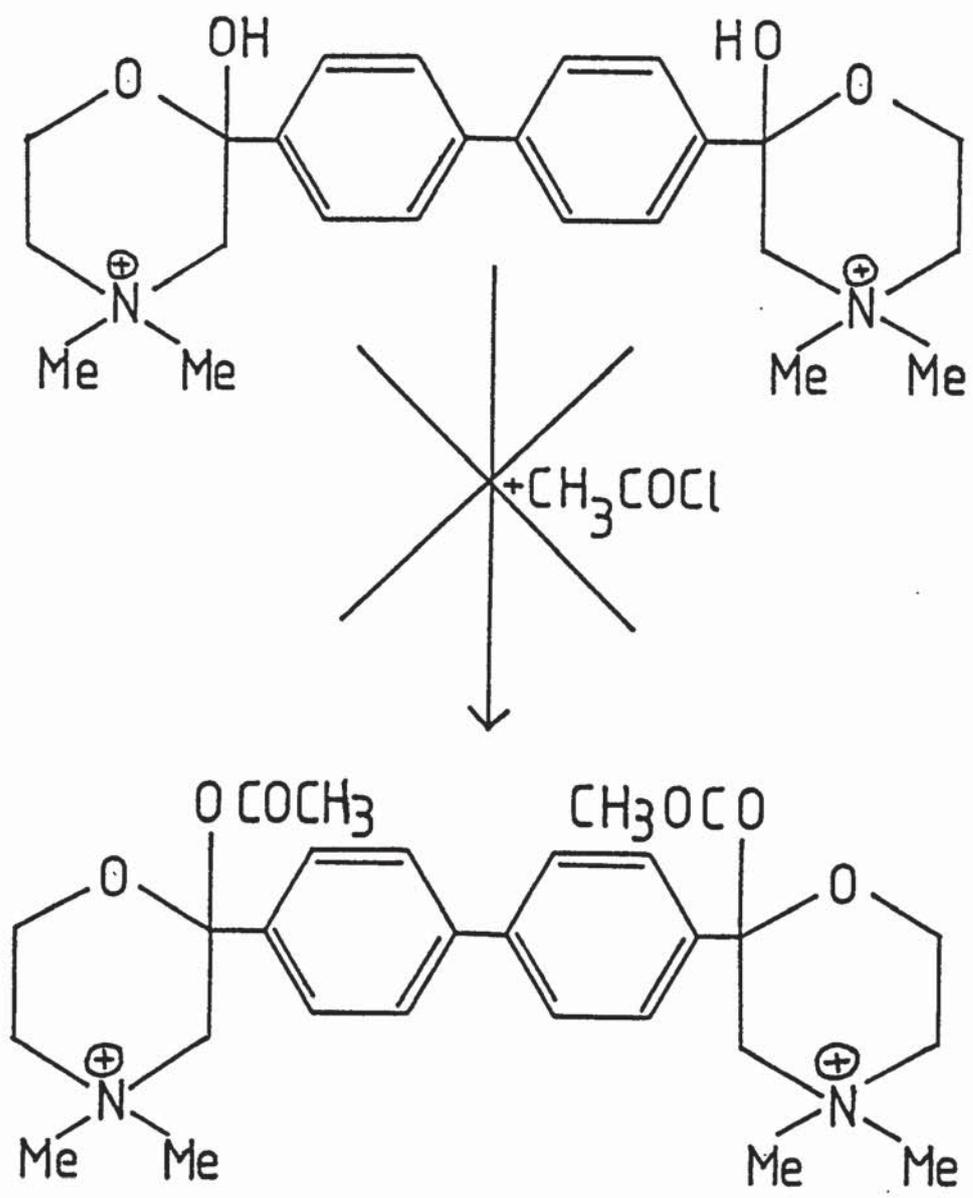
4.2.7.1. Synthesis

Tertiary-HC-3(69) was quaternised with [¹⁴C]-methyl iodide (Menschutkin reaction) (Fig.4.9.). The product was purified on a weak cation-exchange resin, CM-Sephadex. The resin was first eluted with water and as [¹⁴C]-HC-3(70) possesses two quaternary nitrogens (cations) it bound to the resin, whereas the unreacted starting materials, tertiary-HC-3 and methyl iodide, possess no positive charges and were eluted from the column. When no starting materials could be detected in the aqueous eluates [¹⁴C]-HC-3 was eluted from the column by a solution of NaCl (IM). A weak cation-exchange resin and a concentrated solution of NaCl had to be used as [¹⁴C]-HC-3 binds strongly to cation-exchange resins probably due to the fact that each molecule possesses two cations.

4.2.7.2. Specific Activity and Purity

4.2.7.2.1. Specific Activity

The specific activity of [¹⁴C]-HC-3 was calculated from the specific activity of the [¹⁴C]-methyl iodide, bearing in mind that two molecules of methyl iodide react with one molecule of tertiary-HC-3. This was considered to be the most accurate figure practically obtainable.



Cyclic acetyl - HC-3 (45)

Fig. 4.8:- Attempted synthesis of cyclic acetyl-HC-3 (45)

4.2.7.2.2. Radiochemical Purity

Methyl iodide is volatile at room temperature and therefore any unreacted material would be removed by the vacuum distillation process which occurs at the end of the reaction. This was found to be the case as no radioactivity was detected in the aqueous eluates from the ion-exchange column. For practical purposes therefore the product was considered to be radiochemically pure.

4.2.7.2.3. Chemical Purity

Tertiary-HC-3 was eluted from the column by water. Samples of the eluates were run on a TLC plate. As the elution continued iodine stained spots of decreasing colour intensity could be detected on the TLC plate. No tertiary-HC-3 was detected on running a TLC of a sample of the final product, [^{14}C]-HC-3 was considered to be chemically pure.

4.2.8. Experimental

All the reactants and apparatus used in the syntheses were dry.

4.2.8.1. 4,4'-bis(chloroacetyl)1,1'-biphenyl derivatives

The 1,1'-biphenyl analogue (0.07 mole) (51-55) was dissolved in a sufficient quantity of carbon disulphide (60ml). This solution was then cooled and powdered aluminium chloride (52g, 0.039 mole) was added slowly with stirring. Chloroacetyl chloride (18.4g, 0.16 mole) was added dropwise to the mixture which was then allowed to reflux (no heat required) with stirring, until liberation of hydrogen chloride had ceased (about 3 hr). The resulting complex was thoroughly broken up in a mixture of ice, methanol and hydrochloric acid and the product oven dried at 80°. The product was recrystallised from butan-1-ol. A cold solution of the product in acetone was passed through activated charcoal

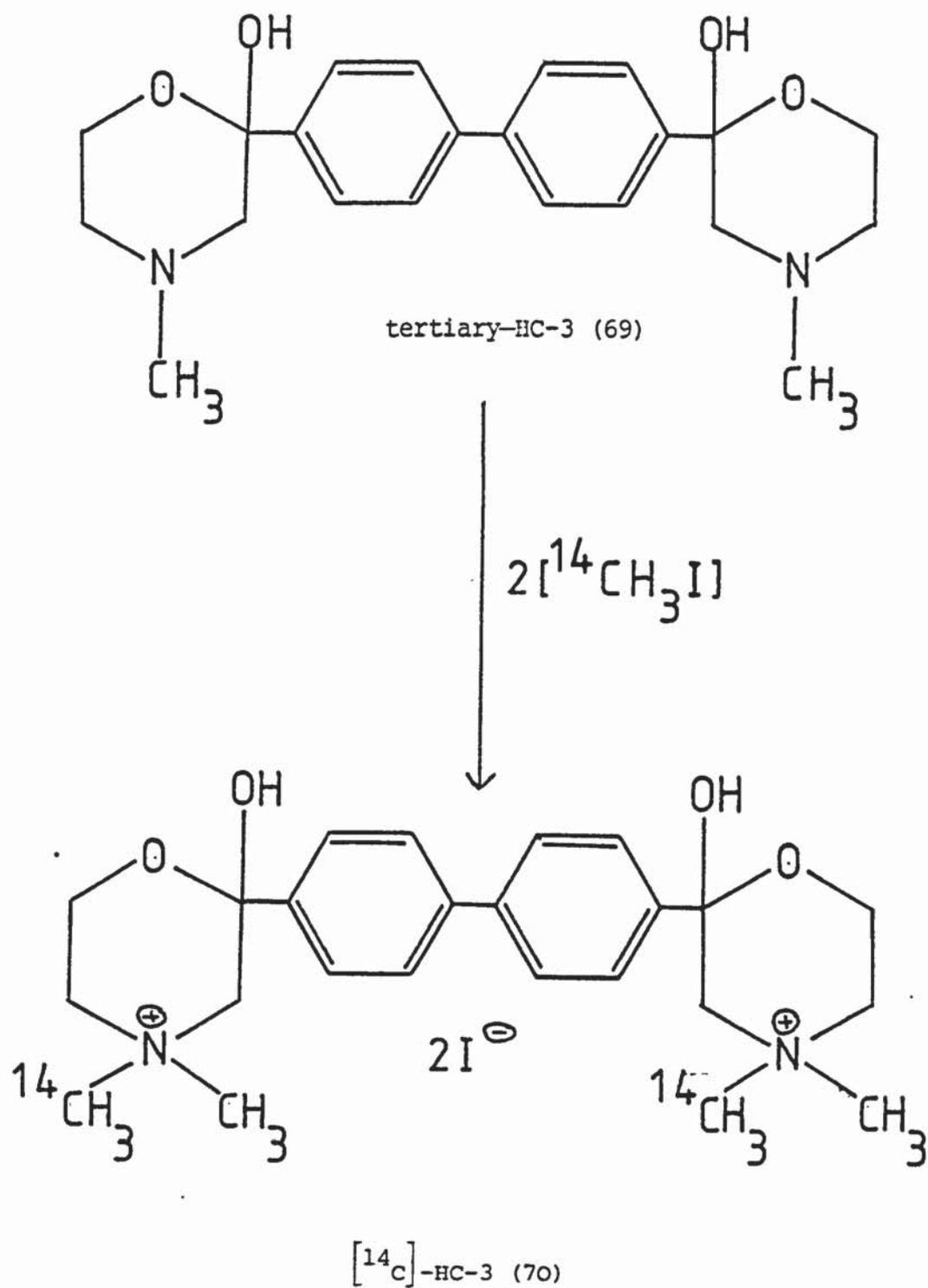


Fig. 4.9:- Synthesis of $[^{14}\text{C}]\text{-HC-3}$

until a clear solution was obtained. This solution was then filtered through Kieselguhr. The acetone was then removed by vacuum distillation and the resulting colourless product crystallised from butan-1-ol.

1,1'-Biphenyl (51) (11g, 0.07mole) gave colourless needles of 4,4'-bis(chloroacetyl)1,1'-biphenyl (58) (19.5g 89%), mp 225-226^o. ν_{\max} (nujol), 2900-2850 (CH₂), 1700 (aromatic ketone), 1600 (aromatic), 1560, 1480, 1420-1380, 1340, 1320, 1220 (aromatic para), 1000 (aromatic), 820 (aromatic para), 780, 760 (C-Cl) cm⁻¹. Anal: (C₁₆H₁₂O₂Cl₂); requires, C, 62.54; H, 3.91; O, 10.42; Cl, 23.13%; found, C, 62.34; H, 4.10; O, 10.21; Cl, 23.35%.

1,1'-Diphenylmethane (52) (11.8g, 0.07mole) gave brown needles (18.7g, 83%) which were decolourised to give colourless crystals of 4,4'-bis(chloroacetyl)1,1'-diphenylmethane (59) mp 115-117^o. ν_{\max} (nujol), 2900-2850 (CH₂), 1700 (aromatic ketone), 1600 (aromatic), 1560, 1480, 1460-1440, 1340, 1320, 1220 (aromatic para), 1180 (aromatic para), 1000, 880, 820 (aromatic para), 780, 760 (C-Cl) cm⁻¹. Anal: (C₁₇H₁₄O₂Cl₂); requires, C, 63.55; H, 4.36; O, 9.97; Cl, 22.12%; found, C, 63.60; H, 4.50; O, 9.87; Cl, 22.04%.

1,1'-Diphenylethane (53) (12.7g, 0.07mole) gave yellow needles (19.2g, 82%) which were decolourised to give colourless crystals of 4,4'-bis(chloroacetyl)1,1'-diphenylethane (60) mp 123^o. ν_{\max} (nujol), 2900-2850 (CH₂), 1700 (aromatic ketone), 1600 (aromatic), 1580, 1480, 1420-1380, 1340-1320, 1220, 1200 (aromatic para), 1000 (aromatic), 940, 880-820 (aromatic para), 800, 760 (C-Cl) cm⁻¹. Anal: (C₁₈H₁₆O₂Cl₂); requires, C, 64.48; H, 4.78; O, 9.55; Cl, 21.19%; found, C, 64.30; H, 4.97; O, 9.34; Cl, 21.39%

1,1'-Diphenylpropane (54) (13.7g, 0.07mole) gave yellow needles (13.9g, 57%) which were decolourised to give colourless crystals of 4,4'-bis(chloroacetyl)1,1'-diphenylpropane (61) mp 131-133^o. ν_{\max} (nujol), 2900-2850 (CH₂), 1700 (aromatic ketone), 1600 (aromatic), 1480, 1420-1380, 1340-1320, 1220 (aromatic), 1180 (aromatic para), 1080, 1000 (aromatic), 840 (aromatic para), 800, 780-740 (C-Cl) cm⁻¹. Anal: (C₁₉H₁₈O₂Cl₂); requires, C, 65.33; H, 5.16; O, 9.17; Cl, 20.34%; found, C, 65.54; H, 5.11; O, 9.09; Cl, 20.26%.

1,1'-Diphenylbutane (55) (14.7g, 0.07mole) gave yellow needles (13.72g, 54%) which were decolourised to give colourless crystals of 4,4'-bis(chloroacetyl)1,1'-diphenylbutane (62) mp 149-151^o. ν_{\max} (nujol), 2900-2850 (CH₂), 1700 (aromatic ketone), 1600 (aromatic), 1480, 1580-1420, 1340-1320, 1220 (aromatic) 1180 (aromatic para), 1000 (aromatic), 940, 880, 820, 780, 740, (C-Cl) cm⁻¹.
Anal: (C₂₀H₂₀O₂Cl₂); requires, C, 66.12; H, 5.51; O, 8.82; Cl, 19.56%; found, C, 66.27; H, 5.60; O, 8.72; Cl, 19.41%.

4.2.8.2. 2,2'-[1,1'-Biphenyl]-4,4'-diylbis[2-hydroxy-4,4-dimethylmorpholinium]dichloride analogues

4,4'-bis(chloroacetyl)1,1'-biphenyl analogue (58-62) (0.03 mole) was dissolved in boiling acetone and 2-dimethylamino-ethanol (7.4g, 0.08 mole) added. The mixture was then allowed to cool, without stirring for 5 hr. The precipitated product was crystallised from ethanol/ether. This ethanol/ether mixture containing the crystals was centrifuged and the precipitate washed twice with fresh ether which was finally evaporated off under vacuum. The product was dried in a vacuum dessicator. The analogues were decolourised by dissolving in ethanol and, before precipitation with ether, the cold solution was passed through charcoal and

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finally through Kieselguhr.

4,4'-bis(chloroacetyl)1,1'-biphenyl (58) (9.2g, 0.03mole) gave colourless microneedles of 2,2'-[1,1'-biphenyl]-4,4'-diylbis[2-hydroxy-4,4-dimethylmorpholinium] dichloride (hemicholinium-3 as a dichloride salt, HC-3) (4), (10.3g, 71%) mp 222^o. ν_{\max} (nujol), 3400-3200 (OH), 2900, 1680, 1600 (aromatic), 1480, 1380, 1345, 1280, 1240, 1180, 1140, 1120, 1080, 1020, 1000 (aromatic), 960, 940, 840, 820 (aromatic para), 740 cm^{-1} . ν_{\max} (water), 3600-3300 (OH), 2900-2700 (CH_2), 1480 (CH_2), 1260, 1120, 1100, 1080 (aromatic), 980, 960 cm^{-1} . λ_{\max} (water), 258nm. $\tau_{(\text{D}_2\text{O})}$, 2.1, 2.32 (8H, d, -C₆H₄-C₆H₄-), 2.53 (d), 5.15 (16H, s, H₂O), 5.3 (2H, sextet), 5.8 (2H, d), 6.03 (H, m), 6.3 (4H, m), 6.4 (6H, s, 2-CH₃), 6.48 (H, m), 6.65 (2H, m), 6.72 (6H, s, 2-CH₃), 6.98 (H, s, -OH) Anal: (C₂₄H₃₄N₂O₄Cl₂) requires; C, 59.38; H, 7.01; N, 5.77; O, 13.20; Cl, 14.64%, found; C, 59.17; H, 6.98; N, 5.85; O, 13.30; Cl, 14.70%.

4,4'-bis(chloroacetyl)1,1'-diphenylmethane (59) (9.63g, 0.03mole) gave brown crystals (7.8g, 52%) which were decolourised to give colourless microneedles of 2,2'-[1,1'-diphenylmethane]-4,4'-diylbis[2-hydroxy-4,4'-dimethyl]morpholinium dichloride (1C-HC) (7) mp 199-202^o. ν_{\max} (KBr disc) 3400-3300 (OH), 3200, 1640, 1480, 1420, 1380, 1260, 1240, 1140, 1100-1080 (aromatic), 980, 945, 925, 880, 840 (aromatic para), 780 cm^{-1} . ν_{\max} (water), 3000 (OH), 2800, 1480, 1280, 1140, 1120, 1080-1060 (aromatic), 980, 960-940 cm^{-1} . λ_{\max} (water), 223, 260nm. $\tau_{(\text{D}_2\text{O})}$, 2.3 (d), 2.5 (4H, d, 4=CH-), 2.7 (4H, d, 4=CH-), 5.2 (8H, s, 4H₂O), 5.42 (2H, sextet), 5.9 (2H, d), 6.08 (2H, s, - ϕ -CH₂- ϕ -), 6.15 (H, m), 6.43 (4H, d), 6.53 (6H, s, 2-CH₃), 6.6 (H, s), 6.80 (H, m), 6.82 (H, s), 6.9 (6H, s, 2-CH₃), 7.05 (H, s, -OH). Anal: (C₂₅H₃₆N₂O₄Cl₂) requires; C, 60.12; H, 7.21; N, 5.61; O, 12.83; Cl, 14.23%; found, C, 60.32; H, 7.40; N, 5.5; O, 12.68; Cl, 14.1%.

4,4'-bis(chloroacetyl)1,1'-diphenylethane (60) (10.1g, 0.03mole) gave yellow crystals (14.5g, 94%) which were decolourised to give colourless microneedles of 2,2'-[1,1'-diphenylethane]-4,4'-diylbis[2-hydroxy-4,4'-dimethyl morpholinium]dichloride (2C-HC) (46) mp 189^o. ν_{\max} (nujol) 3200(OH), 2800, 1680, 1600, 1480, 1420, 1380, 1340, 1260, 1180, 1140, 1100, 1080 (aromatic), 980, 940, 920, 840 (aromatic para), 780cm^{-1} . ν_{\max} (water), 3000(OH), 2800, 1480, 1260, 1140, 1100, 1080-1060 (aromatic), $980, 960\text{cm}^{-1}$, λ_{\max} (water), 218, 258nm. $\tau_{(\text{D}_2\text{O})}$, 2.2 (d), 2.55 (4H, d, 4=CH-), 2.75 (d), 2.8 (4H, d, 4=CH-), 5.25 (16H, s, 8H₂O), 5.35 (2H, sextet), 5.9 (2H, d), 6.15 (H, m), 6.4 (4H, d), 6.5 (6H, s, 2-CH₃), 6.6 (H, t), 6.75 (H, s), 6.78 (H, d), 6.8 (6H, s, 2-CH₃), 7.05 (H, s, -OH), 7.1 (4H, s, $\phi-(\text{CH}_2)_2-\phi$). Anal: (C₂₆H₃₈N₂O₄Cl₂) requires; C, 60.82; H, 7.41; N, 5.46; O, 12.48; Cl, 13.84%; found; C, 61.04; H, 7.20; N, 5.45; O, 12.36; Cl, 13.95%.

4,4'-bis(chloroacetyl)1,1'-diphenylpropane (61) (10.5g, 0.03mole) gave yellow crystals (10.3g, 65%) which were decolourised to give colourless microneedles of 2,2'-[1,1'-diphenylpropane]-4,4'-diylbis[2-hydroxy-4,4'-dimethyl morpholinium]dichloride (3C-HC) (47) mp 176-178^o. ν_{\max} (nujol) 3200-3000(OH), 2900, 1680, 1600, 1420, 1380, 1260, 1240, 1140, 1100 (aromatic), 980, 940, 840 (aromatic para) cm^{-1} . ν_{\max} (water) 3000(OH), 2800, 1480, 1240, 1120, 1080-1060 (aromatic), $980, 960\text{cm}^{-1}$. λ_{\max} 220, 260nm. $\tau_{(\text{D}_2\text{O})}$, 2.25 (d), 2.55 (4H, d, 4=CH-), 2.75 (d), 2.8 (4H, d, =CH-), 5.3 (16H₂O), 5.45 (2H, sextet), 5.95 (2H, d), 6.2 (H, m), 6.35 (4H, d), 6.5 (6H, s, 2-CH₃), 6.7 (2H, m), 6.8 (H, broad s), 6.88 (6H, s, 2-CH₃), 7.1 (H, s, -OH), 7.4 (4H, m, $(-\phi-\text{CH}_2)_2$), 8.4 (2H, m, -CH₂-). Anal: (C₂₇H₄₀N₂O₄Cl₂), requires; C, 61.48; H, 7.59; N, 12.14; O, 5.31; Cl, 13.47%; found; C, 61.34; H, 7.45; N, 12.25; O, 5.49; Cl, 13.57%.

4,4'-bis(chloroacetyl)1,1'-diphenylbutane (62) (10.9g, 0.03mole) using 1,4-dioxan as the solvent, gave yellow crystals (11.2g, 69%) which were decolourised to give colourless microneedles of 2,2'-[1,1'-diphenylbutane]-4,4'-diylbis[2-hydroxy-4,4-dimethyl morpholinium]dichloride (4C-HC) (48) mp 196-198°. ν_{\max} (nujol) 3200-3000 (OH), 2900, 1680, 1600 (aromatic), 1500, 1480, 1420, 1380, 1340, 1260, 1220, 1180, 1140, 1100, 1080, 1040, 1020 (aromatic), 980, 960, 880, 860, 820 (aromatic para), 780 cm^{-1} . ν_{\max} (water) 3000 (OH), 2800, 1480, 1240, 1120, 1080-60 (aromatic), 980, 940 cm^{-1} . λ_{\max} (water) 218, 257 nm. τ (D_2O), 2.2 (broad), 2.5 (4H; \underline{d} , =CH-), 2.72 (broad), 2.75 (4H; \underline{d} , =CH-), 5.2 (16H; \underline{s} , 8H₂O), 5.35 (2H; sextet), 5.85 (2H; \underline{d}), 6.15 (H; \underline{m}), 6.35 (4H; \underline{t}), 6.5 (6H; \underline{s} , 2-CH₃), 6.6 (H; \underline{m}), 6.7 (H; \underline{m}), 6.75 (H; broad \underline{s}), 6.81 (6H; \underline{s} , 2CH₃), 7.1 (H; \underline{s} , -OH), 7.4 (4H; \underline{m} , (ϕCH_2)₂), 8.4 (4H; \underline{m} , -CH₂-CH₂-). Anal: (C₂₈H₄₂N₂O₄Cl₂) requires; C, 62.12; H, 7.76; N, 5.18; O, 11.83; Cl, 13.12%; found; C, 62.30; H, 7.89; N, 5.08; O, 11.72; Cl, 13.01%.

4.2.8.3. 2,2'-[1,1'-biphenyl]-4,4'-diylbis[2-hydroxy-4-methyl morpholinium] dihydrochloride (tertiary-HC-3) (Method follows that described by Domer and Schueler, 1960)

4,4'-bis(chloroacetyl)1,1'-biphenyl (58) (9.2g, 0.03mole) was dissolved in 2-methylaminoethanol (9g, 0.12mole). The mixture was cooled and a three volume excess of water was then added. This mixture was then allowed to stand overnight (12hr) during which time a brown precipitate formed. The precipitate was washed several times with water and collected on a filter and then extracted with boiling benzene using a continuous extractor. The benzene was cooled and the product crystallised. The product was repeatedly crystallised from ethanol giving colourless needles of 2,2'-[1,1'-biphenyl]-4,4'-diylbis[2-hydroxy-4-methyl morpholinium] dihydrochloride (tertiary-HC-3) (69) (6.6g, 48%) mp 230° (dec)

ν_{\max} (nujol) 3400 (OH), 2900, 1620 (aromatic), 1480, 1380, 1280, 1220 (aromatic para), 1080, 1000 (aromatic), 860cm^{-1} . λ_{\max} (water) 258nm, Anal: ($\text{C}_{22}\text{H}_{30}\text{N}_2\text{O}_4\text{Cl}_2$), requires; C, 57.77; H, 6.56; N, 6.13; O, 14.00; Cl, 15.54%; found; C, 57.55; H, 6.42; N, 6.03; O, 14.24; Cl, 15.76%.

4.2.8.4. 4,4'-Dimethyl-2-hydroxy-2-methyl-morpholinium chloride (methylmorpholinium)

2-Dimethylaminoethanol (5.3g, 0.06mole) was dissolved in ethanol (20mls) and the solution was cooled. Chloroacetone (5.5g, 0.06mole) was added with care. The product was recrystallised from ethanol/ether to give colourless plates of 4,4-dimethyl-2-hydroxy-2-methyl-morpholinium chloride. (8.1g, 75%) mp 214-216^o ν_{\max} (nujol) 3200 (OH), 2900, 1460, 1380, 1240, 1180, 1140, 1080, 1040, 1020, 980, 920, 860, 800, 700cm^{-1} . Anal: ($\text{C}_7\text{H}_{16}\text{NO}_2\text{Cl}$) requires; C, 46.28; H, 8.82; N, 7.71; O, 17.63; Cl, 19.56%, found; C, 46.39; H, 8.71; N, 7.81; O, 17.60; Cl, 19.49%.

4.2.8.5 Attempted synthesis of 2,2'-[1,1'-biphenyl]-4,4'-diylbis[2-acetyloxy-4,4-dimethylmorpholinium] dichloride (cyclic acetyl-HC-3)

4.2.8.5.1 Panormow modification of Schotten-Baumann Reaction (Panormow, 1891)

2,2'-[1,1'-biphenyl]-4,4'-diylbis[2-hydroxy-4,4-dimethylmorpholinium] diiodide (hemicholinium-3 as a diiodide salt, HC-3(4) (10mg, 0.02mmole) was dissolved in 20% sodium hydroxide (4ml). Acetylchloride (0.5g, 6mmole) was added dropwise and the resulting solution was refluxed for 12hr. The mixture was allowed to cool, but no crystals were formed. The volume was reduced by distillation *in vacuo* to yield a residue from which starting material was recovered unchanged.

4.2.8.5.2. Einhorn modification of Schotten-Bauman Reaction

2,2'-[1,1'-biphenyl]-4,4'-diylbis[2-hydroxy-4,4-dimethyl morpholinium]diiodide (hemicholinium-3 as a diiodide salt, HC-3 (4)) (10mg, 0.02mmole) was dissolved in dry pyridine (5ml). Acetylchloride (0.5g, 6mmole) was added dropwise and the solution was stirred for 3hr. The mixture was then added to water (10ml). This mixture was allowed to stand for 24hr. The volume was then reduced by distillation *in vacuo* to yield a residue from which starting material was recovered unchanged when the residue had been dried.

4.2.8.6 Synthesis of 2,2'-[1,1'-biphenyl]-4,4'-diylbis[2-hydroxy-4, [14C]methyl-4-methylmorpholinium]dichloride ([14C]-HC-3)

4.2.8.6.1 Reaction with non-radioactive methyl iodide

The reaction was first run using non-radioactive methyl iodide to verify the method used. Tertiary-HC-3 (69) (18.2mg, 0.04mmole) was dissolved in methanol (5mls) and mixed with another 5mls of methanol containing methyl iodide (2.48mg, 0.017mmole). The mixture was refluxed gently with stirring. After 1hr the methanol and any unreacted methyl iodide were removed by vacuum distillation. The residue was dissolved in distilled water (2ml) and passed through an ion-exchange column (CM-Sephadex C-50, swollen in distilled water and supported in a 3" pasteur pipette) and eluted with distilled water. Samples of the aqueous eluates were run on a cellulose thin layer plate (butanol-methanol-acetic acid-water; 8:2:1:3) and developed with iodine. Tertiary-HC-3 (69) has an R_f value of 0.5. When no more starting material could be detected (requires about 100ml of water to elute all the unreacted tertiary-HC-3(69)) the column was eluted with NaCl (1M). Samples of these eluates were run on a TLC plate as before, the quaternary product HC-3(4) has an R_f value of 0.9. No

tertiary-HC-3(69) could be detected in any of these NaCl eluates. Those eluates which were shown by TLC to contain HC-3(4) were pooled. A sample from this pool gave a precipitate with ammonium reinekate indicating the presence of a quaternary nitrogen. The pooled eluates absorbed ultra-violet light giving λ_{\max} 258nm, this indicates the presence of the diphenyl nucleus of HC-3. The pooled eluates were dried in a vacuum. The residue was NaCl and presumably HC-3(4). From this dry residue a NaCl disc was made (in a similar way to a KBr disc) and an ir absorption spectrum of this disc was run. The spectrum showed absorptions characteristic of HC-3 (4). ν_{\max} (NaCl disc) 3,400-3,200 (OH), 2,900, 1680, 1620 (aromatic), 1480, (CH), 1345, 1280, 1240, 1180, 1140, 1120, 1080, 1020, 1000 (aromatic) 960, 940, 840, 820 (aromatic para) 740cm^{-1} .

4.2.8.6.2 Reaction with radioactive $[^{14}\text{C}]$ -methyl iodide

Tertiary-HC-3(69) (18.2mg, 0.04mmole) was dissolved in methanol (5mls) and this was poured into the top of a 'Break-seal' ampoule (Type P1) (Fig. 4.10); which had just been taken from the freezer, and which contained $[^{14}\text{C}]$ -methyl iodide (1.24mg, 0.0086mmole, 59.7mCi/mmole). The ampoule was then surrounded with an ice-salt mixture and cooled. A condenser was fitted to the top of the ampoule. The ampoule seal was then broken with a glass rod and the methanol solution poured into the bottom of the ampoule and mixed with the $[^{14}\text{C}]$ -methyl iodide. This solution was refluxed and purified as described above. No radioactivity could be detected in samples of the aqueous eluates indicating that no $[^{14}\text{C}]$ -methyl iodide had been present in the residue. However, the NaCl eluates did contain radioactivity and these eluates were pooled. The product, $[^{14}\text{C}]$ -HC-3, was detected by paper electrophoresis (Potter and Murphy, 1967) in the NaCl eluates. After being subjected to electrophoresis for 2hr the paper strips were dried and cut into 0.5cm sections. Only the

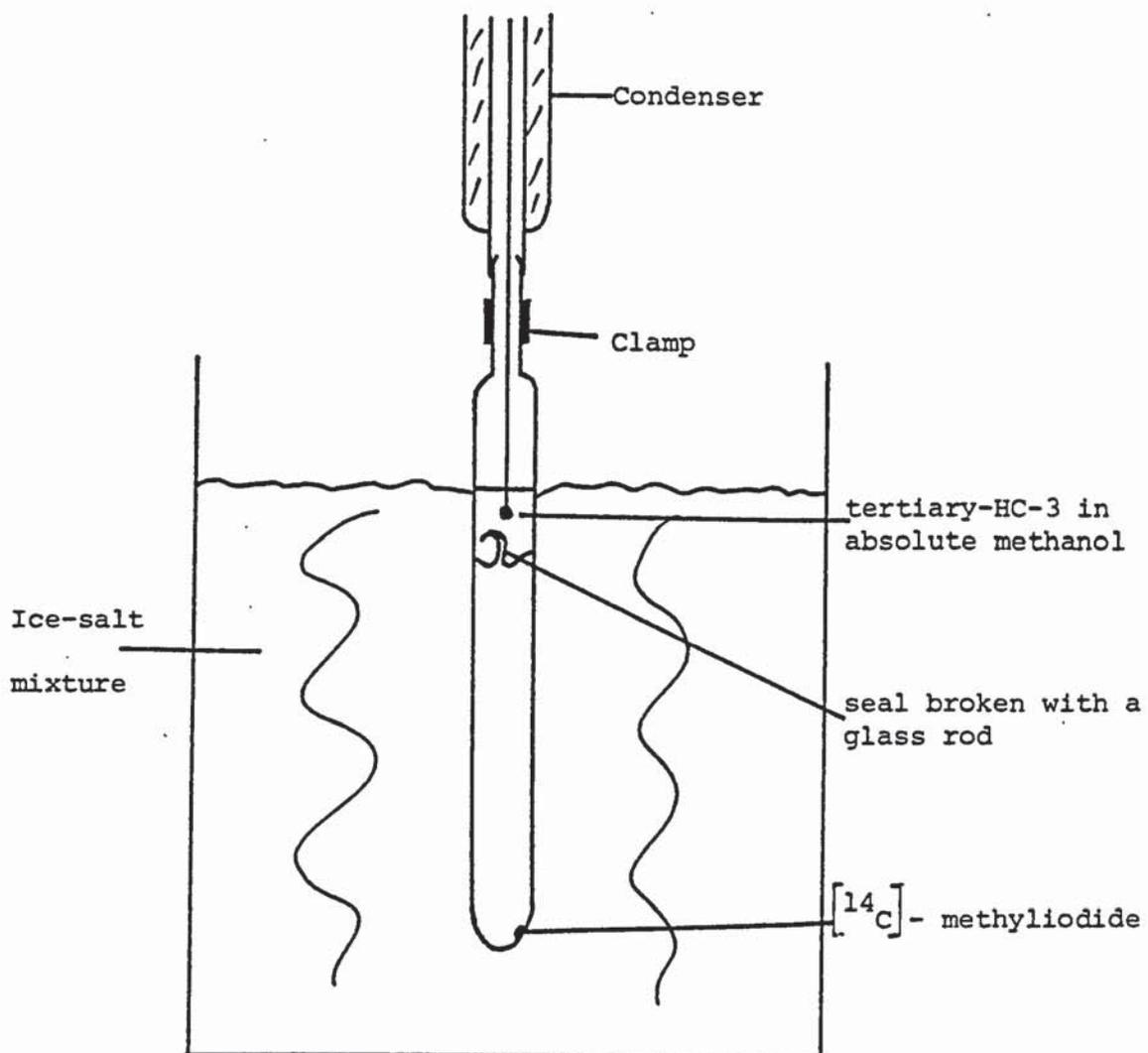


Fig. 4.10:- Break-seal ampoule (Type P1) containing [¹⁴C]-methyl iodide about to be reacted with tertiary-HC-3 in absolute methanol

section 6.5-7cms from the origin gave high counts. This corresponds in position to an iodine stained band of authentic HC-3 similarly subjected to paper electrophoresis in the same cell at the same time.

Specific activity = 119.4mCi/mmole

Yield = 55% in 1M NaCl (500 μ l of NaCl solution
contained 0.160 μ Ci/1.34nmole)

4.3 PHARMACOLOGY

4.3.1. Neuromuscular blocking action

The actions of HC-3 and the hemicholinium analogues, 1CHC, 2CHC, 3CHC and 4CHC were observed on the rat hemidiaphragm and the frog rectus abdominis muscle preparations to determine the extent of pre- and post-junctional block at the neuromuscular junction.

4.3.1.1 Rat phrenic nerve-hemidiaphragm

All of the hemicholinium compounds, in low doses, were found to cause a slowly developing block of the rapidly stimulated muscle (1Hz), but had no effect on the more slowly stimulated muscle (0.1Hz). When choline was added to the incubating medium in the presence of the hemicholinium compound or if the compound was washed out of the bath, the muscle contraction of the rapidly stimulated muscle returned to the control height of contraction. These results suggest that these compounds gave a frequency dependent prejunctional block at these low doses (Table 4.1). 2CHC, 3CHC and 4CHC were all found to be equipotent in causing a prejunctional block and they were approximately 100 times more potent than 1CHC and the parent compound, HC-3. The methylmorpholinium derivative was about 40 times less potent than HC-3

Larger doses of all the hemicholinium compounds except for the methylmorpholinium derivative, caused a block of both the slow and rapidly stimulated muscles which was not reversible with choline. When the drug was washed out of the bath the muscle contractions eventually returned to the control height. It was concluded that these compounds gave a postjunctional block at these high doses (Table 4.1). 3CHC was found to be about 7 times more potent than HC-3 in causing a postjunctional block. 2CHC and 4CHC were both about 5 times more potent than HC-3 and 1CHC was equipotent to HC-3.

Compound	Minimum concentration giving a prejunctional block ($\mu\text{mole/ml}$)	Minimum Concentration giving a postjunctional block ($\mu\text{mole/ml}$)
HC-3	0.124	0.206
1CHC	0.100	0.200
2CHC	0.001	0.039
3CHC	0.001	0.029
4CHC	0.001	0.037
Methylmorpholinium	3.87	No block up to a dose of 10

TABLE 4.1: Effect of the hemicholinium compounds on nerve transmission in the rat phrenic nerve-hemidiaphragm preparation. In each case the prejunctional block was reversed by choline (0.02 to 0.14 $\mu\text{mole/ml}$)

4.3.1.2 Frog rectus abdominis muscle

The activity of the hemicholinium compounds in blocking the effect of ACh on the frog rectus abdominis muscle was observed in order to investigate their postjunctional curare-like blocking action. All of the hemicholinium compounds tested blocked the nicotinic receptor sites. Concentrations producing 50% inhibition of ACh - induced contractions are shown in Table 4.2.

3CHC was found to be the most potent of the hemicholinium compounds which gave a postjunctional block at the rat phrenic nerve-hemidiaphragm preparation and it is interesting to observe that similarly 3CHC was found to be the most effective compound in blocking the ACh induced contractions of the frog rectus abdominis muscle. At equimolar concentrations 3CHC inhibited the action of ACh by 50%. 3CHC was 70 times more potent than HC-3. 2CHC and 4CHC were equipotent in blocking the nicotinic receptors and they were both 35 times more potent than HC-3. 1CHC was 4 times more potent than HC-3.

4.3.2 Anticholinesterase Activity

The effect of the hemicholinium compounds on cholinesterase activity was determined by including the compounds in incubation mixtures containing enzyme, buffer and radioactive substrate as described in the Methods (Section 3.4). The degree of hydrolysis of the substrate was then compared with that obtained in controls containing distilled water in place of the compounds.

In order to determine the effects of the compounds on acetylcholinesterase (AChE) and pseudo-cholinesterase (ChE) the compounds were tested against commercial preparations of these two enzymes obtained from bovine erythrocytes and horse serum respectively, [^{14}C]-ACh was used as the substrate. Also, a 20,000g supernatant from a rat brain homogenate was

Compound	IC ₅₀ (μmole/ml)
HC-3	0.21
1CHC	0.05
2CHC	0.006
3CHC	0.003
4CHC	0.006

TABLE 4.2: Block of acetylcholine (0.003μmole/ml) induced contractions of the frog rectus abdominis muscle. IC₅₀ represents the concentration of the compound which gave a 50% inhibition of ACh induced contraction.

Compound	Enzyme			Substrate
	Bovine Erythrocyte (AChE)	Horse Serum (ChE)	Rat Brain Homogenate	
HC-3	1.9×10^{-3}	1.5×10^{-5}	1.9×10^{-4} 7.0×10^{-5} 8.3×10^{-5}	ACh Ac- β -MeCh BuCh
1C-HC	3.6×10^{-3}	3.9×10^{-4}	4.7×10^{-4} 9.3×10^{-5} 1.0×10^{-3}	ACh Ac- β -MeCh BuCh
2C-HC	2.5×10^{-3}	9.9×10^{-5}	1.4×10^{-4} 3×10^{-4} 4.1×10^{-3}	ACh Ac- β -MeCh BuCh
3C-HC	1.8×10^{-3}	9.6×10^{-3}	2.3×10^{-4} 7×10^{-5} 1.7×10^{-3}	ACh Ac- β -MeCh BuCh
4C-HC	1.9×10^{-3}	5.6×10^{-3}	1.9×10^{-4} 1.7×10^{-5} 4.8×10^{-4}	ACh Ac- β -MeCh BuCh
methyl-morpholinium	2.5×10^{-2}	4.6×10^{-2}	4.6×10^{-3} 2.7×10^{-3} 1.3×10^{-2}	ACh Ac- β -MeCh BuCh
physostigmine	1.3×10^{-6}	3.0×10^{-6}	2.9×10^{-7} 2.7×10^{-7}	ACh Ac- β -MeCh BuCh

TABLE 4.3: IC_{50} values (molar) for the inhibition of cholinesterase activity by the hemicholinium compounds. Incubation mixtures contained enzyme, $[^{14}C]$ -labelled substrates as indicated and a range of concentration of each inhibitor. A Dixon plot was drawn for each compound and the IC_{50} values are calculated from these plots, they represent that concentration of inhibitor which inhibited the hydrolysis of the various substrates by 50%.

For each plot regression lines with correlation coefficients of 0.97 to 0.999 were drawn through at least five points. Each point was the mean of at least three determinations \pm SEM of 10% or less.

used as a source of both AChE and ChE. In this case, $[^{14}\text{C}]$ -ACh was used as the substrate for AChE and ChE combined, $[^{14}\text{C}]$ -acetyl- β -methyl choline was used as a specific substrate for AChE and $[^{14}\text{C}]$ -butyrylcholine was used as a specific substrate for ChE.

The activities of the hemicholinium compounds as anticholinesterases were compared to physostigmine (Table 4.3). All the hemicholinium compounds were shown to inhibit both AChE and ChE. However, they are all much weaker than physostigmine.

HC-3 was the most potent of the compounds tested whereas the methylmorpholinium derivative was the weakest in inhibiting AChE and ChE. Increasing the alkyl chain (HC-3 to 4CHC) did not show any trend in the potency of these compounds as inhibitors of AChE or ChE. The results suggest that HC-3 may be more active against ChE than against AChE.

4.3.3. In Vitro acetylation by ChAc

The rates of acetylation of the hemicholinium compounds were determined by incubating the compounds at 37° for 10min with $[^{14}\text{C}]$ -AcCoA and a partially purified extract of ChAc obtained from rat brain. The acetylated products were routinely isolated and quantitatively assayed by following the method described by Fonnum (1975). The rates of acetylation of the hemicholinium compounds were compared to that of choline which is the natural substrate for ChAc. Blank determinations were obtained by substituting the substrate for distilled water.

The relative rates of *in vitro* acetylation of the hemicholinium compounds were determined and compared to the acetylation of choline (Fig. 4.11). 1CHC, 2CHC, 4CHC and methylmorpholinium were acetylated at less than 5% that of choline. However, HC-3, 3CHC and HC15 were all acetylated at about 28% compared to choline 100%.

Time studies demonstrated that the rates of acetylation of HC-3

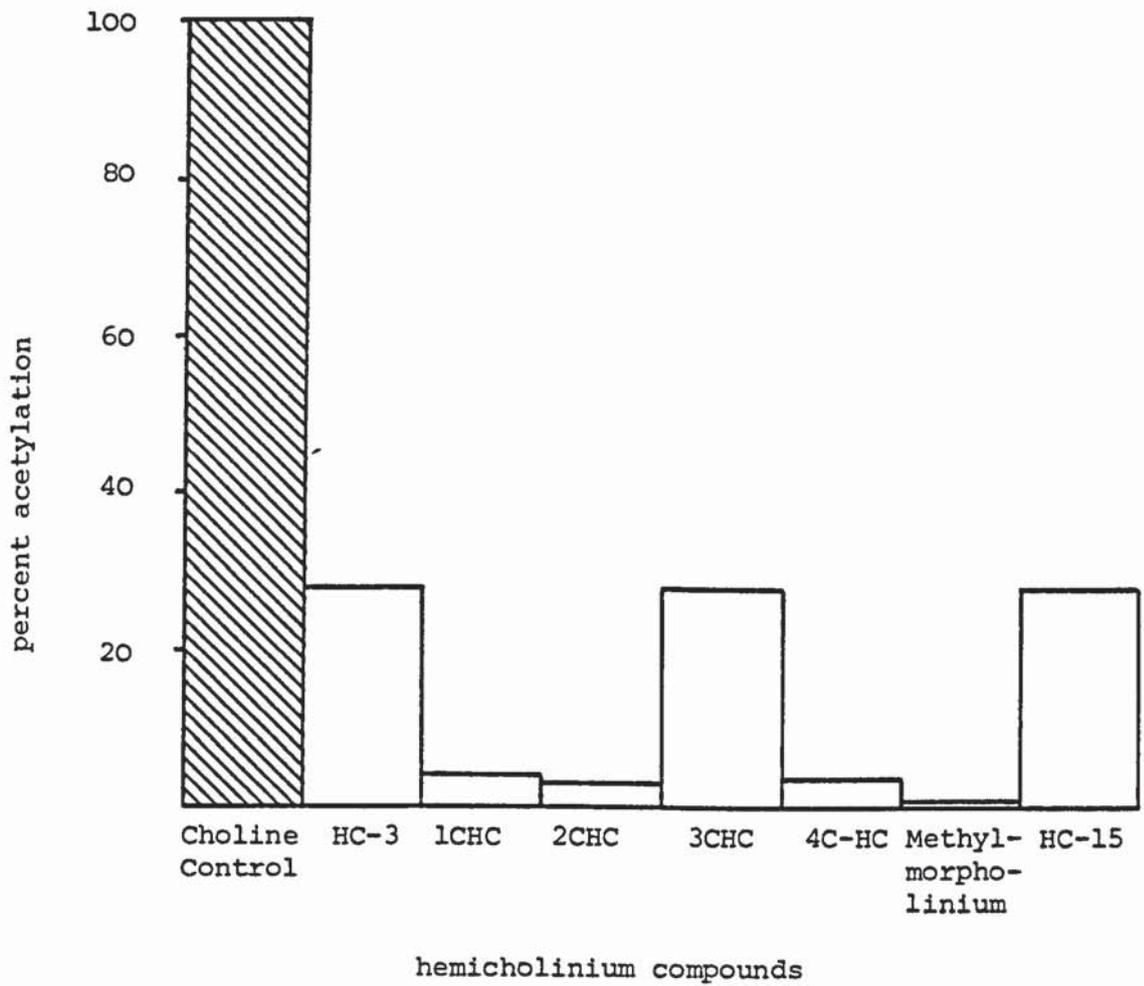


Fig. 4.11:- The rates of acetylation of choline and the hemicholinium compounds by ChAc extracted from rat brain. The incubation was carried out for 10min. at 37°C. The final substrate concentration was 20mM. The experiments were performed in duplicate and repeated five times. SEM are all less than ±5%.

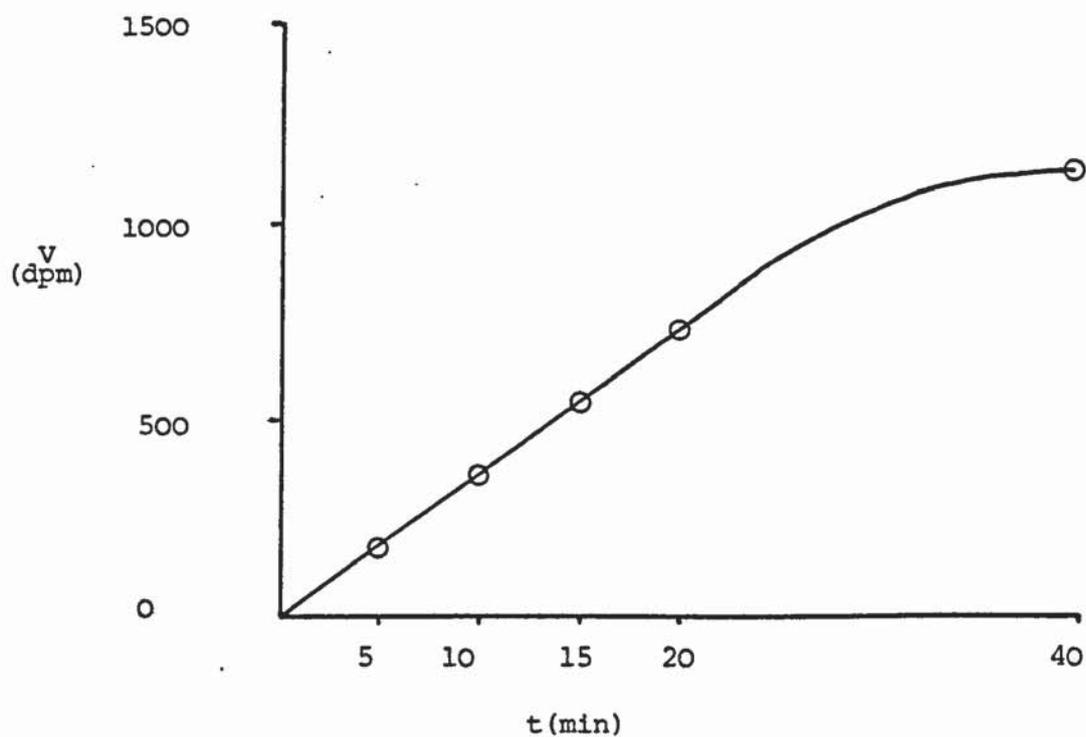
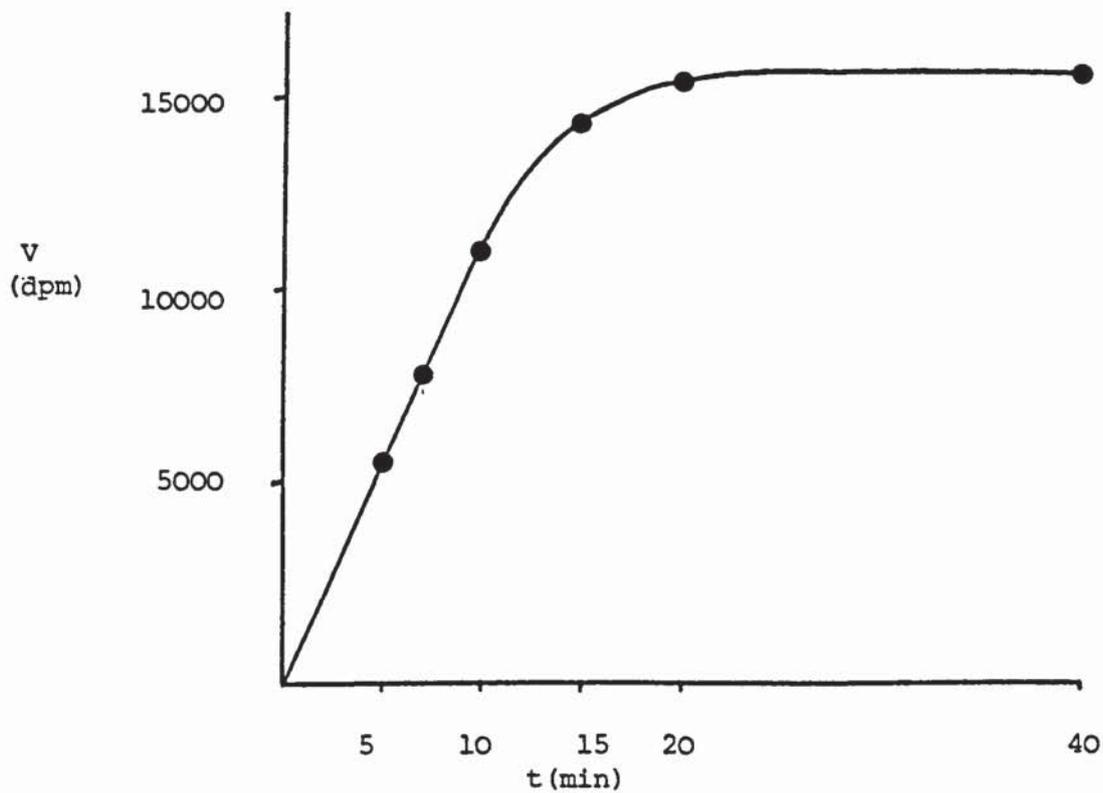


Fig. 4.12:- Rate of acetylation of HC-3 by ChAc *in vitro*. Incubation was at 37°. V is velocity of reaction and t is the incubation time. The concentration of HC-3 was 40mM(●) and 20µM(O). The experiments were performed in duplicate and repeated twice. \pm SEM were all less than 10%.

