

THE COMPARTMENTALIZATION OF ACETYLCHOLINE IN THE
PRESENCE OF THE VESICULAR UPTAKE BLOCKER AH5183

By

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A 5183

To the people of Canada!

For their kindness and for making me feel at home
these many years.

METAMORPHOSIS

Long have I labored with this form and, though it may not be so beautiful or elegant, it has been my state for many a year. I have grown used to it of necessity and now understand its requirements and its rhythm. But life does not stand idle nor does it value consistency for the sake of predictability; life loves diversity and the unexpected. Thus, it is change which is the way of all living things. But how can one give up what one knows without sadness or regret? Does not one fear the untested, the unknown?

Here, I have learned to love that which I cannot predict, to admire that which is different and shows something new with each examination, and I have learned not to fear that which I cannot control. Yet, I do not change without a feeling of longing for what is lost and cast away. I do not know whether I shall fly or crawl when I have left this form I know so well, but at least I know that life will seem different with the new eyes I gain. For me there is no turning back to what was, only the road ahead is open. I trust that what I have learned shall guide me in the new world which my new form will dictate.

From my past I shall have but the memories of my mistakes, for in the end this is perhaps what makes each one of us unique and interesting. Our successes might be matched by others, but our mistakes are very much our own. Here I have been allowed to make my own mistakes and indiscretions and, at times, I have even been granted the luxury of learning something from them. Some might think that I have learned too much, at least in the ways of errors, but such has been my form.

Farewell. Do not forget who I was, for I shall return a lepidopteran to recall the days when my feet clawed the ground in search of a meaning. On that day, perhaps we shall fly and find beauty in all that we are and have been.

SUMMARIES

1
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The Compartmentalization of Acetylcholine in the Presence
of the Vesicular Uptake Blocker AH5183

Abstract

The compound 2-(4-phenylpiperidino)cyclohexanol (AH5183), a potent inhibitor of vesicular acetylcholine (ACh) uptake, blocks the nerve impulse stimulated release of ACh from the superior cervical ganglion of the cat in a use dependent manner, but not until 14% of the initial ganglial ACh content has been released. The first objective of this work was to determine if blockade of ACh release was associated with a depletion of vesicular ACh. By using black widow spider venom (BWSV), a secretagogue which induces vesicular exocytosis, it was shown that depletion of vesicular stores did not likely occur as a result of AH5183 blockade. Furthermore, labeling the ACh synthesized in the presence of the drug, revealed that labeled ACh and pre-existing ACh were equally well released by the venom suggesting that some vesicles were insensitive to AH5183.

Thus, the second objective of these studies was to test whether an AH5183-insensitive vesicular compartment existed and to investigate which subcellular compartments played a role in the release of ACh by the various stimuli. Rat hippocampal slices were stimulated with K^+ , in the presence or absence of AH5183, and then with the purified active toxin of BWSV, α -LTX. The toxin's main effect was to decrease the ACh content of the cytoplasm. Labeling the ACh synthesized in the presence of AH5183 demonstrated that stimulation increased the specific activity of the ACh stored by the denser vesicular compartment, normally associated with a high turnover of transmitter, but not that of the lighter vesicles associated with a storage function.

To investigate how the occluded compartments recovered from AH5183, hippocampal slices were drug-exposed, stimulated, and allowed to recover in the drug's absence. ACh synthesized during the recovery was labeled. Transmitter release and the ACh content of the occluded compartments recovered slowly from the effects of AH5183 and no quantitative relation-

ship was found between the two events. The denser vesicular compartment recovered 1.5 times as quickly as the lighter one. The denser compartment accumulated labeled ACh while the lighter did not, suggesting that different sources of ACh exist within the cytoplasm.

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La Compartimentation de l'Acétylcholine
en Présence du Bloqueur de la Recapture Vésiculaire AH5183

Résumé

Le composé 2-(4-phénylpipéridino)cyclohexanol (AH5183), un inhibiteur puissant de la recapture de l'acétylcholine (ACh) vésiculaire, bloque de façon dépendante à l'usage, la libération de l'ACh du ganglion cervical supérieur du chat, stimulée par l'influx nerveux, mais toutefois, seulement après que 14% du contenu initial en ACh du ganglion ait été libéré. L'objectif premier de ce travail fut de déterminer si le blocage de la libération d'ACh était associé à la déplétion de l'ACh vésiculaire. L'utilisation du venin de l'araignée veuve noire (BWSV), un secrétagogue qui provoque l'exocytose vésiculaire, a démontré que l'épuisement des réserves vésiculaires n'avait probablement pas lieu suite au blocage produit par l'AH5183. De plus, l'ACh radioactive synthétisée en présence de cette drogue a révélé que l'ACh radioactive nouvellement synthétisée ainsi que l'ACh pré-existante étaient toutes deux aussi bien libérées suite à l'action du venin, suggérant que certaines vésicules étaient insensibles à l'AH5183.

Par conséquent, le deuxième objectif de ces travaux fut de déterminer s'il existait un compartiment vésiculaire insensible à l'effet d'AH5183 et d'examiner quels compartiments subcellulaires jouent un rôle dans la libération de l'ACh par différents stimuli. Des sections tissulaires d'hippocampe de rat furent stimulées par le K^+ , en présence ou absence d'AH5183, et par la suite avec la toxine active purifiée du BWSV, soit l' α -latrotoxine. Le principal effet de la toxine fut de

diminuer le contenu en ACh cytoplasmique. Il a été démontré, suite au marquage de l'ACh nouvellement synthétisée en présence de l'AH5183, que la stimulation augmentait l'activité spécifique de l'ACh contenue dans le compartiment vésiculaire le plus dense, celui-ci étant normalement associé à un "turnover" rapide du transmetteur. Cependant, celle des vésicules plus légères, associées à une fonction de stockage, n'était pas affectée.

Pour examiner de quelle façon les compartiments ayant subi un blocage par le AH5183 avait récupéré de celui-ci, des sections tissulaires d'hippocampe ont été exposées à cette drogue, puis stimulées et placées en période de récupération en son absence. L'ACh synthétisée durant la période de récupération était marquée à l'aide d'un précurseur radioactif. La libération de transmetteur et le contenu en ACh des compartiments ayant subi un blocage ont récupérés lentement suite aux effets de l'AH5183, et aucune relation quantitative ne fut observée entre ces deux événements. Le compartiment vésiculaire le plus dense a récupéré 1.5 fois plus rapidement que le plus léger. Le compartiment le plus dense a de plus accumulé l'ACh radioactive, tandis que le plus léger ne le fit pas, suggérant que des sources multiples d'ACh existent à l'intérieur du cytoplasme.

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La Compartimentación de Acetilcolina en
Presencia del Antagonista del Transporte Vesicular AH5183

Resumen

El compuesto 2-(4-fenilpiperidino)ciclohexanol (AH5183), es un potente inhibidor de la captación vesicular de la acetilcolina (ACh) ya que bloquea, de una manera uso-dependiente, la liberación de ACh en el ganglio cervical superior del gato, estimulada por el impulso nervioso. Sin embargo, tal efecto bloqueador no ocurre sino hasta que haya sido liberado un 14% del contenido inicial de la ACh ganglionar. El primer objetivo de este trabajo fue determinar si el bloqueo de la liberación de ACh estaba asociado con una depleción de la ACh vesicular. Con el uso del veneno de la araña Viuda Negra, que es un secretagogo que induce exocitosis vesicular, fue demostrado que la depleción de los depósitos vesiculares problemente no ocurrió como resultado del bloqueador AH5183. Más aún, al marcar la ACh sintetizada en presencia de la droga, fue observado que tanto la ACh liberada como la pre-existente, fueron igualmente bien liberadas por el veneno del arácnido, lo que sugirió que algunas vesículas fueron insensibles al AH5183.

Por consiguiente, el segundo objetivo de estos estudios fue probar si existía un compartimiento vesicular insensible al AH5183, e investigar que compartimientos jugaban un papel en la liberación de la ACh producida por diversos estímulos. Rebanadas de hipocampo de rata fueron estimuladas por K^+ , en presencia y ausencia del bloqueador AH5183, y luego, en presencia de la toxina activada purificada del veneno del arácnido, el α -LTX. El principal efecto de esta toxina fue disminuir el contenido

citoplásmico de ACh. El marcaje de la ACh sintetizada en presencia de AH5183 demostró que la estimulación aumentó la actividad específica de la ACh almacenada por el compartimiento vesicular más denso que está normalmente asociada con un alto recambio del transmisor, pero no tuvo efecto en las vesículas más ligeras asociadas con funciones almacenadoras.

Para investigar cómo los compartimientos ocluidos por el AH5183 se recuperan, las rebanadas del hipocampo fueron expuestas a la droga, estimuladas, y se les permitió recuperarse en ausencia de la droga. La ACh sintetizada durante la recuperación fue marcada. La liberación del transmisor y el contenido de ACh de los compartimientos ocluidos se recuperaron lentamente de los efectos del AH5183, pero no fueron encontradas relaciones cuantitativas entre los dos eventos. El compartimiento vesicular más denso se recuperó 1.5 veces más rápido que el más ligero. Asimismo, el compartimiento más denso acumuló ACh marcada mientras que el más ligero, no. Esto sugirió que existen diferentes fuentes de ACh dentro del citoplasma.

TABLE OF CONTENTS

TABLE OF CONTENTS

	<u>Page</u>
<u>SUMMARIES</u>	i
<u>Abstract</u>	ii
<u>Resume</u>	iv
<u>Resumen</u>	vi
<u>TABLE OF CONTENTS</u>	1
<u>INDEX OF FIGURES</u>	11
<u>INDEX OF TABLES</u>	18
<u>LIST OF ABBREVIATIONS</u>	21
<u>PREFACE</u>	24
<u>ACKNOWLEDGEMENTS</u>	29
<u>INTRODUCTION</u>	36
1.1. THE SYNTHESIS OF ACETYLCHOLINE BY CHOLINERGIC NEURONS.....	37
1.1.1. Choline acetyltransferase.....	37
1.1.2. Choline transport and acetylcholine synthesis.....	39
1.1.3. The source of acetyl-Coenzyme A for acetylcholine synthesis.....	41

1.1.4. Regulation of acetylcholine synthesis.....	42
1.2. THE COMPARTMENTALIZATION AND RELEASE OF ACETYLCHOLINE.....	45
1.2.1. The intracellular acetylcholine compartments.....	45
1.2.2. The release of acetylcholine.....	48
1.2.2.1. The spontaneous release of acetylcholine.....	48
1.2.2.2. The stimulated release of acetylcholine.....	49
1.2.2.3. The quantal nature of stimulated acetylcholine release.....	52
1.3. CHOLINERGIC VESICULAR MECHANISMS OF ACETYLCHOLINE UPTAKE	53
1.3.1. The vesicular ATPase.....	55
1.3.2. Synaptic vesicles of <i>Torpedo</i> electric organ establish a proton gradient important for the uptake of acetylcholine.....	57
1.3.3. The active and passive transport of acetylcholine by synaptic vesicles of <i>Torpedo</i>	60
1.3.4. The aqueous spaces of synaptic vesicles from <i>Torpedo</i> electric organ.....	63

1.4. THE VESICULAR UPTAKE BLOCKER 2-(4-PHENYLPYPERIDINO)CYCLOHEXANOL (AH5183) AS A PHARMACOLOGICAL TOOL FOR CHOLINERGIC TERMINALS.....	66
1.4.1. Early work and description of AH5183 action.....	68
1.4.2. Confirmation of AH5183's action on vesicular uptake of acetylcholine.....	68
1.4.3. AH5183 action on vesicular acetylcholine uptake and structural considerations.....	70
1.4.4. The effect of AH5183 on the release of acetylcholine from various cholinergic preparations.....	83
1.4.5. Sights of AH5183 action other than the vesicular acetylcholine transporter.....	91
1.5. THE VESICULAR VERSUS CYTOPLASMIC ORIGIN OF STIMULATED ACETYLCHOLINE RELEASE.....	93
1.5.1. Intraterminal acetylcholine compartments.....	95
1.5.2. Considerations of the quantal nature of acetylcholine release and presynaptic currents.....	97
1.5.3. Vesicle fusion and mediatophore.....	98

1.6. BLACK WIDOW SPIDER VENOM AS A TOOL FOR STUDYING TRANSMITTER RELEASE AND VESICLES.....	101
1.6.1. Isolation of the active component of black widow spider venom.....	102
1.6.2. The action of black widow spider venom on nerve terminals.....	103
1.6.2.1. Electrophysiological studies.....	103
1.6.2.2. Biochemical studies of transmitter release.....	104
1.6.2.3. Morphological studies.....	107
1.6.3. The α -LTX binding site and its location.....	108
1.6.4. Possible mechanisms of α -LTX action.....	109
<u>STATEMENT OF THE PROBLEM.....</u>	111
<u>MATERIALS AND METHODS.....</u>	114
2.1. <i>IN SITU</i> PERFUSION OF THE CAT SUPERIOR CERVICAL GANGLIA.....	115
2.1.1. Induction and maintainance of anaesthesia....	115
2.1.2. Preparation of the superior cervical ganglia for perfusion.....	115
2.2. DETERMINATIONS INVOLVING BLACK WIDOW SPIDER VENOM.....	117

2.2.1. Determination of the quantity of α -LTX in the black widow spider venom.....	117
2.2.2. Determination of the amount of anti- α -LTX antisera needed to neutralize the black widow spider venom.....	119
2.3. PREPARATION OF RAT BRAIN HIPPOCAMPAL SLICES.....	122
2.4. PREPARATION OF THE P ₂ PELLET AND SUBCELLULAR FRACTIONS.....	125
2.4.1. Preparation of the P ₂ pellet from hippocampal slices.....	125
2.4.2. Preparation of the P ₃ and S ₃ fractions from hippocampal slices.....	129
2.4.3. Preparation of the bound subcellular compartments of acetylcholine from hippocampal slices.....	131
2.5. ENZYME CHARACTERIZATION OF THE TOTAL TISSUE, P ₂ PELLET, AND SUBCELLULAR FRACTIONS FROM HIPPOCAMPAL SLICES.....	133
2.6. EXTRACTION PROCEDURES FOR ACETYLCHOLINE AND [³ H]-ACETYLCHOLINE.....	139
2.7. ACETYLCHOLINE, [³ H]-ACETYLCHOLINE, AND [³ H]-ACETYLDIETHYLHOMOCHOLINE ASSAYS.....	141
2.8. PROTEIN DETERMINATION.....	142

2.9. SYNTHESIS OF DIETHYLHOMOCHOLINE, [³ H]-DIETHYLHOMOCHOLINE, AND [³ H]-ACETYLDIETHYLHOMOCHOLINE.....	143
2.10. CALCULATIONS, STATISTICS, AND MATERIALS....	144
<u>RESULTS</u>	146
3.1. THE EFFECT OF AH5183 AND BLACK WIDOW SPIDER VENOM ON THE RELEASE OF ACETYLCHOLINE FROM THE CAT SUPERIOR CERVICAL GANGLIA	147
3.1.1. The release of acetylcholine from ganglia in the presence and absence of AH5183.....	147
3.1.2. The vesicular release of acetylcholine by black widow spider venom.....	148
3.1.3. The release of acetylcholine by black widow spider venom in the presence of AH5183.....	155
SUMMARY AND DISCUSSION OF SECTION 3.1.....	162
3.2. THE READILY RELEASABLE ACETYLCHOLINE POOL OF HIPPOCAMPAL BRAIN SLICES AND SYNAPTOSOMES.....	165
SUMMARY AND DISCUSSION OF SECTION 3.2.....	167
3.3. THE EFFECT OF AH5183 AND α -LTX ON THE RELEASE AND STORAGE OF ACETYLCHOLINE FROM RAT HIPPOCAMPAL BRAIN SLICES.....	169

3.3.1. The release of acetylcholine from hippocampal slices in the presence and absence of AH5183 $\pm$ α -LTX.....	169
3.3.2. The tissue acetylcholine content and the subcellular acetylcholine distribution from synaptosomes of hippocampal slices exposed to AH5183 and α -LTX.....	173
3.3.3. The acetylcholine content of the bound subcellular compartments from hippocampal slices exposed to AH5183 and α -LTX.....	175
3.3.4. The release of acetyldiethylhomocholine by α -LTX from hippocampal slices.....	179
SUMMARY AND DISCUSSION OF SECTION 3.3.....	180
3.4. THE EFFECT OF AH5183 ON THE STORAGE AND TURNOVER OF ACETYLCHOLINE FROM HIPPOCAMPAL SLICES.....	180
3.4.1. The effect of AH5183 on the release of acetylcholine and its turnover in the cytoplasmic and occluded compartments of rested and stimulated hippocampal slices.....	182
3.4.2. The effect of AH5183 on the acetylcholine content and turnover of the subcellular-bound compartments of stimulated and rested hippocampal slices.....	189

SUMMARY AND DISCUSSION OF SECTION 3.4.....	194
3.5. THE RECOVERY OF ACETYLCHOLINE RELEASE AND OF THE SUBCELLULAR COMPARTMENTS FROM THE BLOCKING ACTIONS OF AH5183.....	194
3.5.1. Recovery time course of the release of acetylcholine from AH5183 blockade.....	195
3.5.2. Total tissue acetylcholine content and acetylcholine specific activity.....	201
3.5.3. Recovery time course of the acetylcholine and [³ H]-acetylcholine contents from the cytoplasmic and occluded fractions of AH5183-treated hippocampal slices.....	207
3.5.4. Recovery of the acetylcholine and [³ H]-acetylcholine content of the subcellular bound pools from AH5183-treated slices.....	212
SUMMARY AND DISCUSSION OF SECTION 3.5.....	218
<u>DISCUSSION</u>	221
4.1. INHIBITION OF ACETYLCHOLINE RELEASE BY AH5183 IS NOT ACCOMPANIED BY A DEPLETION OF VESICULAR ACETYLCHOLINE IN THE SUPERIOR CERVICAL GANGLION.....	222
4.2. THE READILY-RELEASABLE POOL OF ACETYLCHOLINE IN HIPPOCAMPAL SLICES IS ESTIMATED TO BE ABOUT 20-27% OF THE ACh CONTENT.....	227

4.3. ACETYLCHOLINE RELEASED BY α -LTX FROM AH5183-BLOCKED HIPPOCAMPAL SLICES IS MAINLY ASSOCIATED WITH CYTOPLASMIC ACETYLCHOLINE.....	229
4.4. TWO OCCLUDED FRACTIONS ARE CAPABLE OF ACCUMULATING [^{3} H]-ACETYLCHOLINE IN THE PRESENCE OF AH5183.....	231
4.5. RECOVERY OF ACETYLCHOLINE AND [^{3} H]-ACETYLCHOLINE RELEASE FROM AH5183 BLOCKADE IS NOT TEMPORALLY RELATED TO THE RECOVERY OF THE SUBCELLULAR FRACTION'S ACETYLCHOLINE AND [^{3} H]-ACETYLCHOLINE CONTENT.....	234
4.6 THE CHOLINERGIC NERVE TERMINAL.....	241
<u>REFERENCES</u>	246
<u>APPENDIX A</u>	286
<u>APPENDIX B</u>	365

INDEX OF FIGURES

INTRODUCTION

Figure 1I. Model of the active
ACh transport system of cholinergic
vesicles..... 64

Figure 2I. Chemical structures
of AH5183 and AH5954..... 67

Figure 3I. Three dimensional
representation of the active AH5183
analogues in a trans-configuration..... 74

Figure 4I. Chemical structure
of the parent ring compound for the
AH5183 family of analogues..... 75

MATERIALS AND METHODS

Figure 1M. Gel electrophoresis and
densitometric scan of BWSV.....120

Figure 2M. Quantification of antibody
needed to neutralize BWSV activity
on striatal synaptosomes.....123

Figure 3M. Scheme describing the
isolation of the P₂ pellet from
hippocampal slices.....127

Figure 4M. Scheme describing the
isolation of the subcellular
compartments from the P₂ pellet.....130

RESULTS

<u>Figure 1R.</u> Release of ACh $\pm$ AH5183.....	149
<u>Figure 2R.</u> Release of ACh by BWSV.....	150
<u>Figure 3R.</u> Release of ACh by BWSV $\pm$ anti- α -LTX antiserum.....	152
<u>Figure 4R.</u> The release of ACh by BWSV in the presence of naive serum.....	154
<u>Figure 5R.</u> The release of ACh and [³ H]-ADEHCh by BWSV.....	156
<u>Figure 6R.</u> The release of ACh by BWSV in the presence of AH5183 following an AH5183 induced block.....	157
<u>Figure 7R.</u> Total release of ACh by BWSV $\pm$ AH5183.....	160
<u>Figure 8R.</u> The release of ACh and [³ H]-ACh by BWSV.....	161
<u>Figure 9R.</u> Model of the cholinergic terminal.....	164
<u>Figure 10R.</u> The readily releasable pool of ACh in the hippocampus of the rat.....	168
<u>Figure 11R.</u> Outline of the incubation procedure used in the hippocampal slices.....	171