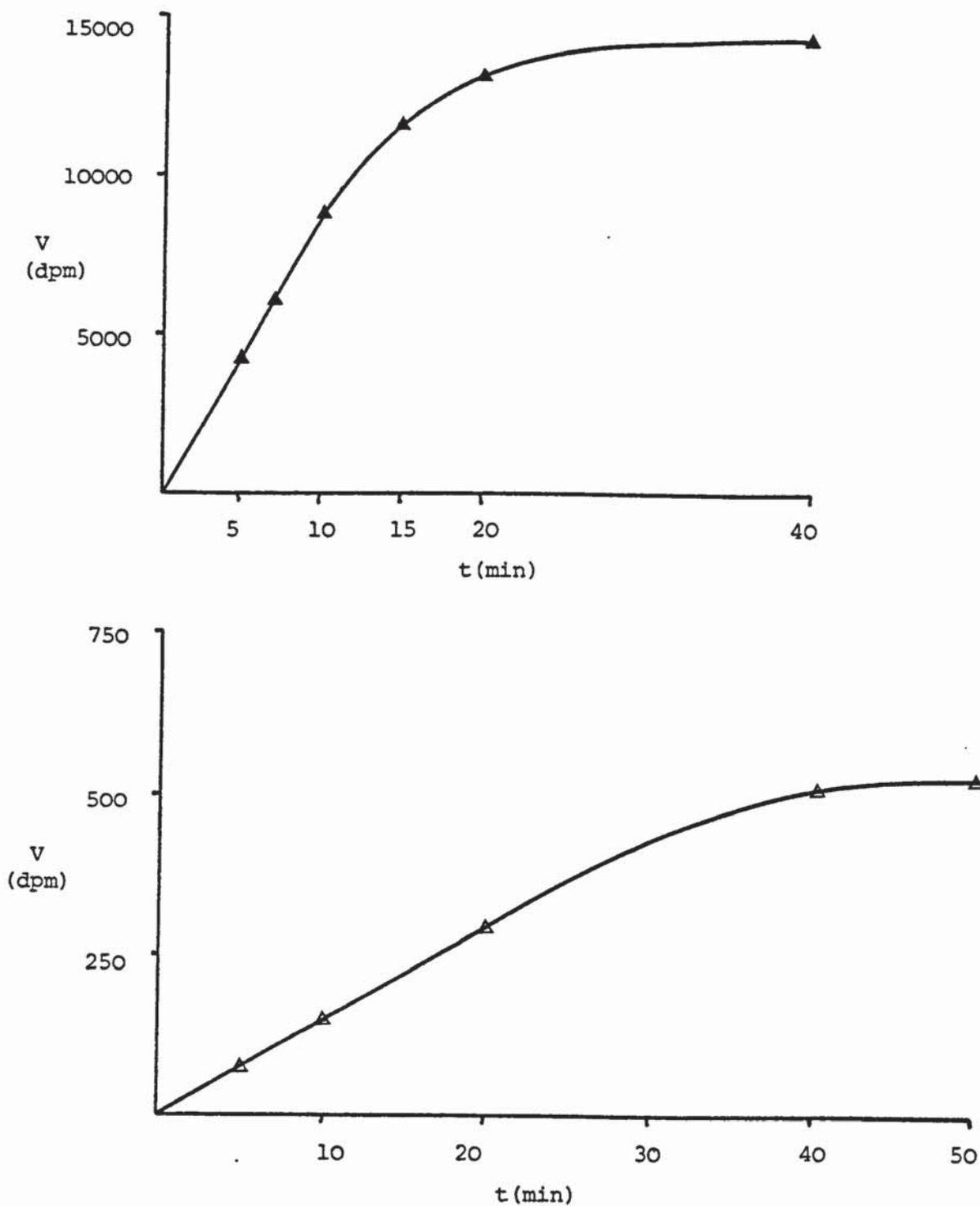


Fig. 4.13:- Rate of acetylation of 3CHC by ChAc *in vitro*. Incubation was at 37°. V is velocity of reaction and t is the incubation time. The concentration of 3CHC was 20mM(▲) and 20µM(△). The experiments were performed in duplicate and repeated twice. ±SEM were all less than 10%.

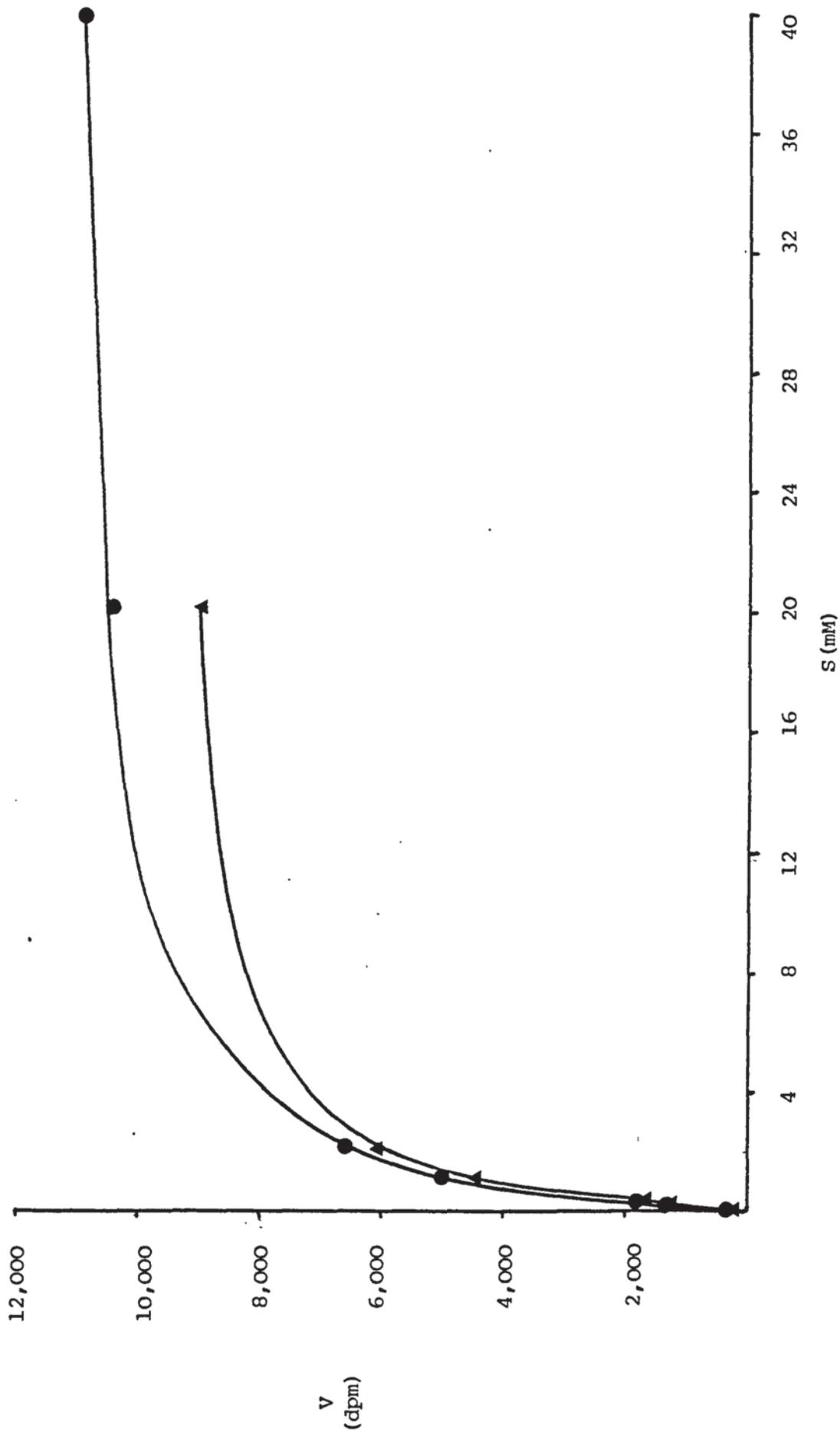


Fig. 4.14:- Velocity/substrate curve of the acetylation of HC-3 (●) and 3CHC (▲) by ChAc *in vitro*. Incubation was at 37° for 10min. Experiments were performed in duplicate and repeated twice. ±SEM were all less than 10%.

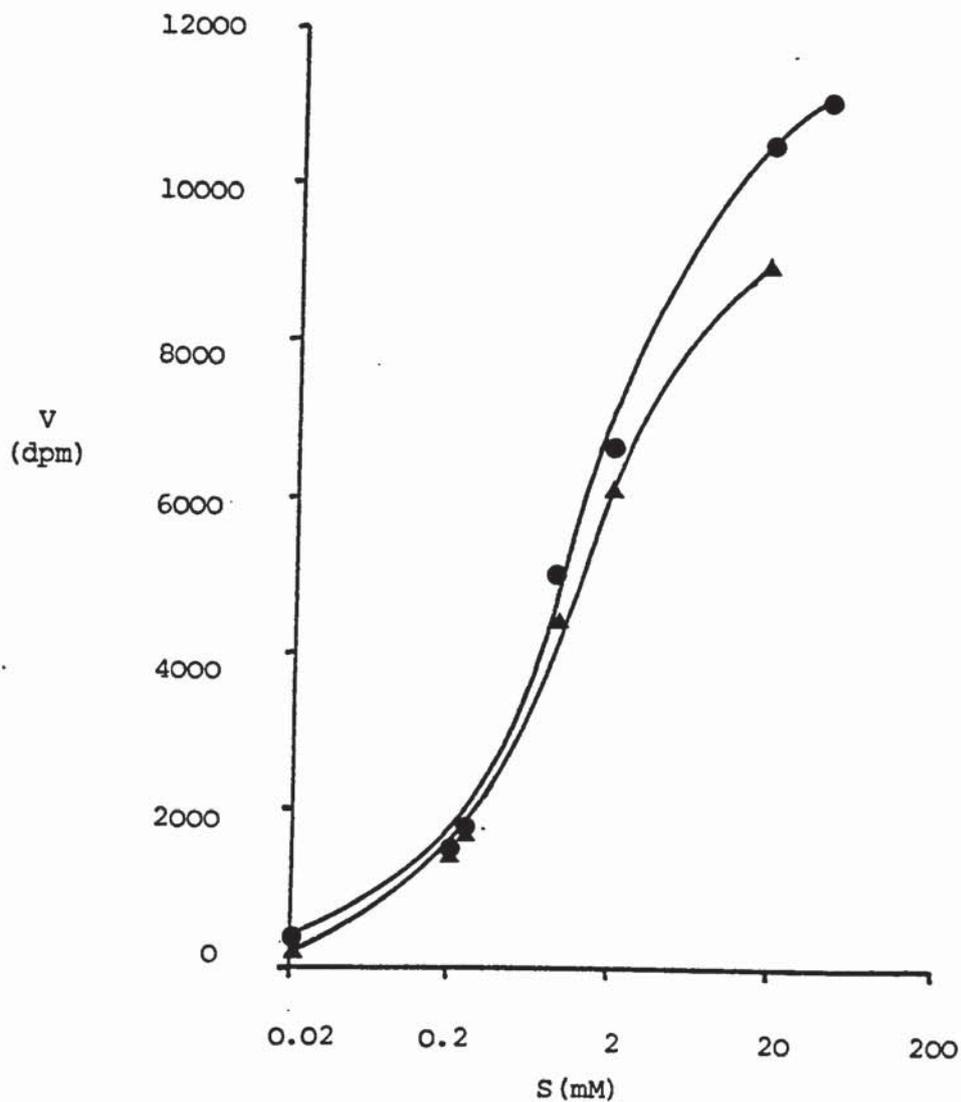


Fig. 4.15:- Velocity/log substrate curve of the acetylation of HC-3 (●) and 3CHC (▲) by ChAc *in vitro*. Incubation was at 37° for 10 min. Experiments were performed in duplicate and repeated twice. ± SEM were all less than 10%.

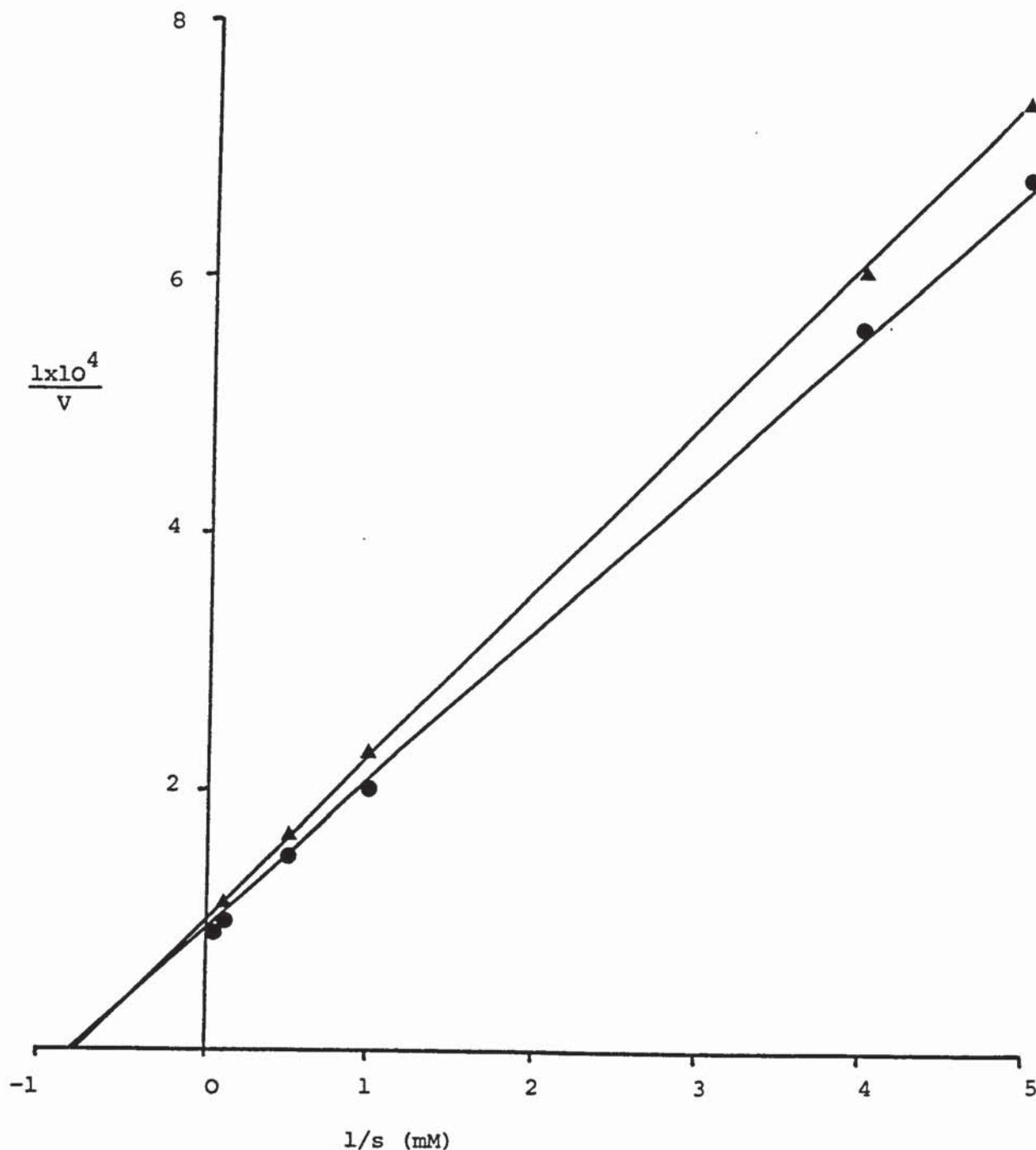


Fig. 4.16:- Lineweaver-Burke (1934) plot of the acetylation of HC-3 (●) and 3CHC(▲) by ChAc *in vitro*. Incubation was at 37° for 10min. S = concentration of substrate and V = velocity of reaction. The apparent K_m (where the concentration of AcCoA was 18 μ M) were HC-3 m = 1.21mM and 3CHC = 1.27mM (Choline, not shown, had a K_m of 0.29mM and a V_{max} 12 μ mole of acetylcholine/g of protein/10min) Experiments were performed in duplicate and repeated twice. \pm SEM were all less than 10%.

and 3CHC were linear for at least 10min at the concentrations employed (Figs. 4.12 and 4.13). The plots of velocity of acetylation as a function of substrate concentration showed typical shaped curves for an enzyme-one substrate dependent reaction (Figs. 4.14 and 4.15). At the concentrations used no substrate inhibition was seen. The apparent Michaelis-Menten constants for each substrate were derived from the plots (Fig. 4.16) according to Lineweaver and Burke (1934). A similar study was carried out with choline as the substrate.

ChAc is a two substrate enzyme and each substrate affects the affinity of the enzyme for the other. However, at a constant concentration of AcCoA, an apparent Michaelis-Menten constant can be determined. The results indicate that choline ($K_m = 0.29\text{mM}$) had a greater affinity for ChAc than the other two analogues. HC-3 (1.21mM) had a slightly greater affinity for ChAc than 3CHC (1.27mM).

Bradshaw (1976), also showed that HC-3 was acetylated by a partially purified extract of ChAc obtained from rat brain at about 28% compared to choline 100%. He showed that although the enzyme extract will contain carnitine acetyltransferase (CarAc) the acetylation of HC-3 is effected by ChAc, since NVPH (40) (a ChAc inhibitor, but not a CarAc inhibitor) inhibited the acetylation of HC-3 by 98% compared to the inhibition of the acetylation of choline, 96%. Bradshaw (1976), suggested that ChAc is probably wholly responsible for the acetylation of HC-3 by the rat brain extract. This conclusion is supported by similar observations made by Barker and Mittag (1975). Therefore it is likely that the acetylation of HC-3 seen in this study is due only to the action of the ChAc enzyme in the rat brain extract. A similar situation may exist for the acetylation of 3CHC.

Purification of ChAc in order to eliminate choline is important since if choline is present in the incubation medium the assay cannot be quantitative since it won't be known how much of the acetylated product

assayed is acetylated hemicholinium compound or ACh. Also, choline the natural substrate for ChAc, if present in the medium will compete with the hemicholinium compound for the enzyme.

It has not been possible to convert dpm of isolated acetylated product to μ mole of isolated acetylated product extracted by the method of Fonnum (1975), since it is not known whether HC-3 is mono or biacetylated by ChAc *in vitro*.

4.3.4. In Vitro inhibition of ChAc

A partially purified extract of ChAc obtained from rat brain and AcCoA were incubated with equimolar concentrations of choline and one of the hemicholinium compounds, and the amount of acetylated product synthesised was assayed by the method of Fonnum (1975). These incubations were compared with control incubations containing the same concentration of choline, but distilled water instead of the hemicholinium compound (Table 4.4). The method of isolation of the acetylated product does not distinguish between ACh and any acetylated hemicholinium compound. Therefore, since HC-3, 3CHC and HC-15 are acetylated *in vitro* by ChAc, as discussed in the previous section (4.3.3.) it cannot be stated that the reduction in acetylation in test incubations with these compounds compared to control incubations is the true amount of inhibition since any synthesis of the acetylated derivatives will mask the inhibition of ACh synthesis. Therefore in the cases of HC-3, 3CHC and HC-15 the rates of inhibition of ChAc could be more than that shown. 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 compete favourably with these hemicholinium compounds for the sites of acetylation on the enzyme.

The most potent inhibitors of ChAc found in this study were

Inhibitor	Percent Inhibition
Water	0
HC-3	7.5
1CHC	2.5
2CHC	40.4
3CHC	18.5
4CHC	34.0
methylnorpholinium	2.5
HC-15	0

TABLE 4.4: Percentage inhibition of the acetylation *in vitro* of choline by ChAc extracted from rat brain. Both choline and the hemicholinium compounds were at a concentration of 14mM. Incubations were carried out at 37° for 10min. Each value is the mean of four determinations. ±SEM was 10% or less.

2CHC and 4CHC although HC-15, HC-3 and 3CHC could be more potent inhibitors than is shown.

4.3.5. Determination of the structure of acetylated HC-3 as synthesised by ChAc *in vitro*

Acetylated HC-3, as synthesised by ChAc *in vitro*, could be in the cyclic form (45), in the open-chain seco form (14) or a mixture of both. Also, acetylated HC-3 could either be monoacetylated or biacetylated.

4.3.5.1 Theory of the experiment

Synthesis of cyclic acetyl-HC-3 (45), as discussed in the chemistry section of this Chapter (Section 4.2), was not possible but it has been presumed that the chromophore in this molecule (45) is identical to that found in the HC-3 (4) molecule, that is the biphenyl moiety. The carbonyl groups of the acetyl moieties are three bonds away from the biphenyl group and they are too far away to have any effect on the chromophore as shown (Fig. 4.17). Open-chain Ac-seco-HC-3(14) is commercially available. The chromophore in this molecule (14) includes the biphenyl moiety and the two carbonyl groups as shown (Fig. 4.17). As in the cyclic acetyl-HC-3 (45), the carbonyl groups of the acetyl moieties are too far away to have any effect on the chromophore. In both isomers (45, 14) the carbonyl groups of the acetyl moieties will absorb uv light but at much shorter wavelengths (higher energies) than those scanned in these experiments.

Therefore cyclic acetyl-HC-3 (45) and open-chain Ac-seco-HC-3(14) have different chromophores and can therefore be identified as they absorb different wavelengths of uv light (Fig. 4.17).

When excess HC-3 was incubated *in vitro* with ChAc and excess

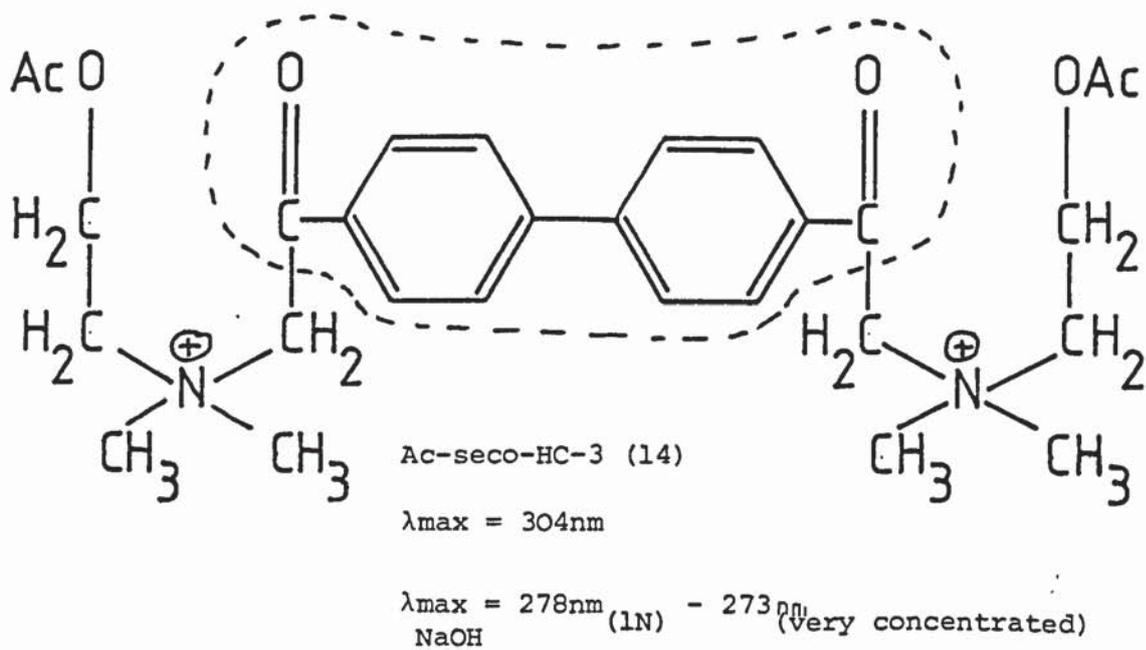
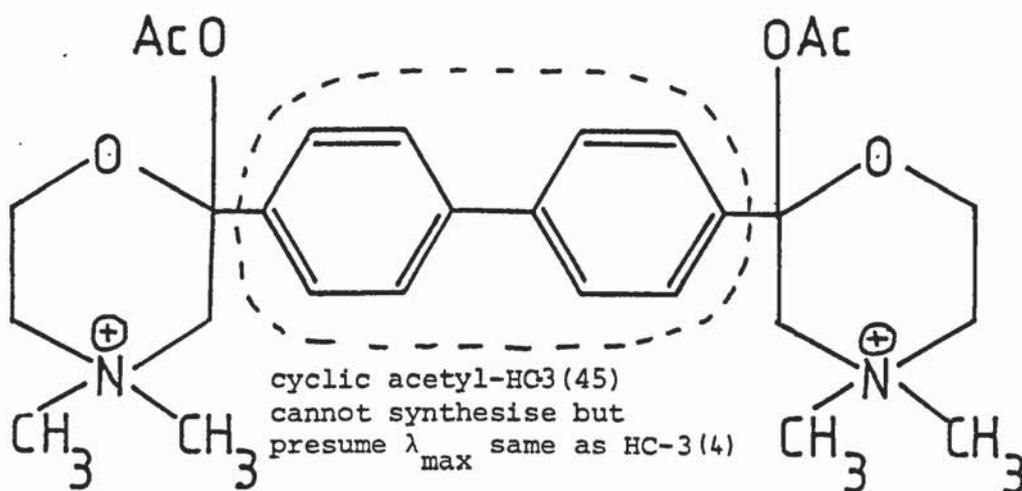
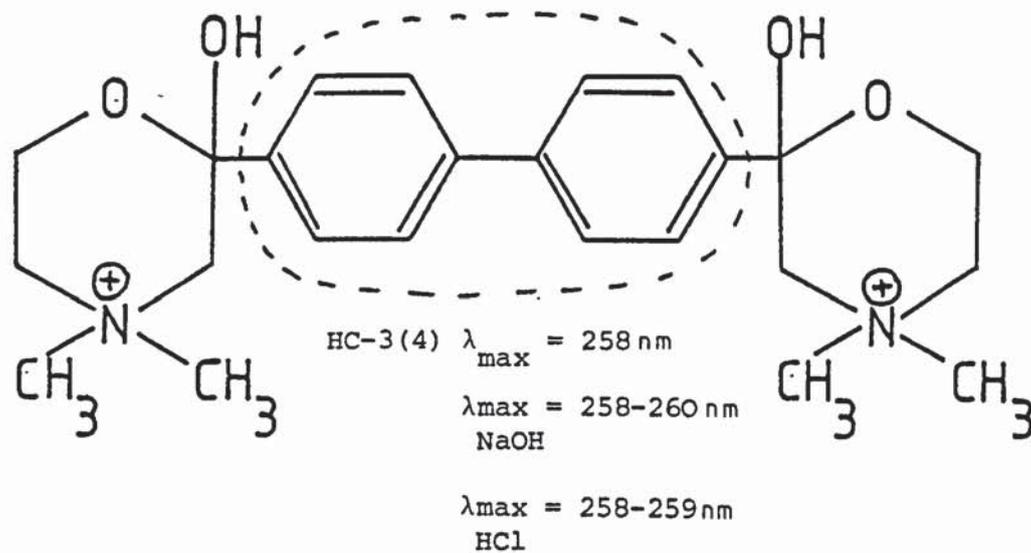


Fig. 4.17:-----Chromophores and λ_{\max} of HC-3 and biacetylated cyclic acetyl-HC3 and Ac-seco-HC-3

$[^{14}\text{C}]$ -AcCoA, as previously described in the Methods (Section 3.5), then $[^{14}\text{C}]$ -acetylated HC-3 was synthesised, as described in the Results section of this chapter (Section 4.3.3). The structure of the acetylated HC-3 should be determinable using uv spectroscopy as discussed above. However, if the acetylated HC-3 is in the cyclic form then the chromophore is the same as that found in the HC-3 molecule. In other words any uv absorption at 258nm could be due to cyclic acetyl-HC-3 or just unreacted HC-3. Similarly aqueous solutions of $[^{14}\text{C}]$ -AcCoA at pH7 absorb at around 258nm in the uv. So to avoid confusion it is necessary to isolate the radiolabelled acetylated HC-3 from the starting materials, since the uv absorption of HC-3 and $[^{14}\text{C}]$ -AcCoA would mask the absorption of any cyclic acetyl-HC-3. The absorption of open-chain Ac-seco-HC-3 would also be masked since a strong absorption by unreacted substrates at 258nm in the uv would mean diluting the mixture so that the absorption would be on the scale of the absorption record. This dilution would make the relatively small amount of product, acetylated HC-3, undetectable in the uv. So, again, in order to identify the structure of acetylated HC-3 it must be isolated from the starting materials, that is HC-3, ChAc and $[^{14}\text{C}]$ -AcCoA.

4.3.5.2 Separation of open-chain Ac-seco-HC-3 from HC-3, ChAc and $[^{14}\text{C}]$ -AcCoA

These control experiments were conducted using only the open-chain Ac-seco-HC-3 since cyclic acetyl-HC-3 could not be chemically synthesised.

Bradshaw and Hemsworth (1976), incubated a mixture of HC-3, ChAc and $[^{14}\text{C}]$ -AcCoA at 37°. Using several methods they showed that this mixture synthesised $[^{14}\text{C}]$ -acetylated HC-3. When the incubated mixture was applied to the top of an ion-exchange column (Amberlite CG-50

[100-200mesh]) unreacted [^{14}C]-AcCoA could be eluted by sodium acetate (1mM), however, unreacted HC-3 and the product [^{14}C]-acetylated HC-3 were not eluted by sodium acetate nor presumably by 1N HCl since these eluates gave no precipitate when ammonium reineckate was added to them (ammonium reineckate gives a pink precipitate in the presence of quaternary nitrogens). These workers also showed that open-chain Ac-seco-HC-3 binds to the ion-exchange resin and is not eluted by water or 1N HCl, but is eluted by 1N NaOH. They detected Ac-seco-HC-3 in the eluates by measuring the uv absorption at 280nm only (Uvicord II spectrophotometer), Ac-seco-HC-3 has a $\lambda_{\text{max}} = 278\text{nm}$, and by the use of ammonium reineckate.

1N NaOH

On the basis of this work control experiments were conducted using an Amberlite CG-50 (100-200mesh) cation exchange resin supported in a 3" pasteur pipette which was plugged with glass wool. The resin was initially equilibrated with distilled water. Fresh resin and columns were used for each control experiment.

Open-chain Ac-seco-HC-3 in an aqueous solution was found to bind to the resin. It was not eluted by water, neither was it eluted by 1N HCl, but it was eluted from the resin by 1N NaOH. This result agrees with the findings of Bradshaw and Hemsworth (1976). Detection of Ac-seco-HC-3 was effected by measuring the uv absorption between 225nm and 400nm, (Fig. 4.18), and by use of ammonium reineckate. Maggio-Cavaliere (1976), found that Ac-seco-HC-3 was stable in aqueous solutions at 27 $^{\circ}$ over a pH range from 1 to 9. At 37 $^{\circ}$ Ac-seco-HC-3 was stable for at least 12hr at pH 7.4. However, between pH 9.0 and 10.4 Ac-seco-HC-3 was shown to be hydrolysed to HC-3, between 27-37 $^{\circ}$, although no time for the conversion was stated, but the rate of conversion was greater at higher temperatures. In the current experiments Ac-seco-HC-3 was stable in 1N NaOH for at least seven days at room temperature (about 20 $^{\circ}$). After this time no HC-3 ($\lambda_{\text{max}} = 258\text{nm}$) was detected (Fig. 4.18). In the following experiments

1N NaOH

where the $[^{14}\text{C}]$ -acetylated HC-3 is a solution in 1N NaOH, it is in this medium for no longer than 6hr at room temperature.

HC-3 applied in an aqueous solution to the Amberlite exchange resin was found to bind to this resin. It was not eluted with water. When 1N HCl was passed through the column the eluates gave no precipitate with ammonium reineckate, but the uv spectrum scanned from 225nm to 400nm showed a λ_{max} at 258nm. So it seems as though HC-3 was eluted from the resin by 1N HCl but at a very slow rate, such that HC-3 was at a concentration where it could be detected by uv but not by ammonium reineckate. In further control experiments it was found that ammonium reineckate will detect HC-3 in aqueous solutions provided that the concentration is no less than 0.5mM. When a volume of this solution equal to 100nmole is added to a concentrated ammonium reineckate solution a pink precipitate forms. However, HC-3 is detectable in the uv as an aqueous solution at a concentration of at least 12 μ M. When a volume of this solution equal to 13nmole is added to a uv cell the solution measurably absorbs light ($\lambda_{\text{max}}=258\text{nm}$). Presumably Bradshaw and Hemsworth (1976) did not observe that HC-3 is eluted by 1N HCl as their methods of detection (ammonium reineckate and measurement of absorption in the uv at 280nm only) were inadequate.

A mixture of Ac-seco-HC-3 (0.5ml, 50mM) and HC-3 (0.5ml, 50mM) in an aqueous solution was applied to the top of an Amberlite ion-exchange resin. 1N HCl was passed through the column and HC-3 was detected in the eluates by uv spectroscopy ($\lambda_{\text{max}}=258\text{nm}$). 100mls of 1N HCl was found sufficient to elute all the HC-3 from the resin (until the eluates showed no λ_{max}). Then the column was eluted with 1N NaOH (20ml). Ac-seco-HC-3 was detected in the eluates by uv spectroscopy ($\lambda_{\text{max}}=278\text{nm}$). Thus it would appear that Ac-seco-HC-3 can be separated from HC-3 using an Amberlite ion-exchange resin.

ChAc in buffer, as described in the Methods (Section 3.5.1.), did not

bind to the Amberlite ion-exchange resin (as it is not a cation) and it was eluted with water. It was crudely detected in the aqueous eluates by lightly tapping the collection tube and observing the froth, which indicates the presence of protein.

$[^{14}\text{C}]$ -AcCoA (1ml, 45 μM) in buffer, as described in the Methods (Table 3.1), was applied to the ion-exchange column. It did not bind to the resin as it was eluted with water. The $[^{14}\text{C}]$ -AcCoA was detected by uv ($\lambda_{\text{max}} = 258\text{nm}$) and by estimating the radioactivity in 200 μl samples of the eluates. The samples were added to NE 260 fluor.

4.3.5.3 Isolation of $[^{14}\text{C}]$ -acetylated HC-3 as synthesised by ChAc *in vitro*

On the basis of these control experiments the following experiment was conducted:-

10 Beckmann microfuge tubes each containing 100 μl of HC-3 (500mM), 100 μl of buffer containing 45 μM of $[^{14}\text{C}]$ -AcCoA and 50 μl of partially purified ChAc were incubated at 37 $^{\circ}$ for 1hr.

(1) At the end of the incubation 25 μl was removed from one tube and $[^{14}\text{C}]$ -acetylated HC-3 was detected by the method of Fonnum (1975) (see Methods, Section 3.5.3). The extract gave 18,000dpm at 85% efficiency. The remainder of the contents of this one tube and the contents of four other tubes were added to the top of an Amberlite ion-exchange resin (CG-50 [100-200 mesh]) supported in a 3" pasteur pipette. The resin had been equilibrated with distilled water.

(2) Distilled water (20ml) was then passed through the column. These eluates showed the presence of ChAc and also the presence of unreacted $[^{14}\text{C}]$ -AcCoA which were detected as in the control experiments.

(3) When no more $[^{14}\text{C}]$ -AcCoA was detected in the eluates 1N HCl (100ml) was passed through the column. These eluates gave no precipitate with ammonium reineckate but absorbed in the uv having a $\lambda = 258\text{nm}$. The
1N HCl

amount of radioactivity in 200 μ l samples of these eluates was estimated using NE260 as the fluor and also glass fibre papers and a toluene fluor, but no radioactivity was detected. It was concluded that these eluates contained unreacted HC-3.

(4) When the HCl eluates were found to contain no HC-3, 1N NaOH (5ml) was passed through the column. These eluates contained radioactivity detected as described in paragraph (3). The eluates containing radioactivity were pooled and they absorbed uv light (Fig. 4.20). It was concluded that these eluates contain acetylated HC-3.

(5) This method was repeated for the contents of the other 5 Beckman microfuge tubes using a fresh ion-exchange column.

This experiment was repeated using 5 Beckman microfuge tubes each containing 250 μ l of reaction as described above.

4.3.5.4. Identification of $[^{14}\text{C}]$ -acetylated HC-3 as synthesised by ChAc *in vitro*

The uv spectrum obtained for acetylated HC-3 in 1N NaOH as synthesised *in vitro* by ChAc is shown (Fig. 4.20) and can be compared to the spectrum of HC-3 (Fig. 4.19) and Ac-seco-HC-3 (Fig. 4.18). Most of the acetylated HC-3 appears to be in the cyclic form ($\lambda_{\text{max}} = 258\text{nm}$) (Fig. 4.20) although the uv record seems to indicate the presence of some open-chain acetylated HC-3 by a λ_{max} at 278nm.
1N NaOH

It is theoretically possible to estimate whether the $[^{14}\text{C}]$ -acetylated HC-3 is momacetylated or biacetylated.

In each experiment

25 μ l of incubation mixture yielded acetylated HC-3 approximately equivalent to 18,000dpm

4 tubes containing 250 μ l should give 720,000dpm

1 tube containing 225 μ l should give 162,000dpm

882,000dpm

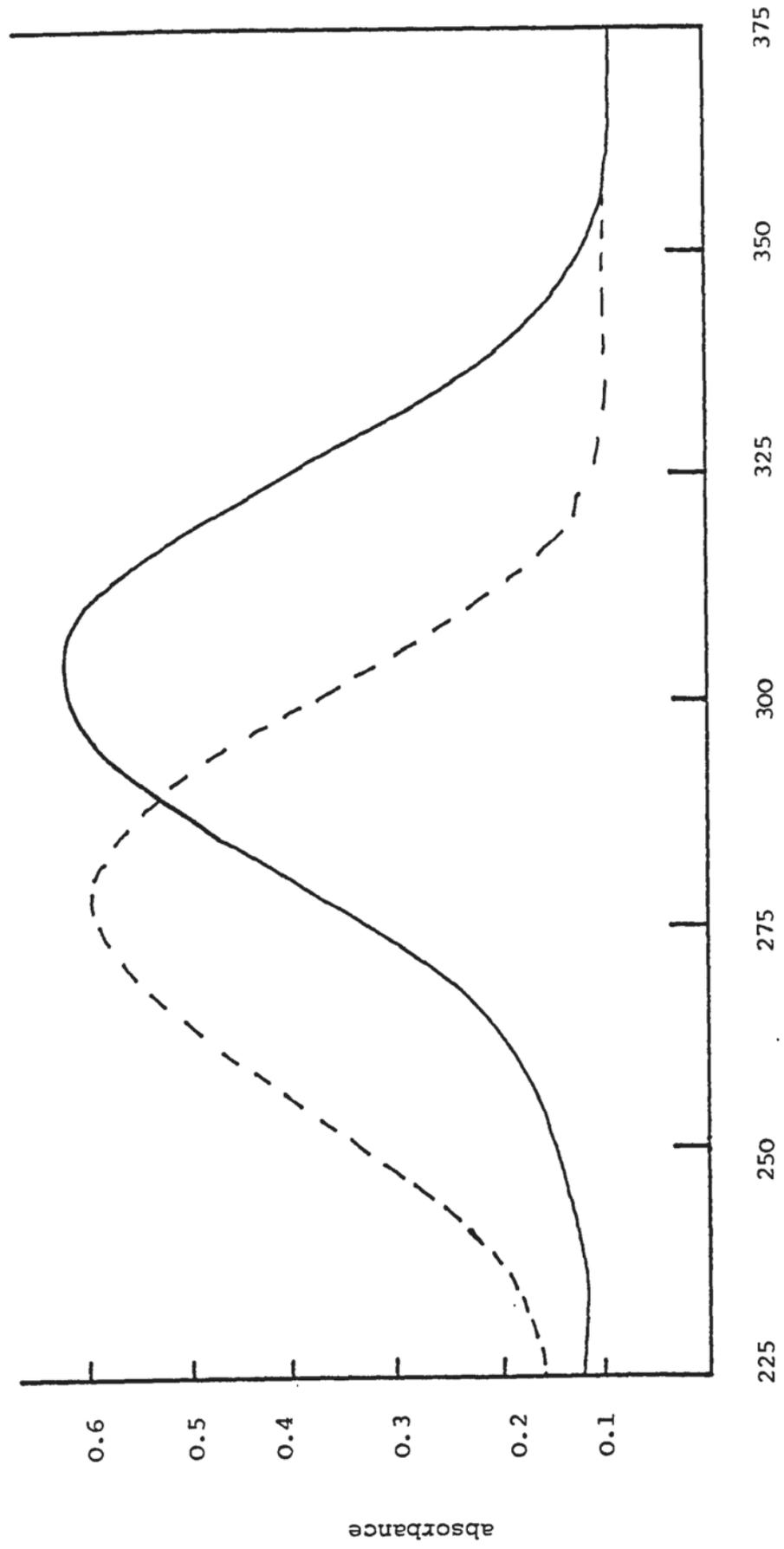


Fig. 4.18:- uv spectrum of Ac-seco-HC-3 (14) — pH7.0, - - - - - in 1N NaOH after 7 days at room temperature

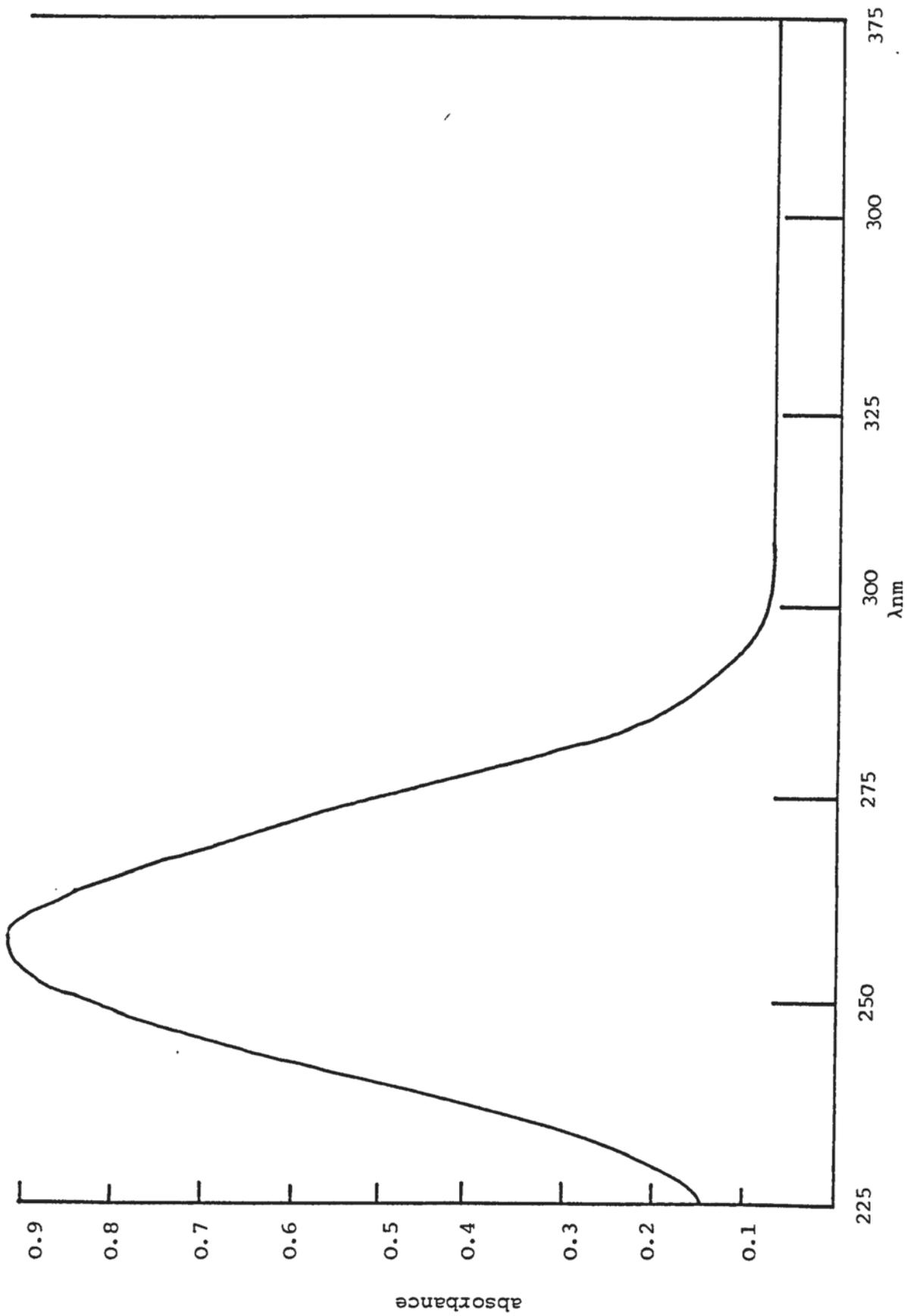


Fig. 4.19:- uv spectrum of HC-3 in water (pH7.0) and in 1N NaOH.

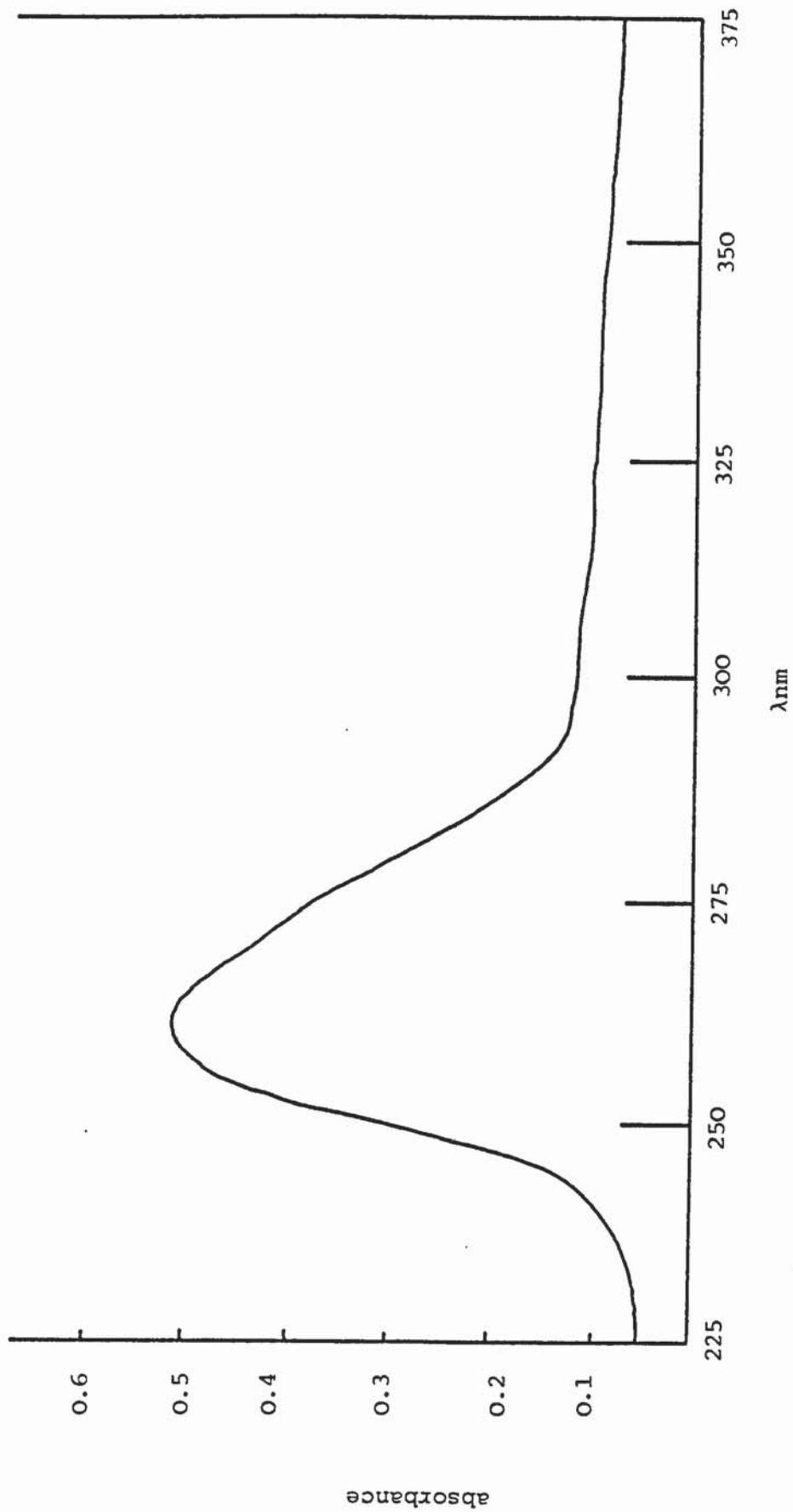


Fig. 4.20:- uv spectrum of acetylated HC-3 as synthesised by ChAc *in vitro* in 1N NaOH.

$[^{14}\text{C}]\text{-AcCoA}$ has a specific activity of $59.5\mu\text{Ci}/\mu\text{mole}$ i.e. $131 \times 10^6 \text{dpm}/\mu\text{mole}$.

a) If 1 molecule of HC-3 combines with 1 molecule of $[^{14}\text{C}]\text{-AcCoA}$ to give monoacetylated HC-3 then

$$\begin{aligned} 882,000\text{dpm} &= \frac{882,000}{131 \times 10^6} \mu\text{mole} \\ &= 0.67 \times 10^{-2} \mu\text{mole} \text{ of monoacetylated HC-3} \end{aligned}$$

M.Wt. of monoacetylated HC-3 dibromide = 616

$$\therefore 0.67 \times 10^{-2} \mu\text{mole} = 4.13\mu\text{g of monoacetylated HC-3}$$

b) If 1 molecule of HC-3 combines with 2 molecules of $[^{14}\text{C}]\text{AcCoA}$ to give biacetylated HC-3 then there would be half the number of μmole of product compared to a), = $0.34 \times 10^{-2} \mu\text{mole}$ of biacetylated HC-3.

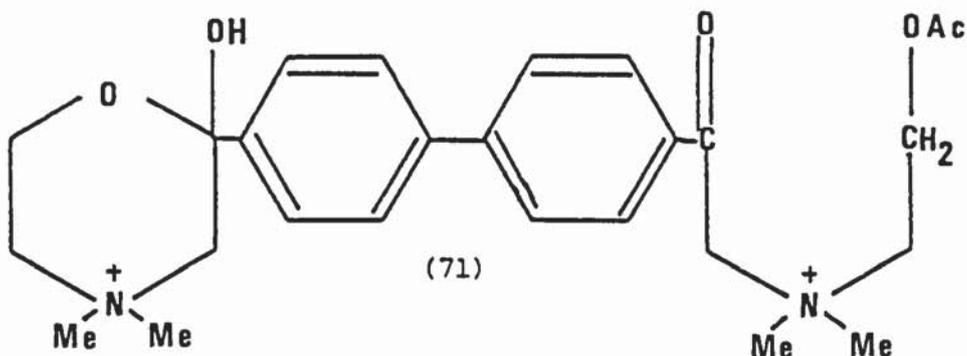
M.wt. of biacetylated HC-3 dibromide = 658

$$\therefore 0.34 \times 10^{-2} \mu\text{mole} = 2.23\mu\text{g of biacetylated HC-3}$$

As 5mls of NaOH was used to elute the acetylated HC-3 from the column, this would give $4.1\mu\text{g}/5\text{mls}$ ($0.82\mu\text{g}/\text{ml}$) for monoacetylated HC-3 and $2.2\mu\text{g}/5\text{mls}$ ($0.44\mu\text{g}/\text{ml}$) for the biacetylated HC-3

From control experiments the amount of absorption of uv at 258nm by the $[^{14}\text{C}]\text{acetylated HC-3}$ (Fig. 4.20), isolated from 5 tubes each containing 250 μl of incubation medium is equal to the absorption of HC-3 at 258nm at a concentration $6.5\mu\text{g}/\text{ml}$. Therefore the maximum theoretically predicted yield of $0.82\mu\text{g}/\text{ml}$ is insufficient to give the yield practically obtained as shown by the uv absorption spectrum (Fig. 4.20). But because of this high yield it seems more likely that the acetylated HC-3 is monoacetylated.

As it is likely that the acetylated HC-3 is monoacetylated it is possible that the structure of acetylated HC-3 is as shown below (71)



This structure would have a λ_{max} between 258-278nm. However the λ_{max} of acetylated HC-3 (Fig. 4.20) ^{1N NaOH} are 258 and 278nm, indicating that the ^{1N NaOH} acetylated molecules are either totally in the cyclic or open-chain forms.

Open-chain Ac-seco-HC-3 has been shown to be stable in 1N NaOH at room temperature for at least seven days. Therefore the absorption at 258nm is unlikely to be due to HC-3 formed as a breakdown product of any open-chain acetylated HC-3. It is possible that a cyclic form of acetylated HC-3 might be more susceptible to hydrolysis by 1N NaOH however, this should make no difference to the uv spectrum since the chromophore is presumably the same.

Ac-seco-HC-3 is not degraded by 1N HCl to HC-3 since in control experiments it could be detected in large amounts in 1N NaOH after the HCl had eluted all the HC-3 from the column. Similarly [¹⁴C]-acetylated HC-3 as synthesised by ChAc was not eluted by 1NHCl. It was only eluted when 1N NaOH was passed through the column showing that it had different binding properties as compared to HC-3.

4.3.5.5 Binding properties of HC-3 and acetylated HC-3 to the Amberlite ion-exchange resin

Apparently open-chain Ac-seco-HC-3 has similar binding properties with regard to the Amberlite resin as the cyclic acetyl-HC-3 synthesised by ChAc.

Amberlite CG-50 is a weakly acidic cation exchange resin and is commonly used for the separation of closely similar materials. This resin is a methacrylic acid polymer and the reacting group is analogous to acetic acid: RCO_2H where R represents the non-mobile part of the ion-exchange resin. All chemical reactions taking place in ion exchanger are reversible reactions. On dissociation of the functional groups into RCO_2^- and H^+ the protons are not attached permanently to the functional group but move about freely within the structure of the swollen grains of the resin and may be regarded as being distributed within the free space of the resin. The protons can be exchanged for an equivalent amount of ions of like charge. An equilibrium will be set up between the protons in the resin and, in the experiment described here, between the quaternary nitrogens of the HC-3 and acetylated HC-3 molecules which are applied to the resin in an aqueous solution. The position of this equilibrium depends on the relative affinities of the ions for the resin and on the relative concentrations of the ions. Ions of low affinity for the resin, if present in high enough concentration, can replace ions of higher affinity already bound to the resin. However, the affinity (or exchange potential) of the ions in dilute aqueous solution at room temperature depends mainly on the size of their charge and increases with it; for ions of the same charge the affinity is inversely proportional to the effective ionic radius (i.e. the radius of the hydrated ion as measured by the Debye-Hückel ion parameter). The effective radius of many cations is inversely proportional to their atomic weight. Therefore the affinity is directly proportional

to the atomic weight.

Therefore according to these rules HC-3 and acetylated HC-3, because of the larger size of their charge (two quaternary nitrogens) and their larger atomic weights, will have a greater affinity for the resin than the protons and HC-3 and acetylated HC-3 will therefore replace the protons and bind to the resin. 1N HCl presumably contains protons in sufficiently high concentration to replace HC-3 very slowly.

Acetylated HC-3 whether cyclic or open-chain, due to its greater affinity for the resin compared to HC-3, presumably because of its larger atomic weight is not replaced by this concentration of HCl. However, 1N NaOH because of the high concentration of sodium ions will replace acetylated HC-3. Under the conditions of the experiment, therefore, sodium ions have a greater affinity for the resin compared to protons possibly due to their larger atomic weight.

4.3.6 Transport of [³H]-choline and [¹⁴C]-HC-3 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 and Snyder, 1972, 1973).

4.3.6.1 Inhibition of high affinity choline transport by the hemicholinium compounds

A crude preparation of synaptosomes, isolated from rat brain, was incubated with [³H]-choline (1 μ M), 0.55 μ Ci/nmole) at 37 $^{\circ}$ and 0 $^{\circ}$. Choline transport into synaptosomes at 37 $^{\circ}$ was time dependent, being linear for 7min, (Fig. 4.21). The amount of choline accumulated by synaptosomes at 37 $^{\circ}$ for 7min was 4-6 times greater than that accumulated at 0 $^{\circ}$ for 7min. Values obtained for choline transport at 37 $^{\circ}$ were corrected by subtracting

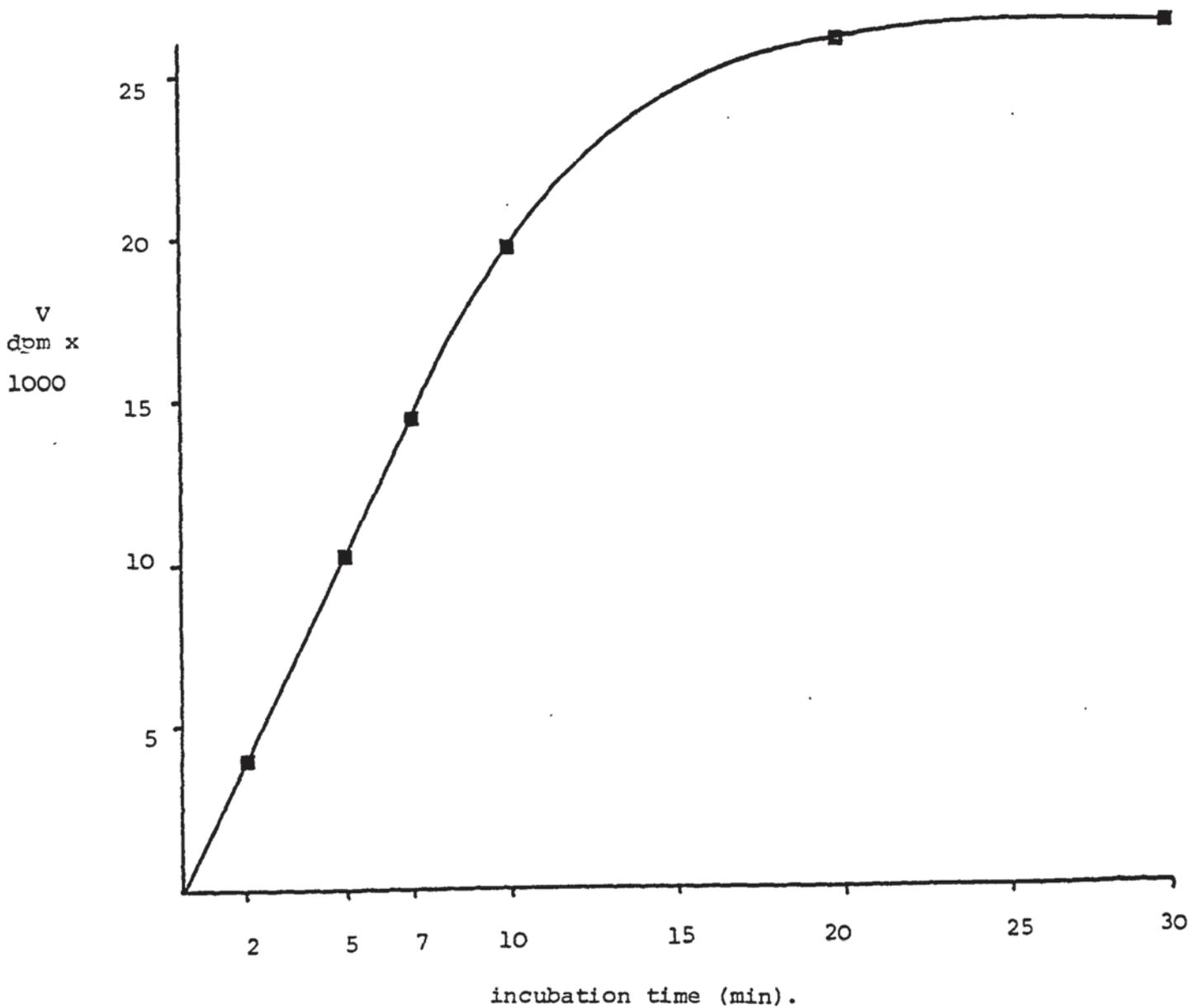


Fig. 4.21:- Velocity/time curve for the transport of $[^3\text{H}]$ -choline ($1\mu\text{M}$) into synaptosomes at 37° . Each point is the mean of 4 determinations. \pm SEM were 10% or less. Blank readings (incubation at 0° for 2,5,7,10,20 and 30min) were subtracted from the appropriate values.

the values obtained from the incubation at 0°.

The inhibition of the hemicholinium compounds on choline transport was measured by adding the compounds to the buffer containing [³H]-choline (1μM) and synaptosomes and incubating at 37° for 7min. The concentration of hemicholinium compound inhibiting the transport of choline into synaptosomes by 50% (IC₅₀) (Table 4.5) was determined graphically from a Dixon plot (1953), and one such plot is shown for 3CHC (Fig. 4.22), of the hemicholinium compounds studied 3CHC was found to be the most potent inhibitor of choline transport, being five times more potent than HC-3. 4CHC was about four times and 2CHC about twice as potent as HC-3. However, HC-3 was about five times more potent than 1CHC and about 300 times more potent than methylmorpholinium. The IC₅₀ of methylmorpholinium was 30 times the concentration of choline.

4.3.6.2 Transport of [¹⁴C]-HC-3 into synaptosomes

Since HC-3 was acetylated *in vitro* by ChAc it was decided to investigate as to whether it can be accumulated by synaptosomes and acetylated *in situ*. [¹⁴C]-HC-3 was prepared as described in the Chemistry section of this chapter (Section 4.2.). [¹⁴C]-HC-3 was in a solution of NaCl (1M) such that 500μl of this solution contained 1.34nm/0.16μCi of [¹⁴C]-HC-3 and 29.25mg of NaCl. Krebs Ringer Phosphate buffer was used routinely as the incubating medium for synaptosome experiments in this study and this solution contains 20.45mg of NaCl and about 1mg of sodium as sodium phosphate in 2mls. In order to compensate for the NaCl in the solution containing [¹⁴C]-HC-3 which was added to the incubating medium, a modified buffer nominally called Tris-Krebs buffer was used. Tris-Krebs buffer differed from Krebs Ringer Phosphate buffer in that it contained no sodium ions and a reduced amount of chloride ions. Therefore the incubating medium which was used in the experiments which determined the rate of transport of

Inhibitor	IC ₅₀ μM
HC-3	0.097
1CHC	0.45
2CHC	0.048
3CHC	0.018
4CHC	0.027
methylmorpholinium	30
Ac-seco-HC-3	0.020
HC-15	6.5

TABLE 4.5: Inhibition of the high-affinity transport of choline into synaptosomes. The IC₅₀ values were determined graphically from Dixon plots (1953). For each plot a regression line with a correlation coefficient of 0.99 was drawn through at least five points. Each point was the mean of at least four determinations ± SEM of less than 10%. The synaptosomes were incubated with [³H]choline (1 μM) and inhibitor for 7min at 37°C. Incubation of the synaptosomes with [³H]choline (1 μM) at 0°C for 7min gave the blank readings.

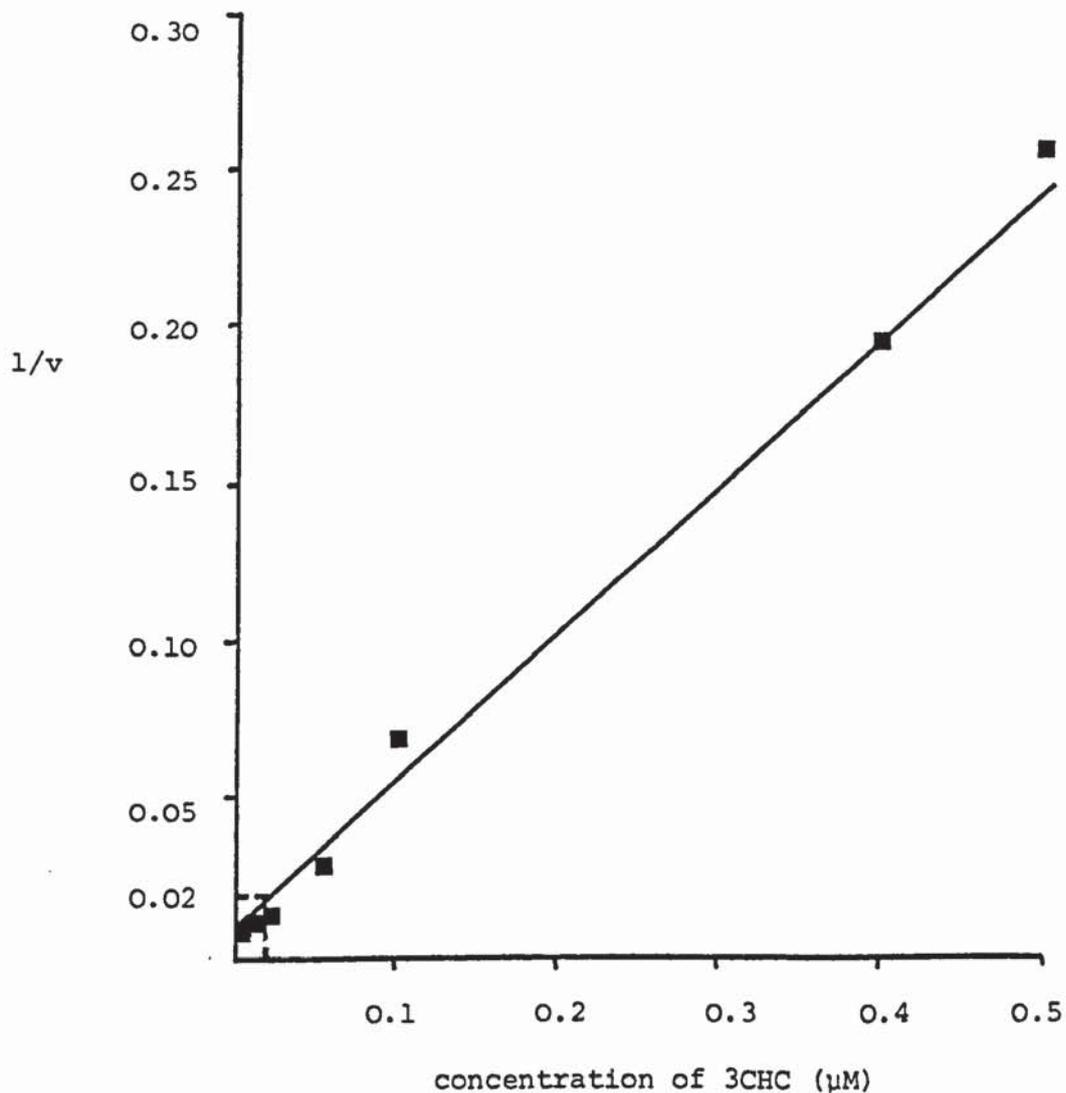


Fig. 4.22:- Dixon (1953), plot of the inhibition of the high affinity transport of choline into synaptosomes by 3CHC. v is the % uptake of choline compared to control (no inhibitor). Each point is the mean of at least 4 determinations. \pm SEM of 10% or less. The correlation coefficient is 0.996. The synaptosomes were incubated with $[^3\text{H}]$ -choline ($1\mu\text{M}$) and inhibitor (0.005 - $0.5\mu\text{M}$) for 7min. at 37° .

[^{14}C]-HC-3 into synaptosomes was prepared by adding 500 μl of the [^{14}C]-HC-3 in NaCl (1M) to the synaptosomes which were suspended in Tris-Krebs buffer.

4.3.6.2.1 Verification of the modified incubation medium

To ensure that this modified incubation medium was a suitable environment in which to measure the transport of [^{14}C]-HC-3 into synaptosomes, the transport of [^3H]-choline was determined in this medium.

[^3H]-choline (0.5 μM , 0.55 $\mu\text{Ci/nmole}$) was incubated in a medium containing synaptosomes, NaCl (500 μl of a 1M solution) and non-labelled choline (0.5 to 99.5 μM ; giving final choline concentrations of 1 to 100 μM) made up to 2mls with Tris-Krebs buffer. The amount of choline accumulated by the synaptosomes was calculated taking into consideration the specific activity of [^3H]-choline at the various concentrations used. It was expressed as μmole of choline transported into synaptosomes/g of protein/7 min. The accumulation of choline by synaptosomes at 37 $^{\circ}$ was about three times that at 0 $^{\circ}$. Values obtained at 0 $^{\circ}$ were subtracted from the values obtained at 37 $^{\circ}$. Choline transport at 37 $^{\circ}$ was shown to be linear for 7min, for the range of concentrations chosen (Fig. 4.23). A Lineweaver-Burke plot was drawn (Fig. 4.24) and the points described a two component transport system. The high affinity transport system had a K_{TH} of 3.4 μM and a V_{max} of 0.15 $\mu\text{mole/g}$ of protein/7min. These values are similar to those found by Yamamura and Snyder (1972, 1973). Hence the incubating medium used in these experiments is suitable for studying the transport of HC-3 into synaptosomes.

4.3.6.2.2 Incubation of synaptosomes with [^{14}C]-HC-3

The incubation medium contained a crude preparation of synaptosomes, [^{14}C]-HC-3 (0.67 μM ; 0.16 $\mu\text{Ci}/1.34\text{nmole}$), non-labelled HC-3 (0.2 μM to 200 μM ;

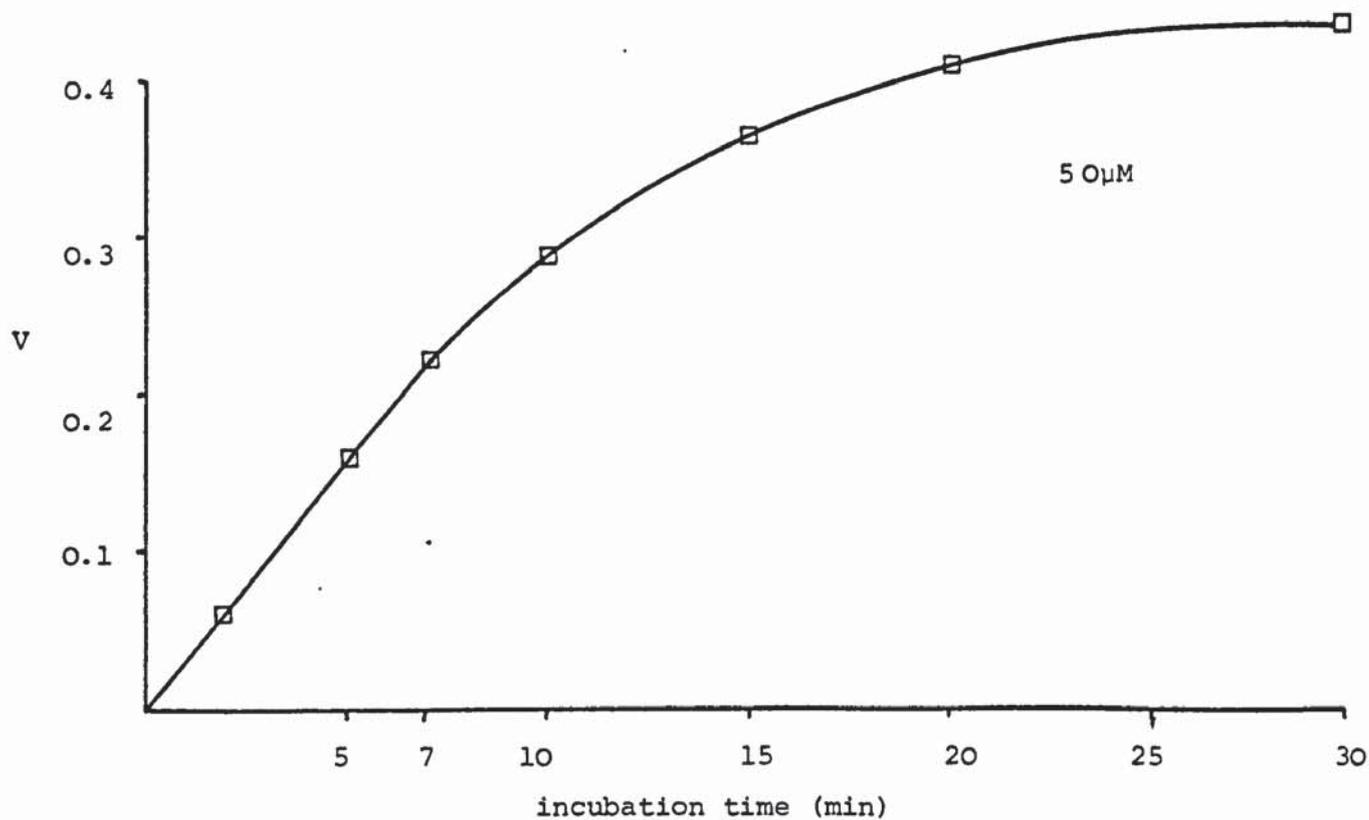
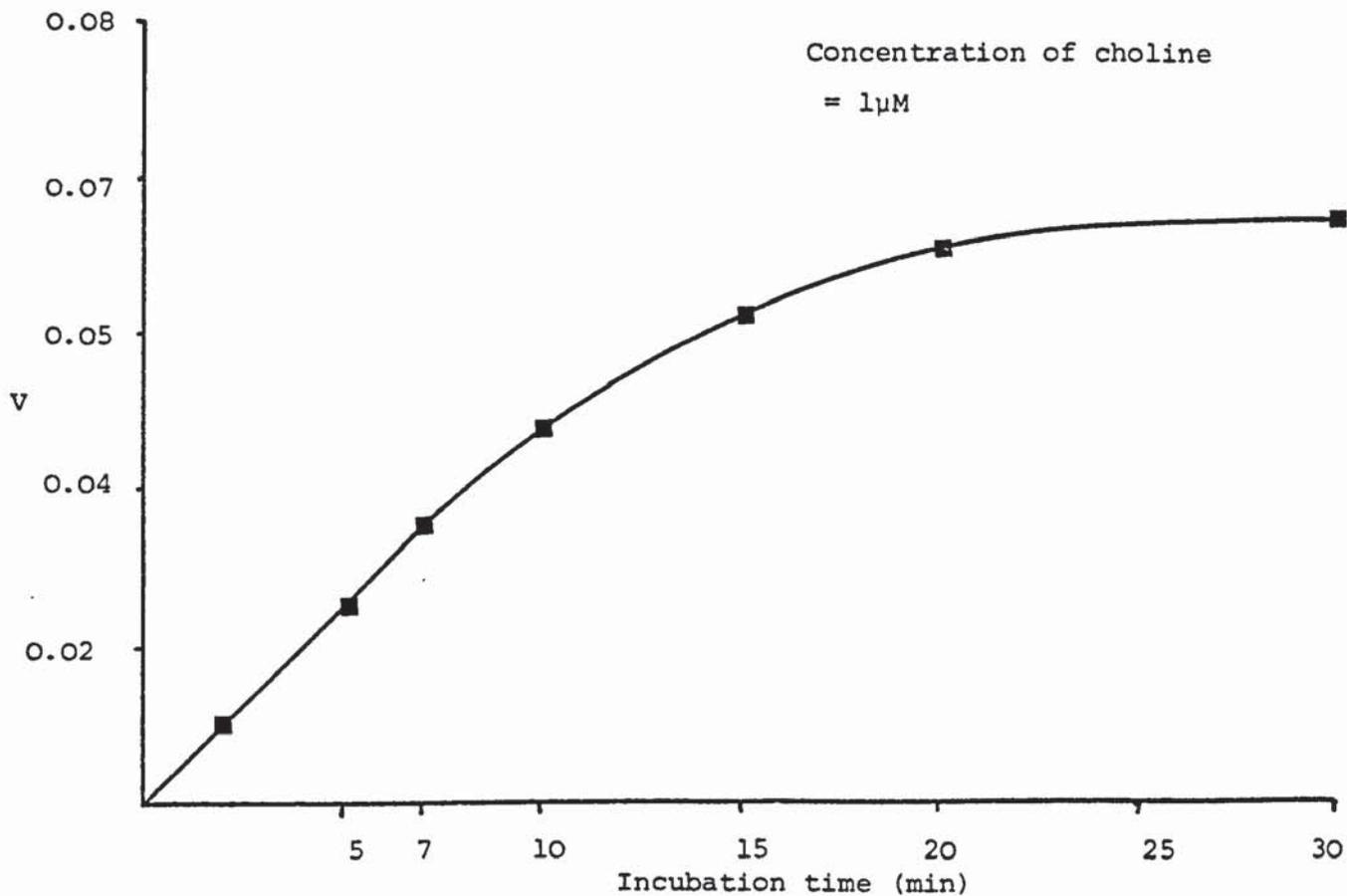


Fig. 4.23:- Velocity/time curve for the transport of $[^3\text{H}]$ -choline into synaptosomes. V is expressed as μmole of choline transported into synaptosomes/g of protein at 37° . Experiments were performed in duplicate and repeated three times. $\pm\text{SEM}$ were less than 10%.

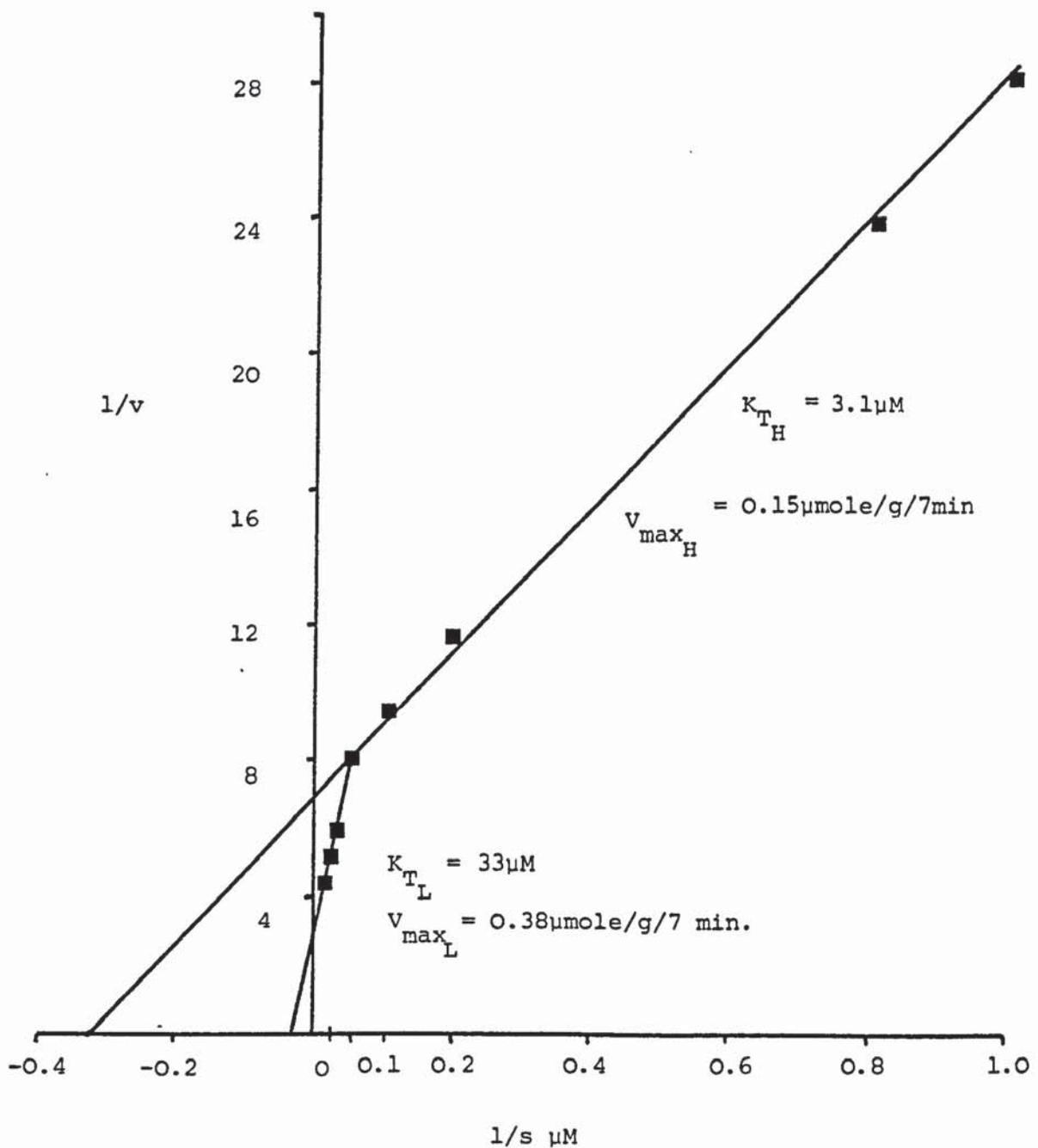


Figure 4.24:- Double reciprocal plot of the transport of ^3H -choline into synaptosomes. Each point is the mean of at least 4 determinations. $\pm\text{SEM}$ less than 10%. K_T is that concentration of substrate which provides one half the maximum velocity of uptake and was calculated for the high affinity (H) and low affinity (L) transport systems using Michaelis-Menten Kinetics. V is expressed as μmole of choline accumulated/g of protein in 7min. Synaptosomes were incubated at 37° for 7min. S is the concentration of choline.

giving final concentrations of HC-3 of 0.87 μ M to 200.67 μ M) and NaCl (29.25mg) made up to 2mls with Tris-Krebs buffer. The amount of HC-3 accumulated by the synaptosomes was calculated taking into consideration the specific activity of [14 C]-HC-3 at the various concentrations used. It was expressed as μ mole of HC-3 transported into the synaptosomes/g of protein/7mins. The accumulation of HC-3 by synaptosomes at 37 $^{\circ}$ was about twice that at 0 $^{\circ}$. Values obtained at 0 $^{\circ}$ were subtracted from the values obtained at 37 $^{\circ}$. HC-3 transport was shown to be linear for 7min, for the range of concentrations chosen (Fig. 4.25). A Lineweaver-Burke plot was drawn (Fig. 4.26) from the results of six experiments. It was possible to draw one straight line through all of the points with a correlation coefficient of 0.9914. The K_T of transport of HC-3 into synaptosomes was 52.2 μ M and the V_{max} was 0.224 μ mole/g of protein/7min. This represents low affinity transport of HC-3.

The effect of some metabolic inhibitors and choline on the transport of HC-3 is shown in Table 4.6. It can be seen that choline at 16 times the concentration of HC-3 inhibits the transport of HC-3 by about 50% and since HC-3 inhibits the transport of choline, as discussed in this Chapter (Section 4.3.6.1), these results indicate that HC-3 is transported by the same mechanism as choline. Metabolic inhibitors, KCN and DNP inhibit the transport of HC-3 to some extent indicating that the transport might be an energy requiring process. Ouabain was found to be a weak inhibitor of HC-3 transport.

It was not possible to determine the transport of HC-3 at a reduced sodium concentration since [14 C]-HC-3 was in an environment of high sodium levels in the form of a NaCl solution (1M)

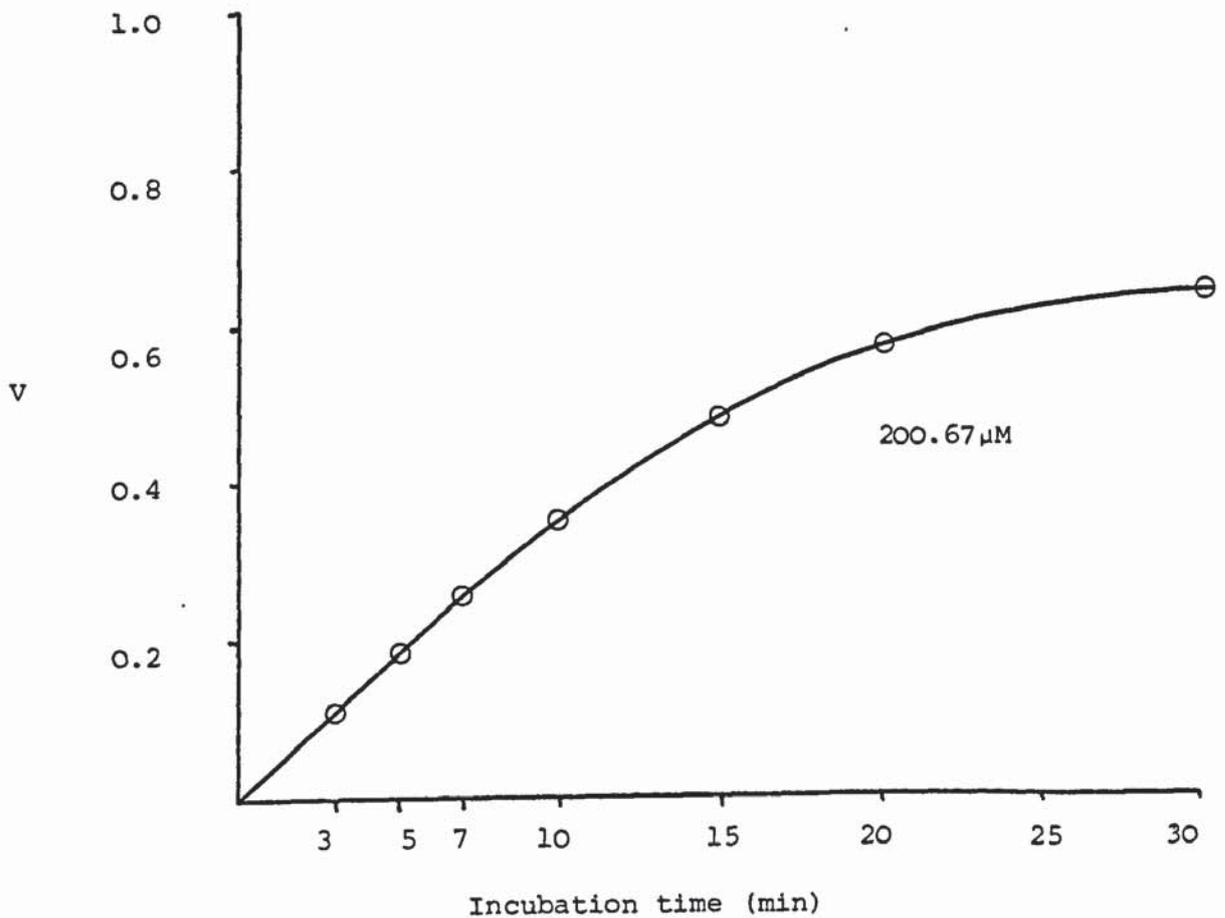
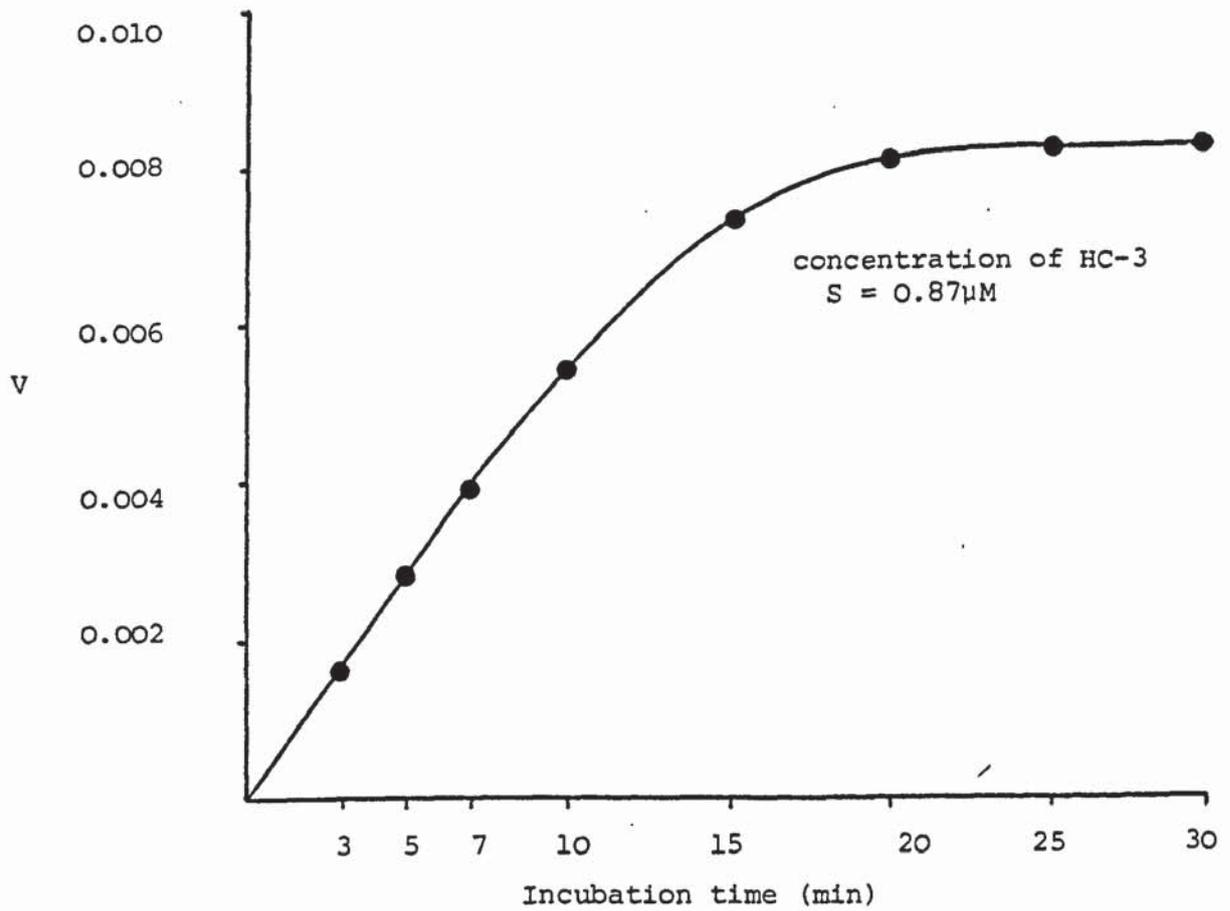


Fig. 4.25:- Velocity/time curve for the transport of $[^{14}\text{C}]$ -HC-3 into synaptosomes. V is expressed as μmole of HC-3 transported into synaptosomes/g of protein at 37° . Each point is the mean of five determinations. $\pm\text{SEM}$ of 10% or less.

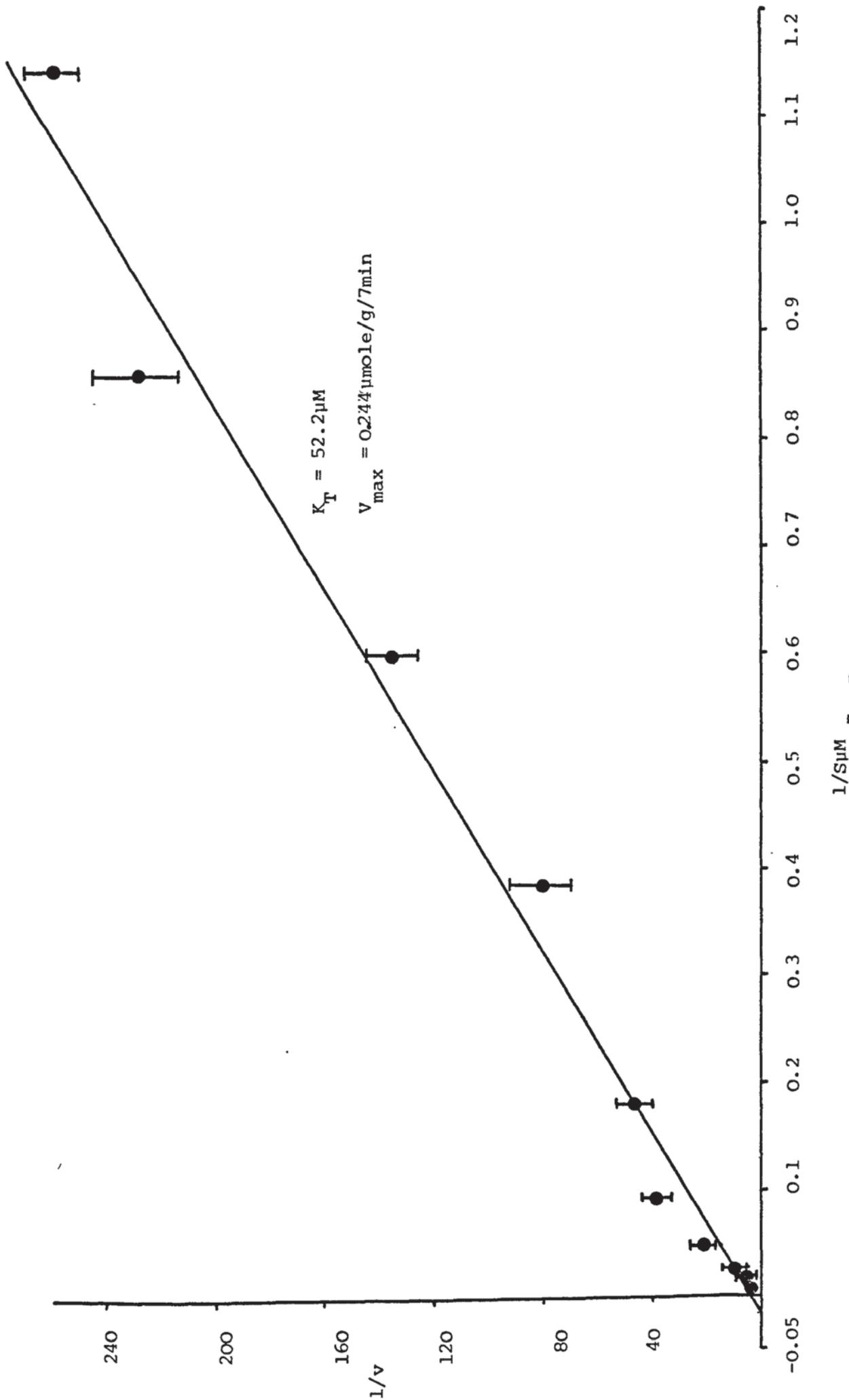


Fig. 4.26:- Double reciprocal plot of the transport of $[^{14}\text{C}]$ -HC-3 into synaptosomes. Each point is the mean of six determinations (\pm SEM). K_T is that concentration of substrate which provides one half the maximum velocity of uptake and was calculated using Michaelis-Menten Kinetics. V is expressed as $\mu\text{mole of HC-3 accumulated/g of protein in 7min}$. Synaptosomes were incubated at 37° for 7min. S is the concentration of HC-3.

Inhibitors		<u>Inhibition</u> (Percent of control)
DNP	200 μ M	44%
KCN	100 μ M	97%
Ouabain	200 μ M	15%
Choline	100 μ M	53%

TABLE 4.6: Effect of some inhibitors on the transport of [14 C]-HC-3 into synaptosomes. Synaptosomes was incubated with [14 C]-HC-3 (6.4 μ M) at 37 $^{\circ}$ for 7min . Results are expressed as percentage inhibition of uptake compared to control value (incubation in the absence of inhibitor) of 0%. Values are the mean of four determinations. \pm SEM were less than 10%. The experiment was replicated twice. Blank readings (incubation at 0 $^{\circ}$ for 7min . in the absence of inhibitor) were subtracted from all values.

4.3.6.3 Identity of radioactive materials accumulated by synaptosomes incubated with $[^{14}\text{C}]$ -HC-3

In these experiments a crude preparation of synaptosomes was incubated with $[^{14}\text{C}]$ -HC-3 (2.67 μM) at 37 $^{\circ}$ for 7min as described above. (Section 4.3.6.2.2), additional experiments involving higher concentrations of HC-3 were not conducted due to a shortage of $[^{14}\text{C}]$ HC-3. After incubation, the contents of the synaptosomes were extracted and non-labelled HC-3 was added to this extract. The compounds now present in the extract were separated by paper electrophoresis. The band corresponding to HC-3 was visualised with iodine vapour. The iodine was then allowed to evaporate off and the electrophoresis paper was cut into 1cm strips. The strip corresponding to the visualised HC-3 band showed a peak of radioactivity 7-8cm from the origin (Fig. 4.27). The other strips possessed less than 15% of the total radioactivity detected. Unfortunately this experiment was only conducted twice due to a shortage of $[^{14}\text{C}]$ -HC-3. It is tentatively concluded that $[^{14}\text{C}]$ -HC-3 is not metabolised to any $[^{14}\text{C}]$ -product by synaptosomes during the incubation.

4.4. DISCUSSION

4.4.1 HC-3

4.4.1.1 Structure

HC-3 was synthesised as the dichloride salt. From the spectroscopic data it was concluded that all of the HC-3 molecules in aqueous solution were in the cyclic-hemiacetal form (4) and non were in the open-chain seco form (3). Therefore, the cyclic form (4), must be the more stable tautomer. Hence HC-3 reaches the biological tissues as the cyclic form (4) although it may subsequently open up to the open-chain seco structure (3), on a membrane surface for example, to effect its biochemical action(s). The results showing the stability of cyclic-HC-3 (4) in aqueous solutions

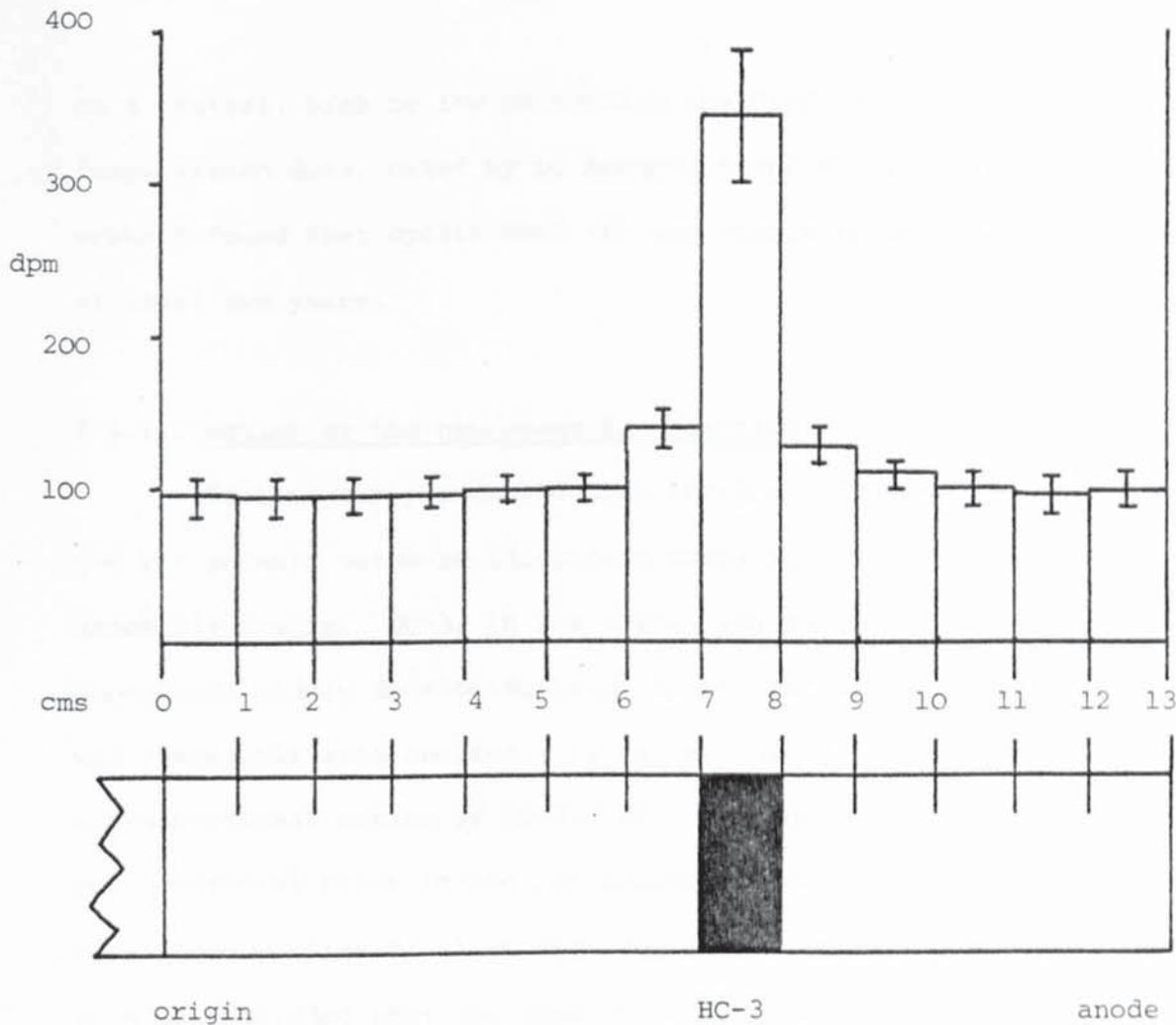


Fig. 4.27:- Paper electrophoresis (for 2hr). following method of Potter and Murphy, (1967) of an extract for synaptosomes that had been incubated with [^{14}C]-HC-3 ($2.67\mu\text{M}$) at 37° for 7min. For each experiment the contents of two incubating tubes were extracted and one half of the pooled extracts was subjected to electrophoresis. The experiment was repeated once. The position of non-labelled HC-3 is shown on the electrophoresis paper as visualised with iodine vapour and the bar graph above shows the radioactivity detected ($\pm\text{SEM}$) on the corresponding paper strips cut from the electrophoresis paper.

at a neutral, high or low pH confirm the findings of Haarstad and Schueler (unpublished data, cited by Di Augustine and Haarstad, 1970). These workers found that cyclic-HC-3 (4) was stable in an aqueous solution for at least two years.

4.4.1.2 Action at the neuromuscular junction

Preliminary pharmacological screening of HC-3 was conducted using the rat phrenic nerve-hemidiaphragm preparation and the frog rectus abdominis muscle. HC-3, in low doses, was shown to give a frequency dependent, slowly developing block in the rat diaphragm preparation which was reversible with choline. It was suggested that this block was due to a prejunctional action of HC-3. HC-3 in high doses, was shown to give a postjunctional block in the rat diaphragm preparation. Similarly HC-3 gave a curare-like block on the frog rectus abdominis muscle. It can therefore be concluded that low doses of HC-3 give a prejunctional block at the neuromuscular junction, whilst higher doses competitively block the nicotinic receptors on the muscle. Similar conclusions were made by Reitzel and Long (1959b) Marshall (1969) and Takagi, Kojima, Nagata and Kuromi (1970).

4.4.1.3 Anticholinesterase activity

HC-3 was shown to possess weak anticholinesterase activity which confirmed the findings of Long and Schueler (1954), Domino, Shellenberger and Frappier (1968), Marshall and Long (1959), Domino, Shellenberger and Frappier (1968) and Hemsworth (1971a). Only the findings of Hemsworth (1971a) are specifically comparable to the results presented in this study, with regard to the substrates used and the IC_{50} values quoted. This study shows that HC-3 is 5 times more potent an inhibitor of AChE and 46 times more potent an inhibitor of ChE than was shown by Hemsworth (1971a). These differences are possibly due to the different methods of assay used.

However, as HC-3 is a weak inhibitor of AChE and ChE when compared to physostigmine for example it is unlikely that its anticholinesterase activity plays any significant part in its neuromuscular blocking action.

4.4.1.4 Mechanism of action of HC-3 in causing a prejunctional block at the neuromuscular junction

Preliminary screening therefore confirmed the pre- and postjunctional action of HC-3 at the neuromuscular junction and its weak anticholinesterase activity. Further study was carried out to try to find the precise site(s) of action of HC-3 which results in the prejunctional block. A presynaptic block by a drug at a cholinergic synapse can be the result of interference at many sites in the neuron as discussed in the Introduction (Section 1.4). However, the specific antagonistic action of choline against the HC-3 induced prejunctional block suggests that *in vivo* HC-3 inhibits the synthesis of ACh by interfering with the supply of choline and/or by competitively inhibiting the reaction of choline with ChAc.

4.4.1.4.1 Inhibition of the supply of choline for ACh synthesis

Impairment of the supply of choline can result from the enhancement of an alternative metabolic pathway at the expense of the pathway involving the synthesis of ACh. Gomez, Domino and Sellinger (1970), found that intraventricular HC-3 reduced the synthesis of ACh but enhanced choline conversion to phosphorylcholine. This indicates that HC-3 diverts free choline away from the synthesis of ACh to the synthesis of phosphorylated choline.

Alternatively, impairment of the supply of choline can result from an inhibition of the transport of choline from extracellular sites to the intracellular sites of acetylation in the neuron. MacIntosh, Birks and Sastry (1958), originally postulated this mechanism of action for HC-3. This hypothesis is supported by many studies which have shown that HC-3 is

a potent inhibitor of the high affinity choline transport system in synaptosomes (Table 1.3). Several workers have shown that HC-3 has a greater affinity for this transport system than choline. The present study has confirmed the potent inhibitory action of HC-3 on the high affinity choline transport system in synaptosomes.