<u>Figure 12R.</u> Release of ACh by high K^+ and α -LTX from hippocampal slices in the presence and absence of AH5183.....	172
<u>Figure 13R.</u> The ACh content of the P_3 and S_3 fractions from slices treated with or without α -LTX.....	177
<u>Figure 14R.</u> The ACh content of the bound-subcellular compartments of slices treated with or without α -LTX.....	178
<u>Figure 15R.</u> The release of ACh and ADEHCh by α -LTX.....	181
<u>Figure 16R.</u> Experimental protocol to test the turnover of ACh in the subcellular compartments of AH5183 exposed slices.....	183
<u>Figure 17R.</u> The release of ACh from hippocampal slices $\pm$ AH5183.....	185
<u>Figure 18R.</u> The effect of stimulation on the turnover of ACh in the P_3 and S_3 fractions of control slices.....	186
<u>Figure 19R.</u> The effect of stimulation on the turnover of ACh in the P_3 and S_3 fractions of slices exposed to AH5183.....	188
<u>Figure 20R.</u> The ratio of the specific activities of ACh from the P_3 and S_3 fractions of hippocampal slices.....	190

<u>Figure 21R.</u> The effect of stimulation on the turnover of ACh in the bound-subcellular fractions of control slices.....	192
<u>Figure 22R.</u> The effect of stimulation on the turnover of ACh from the bound-subcellular compartments of slices exposed to AH5183	193
<u>Figure 23R.</u> Experimental protocol to determine the recovery kinetics of ACh release from AH5183-treated HIPP slices.....	197
<u>Figure 24R.</u> The recovery of ACh release from HIPP slices following the removal of AH5183.....	198
<u>Figure 25R.</u> The specific activity of released ACh from AH5183-treated HIPP slices following the removal of the drug, AH5183.....	200
<u>Figure 26R.</u> The effect of drug removal on total tissue ACh content from AH5183-treated slices.....	203
<u>Figure 27R.</u> Effect of AH5183 removal on the specific activity of ACh in the total tissue from AH5183-exposed slices.....	204
<u>Figure 28R.</u> The effect of time on the ACh and [^3H]-ACh content from the cytoplasmic and occluded pools of control slices.....	209

Figure 29R. The effect of time following the removal of AH5183 on the ACh and [^3H]-ACh content from the cytoplasmic and occluded pools of drugged slices.....210

Figure 30R. Experimental protocol to determine the recovery kinetics of the ACh content of the subcellular occluded fractions from AH5183-treated HIPP slices.....214

Figure 31R. The effect of time on the ACh and [^3H]-ACh content of the subcellular bound pools from control slices.....215

Figure 32R. The effect of time following the washout of AH5183 on the ACh and [^3H]-ACh content of the subcellular bound pools from AH5183-treated slices.....217

DISCUSSION

Figure 1D. Model of the cholinergic terminal determined from experiments where the effects of AH5183 and BWSV were tested in the cat SCG.....226

Figure 2D. Model of the cholinergic terminal derived from experiments done on hippocampal slices to determine the existence of an occluded fraction capable of accumulating newly synthesized ACh in the presence of AH5183.....235

Figure 3D. Model of the cholinergic
terminal derived from experiments done
in hippocampal slices to determine the
recovery of slices from the actions of AH5183.....240

Figure 4D. Model of the cholinergic
terminal hypothesizing the interrelationship
of the ACh compartments.....244

INDEX OF TABLES

INTRODUCTION

<u>Table 1I.</u> Potency of AH5183 and analogues.....	76

<u>Table 2I.</u> Sites of AH5183 action.....	94

MATERIALS AND METHODS

<u>Table 1M.</u> ACh recovery in synaptosomal pellet isolation.....	128

<u>Table 2M.</u> The diffusion of ACh from the soluble compartment to the bound subcellular fractions.....	134

<u>Table 3M.</u> Enzyme characterization of subcellular fractions.....	138

RESULTS

<u>Table 1R.</u> Release of ACh from AH5183 and BWSV-treated ganglia.....	159

<u>Table 2R.</u> Release of ACh and [³ H]-ACh from AH5183 and BWSV-treated ganglia.....	163

<u>Table 3R.</u> Effects of eserine on ACh content.....	166

<u>Table 4R.</u> Effects of AH5183 and α -LTX on ACh release and tissue content.....	174

<u>Table 5R.</u> Accumulation of ^3H in tissue and synaptosomes.....	206

<u>Table 6R.</u> Summary of the specific activity of released ACh and that of various tissue fractions.....	219

LIST OF ABBREVIATIONS

ABBREVIATIONS USED

<u>Abbreviation</u>	<u>Full Name</u>
ACh	Acetylcholine
[³ H]-ACh	Acetylcholine (Tritiated)
AChE	Acetylcholinesterase
ADEHCh	Acetyldiethylhomocholine
[³ H]-ADEHCh	Acetyldiethylhomocholine (Tritiated)
ATP	Adenosine Triphosphate
ANOVA	Analysis Of Variance
BWSV	Black Widow Spider Venom
Ch	Choline
[³ H]-Ch	Choline (Tritiated)
ChAT	Choline Acetyltransferase
cpm	Counts Per Minute
DEHCh	Diethylhomocholine
[³ H]-DEHCh	Diethylhomocholine (Tritiated)
dpm	Dissintegrations Per Minute
³ H-DA	Dopamine (Tritiated)
e.p.p.	End-Plate Potential
EGTA	[Ethylenebis(oxyethylenenitrilo)]tetraacetic Acid
HC-3	Hemicholinium-3
HIPP	Hippocampal
LDH	Lactate Dehydrogenase
α -LTX	Alpha-Latrotoxin
m.e.p.p.	Miniature End-Plate Potential
NADH	β -Nicotinamide Adenine Dinucleotide
Pargyline	N-Methyl-N-2-Propynylbenzylamine
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethansulfonic Acid
PAGE	Polyacrylamide Gel Electrophoresis
NaCac	Sodium Cacodylate
SDS	Sodium Dodecyl Sulfate

SDH	Succinic Dehydrogenase
SCG	Superior Cervical Ganglion
TPB	Tetraphenylboron
TLC	Thin Layer Chromatography
TCA	Trichloroacetic Acid
³ H	Tritium
Tris base	2-Amino-2-(Hydroxymethyl)-Propane-1,3-Diol
AH5183	2-(4-Phenylpiperidino)Cyclohexanol

PREFACE

The aim of this thesis has been to investigate how acetylcholine (ACh) compartmentalizes itself when the vesicular uptake blocker, 2-(4-phenyl-piperidino)cyclohexanol (AH5183), is present and to try and better understand what role this compartmentalization plays in the release of transmitter. In this dissertation I refer to this compound as AH5183, the original name given to this drug by the pharmaceutical company that first synthesized it. Within the past 4 years the name vesamicol has been suggested by S. M. Parsons; though it is becoming the name of choice, much of the literature concerning this compound uses the abbreviation and thus, for the sake of clarity it is preserved here.

It is the policy of many universities that candidates for the doctoral degree claim some originality in their contribution to the human store of knowledge. This of course presupposes that the candidate knows every thought ever put forth concerning the matter to be discussed or that indeed the candidate's sense of self worth is great enough to harbor the belief that this contribution shall in fact be placed in the "knowledge barrel" and that it will not suffer the fate of such insightful work as that of Gregor Mendel. This need for the original contribution of knowledge forgets such men as Aristotle, who stated that all he knew was that he knew nothing, and Issac Newton, who stood on the shoulders of giants to better see what lay on the horizon of natural phenomena. It is very difficult to claim originality when one acknowledges one's total ignorance or the overwhelming influence of past "giants" and this by men who are held to be great and original thinkers. Indeed, if one holds the views of Karl Popper, that man can never know anything with absolute certainty and that science can only provide a better estimate of the possible truth by disproving views rather than by proving them, then one is left with no alternative but to disavow any contribution to original knowledge since such a concept is false. Thus, it would be best that I re-title this section, "A disclaimer of contribution to original knowledge." I wish only to claim that the following thoughts are the result of my imagination, bound and held in check by the constraints of the experiments I have performed and the results that these experiments have yielded. I must acknowledge that these

thoughts, though mine, must be the result of many interactions with better thinkers than myself and from the reading of both relevant and not so relevant works. I cannot claim an absolute knowledge of this or any field of human endeavor and must therefore state that these are reasonably supported, personal thoughts about the workings of the cholinergic nerve terminal and that to the best of my recollection they are relatively new or "original".

Here, thus, are my "original" contributions to the field of cholinergic neurobiology:

1. Preganglionic nerve stimulation of the cat superior cervical ganglion, in the presence of AH5183, leads to a use dependent inhibition in the release of ACh. Because of AH5183's ability to block the vesicular transport of ACh, it was of interest to investigate whether this blockade of release was the result of depleted ACh vesicular stores. The venom of black widow spiders was used to test this question because of its ability to induce the exocytosis of vesicular contents. Two basic observations were made with these experiments:

- A). The venom released large quantities of transmitter in the continued presence of AH5183 at a time when nerve impulses were no longer able to mobilize transmitter for release. This result was interpreted to indicate that all vesicular stores need not be depleted for the inhibition of nerve impulse stimulated ACh release to take place. Furthermore, it is suggested that this result indicates that probably not all of the nerve terminal's vesicles are directly involved in the nerve impulse-evoked release of transmitter to the synaptic cleft.

- B). By perfusing and stimulating ganglia with [^3H]-Ch during drug exposure, it was possible to label that ACh synthesized in the presence of AH5183 while inducing a block in the release of transmitter. Exposure of these ganglia to venom brought about the release of both [^3H]-ACh and pre-existing ACh. This result implies that vesicles may differ in their sensitivity to the inhibitory action of AH5183 on transmitter transport *in vivo*.

2. To directly test the possibility that vesicles differed in their sensitivity to AH5183, rat hippocampal slices were exposed to AH5183 and, while exposed to [^3H]-Ch so as to label newly synthesized transmitter, they were either stimulated or left at rest. Stimulation of the slices by high K^+ increased the turnover of ACh, measured as an increase in the specific activity of the transmitter, in both the cytoplasmic and occluded compartments of drug-exposed slices. When the occluded compartments were further subfractionated, the increased accumulation of [^3H]-ACh seen in drug-exposed, stimulated slices was attributed to two of the occluded subfractions, E&F and G&H, increasing their [^3H]-ACh content while a third fraction, D, did not accumulate labeled transmitter to any appreciable extent. These results are consistent with a differential sensitivity of vesicles to inhibition by AH5183.

3. If different occluded ACh compartments are AH5183-sensitive to varying extents they should recover with different kinetics from the effects of the drug. Thus, slices were exposed to AH5183, stimulated, and subsequently allowed to recover in the drug's absence. Transmitter synthesized during the recovery period was labeled by the use of [^3H]-Ch so as to better assess the recovery of the various fractions. Fractions, E&F and G&H, accumulated ACh at a rate 1.5 times faster than that of fraction D, again consistent with the idea of heterogeneous sensitivity. Though fraction D accumulated ACh with time, it did not accumulate [^3H]-ACh. Thus, it is suggested that compartments of ACh likely exist in the cytoplasm for the source of ACh accumulated by the D fraction appears different from that accumulated by fractions E&F and G&H.

4. The specific activity of ACh released by hippocampal slices stimulated at various times following the removal of AH5183 was compared to the specific activity of transmitter found in the

various subcellular compartments. It was observed that during the recovery from drug-blockade released transmitter always had a higher specific activity than that of the intraterminal compartments. The pattern for released ACh specific activity qualitatively matched the pattern observed in the G&H fraction but not that of any other compartment.

5. In the superior cervical ganglion, nerve stimulation can release 14% of the initial ACh content in the presence of AH5183. This amount of transmitter is the same as that estimated to form the readily releasable pool of ACh in this sympathetic ganglion. The size of the readily releasable transmitter pool was estimated in hippocampal slices by measuring the decrease in ACh content seen upon stimulation of AH5183-exposed hippocampal slices. The synthesis of new transmitter was inhibited in these slices by the use of hemicholinium-3. The readily releasable pool of transmitter was found to be 20% of the ACh content when whole tissue was used for the estimate and 27% when the synaptosomal fraction was measured. Because the synaptosomal fraction contains nerve endings and not cell bodies it was concluded that 27% may be a closer estimate of the pool's size.

6. Hippocampal slices exposed to AH5183 and stimulated with K^+ have a greatly attenuated release of ACh. In these slices the addition of α -Latrotoxin, the active component of black widow spider venom, causes the release of ACh in the continued presence of the drug. The major subcellular compartment affected by toxin exposure is the that associated with the cytoplasm and not the occluded subcellular fraction.

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It is impossible for one to begin and end an endeavor such as a Ph.D. without relying on the help of many friends and colleagues. I have perhaps abused this privilege more than others by staying here so long but people have always been very patient and kind to me. Without the support I received, life would have been dreary and inevitably I would not be taking such fond memories of this department.

Perhaps the bulk of the despair which I must have caused fell on my supervisor, Professor Brian Collier. If patience is a virtue, then he is one of the most virtuous men ever to live. No student could ask for a better supervisor; he gave me the freedom to make my own errors and always brought me back to something resembling reality when my imagination flew too close to the sun. I leave his laboratory a much better investigator than when I went in, but I leave a mentor and special friend behind when I go. Until I came to the Gaffer's lab I did not know how pleasant afternoon teas could be with a bottle of beer. I thank Chris for her hospitality and for those wonderful invitations; I will always remember the domino games at Christmas, the fine port, and the delicious meals.

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INTRODUCTION

This thesis is an investigation of the manner in which acetylcholine (ACh) is compartmentalized in the presence and absence of the vesicular uptake blocker 2-(4-phenylpiperidino)cyclohexanol (AH5183) and to the possible consequences this may have for the release of neurotransmitter. It is my intent that the introduction should serve two functions. The first, is that it should review that literature pertinent to the investigations which are herein described and second, that it should provide the reader with a sense of the limitations inherent in the tools presently available to cholinergic biologists as well as the central controversy about the origin of released ACh and the mechanism by which it is released.

1.1. THE SYNTHESIS OF ACETYLCHOLINE BY CHOLINERGIC NEURONS.

Under physiological conditions when a sufficient stimulus reaches the cholinergic nerve endings these respond by liberating ACh so as to affect the post junctional element. Once released, ACh diffuses across the synaptic cleft and either diffuses away, interacts with a receptor, or is metabolized by the enzyme acetylcholinesterase (AChE), which hydrolyzes the ester bond and liberates acetate and choline (Ch). This enzymatic cleavage inactivates ACh and thus terminates the action of the transmitter. Because ACh action is terminated by its breakdown and not by a re-uptake system, such as that for the catecholamines, the nerve terminal must resynthesize any ACh that it releases, or eventually it faces the depletion of transmitter stores. Work on the synthesis of ACh and its regulation have been recently reviewed (Tucek, 1983, 1985, 1988).

1.1.1. Choline acetyltransferase.

The enzyme responsible for the synthesis of ACh was first discovered by Nachmansohn and Machado (1943) in soluble extracts of cholinergic tissues, and is presently called choline acetyltransferase (ChAT). Though the enzyme is found in non cholinergic tissues, such as

the placenta (Morris, 1966), in neuronal tissues, such as the brain, the distribution of ChAT is well matched by the presence of ACh (early findings reviewed by Feldberg, 1945; Hebb and Morris, 1969). The enzyme synthesizes ACh by transferring the acetyl group of acetyl-Coenzyme A (AcCoA) to Ch such that an ester bond is formed between the acetate and Ch. The enzyme reaction is usually considered to be a sequential ordered reaction (White and Cavallito, 1970; White and Wu, 1973; Ryan and McClure, 1980). In the case of ChAT, this means that AcCoA must first bind to the enzyme before Ch can, and following the binding of Ch the synthesis takes place. Just as the reactants must bind in a sequential order, so too the products are released in sequential order with ACh being released first and the CoA second. The Michaelis-Menten constant for AcCoA is K_m is 15-100 μ M and that for Ch is 0.4-3 mM.

As subcellular fractionation techniques for nervous tissue were developed, Hebb and Smallman (1956) and Hebb and Whittaker (1958) found that both ACh and ChAT activity were co-isolated in a mitochondrial pellet. Further work on fractionation revealed that the ACh and ChAT were in fact not associated with mitochondria (see interesting discussion in Feldberg, 1945) but with synaptosomes, nerve terminals which have resealed following the homogenization of the tissue (Gray and Whittaker, 1962). Within the nerve terminals ChAT was thought to be a soluble enzyme, existing in the cytoplasm, but during isolation procedures that use low ionic strength solutions, ChAT would stick to membranes (Fonnum, 1968). More recent work on the possible forms of ChAT has revealed that 3 major forms of the enzyme exist (Benishin and Carroll, 1983; Eder-Colli and Amato, 1985; Eder-Colli *et al.*, 1986). The largest fractional component of ChAT consists of a soluble form, probably cytoplasmic, which can be collected in a high-speed supernatant by repeated washings of the pellet with 100 mM sodium phosphate. There is an ionically membrane-bound form of ChAT which can be released from membranes by a high-salt wash and then a membrane-bound form which cannot be set free of the membranes unless a detergent is used. It is suspected that it is this third form of ChAT which may have an antigenic site facing the outside of the plasma membrane (Docherty *et al.*, 1982). The 3 forms differ

with regards to pH optimum, affinities for substrates and inhibition by products, heat stability, and substrate specificity (Benishin and Carroll, 1983; Eder-Colli and Amato, 1985; Eder-Colli *et al.*, 1986). Furthermore, stimulation of nerve endings by different means cause differential stimulation of the various ChAT forms (Carroll, 1987).

There is as yet no direct evidence concerning a differential role or function of the various forms of ChAT in the synthesis of ACh, but one report suggests an important role is played by the non-ionically membrane-bound form (Mykita and Collier, 1989). Establishing which form is important to a given process may come from molecular biological studies of ChAT, especially since ChAT has now been cloned and sequenced (Itoh *et al.*, 1986; Berrard, *et al.*, 1987).

1.1.2. Choline transport and acetylcholine synthesis.

In order for nerve terminals to synthesize ACh, they need not only the synthesizing enzyme, ChAT, but the precursors, Ch and AcCoA, as well. Nerve terminals, if they synthesize Ch *de novo* at all do so to a very small extent (Ansell and Spanner, 1967, 1971; Blusztajn and Wurtman, 1981) and must therefore obtain the precursor from extracellular sources. The importance of extracellular Ch to ACh synthesis was well documented for the superior cervical ganglia of the cat by Birks and MacIntosh (1961). They reported that perfusion of the ganglion, in the absence of external Ch and in the presence of an AChE inhibitor, led to an exhaustion of transmitter release. Furthermore, even in the presence of extracellular Ch, when hemicholinium-3 (HC-3) was used, a compound which blocks the high-affinity uptake of Ch by nerve terminals, release of ACh was eventually blocked. Thus, the nerve terminal seems to have an important requirement for external Ch in order to maintain transmitter content at an appropriate level to permit ACh release.

The Ch needed for the synthesis of ACh can be obtained from 3 sources. Perhaps the most abundant of these sources is the plasma, which has a well controlled Ch concentration of approximately 10^{-5} M (Bligh, 1952; Choi *et al.*, 1975). The second source is Ch that is

liberated from the hydrolysis of released ACh. Collier and Katz (1974) reported that 60% of released ACh can be recaptured by the superior cervical ganglion as Ch. It is also possible for the nerve terminal to obtain Ch from certain membrane phospholipids (Jope and Jenden, 1979; reviewed in Ansell and Spanner, 1978). Though this last source of Ch may be available, in the superior cervical ganglion, only small amounts of this Ch is used for the normal synthesis of ACh (Collier and Lang, 1969).

Though Ch is small, it is a quaternary amine and its charge makes it impermeant to the plasma membrane of the nerve terminal. Therefore, some kind of transport system is needed for Ch to enter the terminal (Birks and MacIntosh, 1961). Two different systems for the transport of Ch have been discovered, a high and a low affinity system (Yamamura and Snyder, 1972, 1973; Haga and Noda, 1973; Carroll and Buterbaugh, 1975). The low-affinity transport system has a K_T for Ch of between 30 μ M and 200 μ M and does not appear to be associated with the maintenance of ACh levels in cholinergic terminals (Marchbanks, 1968; Diamond and Kennedy, 1969; Diamond and Milfray, 1972; Pert and Snyder, 1974). The other transport system has a much higher affinity for Ch with a K_T in the range of 1 to 10 μ M and is associated with ChAT and ACh rich areas of the brain; as well, it is the system which is important to the synthesis of ACh given that most of the Ch transported by this system is found converted into ACh in the tissue (Haga, 1971; Yamamura and Snyder, 1972, 1973; Haga and Noda, 1973; Sorimachi and Kataoka, 1974; Barker and Mittag, 1975; Simon and Kuhar, 1975).