4.4.1.4.2 Inhibition of and acetylation by ChAc *in vitro*

A drug can inhibit the synthesis of ACh by inhibiting ChAc. This study has indicated that HC-3 has little direct inhibitory action on ChAc and this finding agrees with the findings of previous workers (Bhatangar, Lam and McColl, 1965; Hemsworth, 1971a; Domino, Mohrman, Wilson and Haarstad, 1973 and Hemsworth and Cholakis, 1978). However, HC-3 is a choline analogue and it has been shown that it can act as a substrate for ChAc *in vitro* (Table 1.2), and thereby compete with choline for the acetylating enzyme. The present study showed that in the absence of choline HC-3 was acetylated by about 28% compared to choline. This confirms the findings of Rodriguez de LoresArnaiz, Zieher and De Robertis (1970), who showed that HC-3 was acetylated by about 20%, Hemsworth (1971a) who showed that HC-3 was acetylated by about 30% and Bradshaw and Hemsworth (1975) who showed that HC-3 was acetylated by about 27%, by ChAc *in vitro* compared to choline 100%. In the present study the K_m of HC-3 for ChAc *in vitro* was four times the K_m of choline. However, Barker and Mittag (1975), and Mann and Hebb (1975), presented results showing that the K_m of HC-3 for ChAc *in vitro* was ten times the K_m of choline. This difference is possibly due to the different methods of assay used. The acetylation of HC-3 by *in vitro* ChAc is interesting from the point of view of structure/activity relationships. Further study has shown that most of the acetylated HC-3 as synthesised by ChAc *in vitro* is in the cyclic form. Hence, ChAc can presumably align with the "hydroxyl choline moiety" which is fixed in the morpholine ring of HC-3 as indicated in Fig. 4.1. The "hydroxyl choline moiety" is a β -substituted

choline analogue. Hemsworth and Smith (1970a), showed that a D- β -Substituted analogue of choline was acetylated *in vitro* by ChAc to a small extent, that is 8% compared to choline 100%. It seems that the cyclic form of the D- β -substituted choline moiety, as found in the HC-3 molecule, confers some favourable rigidity since HC-3 is acetylated *in vitro* by ChAc by 28%. Since acetylated HC-3, synthesised *in vitro* by ChAc was found to be mostly in the cyclic form, this suggests that it is the cyclic form of HC-3 which is the biochemically active structure.

4.4.1.4.3 False transmitter hypothesis

4.4.1.4.3.1 Transport of HC-3 into cholinergic neurons

The acetylation of HC-3 *in vitro* by ChAc suggests that HC-3 could be acetylated by ChAc *in vivo*, and that the acetylated product could be released as a false transmitter. However, in order for HC-3 to be acetylated *in vivo* it must like choline be transported into the nerve terminal to the intracellular sites of acetylation.

Collier (1973), showed that the cat superior cervical ganglia accumulated [^{14}C]-HC-3, but there was no clear difference between the amount of HC-3 accumulated by stimulated and non-stimulated ganglia, whereas choline transport was enhanced by stimulation. Collier (1973) incubated [^{14}C]-HC-3 (15 μM) with a crude preparation of synaptosomes, derived from mouse brain, at 37 $^{\circ}$ for 30min. Little [^{14}C]-HC-3 was accumulated by the synaptosomes, in fact the accumulation of choline was found to be ten times greater than the accumulation of HC-3, when choline was incubated with the synaptosomes at half the concentration of HC-3. Choline (350 μM) was not found to block the accumulation of HC-3.

However, it must be noted that in Collier's experiments some HC-3, albeit a small amount compared to choline, was transported into the cholinergic ganglia and synaptosomes. HC-3 is a bis-quaternary compound

and this charged molecule would not be expected to pass across the nerve membrane by simple diffusion. Therefore any membrane transport of HC-3 is most likely to be due to some kind of mediated diffusion perhaps similar to the transport mechanism of choline.

In contrast to the work of Collier (1973), the present study investigated the transport of HC-3 into synaptosomes at initial rates and using a range of concentrations of HC-3, so that the K_T and V_{max} values could be calculated. HC-3 was shown to be transported into synaptosomes by a low affinity process. A high concentration of choline (100 μ M) was found to inhibit the transport of HC-3 by about 50% and this result disagrees with the findings of Collier (1973). The inhibitory effect of choline suggests that HC-3 is transported by the choline transport system. The V_{max} for the transport of HC-3 was 59% that of the low affinity transport of choline into synaptosomes and the affinity of HC-3 for the low affinity transport mechanism was approximately 1.5 times less than that of choline. KCN and DNP significantly inhibited the transport of HC-3 indicating that HC-3 is transported by some energy requiring process.

Sellinger, Domino, Haarstad and Mohrman, (1969), and Slater and Stonier (1973), also presented work which suggested that HC-3 could be transported into cholinergic neurons.

4.4.1.4.3.2 Acetylation of HC-3 in cholinergic neurons

As it has been shown that HC-3 can be accumulated by nerve ending particles, it is consequently possible that HC-3 could be acetylated *in vivo* by ChAc. However, in the two experiments conducted in this study there was no evidence of any acetylation of HC-3 by synaptosomes, that is *in situ*. This suggests that HC-3 does not cause a presynaptic block at cholinergic neurons by directly competing with choline for ChAc and thereby inhibiting the synthesis of ACh *in vivo*. The lack of acetylation of HC-3 in synaptosomes also suggests that the presynaptic block is not due to the

release of an acetylated false transmitter. This conclusion agrees with the work of Collier (1973), who did not observe any increase in the efflux of radioactivity on electrical stimulation of ganglia which had been pre-loaded with $[^{14}\text{C}]\text{-HC-3}$. Collier concluded that HC-3 appeared not to act as, or to form, a false cholinergic transmitter.

It has been suggested that the high affinity transport system for choline is coupled to the acetylation process in cholinergic neurons (for a review see Haubrich and Chippendale, 1977). If this is the case, and as HC-3 is transported into synaptosomes by a low affinity process only, then this could explain why no acetylated HC-3 was detected in the present experiments.

4.4.1.5 Summary of results

In summary, therefore, HC-3 has a postjunctional curare-like block on the frog rectus abdominus muscle, weak anticholinesterase activity, a prejunctional block in the rat phrenic nerve-hemidiaphragm preparation, is acetylated *in vitro* by ChAc to predominately the cyclic form of acetylated HC-3, does not inhibit ChAc *in vitro*, inhibits the high affinity transport of choline into synaptosomes and is itself accumulated by synaptosomes. There is no evidence for the *in situ* acetylation of HC-3. The results indicate that the prejunctional block of HC-3 is due to the inhibition of the transport of choline into the cholinergic neuron.

4.4.2 Some diphenylalkane analogues of HC-3 and a methyl analogue of HC-15

4.4.2.1 Structure

1CHC, 2CHC, 3CHC and 4CHC were all synthesised as dichloride salts. From the spectroscopic data it was concluded that all of the molecules in aqueous solutions were in the cyclic hemiacetal form (7, 46-48). Therefore these analogues are similar to HC-3 in that the cyclic hemiacetal form is the most stable tautomer. Similarly methylmorpholinium, as a solid,

appears to be in the cyclic form (49). It was not possible to determine the precise structure of this compound in aqueous solutions as it does not absorb in the uv.

4.4.2.2. Action at the neuromuscular junction

Preliminary pharmacological screening of the hemicholinium analogues using the rat phrenic nerve-diaphragm preparation showed that at low doses like HC-3, these compounds caused a frequency dependent slowly developing block in the rat diaphragm preparation which was reversible with choline. It was concluded that this block was due to a prejunctional action of the analogues. In larger doses, 1CHC, 2CHC, 3CHC and 4CHC caused a post-junctional block on the rat diaphragm preparation. No such postjunctional block was seen with methylmorpholinium, up to a dose of 10 μ mole/ml. Similarly 1CHC, 2CHC, 3CHC and 4CHC caused a curare-like block on the frog rectus abdominis muscle. It can therefore be concluded that lower doses of the hemicholinium analogues gave a prejunctional block whilst with the exception of methylmorpholinium higher doses competitively blocked the nicotinic receptors on the muscle. Hence, 1CHC, 2CHC, 3CHC and 4CHC are similar to HC-3 in that they gave both a pre- and postjunctional block, at the neuromuscular junction. 1CHC, 2CHC, 3CHC and 4CHC were all more potent than HC-3 in their prejunctional blocking action, methylmorpholinium, however, was less potent.

4.4.2.3. Anticholinesterase activity

The hemicholinium analogues, like HC-3, possessed weak anticholinesterase activity. It is unlikely that this weak activity plays any significant part in their neuromuscular blocking action.

4.4.2.4 Mechanism of action of the analogues in causing a prejunctional block at the neuromuscular junction

Preliminary screening has suggested that the analogues have a prejunctional blocking action at the neuromuscular junction. Further study was carried out to try to determine more about the mechanism of action of the hemicholinium analogues which results in the prejunctional block. As with HC-3, it was thought that the specific antagonistic action of choline against the prejunctional block induced by the analogues suggested that the analogues inhibit the synthesis of ACh by interfering with the supply of choline and/or by competitively inhibiting the reaction of choline with ChAc.

4.4.2.4.1 Inhibition of the supply of choline for ACh synthesis

Impairment of the supply of choline can result from the enhancement of an alternative metabolic pathway, for example phosphorylation, at the expense of the pathway involving the synthesis of ACh. However, no work, in this study or in the literature, has investigated this possibility for the hemicholinium analogues studied in this thesis.

Alternatively, impairment of the supply of choline can also result from an inhibition of the transport of choline from extracellular sites to the intracellular sites of acetylation in the neuron. MacIntosh, Birks and Sastry (1958), postulated this mechanism of action for HC-3 and experiments in this study suggest this is a possible mechanism of action for the hemicholinium analogues since they are all inhibitors of the high affinity transport of choline into synaptosomes. It is interesting to observe that Holden, Rossier, Beaujouan, Guyenet and Glowinski (1975), reported two series of bis-quaternary compounds and showed that as the interatomic distance between the two quaternary nitrogens increased, so the potency of inhibition of the high affinity transport of choline into synaptosomes

increased (Table 1.3). No such trend was shown by the bis-quaternary HC-3 analogues studied in this thesis. Methylmorpholinium was a weak inhibitor of choline transport compared to HC-15. This suggests that the phenyl-group in the HC-15 molecule plays a role in this type of inhibition.

4.4.2.4.2 Inhibition of and acetylation by ChAc *in vitro*

A drug can inhibit the synthesis of ACh by inhibiting ChAc. This study has indicated that HC-3 has little direct inhibitory action on ChAc and 1CHC, 3CHC and methylmorpholinium also seem to have little inhibitory activity. But 2CHC and 4CHC appear to significantly inhibit the synthesis of ACh.

The analogues possess four choline moieties, and 3CHC, like HC-3 was acetylated by ChAc *in vitro* by about 28% compared to choline 100%, whereas the other hemicholinium analogues were acetylated by less than 5%. The structure of acetylated 3CHC has not been investigated but it is probably similar to the structure of acetylated HC-3. It was proposed, in the case of HC-3 that the ChAc enzyme aligns with the "hydroxyl choline moiety" (Fig. 4.1.). However, all of the hemicholinium analogues studied have this "hydroxyl choline moiety" but they are all not acetylated. This suggests that in these analogues, the interatomic distance between the two phenyl groups in the molecules of the analogues is important for acetylation. This indicates that both halves of the HC-3 molecule are bound to the ChAc enzyme. There was no evidence of *in vitro* acetylation of methylmorpholinium by ChAc, however, HC-15 is acetylated and this suggests that a phenyl group attached to the morpholine ring is important for the acetylation of the cyclic choline moiety found in the ring.

4.4.2.4.3 Inhibition of and acetylation by ChAc *in vivo*

The acetylation of 3CHC *in vitro* by ChAc suggests that this analogue could be acetylated by ChAc *in vivo*. This could cause a presynaptic

block in two ways. Firstly by competing with choline for ChAc and thus inhibiting the synthesis of ACh and secondly by the release of an acetylated false transmitter. However, 3CHC must be transported into cholinergic neurons for these two mechanisms of action to occur and this has not been investigated.

2CHC and 4CHC directly inhibit ChAc *in vitro* and in this way they could inhibit the synthesis of ACh *in vivo*. However, they must be transported into cholinergic neurons for this to occur and again this has not been shown.

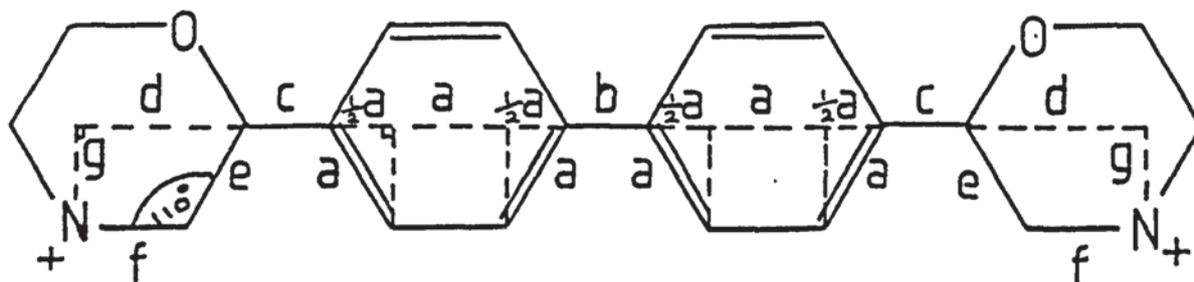
The lack of *in vitro* acetylation of 1CHC, 2CHC, 4CHC and methylmorpholinium does not rule out the possibility that these analogues cannot be acetylated *in vivo* provided they can be transported into the neuron. Since Collier, Lovat, Ilson, Barker and Mittag (1977), found that homocholine was acetylated *in vitro* by ChAc by less than 5% compared to choline 100% but was acetylated at much higher rate *in situ*, to form a false cholinergic transmitter. Similarly it is possible for these hemicholinium analogues to be acetylated *in vivo* and then released as false transmitters. Obviously further experimentation is required.

4.4.2.5 Structure/activity relationships

Bondlengths and bond angles have been taken from Tables of interatomic distances and configuration in molecules and ions (Ed. by Sutton, Jenkin, Mitchell and Cross, 1958). In the case of morpholinium ring in the hemicholinium molecules the bond lengths and angles have been taken from Scheidt, Hanson and Rasmussen (1969), and Bosch and Van Bodegom (1977). All of the bond lengths and angles have been calculated from crystal structures.

The interatomic distance between the two quaternary nitrogens in the HC-3 molecule has been calculated to be approximately 14Å (Fig. 4.28), this agrees with the calculations originally made by Long and Schueler (1954). Since rotation can occur around the 'C' bond it is possible to obtain

$a = 1.397$; $b = 1.50$; $c = 1.52$; $d = 2.00$; $e = 1.54$; $f = 1.479$; $g = 1.44 \text{ \AA}$



(i) Minimum internitrogen distance 14.13 \AA



(ii) maximum internitrogen distance = 14.4 \AA

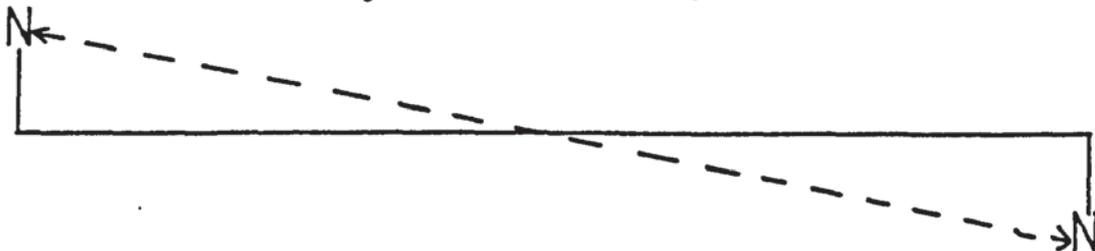
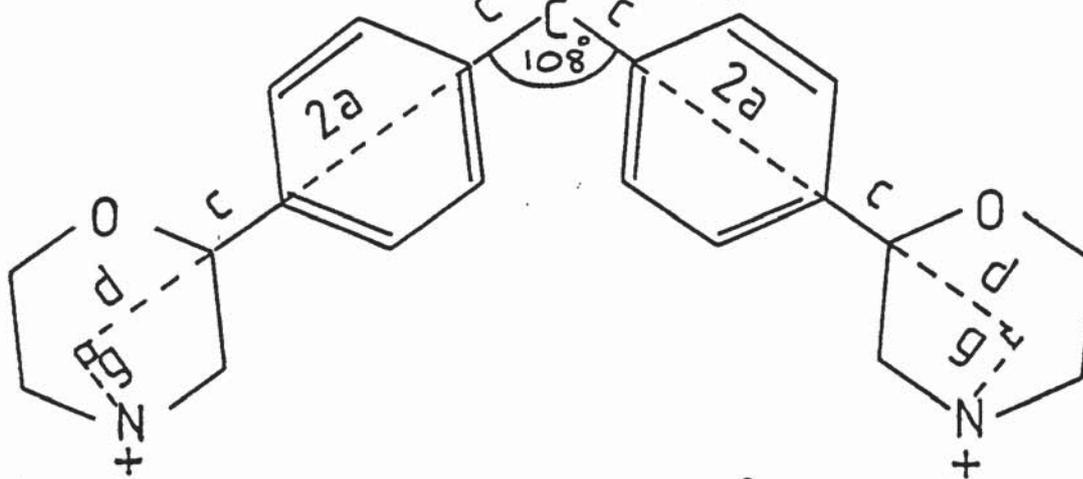
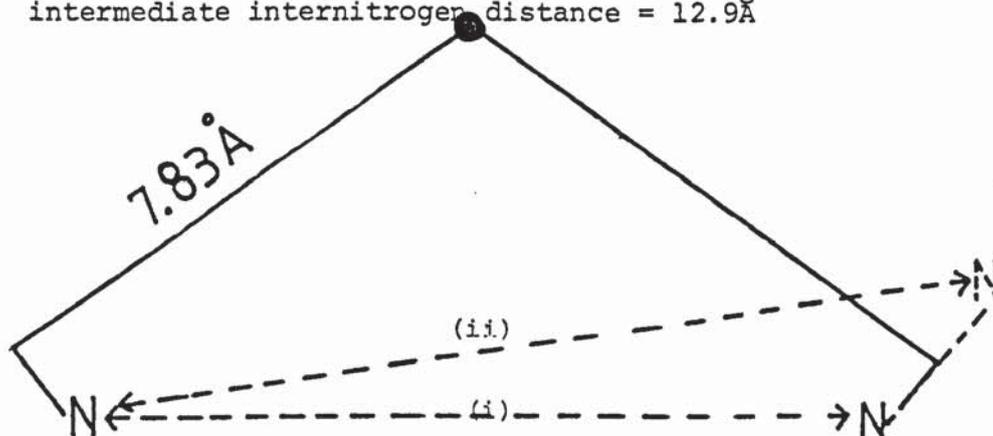


Fig. 4.28:- HC-3

$a = 1.397; c = 1.52; d = 2.00; g = 1.44\text{\AA}$



- (i) minimum internitrogen distance = 11.0\AA
- (ii) intermediate internitrogen distance = 12.9\AA



- (iii) maximum internitrogen distance = 14.4\AA

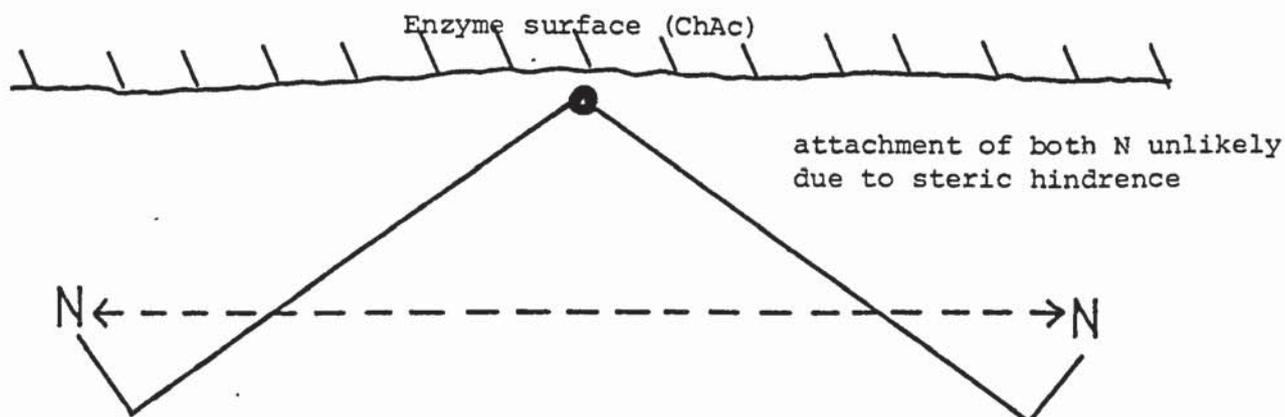
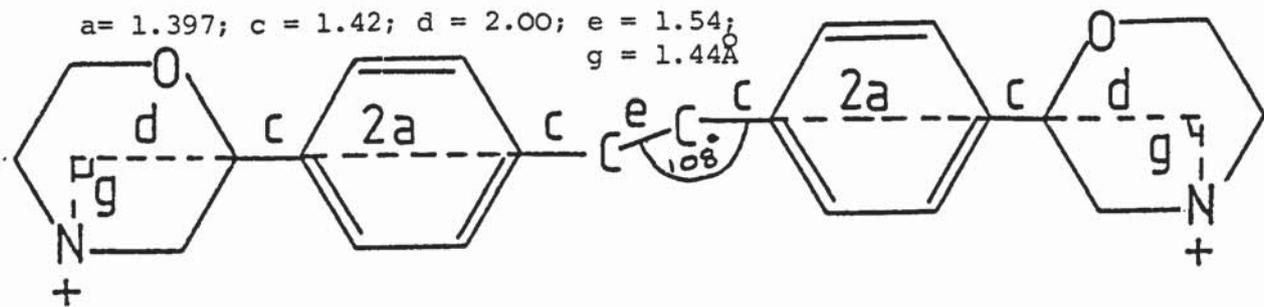
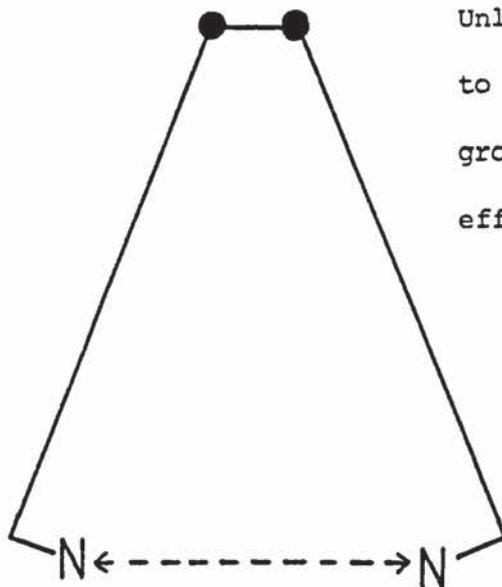


Fig. 4.29:- = 1CHC



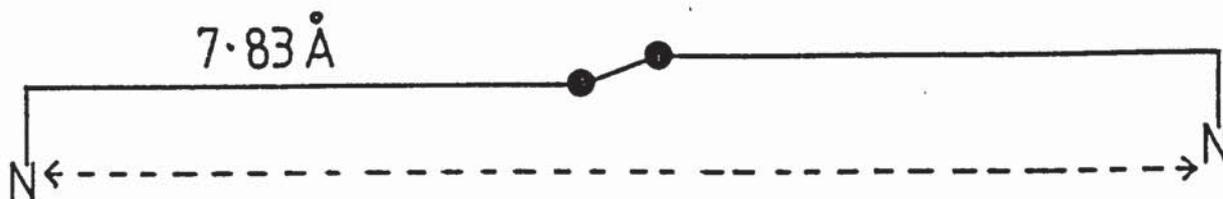
(a) Unstable conformation: minimum internitrogen distance



Unlikely conformation due to closeness of phenyl group (steric and electronic effects) distance = 3.6 \AA

(b) Stable conformation:

(i) minimum internitrogen distance = 16.1 \AA



(ii) maximum internitrogen distance = 16.7 \AA

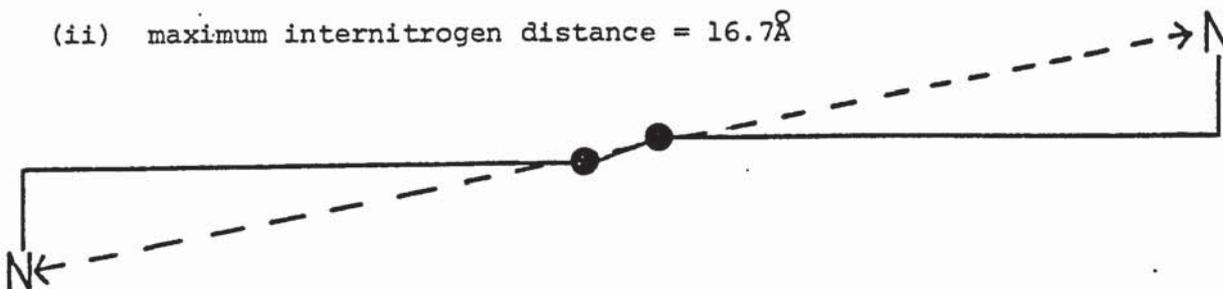
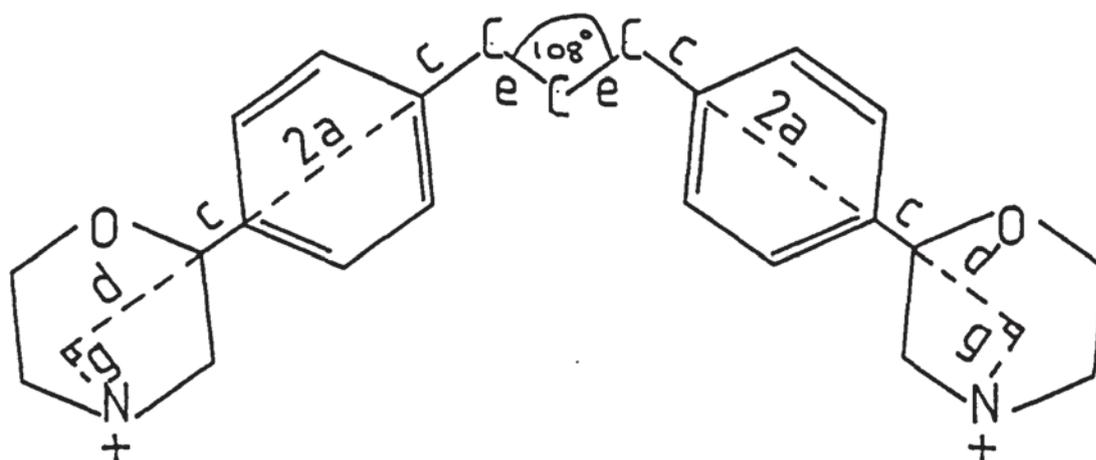


Fig. 4.30: 2CFC

a=1.397; c=1.42; d=2.00; e=1.54; g=1.44Å



(a) Unlikely conformation

due to steric hindrance and electronic repulsion.

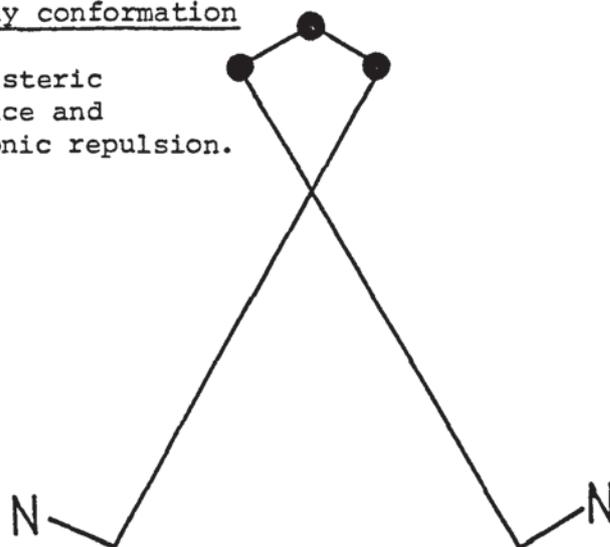
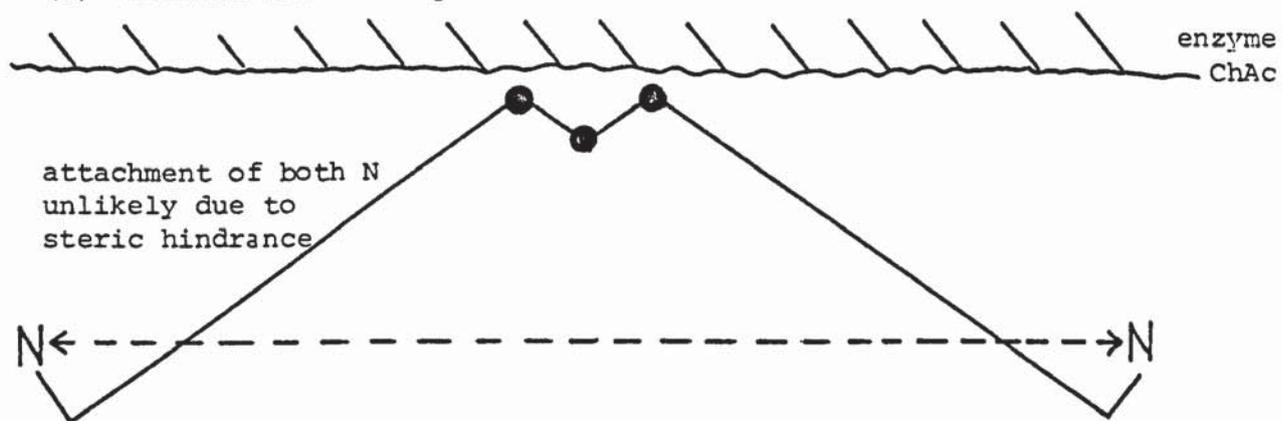


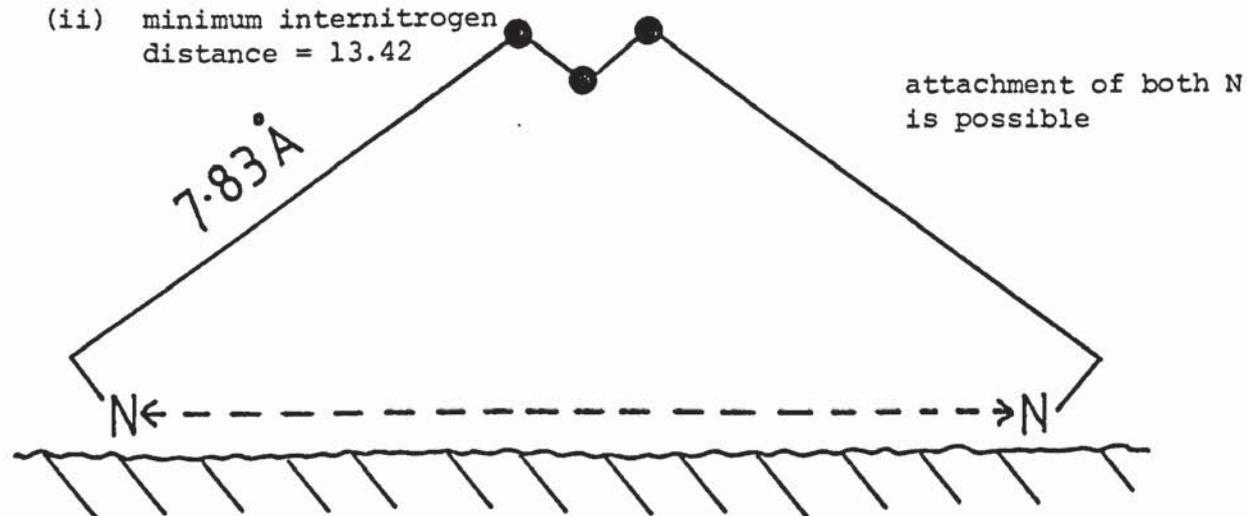
Fig. 4.31:- 3CHC (continued overleaf)

(b) Most stable conformation

(i) maximum internitrogen distance = 16.9\AA



(ii) minimum internitrogen
distance = 13.42



(iii) intermediate internitrogen distance = 15.2\AA

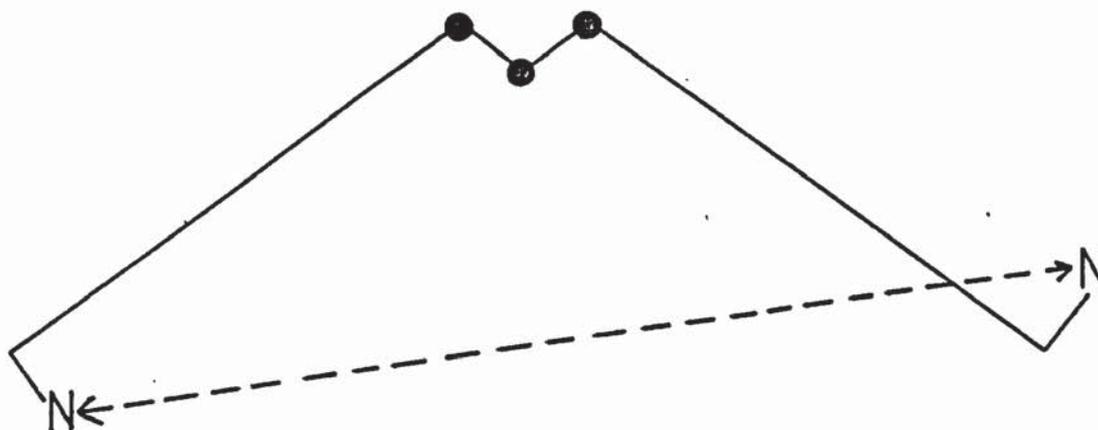
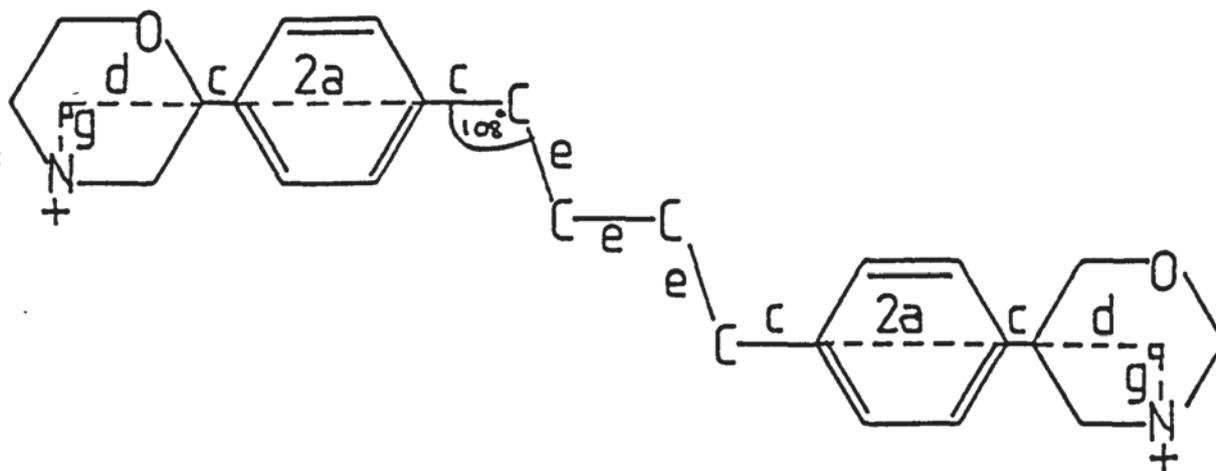
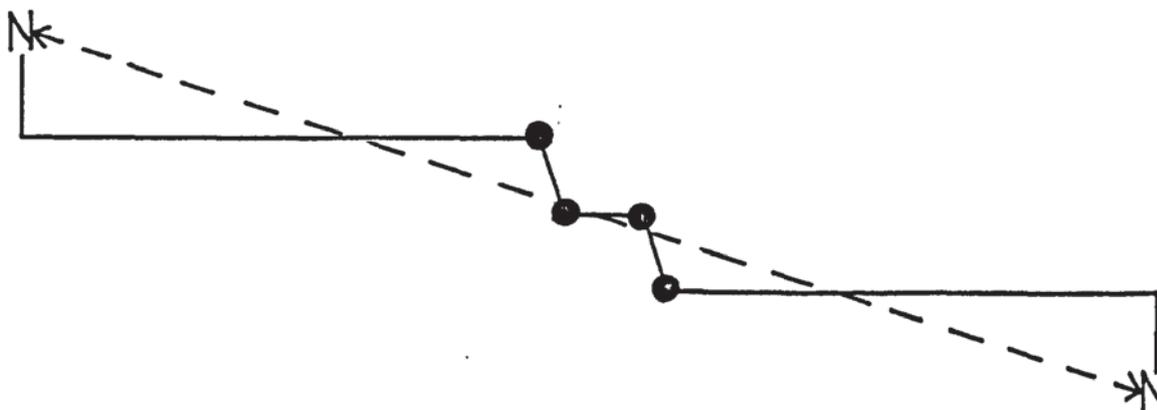


Fig. 4.31:- 3CHC (continued from previous page)

$a=1.397$; $c=1.52$; $d=2.00$; $e=1.54$; $g=1.44\text{\AA}$



(a) maximum internitrogen distance = 19.0\AA



(b) minimum internitrogen distance of this particular conformer = 18.4\AA

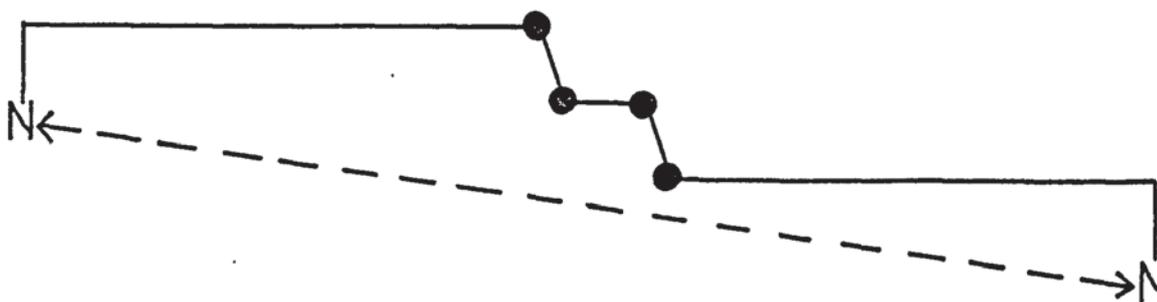
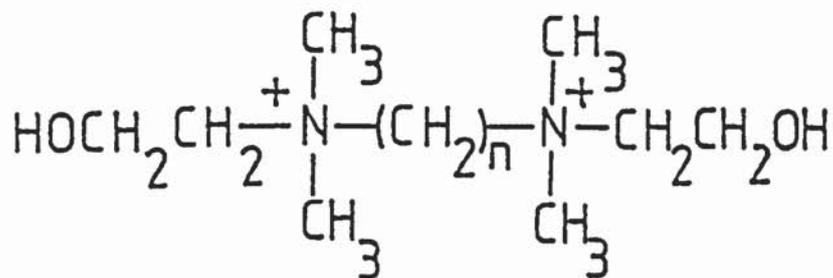
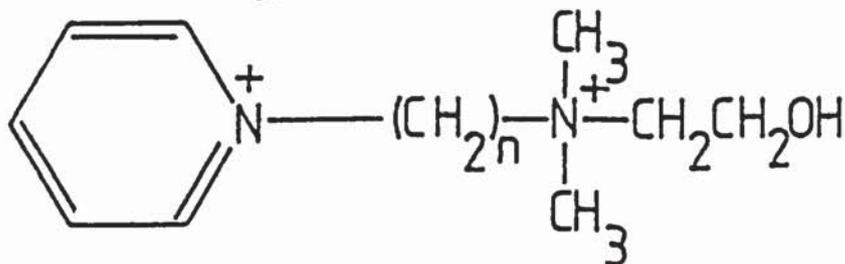


Fig. 4.32:- 4CHC

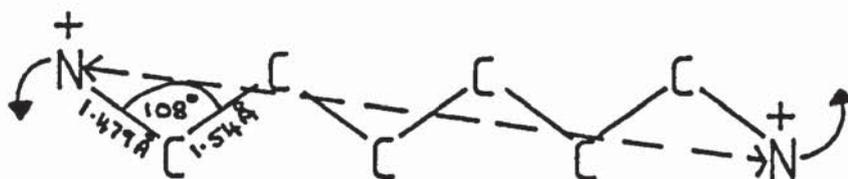
(a) Hemsworth (1971b, 1976)



(b) Sollenberg, Stensiö and Sörbo (1979)



(c) maximum internitrogen distance of (a) and (b) when $n=6$ is 8.7\AA



(d) maximum internitrogen distance of (a) and (b) when $n = 10$ is 13.6\AA

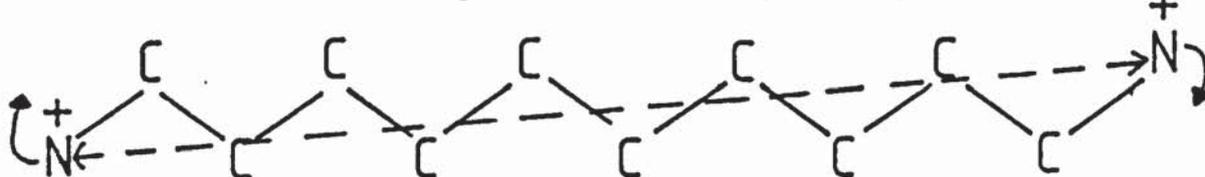


Fig. 4.33:- Bisquaternary analogues studied by Hemsworth (1971b, 1976) and Sollenberg Stensiö and Sörbo (1979). Bending of bonds will allow both N of each molecule to be parallel to the enzyme surface.

a minimum internitrogen distance of 14.13Å and a maximum of 14.4Å.

The insertion of a methylene group between the two phenyl rings in the HC-3 molecule to give the analogue 1CHC reduced the minimum internitrogen distance to 11Å (Fig. 4.29). Again, rotation around the 'C' bond can occur giving a maximum internitrogen distance of 14.4Å.

The insertion of two methylene groups between the two phenyl rings in the HC-3 molecule gave the analogue 2CHC. One conformation of this molecule is shown in Fig. 4.30a, and it can be seen that the phenyl rings lie close to each other. Therefore it is likely that steric and electronic effects will make this an unstable conformation. The conformation shown in Fig. 4.30b, however, is more stable. Rotation about the 'C' bond in this isomer gives a minimum internitrogen distance of 16.1Å, a maximum of 16.7Å.

The 3CHC molecule can exist in the conformation shown in Fig. 4.31a, however, due to steric hindrance and electronic repulsion this is a very unstable conformation. The most stable structure is shown in Fig. 4.31b, again rotation about the 'C' bond gives a maximum internitrogen distance of 16.9Å and a minimum of 13.4Å.

The most stable conformation of the 4CHC molecule is shown in Fig. 4.32. Rotation about the 'C' bond gives a maximum internitrogen distance of 19.0Å and a minimum of 18.4Å.

Both HC-3 and 3CHC are acetylated *in vitro* by ChAc by 28% compared to choline 100% while other analogues 1CHC, 2CHC and 4CHC are acetylated by less than 5%. This and other studies (Hemsworth 1971b, 1976 Sollenberg, Stensiö and Sörbo 1979) suggest that the internitrogen distance is an important factor in determining whether or not bis-quaternary nitrogen choline analogues are acetylated *in vitro* by ChAc. HC-3 and 3CHC have a similar interatomic distance between the quaternary nitrogens in their molecular structures. HC-3 possess a minimum internitrogen distance 14.13Å

(Fig. 4.28), and 3CHC a minimum internitrogen distance of 13.4\AA , (Fig. 4.31b) a difference of 0.7\AA . Bearing in mind the approximations made in calculating the interatomic distances and the bending of bonds that occurs in solutions it seems possible for these two structures to occupy the same sites on the enzyme. Both quaternary nitrogens in either molecule presumably bind to anionic sites approximately 14\AA apart.

1CHC has a conformation in which the internitrogen distance is similar to that of HC-3, this is 14.4\AA (Fig. 4.29(iii)), but this conformation is unlikely to allow both quaternary nitrogen atoms to combine with anionic sites on the same planar surface of the enzyme ChAc, since the bulky phenyl groups will sterically hinder the attachment as shown (Fig. 4.29(iii)). This is presumably why 1CHC is not acetylated.

Due to rotation of groups around simple bonds an infinite number of intermediate conformations is possible between those giving maximum and minimum internitrogen distances, in each of the hemicholinium compounds. It is in agreement with the results to conclude that the two quaternary nitrogen atoms both have to be in the same plane as all the other atoms in the molecule for acetylation to occur, for example 1CHC is not acetylated by ChAc despite there being conformations where the internitrogen distance is approximately 14\AA apart since these conformations do not have the nitrogen atoms in the same plane (the exception to this has been discussed, see above).

Hemsworth, (1971b, 1976), studied a series of dicholine compounds (discussed in the Introduction 1.2.9), while Sollenberg, Stensiö and Sörbo (1979) studied a series of bis-quaternary nitrogen pyridinium choline analogues. Both groups of workers concluded that increasing the internitrogen distance in these analogues improved the compounds as substrates for ChAc *in vitro*. Analogues having an N-N interatomic distance 8.7 to 13.6\AA (Fig. 4.33) had high rates of acetylation (Table 1.2). The later interatomic distance gave substrates which had optimal rates of acetylation.

The hemicholinium compounds studied seem to require a more precise inter-nitrogen distance but again about 14\AA is the optimum distance.

These results indicate that the bis-quaternary nitrogen choline analogues bind to anionic sites on the ChAc enzyme which are about 14\AA apart. Very little is known about the active sites of ChAc but each site is usually located on enzymes in a cleft the depth of which appears to vary, depending upon the enzyme. However, if both quaternary nitrogen compounds combine with active sites this would mean that the active sites are very close to each other on the enzyme. This is unlikely since active sites would be expected to be as far away from each other as possible so that the substrates will have easy access to them, without any steric interference. Sollenberg, Stensiö and Sörbo (1979), concluded from their studies that one quaternary nitrogen of the bis-quaternary choline analogues binds to the active site, while the other binds to some other anionic group located outside the active site of the enzyme. Such a site could be an acidic amino acid group. This suggestion for the binding of bis-quaternary nitrogen choline analogues is supported by the tentative conclusion made earlier (Section 4.3.5) that the acetylated form of HC-3 as synthesised *in vitro* by ChAc is a monacetylated derivative.

The various potencies of the hemicholinium analogues in causing a pre- and postjunctional block and in inhibiting the high affinity choline transport system of synaptosomes cannot be explained in terms of the internitrogen distances. Only the ability of ChAc to acetylate, *in vitro*, the hemicholinium analogues can be rationalised in this way.

4.4.2.6. Summary of results

In general the hemicholinium analogues were each shown to possess a prejunctional blocking action at the neuromuscular junction, a weak anti-cholinesterase activity, an inhibitory action on the high affinity choline

transport mechanism of synaptosomes and a variety of actions on ChAc.
only 3CHC was acetylated *in vitro* by this enzyme.

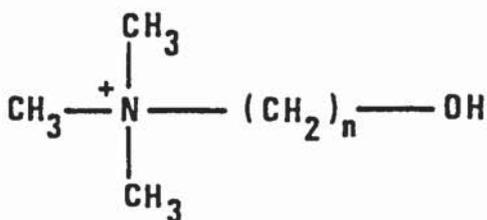
5. RESULTS CHAPTER II:- SOME LONG-CHAIN CHOLINE ANALOGUES

5. RESULTS CHAPTER II:- SOME LONG-CHAIN CHOLINE ANALOGUES

5.1 INTRODUCTION

5.1.1 Historical Introduction

Bowman and Rand, (1962), investigated the neuromuscular blocking properties of choline (1), a three carbon analogue of choline that is 3C-choline (homocholine; 3-trimethylamino-1-propanol; 3-hydroxy-N,N,N-trimethylpropaninium, 29) and a four carbon analogue of choline that is 4C-choline (4-trimethylamino-1-butanol; 4-hydroxy-N,N,N-trimethylbutanaminium, 72). They demonstrated that these three compounds gave a depolarising block at the neuromuscular junction.



n = 2; choline (1)
 n = 3; 3C-choline, homocholine (29)
 n = 4; 4C-choline (72)
 n = 5; 5C-choline (73)

Several groups of workers have shown that both 3C-choline and 4C-choline are acetylated by ChAc *in vitro* by an negligible amount, that is by less than 5% compared to choline 100% (Table 1.2). However, one study by Burgen, Burke and Desbarats-Schonbaum, (1956), showed that 4C-choline was acetylated by 29%.

No further work has been carried out with 4C-choline. However, especially since the start of the present study, the presynaptic actions of 3C-choline have been extensively investigated.

Simon, Mittag and Kuhar, (1975), and Collier, Lovat, Ilson, Barker and Mittag, (1977), showed that 3C-choline inhibited the high affinity transport of choline into synaptosomes (Table 1.3). Collier, Lovat, Ilson, Barker and Mittag, (1977), found that [³H]-3C-choline was itself

transported into synaptosomes by a high and low affinity system and that 64% of the 3C-choline accumulated by the high affinity transport system was acetylated. Collier and Ilson, (1977), showed that the cat superior cervical ganglia also accumulated [³H]-3C-choline and that electrical stimulation of the ganglia increased the accumulation. Collier, Lovat, Ilson, Barker and Mittag, (1977), showed that in the absence of physostigmine 7.1% of the 3C-choline accumulated by the ganglia was acetylated whereas in the presence of physostigmine 52% of the 3C-choline accumulated was acetylated. This difference could be due to the inhibitory effect of physostigmine on the hydrolysis of released ACh. In the absence of physostigmine, choline, so formed, is probably recaptured for ACh synthesis (Collier and Katz, 1974), and it will presumably compete with 3C-choline for uptake and acetylation in ganglia. Collier, Lovat, Ilson, Barker and Mittag, (1977), showed that after preloading the ganglia with 3C-choline, acetyl-3C-choline was released from the ganglia on electrical stimulation as a false cholinergic transmitter.

5.1.2. Long-chain choline analogues investigated and aims of study

The present study synthesised choline (1), 3C-choline (29), 4C-choline (72) and a new analogue 5C-choline (73). An investigation was conducted into their action at cholinergic synapses and in particular at the neuromuscular junction. Therefore this study investigated the compounds with regard to their:-

- (a) prejunctional and postjunctional activity at the neuromuscular junction.
- (b) action on cholinesterase enzymes *in vitro*.
- (c) action on ChAc *in vitro*.
- (d) action on the transport of choline into synaptosomes.

In each case the activities of the analogues were compared to

that of the parent compound, choline, in an attempt to investigate the effect of the insertion of methylene groups between the quaternary nitrogen and the hydroxyl group.

5.2. CHEMISTRY

5.2.1. Synthesis of choline and 3C-choline

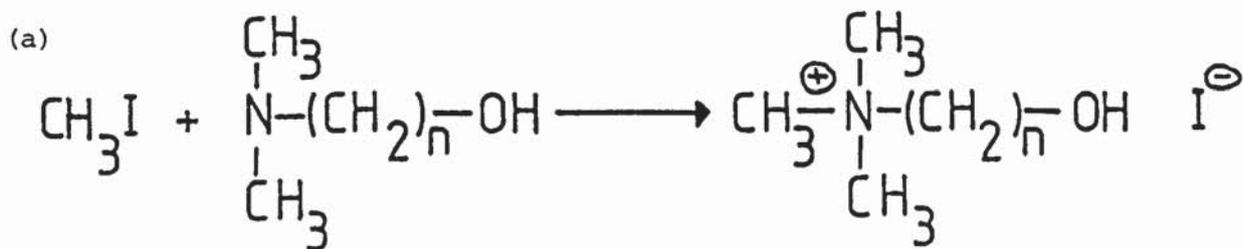
Choline (1) and 3C-choline (29) were synthesised by quaternising the tertiary amine analogues with methyl iodide (Menschutkin Reaction) (Fig. 5.1.a). The methyl iodide undergoes nucleophilic substitution by the tertiary amine.

5.2.2. Synthesis of 4C-choline

4C-choline (72), was synthesised by reacting trimethylethanolamine with 4-iodo- butan-1-ol which undergoes nucleophilic substitution (Fig. 5.1.b).

5.2.3. Synthesis of 5C-choline

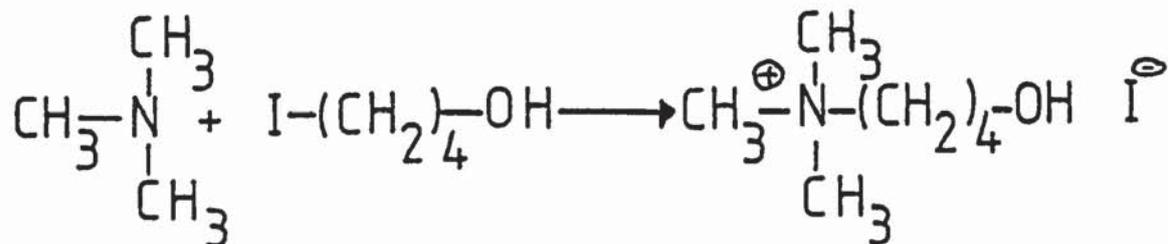
5C-choline (73), was prepared by reacting methyl iodide with the primary amine 5-aminopenta n-1-ol (the required tertiary amine was not commercially available). The methyl iodide undergoes nucleophilic substitution. The reaction yields a mixture of primary, secondary, tertiary and quaternary amines (Fig. 5.1.C). To obtain the pure quaternary amine the mixture was made alkaline and the solution was boiled to dryness. The primary, secondary and tertiary amines are all volatile in alkaline solution and in this way they were removed from the mixture. The quaternary amine (73), was then extracted from the residue with chloroform.



n = 2; choline (1)

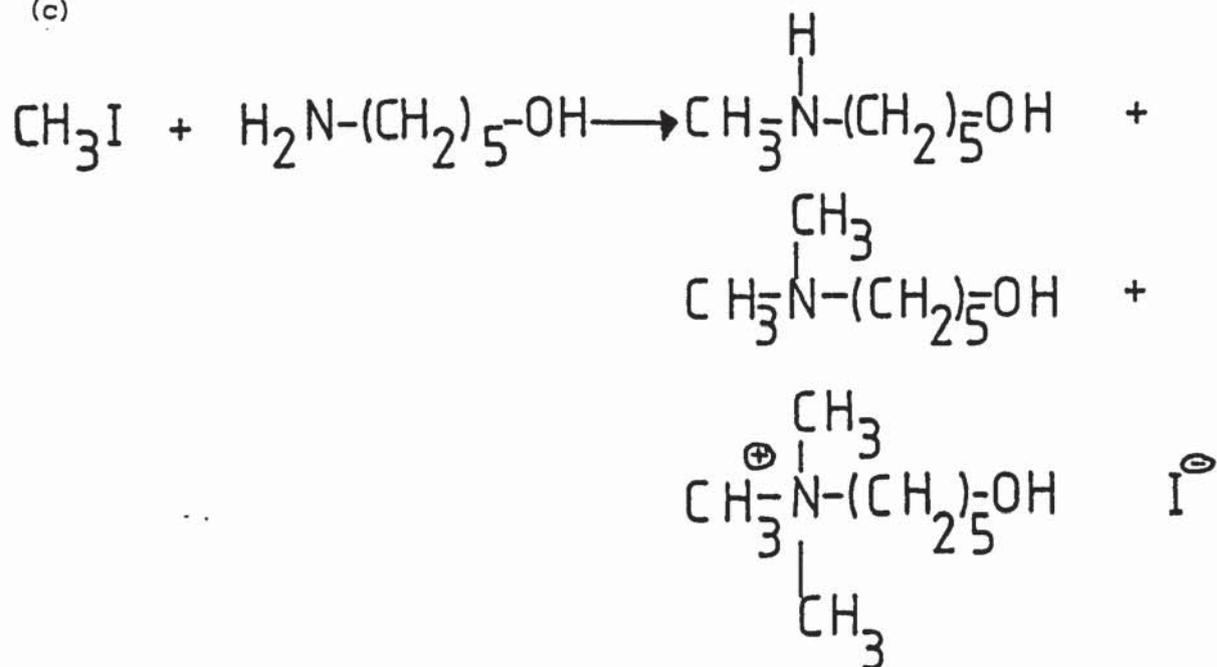
n = 3; 3C-choline (29)

(b)



4C-choline (72)

(c)



5C-choline (73)

Fig. 5.1:- Synthesis of choline, 3C-choline, 4C-choline and 5C-choline

5.2.4. Experimental

5.2.4.1. 2-hydroxy-N,N,N-trimethyl-ethanaminium iodide, ([2-hydroxyethyl]-trimethylammonium iodide, choline as an iodide salt).

2-hydroxy-N,N-dimethyl-ethanaminium (N,N-dimethyl-2-aminomethanol) (5.3g, 0.06mole) was dissolved in a sufficient quantity of 95% ethanol (20ml) and the solution cooled. Methyl iodide (17.0g, 0.12mole) was added dropwise. The product was recrystallised from 95% ethanol to give colourless needles of 2-hydroxy-N,N,N-trimethyl-ethanaminium iodide (choline iodide) (1) (11.8g, 86%) mp 264-266^o. ν_{\max} (nujol), 3,400 (OH), 2900, 1470, 1380, 1280-1200, 1140, 1080, 1060, 1020, 980, 900, 860 cm^{-1} .

Anal: (C₅H₁₄INO) requires; C, 25.97; H, 6.06; I, 54.98; N, 6.06% found; C, 26.32; H, 5.94; I, 54.81; N, 6.04%.

5.2.4.2. 3-hydroxy-N,N,N-trimethyl-propanaminium iodide (3-trimethylamino-1-propanol iodide; homocholine as an iodide salt; 3C-choline)

Using the method described above, 3-hydroxy-N,N-dimethylpropanaminium (3-dimethylamino-1-propanol) (6.18g; 0.06mole) gave colourless needles of 3-hydroxy-N,N,N-trimethyl-propanaminium iodide (3C-choline; homocholine iodide) (29) (12.5g, 85%) mp 179-181^o. ν_{\max} (nujol) 3450 (OH), 2900, 1470, 1380, 1220, 1280, 1140, 1070, 960, 940, 920, 900, 800, 720 cm^{-1} .

Anal: (C₆H₁₆INO) require; C, 29.39; H, 6.53; I, 51.84; N, 5.71% found; C, 29.68; H, 6.64; I, 51.63; N, 5.51%.

5.2.4.3. 4-hydroxy-N,N,N-trimethyl-butanaminium iodide (4-trimethylamino-1-butanol iodide; 4C-choline)

Trimethylamine (5.9g, 0.1mole) was dissolved in a sufficient quantity of ethanol (20mls) and 4-iodobutan-1-ol (12g, 0.06mole) added. The solution was heated gently under reflux with stirring for 1hr. The solvent was then evaporated off under reduced pressure and the product recrystallised

from ethanol to give colourless needles of 4-hydroxy-N,N,N-trimethylbutanaminium iodide (4C-choline) (72) (9.8g,63%) mp 249-250^o. ν_{\max} (nujol) 3400(OH), 2900, 1480, 1380, 1260, 1200, 1080, 1060, 920, 760 cm^{-1} .

Anal: ($\text{C}_7\text{H}_{18}\text{INO}$) requires; C, 32.43; H, 6.95; I, 49.03; N, 5.41% found; C, 32.23; H, 6.73; I, 49.15; N, 5.63%.

5.2.4. 45-hydroxy-N,N,N-trimethyl-pentanaminium iodide (5-trimethylamino-1-pentanol; 5C-choline)

5-hydroxypentamine (5-amino-1-pentanol) (5g, 0.05mole) was dissolved in ethanol (20mls) and methyl iodide (28.4g, 0.2mole) was then added. The solution was heated gently with stirring under reflux for 2hr. An aqueous solution of potassium hydroxide was then added until the solution was just alkaline to litmus. This mixture was evaporated down to dampness and dried in a vacuum oven at 40^o. Sufficient water was then added to dissolve the residue and this solution was continuously extracted with chloroform. The chloroform was evaporated off leaving a yellow liquid. This product was dried and then recrystallised from ethanol to give colourless needle shaped crystals of 5-hydroxy-N,N,N-trimethyl-pentanaminium iodide (5C-choline) (73) mp 127-128^o. ν_{\max} (nujol) 3,400(OH), 2,900, 1480, 1400, 1280, 1260, 1220, 1080, 1020, 780 cm^{-1} .

Anal: ($\text{C}_8\text{H}_{20}\text{INO}$) requires; C, 35.16; H, 7.33; I, 46.52; N, 5.13% found; C, 35.48; H, 7.12; I, 46.43; N, 5.11%.

5.3. PHARMACOLOGY

5.3.1. Neuromuscular blocking action

The actions of the long-chain choline analogues were observed on the rat hemidiaphragm and the frog rectus abdominis muscle to determine the extent of pre- and postjunctional block at the neuromuscular junction.

5.3.1.1 Rat phrenic nerve-hemidiaphragm

3C-choline and 5C-choline, in low doses, were found to cause a slowly developing block of the rapidly stimulated muscle (1Hz) but had no effect on the more slowly stimulated muscle (0.1Hz). The addition of choline to the incubating medium did not reverse the block, but on washing the drug out of the bath, the muscle contractions of the rapidly stimulated muscle returned to the control height of contraction. These results suggest that 3C-choline and 5C-choline gave a prejunctional block in low doses (Table 5.1). Neither choline nor 4C-choline showed this prejunctional blocking action, but they did cause a postjunctional block as indicated by a block of both the slow and rapidly stimulated muscles. Similarly 3C-choline and 5C-choline at higher doses than those giving the prejunctional block, elicited a postjunctional block. 3C-choline, 4C-choline and 5C-choline were all about one and a half times more potent than choline in causing a postjunctional block.

5.3.1.2 Frog rectus abdominis muscle

All of the long-chain choline analogues elicit contractions of the frog rectus abdominis muscle, (Table 5.2). The two most potent compounds in this respect were 3C-choline and 5C-choline which were approximately 6 times more potent than choline. These results suggest that the long-chain choline analogues have a depolarising action at the neuromuscular junction.

Compound	Minimum Effective dose giving a prejunctional block $\mu\text{mole/ml}$	Minimum Effective dose giving a postjunctional block $\mu\text{mole/ml}$
choline	No prejunctional block	0.61
3C-choline	0.08	0.41
4C-choline	No prejunctional block	0.39
5C-choline	0.11	0.37

TABLE 5.1:- Effect of the long-chain choline analogues on nerve transmission in the rat phrenic-nerve hemidiaphragm preparation. In either case the reversal of the prejunctional block by choline could not be shown. Each experiment was repeated twice. S.E.M. were $\pm 10\%$ or less.

Compound	Equipotent molar ratio
acetylcholine	1
choline	779
3C-choline	111
4C-choline	1264
5C-choline	133

TABLE 5.2:- Equipotent molar ratios of the long-chain choline analogues relative to ACh in producing a contracture of the frog rectus-abdominis muscle. Each experiment was repeated twice. S.E.M. were $\pm 10\%$ or less.

Compound	Enzyme Rat Brain Homogenate	Substrate
choline	2.0×10^{-2}	ACh
3C-choline	5.6×10^{-2}	ACh
4C-choline	1.1×10^{-2}	ACh
5C-choline	4.5×10^{-1}	ACh

TABLE 5.3:- IC_{50} (Molar) values for the inhibition of cholinesterase activity by the long-chain choline analogues. Incubation mixtures contained enzyme, [^{14}C]-ACh and a range of concentrations of each inhibitor. A Dixon plot was drawn for each compound and the IC_{50} values are calculated from these plots, they represent that concentration of inhibitor which inhibited the hydrolysis of ACh by 50%. For each plot regression lines with correlation coefficients of 0.997-0.999 were drawn through at least five points. Each point was the mean of at least three determinations \pm SEM of 10% or less.

5.3.2. Anticholinesterase activity

The effect of the long-chain choline analogues on cholinesterase activity was determined by including the analogues in incubation mixtures containing enzyme (a 20,000g supernatant from rat brain homogenate containing both AChE and ChE), buffer and radioactive substrate ($[^{14}\text{C}]$ -Ach) as described in the Methods (Section 3.4). The degree of hydrolysis of the substrate was then compared with that obtained in controls containing distilled water in place of the compound.

All the long-chain choline analogues were shown to possess weak anticholinesterase activity (Table 5.3), and for this reason further experiments with purified enzymes were not conducted.

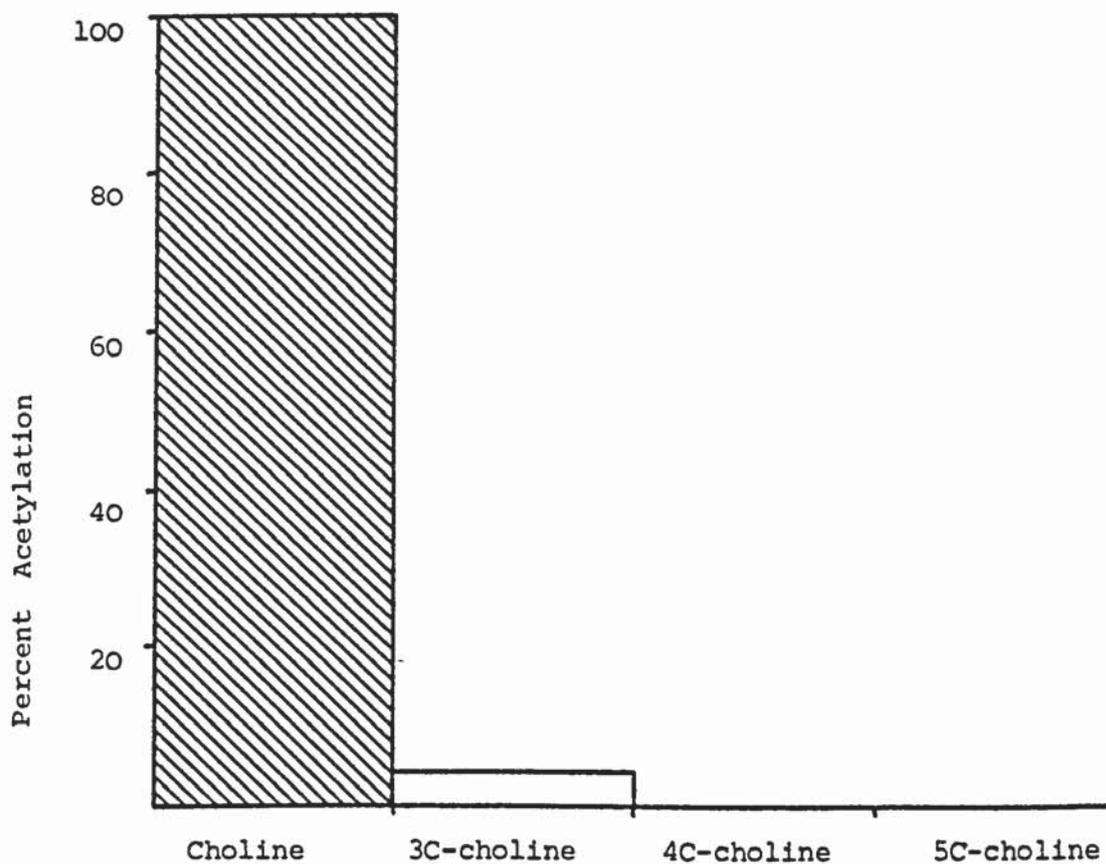
5.3.3 In Vitro acetylation by ChAc

The rates of acetylation of the long-chain choline analogues were determined by incubating the compounds at 37° for 10min. with $[^{14}\text{C}]$ -AcCoA and a partially purified extract of ChAc obtained from rat brain. The acetylated products were routinely isolated and quantitatively assayed by following the method of Fonnum (1975). The rates of acetylation of the long-chain choline analogues were compared to that of choline which is the natural substrate for ChAc. Blank determinations were obtained by substituting the substrate for distilled water.

The relative rates of *in vitro* acetylation of the long-chain choline analogues were determined and compared to the acetylation of choline. (Fig. 5.2), 3C-choline, 4C-choline and 5C-choline were acetylated at rates of less than 5% that of choline. Because of these very low rates no further studies were conducted.

5.3.4. In Vitro inhibition of ChAc

A partially purified extract of ChAc obtained from rat brain and AcCoA were incubated with equimolar concentrations of choline and one of



Long chain choline compounds

Fig. 5.2:- The rates of acetylation of choline and the long-chain choline analogues by ChAc extracted from rat brain. The incubation was carried out for 10min. at 37°. The final substrate concentration was 20mM. Each experiment was performed in duplicate and repeated three times \pm SEM were less than 5%.

Inhibitor	Percent Inhibition
Water	0
3C-choline	0
4C-choline	1
5C-choline	82

TABLE 5.4:- Percentage inhibition of the acetylation of choline by ChAc extracted from rat brain. Both choline and the long-chain choline analogues were at a concentration of 14mM. Incubation was carried out at 37° for 10min. Each value is the average of four determinations. SEM was ±10% or less.

the long-chain choline analogues, and the amount of acetylated product synthesised was assayed by the method of Fonnum, (1975). These incubations were compared with control incubations containing the same concentration of choline, but distilled water instead of the long-chain choline analogue. The method of isolation of the acetylated product does not distinguish between Ach and any acetylated long-chain choline analogue, but this is not an important consideration since no evidence has been found which suggests that the analogues are significantly acetylated, as discussed previously (Section 5.3.3.).

3C-choline and 4C-choline showed a little inhibitory activity against ChAc *in vitro* whereas 5C-choline was a potent inhibitor (Table 5.4).

5.3.5. Inhibition of the high affinity transport of choline into synaptosomes by the long-chain choline analogues

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, the other has a low affinity for choline (Yamamura and Snyder 1972, 1973).

A crude preparation of synaptosomes, isolated from rat brain, was incubated with [^3H]-choline ($1\mu\text{M}$; $0.55\mu\text{Ci/nmole}$) at 37° and 0° . Choline transport into synaptosomes at 37° was time dependent being linear for 7min. (Fig. 4.21). The amount of choline accumulated by synaptosomes at 37° for 7min. was 4-6 times greater than that accumulated at 0° for 7min. Values obtained for choline transport at 37° were corrected by subtracting the values obtained from the incubation at 0° .

The inhibition of the long-chain choline analogues on choline transport was measured by adding the analogues to the buffer containing [^3H]-choline ($1\mu\text{M}$) and synaptosomes and incubating at 37° for 7min. The

Inhibitor	IC ₅₀
<u>Long-chain choline analogue</u>	<u>μM</u>
3C-choline	3.6
4C-choline	7.1
5C-choline	8.5

TABLE 5.5:- Inhibition of the high affinity choline transport into synaptosomes. The IC₅₀ values were determined graphically from Dixon plots (1953). For each plot a regression line with a correlation coefficient of 0.99 was drawn through at least five points. Each point was the mean of at least seven determinations ±SEM of 10% or less. The synaptosomes were incubated with [³H]-choline (1μM) and inhibitor for 7min. at 37°.

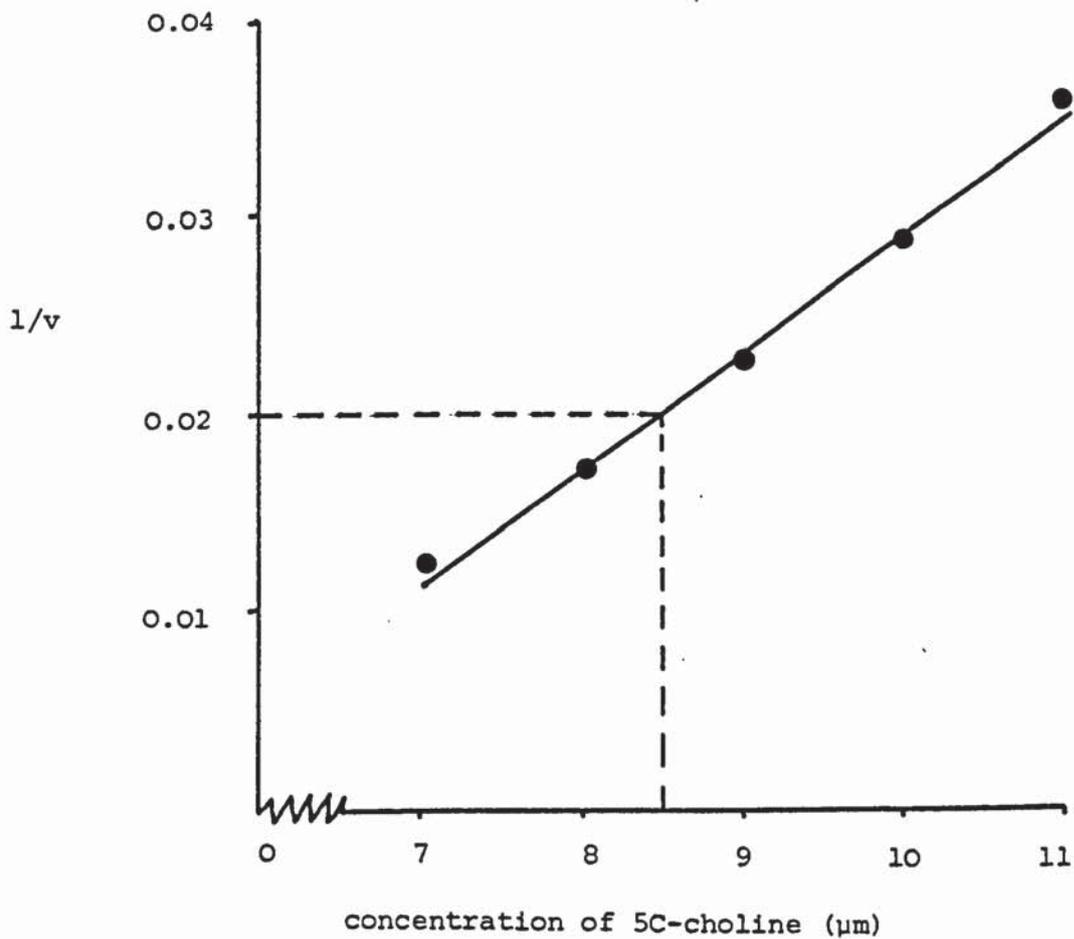


Fig. 5.3:- Dixon (1953) plot of the inhibition of the high affinity transport of choline into synaptosomes by 5C-choline. V is the % uptake of choline compared to control (no inhibitor). Each point is the mean of at least 7 determinations \pm SEM of 10% or less. The correlation coefficient is 0.99. The synaptosomes were incubated with [^3H]-choline ($1\mu\text{M}$) and inhibitor (7-11 μM) for 7 min at 37° .

concentration of the long-chain choline analogues inhibiting the transport of choline into synaptosomes by 50% (IC_{50}) (Table 5.5), was determined graphically from a Dixon plot (1953), and one such plot is shown for the 5C-choline compound. (Fig. 5.3).

5.4. Discussion

5.4.1 Action at the nueromuscular junction

Preliminary pharmacological screening using the rat phrenic nerve-diaphragm preparation showed that low doses of 3C-choline and 5C-choline caused a prejunctional block, whereas choline and 4C-choline gave no such prejunctional block. However, Bowman and Rand, (1962), could find no evidence of a prejunctional blocking action for 3C-choline on this preparation.

In higher doses both 3C-choline and 5C-choline as well as choline and 4C-choline gave a postjunctional block in the rat diaphragm preparation. This postjunctional effect seems to be due to a depolarising action of these analogues since they all caused a contraction of the frog rectus abdominis muscle. 3C-choline and 5C-choline were both more potent than both choline and 4C-choline in this respect, although all the analogues were at least 100 times less potent than ACh. The depolarising blocking action of choline, 3C-choline and 4C-choline agrees with the findings of Bowman and Rand, (1962).

It is interesting to observe that the long-chain analogues which caused the prejunctional block in the rat diaphragm and which had the more potent depolarising action on the frog rectus abdominis muscle possessed an odd number of methylene groups between the quaternary nitrogen atom and the hydroxyl group. This is in contrast to the activity of the analogues which possessed an even number of methylene groups.

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5.4.2. Anticholinesterase activity

All the long-chain choline analogues possessed very weak anticholinesterase activity and it is unlikely that this plays any significant part in their neuromuscular blocking action.

5.4.3. Mechanism of action of the long-chain choline analogues in causing a prejunctional block at the neuromuscular junction

Preliminary screening in the present work has suggested that 3C-choline and 5C-choline have a prejunctional blocking action at the neuromuscular junction. Further studies were carried out to try to determine more about the mechanism of action of these two analogues which results in the prejunctional block. 4C-choline was also routinely studied for comparison.

5.4.3.1 Inhibition of the high affinity transport of choline into synaptosomes

A drug could cause a presynaptic block at the cholinergic neuron by inhibiting the supply of choline for ACh synthesis. This can be achieved by inhibition of the high affinity transport of choline into the neuron.

All the long-chain choline analogues including 4C-choline inhibited the high affinity transport of choline into synaptosomes. 3C-choline had an IC_{50} of $3.6\mu M$ which is in agreement with Simon, Mittag and Kuhar, (1975) who obtained an IC_{50} for this compound of $4\mu M$.

5.4.3.2. Inhibition of and acetylation by ChAc *in vitro*

A drug could cause a presynaptic block at the cholinergic neuron by inhibiting ChAc. Of the long-chain choline analogues studied only 5C-choline significantly inhibited ChAc *in vitro*. Since the synthesis of

ACh was inhibited by 82% it can be concluded that 5C-choline is a potent inhibitor. Several classes of ChAc inhibitors are known, as discussed in the Introduction (Section 1.3). To this list can be added 5C-choline. This compound is of particular interest since unlike most of the styryl-pyridine inhibitors it has weak anticholinesterase activity. However, in order for this analogue to inhibit ChAc *in vivo*, it must be transported into the cholinergic neuron and this has not been investigated.

The present study indicated that all of the long-chain choline analogues studied were insignificantly acetylated *in vitro* by ChAc at less than 5%, compared to choline 100%. This is in agreement with the findings of several workers who investigated the *in vitro* acetylation of 3C-choline by ChAc (Table 1.2). However, 3C-choline has been shown to be acetylated *in situ* (that is in synaptosomes and the cat superior cervical ganglia) by Collier, Lovat, Ilson, Barker and Mittag (1977).

5.4.3.3 False transmitter hypothesis

During the present investigation Collier, Lovat, Ilson, Barker and Mittag, (1977), showed that 3C-choline was transported into the cat superior cervical ganglia and synaptosomes where it was acetylated to acetyl-3C-choline which was released from the ganglia on nervous stimulation as a false transmitter. Thus the prejunctional block of 3C-choline seen in the rat diaphragm preparation could be due at least in part to the synthesis and release of a false transmitter.

4C-choline and 5C-choline have not been investigated to see if they act as, or form false transmitters.

5.4.4. Conclusions

Increasing the distance between the quaternary nitrogen and the hydroxyl group from two methylene groups (choline) to three methylenes gave an analogue (3C-choline) which unlike choline was not acetylated by

ChAc *in vitro*, but which like choline was transported into cholinergic neurons and acetylated to acetyl-3C-choline which is released as a transmitter (Collier, Lovat, Ilson, Barker and Mittag, 1977).

Increasing the methylene groups to four gave a compound (4C-choline) which was not shown to cause a prejunctional block in the rat diaphragm preparation nor to be acetylated by ChAc *in vitro*. However, 4C-choline has been shown to possess an inhibitory action on the high affinity transport of choline into synaptosomes. It is possible that the concentration of 4C-choline necessary to inhibit choline transport, to an extent such that the synthesis of ACh is sufficiently impaired to give a prejunctional block, also elicits a detectable postjunctional block on the rat phrenic nerve diaphragm preparation.

5C-choline was shown to give a prejunctional block in the rat diaphragm preparation. The results of this study indicate that this could be due to its inhibitory action on the choline transport system and/or due to its inhibitory action on ChAc.

6. RESULTS CHAPTER III:- SOME HYDROXYPIPERIDINIUM AND

HYDROXPYRIDINIUM ANALOGUES OF CHOLINE

6. RESULTS CHAPTER III:- SOME HYDROXYPIPERIDINIUM AND
HYDROXYPYRIDINIUM ANALOGUES OF CHOLINE

6.1 INTRODUCTION

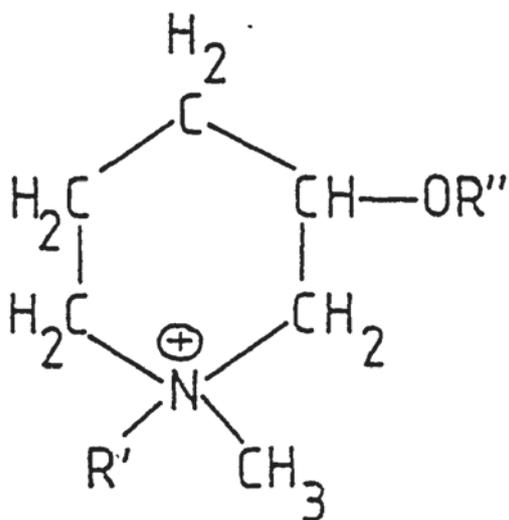
The hydroxypiperidinium and hydroxypyridinium compounds are cyclic analogues of choline. 3-Hydroxypiperidinium (3-hydroxy-N,N-dimethylpiperidinium) (74), 2-hydroxymethylpiperidinium (2-hydroxymethyl-N,N-dimethylpiperidinium) (75), and hydroxypyridinium (5-hydroxy-2-methyl-N-methylpyridinium) (76) are analogues in which a choline skeleton is immobilised by being fixed in a ring. 3-hydroxymethylpiperidinium (3-hydroxymethyl-N,N-dimethylpiperidinium)(77) and 4-hydroxypiperidinium (4-hydroxy-N,N-dimethylpiperidinium) (78) are analogues in which a 3C-choline (homocholine) (29) skeleton is fixed in a ring.

It is interesting to note that the choline moiety of HC-3(4) is fixed in a morpholine ring in a similar way to the choline moiety in the 3-hydroxypiperidinium molecule. Apart from the hemicholiniums very little work has been done on cyclic choline analogues and it was therefore thought of interest, particularly from the point of view of structure/activity relationships, to study these compounds and investigate their actions at the cholinergic synapse and in particular the neuromuscular junction.

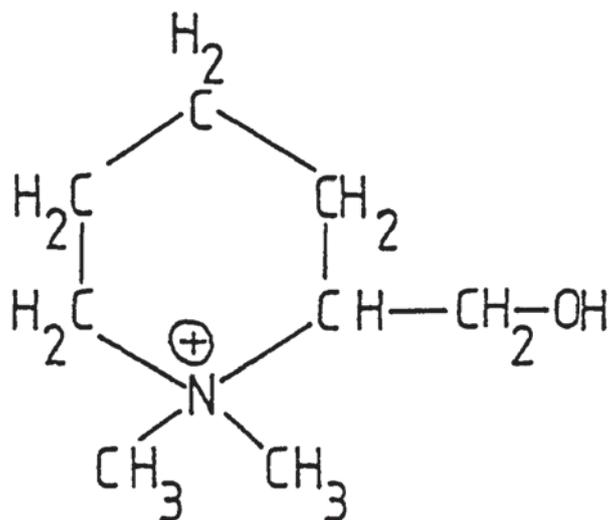
Therefore this study investigated these compounds with regard to their:

- (a) prejunctional and postjunctional activity at the neuromuscular junction
- (b) action on cholinesterase enzymes *in vitro*
- (c) action on ChAc *in vitro*
- (d) action on the transport of choline into synaptosomes.

It was found that 3-hydroxypiperidinium was acetylated *in vitro* by ChAc at a rate of 55% compared to choline 100%. Since this was the highest amount of acetylation of any analogue that had been found in this



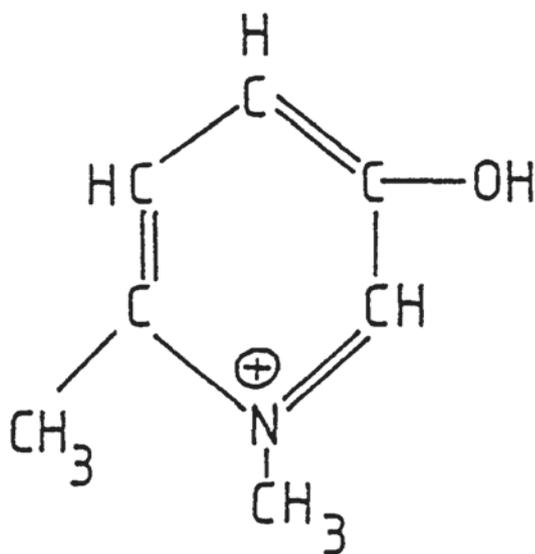
R' = CH₃, R'' = H: 3-hydroxypiperidinium (74)



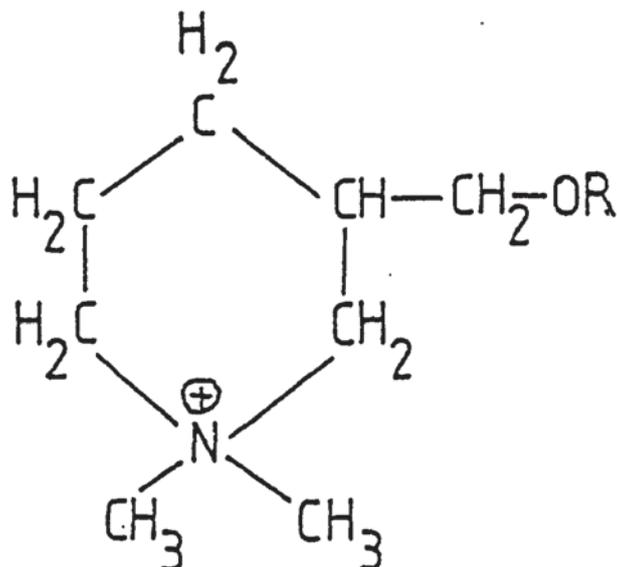
2-hydroxymethylpiperidinium (75)

R' = ¹⁴C, R'' = H: [¹⁴C]-3-hydroxypiperidinium (79)

R' = CH₃, R'' = Ac: 3-acetoxypiperidinium (80)

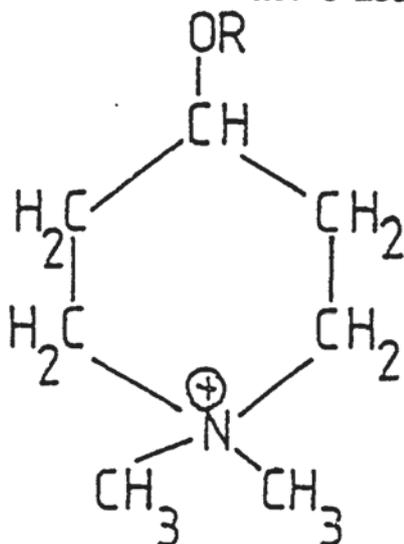


hydroxypyridinium (76)



R = H: 3-hydroxymethylpiperidinium (77)

R = Ac: 3-methacetoxypiperidinium (81)



R = H; 4-hydroxypiperidinium (78)

R = Ac: 4-acetoxypiperidinium (82)

thesis it was decided to investigate this analogue further to see if it could be transported into cholinergic neurons. In order to study this transport radioactively labelled 3-hydroxypiperidinium has to be used. Unfortunately, this is commercially unavailable unless the analogue is "custom made" at a very high cost. It was therefore decided to synthesise $[^{14}\text{C}]$ -3-hydroxypiperidinium (79) using $[^{14}\text{C}]$ -methyl iodide.

Experiments indicated that this analogue (79) was acetylated *in situ* and released as a false cholinergic transmitter. In order to study the action of this false transmitter postfunctionally the acetylated derivative of 3-hydroxypiperidinium that is 3-acetoxypiperidinium (80), and for further interest the acetylated derivatives of other hydroxy-piperidinium compounds, that is, 3-methacetoxypiperidinium (81) and 4-acetoxypiperidinium (82), were studied.

6.2 CHEMISTRY

6.2.1 Synthesis of the hydroxypiperidinium and hydroxypyridinium analogues

The hydroxypiperidinium and hydroxypyridinium analogues (74-78), were prepared by quaternising the appropriate tertiary amine with methyl iodide (Menschutkin Reaction). The tertiary nitrogen acts as a nucleophile.

6.2.2 Synthesis of the acetoxypiperidinium analogues

The acetylated analogues (80-82) were synthesised using either acetylchloride or acetic anhydride as the acetylating agents.

6.2.3 [¹⁴C]-3-hydroxypiperidinium

6.2.3.1 Synthesis

[¹⁴C]-3-hydroxypiperidinium (79) was synthesised by quaternising 3-hydroxy-N-methylpiperidine with [¹⁴C]-methyl iodide. The product was purified on a strong cationic exchange resin SP-Sephadex. The resin was first eluted with water and as [¹⁴C]-3-hydroxypiperidinium possesses one quaternary nitrogen (cation) it bound to the resin, whereas the unreacted starting materials possess no positive charges and were eluted from the column. When no starting materials could be detected in the aqueous eluates, [¹⁴C]-3-hydroxypiperidinium was eluted from the column by NaCl (300mM).

6.2.3.2 Specific activity and purity

6.2.3.2.1 Specific activity

The specific activity of [¹⁴C]-3-hydroxypiperidinium was calculated from the specific activity of the [¹⁴C]-methyl iodide, bearing in mind that one molecule of methyl iodide reacts with one molecule of 3-hydroxy-N-methylpiperidine.

6.2.3.2.2 Radiochemical purity

Methyliodide is volatile at room temperature and therefore any unreacted material would be removed by the vacuum distillation process which occurs at the end of the reaction. This was found to be the case as no radioactivity was detected in the aqueous eluates from the ion-exchange column. For practical purposes, therefore, the product was considered to be radiochemically pure.

6.2.3.2.3 Chemical purity

3-hydroxy-N-methylpiperidine was eluted from the column by water. Samples of the eluates were run on a TLC plate. As the elution continued iodine stained spots of decreasing colour intensity were detected on the TLC plate. No 3-hydroxy-N-methylpiperidine was detected on running a TLC of a sample of the final product. For practical purposes, [^{14}C]-3-hydroxypiperidinium was considered to be chemically pure.

6.2.4 Experimental

6.2.4.1 3-hydroxy-N,N-dimethylpiperidinium iodide (3-hydroxypiperidinium)

3-hydroxy-N-methylpiperidine (5.8g, 0.05 mole) was dissolved in ethanol (30mls) and the solution cooled. Methyliodide (14.2g, 0.1mole) was added dropwise. The solution was then refluxed gently for 1hr and then allowed to cool. The product was recrystallised from alcohol to give colourless needles of 3-hydroxy-N,N-dimethylpiperidinium iodide (3-hydroxypiperidinium) (74) (11.0g, 85%) mp $313^{\circ}\text{v}_{\text{max}}$ (nujol) 3400(OH), 2900, 1480, 1380, 1320, 1280, 1240, 1220, 1120, 1080, 1020, 1000, 980, 940, 920, 900 cm^{-1} .

Anal: ($\text{C}_7\text{H}_{16}\text{INO}$) requires; C, 32.68; H, 6.23; I, 49.42; N, 5.45% found; C, 32.87; H, 6.11; I, 49.43; N, 5.42%.

6.2.4.2. 2-hydroxymethyl-N,N-dimethylpiperidinium iodide (2-hydroxymethyl-piperidinium)

Using the method described above 1-methyl-2-piperidinemethanol (6.5g, 0.05mole) gave colourless needles of 2-hydroxymethyl-N,N-dimethylpiperidinium iodide (2-hydroxymethylpiperidinium) (75) (11.3g, 83%) mp 295^o (dec) ν_{\max} (nujol) 3400 (OH), 2900, 1480, 1380, 1320, 1260, 1220, 1100, 1060, 940 cm^{-1} .
Anal: (C₈H₁₈INO) requires; C, 35.42; H, 6.64; I, 46.86; N, 5.17% found; 35.31; H, 6.65; I, 46.97; N, 5.33%.

6.2.4.3 3-hydroxymethyl-N,N-dimethylpiperidinium iodide (3-hydroxymethyl-piperidinium)

Using the method described above 1-methyl-3-piperidinemethanol (6.5g, 0.05mole) gave colourless needles of 3-hydroxymethyl-N,N-dimethylpiperidinium iodide (3-hydroxymethylpiperidinium) (77) (10.7g, 78%) mp 203-204^o ν_{\max} (nujol) 3400 (OH), 2900, 1460, 1380, 1300, 1260, 1220, 1160, 1120, 1080, 1040, 1000, 960, 940, 900, 880 cm^{-1} .
Anal: (C₈H₁₈INO) requires; C, 35.42; H, 6.64; I, 46.86; N, 5.17% found; C, 35.61; H, 6.73; I, 46.64; N, 5.05%.

6.2.4.4 4-hydroxy-N,N-dimethylpiperidinium iodide (4-hydroxypiperidinium)

Using the method described above 4-hydroxy-N-methylpiperidine (5.8g, 0.05mole) gave colourless needles of 4-hydroxy-N,N-dimethylpiperidinium iodide (4-hydroxypiperidinium) (78) (11.3g, 87%) mp 310^o (dec) ν_{\max} (nujol) 3300 (OH), 2900, 1480, 1380, 1280, 1180, 1160, 1060, 1020, 1000, 940, 920, 900, 820 cm^{-1} .
Anal: (C₇H₁₆INO) requires; C, 32.68; H, 6.23; I, 49.42; N, 5.45% found; C, 32.91; H, 6.33; I, 49.40; N, 5.31%.

6.2.4.5 5-hydroxy-2-methyl-N-methylpyridinium iodide (hydroxypyridinium)

Using the method described above 3-hydroxy-6-methylpyridine (5.5g, 0.05mole) gave colourless needles of 5-hydroxy-2-methyl-N-methylpyridinium iodide (hydroxypyridinium) (76) (8.5g, 67%) mp 224° (dec) ν_{\max} (nujol) 3400 (OH), 2900, 1580, 1540, 1480, 1400, 1380, 1340, 1320, 1260, 1220, 1160, 1040, 960, 860, 740, 700 cm^{-1} .

Anal: ($\text{C}_7\text{H}_{10}\text{INO}$) requires; C, 33.47; H, 3.98; I, 50.60; N, 5.58% found; C, 33.49; H, 3.97; I, 50.63; N, 5.55%.

6.2.4.6 3-acetoxy-N,N-dimethylpiperidinium iodide (3-acetoxypiperidinium)

3-hydroxy-N-methylpiperidine (4.6g, 0.04mole) was dissolved in anhydrous ether (30mls) and anhydrous pyridine (2.3mls). The mixture was cooled and acetylchloride (4g, 0.05mole) was added dropwise, with occasional stirring. This mixture was then refluxed for 4hr. After cooling the mixture was filtered. The precipitate was partitioned between chloroform and 10% sodium carbonate. The chloroform extract was dried over magnesium sulphate and the chloroform was then distilled off leaving a brown oil (4.2g). The brown oil was dissolved in the minimum quantity of anhydrous ether, and methyl iodide (14.2g, 0.1mole) was added. The mixture was allowed to stand for 2 days. The product was recrystallised once from ethanol and then twice from diacetone alcohol to give colourless needles of 3-acetoxy-N,N-dimethylpiperidinium iodide (3-acetoxypiperidinium) (80) (4.9g; 40%) mp 140-141 (lit. 140.5-142°) ν_{\max} (nujol) 2900, 1740 (acetyl ester) 1480, 1380, 1260 1220, 1100, 1040, 980, 960, 920, 880, 820 cm^{-1} .

Anal: ($\text{C}_9\text{H}_{18}\text{INO}_2$) requires; C, 36.12; H, 6.02; I, 42.48; N, 4.68, O, 10.70%; found; C, 36.28; H, 6.14; I, 42.26; N, 4.51%.

6.2.4.7 3-methacetoxy-N,N-dimethylpiperidinium iodide (3-methacetoxy-piperidinium)

1-methyl-3-piperidinemethanol (4g, 0.03mole) was dissolved in pyridine (100mls) and acetic anhydride (25mls). The mixture was warmed on a steambath for 5min. and then stirred at 20-25° for 24hr. Activated charcoal (2.5g) was then added and the mixture stirred for a further 12hr. The solution was then filtered and the excess acetic anhydride and pyridine were removed by vacuum distillation, leaving a brown oil. Sodium carbonate solution (10%) was added to the oil and the mixture extracted with chloroform. The chloroform extract was dried over magnesium sulphate and the chloroform was then distilled off leaving a brown oil (6g). The brown oil was dissolved in a minimum quantity of anhydrous ether and methyl iodide (14.2g, 0.1mole) was added. The mixture was allowed to stand for 2 days. The product was recrystallised once from ethanol and twice from diacetone alcohol to give colourless needles of 3-methacetoxy-N,N-dimethylpiperidinium iodide. (3-methacetoxy-piperidinium) (81) (7.8g, 80%) mp 115°-116° (lit. 117-119°) ν_{\max} (nujol) 2900, 1720 (acetyl ester), 1460, 1380, 1300, 1240, 1180, 1120, 1080, 1060, 1040, 1000, 980, 940, 900, 880 cm^{-1} .
Anal: (C₁₀H₂₀INO₂) requires; C, 38.34; H, 6.39; I, 40.58; N, 4.47% found, C, 38.44; H, 6.42; I, 40.47; N, 4.48%.

6.2.4.8 4-acetoxy-N,N-dimethylpiperidinium iodide (4-acetoxypiperidinium)

4-hydroxy-N-methylpiperidine (2.3g, 0.02mole) was dissolved in acetic anhydride (15mls) and the solution was refluxed for 4hr. on a steam bath. The excess acetic acid was then removed by vacuum distillation. 10% sodium carbonate (20mls) was added to the residue and this mixture was then extracted with chloroform. The chloroform extract was dried over magnesium sulphate and the chloroform was then distilled off leaving a brown oil (2.3g). The brown oil was dissolved in the minimum quantity of

anhydrous ether, and methyl iodide (14.2g, 0.1mole) was added. The mixture was allowed to stand for 2 days. The product was recrystallised once from ethanol and then twice from diacetone alcohol to give colourless needles of 4-acetoxy-N,N-dimethylpiperidinium iodide (4-acetoxypiperidinium) (82) (2.5g, 42%) mp 161-162 (lit. 164^o) ν_{\max} (nujol) 2900, 1740 (acetyl ester), 1480, 1380, 1340, 1260-1220, 1160, 1100, 1040, 980, 920, 830 cm^{-1} .

Anal: (C₉H₁₈INO₂) requires; C, 36.12; H, 6.02; I, 42.48; N, 4.68% found; C, 36.31; H, 6.21; I, 42.26; N, 4.7%.

6.2.4.9 Synthesis of 3-hydroxy-N-[¹⁴C]-methyl-N-methylpiperidinium iodide ([¹⁴C]-3-hydroxypiperidinium)

The methods of synthesis and purification followed those described by Barker and Mittag, (1975). To verify the methods used, the reaction was first run using 'cold' methyl iodide.

6.2.4.9.1 Reaction with non-radioactive methyl iodide

3-hydroxy-N-methylpiperidine was diluted with methanol such that 5ml contained 4.6mg (0.04mmole). Methyl iodide was treated in the same way to give 2.48mg (0.017mmole) in 5ml of methanol. The two reactants were then mixed and refluxed gently with stirring. After 1hr the methanol was removed by vacuum distillation. The residue was dissolved in distilled water (2ml) and passed through an ion-exchange column (SP-Sephadex C-25, swollen in distilled water and supported in a 3" pasteur pipette) and eluted with distilled water (20ml). This procedure elutes any unreacted starting material. Samples of the aqueous eluates were run on a cellulose thin layer plate (butanol-methanol-acetic acid-water; 8:2:1:3) and developed with iodine. The starting material 3-hydroxy-N-methyl piperidine, had an R_f value of 0.5. When no more starting material could be detected the column was eluted with NaCl (300mM). Samples of these

eluates were also run on the TLC plate as before until the quaternary product, 3-hydroxy-piperidinium (74) could be detected, Rf value of 0.6. Another sample of this eluate gave a precipitate with Reineckate, indicating the presence of a quaternary nitrogen. No starting material could be detected in any of these eluates.

6.2.4.9.2 Reaction with radioactive $[^{14}\text{C}]$ -methyl iodide

3-hydroxy-N-methylpiperidine was diluted with methanol such that 5ml contained 2.30mg (0.02mmole). This was poured into the top of a 'Break-seal ampoule (Type P1)', which had just been taken from the freezer, and which contained $[^{14}\text{C}]$ -methyl iodide (1.24mg, 0.0086mmole, 58mCi/mmole). The ampoule was then surrounded with an ice-salt mixture and cooled. A condenser was fitted to the top of the ampoule. The ampoule seal was then broken with a glass rod and the methanol solution poured into the bottom of the ampoule and mixed with the $[^{14}\text{C}]$ -methyl iodide. This solution was refluxed and purified as described above. No radioactivity could be detected in samples of the aqueous eluates indicating that no $[^{14}\text{C}]$ -methyl iodide had been present in the residue. However, the NaCl eluates did contain radioactivity and these eluates were pooled. The product 3-hydroxy-N- $[^{14}\text{C}]$ -methyl-N-methylpiperidinium iodide (79) was detected by paper electrophoresis as described by Potter and Murphy, (1967). After being subjected to electrophoresis for 2hr the paper strips were dried and cut into 1cm sections. Only the section 7-8cm from the origin gave high counts. This corresponds to 3-hydroxypiperidinium iodide (74) (7.5cms). (Fig.6.8).

Specific Activity of Product = 58mCi/mmole

Yield = 98% in 300mM NaCl (50 μ l of NaCl solution contained 0.55 μ Ci/9 nmole)

6.3 PHARMACOLOGY

6.3.1 Neuromuscular blocking action

The actions of the hydroxypiperidinium and hydroxypyridinium analogues were observed on the rat hemidiaphragm and the frog rectus abdominis muscle to determine the extent of pre- and postjunctional block at the neuromuscular junction.

6.3.1.1 Rat phrenic nerve-hemidiaphragm preparation

3-hydroxypiperidinium, 4-hydroxypiperidinium and 3-hydroxymethylpiperidinium were found to cause a slowly developing block of the rapidly stimulated muscle (1Hz) but had no effect on the more slowly stimulated muscle (0.1Hz) at the doses employed. When choline was added to the incubating medium in the presence of one of the hydroxypiperidinium 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 these analogues gave a frequency dependent prejunctional block at the doses employed (Table 6.1). 4-hydroxypiperidinium was about twice as potent in this respect as 3-hydroxypiperidinium and 3-hydroxymethylpiperidinium.

Hydroxypyridinium did not give a prejunctional block. Instead this analogue caused a block of both the slow and rapidly stimulated muscles which was not reversible with choline. When the drug was washed out of the bath the muscle contractions eventually returned to the control height of contraction. It was concluded that this analogue gave a postjunctional block (Table 6.1).

6.3.1.2 Frog rectus abdominis muscle

3-hydroxypiperidinium, 4-hydroxypiperidinium and hydroxypyridinium (0.4 to 1.9 $\mu\text{mole/ml}$) all slightly enhanced the ACh induced contraction of

Analogue	Minimum effective dose giving a prejunctional block ($\mu\text{mole/ml}$)	Minimum effective dose giving a post-junctional block ($\mu\text{mole/ml}$)
3-hydroxypiperidinium	2.7	
4-hydroxypiperidinium	1.2	
3-hydroxymethylpiperidinium	2.6	
4-hydroxypyridinium	No block	3.2

TABLE 6.1:- Effect of the hydroxypiperidinium and hydroxypyridinium analogues on nerve transmission in the rat phrenic nerve-hemidiaphragm preparation. Any prejunctional block could be reversed by choline (0.02 to 0.14 $\mu\text{mole/ml}$). Those compounds giving a prejunctional block were not tested for postjunctional activity in this preparation. Each experiment was repeated twice. $\pm\text{SEM}$ were 10% or less.