Though they may be found simultaneously in the same tissue, the two transport processes do not appear to be two variants of the same system since the high-affinity process requires Na^+ , Cl^- , and an energy source, suggesting that this is an active uptake process, while the low-affinity process appears to require neither Na^+ nor an energy source and is a facilitative transport process (Haga and Noda, 1973; Yamamura and Snyder, 1973; Simon and Kuhar, 1976; Simon *et al.*, 1976). The high-affinity system of Ch transport is associated with nerve terminals and not cell bodies (Ruch *et al.*, 1982), thus it is well situated to

supply Ch for the synthesis of ACh. An increase in nerve terminal activity, in the form of transmitter release, leads to an increased accumulation of labeled ACh in sympathetic ganglia and this increased accumulation is associated with the high-affinity transport of Ch (Collier and Lang, 1969; Collier and MacIntosh, 1969; O'Regan and Collier, 1981a).

1.1.3. The source of acetyl-Coenzyme A for acetylcholine synthesis.

The other precursor for the synthesis of ACh is AcCoA (Balfour and Hebb, 1952). This precursor can be generated intracellularly by several pathways, the most studied source being that produced in mitochondria by pyruvate dehydrogenase, a mitochondrial matrix enzyme (reviewed by Wieland, 1983). The source of AcCoA needed for the synthesis of ACh in brain appears to start with glucose and lead to pyruvate (Quastel *et al.*, 1936; Browning and Schulman, 1968; Tucek and Cheng, 1970, 1974; Lefresne *et al.*, 1973). Since glucose is easily converted to pyruvate by the cytoplasmic, glycolysis enzyme pathway, it appears that pyruvate may be the important precursor of AcCoA production for ACh synthesis. When brain slices are incubated with labeled glucose or pyruvate it was found that pyruvate could label ACh to a greater extent (Browning and Schulman, 1968; Lefresne *et al.*, 1973; Dolezal and Tucek, 1981). In the case of mammalian brain and sympathetic ganglia, acetate does not form a significant amount of AcCoA for ACh synthesis (Browning and Schulman, 1968; Tucek and Cheng, 1974; Dolezal and Tucek, 1981; Kwok and Collier, 1982) but in the *Torpedo* electric organ, acetate does function in this way and ACh can be efficiently labeled by extracellular labeled acetate (Israel and Tucek, 1974; Michaelson *et al.*, 1986; Suszkiw and Manalis, 1987; Gaudry-Talarmain *et al.*, 1989).

If the AcCoA needed for ACh synthesis is produced by mitochondria, a new problem arises. The inner mitochondrial membrane is not permeable to AcCoA and thus this formed precursor cannot combine with ChAT, which is mainly located in the cytoplasm, to support ACh synthesis. When

brain slices are incubated with a specific inhibitor of ATP citrate lyase, an enzyme which uses citrate to produce AcCoA, ACh synthesis is inhibited by 30% (Tucek *et al.*, 1981; Ricny and Tucek, 1982). The figure of 30% must be viewed as a lower estimate since other systems may partially compensate for a non-functioning ATP citrate lyase. Dolezal and Tucek (1981) also reported that acetylcarnitine could function as an acetyl donor for ACh synthesis. Given that citrate and acetylcarnitine are both produced in mitochondria and that they can be transported out, they could be important intermediates in shuttling high energy acetyl groups from the mitochondria to the cytoplasm. A very interesting observation which suggests that AcCoA could directly leak out of mitochondria in response to nerve terminal activity is that made by Ricny and Tucek (1983). In this report, isolated mitochondria released AcCoA when the Ca^{+2} concentration of the medium was between 10^{-8} and 10^{-7} M. This could be an important and direct source of AcCoA because as the nerve impulse arrives at the terminal to trigger transmitter release, Ca^{+2} channels allow an influx of Ca^{+2} to occur. In this way transmitter release could be linked to ACh synthesis.

1.1.4. Regulation of acetylcholine synthesis.

In cholinergically innervated tissues the content of ACh is very carefully maintained. Release of transmitter does not deplete the stores of transmitter because as the nerve terminal releases ACh the synthesis of transmitter is turned on to maintain release. Thus, in the superior cervical ganglion even during stimulation by nerve impulses, the content of ACh in the stimulated ganglion is not different from that of the unstimulated contralateral ganglion (Birks and MacIntosh, 1961). As the ganglion is stimulated to release ACh, the synthesis of new ACh is accelerated to meet the demand (Collier and MacIntosh, 1969) and this same effect is seen in other tissues (Browning and Schulman, 1968; Potter, 1970; Richter and Marchbanks, 1971a; Lefresne *et al.*, 1973).

When the availability of one of the precursors is compromised, the synthesis of ACh is inhibited (see preceding sections). In this way,

inhibition of Ch uptake by HC-3 or by removal of Na^+ inhibits the synthesis of ACh (Birks and MacIntosh, 1961; Haga and Noda, 1973; Yamamura and Snyder, 1973; Barker and Mittag, 1975; Simon *et al* , 1976). By removing glucose and pyruvate, the main precursors of AcCoA, from the extracellular medium, though Ch uptake is maintained, the synthesis of ACh is inhibited (Johe and Jenden, 1977, 1980; Johe, 1978; Johe *et al* , 1978). It is important to note that severe restrictions on the availability of the ACh precursors should affect synthesis but such manipulations do not tell us much about what regulates this process. More limited restrictions in the availability of AcCoA do have an effect on the levels of ACh found in rat brain slices (Rigny and Tucek, 1980, 1981). O'Regan and Collier (1981b) reported that increasing the perfusion medium's Ch concentration causes an increase in the ACh content of the superior cervical ganglion. This excess ACh was not releasable by nerve impulses, indicating that it is in a compartment which may not play an important physiological role. Although, under experimental conditions the supply of precursors may be curtailed and in this way influence ACh synthesis, it has yet to be established whether precursor availability at any given moment is the physiological mechanism by which the synthesis of ACh is controlled.

It is possible that the regulation of ACh levels is related to ChAT activity. At first, this seemed very unlikely since the activity of ChAT in tissues was found to be greater than that needed to synthesize ACh that is released (reviewed by Hebb, 1972; see also Hanin and Costa, 1976). However, these conclusions are based on measurements of total ChAT activity in the presence of saturating concentrations of both Ch and AcCoA. It is known that ACh can inhibit ChAT activity and thus it would seem, at least in principle, that ACh levels could regulate enzyme activity. The inhibition of ChAT by ACh has a K_i of 45-200 mM and thus requires high concentrations of ACh to inhibit, levels which are unlikely to exist in the terminal cytoplasm (Kaito and Goldberg, 1969; Glover and Potter, 1971; Ryan and McClure, 1980). There are of course other means by which ChAT activity can be regulated. Cozzari and Hartman (1983) isolated certain endogenous factors which inhibited ChAT

in vitro, but what role these might play in the normal regulation of ACh synthesis, if any, remains to be investigated. At this time, not enough is known about the regulators of ChAT or of its several forms to determine whether ChAT is the regulatory step in the synthesis of ACh (see section on ChAT above).

Since ChAT is an enzyme it cannot affect the equilibrium point of the reaction, $\text{Ch} + \text{AcCoA} \rightarrow \text{ACh} + \text{CoA}$; all it can do is to affect the rate of the reaction. As mentioned above, there appears to be more than enough ChAT to meet the needs of even the highest rates of ACh release; thus, Potter *et al.* (1968) and Glover and Potter (1971) suggested that the formation of ACh was at equilibrium and that its release, during stimulation, is what triggered its synthesis. This hypothesis is based on the law of mass action and suggests that this is the regulatory mechanism of ACh synthesis. According to the law of mass action, the concentration of ACh is determined by the relationship:

$$[\text{ACh}] = K_{\text{eq}} \times [\text{Ch}] \times [\text{AcCoA}]/[\text{CoA}]$$

where K_{eq} is the equilibrium constant and $[*]$ refers to the concentration of any given molecular species. This hypothesis has not been directly tested because the cytoplasmic concentrations of the reactants and products cannot yet be measured in purely cholinergic nerve terminals such that no contamination from mitochondria and vesicles occur (see section 1.2.1.), and because the levels of any given compound are expected to change with different levels of activity (that is, the concentrations are likely to be dynamic and not static) (see Tucek, 1985 for an interesting review of mass action). Interesting results by Collier *et al.* (1986) and Mykita and Collier (1989) bring into question whether the law of mass action is the determining principle in ACh synthesis. Collier *et al.* (1986) found that, in the presence of a vesicular uptake blocker, release of ACh by nerve impulses was blocked in ganglia, though synthesis remained unaffected in 30 min of stimulation. In this study the ganglial ACh content increased by that amount of ACh that was not released. Mykita and Collier (1989) then observed that this increase in

ACh content could be blocked by a compound which reacts with outwardly oriented (extracellularly oriented) amine groups, but this treatment had no effect on the uptake of Ch. Though these results do not disprove the hypothesis of mass action in the regulation of ACh synthesis, they do suggest that regulation of ChAT activity may indeed be the important regulatory step.

1.2. THE COMPARTMENTALIZATION AND RELEASE OF ACETYLCHOLINE.

With the discovery by Fatt and Katz (1950, 1952) that transmitter release at the neuromuscular junction had a quantal nature, that is, that the muscle end-plate potential in response to nerve stimulation represented a multiple of one base quantity, the interest of many neurobiologists turned to the elucidation of how this quantum was generated. Following the observation by de Robertis and Bennett (1955) that presynaptic endings were filled with small spherical structures (vesicles), del Castillo and Katz (1954, 1955) proposed that the quantal nature of ACh release observed at the neuromuscular junction could result from the release, by exocytosis, of ACh possibly contained within those spherical structures. This hypothesis concerning the nature of the release of ACh has come to be called the vesicular hypothesis of ACh release. Because the hypothesis supposes that ACh must be in a special compartment (the vesicles) for release to occur, the work on the nature of ACh release has since been integrally connected to the ways in which ACh is compartmentalized within the nerve terminal. Though most investigators of this phenomenon believe in the vesicular origin of ACh release, there is as yet not a universal acceptance of this hypothesis nor can all experimental results easily be explained by it. The literature dealing with ACh compartments and release is therefore reviewed here, and the controversy, known today as the cytoplasmic versus vesicular origin of released ACh, will be reviewed in section 1.5.

1.2.1. The intracellular acetylcholine compartments.

Early studies which attempted to subfractionate tissues containing high levels of ACh found that the content of transmitter was not evenly distributed throughout the various fractions (Hebb and Whittaker, 1958; Bellamy, 1959; Whittaker, 1959; de Robertis *et al.*, 1961; Crossland and Slater, 1968; Israel *et al.*, 1970; Marchbanks and Israel, 1970; Richter and Goldstein, 1970; Slater, 1971; Whittaker *et al.*, 1972; Wilson and Cooper, 1972). Thus, homogenization lead to the loss of some 20-40% of the ACh content of the various tissues while the rest could be isolated in high speed pellets associated with mitochondria. This labile form of ACh, which could be preserved if AChE inhibitors were used, was called free ACh, while that preserved in the high speed pellets was named bound ACh because it was not free to be hydrolyzed by AChE (for an interesting discussion of early ideas concerning bound ACh see Feldberg, 1945). The bound ACh was found to be preserved in fractions which were interpreted, by electron microscopy, to contain nerve endings, called synaptosomes.

Because these nerve endings contain many subcellular inclusions, synaptosomes were disrupted and further subfractionated, and another two compartments of ACh could be identified (de Robertis *et al.*, 1963; Whittaker *et al.*, 1964; Whittaker and Sheridan, 1965; Wilson and Cooper, 1972; Salehmoghaddam and Collier, 1976; von Schwarzenfeld, 1979; Boksa and Collier, 1980 a and b; Collier and Kwok, 1982; Suszkiw and O'Leary, 1982). These studies established that approximately 20 to 50% of the ACh present in the nerve endings of cholinergic tissues was associated with intracellular occluded compartments and was, thus, safe from hydrolysis by AChE. This fraction of ACh could be isolated by ultrahigh speed centrifugation and was present in the pellet. The remaining ACh was lost to hydrolysis unless an AChE inhibitor was used. Therefore the synaptosome-containing fraction was found to contain a labile fraction of ACh, thought to be cytoplasmic and comprising 50 to 80% of the ACh content, and a stable-bound fraction of ACh, associated with vesicles. The possibility that the cytoplasmic ACh arises as a result of synaptosomal vesicle disruption during the isolation of the various fractions seems unlikely since, when the transmitter was labeled from radioactive precursors, this component was found to have ACh with a higher specific

activity (S.A.) than that found in the stable-bound compartment and, by use of anti-AChE agents, it could increase its content of ACh by a larger amount than that found in the stable-bound fraction (Chakrin and Whittaker, 1969; Richter and Marchbanks, 1971b; Collier *et al.*, 1972; Dunant *et al.*, 1972; Marchbanks and Israel, 1972; Aquilonius *et al.*, 1973; Molenaar and Polak, 1973; Molenaar *et al.*, 1973; von Schwarzenfeld, 1979; Suszkiw and O'Leary, 1982). Though it is unlikely that all of the labile ACh found in disrupted synaptosomes is an artifact of preparation, it is not yet possible to estimate whether some of this ACh arises from a disturbed occluded compartment and if so, what proportion of the labile fraction is really from a vesicular source. The use of false transmitters has also shown that these are accumulated differently by the labile and stable-bound compartments (von Schwarzenfeld, 1979; von Schwarzenfeld *et al.*, 1979; Luqmani *et al.*, 1980; Boksa and Collier, 1980 a and b; Welner and Collier, 1984), but these studies, though supporting the idea of two intraterminal pools of ACh, cannot address the problem of contamination of the labile pool by the stable-bound one.

The stable-bound ACh was found not to represent one compartment but several, two of which have been associated with vesicular structures (Whittaker *et al.*, 1964; Barker *et al.*, 1972; Wilson *et al.*, 1973; Zimmermann and Whittaker, 1977; Suszkiw *et al.*, 1978; von Schwarzenfeld, 1979; Zimmermann, 1979b; Giompres *et al.*, 1981 a and b; Agoston *et al.*, 1985). The various compartments could be separated by buoyant density centrifugation, zonal gradient centrifugation, or by glass bead chromatography. Thus, the internal bound compartments of ACh differ in both size and density, but again one cannot be sure that these differences are not the result of changes caused by the isolation techniques used (see Whittaker, 1987 for some comments concerning this). This possibility seems unlikely because the two vesicular ACh compartments were found to have different turnover rates, as determined by the use of labeled Ch. One vesicular ACh compartment, associated with less dense and larger vesicles, contains ACh which has a slow rate of turnover and is referred to as the VP₁ compartment. The other vesicular ACh

compartment, denser and smaller vesicles than those of VP_1 , has a much faster turnover of ACh and is referred to as the VP_2 compartment.

Thus, in cholinergic nerve terminals at least 3 important compartments of ACh have been identified. The larger compartment is that associated with the cytoplasm. The other two ACh compartments are smaller and contain ACh trapped in vesicles which appear to have different functions (see section 1.2.2. for more discussion of these 3 compartments of ACh).

1.2.2. The release of acetylcholine.

Though it is tempting to think that all cholinergic nerve terminals use the same mechanisms to release ACh, one must be cautious not to extend the findings for one tissue to the next without consideration of possible differences (Collier, Boksa, and Ilson, 1978). There are important differences with respect to the modulation of ACh release even within mammalian tissues (Szerb and Somogyi, 1973; Kato et al., 1975) and there is some indirect evidence that important differences exist in the way in which ACh is released in response to stimulation between the electric organ of *Torpedo* and the mammalian neuromuscular junction (Suszkiw and Manalis, 1987). Having said this, it is also true that certain common aspects also exist between tissues and these will be discussed below.

1.2.2.1. The spontaneous release of acetylcholine.

Cholinergic nerve endings release ACh both during rest and during periods of stimulation. The release of ACh when the neuron is at rest is referred to as the basal or spontaneous release of transmitter and is a well established phenomenon of cholinergic tissues (Birks and MacIntosh, 1961; Collier 1969; Dawes and Vizi, 1973; Carroll and Goldberg, 1975; Katz and Miledi, 1977; Vyskocil and Illes, 1977; Gundersen and Howard, 1978; Vizi and Vyskocil, 1979; Boksa and Collier, 1980b; Dunant et al., 1980). The basal release of ACh is insensitive to the presence

of extracellular Ca^{+2} and can be increased or decreased by the inhibition or stimulation, respectively, of the $\text{Na}^{+}/\text{K}^{+}$ ATPase. Electrophysiologically, the spontaneous release of transmitter at the neuromuscular junction causes a constant but slight depolarization of the post-junctional plasma membrane ranging from 40 μV to 2 mV in amplitude (Katz and Miledi, 1977; Vyskocil and Illes, 1977) and can be demonstrated by blocking the nicotinic receptors in the presence of an AChE inhibitor. Vizi and Vyskocil (1979) have estimated that only about 1% of spontaneous ACh release is due to quanta, such as those measured by Fatt and Katz (1950, 1952) and referred to as miniature end-plate potentials (m.e.p.p.s) (see below for further comments on the quantal nature of stimulated ACh release). This non-quantal, Ca^{+2} insensitive, basal release of transmitter appears to originate from the cytoplasmic compartment of ACh. This conclusion was reached by three studies which looked at the ratio of true transmitter to a false transmitter, acetylhomocholine, released during rest and the ratio of these two compounds found in the subcellular fractions (Boksa and Collier, 1980b; Carroll and Aspry, 1980; Carroll, 1983).

1 2.2.2. The stimulated release of acetylcholine.

Perhaps the most complete and interesting study of the stimulated release of ACh from cholinergic terminals by a physiological stimulus is that of Birks and MacIntosh (1961) on the superior cervical ganglion of the cat. These authors found that the ganglion could release 85% of its ACh content in response to nerve impulses, when the synthesis of ACh was blocked. The remaining 15% of ACh could not be released and was hypothesized to be in a part of the ganglion far from the release sites of the nerve terminals, and so it was called "stationary ACh". By analyzing the kinetic profile of ACh released from ganglia stimulated at fast rates (20 Hz) these authors were able to identify two components. The first component of released ACh was estimated to be about 14% of the ganglial ACh content and because this was easily released by nerve impulses it was referred to as the "readily-releasable" pool of ACh. The

second component of released ACh appeared to require mobilization to be releasable, was estimated to consist of 60% of the ganglion's transmitter content, and was called the "storage" pool of ACh. If an AChE inhibitor, such as eserine, was present in the perfusion medium the ACh content of the ganglion could double. This extra ACh was named "surplus" ACh by these investigators and later work by Collier and Katz (1970, 1971) found that this accumulated ACh was not releasable by nerve impulses though high K^+ solutions and high concentrations of extracellular ACh could mobilize this ACh to be released. Thus, from this work, it appears that there are two pools of ACh that can be released by physiological stimuli and likely constitute what is generally considered the evoked release of ACh.

The stimulated release of ACh from the superior cervical ganglion is dependent on the presence of extracellular Ca^{+2} (Harvey and MacIntosh, 1940; Hutter and Kostial, 1954; Douglas *et al.*, 1961; MacIntosh and Collier, 1976; Vella Lamarca and Collier, 1983). The same is true in mammalian brain whether electrical stimulation or high K^+ are used to evoke the release of ACh (Randic and Padjen, 1967; Hemsworth and Mitchell, 1970; Carroll and Goldberg, 1975; Gundersen and Howard, 1978; Boksa and Collier, 1980b). Studies of the neuromuscular junction and *Torpedo* electric organ have also demonstrated the need for extracellular Ca^{+2} to increase ACh release in response to the stimulus (Vizi and Vyskocil, 1979; Dunant *et al.*, 1980). Furthermore, at the cat superior cervical ganglion, increasing the entry of Ca^{+2} by 4-aminopyridine caused an increase in nerve stimulated ACh release (Vella Lamarca and Collier, 1983). A similar effect on the evoked release of ACh by electrical stimulation or high K^+ was noted in the guinea-pig myenteric plexus by increasing extracellular Ca^{+2} concentrations (Yokoyama *et al.*, 1986). Though Ca^{+2} is absolutely necessary for the evoked release of ACh by several stimuli (see section 1.6 for the Ca^{+2} independent release of ACh induced by black widow spider venom), its role in this process has yet to be elucidated. There is some evidence that the termination of evoked ACh release is not due to a decrease of intraterminal Ca^{+2} concentration (reviewed in Reichardt and Kelly, 1983) and theoretical

considerations imply that another factor is involved in the initiation and termination of ACh release, though Ca^{+2} is needed for initiation of evoked release of transmitter (Parnas and Segel, 1984).