Analogue	Enzyme			Substrate
	Bovine Erythrocyte (AChE)	Horse Serum (ChE)	Rat Brain Homogenate	
3-hydroxy-piperidinium	1.7×10^{-2}	5.7×10^{-2}	5.6×10^{-3} 2.1×10^{-3} 8.9×10^{-2}	ACh Ac- β -MeCh BuCh
4-hydroxy-piperidinium			1.6×10^{-2}	ACh
3-hydroxy-methyl-piperidinium			1.7×10^{-2}	ACh
hydroxy-pyridinium	2.4×10^{-3}	2.2×10^{-3}	6.7×10^{-3} 1.4×10^{-3} 4.9×10^{-2}	ACh Ac- β -MeCh BuCh
4-acetoxy-piperidinium	2.7×10^{-2}	9.4×10^{-2}	9.1×10^{-3} 8.2×10^{-3} 6.2×10^{-2}	ACh Ac- β -MeCh BuCh

TABLE 6.2:- IC₅₀ (molar) values for the inhibition of cholinesterase activity by the hydroxypiperidinium+hydroxypyridinium analogues.

Incubation mixtures contained enzyme, [¹⁴C]-labelled substrate and a range of concentrations of each inhibitor. A Dixon plot was drawn for each compound and the IC₅₀ values are calculated from these plots, they represent that concentration of inhibitor which inhibited the hydrolysis of the various substrates by 50%. For each plot regression lines with correlation coefficients of 0.98 to 0.99 were drawn through at least five points. Each point was the mean of at least three determinations \pm SEM of 10% or less.

the frog rectus abdominis muscle. However, 3-hydroxymethylpiperidinium was shown to have a direct depolarising action on the muscle at a dose of 0.7 μ mole/ml and above. The equipotent molar ratio of this compound compared to ACh was 887:1.

6.3.2. Anticholinesterase activity

The effect of the hydroxypiperidinium and hydroxypyridinium analogues on cholinesterase activity was determined by including these analogues in incubation mixtures containing enzyme, buffer and radioactive substrate as described in the Methods (Section 3.4). The degree of hydrolysis of the substrate was then compared with that obtained in controls containing distilled water in place of the analogues.

The activities of the hydroxypiperidinium and hydroxypyridinium analogues are shown in Table 6.2. All the analogues were shown to inhibit both ChE and AChE. However, they were all very much weaker in this respect than physostigmine (Table 4.3).

6.3.3 In vitro acetylation by ChAc

The rate of acetylation of the hydroxypiperidinium and hydroxypyridinium analogues was determined by incubating the analogues at 37° for 10min. with [¹⁴C]-AcCoA and a partially purified extract of ChAc obtained from rat brain. The acetylated products were routinely isolated and quantitatively assayed by following the method of Fonnum, (1975). The rates of acetylation of the analogues were compared to that of choline. Blank determinations were obtained by substituting the substrate for distilled water.

The relative rates of *in vitro* acetylation of the hydroxypiperidinium and hydroxypyridinium analogues were determined and compared to the acetylation of choline (Fig. 6.1), 3-hydroxypiperidinium was acetylated at a rate of 55% compared to choline 100%.

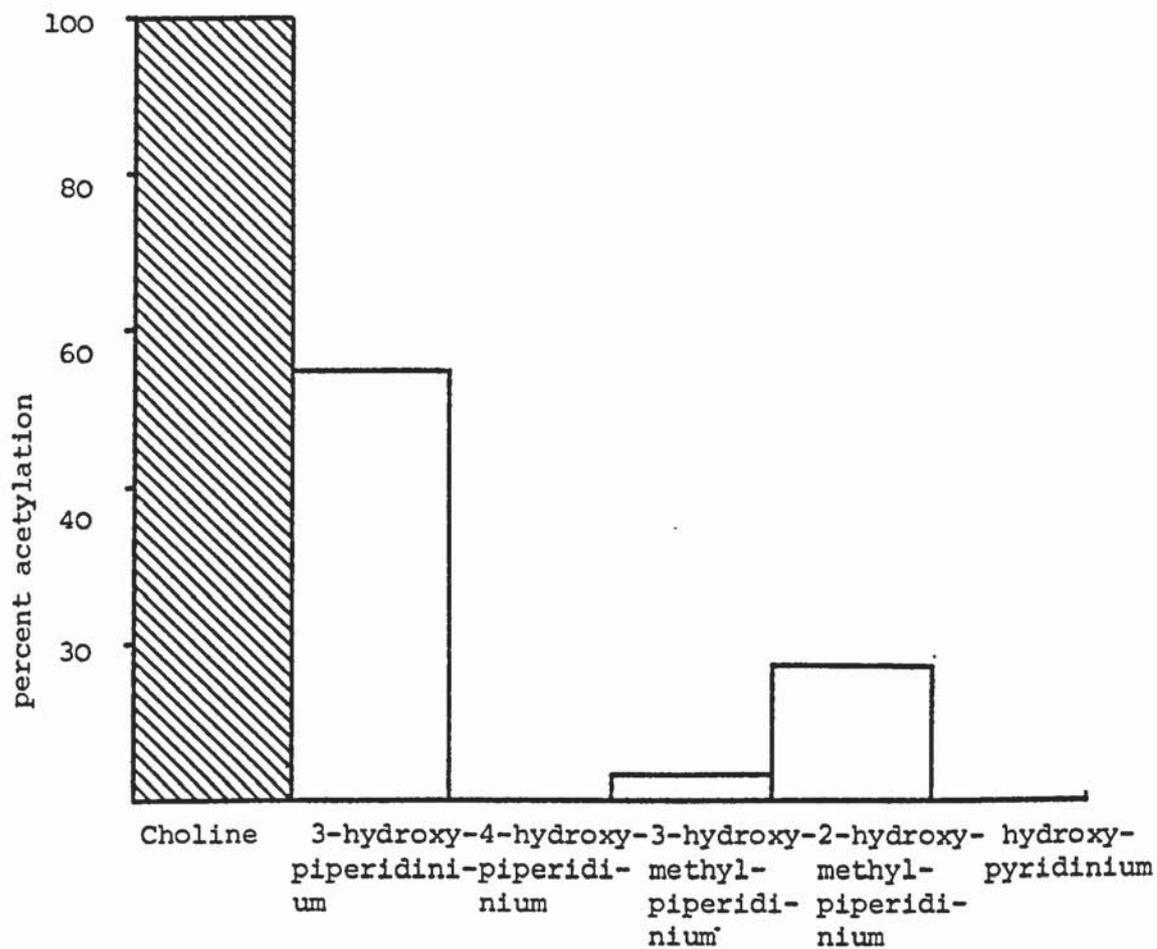


Fig. 6.1:- The rates of acetylation of choline and the hydroxypiperidinium and hydroxypyridinium analogues by ChAc extracted from rat brain. The incubation was carried out for 10min. at 37°. The final substrate concentration was 20mM. Each experiment was performed in duplicate and repeated six times \pm SEM were less than 5%.

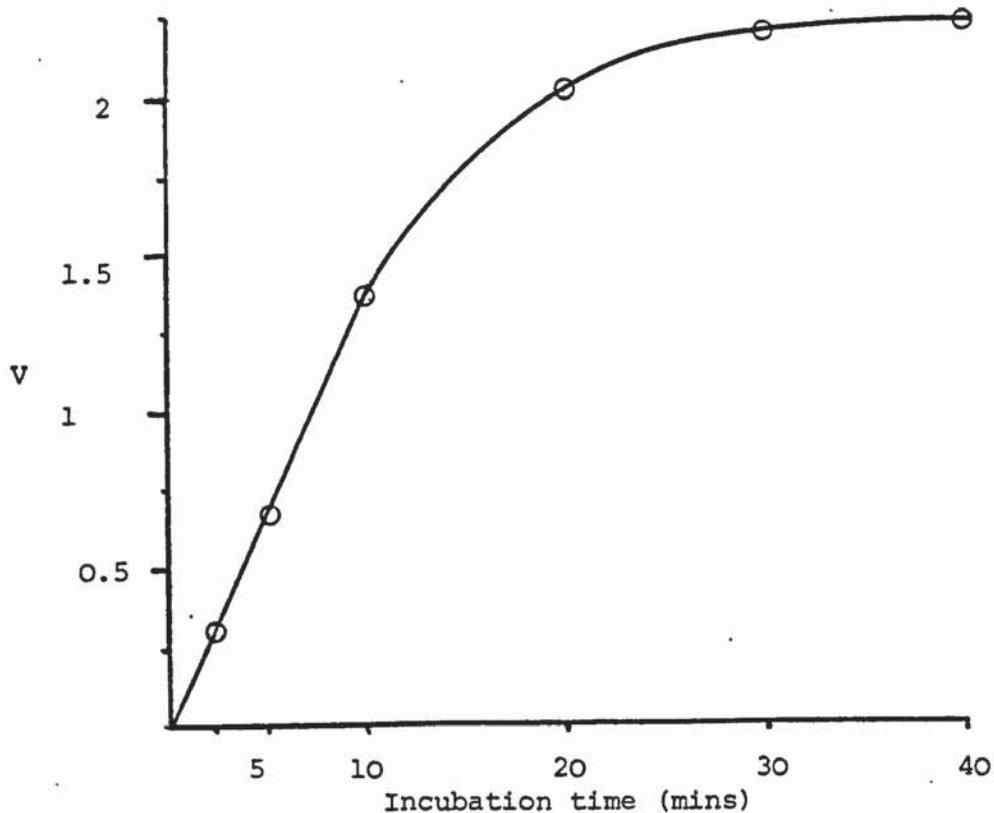
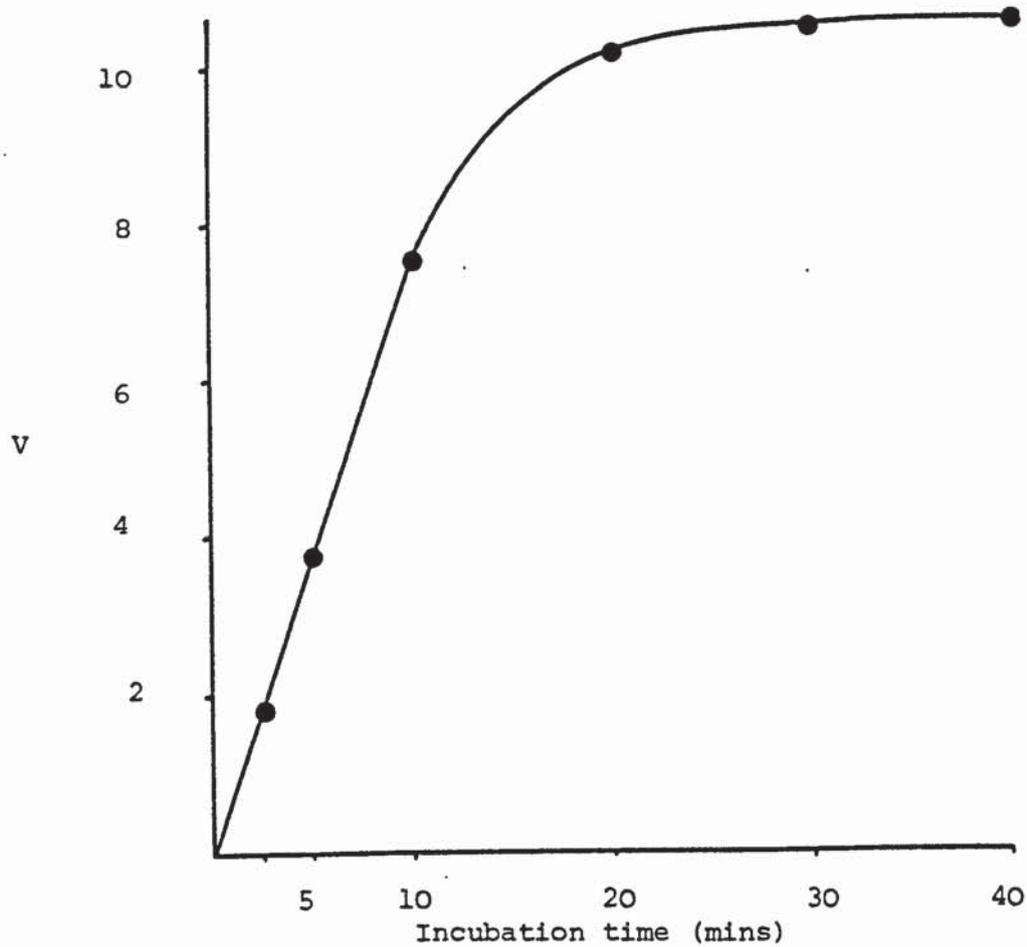


Fig. 6.2:- The rate of acetylation of 3-hydroxypiperidinium by ChAc extracted from rat brain. Incubation was at 37°. V=μmole of [¹⁴C]-acetylated-3-hydroxypiperidinium/g of protein. Concentration of 3-hydroxypiperidinium 200mM(**●**), 200μM(**○**) The experiments were performed in duplicate and repeated twice. ±SEM were less than 10%.

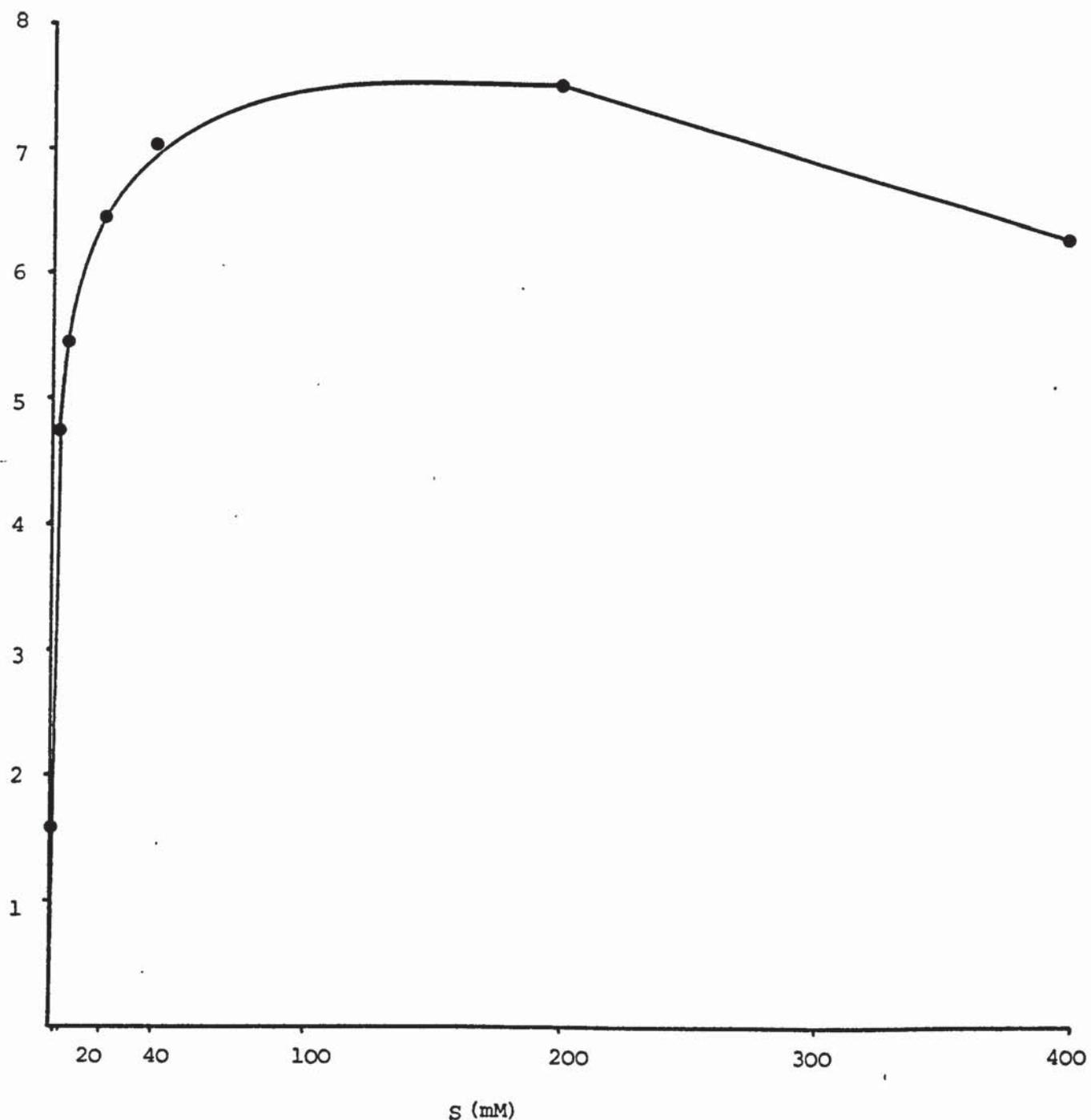


Fig. 6.3:- Velocity/substrate curve of the acetylation of 3-hydroxypiperidinium by ChAc extracted from rat brain. Incubation was at 37° for 10min. $v = \mu\text{mole of } [^{14}\text{C}]\text{-acetylated-3-hydroxypiperidine/g of protein/10min.}$ The experiment was performed in duplicate and repeated twice $\pm\text{SEM}$ were less than 10%.

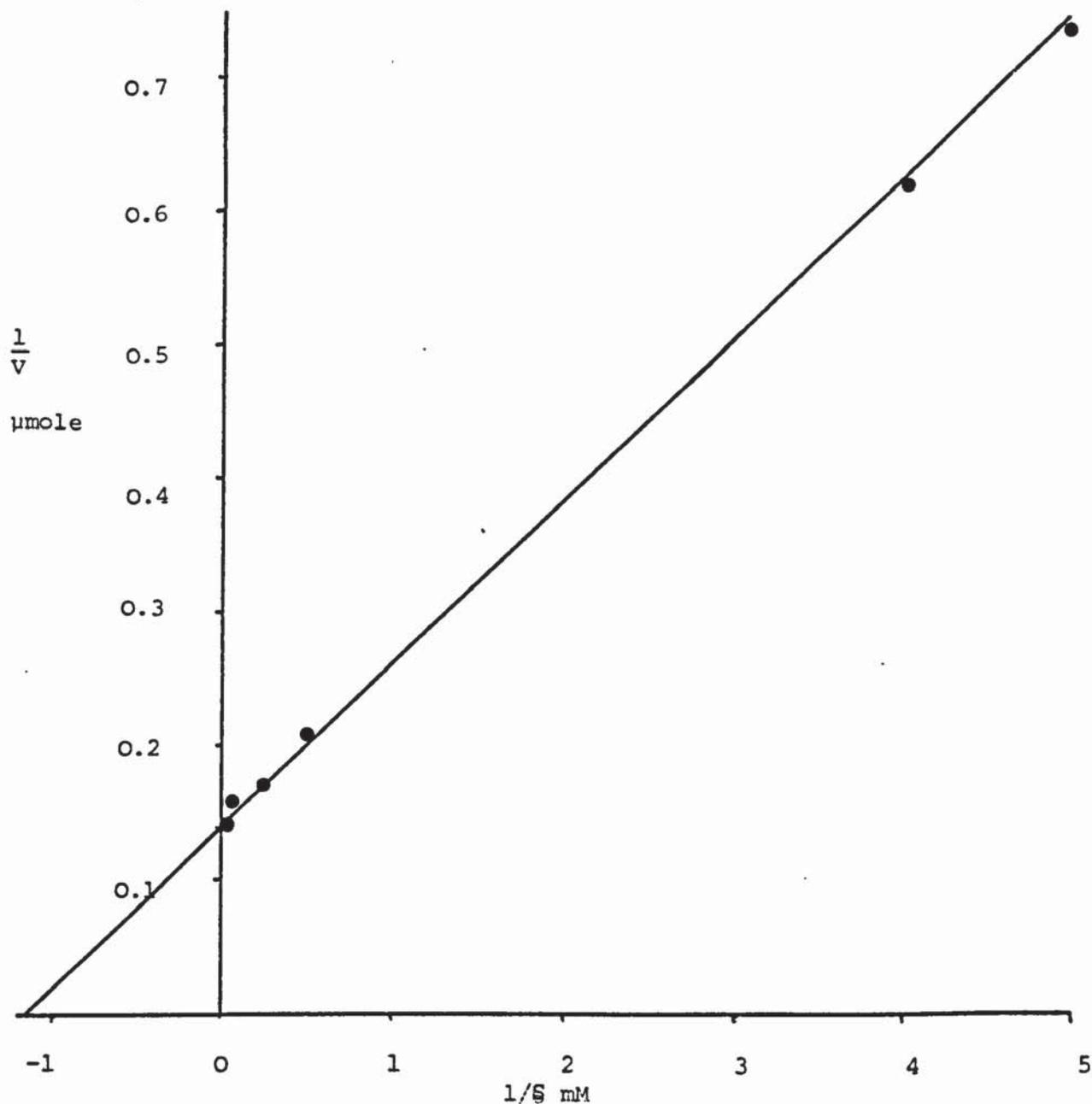


Fig. 6.4:- Acetylation of 3-hydroxy-piperidinium by ChAc extracted from rat brain. Derivation of Michaelis-Menten constants from a Lineweaver-Burke (1934), plot. Incubation was at 37° for 10min $V = \mu\text{mole}$ of [^{14}C]-acetylated-3-hydroxypiperidinium/g of protein/10min. The apparent K_M for 3-hydroxypiperidinium (concentration of AcCoA was 18 μM) was 0.88mM and the V_{max} was 7.4 $\mu\text{mole/g/10min}$ (choline, not shown had a K_M of 0.29mM). Each point is the mean of six determinations. $\pm\text{SEM}$ less than 10%.

Because of the relatively high rate of acetylation seen, the acetylation of 3-hydroxypiperidinium by ChAc was further investigated. Time studies demonstrated that the rate of acetylation of 3-hydroxypiperidinium was linear for at least 10min. at the concentrations employed. (Fig. 6.2). The plot of velocity of acetylation against substrate concentrations showed a typically shaped curve for an enzyme-one substrate reaction (Fig. 6.3). However, at a substrate concentration of 400mM substrate inhibition was seen. The apparent Michaelis-Menten constants were derived from the plot (Fig. 6.4), according to Lineweaver and Burke (1934), at a constant concentration of AcCoA. 3-hydroxypiperidinium had a K_m of 0.88mM which is about three times that of choline.

6.3.4 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 or hydroxypyridinium analogues and the amount of acetylated product synthesised was assayed. These incubations were compared with control incubations containing the same concentration of choline, but distilled water instead of the analogue, (Table 6.3). The method of isolation of the acetylated 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-hydroxymethyl piperidinium which have been shown to be acetylated by ChAc (Section 6.3.3). However, it should be remembered that in these experiments choline, the natural substrate for ChAc, is present in relative high concentrations and therefore will presumably favourably compete with these choline analogues for the enzyme. In this study the most potent inhibitor of ChAc is hydroxypyridinium.

Inhibitor	Percent Inhibition
3-hydroxypiperidinium	5 (\pm 0.4)
4-hydroxypiperidinium	0 (\pm 0.01)
3-hydroxymethylpiperidinium	0 (\pm 0.01)
2-hydroxymethylpiperidinium	5 (\pm 0.02)
hydroxypyridinium	23 (\pm 1.2)

TABLE 6.3:- Percentage inhibition of the acetylation of choline by ChAc extracted from rat brain. Both choline and the hydroxypiperidine and hydroxypyridine analogues were at a concentration of 14mM. Incubation was carried out at 37^o for 10min. Each value is the average of four determinations (\pm SEM).

6.3.5 Transport of [^3H]-choline and [^{14}C]-3-hydroxypiperidinium 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 and Snyder, 1972; 1973).

6.3.5.1 Inhibition of the high affinity transport of choline by the hydroxypiperidinium and hydroxypyridinium analogues

A crude preparation of synaptosomes was incubated with [^3H]-choline ($1\mu\text{M}$, $0.55\mu\text{Ci/nmole}$) at 37° and 0° . The transport of choline into synaptosomes at 37° was linear for 7min. (Fig. 4.21). The amount of choline accumulated by synaptosomes at 37° was 4-6 times greater than that accumulated at 0° for 7min. The inhibition by the hydroxypiperidinium and hydroxypyridinium analogues was determined by adding the compounds to the buffer containing [^3H]-choline ($1\mu\text{M}$) and synaptosomes and incubating at 37° for 7min. The concentration of the analogues inhibiting the transport of choline into synaptosomes by 50% (IC_{50}) (Table 6.4) was determined graphically from a Dixon plot, (1953), and one such plot is shown for 3-hydroxypiperidinium (Fig. 6.5). In this study 3-hydroxymethylpiperidinium was the most potent inhibitor of choline transport.

6.3.5.2 Transport of [^{14}C]-3-hydroxypiperidinium into synaptosomes

[^{14}C]-3-hydroxypiperidinium ($9\mu\text{M}$; $0.55\mu\text{Ci/9nmole}$), was prepared as described in Section 6.2. and it was incubated with a crude preparation of synaptosomes in KRP buffer. Non-radioactively labelled 3-hydroxypiperidinium ($2\mu\text{M}$ to $500\mu\text{M}$) was added to the incubation medium to give final concentrations of $11\mu\text{M}$ to $509\mu\text{M}$. The final incubation volume was 1ml.

Inhibitor	IC ₅₀ (μM)
<u>Hydroxypiperidinium and hydroxypyridinium analogues</u>	
3-hydroxypiperidinium	31
4-hydroxypiperidinium	33
2-hydroxymethylpiperidinium	14
3-hydroxymethylpiperidinium	8
hydroxypyridinium	76

TABLE 6.4:- Inhibition of high-affinity choline transport into synaptosomes. The IC₅₀ values were determined graphically from Dixon plots (1953). 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.M. of 10% or less. The synaptosomes were incubated with [³H]choline (1μM) and inhibitor for 7min. at 37°.

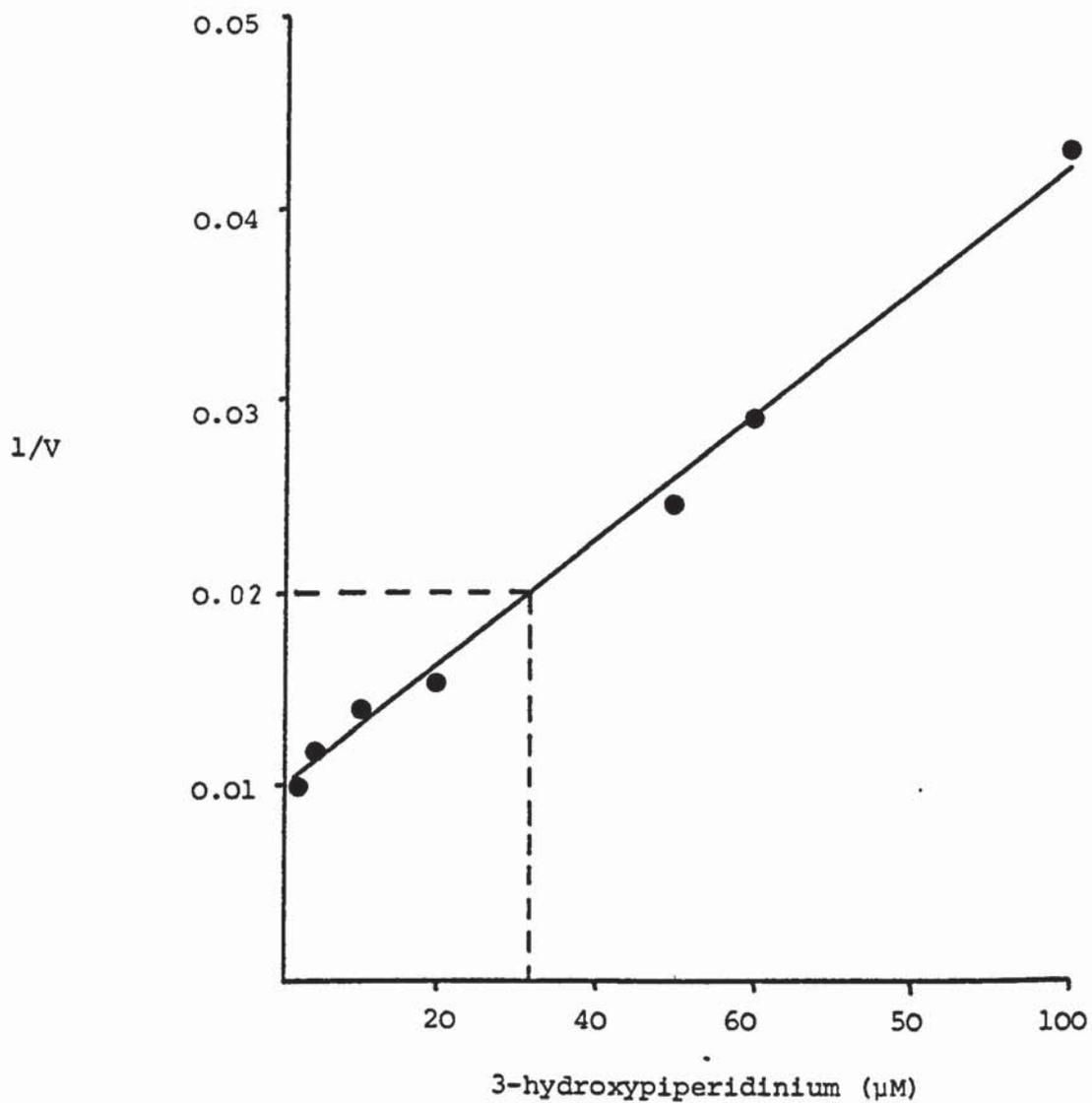


Fig. 6.5:- Dixon (1953) plot of the inhibition of the high affinity transport of choline into synaptosomes by 3-hydroxypiperidinium V is the % uptake compared to control (no inhibitor). Each point is the mean of at least five determinations \pm SEM of 10% or less. The correlation coefficient is 0.98. The synaptosomes were incubated with [3 H]-choline (1 μ M) and inhibitor for 7 min at 37 $^{\circ}$.

The amount of 3-hydroxypiperidinium accumulated by the synaptosomes was calculated by taking into consideration the specific activity of the $[^{14}\text{C}]$ -3-hydroxypiperidinium at the various concentrations used, it was expressed as μmole of $[^{14}\text{C}]$ -3-hydroxypiperidinium transported into synaptosomes/g of protein/7min. The accumulation of 3-hydroxypiperidinium by synaptosomes at 37° was about three times that at 0° . Values obtained at 0° were subtracted from the values obtained at 37° . The transport of 3-hydroxypiperidinium was shown to be linear for 7min. for the range of concentrations chosen (Fig. 6.6).

A Lineweaver-Burke plot was drawn (Fig. 6.7). The curve could be resolved into two distinct components. This is similar to the transport of choline into synaptosomes (Yamamura and Snyder, 1973). Lower concentrations of 3-hydroxypiperidinium (11-33 μM) gave a straight line as calculated by regression analysis, with a correlation coefficient of 0.98. This component presumably represents the high affinity transport of 3-hydroxypiperidinium into synaptosomes, the K_{T_H} value was 6.7 μM and the V_{max} was 0.039 $\mu\text{mole/g}$ of protein/7min. Higher concentrations (49-509 μM) gave the other straight line, with a correlation coefficient of 0.99. This component represents the low affinity transport of 3-hydroxypiperidinium into synaptosomes, the K_{T_L} was 192.9 μM and the V_{max} was 0.235 $\mu\text{mole/g}$ of protein/7min.

Studies with $[^3\text{H}]$ -choline concurred with the work of Yamamura and Snyder (1973), in that the transport of choline into a crude preparation of synaptosomes could be resolved into two components. The K_{T_H} value was 3.5 μM , the V_{max} was 0.13 $\mu\text{mole/g}$ of protein/7min., the K_{T_L} was 35 μM and the V_{max} was 0.4 $\mu\text{mole/g}$ of protein/7 min. Thus the K_{T_H} of 3-hydroxypiperidinium is twice that of choline.

The biphasic plot (Fig. 6.7), is only an indication that there are two distinct transport mechanisms for 3-hydroxypiperidinium. Further

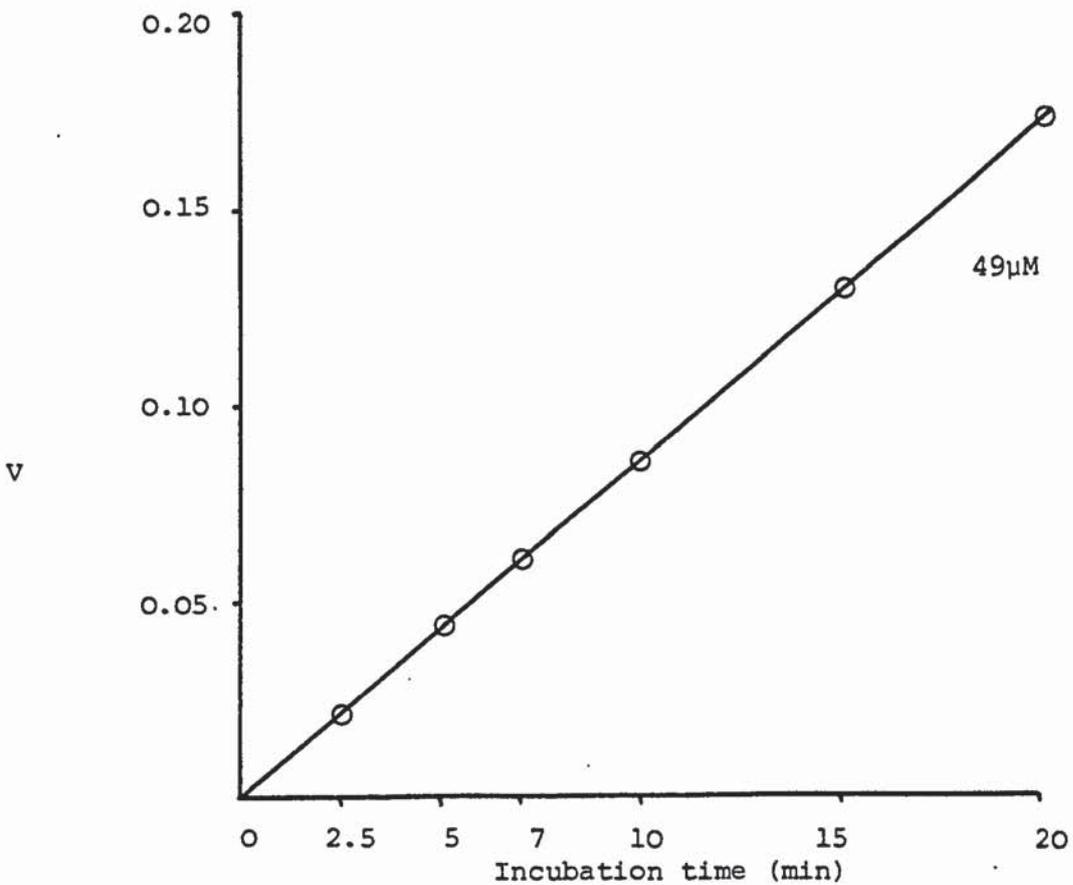
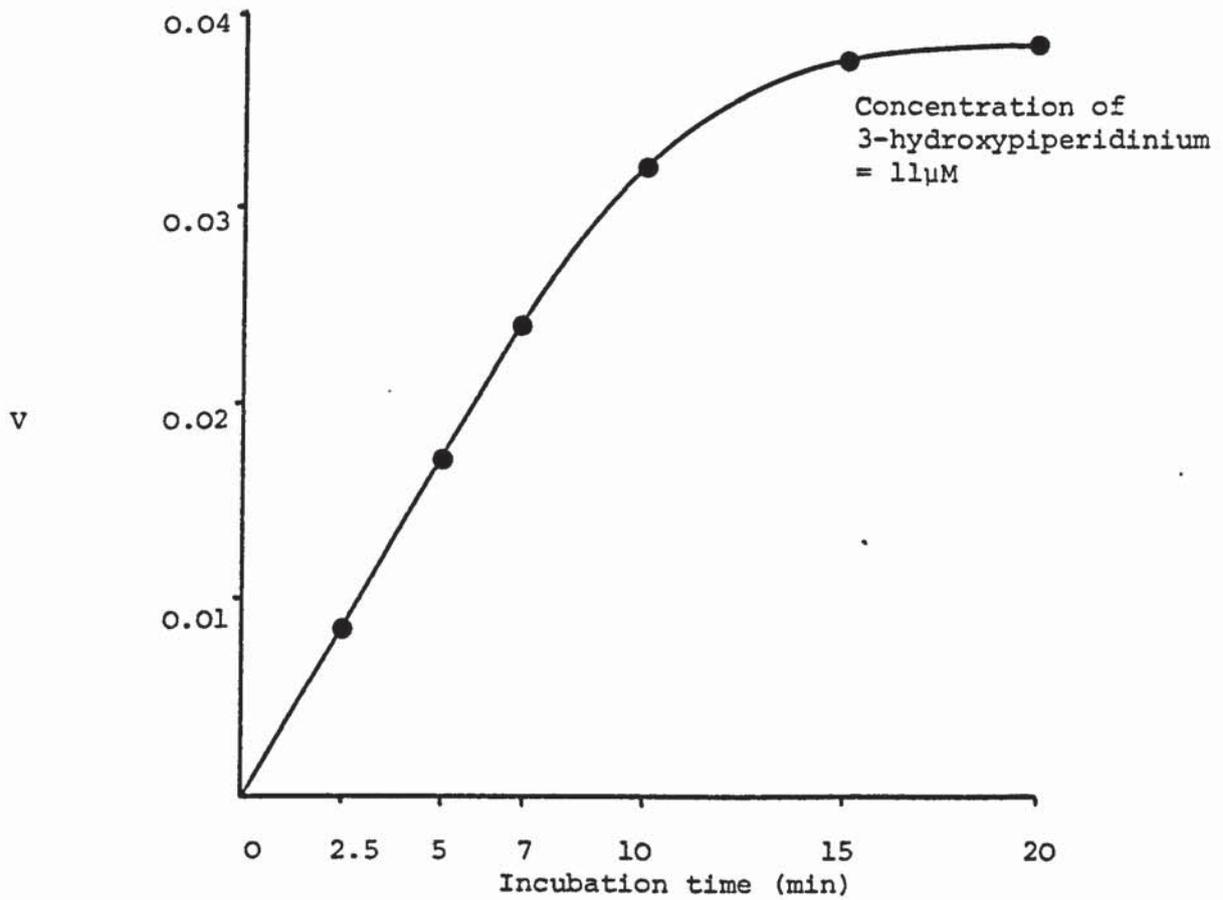


Fig. 6.6:- Velocity/time curves for the transport of $[^{14}\text{C}]$ -3-hydroxypiperidinium into synaptosomes. V is expressed as μmole of $[^{14}\text{C}]$ -3-hydroxypiperidinium accumulated/g of protein at 37° . Each point is the mean of six determinations. $\pm\text{SEM}$ of 10% or less.

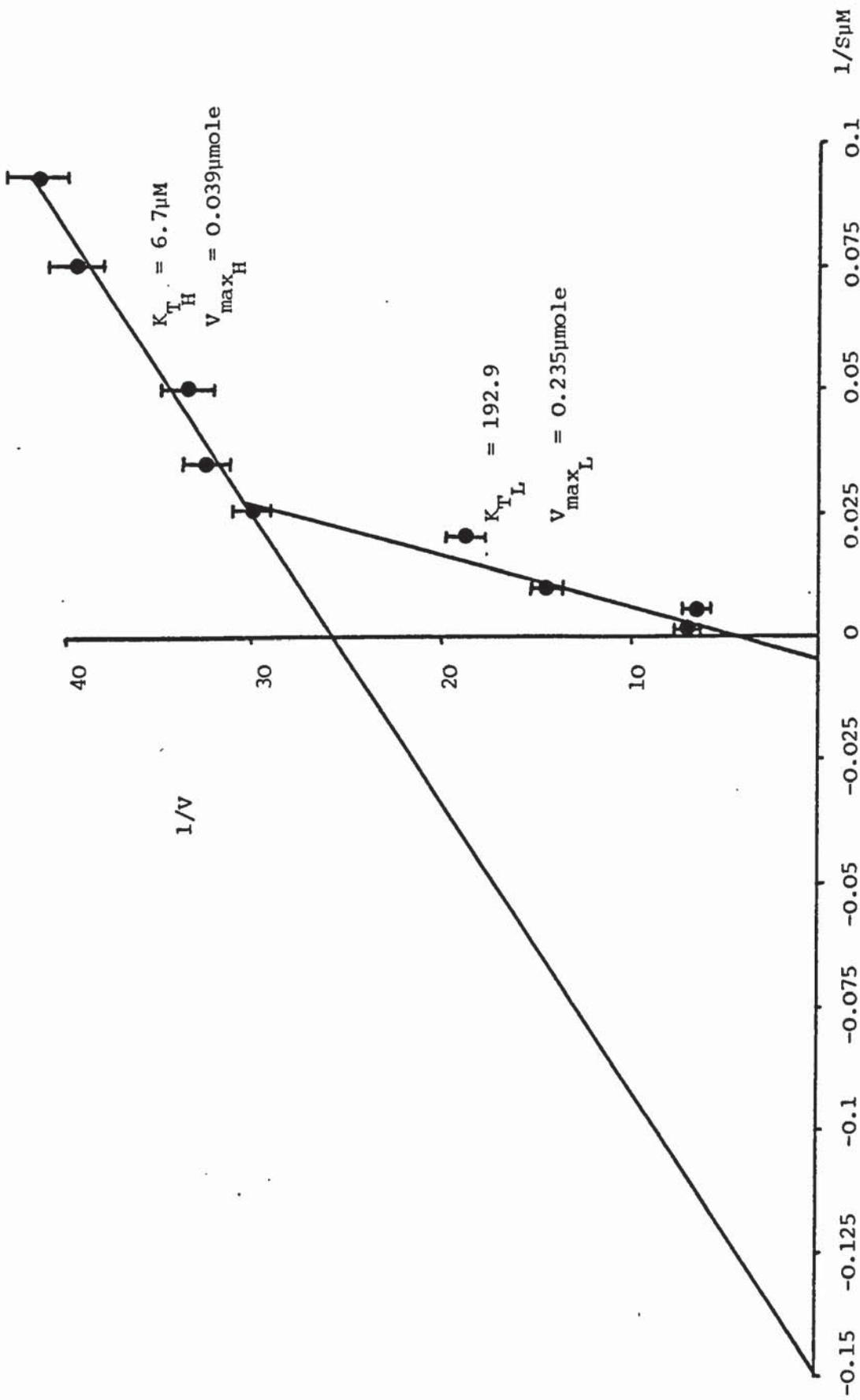


Fig. 6.7:- Double-reciprocal plot of the transport of [¹⁴C]-3-hydroxy-piperidinium into synaptosomes. Each point is the mean of at least four determinations (\pm SEM). K_T is that concentration of substrate which provides one half the maximum velocity uptake, and was calculated for the high affinity (H) and low affinity (L) transport systems using Michaelis-Menten Kinetics. V is expressed as μmole of [¹⁴C]-3-hydroxypiperidinium accumulated/g of protein in 7min. Synaptosomes were incubated at 37° for 7 min.

confirmation of the two component system would come from a study of metabolic inhibitors and a reduction in sodium concentration; since the high affinity transport system for choline, unlike the low affinity system, has been shown by some workers to be more sensitive to metabolic inhibitors and to be sodium dependent (Yamamura and Snyder, 1973). Consequently further study was conducted taking into consideration the fact that the proposed high affinity transport would contribute more to the uptake seen at lower concentrations, whereas the proposed low affinity transport would contribute more to the uptake seen at higher concentrations. Hence, the effect of some metabolic inhibitor and other drugs on the proposed high and low affinity transport systems was measured at 11 μ M and 49 μ M respectively. (Table 6.5). DNP, KCN and ouabain seemed to inhibit the transport of 3-hydroxypiperidinium at both concentrations to about the same extent. Haga and Noda (1973), unlike Yamamura and Snyder (1973), did not find that NaCN or DNP significantly affected the high affinity transport of choline into synaptosomes. Hence, the results presented in this thesis are not incompatible with the hypothesis that 3-hydroxypiperidinium is transported into synaptosomes by a high and low affinity mechanism. Ouabain was a weak inhibitor of the transport of 3-hydroxypiperidinium into synaptosomes and it is possible that a sufficiently high concentration was not used.

HC-3 and choline, however, both inhibited the high affinity transport more than the low affinity transport as shown (Table 6.5). The latter results indicate that 3-hydroxypiperidinium is transported by the two component choline transport system.

The transport of 3-hydroxypiperidinium into synaptosomes was studied at reduced sodium concentrations (Table 6.6). The high affinity transport system was more sensitive to sodium than the low affinity system. This is similar to the transport of choline where again the high affinity system was more sodium dependent than the low affinity system (Yamamura and Snyder, 1973; Haga and Noda 1973).

Inhibitor	Concentration of $[^{14}\text{C}]-3\text{-hydroxypiperidinium}$	
	11 μM	49 μM
DNP 200 μM	30%	37%
KCN 100 μM	55%	64%
Ouabain 100 μM	5%	11%
HC-3 2 μM	72%	40%
" 100 μM	77%	75%
Choline 100 μM	100%	33%

TABLE 6.5:- Effect of some metabolic inhibitor and other drugs on the transport of $[^{14}\text{C}]-3\text{-hydroxypiperidinium}$ into synaptosomes.

Synaptosomes were incubated with $[^{14}\text{C}]-3\text{-hydroxypiperidinium}$ at 37° for 7min. Results are expressed as percentage inhibition of uptake compared to control value (incubation in the absence of inhibitor) of 0%. Values are the mean of four determinations. $\pm\text{S.E.M.}$ were less than 10%.

Incubation Conditions	$[^{14}\text{C}]$ -3-hydroxypiperidinium accumulated at the following concentrations			
	13 μM	19 μM	59 μM	209 μM
NaCl (185mM) (control)	100%	100%	100%	100%
NaCl (10mM) and LiCl (175mM)	53%	58%	89%	91%

TABLE 6.6:- Influence of sodium ions on the transport of $[^{14}\text{C}]$ -3-hydroxypiperidine into synaptosomes. Synaptosomes were incubated with $[^{14}\text{C}]$ -3-hydroxypiperidinium 37° for 7min. in the appropriate Krebs-phosphate buffer. The sodium chloride concentration was as shown. Values are the mean of four determinations. \pm S.E.M. were less than 10%.

It is concluded that 3-hydroxypiperidinium was transported by both a high and low affinity mechanism into a crude preparation of synaptosomes.

6.3.5.3 Identification of the radioactive materials accumulated by synaptosomes

In these experiments a crude preparation of synaptosomes was incubated with $[^{14}\text{C}]$ -3-hydroxypiperidinium ($11\mu\text{M}$) at 37° for 7min. as described (Section 3.6). After incubation, the contents of the synaptosomes were extracted and non-labelled 3-hydroxypiperidinium and 3-acetoxypiperidinium (synthesised as described in Section 6.2), were added to this extract. The compounds now present in the extract were separated by paper electrophoresis. Two bands corresponding to 3-hydroxypiperidinium and 3-acetoxypiperidinium were visualised with iodine vapour. The electrophoresis paper was cut into strips and any radioactivity on the strips was detected. Two peaks of radioactivity corresponding to the two bands previously visualised with iodine vapour were detected (Fig. 6.9). It is concluded that the $[^{14}\text{C}]$ -3-hydroxypiperidinium is metabolised to $[^{14}\text{C}]$ -3-acetoxypiperidinium by synaptosomes. Apart from the acetylated derivative no other metabolite was detected. Approximately 50% of the accumulated analogue is metabolised.

6.3.6 Mouse phrenic nerve-hemidiaphragm preparation

6.3.6.1 Introduction

3-hydroxypiperidinium, like choline, has been shown to be transported into synaptosomes where, again like choline, it was acetylated, to an ACh analogue the 3-acetoxypiperidinium compound. It was thought possible that 3-acetoxypiperidinium could behave as a false transmitter and be released from the cholinergic nerve terminals by nerve stimulation.

Potter, (1970), showed that by incubating the rat phrenic nerve-

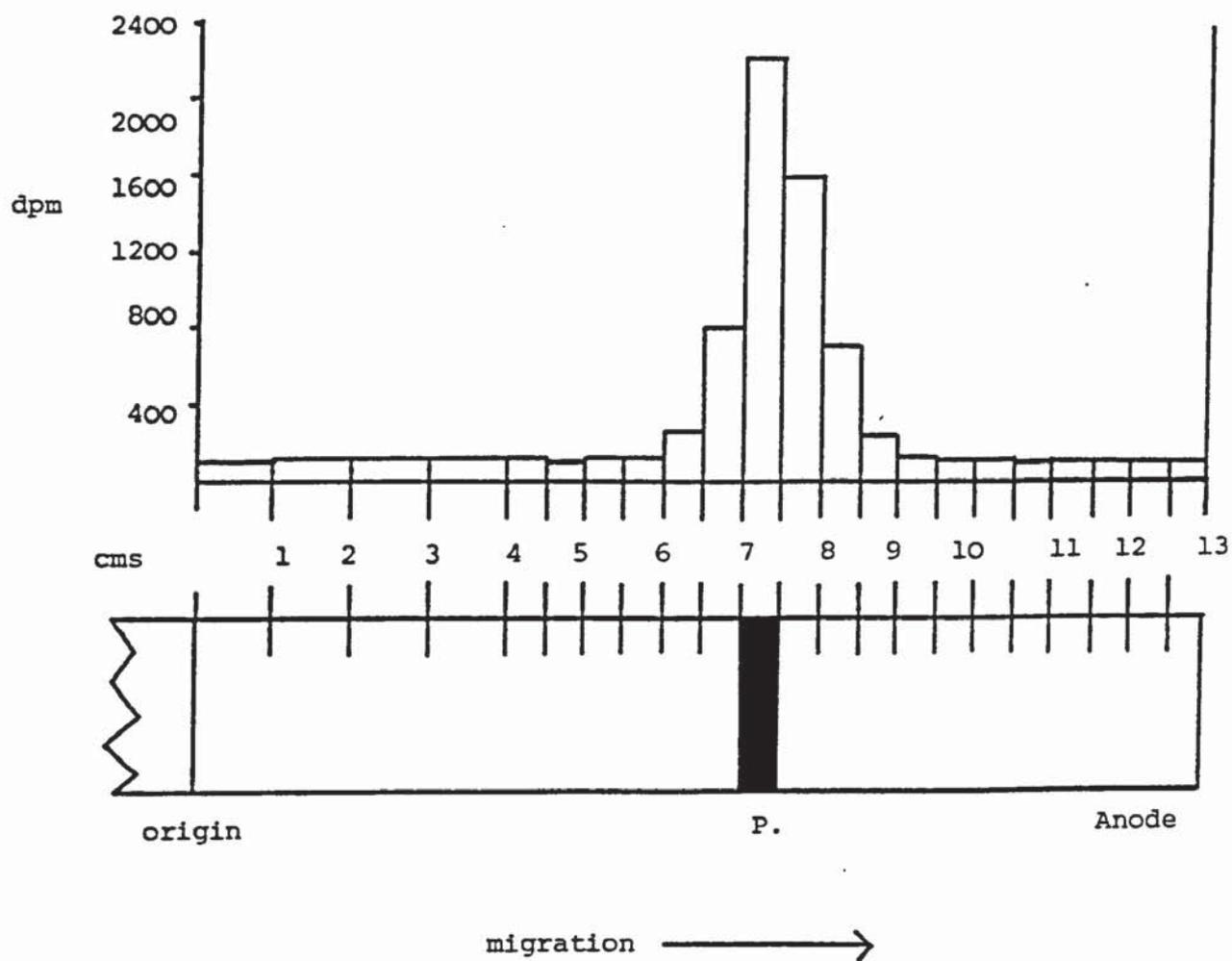


Fig 6.8:- Paper electrophoresis of $[^{14}\text{C}]$ -hydroxypiperidinium (P) after 2hr. following the method of Potter and Murphy (1967).

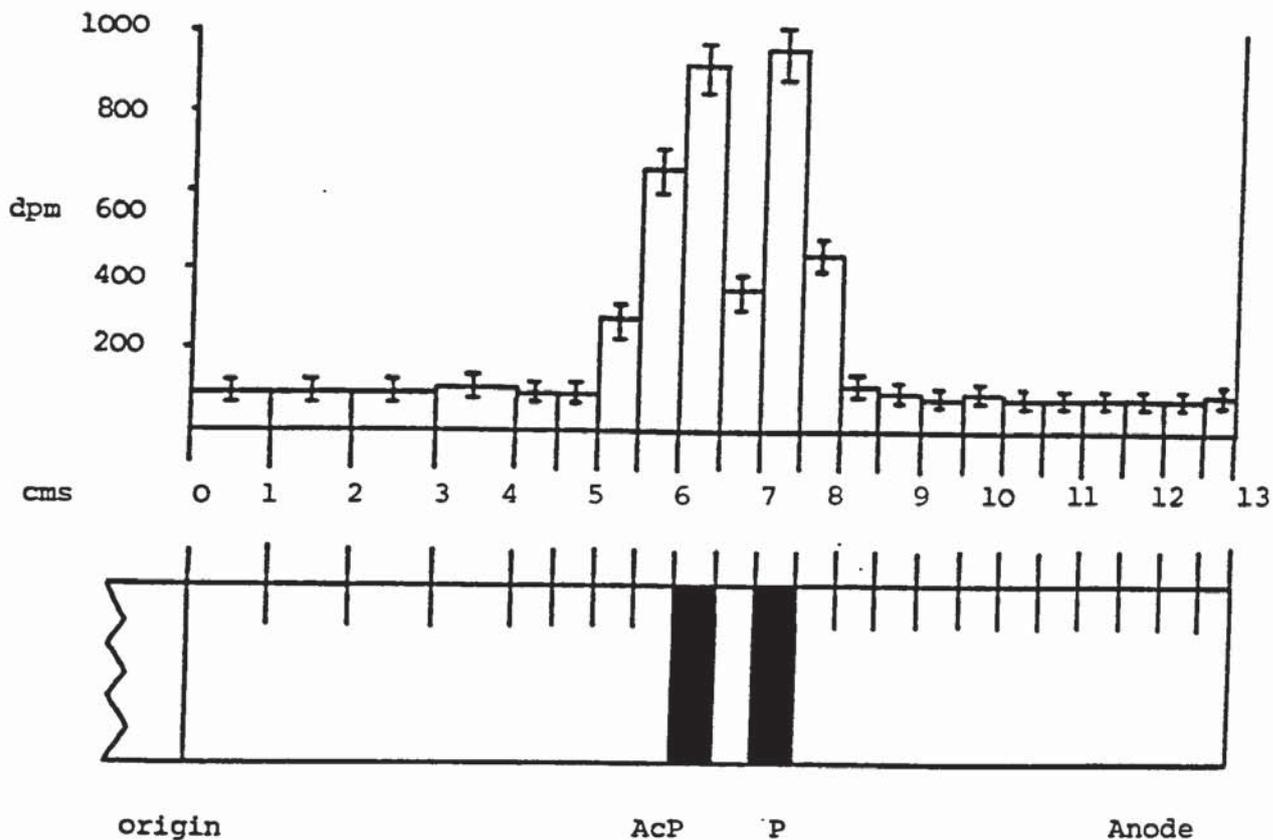


Fig. 6.9:- Paper electrophoresis (as for figure 6.8), of an extract of from synaptosomes that had been incubated with [^{14}C]-3-hydroxy-piperidinium ($11\mu\text{M}$) at 37° for 7min. For each experiment the contents of three incubating tubes were extracted and one third of the pooled extracts was subjected to electrophoresis. The experiment was repeated three times. AcP and P show the position of non-labelled 3-acetoxypiperidinium and 3-hydroxy-piperidinium respectively on the electrophoresis paper and the bar graph above shows the radioactivity detected ($\pm\text{SEM}$) on the corresponding paper strips cut from the electrophoresis paper.

diaphragm in a medium containing $[^{14}\text{C}]$ -choline (30 μM) at 37 $^{\circ}$, the synthesis, storage and release of $[^{14}\text{C}]$ -ACh by this muscle preparation could be studied. Since 3-hydroxypiperidinium is a choline analogue it was thought of interest to incubate this tissue in a medium containing $[^{14}\text{C}]$ -3-hydroxypiperidinium. It was thought possible that $[^{14}\text{C}]$ -3-acetoxypiperidinium could be synthesised by the rat phrenic nerve-diaphragm tissue and then released as a false transmitter. If this was shown to occur then it would be interesting at some future date to study the postsynaptic action of 3-acetoxypiperidinium on the diaphragm muscle using electrophysiological techniques. In our laboratories the mouse diaphragm is used routinely in electrophysiological experiments. Therefore, in order to be able to directly relate the biochemical work presented in this thesis with the proposed electrophysiological study it was decided to use the mouse phrenic nerve-diaphragm preparation in this study, as opposed to the rat preparation used by Potter, (1970).

Potter, (1970), showed that the diaphragm muscle accumulated choline from the incubation medium and there is some evidence that ChAc is present in small amounts in skeletal muscle (Tuček, 1973^vab). Therefore, to increase the likelihood that any acetylation of the 3-hydroxypiperidinium is effected by neuronal ChAc only the nerve-end plate region of the muscle was used.

6.3.6.2 Verification of method

In preliminary experiments the phrenic nerve-end plate region dissected from a mouse diaphragm was preincubated in Krebs containing $[^3\text{H}]$ -choline, washed and then incubated in fresh Krebs as described in the Methods (Section 3.7). The tissue was allowed to rest for 10min. during which time a 100 μl sample of the Krebs was taken at time 0, 5 and 10min. Then the phrenic nerve was stimulated and 100 μl samples subsequently taken at 5, 10 and 20min. after the commencement of stimulation. The amount of

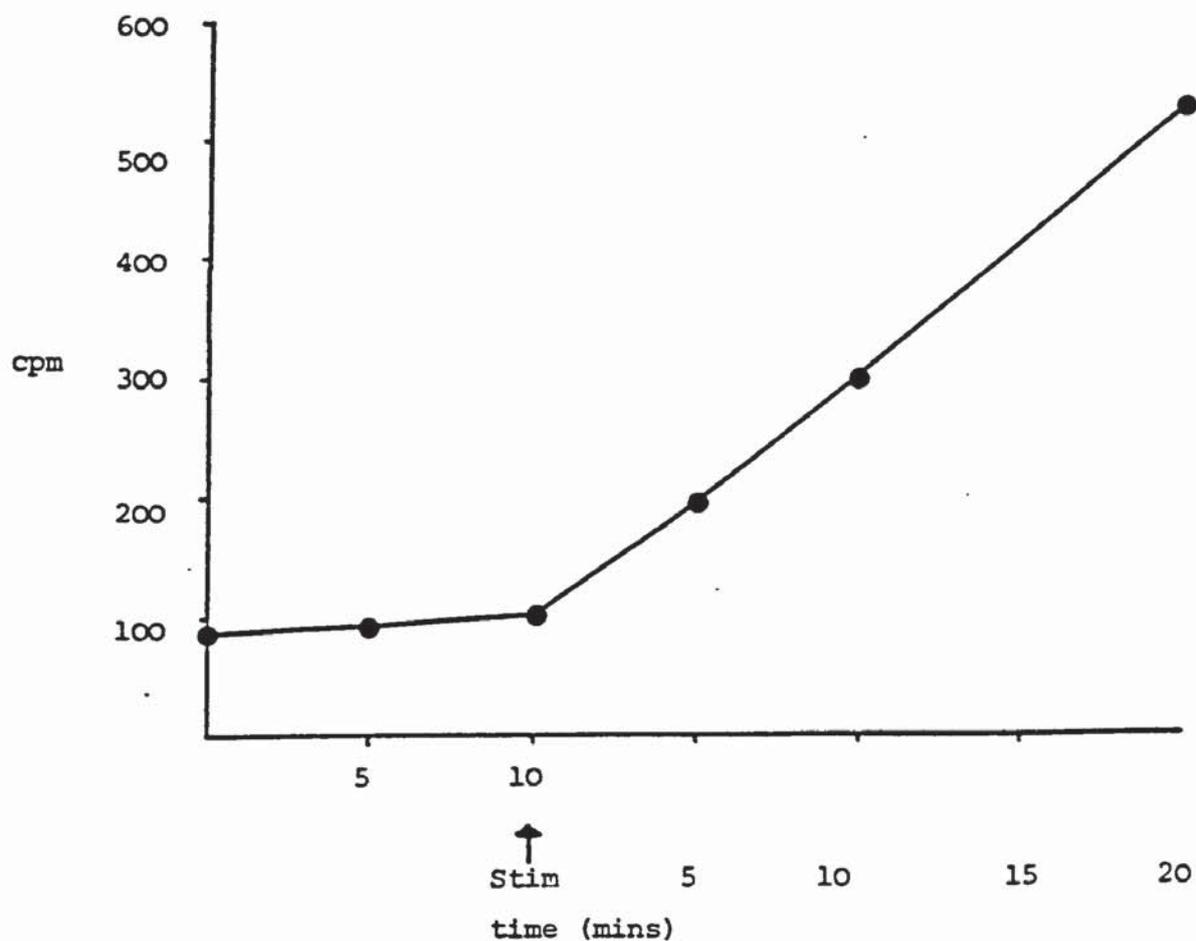


Fig. 6.10:- Radioactivity detected in 100 μ l samples of Krebs bathing the phrenic nerve-end plate region of a mouse hemidiaphragm taken at the times indicated, before and after nervous stimulation. The tissue was preincubated in Krebs containing [3 H]-choline

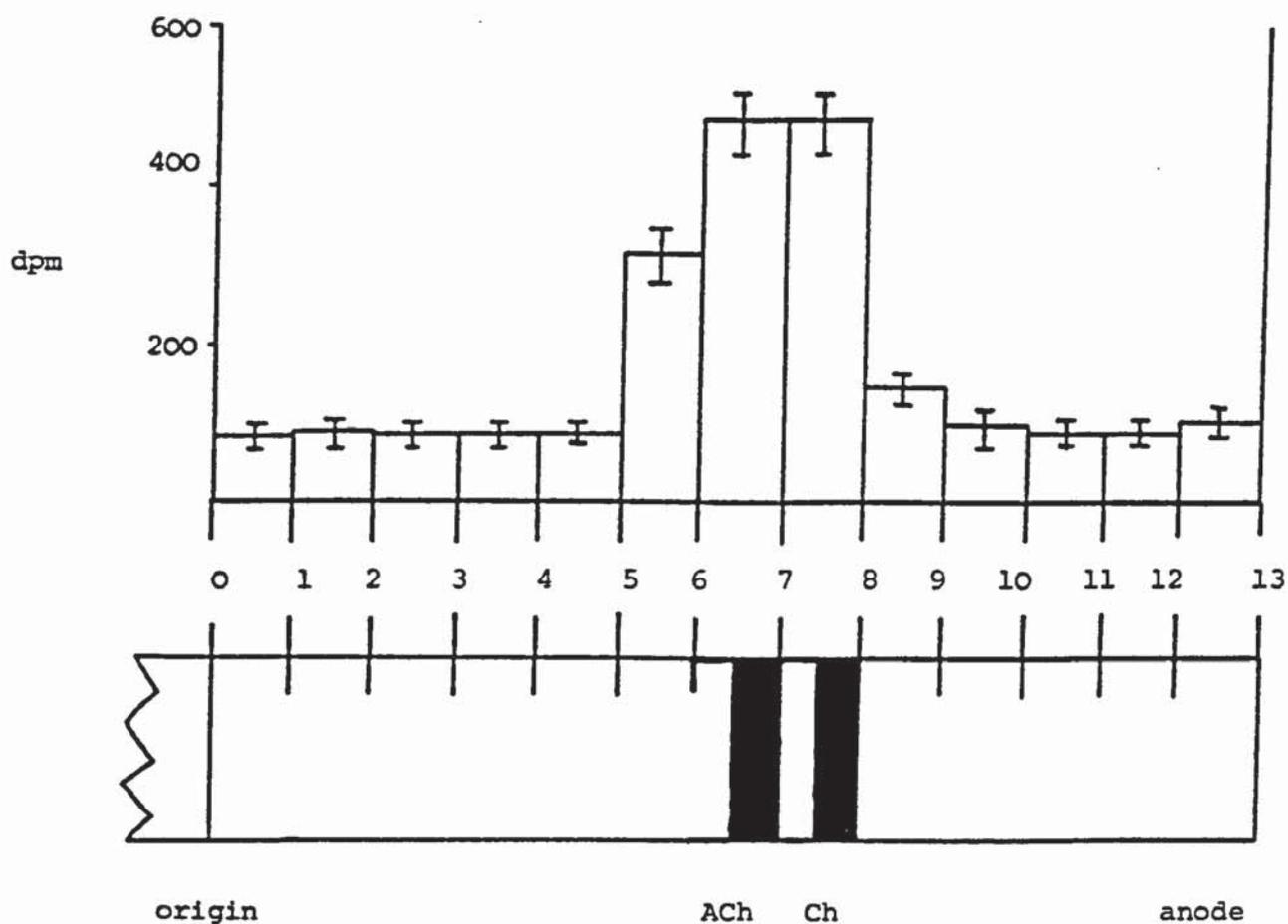


Fig 6.11:- Paper electrophoresis (See Fig. 6.8) of an extract from the phrenic nerve-end plate region of a mouse hemidiaphragm that had been preincubated with [^3H]-choline. One third of the extract has been subjected to electrophoresis. The experiment was repeated three times. ACh and Ch show the position of non-labelled acetylcholine and choline respectively on the electrophoresis paper and the bargraph above shows the radioactivity detected (\pm SEM) on the corresponding paper strips cut from the electrophoresis paper.

radioactivity in these aqueous samples was determined using NE260 as the fluor. A plot of cpm against time is shown (Fig. 6.10), it can be seen that stimulation increased the amount of radioactivity released into the Krebs. At the end of the incubation period (30 min) the tissue was extracted and the compounds present in the extract were identified by paper electrophoresis. Unfortunately in these preliminary experiments the radioactivity present on lcm strips of the electrophoresis paper was determined and two separate peaks of radioactivity could not be seen (Fig. 6.11). However, the results did indicate the presence of ACh. It can be concluded from these experiments that some radioactively labelled compound(s) is released from the tissue on electrical stimulation of the phrenic nerve and that the tissue metabolises choline to ACh. On the basis of this preliminary work and the work of Potter, (1970), further experimentation was conducted with the 3-hydroxypiperidinium compound.

6.3.6.3 Incubation with $[^{14}\text{C}]$ -3-hydroxypiperidinium

The phrenic nerve-endplate region dissected from a mouse diaphragm was pre-incubated in Krebs containing $[^{14}\text{C}]$ -3-hydroxypiperidinium, washed and then incubated in fresh Krebs. After nerve stimulation the surrounding Krebs medium was found to contain $[^{14}\text{C}]$ -3-hydroxypiperidinium and $[^{14}\text{C}]$ -3-acetoxypiperidinium (Fig. 6.12). An extract of the tissue, after nerve stimulation also contained both $[^{14}\text{C}]$ -3-hydroxypiperidinium and $[^{14}\text{C}]$ -3-acetoxypiperidinium (Fig. 6.13). In control experiments where the tissue had been pre-incubated with $[^{14}\text{C}]$ -3-hydroxypiperidinium and then incubated in fresh Krebs without any nervous stimulation, no $[^{14}\text{C}]$ -3-acetoxypiperidinium was detected in the Krebs. Therefore, it can be concluded that the phrenic nerve-endplate region of a mouse diaphragm accumulated 3-hydroxypiperidinium, this compound was then acetylated by the tissue to 3-acetoxypiperidinium which was released as a false transmitter at the neuromuscular junction. There is no evidence of any other radiolabelled metabolite.

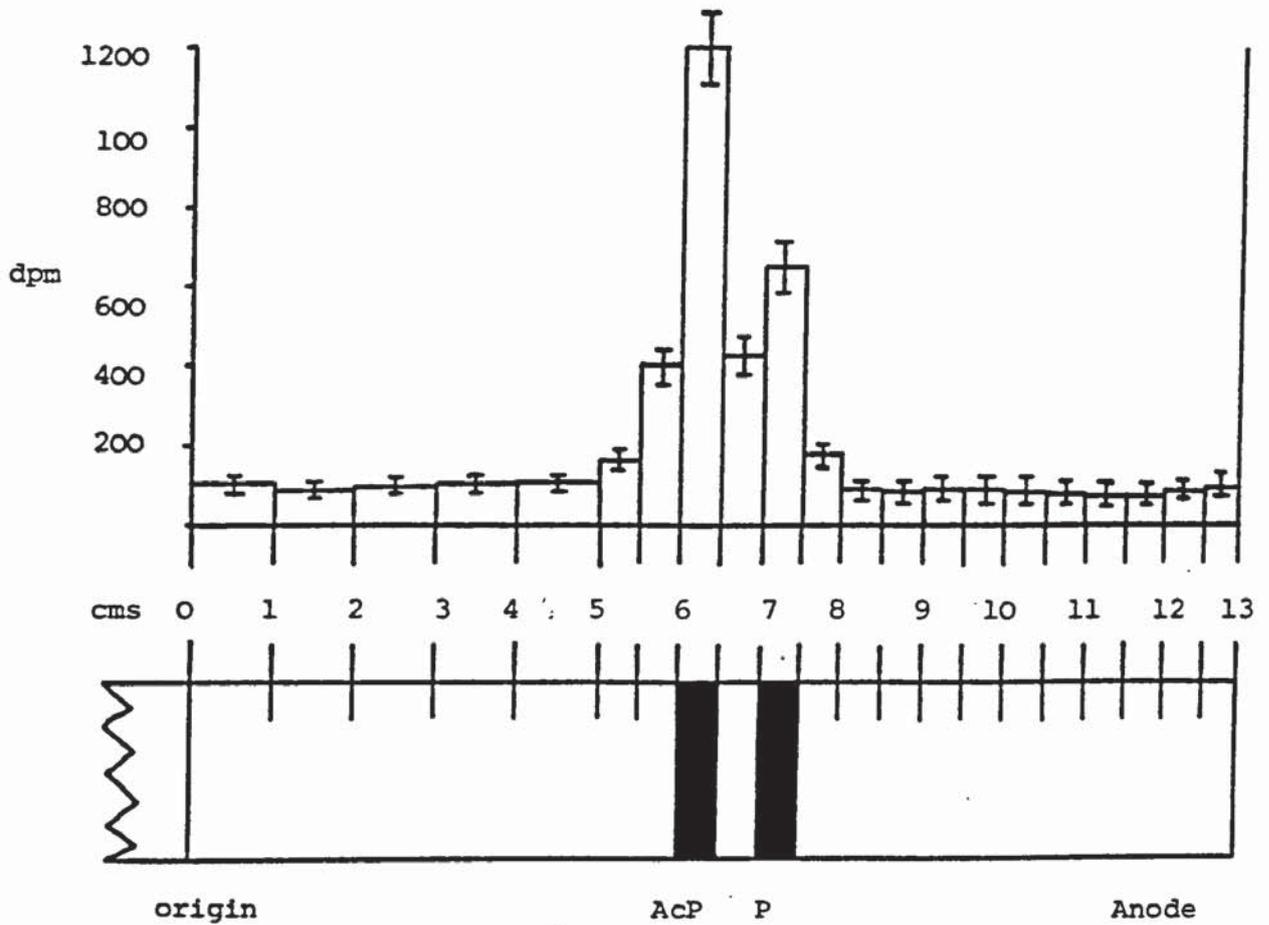


Fig. 6.12:- Paper electrophoresis (See Fig. 6.8) of the Krebs which bathed the electrically stimulated mouse phrenic nerve-diaphragm preparation. This tissue had been preincubated with [^{14}C]-3-hydroxypiperidinium. One third of the Krebs has been subjected to electrophoresis. The experiment was repeated six times. AcP and P show the position of non-labelled 3-acetoxypiperidinium and 3-hydroxypiperidinium respectively on the electrophoresis paper and the bar graph above shows the radioactivity detected (\pm SEM) on the corresponding paper strips cut from the electrophoresis paper.

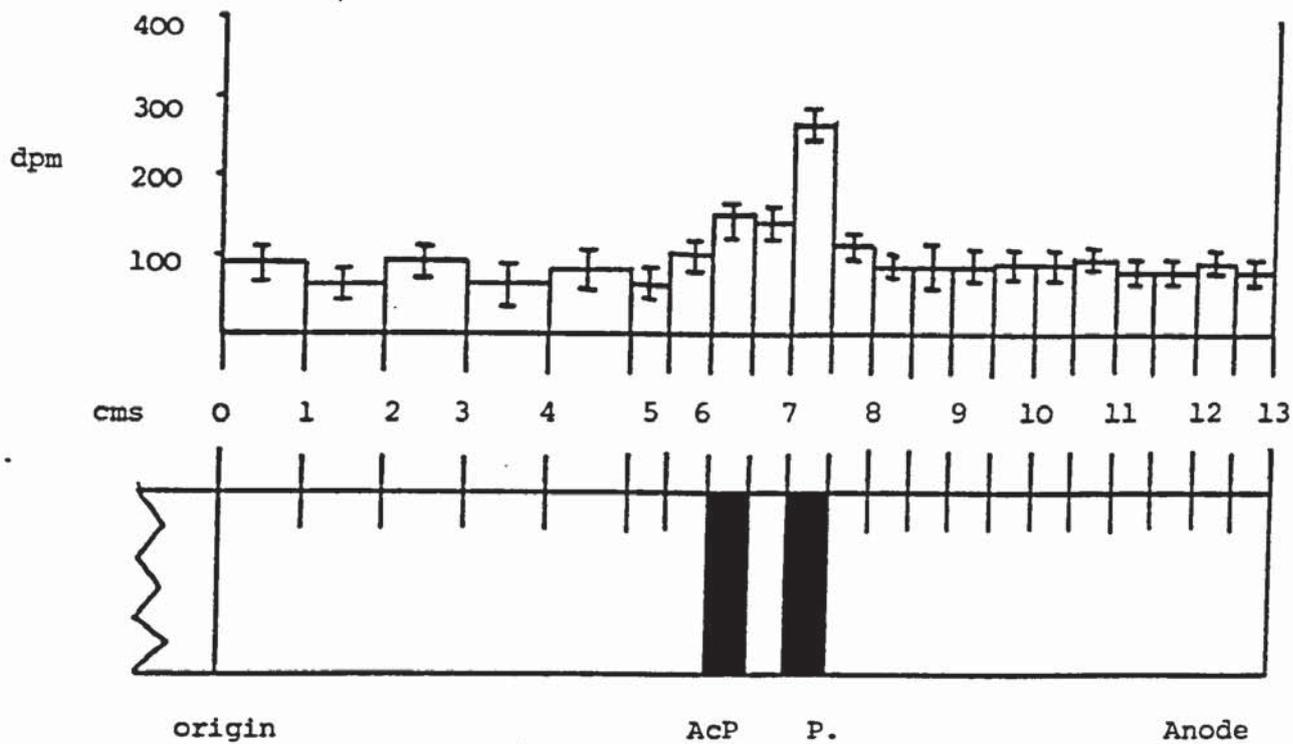


Fig. 6.13:- Paper electrophoresis (See Fig. 6.8) of an extract from the electrically stimulated mouse phrenic nerve-diaphragm preparation. One third of the extract has been subjected to electrophoresis. The experiment was repeated six times. See Fig. 6.12 for further details.

The 3-acetoxypiperidinium compound presumably diffuses from the synaptic gap into the surrounding incubation medium. It is possible that the tissue extract contains some 3-acetoxypiperidinium which has been released into the synaptic gap but which has not diffused into the Krebs, as well as intracellular 3-acetoxypiperidinium. It is also possible that the washing procedure after the pre-incubation with $[^{14}\text{C}]$ -3-hydroxypiperidinium does not remove all of this compound from the extracellular spaces, therefore the $[^{14}\text{C}]$ -3-hydroxypiperidinium detected in the Krebs and tissue extracts may not all be intracellular in origin. These observations may affect the calculations described below.

Fig. 6.12 and 6.13, shows the average results of six separate experiments conducted, in which one third of the tissue extract and one third of the Krebs were subjected to electrophoresis. From these results it can be tentitively calculated:

When specific activity of 3-hydroxypiperidinium = $1\mu\text{Ci}/0.018\ \mu\text{mole}$	
Total amount of $[^{14}\text{C}]$ -3-hydroxypiperidinium accumulated by the tissue	= $1.3\mu\text{mole/kg}$ of tissue (wet weight)
% of the accumulated $[^{14}\text{C}]$ -3-hydroxypiperidinium that is acetylated	= 60% ($0.8\mu\text{mole/kg}$ of tissue (wet weight))
% of the total amount of $[^{14}\text{C}]$ -3-acetoxypiperidinium released from intracellular sites with no nervous stimulation	= 0%
with nervous stimulation	= 90%

6.3.7 Pharmacological actions of the acetoxypiperidinium compounds

6.3.7.1 Guinea-pig ileum

The acetoxypiperidinium compounds caused contractions of the guinea-pig ileum. Their log dose - response curves were parallel to that of ACh. The equipotent molar ratio of these compounds compared to ACh is

Compound	Equipotent Molar Ratio	Equipotent Molar Ratio (Abramson's Results)
Acetylcholine	1	1
3-acetoxypiperidinium	162	156
4-acetoxypiperidinium	68	77
3-methacetoxypiperidinium	3760	1703

TABLE 6.7:- Action of acetoxypiperidinium compounds on the guinea-pig ileum. Results from previous work by Abramson (1964) are shown for comparison. The response of the tissue to the analogues was no different in the presence of hexamethonium

Compound	Equipotent Molar Ratio
ACh	1
3-acetoxypiperidinium	57
4-acetoxypiperidinium	57
3-methacetoxypiperidinium	105

TABLE 6.8:- Action of the acetoxypiperidinium compounds on the frog rectus abdominis muscle

Compound	K_m (μ M)	V_{max}
ACh	0.56	9.09
3-acetoxypiperidinium	1.13	3.13
4-acetoxypiperidinium	0.27	1.07
3-methacetoxypiperidinium	0.63	1.52

TABLE 6.9:- Hydrolysis of the acetoxypiperidinium compounds by AChE

Compound	K_m (μ M)	V_{max}
ACh	0.84	3.2
3-acetoxypiperidinium	0.90	2.5
4-acetoxypiperidinium	0.29	1.2
3-methacetoxypiperidinium	0.97	1.6

TABLE 6.10:- Hydrolysis of the acetoxypiperidinium compounds by ChE.

V_{max} is the maximum rate of hydrolysis (number of mole of analogue hydrolysed by 1mg of protein in 1 min) at infinite substrate concentration.

Correlation coefficients are at least 0.90 .

shown in Table 6.7.

6.3.7.2 Frog rectus abdominis muscle

The acetoxypiperidinium compound caused a contracture of the frog rectus abdominis muscle. The equipotent molar ratios compared to ACh is shown in Table 6.8.

6.3.7.3 Degradation of the acetoxypiperidinium compounds by cholinesterase enzymes

The degradation of the acetoxypiperidinium compounds by AChE from bovine erythrocytes and pseudocholinesterase (ChE) from horse serum was determined using the Null point-titration method as discussed in the Methods (Section 3.4.2.2.). All the analogues were substrates for these enzymes. It was found that the rate of hydrolysis of the analogues decreased significantly after 2½ min and the results obtained after this time were not representative of the initial rate of hydrolysis.

Therefore, the number of moles of analogue hydrolysed by 1 mg of enzyme in 1 min (rate of hydrolysis) were calculated. Lineweaver-Burke, (1934), plots of the reciprocal of the rate of hydrolysis against the reciprocal of the substrate concentration were calculated using regression analysis, and the K_m and V_{max} for each analogue is shown in Table 6.9 and 6.10.

6.4 Discussion

6.4.1. Action at the neuromuscular junction

Preliminary pharmacological screening using the rat phrenic nerve-diaphragm preparation showed that the hydroxypiperidinium analogues caused a prejunctional block on the rat phrenic nerve-diaphragm preparation. They also had some depolarising action on the nicotinic receptors of the

frog rectus abdominis muscle. The hydroxypyridinium compound however, did not give a prejunctional block on the rat-diaphragm preparation.

6.4.2 Anticholinesterase activity

All the hydroxypiperidinium and hydroxypyridinium analogues possessed very weak anticholinesterase activity and it is unlikely that this plays any significant part in their action at cholinergic synapses.

6.4.3 Mechanism of action of the hydroxypiperidinium analogues in causing a prejunctional block at the neuromuscular junction

Preliminary screening has suggested that the hydroxypiperidinium analogues have a prejunctional blocking action at the neuromuscular junction. Further study was carried out to try to determine more about the mechanism of action of these analogues which results in this prejunctional block.

6.4.3.1 Inhibition of the high affinity transport of choline into synaptosomes

A drug can cause a presynaptic block at the cholinergic neuron by inhibiting the supply of choline for ACh synthesis. This can be achieved by inhibition of the high affinity transport of choline into the neuron.

All the hydroxypiperidinium compounds inhibited the high affinity transport of choline into synaptosomes. 3-hydroxymethylpiperidinium was the most potent in this respect, and this cyclic three carbon analogue of choline was half as potent as 3C-choline (homocholine) which had an IC_{50} of $3.6\mu M$ (Results Chapter II section 5.3.5.). The other 3C-choline analogue, 4-hydroxypiperidinium was about nine times less potent than 3C-choline. The cyclic choline analogues, 2-hydroxymethylpiperidinium and 3-hydroxypiperidinium were at least nine times less potent than choline

itself, which has an IC_{50} of $1.6\mu M$. (Simon, Mittag and Kuhar, 1975), in inhibiting the high affinity transport of choline into synaptosomes.

Hydroxypyridinium was a weak inhibitor of the choline transport and this could account for the fact that it did not give a prejunctional block on the rat diaphragm muscle.

6.4.3.2 Inhibition of and acetylation by ChAc *in vitro*

A drug could cause a presynaptic block at the cholinergic neuron by inhibiting ChAc. Of the hydroxypiperidinium and hydroxypyridinium analogues studied, the hydroxypyridinium compound was the most potent direct inhibitor of ChAc.

A drug can inhibit the synthesis of ACh and therefore cause a presynaptic block by acting as a substrate for ChAc and thereby compete with choline for the acetylating enzyme.

4-hydroxypiperidinium and 3-hydroxymethylpiperidinium, the cyclic 3C-choline (homocholine) analogues, were acetylated *in vitro* by ChAc by less than 5%, compared to choline, 100% and compared to 3C-choline, less than 5% (see results Chapter II, Section 5.3.3.). However, the cyclic choline analogues were acetylated. 2-hydroxymethylpiperidinium was acetylated by 18% and 3-hydroxypiperidinium by 55% compared to choline 100%.

Hydroxypyridinium, however, was not acetylated, this agrees with a similar finding made by Barker and Mittag (1975), who also studied the acetylation of a hydroxypyridinium compound (Table 1.2).

6.4.3.3 False transmitter hypothesis

6.4.3.3.1 3-hydroxypiperidinium

Since 3-hydroxypiperidinium was acetylated by ChAc *in vitro* at the highest rate compared to all the other choline analogues investigated in this study, and because there has been no reports of any investigation of

the piperidinium compounds as cyclic analogues of choline it was thought of interest to investigate the 3-hydroxypiperidinium compound further. These further studies required the use of radioactively labelled 3-hydroxypiperidinium. This was commercially unavailable and therefore $[^{14}\text{C}]$ -3-hydroxypiperidinium was synthesised from the readily available $[^{14}\text{C}]$ -methyl-iodide.

6.4.3.3.1.1 Transport into synaptosomes

Due to the *in vitro* acetylation of 3-hydroxypiperidinium by ChAc it was thought possible that 3-hydroxypiperidinium could be acetylated *in vivo* by the cholinergic neuron to 3-acetoxypiperidinium which could behave as a false transmitter. However, in order for 3-hydroxypiperidinium to be acetylated *in vivo* it has, like choline, to be transported into the nerve terminal, from the extracellular fluid to the intracellular sites of acetylation.

Therefore, in order to determine whether 3-hydroxypiperidinium can be transported into cholinergic neurons radioactively labelled 3-hydroxypiperidinium was incubated with a crude preparation of synaptosomes. 3-hydroxypiperidinium was found to be transported into the synaptosomes. The transport could be resolved into a high and low affinity component. The high affinity transport was more dependent upon Na^{\oplus} , and more sensitive to the inhibitory action of HC-3, than the low affinity transport. This is similar to the transport of choline as discussed in the Introduction (Section 1.1), and similar to some other choline analogues which have been shown to be transported into synaptosomes (Barker and Mittag, 1975; Collier, Lovat, Ilson, Barker and Mittag, 1977). The apparent K_T of the high affinity transport of 3-hydroxypiperidinium was twice that of choline, and the maximum velocity of transport was about 26% that of choline. Choline and 3-hydroxypiperidinium antagonised each others transport, therefore, the high affinity transport of 3-hydroxypiperidinium appears to be by the

same mechanism as that responsible for the high affinity transport of choline into the cholinergic neuron. The difference between the K_{T_L} and the K_{T_H} of 3-hydroxypiperidinium (about 29 fold) was greater than the difference between the K_{T_L} and the K_{T_H} of choline (about 10 fold), but the present experiments did not try to characterize this low affinity transport process further.

6.4.3.3.1.2 Metabolism by synaptosomes

It has been previously discussed, in the Introduction (Section 1.1), that the acetylation of choline *in vivo* by cholinergic neurons might be coupled to its high affinity transport system. On incubating synaptosomes with concentrations of choline, MEC and pyrrolcholine which favour the high affinity transport system Barker and Mittag (1975) showed that in each case 45% of the total amount of choline and choline analogue transported into synaptosomes was acetylated. Similarly the results presented in the present study show that about 50% of the accumulated 3-hydroxypiperidinium was acetylated. Therefore the difference between the rates of acetylation (and the K_m and V_{max} values) of choline and these analogues seen with ChAc *in vitro* is apparently not seen *in situ*. Thus the results of the present study are consistent with the hypothesis that the transport of choline into synaptosomes is coupled to its acetylation.

6.4.3.3.1.3 Mouse phrenic nerve-diaphragm preparation

It has therefore been shown that 3-hydroxypiperidinium can be accumulated by isolated cholinergic nerve terminals (synaptosomes) and acetylated to 3-acetoxypiperidinium. It is possible that this acetylated derivative could behave as a false transmitter, but to be a false transmitter it must like ACh be released from the cholinergic neuron on electrical stimulation. Potter, (1970), showed that by incubating the rat phrenic nerve-diaphragm (a neuromuscular junction) in a medium containing

[¹⁴C]-choline, the synthesis, storage and release of [¹⁴C]-ACh by this muscle preparation could be studied. In a similar way, since 3-hydroxypiperidinium is a choline analogue and since it has been shown to be accumulated by synaptosomes and acetylated, it was thought of interest to incubate the phrenic-nerve endplate region of a mouse diaphragm in a medium containing [¹⁴C]-3-hydroxypiperidinium. The experiments showed that this tissue accumulated 3-hydroxypiperidinium where it was acetylated to 3-acetoxypiperidinium which was released, on electrical stimulation, as a false transmitter. Due to a shortage of material, no time studies were carried out and therefore the turnover of 3-acetoxypiperidinium was not determined. For the same reason the effect of HC-3 on the accumulation of 3-hydroxypiperidinium into this tissue was not determined. However, it was possible to calculate from these experiments how much of the accumulated 3-hydroxypiperidinium was acetylated, that is 60% (0.8 μmole/Kg of tissue). This rate of acetylation is similar to that found in synaptosomes. Potter, (1970), calculated from his experiments in which he used the phrenic nerve-muscle preparation from a rat, that the total content of ACh in his tissue was approximately 0.9 μmole/Kg. On the basis of this result it can be concluded that if the 3-acetoxypiperidinium compound replaces the endogenous ACh on a molar basis, then 3-acetoxypiperidinium replaces 88% of the endogenous ACh, although this calculation must be considered with caution since a different preparation has been used. Obviously further experiments are required to determine the turnover of choline and the turnover of 3-hydroxypiperidinium in the phrenic-nerve endplate region of a mouse diaphragm. This preparation can provide an alternative tissue to the superior cervical ganglion of the cat for studying the synthesis, storage and release of ACh and other analogues.

6.4.3.3.1.4 Conclusion

It can be concluded that like choline, 3-hydroxypiperidinium is accumulated by cholinergic neurons by a high and low affinity transport system, where it is acetylated to the ACh analogue, 3-acetoxypiperidinium. This compound can be released from the phrenic nerve, on electrical stimulation as a false cholinergic transmitter.

6.4.3.3.2 2-hydroxymethylpiperidinium, 3-hydroxymethylpiperidinium 4-hydroxypiperidinium

The small rate of acetylation of 2-hydroxymethylpiperidinium and the lack of acetylation of 3-hydroxymethylpiperidinium and 4-hydroxypiperidinium does not rule out the possibility that these analogues cannot be acetylated *in vivo* provided they can be transported into the neuron. Since Collier, Lovat, Ilson, Barker and Mittag (1977), found that 3C-choline (homocholine) was acetylated *in vitro* by ChAc by less than 5% compared to choline 100%, but was acetylated at a much higher rate *in situ* to form a false cholinergic transmitter. Similarly it is possible for these three piperidinium analogues to be acetylated *in vivo* and then released as a false transmitter. Obviously further experimentation is required.

6.4.4 Structure/activity relationships

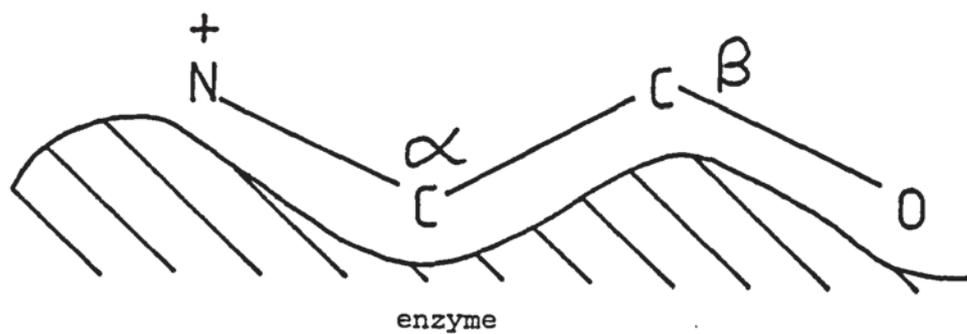
Bond lengths and bond angles have been taken from Tables of interatomic distances and configuration in molecules and ions (ed by Sutton, Jenkin, Mitchell and Cross, (1958). In the case of the choline moiety additional reference has been made to Hjortas and Sorum (1971). In the present work certain interatomic distances have been calculated by use of a Fieser model. The maximum and minimum interatomic distance between the nitrogen and oxygen atoms has been calculated for all the hydroxypiperidinium molecules although there are obviously an infinite number of conformations between these two extremes.

The maximum interatomic distance between the quaternary nitrogen

and the hydroxyl group of choline, is approximately 3.7\AA (Fig. 6.14). This distance is similar to that found in the choline moieties of the two cyclic analogues, 3-hydroxypiperidinium and 2-hydroxymethylpiperidinium (Fig. 6.15 and 6.16). These two analogues can be considered to be β and α substituted choline analogues respectively. Hemsworth and Smith (1970a) studied methyl substituted choline analogues and found that the α substituted analogue was acetylated at a higher rate than the β . This trend is not followed by these cyclic choline analogues since the β substituted 3-hydroxypiperidinium is acetylated at a higher rate than the α substituted 2-hydroxymethylpiperidinium compound. However, in the choline analogues studied by Hemsworth and Smith (1970a) free rotation is allowed around all of the bonds, whereas in the cyclic choline analogues examined in this study rotation is prevented around some bonds.

The choline moiety in the 3-hydroxypiperidinium and 2-hydroxymethylpiperidinium molecules is shown (Fig. 6.15 and 6.16). ChAc can presumably combine with the choline moieties in both molecules, since both analogues are acetylated to some extent as shown. However, 3-hydroxypiperidinium is acetylated at about three times the rate of 2-hydroxymethylpiperidinium, so presumably a choline moiety in which the α methylene group and the hydroxyl group are directed towards the enzyme surface and the β methylene group directed away from the enzyme surface is a more favourable conformation for acetylation by ChAc *in vitro*. Such a conformation is fixed in the 3-hydroxypiperidinium compound since the piperidinium ring prevents rotation around the N-C-C bonds (Fig. 6.15). The enzyme is not able to combine with such a conformation in the 2-hydroxymethylpiperidinium due to both steric hindrance of the piperidinium ring and because the ring prevents rotation around the N-C bond. However, rotation can occur around the C-O bond in this molecule and a cis conformation can exist where the α methylene

(a) maximum distance = 3.7\AA



(b) minimum distance = 2.4\AA

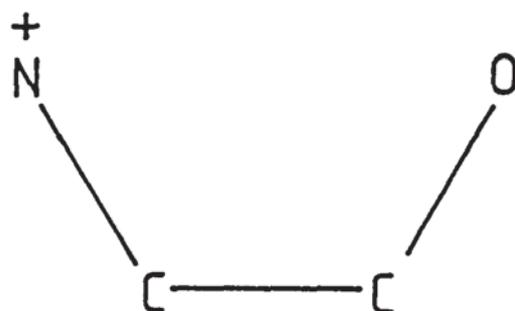
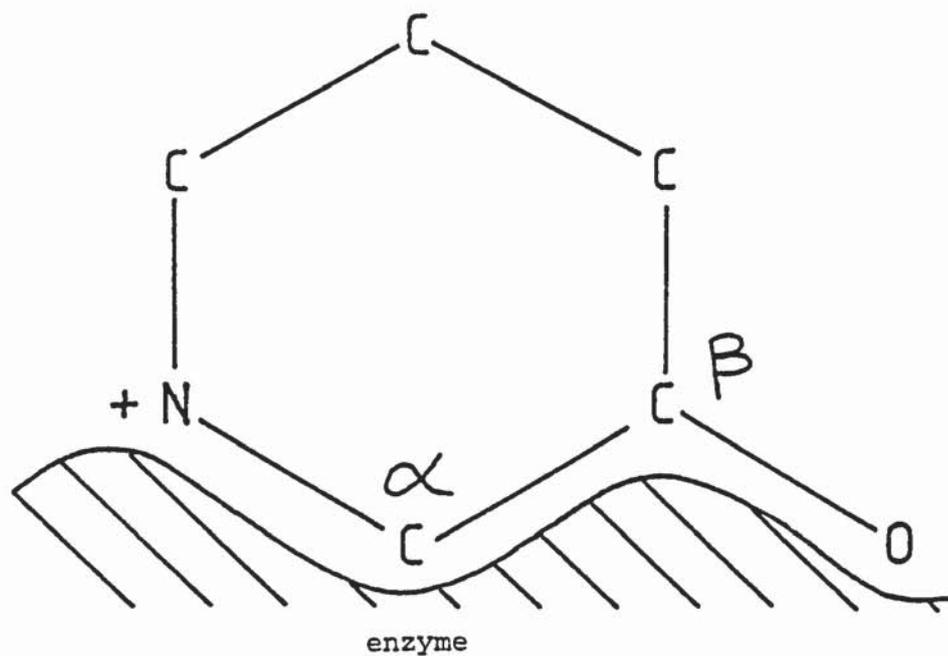


Fig. 6.14:- Choline, N-O interatomic distances (100% acetylation by ChAc *in vitro*.)

(a) maximum distance = 3.7\AA



(b) minimum distance = 2.9\AA

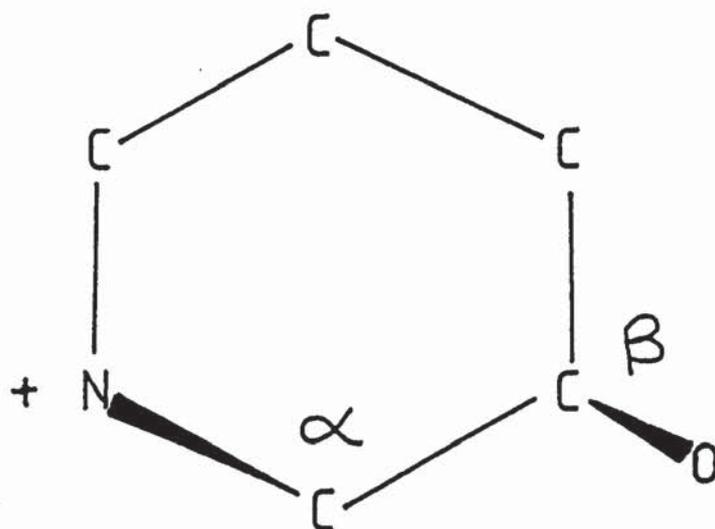
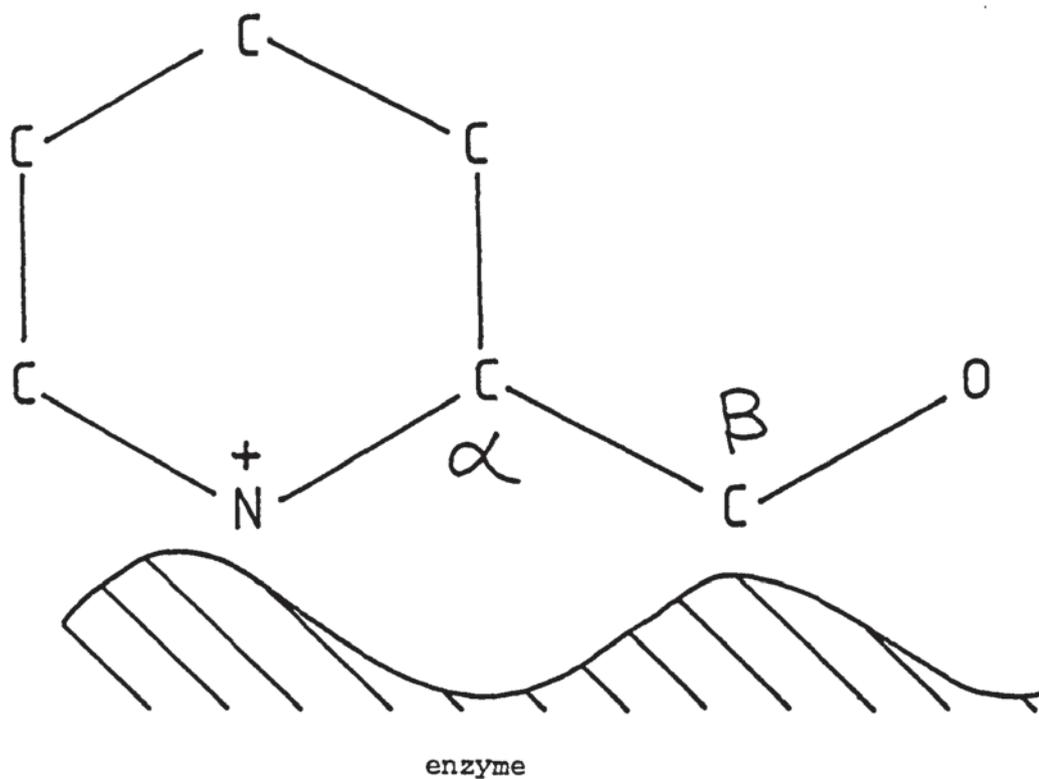
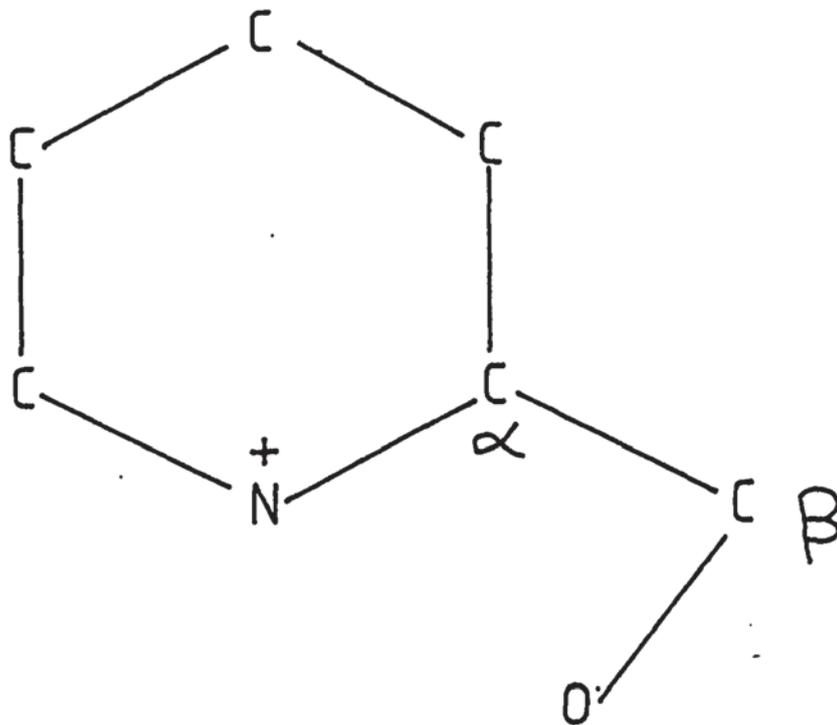


Fig. 6.15:- 3-hydroxypiperidinium, N-O interatomic distances (55% acetylation by ChAc *in vitro*.)

(a) maximum distance = 3.7\AA



(b) minimum distance = 2.4\AA



, Fig. 6.16:- 2-hydroxymethylpiperidinium, N-O interatomic distances (18% acetylation by ChAc *in vitro*.)

- (a) minimum distance (boat, as drawn) = 3.3\AA
(b) maximum distance (chair) = 3.5\AA

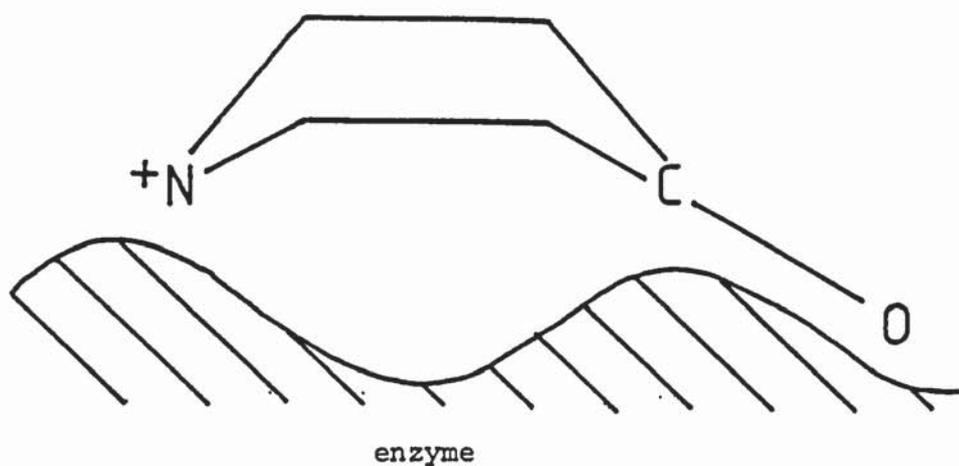
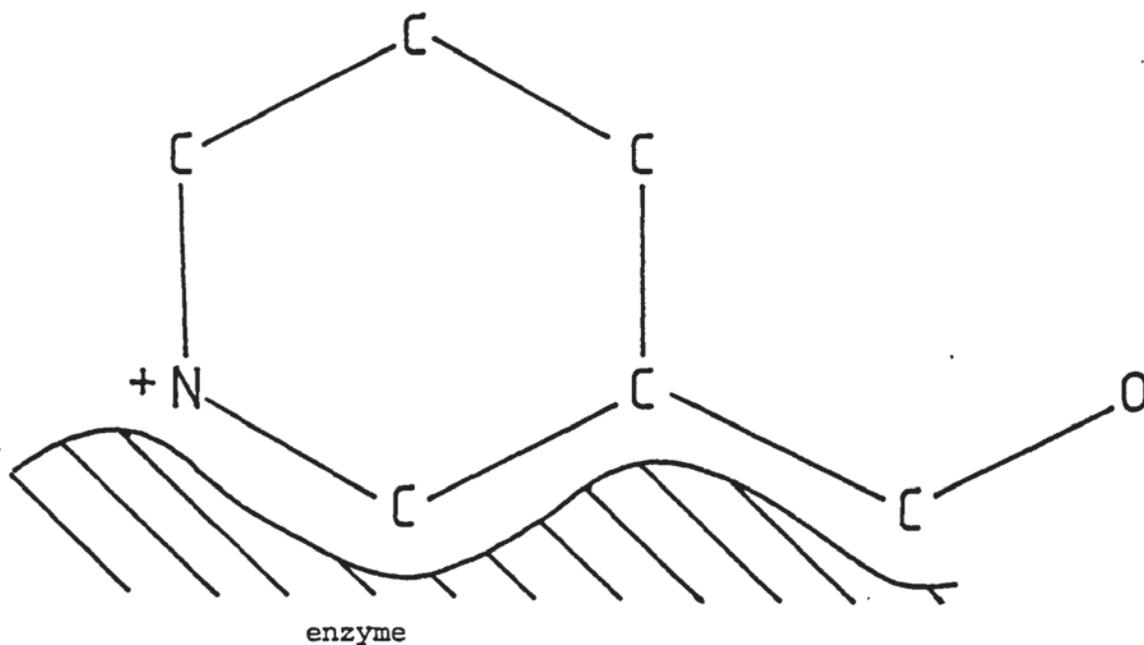
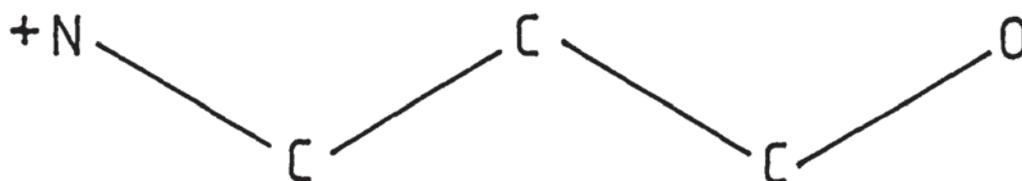


Fig. 6.17:- 4-hydroxypiperidinium = N-O interatomic distance
(less than 5% acetylation by ChAc *in vitro*.)