Collier (1969) was the first to note that newly synthesized ACh was preferentially released by nerve impulses, not the older ACh stores contained in the superior cervical ganglion of the cat. This was found to be the case in *Torpedo* electric organ (Dunant *et al.*, 1972) and in brain (Richter and Marchbanks, 1971b; Chakrin *et al.*, 1972; Molenaar *et al.*, 1973; Suszkiw and O'Leary, 1982). This interesting observation led investigators to attempt to establish which of the 3 main subcellular fractions of ACh were the source of the evoked ACh release. Some of the initial attempts in brain and superior cervical ganglion found that the S.A. of released ACh was higher than the S.A. of ACh in either the cytoplasmic or the stable-bound fractions (Richter and Marchbanks, 1971 a and b; Chakrin *et al.*, 1972; Molenaar *et al.*, 1973; Collier *et al.*, 1978; Suszkiw and O'Leary, 1982). Carroll and Aspry (1980) exploited their finding that incubation of brain minces in Li^+ instead of Na^+ led to the preferential loss of stable-bound ACh. By allowing the minces to recover in the presence of $[^{14}\text{C}]$ -homocholine, they were able to differentially label the cytoplasmic and stable-bound fractions of transmitter. Under resting conditions the ratio of the basal release of $[^{14}\text{C}]$ -acetylhomocholine ($[^{14}\text{C}]$ -AChCh) to ACh resembled that found in the cytoplasmic fraction but upon stimulation by high K^+ the released ratio of $[^{14}\text{C}]$ -AChCh to ACh was much closer to that found in the stable-bound fraction. If further fractionation of the stable-bound fraction is undertaken, one of the vesicular fractions of ACh (VP_2) is found to have a similar ACh S.A. or ratio of false transmitter to ACh as that found in the released transmitter (Suszkiw *et al.*, 1978; von Schwarzenfeld, 1979). Though these results would indicate that a metabolically active group of vesicles are the source of released ACh, objections have been raised to the interpretation of the results (see letters to the editor of *Neuroscience* by Marchbanks, 1984 and the replies by Collier, 1984, by Zimmermann, 1984, and by Whittaker, 1984).

1.2.2.3. The quantal nature of stimulated acetylcholine release.

The release of ACh from the neuromuscular junction was found to appear as discreet packets of one average size causing a subthreshold depolarization of the post junctional membrane (Fatt and Katz, 1950, 1952; del Castillo and Katz, 1954). These unit subthreshold depolarizations are known as miniature end-plate potentials or m.e.p.p.s. When the motoneuron is stimulated, it releases ACh in such a way that it causes an end-plate potential (E.P.P.) to develop in the muscle, capable of triggering an action potential and muscle contraction. When the size of the induced E.P.P. was compared to that of the m.e.p.p. it was found that the former was always a multiple of the latter and thus, the quantal nature of ACh release from motoneurons was discovered. Unfortunately, upon more careful observation of these electrical phenomenon, under a variety of conditions, two other classes of m.e.p.p.s were discovered; one type had a smaller amplitude than the original m.e.p.p. and was named a sub or mini m.e.p.p. and the second class, larger than the original m.e.p.p., was named a giant m.e.p.p. (see review and model by Vautrin *et al.*, 1991). The normal m.e.p.p. is not a multiple of the sub m.e.p.p., nor is the giant m.e.p.p. a multiple of the normal m.e.p.p.. Based on this and other characteristics of the m.e.p.p. subclasses, such as a skew in the amplitude distribution about the mean for mini m.e.p.p.s, it is unlikely that these represent the released ACh from one type of vesicle. Thus, the vesicular hypothesis of transmitter release seems not to account for these findings, unless different vesicles are found capable of storing different amounts of ACh or a mechanism is discovered which permits partial filling or over filling of a vesicle. The same problem arises for the cytoplasmic hypothesis of ACh release because a system is needed which can generate all three classes of m.e.p.p.s under the appropriate conditions. Work by Van der Kloot and Van der Kloot (1985, 1986) has demonstrated that the amplitude of the classical m.e.p.p. may be altered. Thus, repeated stimulation of the motoneurons in the frog, or treatment of the neuromuscular junction with cAMP analogues, noradrenaline, or adrenaline, caused the amplitude

of the standard m.e.p.p.s to double or triple. Though this suggests that packaging may be modulated, since the increase may be blocked by an inhibitor of the vesicular ACh transport system, it cannot yet explain the subclasses of m.e.p.p.s described above. Our present understanding of ACh packaging by synaptic vesicles does not offer us the necessary mechanism by which a vesicle may fill itself to different specified levels (see section 1.3. for a discussion of this topic).

When the neuromuscular junction is stimulated in the presence of HC-3, synthesis of ACh can be blocked, and the quantal release of ACh studied (Elmqvist and Quastel, 1965). Under these conditions it was observed that the number of quanta released by stimulation was normal, but that the size of the m.e.p.p.s (amplitude) declined after a period of normality until they could no longer be measured. The authors concluded that there was a quick equilibration of available ACh to form smaller and smaller quanta. This idea of a rapid equilibration was supported by Large and Rang (1978) using the false transmitter, acetylmonoethylcholine. These results can be interpreted by either the vesicular or the cytoplasmic theories of transmitter release equally well.

Biochemical studies have also shown that a specific amount of ACh is released by a single impulse or a train of impulses. This appears to be the case in the superior cervical ganglia (Birks and MacIntosh, 1961), in *Torpedo* electric organ (Dunant *et al.*, 1980), and in cerebral cortex (Bourdois *et al.*, 1974). Because of technical difficulties, only the ACh release by the superior cervical ganglion and the *Torpedo* electric organ has been found, electrophysiologically, to have a quantal nature similar to that of the neuromuscular junction (Blackmann *et al.*, 1963; Sacchi and Perri, 1973; McLachlan, 1975; Muller *et al.*, 1985).

1.3. CHOLINERGIC VESICLES: MECHANISMS OF ACETYLCHOLINE UPTAKE.

One critical aspect of the vesicular hypothesis is for ACh to be localized in vesicles for possible release upon the arrival of nerve impulses (del Castillo and Katz, 1955). The finding that ACh was stored in vesicles within cholinergic terminals was of critical importance not

only to the vesicular hypothesis but to the biochemical study of cholinergic transmission as well (Sheridan *et al.*, 1966; Whittaker and Sheridan, 1965; Wilson *et al.*, 1973). The mechanism by which ACh is stored by vesicles has received much attention by cholinergic biologists working on the electric organ of *Torpedo* because, so far, this is the only purely cholinergic tissue yielding large enough quantities of vesicles. Several reviews concerning this topic and other measures of cholinergic vesicles have been published (Parsons *et al.*, 1982; Whittaker, 1982 and 1984; Marshall and Parsons, 1987; Parsons *et al.*, 1987).

Vesicles derived from *Torpedo* electric organ are about twice as large in diameter as cholinergic vesicles of other tissues, such as the neuromuscular end-plate of *Torpedo* itself (80-90 nm versus 45-50 nm), and they are known to encapsulate several compounds. These vesicles contain about 40,000 to 200,000 molecules of ACh per vesicle; the larger figure is more likely and gives a theoretical concentration of ACh in the vesicle of about 0.5 to 0.9 M (Sheridan and Whittaker, 1964; Ohsawa *et al.*, 1979). Along with the store of transmitter, electric organ vesicles contain ATP in a ratio of about 1 ATP to every 4-5 ACh molecules, making the ATP concentration in the vesicle's interior about 0.1 to 0.25 M (Dowdall *et al.*, 1974; Giompres and Luqmani, 1980; Michaelson and Angel, 1981; Anderson *et al.*, 1986). One possibility is that ATP is present in order to neutralize the charge potential of the vesicle which would result if such a large concentration of ACh, in solution, were present alone inside the vesicle. Finally, vesicles from *Torpedo* electric organ are known to take up Ca^{+2} such that isolated vesicles contain around 40,000 Ca^{+2} molecules per vesicle. Why Ca^{+2} is sequestered by these vesicles is not yet known but proponents of the cytoplasmic hypothesis have argued that this is the mechanism by which terminals can extrude much of the Ca^{+2} that enters the terminal during stimulation (Israel *et al.*, 1980). Though some of the internal constituents of the vesicle may be complexed, it appears that most of the ACh and ATP are in solution. This was determined by NMR analysis of vesicles (Stadler and Fuldner, 1980; Fuldner and Stadler, 1982) and is in agreement with studies of the vesicular water spaces and estimates of

osmolarity (reviewed by Whittaker, 1982 and 1984). Because the vesicle behaves as an osmometer it is believed that the vesicle's interior is iso-osmotic or just slightly hyperosmotic with respect to the surrounding cytoplasm.

Cholinergic vesicles purified from this special organ of *Torpedo* have a simple protein composition. Five major protein bands appear when the vesicle membrane composition is studied using polyacrylamide gel electrophoresis. There is one protein of 160,000 molecular weight (M.W.) with no known or suspected function, one protein of 147,000 M.W. which could be the Mg^{+2} activated ATPase responsible for establishing the proton gradient, one protein of 42,000 M.W. which appears to be a unique actin different from that found in muscle, a protein of M.W. 34,000 which could be the ATP translocase or an ADP/ATP exchanger, and then there is a 25,000 M.W. protein which might be the actual ACh transporter (reviewed by Zimmermann, 1982; Whittaker, 1984). More work is needed to establish the respective functions of these vesicle membrane proteins but it is hoped that, with modern methods of analysis utilizing molecular biology, these functions may be ascertained.

1.3.1. The vesicular ATPase.

When synaptic vesicles of *Torpedo* electric organ are purified by differential density centrifugation and size exclusion glass bead chromatography, most enzymatic activities, such as AChE, can be separated from the vesicles, implying that these are not intrinsic activities of the vesicle (Breer *et al.*, 1977). However, one enzyme activity that remains and is intrinsic to the vesicle is an ATPase activity which is not like that of other organelles (Breer *et al.*, 1977, Rothlein and Parsons, 1979; Michaelson and Ophir, 1980; Harlos *et al.*, 1984; Diebler and Lazereg, 1985; Stelz *et al.*, 1987). The vesicular ATPase activity has an interesting pharmacology which indicates that it is not a contaminant activity of plasma membrane Na^{+}/K^{+} ATPase, of sarcoplasmic reticulum ATPase, or of mitochondrial ATPase. This unique ATPase is not inhibited by ouabain, oligomycin, valinomycin, or carbonyl

cyanide p-trifluoromethoxyphenyl-hydrazone (FCCP), compounds which can potentially inhibit other ATPase. However, the vesicular ATPase is sensitive to blockade by compounds such as 4-chloro-7-nitrobenza-oxadiazole and dicyclohexylcarbodiimide (DCCD) which are both lipid soluble. A water soluble compound related to DCCD had no effect on the ATPase and this suggests that the protein is membrane bound (Rothlein and Parsons, 1979). Though the ATPase activity cannot be separated from the vesicle it is rather labile, 30% disappearing after 24 hr of storage of the vesicles (Breer *et al.*, 1977).

The ATPase of synaptic vesicles was found to be stimulated by Mg^{+2} or Ca^{+2} , though stimulation by Mg^{+2} was greater (Breer *et al.*, 1977; Rothlein and Parsons, 1980). Stimulation by these ions was not additive, suggesting that they interact at the same site and can replace each other (Rothlein and Parsons, 1980). The ATPase activity, when stimulated by Mg^{+2} , could be inhibited by Na^{+} and K^{+} , unlike the plasma membrane Na^{+}/K^{+} ATPase which is stimulated by these monovalent ions. Furthermore, the vesicular ATPase activity was not inhibited by ouabain, consistent with the view that it is not a contaminant activity but a unique and integral activity of vesicles. The Mg^{+2} stimulated activity of the vesicular ATPase can be further stimulated by 20% more when ACh is present at 20 mM concentrations while Ch has no effect on the activity (Breer *et al.*, 1977). The observed stimulation of ATPase activity by ACh is intriguing, for it suggests that ACh may modulate the vesicle's own processes to accumulate transmitter. A third substance which may be important in the regulation of the vesicular ATPase is the bicarbonate anion. Rothlein and Parsons (1980) observed that bicarbonate could stimulate the ATPase activity with a K_m of 5 mM. Bicarbonate alone was not sufficient for the stimulation, but when present with Mg^{+2} , the highest activities were obtained (Ca^{+2} also worked but to a lower stimulation level). The stimulation by bicarbonate and Mg^{+2} was also found in vesicle ghosts suggesting that no internal factor is needed for enzyme activation.

A recent report by Yamagata and Parsons (1989b) has established that synaptic vesicles from *Torpedo* have not one type of ATPase activity

but two. One of the ATPases is a phosphointermediate-forming ATPase, named such because the protein is phosphorylated by ATP (Yamagata *et al.*, 1989), and appears to constitute about 58% of the ATPase activity of the vesicle (Yamagata and Parsons, 1989b). This type of ATPase is inhibited by vanadate (Diebler and Lazereg, 1985; Yamagata and Parsons, 1989b). Since ACh uptake is slightly activated by vanadate, Yamagata and Parsons (1989b) concluded that this ATPase activity is not important in ACh transport. This ATPase has been found by Yamagata and Parsons (1989a) to be glycosylated and to have a hydrodynamic molecular weight, for the complex, of 210,000. The second type of ATPase found in these vesicles is inhibited by N-ethylmaleimide (NEM) and it cannot be protected from this inhibition by vanadate as the first type can (Yamagata and Parsons, 1989b). Since the NEM inhibits ACh uptake, even when the phospho-intermediate ATPase is protected and reactivated, it is believed that this second ATPase type is the one important for ACh uptake (Yamagata and Parsons, 1989b).

Given that ATP is present both inside of the vesicle and in the cytoplasm the question arose as to how the enzyme was oriented on the vesicle's membrane. By using [^3H]-ATP, Michaelson and Ophir (1980) found that ATPase stimulation by ATP and Mg^{+2} during 15 min did not lead to any uptake of [^3H]-ATP by the vesicles. Furthermore, during this time no vesicle internalized ACh or ATP was lost. Because of these observations, and because lysed vesicles do not differ from intact vesicles, the authors concluded that the enzyme's catalytic site must lie on the outside of the vesicle pointing towards the cytoplasm. Thus, it is unlikely that the vesicular ATP content is needed for the activity of the ATPase but probably serves some other function such as that previously mentioned.

1.3.2. Synaptic vesicles of *Torpedo* electric organ establish a proton gradient important for the uptake of acetylcholine

The ATPase activity of the inner mitochondrial membrane, when functioning under normal conditions is not involved in the breakdown of

ATP to ADP to translocate a proton, but in fact uses a proton gradient to form ATP from ADP. In the case of the vesicular ATPase it is more likely that, once a vesicle has lost its contents, the enzyme uses ATP to establish a proton gradient across the vesicular membrane, thereby acidifying the vesicle's interior. There is much evidence that secretory vesicles contain an acidic internal milieu (see review by Forgac, 1989 for a discussion of the ATPases that establish these proton gradients) as has been discovered to be the case in synaptic vesicles of the *Torpedo* electric organ.

Michaelson and Angel (1980) used 9-aminoacridine (9AA), a compound which can cross membranes and partitions itself across a membrane according to the pH difference, to investigate the pH of the internal vesicular milieu. Because of the simple partitioning relationship of 9AA with pH, the authors only had to measure the fractional fluorescence quench associated with vesicles and the vesicles internal volume to establish the internal pH. The following equation describes the relationship:

$$\frac{[H^+]_{in}}{[H^+]_{out}} = \frac{[9AA]_{in}}{[9AA]_{out}} = \frac{Q}{(1-Q) \times (V_{in})}$$

Where:

- $[H^+]$ - hydronium ion concentration
- $[9AA]$ - concentration of 9AA
- Q - fractional quench
- V_{in} - internal vesicular volume

By simple mathematical transformation and keeping in mind that pH is equal to the $-\log [H^+]$, the internal pH may be calculated by:

$$pH_{in} = (\log (1-Q) + \log (V_{in}) + pH_{out}) - \log Q$$

Using this method and assuming an internal volume of 1.9 μ l/mg protein, Michaelson and Angel (1980) estimated the internal pH to be around 5.2. Of course, if the internal volume is larger than assumed, the calculated internal pH will be higher (less acidic) and vice versa. Any treatment that disrupts the vesicular membrane abolishes the quenching of 9AA by vesicles. When the proton translocator FCCP was used, the quench caused by vesicles was only decreased by a small amount, but when added together with valinomycin, a K^+ ionophore which by itself has no effect on fluorescence quenching, fluorescence is returned to 90% of that in the vesicle's absence indicating a breakdown of the pH gradient. Anderson *et al.* (1982) found that addition of these two ionophores to isolated vesicles resulted in a stimulation of the vesicular ATPase activity by 30 to 40%, consistent with the idea that the pH gradient is disrupted (as the pH rises the ATPase becomes active to pump protons back in so as to re-establish the pH gradient).

That the proton gradient is important to the uptake of ACh by vesicles has been established by the use of a variety of ionophores and ammonium (Toll and Howard, 1980; Anderson *et al.*, 1982). In PC12 cells, most of the cellular ATP is derived from glycolysis and not from mitochondrial respiration (oxidative phosphorylation), so the use of most of the ionophores had little effect on cellular ATP which is expected to be important to vesicular transmitter packaging and to cellular function (Toll and Howard, 1980). Nigericin, a H^+/K^+ exchanger which should collapse the vesicular proton gradient, blocked the incorporation of [^3H]-ACh by PC12 granules without affecting [^3H]-ACh synthesis. The same effect was found when FCCP was used. The potent mitochondrial ATPase inhibitor oligomycin had no effect on the packaging of [^3H]-ACh. In purified *Torpedo* synaptic vesicles, under controlled *in vitro* conditions, Anderson *et al.* (1982) found that the proton gradient disrupting ionophores were also capable of blocking the transport of [^3H]-ACh by vesicles, and further extended the list of such ionophores to include A23187, a Ca^{+2}/H^+ exchanger, and gramicidin, a non specific H^+ , K^+ , Na^+ ionophore. Furthermore, as increasing concentrations of FCCP blocked ACh transport they concomitantly raised ATPase activity

(Parsons *et al.*, 1987). If the pH potential is an important component of ACh uptake, then making the medium that the vesicles are in slightly alkaline should increase uptake, because the pH potential difference increases between the acidic vesicle interior and the alkaline vesicle exterior. Raising the external pH from 6.6 to 7.6 increased the transport of [^3H]-ACh by vesicles. Furthermore, by using ammonium ions (NH_4^+) in the medium, which can enter the vesicle as NH_3 and then react with the internal protons to again form NH_4^+ , these authors found that [^3H]-ACh uptake could be completely inhibited. Thus the importance of the proton gradient to the vesicular uptake of ACh seems to be reasonably well established. Anderson *et al.* (1982) also found that the K^+ -specific ionophore, valinomycin, could inhibit the transport of [^3H]-ACh. Since this ionophore appears not to disrupt the proton gradient by itself (Michaelson and Angel, 1980), it suggests that an electric potential is also important to ACh vesicular transport (Carpenter and Parsons, 1978).

1.3.3. The active and passive transport of acetylcholine by synaptic vesicles of *Torpedo*.

The effects of ionophores on the transport of ACh by purified vesicles discussed in the above section refer to the active uptake of ACh. Purified vesicles from the *Torpedo* electric organ are, however, known to be able to take up ACh by two different processes. In addition to the activated transport of ACh there is a passive process with different kinetics and a different pharmacological sensitivity from that of the active process. So far, the active process is associated with an active ATPase and a proton gradient while the passive process does not appear to require a functionally active ATPase.

Purified vesicles from *Torpedo* do not demonstrate active uptake of ACh unless the vesicles are placed in a solution containing Mg^{+2} , ATP, and bicarbonate (Koenigsberger and Parsons, 1980). Under passive conditions ACh and Ch are almost equally well transported but they are not concentrated much more beyond that concentration found in the medium bathing the vesicles (Koenigsberger and Parsons, 1980; Parsons and

Koenigsberger, 1980). The passive uptake of ACh was reported to be very rapid, taking only a few minutes to reach maximum, but after the small and rapid uptake of [^3H]-ACh, about 11 [^3H]-ACh/vesicle, no further increase in the accumulated [^3H]-ACh was seen with time (Anderson et al., 1986). The possibility existed that this supposedly accumulated [^3H]-ACh was not actually transported but adsorbed to the vesicles. Parsons and Koenigsberger (1980) reported that when vesicles were preloaded with [^{14}C]-mannitol, allowed to take up [^3H]-ACh, and were then re-isolated on a gradient, the vesicular fraction contained most of the [^{14}C]-mannitol, [^3H]-ACh, and ATP. This finding confirmed that the accumulated [^3H]-ACh was associated with vesicles and that if adsorbed, it could not be lightly adsorbed to the membrane. Giompres and Luqmani (1980) and Michaelson and Angel (1981) observed that transported [^3H]-ACh could be released by hypo-osmotic lysing of the vesicles (both reports) or dilution of vesicles with iso-osmotic buffer containing cold ACh (Michaelson and Angel, 1981). Though it is not clear whether the observed transport in these two reports involved passive or active process or both, the finding supports the idea that the [^3H]-ACh was located inside vesicles, if only weakly held there given its release by cold ACh. Furthermore, if only Mg^{+2} and ATP were added without the bicarbonate, there was an inhibition of passive [^3H]-ACh transport, while bicarbonate alone had no effect. The inhibition was not dependent on Cl^- , Na^+ , or K^+ ions nor did the ionic strength of the medium affect the inhibition (Koenigsberger and Parsons, 1980).

Under active vesicular transport conditions, ACh is better transported than Ch by at least 4 fold and the vesicles concentrate ACh by a factor of 2 or more, as compared to the medium ACh concentration (Koenigsberger and Parsons, 1980; Parsons and Koenigsberger, 1980). In order to achieve this active uptake of [^3H]-ACh, Mg^{+2} , ATP, and bicarbonate were needed, with no ion being able to replace the bicarbonate in the stimulation (Koenigsberger and Parsons, 1980). Unlike the passive uptake process, the active process accumulated [^3H]-ACh over time following about a 2 min lag and reached maximal accumulation 30 to 40 min after it began. In this time approximately 320 [^3H]-ACh molecules per

vesicle have been accumulated, representing a [^3H]-ACh concentration of 3 mM in the vesicle (medium [^3H]-ACh being 50 μM) (Anderson *et al.*, 1986). Because the active process is stimulated by reagents that reduce sulfhydryl groups, such as dithiothreitol, and inhibited by reagents that oxidize these groups, such as p-chloromercuribenzenesulfonate, it appears that a reduced cysteine is needed for active transport (Parsons and Koenigsberger, 1980; Parsons *et al.*, 1987). Bahr and Parsons (1986) reported that the K_T for active transport was 0.46 mM for ACh with a V_{max} of 0.6 nmoles [^3H]-ACh/min/mg protein and found no cooperativity of ACh for the active transport process.

There are two early reports of labeled ACh accumulation by purified vesicles, where the process being observed is hard to judge as being active or passive (Giompres and Luqmani, 1980; Michaelson and Angel, 1981). In Michaelson and Angel's (1981) report, vesicle ghosts were used in an iso-osmolar medium. The accumulation of [^3H]-ACh was very rapid, reaching maximum uptake in 2 min and concentration of the ACh by the ghosts was 12 fold. The process was inhibited by Mg^{+2} and ATP while stimulated by Mg^{+2} , ATP, and bicarbonate. The observed kinetics of transport were best fit by a 2 transport process, one of high affinity and low capacity ($K_T = 0.3$ mM and $V_{\text{max}} = 4.5$ nmoles of [^3H]-ACh/mg protein/min) and a second of low affinity and high capacity ($K_T = 10$ mM and a $V_{\text{max}} = 130$ nmoles [^3H]-ACh/mg protein/min). Giompres and Luqmani (1980) reported that purified vesicles took up both [^3H]-ACh and [^{14}C]-Ch, though [^3H]-ACh was better transported, and required 4-5 hr to reach maximum uptake. Kinetic analysis in this study showed only one process with a K_T of 3 mM and a V_{max} of 0.2 nmoles [^3H]-ACh/min/mg protein for ACh and the same V_{max} with a K_T of 10 mM for Ch. Thus, ACh is preferred by about 3 to 1 over Ch, but the V_{max} for both ACh and Ch are the lowest reported among the studies cited here. Osmotic shock reduced the vesicle's ability to concentrate ACh and transport of [^3H]-ACh could be inhibited by 500 μM HC-3. Thus, in both reports there are aspects of the transport process which seem more appropriate to the passive process and some to the active process. It is likely that both processes were simultaneously observed and not differentiated.

Figure 11 summarizes some of the data dealing with the active uptake of ACh by vesicles. It appears that the passive process of ACh transport depends on a negative vesicle interior, caused by the presence of ATP and proteoglycan, while the active process requires a positive, acidified, vesicle interior (Parsons *et al.*, 1987). Though the qualitative aspects of this model seem well established, there are some quantitative discrepancies between studies which may be due to different vesicle purities and concentrations and to differing initial states of the isolated vesicles.

1.3.4. The aqueous spaces of synaptic vesicles from *Torpedo* electric organ.

Given that there are a variety of components in the isolated synaptic vesicles of *Torpedo* electric organ, the question arises as to what proportion of these components are free in solution and what proportion may be crystallized in the vesicle's interior. Whittaker and his coworkers have studied this question in great detail because of the importance to such questions as the ACh concentrating power of cholinergic vesicles and the relative importance of the other internally located substances to this supposedly prime function of the vesicles. Whittaker (1982) has reviewed the findings of his group.

Breer *et al.* (1978) found that isolated vesicles behaved as osmometers between 800 and 1400 m osmolar, by measuring the shifts in vesicle density caused by changes in the vesicle water content. They reported that below 800 m osmolar, vesicles experience an osmotic pressure that causes them to lyse, thereby losing their contents of ATP and ACh and becoming lighter. From this they concluded that the substances had to be in solution because, if crystallized, they would not exert any osmotic pressure on the vesicle. In other words, if the constituents of the vesicles were not in solution the osmolarity of the medium bathing them would have a minimal effect on the vesicle volume and density because, at low osmolarities, H₂O would not enter the vesicle to maintain equal dissolved particle concentrations (osmolarity)

Cholinergic Vesicle

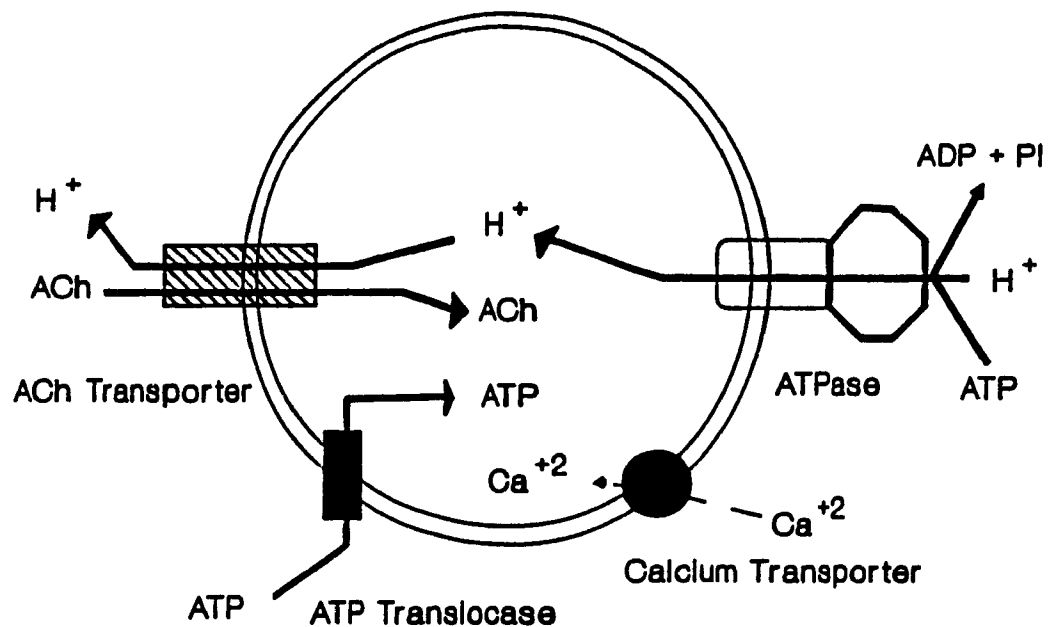


Figure 11 Model of the active ACh transport system of cholinergic vesicles. The model illustrates the ATPase which can acidify the vesicle interior for active transport of ACh and a transporter which is thought to transport ACh in while transporting H⁺ ions out. The possible existence of an ATP translocator, which would account for the vesicular content of ATP, and a Ca⁺² transporter are shown. See section 1.3 for details.

between the inside and outside of the membranous structure, thus not lysing the vesicles. These authors report the density of native vesicles as 1.058 g/ml and that of vesicle membrane as 1.132 g/ml. Because vesicles are small structures, the thickness of the membrane forms a sizable proportion of the volume which they can contain. Breer *et al.* (1978) reported the membrane thickness to be about 8-9% of the total possible vesicle volume, leaving about 90% of the remaining volume for solute and water. This initial estimate of the fractional volume made up by the membrane appears to have been too low and probably resulted from the small changes in density that were observed. Thus, Giompres *et al.* (1981) further refined these measures by using permeating substances that could affect density by greater amounts than H₂O.

When [²H]-H₂O is used as a permeating agent and replaces H₂O, it can increase the density of vesicles. These authors found that [²H]-H₂O could occupy 83% of the vesicle's fractional volume. If dimethylsulfoxide (DMSO) is used as the permeant substance, the fractional vesicle volume occupied is 72%. Lysis of the vesicles does not affect these estimates of the fractional volume occupied by each. However, if glycerol is used as the permeant substance it can only occupy 65% of the vesicle volume and upon lysing the vesicles, it occupies 75%. Thus it appears that DMSO occupies most of the vesicle's internal volume while glycerol can only exchange with osmotically active H₂O but not that needed for solute hydration. By this rationale, [²H]-H₂O can replace all H₂O and some must also be bound to the vesicle membrane itself. These findings, as well as those of Breer *et al.* (1978), imply that the solutes are in solution since the vesicle is osmotically active and most of the theoretical volume can be replaced with these permeant substances. A vesicle of 90 nanometers diameter and a membrane thickness of 4 nanometers has a theoretical internal volume of about 75% of the vesicle volume, in agreement with these measures.

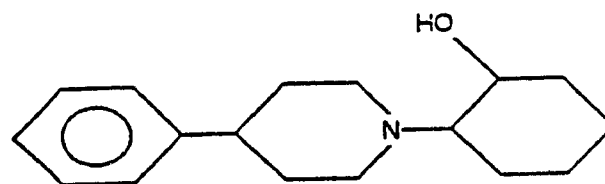
Studies of the ATP and ACh NMR spectra in vesicles further confirmed these solutes are in solution (Stadler and Fuldner, 1980; Fuldner and Stadler, 1982). By measuring the proton NMR spectra of ACh, free in solution, following lysis of vesicles, and in intact vesicles, Stadler

and Fuldner (1980) concluded that the line widths of the spectrum were similar enough between these conditions and, thus, that the internal ACh was in solution. When the ^{31}P -NMR spectrum of ATP was measured in vesicles, the band widths and shifts were best reproduced by a solution of ATP containing Mg^{+2} and ACh in an acidic pH. Thus, Fuldner and Stadler (1982) concluded that the ATP and ACh are in solution in an environment of about pH 5.5 (similar to the internal pH estimated by Michaelson and Angel, 1980). From these observations it appears that all transported ACh is hydrated and therefore represents a true concentrating power of the vesicular transport system.

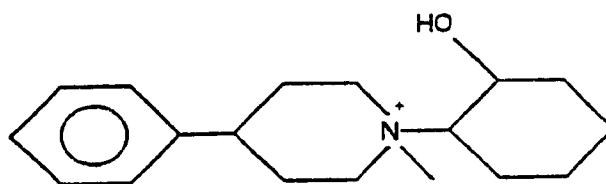
From the above results on the permeant substance measures, one can conclude that about 33% of the membrane's volume is due to H_2O (from $[\text{}^2\text{H}]\text{-H}_2\text{O}$ measures). The internal volume of the vesicle should be about 75%, as determined with lysed vesicles in the presence of glycerol. This volume seems divided into 3% for solutes (non hydrated) and 72% for H_2O , but given that the solutes are in solution, they are estimated to occupy about 10% of the vesicle's volume when fully hydrated (from DMSO and glycerol measures).

1 4. THE VESICULAR UPTAKE BLOCKER 2-(4-PHENYLPYPERIDINO)CYCLOHEXANOL (AH5183) AS A PHARMACOLOGICAL TOOL FOR CHOLINERGIC TERMINALS.

In the search for compounds that could be used as analgesics, Allen and Hanburys Ltd. produced a series of piperidino derivatives of which 2-(4-phenylpiperidino)cyclohexanol (AH5183) was one (see figure 2I). Toxicological tests found AH5183 to be toxic both orally (LD_{50} 35.5 mg/kg) and by intravenous (I.V.) (LD_{50} 2.7 mg/kg) administration in mice and produced a flaccid paralysis in both mice and chicks (LD_{50} 1-2 mg/kg) (Brittain et al., 1969).



trans - AH5183
VESAMICOL



AH5954

Figure 2I Chemical structures of AH5183 and AH5954. The structure of AH5183 is shown with no stereochemical specifics since the compound used in experiments presented below was a racemic mixture. The structures of these related compounds is shown to illustrate the similarity.

1.4.1. Early work and description of AH5183 action.

The AH5183-induced flaccid paralysis was evident after a 3-5 min delay, when the drug was given intravenously, and lasted from 12-20 min. The paralysis was not due to an effect on the muscle contractile elements, as determined by direct stimulation of the muscle, but resulted from an inhibition of nerve stimulated muscle contractions, whose onset was dependent on the rate of nerve stimulation. Furthermore, an attenuation in the force of muscle contraction was produced at 1 Hz but not at 0.1 Hz when 150 μ g/kg of AH5183 was given to anesthetized cats. To produce an inhibition of muscle contraction at a rate of 0.1 Hz required much higher doses of AH5183 (1 mg/kg) (Brittain *et al.*, 1969).

Marshall (1970 a and b) extended these initial observations. He noted, that when an AH5183-induced block of the rat hemidiaphragm was established by stimulating the phrenic nerve at 1 Hz, switching to 0.1 Hz caused a transient reversal in the blockade, although to produce a block at 0.1 Hz stimulation normally required 10 to 20 times more drug. As in the earlier studies, Ch was found not to reverse the drug's effect and any manipulation that should increase the rate of transmitter release induced the block sooner. By comparing the effects of AH5954, the N-methyl quaternary ammonium analogue of AH5183 (see figure 2I), with those of AH5183, Marshall concluded that AH5183 had a presynaptic site of action that did not involve the inhibition of Ch uptake (Marshall, 1970b). From these studies it was first proposed that the site of action of AH5183 could involve the packaging of ACh into a compartment for release, thus supposedly vesicular storage (Marshall, 1970a). A direct confirmation of an effect on ACh release by AH5183 was reported by Gandiha and Marshall (1973a). In the chick biventer cervicis muscle, exposure to AH5183 caused a reduction in the release of ACh in response to tetanic stimulation. The effect of AH5183 was shown to be both dose and frequency dependent.

1.4.2. Confirmation of AH5183's action on vesicular uptake of acetylcholine.

Confirmation of Marshall's hypothesis about the site of action of AH5183 came from Toll and Howard (1980) who demonstrated that AH5183 was a potent inhibitor of [^3H]-ACh accumulation by vesicles of the cell line PC12. In these cells there are two different kinds of vesicles, those that store ACh and those that store dopamine. While AH5183 had a small effect on the cell's accumulation of [^3H]-ACh, it had a potent ability to block the accumulation of vesicular [^3H]-ACh. At the doses of AH5183 used for this study (10 $\mu\text{g/ml}$, which is approximately 40 μM), the drug also inhibited the accumulation of ^3H -dopamine by the cells.

The first insights into how AH5183 interfered with the vesicle's uptake of ACh came from the report by Anderson *et al.* (1983), where the drug's action was investigated in purified vesicles from the *Torpedo* electric organ. AH5183 was found to be the most potent inhibitor of the vesicular transport system (IC_{50} of 40 nM) while other cholinergic agents such as HC-3, atropine, and d-tubocurarine were found to be weak inhibitors. There was no relationship between the potency of inhibition by these agents at their traditional sights of action (HC-3 - Ch transport, atropine - muscarinic receptors, and d-tubocurarine - nicotinic receptors) and in the transport of ACh by vesicles, suggesting that the transport system is pharmacologically distinct from other sights affected by ACh and Ch (see table 1I, compound #s 2-4). AH5183 was not an uncoupler of the ATPase responsible for setting up a proton gradient across the vesicular membrane (the proton pump) (Anderson *et al.*, 1983; Diebler and Gaudry-Talarmain, 1989), nor did it act as a base which could neutralize the acidic interior needed for active ACh transport. Furthermore, AH5183 did not inhibit the ATP dependent uptake of Ca^{+2} by isolated vesicles (Diebler and Gaudry-Talarmain, 1989). Structural analogues of the drug were poor inhibitors of the vesicular uptake system and thus the action of AH5183 was not due solely to a physical property of the molecule. The potency of inhibition and the lack of effect at these other sights implied that AH5183 interfered either directly with the ACh transporter or with an important regulator of active transport.

1 4.3. AH5183 action on vesicular acetylcholine uptake and structural considerations.

Parsons and coworkers have pursued the chemistry of AH5183's interaction with the vesicular ACh transport system with great interest. They have characterized most of what we understand about this interaction both in terms of the phenomenon of ACh transport and in terms of the structural requirements of AH5183 to interfere with this process. Much of the information concerning these aspects of AH5183 action are reviewed by Parsons and colleagues (Marshall and Parsons, 1987; Parsons *et al.*, 1987).

Under passive ACh uptake conditions, with no ATP present, isolated vesicles from *Torpedo* take up approximately 11 [^3H]-ACh molecules per vesicle very quickly but then the accumulation of [^3H]-ACh does not increase any further with time, whereas in conditions of active uptake, there is a time dependent uptake of transmitter by vesicles such that after 30-40 min the vesicles have accumulated about 320 [^3H]-ACh molecules per vesicle. Another important difference between the passive and active transport systems is that in the passive process [^3H]-Ch and [^3H]-ACh are almost equally transported but not concentrated, while during active transport [^3H]-ACh is greatly preferred and concentrated (Parsons and Koenigsberger, 1980). AH5183 is a much more potent inhibitor of the active uptake process, IC_{50} of 41 nM (Anderson *et al.*, 1986), than of the passive process, IC_{50} of 20 μM (Anderson *et al.*, 1983).

The inhibition of active ACh transport by AH5183 was found to be non-competitive. Active transport of [^3H]-ACh or [^{14}C]-ACh was reported as having a K_T of 460 μM and a V_{max} of 600 pmoles [^3H]-ACh/min/mg protein by Bahr and Parsons (1986) while Diebler and Gaudry-Talarnain (1989) reported a K_T of 1.21 mM and a V_{max} of 163 pmoles [^{14}C]-ACh/min/g original tissue (after correcting for recovery and protein, V_{max} was 5.4 nmoles [^{14}C]-ACh/min/mg vesicular protein). In the presence of AH5183 the K_T shifted to 220 mM and the V_{max} to 1.62 nmoles [^3H]-ACh/min/mg

protein (Bahr and Parsons, 1986). Diebler and Gaudry-Talarmain (1989) found almost no change in V_{max} but reported an increase in the K_T to 2.2 mM when the vesicles were exposed to AH5183. Both studies reported similar K_1 's of around 45 to 47 nM. The causes for the difference in the measured constants and the qualitative changes seen with exposure to AH5183 are not easily explained (the concentrations of radiolabeled ACh used in these studies only varied 2 fold, 50 μ M in Bahr and Parsons' report and 100 μ M in Diebler-Talarmain's report). Bahr and Parsons (1986) reported that the AH5183 inhibition of [^{3} H]-ACh transport was reversible.

Exposure of [^{3} H]-ACh labeled vesicles to AH5183 had no effect on the efflux of the transmitter from vesicles, suggesting that AH5183's action on the vesicle is not due to either lysing or an effect on vesicular extrusion of ACh. When AH5183 is added during the process of active [^{3} H]-ACh uptake, vesicles lose a small amount of their accumulated [^{3} H]-ACh content and transport ceases. This loss of accumulated [^{3} H]-ACh was calculated to represent 30 ACh molecules per vesicle (Anderson *et al.*, 1986). In this study the number of AH5183 binding sites were calculated to be about 3 per vesicle (in Parsons *et al.*, 1987, the estimate was 4 per vesicle). Since this publication further work by Parsons and coworkers has established the number of binding sites per vesicle to be closer to 10 to 15, suggesting that there might be a 1:1 binding of AH5183 to each vesicle transporter of ACh (Parsons, personal communication).

The original estimates of AH5183 binding sites per vesicle were done using purified vesicles of the VP₁ type. This was necessary because purification of the VP₂ type vesicles from other membranous constituents has been more difficult. Initial estimates of AH5183 binding to these two vesicle populations show that 3.5 times more binding sites per vesicle exist in VP₁ vesicles than in VP₂ vesicles (Parsons *et al.*, 1987). Because of the smaller size of VP₂ vesicles it is possible that the decreased number of binding sites is due to the decreased surface area and not to a decrease in the binding site density. Parsons and coworkers have calculated that the decrease in binding sites is in the

same range as the decrease in surface area, thus suggesting that the AH5183 binding site density is similar between vesicles (Parsons, personal communication). Furthermore, AH5183 blocks the transport of [^3H]-ACh by isolated vesicles of both types equally well (Parsons *et al.*, 1987).