- (a) (i) maximum distance (as drawn) = 4.8\AA
 (ii) minimum distance = 4\AA



- (b) (i) maximum distance = 4.8\AA



- (ii) minimum distance = 0.7\AA

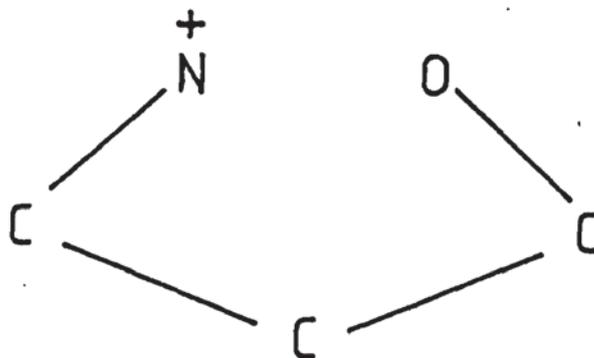


Fig.6.18(a):- 3-hydroxymethylpiperidinium, N-O interatomic distance

(b) 3C-choline (homocholine), N-O interatomic distance
 (both analogues are acetylated by less than 5% by ChAc
in vitro).

group is directed away from the enzyme surface, while the hydroxyl group is directed towards the enzyme surface (Fig. 6.16b). This cis conformation can occur in the choline molecule itself (Fig. 6.14b), but not in the 3-hydroxypiperidinium molecule and since this latter molecule is acetylated by 55%, the cis conformation is unlikely to be the conformer that reacts with the enzyme, ChAc. Apart from the conformational difference just described, there seems to be no other difference between the two moieties in these cyclic choline analogues to account for the varying rates of acetylation by ChAc *in vitro*. Therefore, it seems possible, that the role of the alkyl chain in the choline group is not only to maintain the correct interatomic distance between the quaternary nitrogen and the hydroxyl group but could itself bind to the enzyme and/or ensure that the hydroxyl group is in a favourable conformation for acetylation to occur. Possibly ChAc has a site in which the α methylene group can fit, as shown in Fig.6.15a.

The importance of the alkylchain is again shown on consideration of the 4-hydroxypiperidinium compound. In this analogue the quaternary nitrogen atom and the hydroxyl group are separated by an interatomic distance of between 3.3\AA and 3.5\AA depending upon the conformation of the piperidinium ring (Fig. 6.17). This is about the same distance as that in the choline molecule (Fig. 6.14) yet the 4-hydroxypiperidinium analogue is acetylated by ChAc *in vitro* at less than 5% the rate of choline. This suggests that the alkylchain between the nitrogen atom and the hydroxyl group in this cyclic analogue is not of the correct conformation to allow the acetylation of the compound by ChAc *in vitro*.

The 3-hydroxymethylpiperidinium compound is a β substituted homocholine analogue, and like homocholine it is not significantly acetylated by ChAc *in vitro*. The maximum interatomic distance between the quaternary nitrogen atom and the hydroxyl group of the cyclic homocholine analogue is about the same as that of homocholine itself that is 4.8\AA (Fig. 6.18).

This distance is presumably too large to allow the ChAc enzyme to acetylate these analogues. The minimum interatomic distance (4\AA) is still presumably too large and/or the conformation of the molecule is wrong.

There seems to be no relationship between the interatomic distances or conformation of the hydroxypiperidinium analogues and the potencies of these analogues in causing a prejunctional block at the rat phrenic nerve diaphragm preparation and their potency of inhibition of the high affinity transport of choline into synaptosomes. Only the acetylation of the compounds by ChAc *in vitro* can be rationalised in this way.

6.4.5 Acetylated derivatives of the hydroxypiperidinium compounds

Since 3-acetoxypiperidinium has been shown to be a false cholinergic transmitter, it was thought of interest to study the postjunctional action of this compound on the muscarinic receptors of the guinea-pig ileum and on the nicotinic receptors of the frog rectus abdominis muscle. Its breakdown by AchE and ChE was also investigated. For comparison, and since it was thought that the other piperidinium analogues investigated in this study might be tested in the future to see if they are precursors of false transmitters, 3-methacetoxypiperidinium and 4-acetoxypiperidinium were also studied.

6.4.5.1 Guinea-pig ileum

Previous work on the acetylated analogues by Abramson (1964), showed that all three of the acetoxypiperidinium compounds caused a contracture of the guinea pig-ileum and his results are shown, (Table 6.7), which are slightly different from the results of the present study. Lambrecht (1976), found that 3-acetoxypiperidinium was an agonist on the rat ileum. Lambrecht (1979) showed that 4-acetoxypiperidinium was an agonist on the guinea pig ileum and heart.

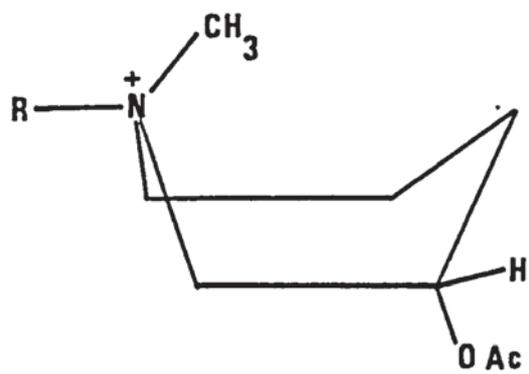
Ing (1949), stated that for active cholinergic drugs, the following skeleton was necessary N-C-C-O-C-C and with a few exceptions any deviation from this structure results in a marked loss of potency.

In the present study, all three acetoxypiperidinium compounds gave a maximal response on the guinea pig ileum showing that they all have the same intrinsic activity as ACh. The 4-acetoxypiperidinium analogue was found to be more active than the 3-acetoxypiperidinium which is different to what would be expected on consideration of the conclusions of Ing, (1949).

Lambrecht and Mutschler (1974), found that the conformation of the N-methyl-3-acetoxypiperidine ion needed for muscarinic action is one in which the N-C-C-O atoms are in a boat conformation as shown in Fig. 6.19a. Lambrecht and Mutschler, (1974), concluded from thermodynamic work that only 0.15% of the molecules of the N-methyl-3-acetoxypiperidine ion exists in the boat conformation (Fig. 6.19a), at 35°. In other words the N-methyl-3-acetoxypiperidine ion exists predominantly in the chair conformation (Fig. 6.19b). Lambrecht and Mutschler, (1974), also concluded that the free energy difference between the boat and chair conformers was 5-6kcal/mole and they suggested that this could not be overcome drug receptor interactions. Therefore, only the small percentage of molecules of the N-methyl-3-acetoxypiperidine ion in the boat conformation (Fig. 6.19a), can activate muscarinic receptors. A similar situation presumably exists for 3-acetoxypiperidinium.

However, if 4-acetoxypiperidinium is considered, (Fig. 6.20), the boat conformation of this molecule can be stabilized by attractive forces between the positively charged nitrogen atom and the lone pair of electrons (δ^-) on the ester oxygen atom. This presumably results in a higher proportion of molecules being in the boat conformation, compared to 3-acetoxypiperidinium where no such stabilisation can occur. Since the boat conformation is necessary for muscarinic activity (Lambrecht and Mutschler, 1974),

(a) boat conformation



(b) chair conformation

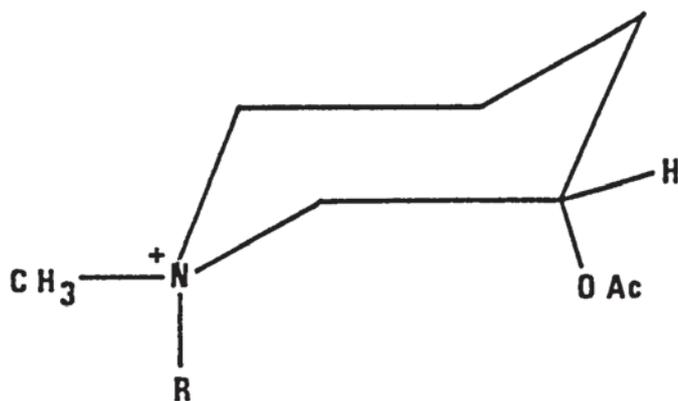


Fig. 6.19:- R = H: N-methyl-3-acetoxypiperidine ion
R = CH₃: 3-acetoxy-piperidinium

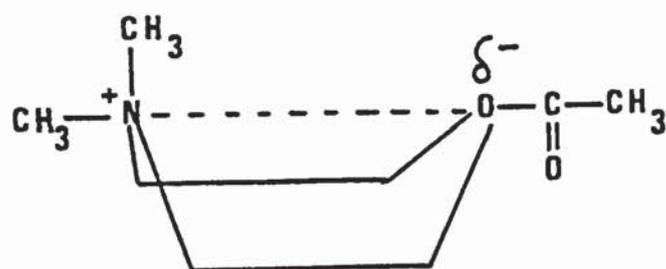


Fig. 6.20:- boat conformation of 4-acetoxypiperidinium

this is presumably why, despite the conclusions of Ing (1949), 4-acetoxypiperidinium is more active than 3-acetoxypiperidinium.

The interatomic distance between the quaternary nitrogen atom and the ester oxygen atom in 3-acetoxypiperidinium is about the same as that in ACh. However, in 3-methacetoxypiperidinium, this distance is greater (because of extra CH₂) and this is presumably why this compound is less active on muscarinic receptors than 3-acetoxypiperidinium.

6.4.5.2 Frog-rectus abdominus muscle

In experiments with the frog rectus abdominus muscle 3-acetoxypiperidinium was found to be equipotent with 4-acetoxypiperidinium. 3-methacetoxypiperidinium was less active than the other two analogues but not to the same extent as that found on the guinea-pig ileum. These results indicate that the stereochemical requirements for drug combination with the nicotinic receptor on skeletal muscle are less stringent than ^{the} for muscarinic receptor.

6.4.5.3 Cholinesterase enzymes

AChE hydrolysed 3-acetoxypiperidinium faster than the 3-methacetoxypiperidinium which in turn was hydrolysed faster than the 4-acetoxypiperidinium compound. ChE was less active on the three analogues than AChE, but showed the same trend. These results indicate that the active conformation for these enzymes is the chair form, which predominates in aqueous solutions of both 3-acetoxypiperidinium and 3-methacetoxypiperidinium but not of 4-acetoxypiperidinium.

6.4.6 Conclusions on the mechanism of action of the hydroxypiperidinium analogues in causing a prejunctional block at the neuromuscular junction

The prejunctional blocking action of the hydroxypiperidinium com-

pounds is probably due, at least in part, to their inhibitory action on the high affinity transport of choline into nerve terminals. 3-hydroxypiperidinium has been shown to be transported into cholinergic neurons and metabolised by them to the false transmitter 3-acetoxypiperidinium. Therefore, part of the presynaptic blocking action of 3-hydroxypiperidinium is probably due to the release of a false transmitter which has been shown to be 57 times weaker than ACh as an agonist of the nicotinic receptor of the frog rectus abdominis muscle.

6.4.7 Summary of Results

The hydroxypiperidinium analogues were shown to possess a prejunctional blocking action at the neuromuscular junction, weak anticholinesterase activity and inhibitory activity on the high affinity transport of choline into synaptosomes. Only 3-hydroxypiperidinium and 2-hydroxymethylpiperidinium were acetylated by ChAc *in vitro* by more than 5% (compared to choline, 100%). It has been demonstrated that 3-hydroxypiperidinium is transported into cholinergic neurons, acetylated to 3-acetoxypiperidinium which is then released as a false transmitter. 3-acetoxypiperidinium was shown to be a weaker cholinergic agonist than ACh.

The hydroxypyridinium analogue did not possess a prejunctional block on the rat diaphragm preparation and possessed a weak anticholinesterase action, a weak inhibitory action on the high affinity transport of choline into synaptosomes and was a poor substrate for ChAc *in vitro*.

7. GENERAL DISCUSSION

7. GENERAL DISCUSSION

Three series of choline analogues have been studied and their action at cholinergic synapses has been investigated especially with regard to their presynaptic action.

Structure/activity relationships have been discussed but only with regard to the acetylation of the analogues by ChAc *in vitro*. It appears as though *in vitro* ChAc is more specific in its structural requirements than any of the other preparations used in this investigation. The long-chain choline analogues were not acetylated by ChAc *in vitro* but the five carbon analogue (5C-choline) was a potent inhibitor of *in vitro* ChAc. Cyclic HC-3 is apparently a stable conformer and most of the acetylated-HC-3, as synthesised by ChAc *in vitro*, was in the cyclic form so it seems as though a cyclic choline moiety can be acetylated by ChAc. Similarly, 3C-HC, 3-hydroxypiperidinium and 2-hydroxymethylpiperidinium were acetylated by ChAc *in vitro*. It is likely that the structure/activity relationships discussed in relation to 3-hydroxypiperidinium could equally apply to HC-3 and 3C-HC, since the choline moiety in all three cyclic analogues is in the same conformation.

All of the analogues studied inhibited, to some extent, the high affinity transport of choline into synaptosomes but the bisquaternary hemicholinium analogues were the most potent in this respect.

Both HC-3 and 3-hydroxypiperidinium have been shown to be accumulated by a crude preparation of synaptosomes. A two component transport system (high affinity and low affinity transport) has been demonstrated for 3-hydroxypiperidinium which is similar to that of choline, whereas HC-3 seems only to be transported by a low affinity mechanism. Only 3-hydroxypiperidinium was acetylated *in situ* by synaptosomes whereas HC-3 was not acetylated despite the fact that HC-3 was acetylated *in vitro* by ChAc. This supports the hypothesis that high affinity transport and ace-

tylation *in situ* are coupled. 3-acetoxypiperidinium has been shown to be released as a false cholinergic transmitter at the neuromuscular junction.

8. REFERENCES

REFERENCES

- Abramson, F.B. (1964). The synthesis and pharmacological properties of some alicyclic compounds related to acetylcholine. Ph.D. Thesis, University of Edinburgh.
- Acara, M., Kowalski, M., Rennick, B. and Hemsworth, B. (1975). Renal tubular excretion of triethylcholine (TEC) in the chicken: Enhancement and inhibition of renal excretion of choline and acetylcholine by TEC. *Br. J. Pharmac.*, 54, 41-48.
- Ansell, G.B. and Spanner, S.G. (1974). The inhibition of brain choline kinase by hemicholinium-3. *J. Neurochem.*, 22, 1153-1155.
- Ansell, G.B. and Spanner, S. (1975). The origin and metabolism of brain choline. In: *Cholinergic Mechanisms*. ed. Waser, P.G. pp. 117-129. New York: Raven Press.
- Appel, W.C. and Vincenzi, F.F. (1970). Effects of hemicholinium and bretylium on the release of autonomic transmitters in the isolated sino atrial-node. *Br. J. Pharmac.*, 40, 268-274.
- Aquilonius, S.M., Flentge, F., Schuberth, J., Sparf, B. and Sundwall, A. (1973). Synthesis of acetylcholine in different compartments of brain nerve terminals *in vivo* as studied by the incorporation of choline from plasma and the effect of pentobarbital on this process. *J. Neurochem.*, 20, 1509-1521.
- Baker, B.R. and Gibson, R.E. (1971). Irreversible enzyme inhibitors. 181. Inhibition of brain choline acetyltransferase by derivatives of 4-stilbazole. *J. Med. Chem.*, 14, 315-322.
- Baker, B.R. and Gibson, R.E. (1972). Irreversible enzyme inhibitors. 193. Inhibition of choline acetyltransferase. Mode of binding by 4-stilbazoles. *J. Med. Chem.*, 15, 639-642.
- Barker, L.A. (1976). Subcellular aspects of acetylcholine metabolism. In: *Biology of cholinergic function*. eds. Goldberg, A.M. and Hanin, I. pp. 203-238. New York: Raven Press.
- Barker, L.A., Dowdall, M.J. and Mittag, T.W. (1975). Comparative studies on synaptosomes: high affinity uptake and acetylation of N-[Me-³H]-choline and *Brain Res.*, 86, 343-348
- Barker, L.A. and Mittag, T.W. (1975). Comparative studies of substrates and inhibitors of choline transport and choline acetyltransferase. *J. Pharmac. exp. Ther.*, 192, 86-94.
- Barlow, R.B. (1955). A series of polymethylene bis-acetoxyethyl dimethylammonium salts. *Br. J. Pharmac. Chemother.*, 10, 168-172.
- Barlow, R.B. and Zoller, A. (1962). Activity of analogues of decamethonium on the chick biventer cervicis preparation. *Br. J. Pharmac. Chemother.*, 19, 485-491.

- Barrass, B.C., Brimblecombe, R.W., Rich, P. and Taylor, J.V. (1970). Pharmacology of some acetylcholine analogues. *Br. J. Pharmacol.*, 39, 40-48.
- Bartels, E. (1962). Structure-activity relationship studied on the isolated single electroplax. *Biochim. biophys Acta.*, 63, 365-373.
- Benz, F.W. and Long, J.P. (1969, a). Investigations on a series of heterocyclic hemicholinium-3 analogs. *J. Pharmac. exp. Ther.*, 166, 225-236.
- Benz, F.W. and Long, J.P. (1969, b). Structure-activity relationships of N-alkyl and heterocyclic analogs of hemicholinium-3. *J. Pharmac. exp. Ther.*, 168, 315-321.
- Benz, F.W. and Long, J.P. (1970). Further structure-activity relationships of heterocyclic analogues of hemicholinium-3. *J. Pharm. Pharmacol.*, 22, 20-25.
- Bhatnagar, S.P., Lam, A. and McColl, J.D. (1965). Inhibition of acetylcholine synthesis in nervous tissue by some quaternary compounds. *Biochem. Pharmacol.*, 14, 421-434.
- Bhatnagar, S.P. and MacIntosh, F.C. (1967). Effects of quaternary bases and inorganic cations on acetylcholine synthesis in nervous tissue. *Can. J. Physiol. Pharmacol.*, 45, 249-268.
- Birks, R. and MacIntosh, F.C. (1961). Acetylcholine metabolism of a sympathetic ganglion. *Can. J. biochem. physiol.*, 39, 787-827.
- Blase, B.W., Loomis, T.A., Collins, J.A., and Sommer, H.Z. (1974). Two active structures for hemicholinium-like action. *Toxicol. Appl. Pharmacol.*, 27, 676-679.
- Borison, H.L. (1961). Respiratory depressant effect of HC-3. *Fedn. Proc.*, 20, 594-599.
- Bosch, A. and Van Bodegom, B. (1977). The crystal structure of the 1:2 complex of N-methyl-N-ethylmorpholinium and bis (7,7,8,8-tetracyanoquinodimethane) MEM(TCNQ)₂ at -160°C. *Acta. Cryst., Sect. B.*, 33, 3013-3021.
- Bowman, W.C. and Hemsworth, B.A. (1965 a). Effects of some polymethylene bis (hydroxymethyl)dimethylammonium salts on neuromuscular transmission. *Br. J. Pharmac. Chemother.*, 25, 392-404.
- Bowman, W.C. and Hemsworth, B.A. (1965b). Effects of triethylcholine on the output of acetylcholine from the isolated diaphragm of the rat. *Br. J. Pharmac. Chemother.*, 24, 110-118.
- Bowman, W.C., Hemsworth, B.A. and Rand, M.J. (1962). Triethylcholine compared with other substances affecting neuromuscular transmission. *Br. J. Pharmac. Chemother.*, 19, 198-218.
- Bowman, W.C., Hemsworth, B.A. and Rand, M.J. (1967). Effects of analogues of choline on neuromuscular transmission. *Ann. N.Y. Acad. Sci.*, 144, 471-482.

- Bowman, W.C. and Rand, M.J. (1961). Actions of triethylcholine on neuromuscular transmission. *Br. J. Pharmac. Chemother.*, 17, 176-195.
- Bowman, W.C. and Rand, M.J. (1962). The neuromuscular blocking action of substances related to choline. *Int. J. Neuropharmac.*, 1, 129-132.
- Bradshaw, D. (1976). Enzymic aspects of acetylcholine metabolism. Ph.D. Thesis. University of Aston in Birmingham.
- Bradshaw, D. and Hemsworth, B.A. (1976). The acetylation of hemicholinium-3 by choline acetyltransferase. *Biochem. Pharmac.*, 25, 1589-1594.
- Bray, G.A. (1960). A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Analyt. Biochem.* 1, 279-285.
- Browning, E.T. (1976). Acetylcholine synthesis: substrate availability and the synthetic reaction. In: *Biology of cholinergic function*. eds. Goldberg, A.M. and Hanin, I. pp. 187-201. New York: Raven Press.
- Bülbring, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Br. J. Pharmac. Chemother.*, 1, 38-61.
- Bull, G. and Hemsworth, B.A. (1963). Inhibition of biological synthesis of acetylcholine by triethylcholine. *Nature*, 199, 487-488.
- Burgen, A.S.V., Burke, G. and Desbarats-Schonbaum, M.L. (1956). The specificity of brain choline acetylase. *Br. J. Pharmac. Chemother.*, 11, 308-312.
- Buterbaugh, G.G., Figge, P.K. and Spratt, J.L. (1968). A comparison of the effects of tetraethylammonium, triethylcholine and hemicholinium on choline uptake by the isolated perfused rabbit heart. *Archs. int. Pharmacodyn.*, 175, 179-185.
- Campbell, C.W.B. (1976). The Na^+ , K^+ , Cl^- contents and derived membrane potentials of presynaptic nerve endings *in vitro*. *Brain Res.*, 101, 594-599.
- Chang, C.C. and Lee, C. (1970). Studies on the [^3H]-choline uptake in rat phrenic nerve-diaphragm preparations. *Neuropharmac.*, 9, 223-233.
- Chang, V. and Rand, M.J. (1960). Transmission failure in sympathetic nerves produced by hemicholinium. *Br. J. Pharmac.*, 15, 588-600
- Chiou, C.Y. (1974). Studies on action mechanisms of a possible false cholinergic transmitter (2-hydroxyethyl)methyldimethylammonium. *Life. Sci.*, 14, 1721-1733.
- Chiou, C.Y. and Long, J.P. (1969). Effects of α, α' -bis-(dimethylammoniumacetaldehyde diethylacetal)-*p, p'*-diacetylbiphenyl bromide (DMAE) on neuromuscular transmission. *J. Pharmac. exp. Ther.*, 167, 344-350.

- Cho, A.K., Jenden, D.J. and Lamb, S.I. (1972). Rates of alkaline hydrolysis and muscarinic activity of some aminoacetates and their quaternary ammonium analogs. *J. Med. Chem.* 15, 39-394.
- Collier, B. (1969). The preferential release of newly synthesised transmitter by a sympathetic ganglion. *J. Physiol.*, 205, 341-352.
- Collier, B. (1973). The accumulation of hemicholinium by tissues that transport choline. *Can. J. Physiol. Pharmac.*, 51, 491-495.
- Collier, B., Barker, L.A. and Mittag, T.W. (1976). The release of acetylated choline analogues by a sympathetic ganglion. *Molec. Pharmac.*, 12, 340-344.
- Collier, B. and Ilson, D. (1977). The effect of preganglionic nerve stimulation on the accumulation of certain analogues of choline by a sympathetic ganglion. *J. Physiol.*, 264, 489-509.
- Collier, B. and Katz, H.S. (1974). Acetylcholine synthesis from recaptured choline by a sympathetic ganglion. *J. Physiol.*, 238, 639-655.
- Collier, B. and Lang, C. (1969). The metabolism of choline by a sympathetic ganglion. *Can. J. Physiol. Pharmac.*, 47, 119-126.
- Collier, B., Lovat, S., Ilson, D., Barker, L.A. and Mittag, T.W. (1977). The uptake, metabolism and release of homocholine: studies with rat brain synaptosomes and cat superior cervical ganglion. *J. Neurochem.*, 28, 331-339.
- Collier, B. and MacIntosh, F.C. (1969). The source of choline for acetylcholine synthesis in a sympathetic ganglion. *Can. J. Physiol. Pharmac.*, 47., 127-135.
- Collier, B., Poon, P and Salehmoghaddam, S. (1972). The formation of choline and acetylcholine by brain *in vitro*. *J. Neurochem.*, 19, 51-60.
- Cornwell, N.H. and Tsou, K.C. (1949). Keto-hemiacetal tautomerism of some N-phenacyl-N-substituted ethanololamines. *J. Am. Chem. Soc.*, 71. 993-996.
- Currier, S.F. and Mautner, H.G. (1974). On the mechanism of action of choline acetyltransferase. *Proc. Nat. Acad. Sci. U.S.A.* 71, 3355-3359.
- Dauterman, W.C. and Mehrotra, K.N. (1963). The N-alkyl group specificity of choline acetylase from rat brain. *J. Neurochem.*, 10, 113-117.
- Diamond, I. and Kennedy, E.P. (1969). Carrier mediated transport of choline into synaptic nerve endings. *J. Biol. Chem.*, 244, 3258-3263.
- Diamond, I. and Milfay, D. (1972). Uptake of [³H-methyl] choline by microsomal, synaptosomal, mitochondrial and synaptic vesicle fractions of rat brain. The effects of hemicholinium. *J. Neurochem.* 19, 1899-1909.

- Di Augustine, R.P. and Haarstad, V.B. (1970). The active structure of hemicholinium inhibiting the biosynthesis of acetylcholine. *Biochem. Pharmac.*, 19, 559-580.
- Dixon, M. (1953). The determination of enzyme inhibitor constants. *Biochem. J.* 55, 170-171.
- Domer, F.R., Chihal, D.M., Charles, H.C. and Rège, A.B. (1977). Effect of sulfur substitution for the noncarbonyl oxygen in hemicholinium-3 and acetyl-seco-hemicholinium-3. Synthesis, biological activity and structure-toxicity relationships. 2. *J. Med. Chem.*, 20, 59-62.
- Domer, F.R. and Schueler, F.W. (1960). Synthesis and metabolic studies of C¹⁴-labelled hemicholinium number three. *J. Am. Pharm. Assoc.*, 49, 553-556.
- Domino, E.F., Mohrman, M.E. Wilson, A.E. and Haarstad, V.B. (1973). Acetylseco hemicholinium-3, a new choline acetyltransferase inhibitor useful in neurpharmacological studies. *Neuropharmac.*, 12, 549-561.
- Domino, E.F., Shellenberger, M.K. and Frappier, J. (1968). Inhibition of acetylcholinesterase *in vitro* by hemicholinium-3 *Arch. int. Pharmacodyn.*, 176, 42-49.
- Dowdall, M.J., Fox, G. Wachtler, K., Whittaker, V.P. and Zimmerman, H. (1975). Recent studies on the comparative biochemistry of the cholinergic neuron. Cold Spring Harbor symp. quant. Biol., 40, 65-81.
- Elmqvist, D. and Quastel, D.M.J. (1965). Presynaptic action of hemicholinium at the neuromuscular junction. *J. Physiol.*, 177, 463-482.
- Evans, E.R. and Wilson, H. (1964). Action of hemicholinium (HC-3) on neuromuscular transmission. *Br. J. Pharmac.* 22, 441-452.
- Everett, S.D. (1968). Pharmacological responses of the isolated innervated intestine and rectal caecum of the chick. *Br. J. Pharmac. Chemother.*, 33, 342-356.
- Fonnum, F. (1975). A rapid radiochemical method for the determination of choline acetyltransferase. *J. Neurochem.*, 24, 407-409.
- Frankenberg, L., Heimbürger, G., Nilsson, C. and Sörbo, B. (1973). Biochemical and pharmacological studies on the sulfonium analogues of choline and acetylcholine. *Eur. J. Pharmac.*, 23, 37-46.
- Gardiner, J.E. (1957). Experiments on the site of action of a choline acetylase inhibitor. *J. Physiol.*, 138, 13P-14P.
- Gardiner, J.E. (1961). The inhibition of acetylcholine synthesis in brain by a hemicholinium. *Biochem. J.*, 81, 297-303.
- Gardiner, J.E. and Sung, L.H. (1969). A p-terphenyl hemicholinium compound. *Br. J. Pharmac.*, 36, 171P-172P.

Giovinco, J.F. (1957). Hemicholinium: a new aid to the study of cholinergic mechanisms. Bull. Tulane Univ. Med. Fac. 16, 177-184.

Glick, S.D., Craine, A.M., Barker, L.A. and Mittag, T.W. (1975) Effects of N-hydroxyethyl-pyrrolidinium methiodide, a choline analogue, on passive avoidance behaviour in mice. Neuropharmac., 14, 561-564.

Goodman, L.S. and Gilman, A. (1971). The pharmacological basis of therapeutics. (4th ed.) p.1660. London: The Macmillan Co.

Gomez, M.V., Domino, E.F. and Sellinger, O.Z. (1970). Effect of hemicholinium-3 on choline distribution *in vivo* in the canine caudate nucleus. Biochem. Pharmac., 19, 1753-1760.

Gray, E.G. (1977). Presynaptic microtubules, agranular reticulum and synaptic vesicles. In: synapses. eds. Cottrell, G.A. and Usherwood, P.N.R. pp6-18. Glasgow and London: Blackie.

Gray, E.G. and Whittaker, V.P. (1962). The isolation of nerve endings from brain: an electron-microscope study of cell fragments derived by homogenization and centrifugation. J. Anat., Lond. 96, 79-88.

Guyenet, P., Lefresne, P., Rossier, J., Beaujouan, J.C. and Glowinski, J. (1973a). Inhibition by hemicholinium-3 of [¹⁴C]-acetylcholine synthesis and [³H]choline high affinity uptake in rat synaptosomes. Mol. Pharmac., 9, 630-639.

Guyenet, P., Lefresne, P., Rossier, J., Beaujouan, J.C. and Glowinski, J. (1973b). Effect of sodium, hemicholinium-3 and antiparkinson drugs on [¹⁴C]acetylcholine synthesis and [³H]choline uptake in rat striatal synaptosomes. Brain Res., 62, 523-529.

Guyenet, P., Lefresne, P., Rossier, J., Beaujouan, J.C. and Glowinski, J. (1975). The role of newly taken up choline in the synthesis of acetylcholine in rat striatal synaptosomes. In: Cholinergic Mechanisms. ed. Waser, P.G. pp.137-144. New York: Raven Press.

Haarstad, V.B., Domer, F.R., Chihal, D.M., Rege, A.B. and Charles, H.C. (1976). Synthesis and structure-toxicity relationships of three new stable analogues of acetyl-seco-hemicholinium-3. J. Med. Chem., 19, 760-763.

Haga, T. (1971). Synthesis and release of [¹⁴C]acetylcholine in synaptosomes. J. Neurochem., 18, 781-798.

Haga, T. and Noda, H. (1973). Choline uptake systems of rat brain synaptosomes. Biochim. biophys. Acta., 291, 564-575.

Haubrich, D.R. (1976). Choline acetyltransferase and its inhibitors. In: Biology of cholinergic function. eds. Goldberg, A.M. and Hanin, I. pp.239-268. New York: Raven Press.

Haubrich, D.R. and Chippendale, T.J. (1977). Regulation of acetylcholine synthesis in nervous tissue. Life. Sci. 20, 1465-1478.

- Haubrich, D.R. and Wang, P.F.L. (1976). Inhibition of acetylcholine synthesis by juglone and 4-(1-naphthylvinyl) pyridine. *Biochem. Pharmac.*, 25, 669-672.
- Hebb, C.O. Krnjević, K. and Silver, A. (1964). Acetylcholine and choline acetyltransferase in the diaphragm of the rat. *J. Physiol.* 171, 504-513.
- Hebb, C.O. and Smallman, B.N. (1956). Intracellular distribution of choline acetylase. *J. Physiol.*, 134, 385-392.
- Hebb, C.O. and Waites, G.M.H. (1956). Choline acetylase in antero- and retro-grade degeneration of a cholinergic nerve. *J. Physiol.*, 132, 667-671.
- Hemsworth, B.A. (1971, a). The effects of a hemicholinium analogue, HC-15, on neuromuscular transmission. *Eur. J. Pharmac.*, 15, 91-100.
- Hemsworth, B.A. (1971, b). Effects of some polymethylene bis (hydroxyethyl)dimethylammonium compounds on acetylcholine synthesis. *Br. J. Pharmac.*, 42, 78-87.
- Hemsworth, B.A. (1972). The effects of some hemicholinium analogues on acetylcholine metabolism and neuromuscular transmission. *Archs. int. Pharmacodyn.*, 199, 272-281.
- Hemsworth, B.A. (1976). The pharmacological actions of some polymethylene-bis-(hydroxyethyl)-dimethyl-ammonium compounds on cholinergic transmission. *Eur. J. Pharmac.*, 35, 127-134.
- Hemsworth, B.A. and Bosmann, H.B. (1971). The incorporation of triethylcholine into isolated guinea pig cerebral cortex synaptosomal and synaptic vesicle fractions *Eur. J. Pharmac.*, 16, 164-170.
- Hemsworth, B.A. and Cholakis, J.M. (1978). Some pharmacological actions of acetylsecohemicholinium. *J. Pharm. Pharmac.*, 30, 291-296.
- Hemsworth, B.A. Darmer, K.I. and Bosmann, H.B. (1971). The incorporation of choline into isolated synaptosomal and synaptic vesicle fractions in the presence of quaternary ammonium compounds. *Neuropharmac.*, 10, 109-120.
- Hemsworth, B.A. and Foldeš, F.F. (1970). Preliminary pharmacological screening of styrylpyridine choline acetyltransferase inhibitors. *Eur. J. Pharmac.*, 11, 187-194.
- Hemsworth, B.A. and Morris, D. (1964). A comparison of the N-alkyl group specificity of choline acetyltransferase from different species. *J. Neurochem.*, 11, 793-803.
- Hemsworth, B.A. and Smith, J.C. (1970, a). Enzymic acetylation of the stereoisomers of α - and β -methylcholine. *Biochem. Pharmac.*, 19, 2925-2927.
- Hemsworth, B.A. and Smith, J.C. (1970, b). The enzymic acetylation of choline analogues. *J. Neurochem.*, 17, 171-177.

- Hersh, L.B., Barker, L.A. and Rush, B. (1978). Effect of sodium chloride on changing the rate-limiting step in the human placental choline acetyltransferase reaction. *J. biol. Chem.*, 253, 4966-4970.
- Hjortas, J. and Sorum, H. (1971). A re-investigation of the crystal structure of choline choride. *Acta. Cryst.*, B27, 1320-1323.
- Hodgkin, A.L. and Martin, K. (1965). Choline uptake by giant axons of *Loligo*. *J. Physiol.*, 179, 26P-27P.
- Holden, J.T., Rossier, J., Beaujouan, J.C., Guyenet, P. and Glowinski, J. (1975). Inhibition of high-affinity choline transport in rat striatal synaptosomes by alkyl bisquaternary ammonium compounds. *Mol. Pharmac.*, 11, 19-27.
- Holton, P. and Ing, H.R. (1949). Specificity of the trimethylammonium group in acetylcholine. *Br. J. Pharmac. Chemother.*, 4, 190-196.
- Howard-Butcher, S., Cho, A.K. and Schaeffer, J.G. (1974). The effect of triethylcholine on choline uptake and acetylation *in vivo* and *in vitro*. *Fedn. Proc.*, 33, 505.
- Hunt, R., Taveau R. DeM. (1909). On the relation between the toxicity and chemical constitution of a number of derivatives of choline and analgous compounds. *J. Pharmac. exp. Ther.*, 1, 303-339.
- Hutter, O.F. (1952). Effect of choline on neuromuscular transmission in the cat. *J. Physiol.*, 117, 241-250.
- Ilson, D., Collier, B. and Boksa, P. (1977). Acetyltriethylcholine: a cholinergic false transmitter in cat superior cervical ganglion and rat cerebral cortex. *J. Neurochem.*, 28, 371-381.
- Ing, H.R. (1949). The structure-action relationships of the choline group. *Science.*, 109, 264-266.
- Jones, D.G. (1975). Synapses and synaptosomes, morphological aspects. London: Chapman and Hall.
- Jukes, T.H. and Welch, A.D. (1942). The effect of certain analogues of choline on perosis. *J. biol. Chem.*, 146, 19-24.
- Kaita, A.A. and Goldberg, A.M. (1969). Control of acetylcholine synthesis-the inhibition of choline acetyltransferase by acetyltransferase by acetylcholine. *J. Neurochem.*, 16, 1185-1191.
- Kasé, Y. and Borison, H.L. (1958). Central respiratory depressant action of "hemicholinium" in the cat. *J. Pharmac. exp. Ther.*, 122, 215-233.
- Keston, A.S. and Wortis, S.B. (1946). The antagonistic action of choline and its triethyl analogue. *Proc. Soc. exp. Biol. Med.*, 61, 439-440.
- Kilbinger, H., Wagner, A. and Zerban, R. (1976). Some pharmacological properties of the false cholinergic transmitter acetylpyrrolidinecholine and its precursor pyrrolidinecholine. *Naunyn-Schmiedeberg's Arch. Pharmac.*, 295, 81-87.

Korey, S.R., Braganza, B. de and Nachmansohn, D. (1951). Choline acetylase. V. esterifications and transacetylations. *J. biol. Chem.*, 189, 705-715.

Krell, R.D. and Goldberg, A.M. (1975). Effect of choline acetyltransferase inhibitors on mouse and guinea-pig brain choline and acetylcholine. *Biochem. Pharmacol.*, 24, 391-396.

Kuhar, M.J. (1976). The anatomy of cholinergic neurons. In: *Biology of cholinergic function*. eds. Goldberg, A.M. and Hanin, I. pp3-27. New York: Raven Press.

Kuhar, M.J. and Murrin, L.C. (1978). Sodium-dependent, high affinity choline uptake. *J. Neurochem.*, 30, 15-21.

Kuhar, M.J., Sethy, V.H., Roth, R.H. and Aghajanian, G.K. (1973). Choline: Selective accumulation by central cholinergic neurons. *J. Neurochem.*, 20, 581-593.

Lambrecht, G. (1976). Struktur und konformations-wirkungs-beziehungen heterocyclischer acetylcholinanaloga. 1. Muskarinwirkung enantiomerer 3-acetoxychinuclidine und 3-acetoxypiperidine. *Eur. J. Med. Chem.-Chim. Therap.* 11. 461-466.

Lambrecht, G. (1979). The rate of action of cyclic ammonium and sulfonium analogues of acetylcholine. *Experientia*, 35, 75-76.

Lambrecht, G. and Mutschler, E. (1974). Conformational isomerism in drug action: does the free energy of binding induce the pharmacophoric conformation of semi-rigid muscarinic agonists? In: *Molecular and Quantum Pharmacology* eds. Bergmann, E. and Pullman, B. pp.179-187. Holland:Reidel Co.

Large, W.A. and Rang, H.P. (1978,a). Factors affecting the rate of incorporation of a false transmitter into mammalian motor nerve terminals. *J. Physiol.*, 285, 1-24.

Large, W.A. and Rang, H.P. (1978,b). Variability of transmitter quanta released during incorporation of a false transmitter into cholinergic nerve terminals. *J. Physiol.* 285, 25-34.

Lineweaver, H. and Burke, D. (1934). The determination of enzyme dissociation constants. *J. Am. Chem. Soc.*, 56, 658-666.

Long, J.P. (1961). Hemicholiniums: structure activity relationships and actions on the peripheral nervous system. *Fedn. Proc.*, 20, 583-586.

Long, J.P., Evans, G.T. and Wong, S. (1967). A pharmacologic evaluation of hemicholinium analogs. *J. Pharmac. exp. Ther.*, 155, 223-230.

Long, J.P. and Schueler, F.W. (1954). A new series of cholinesterase inhibitors. *J. Am. Pharm. Assoc. (Sci. Ed.)*, 43, 79-86.

Longo, V.G. (1958). Action of hemicholinium no. 3 on phrenic nerve action potentials. *J. Pharmac. exp. Ther.*, 122, 45A.

- Longo, V.G. (1959). Actions of hemicholinium No.3 on phrenic nerve action potentials. *Archs. int. Pharmacodyn.*, 119, 1-9.
- Loomis, T.A., Sommer, H.Z. and Collins, J.A. (1974). Lethality, antiesterase and hemicholinium-like actions of a series of bisquaternary ammonium compounds. *Toxicol. Appl. Pharmacol.*, 27, 666-675.
- Lowry, O.H. Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. chem.*, 193, 265-275.
- Lutz, R.E. and Jordan, R.H. (1949). Ring-chain tautomerism of α -(ethylethanolamino)-acetophenone. *J. Am. Chem. Soc.*, 71, 996-999.
- MacIntosh, F.C. Birks, R.I. and Sastry, P.B. (1956). Pharmacological inhibition of acetylcholine synthesis. *Nature*, 178, 1181.
- MacIntosh, F.C., Birks, R.I. and Sastry, P.B. (1958). Mode of action of an inhibitor of acetylcholine synthesis. *Neur.*, 8, suppl., 1, 90-91.
- MacIntosh, F.C. (1961). Effect of HC-3 on acetylcholine turnover. *Fedn. Proc.*, 20, 562-568.
- Maggio-Cavaliere, M.E. (1976). Acetylscophemicholinium? Chemical and pharmacological evaluation of an open ring hemicholinium. *Pharmac.*, 14, 274-287.
- Mann, S.P. and Hebb, C. (1975). Inhibition of choline acetyltransferase by quaternary ammonium analogues of choline. *Biochem. Pharmac.*, 24, 1013-1017.
- Mann, S.P. and Hebb, C. (1977). Free choline in the brain of the rat. *J. Neurochem.*, 28, 241-244.
- Marchbanks, R.M. (1968). The uptake of [14 C]choline into synaptosomes *in vitro*. *Biochem. J.*, 110, 533-541.
- Marshall, F.N. and Long, J.P. (1959). Pharmacologic studies on some compounds structurally related to the hemicholinium HC-3. *J. Pharmac. exp. Ther.*, 127, 236-240.
- Marshall, I.G. (1969). The effects of some hemicholinium-like substances on the chick biventer cervicis muscle preparation. *Eur. J. Pharmac.*, 8, 204-214.
- Martin, K. (1968). Concentrative accumulation of choline by human erythrocytes. *J. Gen. Physiol.*, 51, 497-516.
- Martin, K. (1969). Effects of quaternary ammonium compounds on choline transport in red cells. *Br. J. Pharmac.*, 36, 458-469.
- Martin, A.R. and Orkand, R.K. (1961). Postsynaptic effects of HC-3 at the neuromuscular junction of the frog. *Can. J. biochem. physiol.* 39, 343-349.

- Matthews, E.K. (1966). The presynaptic effects of quaternary ammonium compounds on the acetylcholine metabolism of a sympathetic ganglion. *Br. J. Pharmac. Chemother.*, 26, 552-556.
- Merck Index (1968). 8th edition. (Stecher, P.G. ed.) Merck and Co. Inc. USA.
- Morris, G. and Grewaal, D.S. (1969). Halogen substituted derivatives of acetylcholine as inhibitors of choline acetyltransferase. *Life Sci.*, 8, 511-516.
- Nachmansohn, D. and Machado, A.L (1943). The formation of acetylcholine: A new enzyme "choline acetylase". *J. Neurophysiol.*, 6, 397-403.
- Panormow, A. (1891). Benzoylirung einiger Kohlenhydrate und alko- hole. *Berichte Deut. Chem. Ges.* 24, pt.4., 971.
- Persson, B.O., Larsson, L., Schuberth, J. and Sörbo, B. (1967). 3-Bromacetyltrimethylammonium bromide, a choline acetylase inhibitor. *Acta Chemica. Scand.*, 21, 2283-2284.
- Potter, L.T. (1968). Uptake of choline by nerve endings isolated from the rat cerebral cortex. In: *The interaction of drugs and subcellular components.* ed. Campbell. pp293-304 London:Churchill, J.A. Ltd.
- Potter, L.T. (1970). Synthesis, storage and release of [^{14}C] acetylcholine in isolated rat diaphragm muscles. *J. Physiol.*, 206, 145-166.
- Potter, L.T. and Murphy, W. (1967). Electrophoresis of acetylcholine, choline and related compounds. *Biochem. Pharmac.*, 16, 1386-1388.
- Powers, M.F., Kruger, S. and Schueler, F.W. (1962). Synthesis and pharmacological studies of some aliphatic hemicholinium analogs. *J. Pharm. Sci.*, 51, 27-31.
- Rand, M.J. and Ridehalgh, A. (1965). Actions of hemicholinium and triethylcholine on responses of guinea-pig colon to stimulation of autonomic nerves. *J. Pharm. Pharmac.* 17, 144-156.
- Reisberg, R.B. (1957). Properties and biological significance of choline acetylase. *Yale J. Biol. Med.*, 29, 403-435.
- Reitzel, N.L. and Long, J.P. (1959,a). Hemicholinium antagonism by choline analogues. *J. Pharmac. exp. Ther.*, 127, 15-21.
- Reitzel, N.L. and Long, J.P. (1959, b). The neuromuscular blocking properties of α,α' dimethylethanolamino. 4,4'-biacetophenone. (Hemicholinium). *Archs. Int. Pharmacodyn.*, 119, 20-30.
- Rodríguez de Lores Arnaiz, G., Zieher, L.M. and De Robertis, E. (1970). Neurochemical and structural studies on the mechanism of action of hemicholinium-3 in central cholinergic synapses. *J. Neurochem.*, 17, 221-229.

- Roskoski, R. (1974). Choline acetyltransferase: reversible inhibition by bromoacetyl coenzyme A and bromoacetylcholine. *Biochem.*, 13, 2295-2298.
- Rossier, J. (1977). Choline acetyltransferase: A review with special reference to its cellular and subcellular localization. *Int. Rev. Neurobiol.*, 20, 283-337.
- Rowell, P.P. and Chiou, C.Y. (1974). Pharmacology of a false cholinergic transmitter. *Fedn. Proc.*, 33, 505.
- Saelens, J.K., Simke, J.P., Schuman, J. and Allen, M.P. (1974). Studies with agents which influence acetylcholine metabolism in mouse brain. *Archs. int. Pharmacodyn.*, 209, 250-258.
- Sanford, P.A. and Smyth, D.H. (1969). Choline transport by the small intestine. *J. Physiol.*, 205, 16P-17P.
- Scheidt, W.R., Hanson, J.C. and Rasmussen, P.G. (1969). The crystal and molecular structure of dichloro(ethylenedimorpholine) cobalt (II). *Inorg. Chem.*, 8, 2398-2401.
- Schuberth, J., Sundwall, A., Sörbo, B. and Lindell, J.-O. (1966). Uptake of choline by mouse brain slices. *J. Neurochem.*, 13, 347-352.
- Schueler, F.W. (1955). A new group of respiratory paralyzants I. The "hemicholiniums". *J. Pharmac. exp. Ther.*, 115, 127-143.
- Sellinger, O.Z., Domino, E.F., Haarstad, B. and Mohrman, M.E., (1969). Intracellular distribution of ^{14}C -hemicholinium-3 in the canine caudate nucleus and hippocampus. *J. Pharmac. exp. Ther.*, 167, 63-76.
- Siakotos, A.N., Filbert, M. and Hester, R. (1969). A specific radio-isotopic assay for acetylcholinesterase and pseudo-cholinesterase in brain and plasma. *Biochem. Med.* 3, 1-12.
- Simon, J.R., Mittag, T.W. and Kuhar, M.J. (1975). Inhibition of synaptosomal uptake of choline by various choline analogs. *Biochem. Pharmac.*, 24, 1139-1142.
- Slater, P. and Stonier, P.D. (1973). The uptake of hemicholinium-3 by rat brain cortex slices. *J. Neurochem.*, 20, 637-639.
- Smith, J.C., Cavallito, C.J. and Foldes, F.F. (1967). Choline acetyltransferase inhibitors: a group of styryl-pyridine analogs. *Biochem. Pharmac.*, 16, 2438-2441.
- Sollenberg, J., Stensiö, K.E. and Sörbo, B. (1979). N-substituted choline analogues as substrates for choline acetyltransferase. *J. Neurochem.* 32, 973-977.
- Spanner, S. and Ansell, G.B. (1973). Choline metabolism in discrete areas of the brain: the effect of hemicholinium-3. *Biochem. Soc. Trans.*, 1, 475-477.

- Spanner, S., Hall, R.C. and Ansell, G.B. (1976). Arterio-venous differences of choline and choline lipids across the brain of rat and rabbit. *Biochem. J.*, 154, 133-140.
- Stekol, J.A. and Weiss, K. (1950). The inhibition of growth of rats by triethylcholine. *J. biol. Chem.*, 185, 585-587.
- Sung, C.P. and Johnstone R.M. (1965). Evidence for active transport of choline in rat kidney cortex slices. *Can. J. Biochem.*, 43, 1111-1118.
- Tables of interatomic distances and configuration in molecules and ions. (1958). ed. Sutton, L.E., Jenkin, D.G.: Mitchell, A.D. and Cross, L.C. London: The Chemical Society.
- Takagi, H., Kojima, M., Nagata, M. and Kuromi, H. (1970). On the site of action of hemicholinium-3 at the rat phrenic nerve-diaphragm preparation with special reference to its multiple presynaptic actions. *Neuropharmac.*, 9, 359-367.
- Thampi, S.N., Domer, F.R., Haarstad, V.B. and Schuler, F.W. (1966). Pharmacological studies of α -phenyl hemicholinium 3. *J. Pharm. Sci.*, 55, 381-386.
- Thies, R.E. and Brooks, V.B. (1961). Postsynaptic neuromuscular block produced by hemicholinium No. 3. *Fedn. Proc.*, 20, 569-578.
- Trimble, M.E., Acara, M. and Rennick, B. (1974). Effect of hemicholinium-3 on tubular transport and metabolism of choline the perfused rat kidney. *J. Pharmac. exp. Ther.*, 189, 570-576.
- ^vTuček, S. (1973, a). Choline acetyltransferase activity in skeletal muscles after denervation *Exp. Neurol.*, 40, 23-25.
- ^vTuček, S. (1973, b). Choline acetyltransferase activity in skeletal muscles during postnatal development. *Expl. Neurol.*, 36, 378-388.
- ^vTuček, S. (1978). Acetylcholine synthesis in neurons. London: Chapman Hall.
- Volle, R.L. (1973). Frequency dependent decrease of quantal content in a drug-treated neuromuscular junction. *Naunyn-Schmeideberg's Arch. Pharmac.*, 278, 271-284.
- Von Schwarzenfeld, I. and Whittaker, V.P. (1977). The pharmacological properties of the cholinergic false transmitter N-2-acetoxyethyl-N-methylpyrrolidinium and its precursor N-2-hydroxyethyl-N-methylpyrrolidinium. *Br. J. Pharmac.*, 59, 69-74.
- White, H.L. and Cavallito, C.J. (1970). Choline acetyltransferase. Enzyme mechanism and mode of inhibition by a styrylpyridine analogue. *Biochim. biophys. Acta.*, 206, 343-358.
- Wilson, H. and Long, J.P. (1959). The effect of hemicholinium (HC-3) at various peripheral cholinergic transmitting sites. *Archs int. Pharmacodyn.*, 120, 343-352.

Yamamura, H.I. and Snyder, S.H. (1972). Choline:high affinity uptake by rat brain synaptosomes. *Science*, 178, 626-628.

Yamamura, H.I. and Snyder, S.H. (1973). High affinity transport of choline into synaptosomes of rat brain. *J. Neurochem.*, 21, 1355-1374.