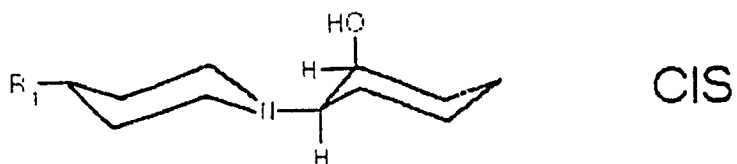
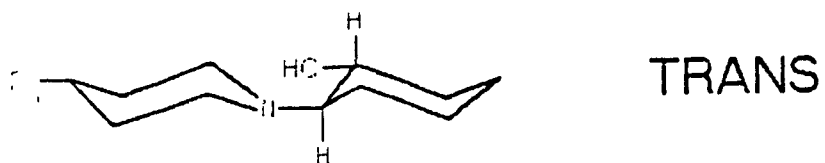
The tertiary amine group of AH5183 can exist in both a charged state and in a neutral state, the ratio of which depends on the pH of the medium. At physiological pH levels enough uncharged drug exists such that it can enter the vesicle and once inside it can become charged again. Because of this physical characteristic it is not possible, using only AH5183, to determine whether the binding site for AH5183 is located on the vesicle's interior (luminal face) or exterior (cytoplasmic face) or both. Parsons *et al.* (1987) used a relatively impermeant sulfhydryl reagent (p-chloromercuribenzenesulfonate) and found that modification of an externally oriented sulfhydryl group abolished AH5183 binding. However, by using proteinase K, a carboxypeptidase, they also established that the binding of AH5183 was only inhibited when the vesicle membrane was permeabilized by sodium cholate, thus implying that an internally oriented carboxy terminal is also important to AH5183 binding. These protein structure altering reagents cannot establish the site of AH5183 binding since quaternary structure may be changed by alterations occurring at a distance from the actual binding site. By using an analogue of AH5183 that retains a high potency (IC_{50} of 51 nM) for inhibition of [^3H]-ACh transport, but is a zwitter-ion at physiological pH (see table 1I compound 22), rendering it impermeant to the vesicle's membrane, it was possible to establish the cytoplasmic face of the vesicle membrane as the most likely location of the AH5183 binding site (Parsons *et al.*, 1987; Rogers *et al.*, 1987).

AH5183 inhibits the active uptake of ACh by vesicles in a non-competitive fashion and early studies were unable to establish any effect of ACh on the drug's action. However, if vesicle ghosts are prepared, several interesting effects were noted with regards to AH5183 binding (Noremberg and Parsons, 1989). Vesicle ghosts were considered to have resealed in a right-side-out configuration because all of the

bound AH5183 could be displaced by an impermeant analogue of the drug (compound 22, Table 1I). Ghosts were found to have higher B_{max} 's and have lower K_D 's for AH5183 than native vesicles, though ghosts resealed in such a way that they retained their normal internal volume and supposedly lost only internal contents. ACh (60 mM) interfered with AH5183 binding by raising the AH5183 K_D of binding from 7 nM to 99 nM. The calculated K_i for this ACh effect on drug binding was reported to be 18 mM, however due to the large amounts of ACh required for this effect it was not possible for the investigators to establish whether ACh was a competitive or non-competitive inhibitor of AH5183 binding. Because ACh is more rapidly taken up by vesicle ghosts than AH5183 could be displaced, it was not possible to ascertain whether the effect of ACh was from the inside or outside of the vesicle. The authors argued that since the number of AH5183 binding sites increased and the K_D was lowered when ghosts were prepared, it was likely that the action of ACh was taking place from the vesicle's interior. Given that the ACh concentration inside the vesicles of *Torpedo* is high, there should be no binding of AH5183 to native vesicles, thus the conclusion was that there must be some endogenous substance that can regulate this binding site of the drug (Noremberg and Parsons, 1989).

By analyzing a series of AH5183 analogues containing structural changes to different parts of the drug molecule, Parsons and coworkers have established certain structural requirements for the action of AH5183 on the active vesicular ACh transport system (Rogers *et al.*, 1987 and 1989). The first requirement of all analogues was that the N of the piperidino ring be in a trans position to the alcohol group of the adjacent ring (see figure 3I). The interaction of AH5183 with its site is also stereoselective given that 1-AH5183 is twice as potent as the racemic mixture (see Table 1I, compound 1a) (Rogers *et al.*, 1989).

Figure 4I contains the structure of the parent compound from which all the analogues studied by Parsons and coworkers are derived (Rogers *et al.*, 1987 and 1989). As with AH5183, ring D is missing in some of the analogues. Ring A is important to the interaction of the drugs with the transport system. While the ring need not be aromatic or planar,



POTENCY
TRANS >>> CIS

Figure 3I. Three dimensional representation of the active AH5183 analogues in a trans-configuration. The piperidino and cyclohexanol rings, and their constituents, are illustrated to show the difference between the structural isomers of these compounds in the cis and trans configurations. The trans-configuration is much more potent than the cis-configuration.

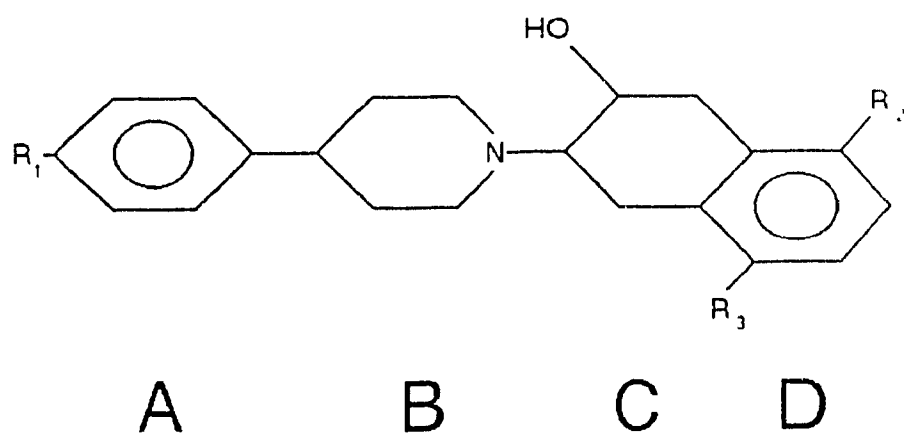
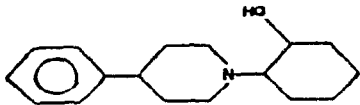
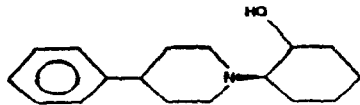
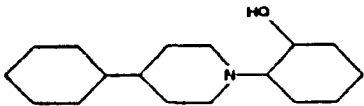
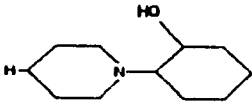
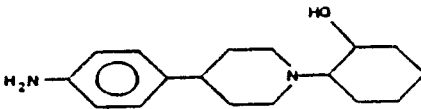
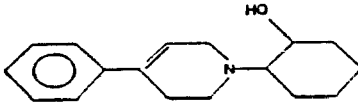


Figure 4I. Chemical structure of the parent ring compound for the AH5183 family of analogues. All of the compounds described in table 11 are derived from this basic ring structure.

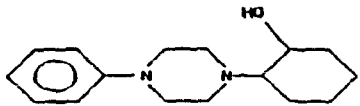
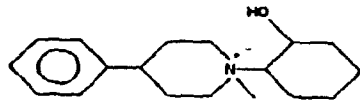
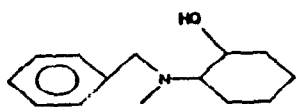
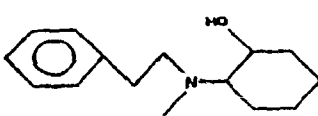
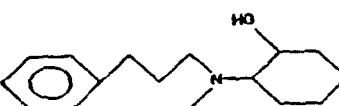
TABLE 1I. POTENCY OF AH5183 AND ANALOGUES

Number	Structure and/or name	IC ₅₀ in nM	Reference
1	 trans - AH5183 VESAMICOL	40	1,2,3
1a	 AH5183	20	3
2	HC-3	300,000	1
3	Atropine	10,000	1

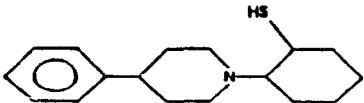
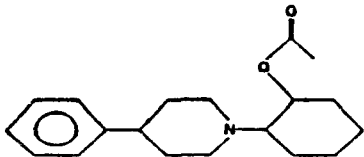
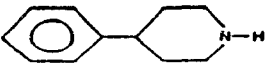
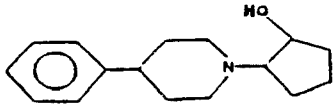
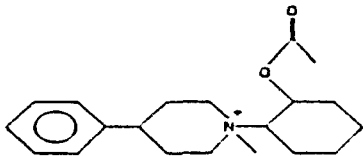
CONTINUATION OF TABLE 11.

Number	Structure and/or name	IC ₅₀ in nM	Reference
4	d-Tubocurarine	10,000	1
5		40	2,3
6		70,000	2,3
7		2,000-500	2,3
8		200	2,3

CONTINUATION OF TABLE 11.

Number	Structure and/or name	IC ₅₀ in nM	Reference
9		300	2,3
10	 AH5954	1,000-2,000	2,3
11		10,000	3
12		20,000	3
13		2,000	3

CONTINUATION OF TABLE 1I.

Number	Structure and/or name	IC ₅₀ in nM	Reference
14		2,000-4,000	2,3
15		2,000	2,3
16		90,000	2,3
17		2,000	2,3
18		5,000	3

CONTINUATION OF TABLE 1I.

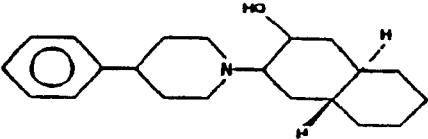
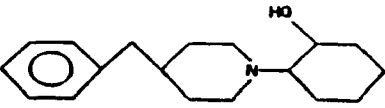
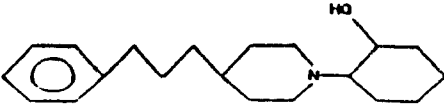
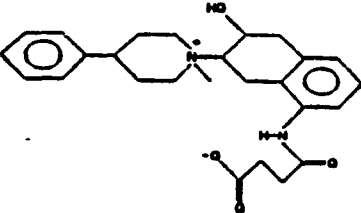
Number	Structure and/or name	IC_{50} in nM	Reference
19		10	2,3
20		300	3
21		70	3
22		50	3

Table 11. Potency of AH5183 and analogues. The table presents the chemical structures of AH5183 and some of its analogues, along with their IC₅₀s of ACh transport by isolated vesicles. Structures and IC₅₀s were obtained from the literature. References used are as follows: 1.- Anderson *et al.*, 1983; 2.- Rogers *et al.*, 1987; 3.- Rogers *et al.*, 1989.

since the substitution of the phenyl group by cyclohexyl had no effect on the IC_{50} as compared to AH5183 (compound 5, Table 1I), removing the ring entirely greatly reduces potency (compound 6, Table 1I). Changes in R_1 to more hydrophilic groups also reduces potency (compound 7, Table 1I) but as the groups become more hydrophobic, potency is improved. If the phenyl ring is made coplanar to ring B, potency is lowered (compound 8 and 9, Table 1I). As well, by methylating the piperidino ring at the N, so as to resemble Ch, one forms AH5954, a compound with a substantially lowered IC_{50} (compound 10, Table 1I). Substituting ring B for a hydrocarbon chain also reduces the drug's ability to block the active uptake of ACh by vesicles (compound 11, 12 and 13, Table 1I). These changes to ring B suggest that the angle existing between the N of ring B and the phenyl group are important to the interaction of the drug with the transport process. All attempted alterations to either the free hydroxyl group of ring C (compound 14 and 15, Table 1I) or the removal or alteration to ring C (compound 16 and 17, Table 1I) greatly reduces the analogue's potencies. Compound #18 of table 1I was interesting because of its resemblance to ACh, but as is seen by the low potency, these changes were detrimental to drug action. This finding should not be too surprising given that AH5183's inhibition of ACh transport is non-competitive and that ACh does not greatly affect AH5183 binding to isolated vesicles (Parsons *et al.*, 1987). Another interesting analogue that forces the N of the piperidino ring and the alcohol of the cyclohexanol ring into a coaxial position is compound # 19 of table 1I. This analogue is the most potent compound, so far reported, at inhibiting the active uptake of ACh by vesicles and thus suggests that a coaxial configuration is one that is preferred for action and or binding. However, in solution, 99% of AH5183 exists in a diequatorial positioning of the piperidino N and the alcohol group of the cyclohexanol, also suggesting that this configuration is a preferred orientation (Rogers *et al.*, 1989).

One other set of interesting analogues are those which lengthen the distance between the A and B rings. These compounds (20 and 21, Table 1I) demonstrate that there are two possible ways of interacting

with the binding site, since adding a CH_2 decreases potency, but adding a $(\text{CH}_2)_3$ chain between ring A and B improves potency over the first analogue and results in an IC_{50} only about twice as large as AH5183 (Rogers *et al.*, 1989).

It is not yet known whether AH5183 interacts with the transporter itself or with a regulatory peptide closely associated with the vesicular ACh transporter. The regulation of transport and of drug binding appear complicated. For example, the specific binding of AH5183 to vesicles shows positive cooperativity (Parsons *et al.*, 1987). Furthermore, a recent abstract (Parsons and Hicks, 1990) has reported the isolation of a non-vesicular protein which can bind AH5183, is located in a variety of tissues both cholinergic and non-cholinergic, and for which no function is known, thus complicating the investigations of AH5183 action.

1.4.4. The effect of AH5183 on the release of ACh from various cholinergic preparations.

It is now known that AH5183 can inhibit the stimulated and basal release of ACh from a variety of tissues and by a variety of stimulation methods. Until now, most reports have attributed this effect of the drug as being due to its effect on the vesicular transport system and in some models this indeed appears to be the best explanation. Some reservations are in order, however, given that AH5183 has a variety of other effects and given that its possible interaction with such proposed ACh translocators as mediatophore have yet to be studied in detail (see section 1.5 for a discussion of the vesicular versus cytoplasmic hypotheses of ACh release).

In PC12 cells, the first system where AH5183 was found to inhibit the uptake of $[^3\text{H}]\text{-ACh}$ by vesicles, treatment with up to $40\text{ }\mu\text{M}$ AH5183 had only a small effect on the synthesis and accumulation of $[^3\text{H}]\text{-ACh}$ by the cells though by $0.4\text{ }\mu\text{M}$ the drug had completely blocked the accumulation of $[^3\text{H}]\text{-ACh}$ by vesicles (Melega and Howard, 1984). Stimulation by 2 mM Ba^{+2} , a secretagogue in PC12 cells, caused the release of 9% of

[³H]-ACh in cells not exposed to AH5183 but in those cells where the loading period was carried out in the presence of 4 μ M AH5183 only 2% of [³H]-ACh was released. Drug treatment only caused slightly more of the prepackaged ACh, to be released in response to the Ba⁺² stimulation. The effect of AH5183 was not restricted to ACh uptake since 40 μ M of the drug affected the accumulation of [³H]-dopamine by the cells but not by the dopaminergic vesicles (Melega and Howard, 1984). Since only 30% of cellular ACh is present in vesicles, the effect of AH5183 on the release and packaging by vesicles of [³H]-ACh by PC12 cells showed a reasonable correlation.

In the *Torpedo* electric organ, a specialized organ which evolved from a neuromuscular precursor and which is solely cholinergic, AH5183 also potently blocks the release of [³H]-ACh. When the inhibitor is added at a concentration of 5 μ M 15 min prior to a loading period with [³H]-acetate, used to label newly synthesized ACh, no effect was noted on either the entry of [³H]-acetate or on ACh synthesis. However, upon stimulation of the *Torpedo* synaptosomes by 125 mM K⁺, while the release of unlabeled ACh, supposedly prepackaged transmitter stores, was not affected by drug exposure, that of [³H]-ACh, transmitter that would have been synthesized and packaged during the drug exposure, was only 15% of that measured in the drug's absence (Michaelson and Burstein, 1985; Michaelson et al., 1986; Michaelson et al., 1987). If AH5183 was added after the loading period, no effect of the drug was seen on the release of either ACh or [³H]-ACh. The effect of 5 μ M AH5183 on the release of [³H]-ACh by K⁺ was dependent on when it was added during the loading period with [³H]-acetate. Inhibition of [³H]-ACh release followed a sigmoidal relationship with time of drug addition and thus suggested that the synthesis of transmitter is not directly coupled to its being packaged but rather that a lag in time occurs between these events (Michaelson et al., 1986). In these reports from Michaelson's laboratory, AH5183 was also found to inhibit the basal release of ACh by 38% (Michaelson et al., 1987). Suszkiw and Manalis (1987) reported similar effects of AH5183 on the nerve impulse stimulated release of transmitter by *Torpedo* electric plaques. Exposure of the tissue to 10

μ M AH5183 during a loading period had no effect on ACh release or on the synthesis of [^{3} H]-ACh from [^{3} H]-acetate, but release of [^{3} H]-ACh and its accumulation by vesicles was 90% inhibited. These results suggest that AH5183 does not affect vesicle mobilization but that its effect on release is based on its effect on the vesicular accumulation of transmitter.

An interesting comparison was made by Gaudry-Talarmain et al (1989) between the effects of AH5183 and cetiedil (α -3-thienylacetic acid 2-(hexahydro-1H-azepin-1-yl) ethyl citrate ester) on the packaging and release of ACh by *Torpedo* synaptosomes. Both compounds inhibit the vesicular uptake of ACh but whereas AH5183 has no effect on the release of ACh (only the release of total ACh was determined) by either 60 mM K⁺ or 3.5 μ M A23187, a Ca⁺² ionophore, cetiedil could attenuate the release of transmitter by both secretagogues. Thus it appears that AH5183 is a more specific agent in its cholinergic effects than cetiedil.

The effects of AH5183 have been most widely studied at the neuromuscular junction because these types of preparations readily lend themselves to electrophysiological as well as biochemical measurements. Gandiha and Marshall (1973a) were the first to report an inhibitory effect of AH5183 on the release of nerve impulse stimulated ACh release. In a second publication, Gandiha and Marshall (1973b), noted that AH5183 did not inhibit the release of ACh by such secretagogues as nicotine and carbachol from the chick biventer cervicis muscle preparation. This finding suggests that AH5183 does not affect the release of surplus ACh, since Collier and Katz (1970) found that ACh can release surplus ACh from the superior cervical ganglia of the cat. Though AH5183 can block the release of ACh from the motor end plate after long periods of stimulation (Gandiha and Marshall (1973a) measured release after 2-3 hr of drug exposure and stimulation), Vizi (1989) noted that when AH5183 is added to the hemidiaphragm after a loading period with [^{3} H]-Ch and a control stimulation of 50 Hz for 0.8 sec every 10 sec for 3 min, the drug has no effect on the release of [^{3} H]-transmitter by a second nerve stimulation train 17 min following the first train. Thus it appears that in the neuromuscular junction, as with *Torpedo* electric organ,

AH5183 has no effect on prepackaged transmitter stores. However, in the presence of a compound that increases ACh release, such as 4-aminopyridine, the normal increased release of transmitter can be blocked by AH5183, suggesting that the increased release transmitter was newly compartmentalized (Vizi, 1989).

While in *Torpedo* electric organ and the neuromuscular junction, AH5183 appears to have no effect on the release of preformed transmitter available for release, in the mammalian brain this does not seem to be the case, though some similarities do exist and some of the differences in the way different cholinergic models respond may be due to the tissue and some to the method of stimulation. Otero *et al.* (1985) reported that the drug had an inhibitory effect on the basal and K^+ stimulated release of $[^3H]$ -ACh. Under the same conditions these authors found that the drug substantially decreased the level of $[^3H]$ -ACh in the P_3 fraction (occluded ACh pools) while raising that found in the S_3 fraction (cytoplasmic ACh), consistent with the idea that AH5183 inhibits the active uptake of ACh by vesicles. Ricny and Collier (1986) also reported an inhibition of ACh release, both basal and K^+ stimulated, and that the total ACh content of the P_3 fraction decreased by 50% while that of the S_3 fraction doubled following AH5183 exposure and K^+ stimulation. In the report by Otero *et al.* (1985) when the occluded subcellular fractions were further subdivided the D (monodispersed vesicles) and H (vesicles associated with the plasma membrane) fractions were inhibited from accumulating $[^3H]$ -ACh by AH5183. The level of inhibition by the drug cannot be easily ascertained for the D fraction because the authors used eserine to preserve the cytoplasmic ACh and no mention is made of any attempts to correct for the diffusion of $[^3H]$ -ACh from the soluble fraction to the first sucrose layer (the D fraction). Ricny and Collier (1986) did not investigate the accumulation of labeled transmitter by striatal slices' occluded ACh compartments, but they did report only finding a significant decrease in the D fraction's content of ACh. Carroll (1985) reported that much lower doses of AH5183, 34 nM, could inhibit the release of ACh from brain minces whether the minces were stimulated by K^+ or veratridine, a secretagogue which maintains sodium

channels open for a longer time. He noted that at levels of the drug ranging from 170 nM to 17 μ M there was not only an inhibition in the [^{14} C]-ACh accumulation by the P_3 fraction but also a decrease in the preformed ACh levels of this fraction. Although this finding could indicate that AH5183 can affect prepackaged ACh, for which no direct measure has been found to indicate such an effect, it is possible that the more likely reason for this effect is that some of the occluded ACh was released into the cytoplasm as a response to stimulation and that, due to the presence of the drug, this transmitter could not be taken back up. This interpretation is consistent with the idea that terminal stores of transmitter can equilibrate rapidly and, in the case of the occluded transmitter stores, this would require the exchange of ACh with the cytoplasm (Elmqvist and Quastel, 1965; Large and Rang, 1978).

The inhibition of newly synthesized ACh release in mammalian brain by AH5183 is reported by a variety of studies (Joep and Johnson, 1986; Suszkiw and Toth, 1986; Suszkiw and Manalis, 1987; Adam-Vizi *et al.*, 1991) but an inhibitory effect is also noted on the release of preformed ACh from brain when exposed to AH5183 (Joep and Johnson, 1986; Ricny and Collier, 1986). This inhibitory effect on the release of performed transmitter in mammalian brain, different from that found in *Torpedo* and with short stimulation in the neuromuscular junction, would indicate that either the whole of the vesicular transmitter stores must turnover more than once during the period of stimulation, or that some fraction of the vesicular stores is responsible for release and that this fraction recycles more than once during the stimulation. Reports by Suszkiw and coworkers (Suszkiw and Toth, 1986; Suszkiw and Manalis, 1987) where AH5183 was used both during and after a loading period with either [^{3} H]-Ch or [^{14} C]-Ch, found that the effect on the release of labeled transmitter was greater than the effect on the accumulation of the labeled transmitter by the occluded fractions and therefore suggest that a fraction of the vesicular ACh content is involved in the K^+ stimulated ACh release. Adam-Vizi *et al.* (1991) reported that while the Ca^{+2} dependent veratridine-evoked release of [^{14} C]-ACh from striatal slices was blocked when AH5183 was present during the loading period, the Ca^{+2} in-

dependent release of transmitter by veratridine was not affected by the drug. Thus, they suggest that the source of this Ca^{+2} independent released transmitter is not likely to be vesicular. Release of prepackaged [^{14}C]-ACh by veratridine, in the presence of Ca^{+2} , was only slightly inhibited by AH5183.

Several of these studies on the mammalian brain found AH5183 to inhibit the synthesis of ACh when slices were stimulated (Jope and Johnson, 1986; Ricny and Collier, 1986; Suszkiw and Toth, 1986; Carroll and Ivy, 1988). Where measured, the inhibition of ACh during drug exposure was not the result of a direct inhibition of ChAT (Carroll, 1985; Jope and Johnson, 1986) or to an inhibition of Ca^{+2} entry (Suszkiw and Manalis, 1987). There was, however, an effect on the uptake of Ch by AH5183 (Otero *et al.*, 1985; Jope and Johnson, 1986; Suszkiw and Toth, 1986) and though the inhibition was found to be weak and of a competitive nature, at least Suszkiw and Toth (1986) were able to attribute the inhibition of ACh synthesis to the inhibition of Ch uptake.

Ricny and Collier (1986) noted that the inhibitory effect of AH5183 on the release of ACh from striatal slices was reversible. As in previous studies (Brittain *et al.*, 1969; Marshall, 1970a; Behr and Parsons, 1986) the effect was slow to revert, such that 60 min after drug washout release by K^{+} stimulation was only 80% of control. The relationship between the recovery of ACh release from drug inhibition and the recovery of the occluded ACh compartments is presented in this thesis but remains the only such study.

The superior cervical ganglia of the cat (SCG) affords the best cholinergic biochemical model for investigating the release and synthesis of ACh in mammalian synapses. The SCG can be perfused *in situ*, the effluent collected and its ACh measured, and it can be stimulated at physiological rates via its preganglionic nerve (Birks and MacIntosh, 1961; Collier, 1969; Collier and Kwok, 1982). The study of Collier *et al.* (1986) on the effects of AH5183 on the synthesis and release of ACh by this sympathetic ganglion illustrates this point. In this study 10 and 40 μM AH5183 was reported to have an effect on the rate of ACh release. During the first 2 min of 5 Hz stimulation the release of ACh

by nerve impulses is normal, but this rate of transmitter release cannot be maintained at the steady rates of controls, and begins to drop after the initial normal release until it reaches basal levels of transmitter efflux, while drug unexposed ganglia are still able to release ACh at 10 to 12 times the basal rate. The amount of ACh released by nerve impulses in the presence of AH5183, regardless of whether 5 or 20 Hz stimulation was used, was reported to be about 14% of the ganglion's initial ACh content, which quantitatively agrees extremely well with Birks and MacIntosh's (1961) "readily releasable pool" of ACh in the cat SCG. Thus, in the presence of AH5183 it would appear that the mobilization of the less readily releasable ACh to a releasable form is blocked, and that the ACh which is already available for release (supposedly prepackaged ACh in a special fraction of vesicles) is free from drug interference. Again the drug effect was found to be slowly reversible, recovering 60% of control transmitter release by 60 min. Compounds such as 4-aminopyridine, which is known to increase stimulated ACh release by lengthening the action potential duration and thus increasing Ca^{+2} entry (Vella Lamarca and Collier, 1983), could not reverse the effect of AH5183 on transmitter release.

One of the most interesting observations of the Collier *et al.* (1986) study was the observation on ACh synthesis. As in some of the mammalian brain reports, AH5183 was not found to inhibit the synthesis of ACh, though in the ganglia this was not estimated using [^3H]-Ch as a precursor. The fundamental observation was that the ACh content of AH5183-treated and stimulated ganglia increased by that amount of ACh which could not be released by nerve impulses in the presence of the drug, a finding which implies that the release of transmitter and its synthesis may not be linked directly (mass action law of ACh synthesis). Furthermore, this increase in ACh content was attenuated or blocked when Ca^{+2} entry, in response to stimulation, was decreased or inhibited. Because the increase in ACh content was observed even in the absence of an AChE inhibitor, Collier *et al.* (1986) concluded that the increased transmitter stores had to be in a compartment protected from the action

of internal AChE. Thus, in the case of the cat SCG the effects of AH5183 could be readily explained by an action on vesicular ACh uptake.

If one accepts that stimulated ACh release is quantal and results from the release of a vesicle's content of ACh, then AH5183 should also have an effect on the size and distribution of miniature-end-plate potentials (m.e.p.p.s) as measured at the neuromuscular junction. Several studies have reported that AH5183 can decrease the amplitude of measured m.e.p.p.s when drug poisoned preparations are stimulated (Van der Kloot, 1986; Whitton *et al.*, 1986; Estrella *et al.*, 1988). The decrease in m.e.p.p. size was dependent on the dose and rate of stimulation. Furthermore, Van der Kloot (1987) noted that treatments which can double the m.e.p.p. amplitude, such as 1 hr exposure to adrenaline or insulin, were ineffective if AH5183 was present during the treatment period. This observation suggests that the increase in m.e.p.p. size seen with these treatments results from a regulation of the ACh content present in the vesicle, though there are many reservations to the equating of one m.e.p.p. to one vesicle. An interesting aspect of the effect of AH5183 on m.e.p.p. amplitude is that it causes m.e.p.p. size to decrease by what appears to be a continuum and not an all or none fashion (Whitton *et al.*, 1986; Van der Kloot, 1986; Estrella *et al.*, 1988). One possible interpretation is that vesicles can be partially inhibited, and thus their rate of filling slows, causing partially filled vesicles to interact with the release process to give a smaller m.e.p.p., formed by the release of less than a normal quantum of ACh. It has yet not been possible to determine whether empty vesicles continue to attempt to exocytose when AH5183 has blocked the stimulated efflux of transmitter because such parameters as m.e.p.p.s disappear.

A rather common finding is that AH5183 not only blocks the evoked release of ACh but that it also inhibits the basal efflux of the transmitter (Carroll, 1985; Edwards *et al.*, 1985; Otero *et al.*, 1985; Vyskocil, 1985; Ricny and Collier, 1986; Vizi, 1989). This was a bit surprising since most of the basal efflux of ACh is the result of non-quantal transmitter release which is not calcium sensitive and thus, one would expect that this type of release would not be affected by AH5183.

Two studies have looked directly at electrophysiological changes at the neuromuscular junction under resting conditions in the presence and absence of AH5183 (Edwards *et al.*, 1985; Vyskocil, 1985). Normally, addition of d-tubocurarine, a nicotinic antagonist, causes a hyperpolarization of the endplate, and this has been interpreted as indicating that the depolarizing effect of basally released ACh is blocked, thus producing a hyperpolarization. In both studies, AH5183 was found to cause a hyperpolarization of its own which was not accompanied by any change in m.e.p.p. size or shape, suggesting that the effect was not due to blockade of nicotinic receptors. The studies hypothesize that basal ACh release is the result of the vesicular ACh transporter inserting itself into the plasma membrane following vesicle fusion and that inhibiting the ACh transporter inhibits basal ACh release. The central problem with this interpretation is that the transporter is known to require a proton gradient and a pH of around 5 is unlikely to exist in the cleft between muscle and motor-end-plate. Edwards *et al.* (1985) raised the pH of the medium bathing the hemidiaphragm to 8.4 and 9.4 and found that this treatment also caused a hyperpolarization of the end-plate region. This finding should be interpreted with caution for two important considerations are not raised by the authors; the first is what happens to the motor-end-plate and muscles at these pHs and the second is the fact that ACh is very sensitive to alkaline hydrolysis, thus perhaps being destroyed before ever reaching the muscle. Though, these results are consistent with the hypothesis that basal release of ACh is caused by the vesicular ACh transporter, much needs to be done before one can reasonably conclude this. One possibility that has yet to be raised is whether the basal efflux is related to the passive transport process.

1.4.5. Sites of AH5183 action other than the vesicular acetylcholine transporter.

Though AH5183 is a very potent inhibitor of active vesicular ACh transport, and though, at low concentrations, its effect on the release of ACh does indeed seem to be the result of this action, a variety of

other effects have been noted. Perhaps the most important effect, for presynaptic cholinergic mechanisms, is AH5183's competitive inhibition of Ch transport (Otero *et al.*, 1985; Suszkiw and Toth, 1986) which can be overcome by an equal concentration of Ch in the medium (determined at 10 μ M AH5183 and Ch). Suszkiw and Toth (1986) accounted for the decreased synthesis of ACh, in the presence of AH5183, by the effect of the drug on Ch transport. Several studies have determined that AH5183 does not block ChAT directly (Brittain *et al.*, 1969; Carroll, 1985) but at least two studies indicate that AH5183 could interact in some way with ChAT. Carroll and Ivy (1988) found that AH5183 could stimulate ChAT activity from drug-exposed slices of the hippocampus. The drug was found to increase the basal rates for the soluble ChAT, the ionically-membrane bound ChAT, and for the membranous ChAT form which can only be liberated by detergent treatment. Mykita and Collier (1989) noted that AH5183 made the increase in SCG ACh content vulnerable to inhibition by picrylsulfonic acid (TNBS), an agent which is impermeant to the plasma membrane and reacts with free amine groups. The very interesting phenomenon here is that AH5183 must be present prior to TNBS treatment or synthesis of ACh is not inhibited; furthermore, Ch uptake does not appear to be inhibited under these conditions, suggesting that AH5183 somehow alters ChAT to make it vulnerable to TNBS modification from the outside.

AH5183 is known not to interfere with Ca^{+2} entry in response to stimulation (Collier *et al.*, 1986; Suszkiw and Toth, 1986) and thus does not block the release of transmitter this way. However, some other effects at cholinergic sites have been noted, such as inhibition of AChE (Van der Kloot, 1986) and inhibition of nicotinic receptors (Brittain *et al.*, 1969; Marshall, 1970a; Enomoto, 1988) but no evidence exists for an effect at muscarinic receptors (Brittain *et al.*, 1969; Marshall, 1970a). At higher doses, AH5183 can act as an anesthetic (Marshall, 1970a; Estrella *et al.*, 1988) and as an α -adrenergic receptor blocker (Marshall, 1970a). A non-cholinergic interaction which could be of importance in studies of brain function is the inhibition of dopamine up-

take noted in PC12 cells at relatively low concentrations (40 μ M) (Toll and Howard, 1980; Melega and Howard, 1984).

Although AH5183 has a variety of actions other than its most potent action of blocking ACh active uptake by vesicles, most studies support the idea that at low concentrations the drug's effects are due to this one potent action. AH5183's interaction with a variety of other cholinergic sites, such as the ACh translocator, mediatophore, are still left to be determined in detail and may be important in the overall effect of this drug on cholinergic transmission. Israel and Morel (1990) have made some preliminary observations on the action of AH5183 on mediatophore's ability to translocate ACh and they find that the drug has no effect. The study is by no means complete, some aspects of this observation are discussed in section 1.5. Table 2I summarizes the known effects of AH5183.

1.5. THE VESICULAR VERSUS CYTOPLASMIC ORIGIN OF STIMULATED ACETYLCHOLINE RELEASE.

The debate about the origin of evoked ACh release is more than 15 years old. The topic has been extensively reviewed by members on both sides of the issue but little common ground has been reached with respect to the interpretation of experimental results (Marchbanks, 1975; MacIntosh and Collier, 1976; Ginsborg and Jenkinson, 1976; Van der Kloot, 1977; Collier, 1979; Israel et al., 1979; Kelly et al., 1979; Zimmermann, 1979a; Ceccarelli and Hurlbut, 1980; MacIntosh, 1980; Tauc, 1982; Tremblay et al., 1983; Israel and Manaranche, 1984; Dunant, 1986). Perhaps part of the reason that a consensus has yet to be reached is that so far all experimental approaches have been open to alternative explanations and no revised hypothesis by either the vesicular camp or the cytoplasmic camp has yet explained all of the observed phenomena satisfactorily. No experimental approach has yet been taken that can exclude one hypothesis while confirming the other, and thus the controversy continues. In this introduction to the debate I will review the major points raised by both schools of thought and refer the reader

Table 2I. Sites of AH5183 Action.

Site of action	Effect
Vesicular ACh Transport Active Passive	Potent non-competitive inhibition (20-40 nM) Weak non-competitive inhibition (20 μ M)
ACh Release Stimulated Basal	Inhibition (time and use dependent) Inhibition (time dependent)
ACh Synthesis ChAT activity Ch Transport	Small or no inhibition No effect or slight stimulation Weak competitive inhibition
MEPP Size	Decreases size with stimulation
Ca²⁺ Entry	No effect
Receptors Nicotinic Muscarinic α -Adrenergic	Antagonist (open channel antagonist) No Effect Weak competitive antagonist
Anesthetic	Weak anesthetic effect
Dopaminergic System Cellular Dopamine Uptake Vesicular Dopamine Uptake	Inhibition No direct effect known

Table 2I Sites of AH5183 action The table summarizes important aspects of AH5183 action at cholinergic and non-cholinergic sites, discussed in section 1.4.

to the above cited reviews, except where new evidence exists or where similar approaches have yielded apparently opposite results.

In its simplest form, the vesicular hypothesis states that vesicles are the source of the quantally released transmitter and that they accomplish this by exocytosis. This hypothesis requires that a vesicle contain the equivalent of one quantum of ACh, that it fuse to the plasma membrane during the release of transmitter, that it recycles following the fusion, and that it refill with one quantum of ACh so as to be ready to release ACh again. For its part, the cytoplasmic hypothesis states that the source of quantally released ACh is the cytoplasm and that a plasma membrane protein, or proteins, form the quantum during release. In this version of ACh release there is no need for vesicle recycling, just for a quantum generating plasma membrane system. This is the hypothesis originally proposed by Fatt and Katz (1952, 1953) to explain their observation of quantal ACh release. Various refinements to these hypotheses have been proposed but in essence these changes have the same broad requirements outlined above.

1.5.1. Intraterminal acetylcholine compartments.

As already mentioned in section 1.2.1., there is reasonable evidence that ACh exists both in the cytoplasm and in the vesicles of cholinergic nerve terminals. The purity of these fractions, with respect to the ACh present in them, cannot be determined because of the invasive nature of the tissue fractionation schemes, but no method has yet been devised for the *in situ* determination of ACh. These observations of the compartmentalization of ACh can support both hypotheses; the vesicular school states that cytoplasmic ACh is needed for vesicle refilling while the cytoplasmic school refers to the vesicular ACh as a storage site to help replenish cytoplasmic ACh levels during release.

Several attempts were made, using choline analogues, to distinguish the different pools of ACh (reviewed in section 1.2.). Studies where the releasability of any given false transmitter was studied were open to criticism. Welner and Collier (1984, 1985) reported that the

false transmitter, acetyldiethylhomocholine, was very poorly incorporated by vesicles and that stimulation did not lead to an increased release of this transmitter analogue. These findings, along with those which identified the transmitter release ratios of false transmitter to ACh as originating from a small population of vesicles, support the concept of vesicular release. However, the plasma membrane mechanism for transmitter release may have similar structural requirements for substrates as the vesicle uptake system has for transport. These results are more easily interpretable by the vesicular hypothesis but cannot rule out cytoplasmic release (see discussion below of the plasma membrane translocating protein called mediatophore).

An elegant attempt by Tauc *et al.* (1974) to distinguish the role of the cytoplasmic from the vesicular ACh in the release of transmitter, involved the injection of AChE into one of two identified and cholinergic interneurons of the buccal ganglion in *Aplysia*. The thinking was that since vesicular ACh is protected from hydrolysis by AChE, this compartment should not be touched by the exogenously added AChE, while the labile cytoplasmic ACh should be destroyed. These manipulations abolished the post synaptic response to the injected neuron but not to that not injected with the enzyme. These authors interpreted this finding to indicate that the source of released transmitter was cytoplasmic and not vesicular. However, Politoff *et al.* (1975) did a similar experiment which had a very different conclusion. These investigators bathed the frog neuromuscular junction in a solution containing AChE and stimulated the motorneuron so as to incorporate the enzyme in the vesicles (see section 1.4.3.). They were able to document this and hypothesized that since the cytoplasmic compartment seemed to be free of exogenous AChE, this treatment could look specifically at the ACh located in the vesicles without affecting that found in the cytoplasm. These manipulations also abolished neuromuscular transmission in a way that could be shown not to be due to the remaining presence of exogenous AChE, supposedly being extruded from vesicles during exocytosis. Thus the conclusion here is that vesicular ACh is important to the release of transmitter during stimulation. Both studies present an interesting

problem of interpretation. The importance of one or both compartments to the release of ACh may be critical without indicating which is the initial source of release. As already noted above, and consistent with the work of Elmqvist and Quastel (1965) and Large and Rang (1978), it is likely that there is a very quick equilibration of cytoplasmic and vesicular ACh during the continued release of transmitter. These studies cannot establish whether the readily releasable pool of ACh has been affected or not and thus little that is unequivocal can be concluded. Both results can be incorporated into the mechanistic schemes of both schools of thought.

1.5.2. Considerations of the quantal nature of acetylcholine release and presynaptic currents.

As mentioned in section 1.2.2.3. there are at least three subclasses of m.e.p.p.s that can be detected at the neuromuscular junction. Proponents of neither hypothesis have been able to describe a feasible mechanism by which these three classes of events can be generated. This is partly due to the lack of understanding still existing about how vesicles regulate their content of ACh and about how mediatophore, the plasma membrane translocating protein (see below for more about mediatophore), might generate various sizes of quanta.

If a quanta is generated by a vesicle fusing with the plasma membrane or by the opening of some channel or translocation of a large amount of ACh to allow release from the cytoplasm, we would expect to detect the transient occurrence of a current. Breckenridge and Almers (1987) have shown in mast cells that when the cytoplasmic vesicles were induced to fuse there was a transient large current created by the fusing vesicles. Young and Chow (1987) failed to detect any transient current that could be associated with transmitter release from *Xenopus* cultured cells and thus concluded that the release of quanta (822 m.e.p.p.s were recorded with no current being noticed in the cholinergic cell) is not associated with any transient current bigger than 2.5 pA, which was the level of detection in the system. These findings imply

that vesicle fusion is not needed for the release of quanta to occur or the fusion is such that the cytoplasm is never open to the extracellular environment during the fusing process. Furthermore, it implies that if ACh is released from the cytoplasm to the exterior it cannot be via a channel or pore and that even a translocator must be electronically neutral. To date neither hypothesis can address this point experimentally to rule out any of the different possibilities.

One recent publication has made the observation that one quantum is equivalent to one vesicle. This study by Hurlbut *et al.* (1990) noted the number of quanta released by the venom of the black widow spider, under conditions that inhibit vesicle recycling, and the disappearance of vesicles. They observed that the relationship of quanta released to the remaining vesicles in the terminal was linear and had a slope of 1/2 vesicles per quantum. Thus, they concluded that within the experimental error of the experiment it was most likely that the appearance of one quantum at the post junctional site of the neuromuscular preparation was the result of one vesicle emptying its contents. This result is clearly in favor of vesicles being the elements which contain a quantum of transmitter (see section 1.5. for a detailed introduction of the effects of black widow spider venom on neurons).

1.5.3. Vesicle fusion and mediatophore.

It is a well accepted observation that stimulation of the neuromuscular junction leads to vesicle recycling. This has mainly been observed by the use of membrane impermeant extracellular materials which either are electron dense, or can be made so, for examination under the electron microscope, or can alter the density of any structure that contains them. Stimulation has been found to increase the number of vesicles or vesicle profiles containing these materials (Hurlbut and Ceccarelli, 1974; Ceccarelli and Hurlbut, 1975; Zimmermann and Denston, 1970; Zimmermann and Whittaker, 1977); since these materials cannot get into these structures unless they have fused to the plasma membrane and opened their interiors to the extracellular environment and since the

cytoplasm of the terminals does not appear to contain this material, the interpretation has been that vesicle fusion is stimulated during periods of transmitter release. Both camps are in general agreement about this phenomenon of vesicle recycling but the interpretation of its significance is quite different. The vesicular school of thought attributes the recycling to the natural requirement of its hypothesis for vesicles to exocytose their content of ACh, while the cytoplasmic school attributes this phenomenon as being part of the mechanism by which terminals extrude Ca^{+2} that has entered during the period of stimulation (Israel *et al.*, 1980). As discussed in section 1.3. synaptic vesicle from *Torpedo* contain both ACh and Ca^{+2} and both can be transported to the interior, thus, neither interpretation is contradicted by the observations.

If vesicles fuse to release their content of ACh, and this ACh is what forms a quantum, it would be expected that vesicle fusion profiles should match the rate of quanta released at any instant. Most studies have not been able to demonstrate this. The fusion profiles are always far too few to account for the rate of quantal release and pharmacological tools are needed, such as 4-aminopyridine which increases Ca^{+2} entry into the terminal by prolonging the action potential duration, to stimulate release for reasonable numbers of profiles to be observed. Elegant studies using *Torpedo* electroplaques have revealed that vesicle fusion profiles, or pits, begin to appear after transmitter release has already begun (see review by Dunant, 1986). Instead, what appears simultaneous to transmitter release are plasma membrane particles, which the cytoplasmic school interprets to be the ACh translocator.

Work by Israel and coworkers has led to the discovery of a plasma membrane protein capable of translocating ACh. When this protein was purified and inserted into proteoliposomes that had ACh entrapped in them, it was found that ACh was released in a Ca^{+2} dependent fashion. Israel's laboratory has named this protein mediatophore and has since found that mediatophore is present in *Torpedo* electroplaques, mammalian brain, and in the neuromuscular junction (Israel *et al.*, 1983, 1988, 1990). Analysis of the pharmacological action of several compounds that

can alter release of ACh was tested on the ability of mediatophore, located in a proteoliposome, to cause ACh release. The profile of activities was the same for any tested drug on *Torpedo* synaptosomes and on the mediatophore containing proteoliposome (Israel and Morel, 1990). The effect of AH5183 was tested in this system and was found not to interfere with the release of ACh in either the synaptosomes or the proteoliposomes. This observation is in agreement with that of Michaelson *et al.* (1986) who found that in *Torpedo* synaptosomes AH5183 had no effect on the K^+ stimulated release of prepackaged ACh. Collier *et al.* (1986) also noted that initial release of ACh by the superior cervical ganglia was not affected by AH5183. The major problem that has not been investigated is whether AH5183 can eventually block the ability of mediatophore to maintain ACh release. Since AH5183 eventually interferes with stimulus evoked release, this point is critical to the possible distinction between hypotheses.

It is important to note that mediatophore seems to desensitize when small amounts of calcium are used to stimulate release from the proteoliposomes containing the protein (Israel *et al.*, 1987). Israel and coworkers have used this to explain how mediatophore may generate a quantum but they have not discussed the possible problem that prolonged stimulation, and thus Ca^{+2} entry, may have to this mechanism of release. The work with this protein seems very promising but as yet not enough is known about the protein to conclude that it is the plasma membrane mechanism assumed to exist by the cytoplasmic hypothesis. The existence of mediatophore supports the cytoplasmic hypothesis of release but its direct role in transmitter release has yet to be determined directly with better secretagogues than compounds which directly form Ca^{+2} channels.

Though neither hypothesis can explain all of the phenomena in a satisfactory way, it is generally accepted that the vesicular hypothesis better fits the present data. One possibility that is now being considered is that the stimulated release of ACh involves both vesicular ACh and a special docking molecule for vesicles to release their contents to the outside with no need to fuse (mediatophore could be such a

molecule). This view has even been mentioned by proponents of the cytoplasmic hypothesis (Israel and Morel, 1990). In this synthesis of both hypotheses most problems with rapid kinetics, formation of a normal quantum, false transmitter release, blockade of release by AH5183, and the importance of both cytoplasmic and vesicular ACh to the continued release of transmitter are surmounted. Such a revised hypothesis would place the origin of stimulated ACh release at the vesicle but the release itself would depend on a non-exocytotic event. At this date more critical tests need to be devised before such a synthesis of hypotheses can be realistically put forward.

1.6. BLACK WIDOW SPIDER VENOM AS A TOOL FOR STUDYING TRANSMITTER RELEASE AND VESICLES.

The consequences of a bite from one of the several species of *Latrodectus* spiders (black widow spiders), have been well known since ancient times. There have been 8 species of black widow spiders that have been classified, some as long ago as 1770 (Sampayo, 1944). The symptomatology resulting from the bite of any one of these spiders is quite similar and consists of a burning sensation that spreads from the site of the bite to include the whole body. The agonizing pain is accompanied by restlessness and irritability. The dominant tones of the sympathetic or parasympathetic systems become exaggerated, causing profuse sweating, bradycardia, hypertension, salivation and lacrimation in large amounts, and exaggerated reflexes. After a few hours the symptoms lessen but return to varying extents in a few days and last for 2 to 3 days (Sampayo, 1944).

The pharmacological action of black widow spider venom (BWSV) on a variety of systems has been studied since the turn of the century; for the sake of brevity I will refer to some of the pharmacological actions of the venom from *Latrodectus mactans* reported by Rafael Sampayo (1944). Animals (dogs, cats and guinea pigs) injected intravenously with the venom showed contractions of the skeletal muscles and the abdomen but if the muscles were denervated prior to invenomation, the venom had no ef-

fect on the muscles. In animals with one denervated leg and an intact one, and instrumented for recording of the electroencephalogram (EEG) and electromyogram (EMG), the injection of venom induced changes in the cortical EEG which were closely correlated with activity in the EMG of the intact leg. The venom also caused the retraction of the nictitating membrane and dilation of the pupil in cats, but if the superior cervical ganglia had been extirpated, no effect was noted on either response. These studies indicated that the action of BWSV is dependent on the existence of a functional neural system and suggested that the venom's primary site of action is the nervous system and not the target organs. Several aspects of BWSV action and its mechanism of action have been recently reviewed (Scheer *et al.*, 1984; Meldolesi *et al.*, 1986).

1.6.1. Isolation of the active component of black widow spider venom.

Early attempts to separate the components of BWSV by electrophoresis on paper and cellulose produced low yields with regards to the toxicity of the native venom. Frontali and Grasso (1964) used low temperatures and separated several active fractions from the BWSV using cellulose column electrophoresis and ion exchange chromatography. These authors were able to separate 3 active fractions by either method, two of which were active on houseflies and one which was lethal in guinea pigs. Because the fractions causing lethality in the invertebrate model were different from that fraction which was lethal to the mammalian model, the venom must contain a variety of components with different targets. The lethal fractions were more active on a mg protein basis than the native toxin.

Grasso (1976) further purified the fraction lethal to mammals and found what appeared to be a single protein band by polyacrylamide gel electrophoresis. The protein was found not to be glycosylated and was estimated to have a molecular weight of 130,000. Almost all of the native venom's toxicity to mammals could be isolated in this fraction, containing one protein, and suggested that mammals are sensitive to a

single toxin in the venom mixture. Frontali *et al.* (1976) also found a fraction which was active in vertebrates, but in this study the fraction was found to contain 4 proteins, all having the same approximate molecular weight of 130,000. The difference in these proteins was only in their iso-electric points but they were otherwise indistinguishable. The proteins were also found to be free of sugar residues and contained no lipolytic or proteolytic activity. Grasso (1976) found the active fraction could release noradrenaline from rat cortical synaptosomes and Frontali *et al.* (1976) noted that their venom component could increase the m.e.p.p. frequency at the frog neuromuscular junction and caused the depletion of synaptic vesicles along with nerve terminal swelling. Tzeng and Siekevitz (1978) were the first to propose calling this vertebrate active component α -latrotoxin (α -LTX).

1.6.2. The action of black widow spider venom on nerve terminals.

Early pharmacological studies of the action of BWSV established the nervous system of vertebrates as the target of action (Sampayo, 1944). It was felt in these early studies that the action was more restricted to the central nervous system and ganglia and not to the nerve terminals on the target organs. Later work has demonstrated that the main site of action for the venom, and thus α -LTX, is the nerve terminals, both central and peripheral.

1.6.2.1. Electrophysiological studies.

The frog neuromuscular junction and the rat hemidiaphragm have been exploited to investigate the effect of BWSV on the release of transmitter as determined electrophysiologically by measuring the number of quanta released. Longenecker *et al.* (1970) reported that exposing the frog sartorius muscle to BWSV caused a transient, massive increase in the number of m.e.p.p.s measured and by 80 minutes the rate of m.e.p.p. events dropped to less than 1 per sec. It was also noted that the BWSV had no direct effect on the muscle, thus the effect was purely

prejunctional. The ability of the venom to release transmitter was also found not to require the presence of external Ca^{+2} in the medium. Once begun, the action of the venom could not be stopped by washing the muscle with venom free medium. The same effect of BWSV on m e p p. frequency was obtained at the mouse hemidiaphragm by Gorio *et al* (1978) but exhaustion of transmitter release occurred as quickly as 20 minutes following venom addition. Paggi and Toschi (1972) also found that BWSV caused the appearance of asynchronous discharges in the superior cervical ganglia of rats *in vitro*, but only when the ganglia is presynaptically innervated. Removing Ca^{+2} had no effect on the BWSV's ability to induce the asynchronous discharges. Fesce *et al.* (1986) not only confirmed these findings but extended the observations of Ca^{+2} independence. When Ca^{+2} is replaced by Mg^{+2} , an ion which does not support nerve impulse stimulated release of transmitter, BWSV can release approximately 7×10^5 quanta from the frog neuromuscular junction, while in the presence of Ca^{+2} about 1.5×10^6 quanta are released by BWSV. Thus the authors concluded that in the presence of Ca^{+2} the quantal stores can undergo an average of two cycles of release, while Mg^{+2} only allows one cycle. This conclusion is based on the observation that BWSV causes vesicle depletion from terminals once transmitter release is exhausted (see below).

One very interesting investigation of BWSV action at the frog neuromuscular junction exploited the lack of vesicle recycling seen with Mg^{+2} and the absence of Ca^{+2} to test the notion that 1 quantum is equivalent to 1 vesicle. Hurlbut *et al.* (1990) estimated the number of quanta released by BWSV at any given time and simultaneously estimated (by electron microscopy) the number of vesicles still present in the nerve terminal. In this way they plotted the relationship of quanta released versus the number of vesicles remaining. The slope of this correlation yielded a linear relationship of 1 quantum for the disappearance of every 1.2 vesicles. Thus this study is in agreement with the original vesicular hypothesis, that equates the ACh necessary to produce 1 quantum with the ACh present in one vesicle.

1.6.2.2. Biochemical studies of transmitter release.

The action of BWSV on the nerve terminals has been most actively studied with biochemical methods, measuring the release of neurotransmitters from a variety of tissues and the PC12 cell line. Two studies have investigated the effect of BWSV on the release of ACh from the rat superior cervical ganglion. Pumplin and McClure (1977) found that BWSV released ACh from the ganglia with first order kinetics and suggest that the venom reduced release of transmitter once maximum activity was reached because the ACh content of the ganglia was not affected by the venom. When these researchers prelabeled the transmitter stores by [^3H]-Ch, they found that the specific activity (S.A.) of released transmitter was the same at the beginning of the venom induced release as at the end. Thus it appears that venom did not mobilize transmitter release by the same mechanism as nerve impulses (Elmqvist and Quastel, 1965; Large and Rang, 1978; Collier, 1969), which in the study of Pumplin and McClure (1977) showed a decreased S.A. of released ACh late in the stimulation as compared to the beginning of stimulation. This effect of venom on [^3H]-ACh release was also noted earlier, indirectly, by Paggi and Toschi (1972) who noted that the [^3H]-ACh content of rat SCG decreased by 70% with BWSV treatment. It would be expected that with continuous stimulation, such as nerve impulses, all of the labeled stores should be released (Large and Rang, 1978). The two studies suggest that BWSV mobilize the ACh from one compartment, being similarly labeled and constituting no more than 70% of the ganglial ACh content. Both studies on the ganglion noted that lack of Ca^{+2} did not block venom induced release but Pumplin and McClure (1977) did find that the venom induced release was decreased by 50% under these Ca^{+2} free conditions. This could be related to the above noted effect, that in the presence of Ca^{+2} the quantal stores may turn over twice.

Brain slices and synaptosomes derived from them have also been used to study BWSV action. In cortical slices the addition of BWSV causes the release of ACh into the medium and a concomitant decrease in tissue ACh content (Frontali *et al.*, 1972). Baba *et al.* (1977) noted

that cortical synaptosomes released prepackaged [^3H]-ACh when exposed to BWSV and that the release had a very short lag time of about 20 sec. They also observed that BWSV could inhibit the high-affinity Ch uptake system, perhaps as a consequence of cell depolarization. In a later publication Baba and Cooper (1980) extended these observations on synaptosomes to include [^3H]-noradrenaline. The venom released high amounts of [^3H]-noradrenaline by depolarizing the synaptosomes. The depolarization of the synaptosomes was measured using a voltage sensitive dye and BWSV depolarized synaptosomes whether Ca^{+2} or K^{+} were present or absent in the medium. The inhibitory effect on the uptake of [^3H]-Ch, [^3H]-noradrenaline, and [^3H]-tyrosine, by BWSV, were thought to be due to the massive venom-induced depolarization and not to a direct action on the transport systems. The release of [^3H]-ACh by venom could be blocked if concanavalin A was used to pretreat the synaptosomes before BWSV exposure. Pre-incubation of the venom with gangliosides also prevented the release of [^3H]-ACh. These findings thus suggested that the toxin binds a sugar moiety and that this is needed for it to interact with the terminal. The effects of BWSV on the release of all studied neurotransmitters can be achieved by α -LTX and toxin action is also independent of extracellular Ca^{+2} (Tzeng *et al.*, 1978).

The PC12 cell line is a clonal line derived from a mouse pheochromocytoma. These cells are not neurons but they have been shown to respond to α -LTX by releasing dopamine and ACh (Grasso *et al.*, 1980). Meldolesi *et al.* (1983) reported that α -LTX bound to these cells with high-affinity and in a saturable fashion. The binding was enhanced by the presence of Ca^{+2} and certain other divalent cations while Mg^{+2} had no effect. When PC12 cells were exposed to α -LTX in the presence of Ca^{+2} , a rapid release of [^3H]-dopamine occurred, while exposure to α -LTX in the presence of Mg^{+2} , and no Ca^{+2} , resulted in a slower release of transmitter. Interestingly, if toxin binding occurred under Ca^{+2} -free conditions, while in the presence of Mg^{+2} , adding Ca^{+2} caused transmitter release to shift from slow to fast kinetics. Thus the binding conditions were not responsible for transmitter release kinetics but rather, the kinetics were dependent on the ionic composition of the

medium once toxin was bound. Eliminating all divalent cations from the medium blocked α -LTX's ability to release transmitter. Meldolesi *et al.* (1983) confirmed that concanavalin A could block toxin action and also found trypsin treatment to be effective at blocking the response while calmodulin inhibitors and Na^+ and Ca^{+2} channel blockers were without effect. These results suggest that a receptive substance for α -LTX exists on target sites and that this substance is most likely a glycosylated protein. Furthermore, given that calmodulin inhibitors have no effect, it is likely that release of transmitter is not via a "normal" pathway. This latter idea was further supported by Meldolesi *et al.* (1984) who found that transmitter release caused by α -LTX did not require internal Ca^{+2} concentrations to rise nor was there a need for synapsin I phosphorylation to obtain the effect on transmitter release.

Investigation of the effect of BWSV on transmitter stores in mouse diaphragm found that 65% of the ACh content could be released by the venom (Gorio *et al.*, 1978). Following venom exposure and depletion of the depletable transmitter stores basal ACh release was only 10-40% lower than controls and K^+ depolarization was unable to increase this release. Though no direct measurements were made of the subcellular compartments' ACh content, the authors estimate that venom depletes the vesicular compartment and only slightly affects the extravesicular compartment, which in the absence of venom treatment, can be mobilized to release its transmitter content by K^+ in the presence of blocking concentrations of HC-3.

1.6.2.3. Morphological studies.

Most functional studies have also investigated the effect of BWSV or α -LTX on the intracellular structures of nerve terminals, synaptosomes, and PC12 cells. The most consistent finding, though not ubiquitous, is that the venom and toxin cause the depletion of synaptic vesicles from these systems (Clark *et al.*, 1970; Longenecker *et al.*, 1970; Clark *et al.*, 1972; Gorio *et al.*, 1978; Tzeng *et al.*, 1978; Caccarelli *et al.*, 1979; Watanabe *et al.*, 1983; for exception to this find-

ing see Baba and Cooper, 1980). It appears that the venom interferes with the vesicle recycling system while also inducing vesicles to fuse. Thus with time the profiles of synaptic vesicles decrease upon electron microscopic examination. Between the time of toxin or venom treatment and the point of transmitter release exhaustion, many profiles of fusing vesicles are seen in cross section (Clark *et al.*, 1972; Ceccarelli *et al.*, 1979; Watanabe *et al.*, 1983) and at least in one study, there is clear evidence of mitochondrial swelling as a result of venom treatment (Gorio *et al.*, 1978). At the frog neuromuscular junction almost all vesicle fusion profiles seen with freeze fracture were associated with active zones, areas thought to normally be involved in transmitter release (Ceccarelli *et al.*, 1979). The fusion of vesicles to the plasma membrane should effect the surface area of the plasma membrane. Several changes in the plasma membrane have been observed and at the end of transmitter release there are many infoldings of the outer membrane and a separation of the axonal membrane from the Schwann cell (Clark *et al.*, 1972) and swelling of the motor end plate region (Clark *et al.*, 1970; Gorio *et al.*, 1978).

1.6.3. The α -LTX binding site and its location

Because all of the venom's effects at vertebrate nerve endings can be attributed to one factor, α -LTX, studying this protein's binding should provide important information about the venom's site of action (Frontali *et al.*, 1976; Grasso, 1976). The toxin can be iodinated with little loss of activity (Tzeng and Siekevitz, 1979). [^{125} I]- α -LTX was found to interact with cortical synaptosomal membranes but not with liver membranes. Pre-treatment of the membranes with trypsin or with 2% β -mercaptoethanol completely blocked binding, strongly indicating a protein as the site of binding. The binding of [^{125} I]- α -LTX to membranes showed no cooperativity (Hill slope not different from 1) and gave an apparent K_d of 1 nM. The labeled toxin dissociated with two rates, one having a half-life of 8.2 min and a second with a half-life of 3.7 hr (Tzeng and Siekevitz, 1979). Meldolesi (1982) also used

[^{125}I]- α -LTX and found a K_d of 0.1 nM. He reported a very fast toxin association rate ($1.2 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ versus $8.2 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ for Tzeng and Siekevitz, 1979) and only one slow dissociation rate ($t_{1/2} = 6.4 \text{ hr}$). In both studies, specific binding was saturable.

Valtorta *et al.* (1984) report very similar binding data for [^{125}I]- α -LTX. They used a mono-specific, polyclonal, anti- α -LTX antibody, tagged with a fluorescent probe, to localize its binding site at the frog neuromuscular junction. Any treatment that inhibits venom binding, such as trypsin, yielded no fluorescence, as did muscles that had been denervated. However, in a normal nerve-muscle preparation the endplate region was found to fluoresce but not portions of unmyelinated axons near the endplate. Thus it appears that the binding site for α -LTX is restricted to the nerve endings themselves and not other adjacent parts of the neuron.

1.6.4. Possible mechanisms of α -LTX action.

Due to concanavalin A's ability to block venom action and the fact that colchicine pre-treatment (prior to concanavalin A exposure) could reverse the inhibition on toxin action, Rubin *et al.* (1978) suggested that the venom induced transmitter release was the result of a redistribution of surface proteins. Baba and Cooper's (1980) observation that venom premixing with gangliosides prevented the venom from acting suggested that the inhibition by concanavalin A was not due to capping of cell surface proteins but rather to a direct inhibition of venom binding to its target. This possibility was directly demonstrated by Tzeng and Siekevitz (1979) when concanavalin A was found to inhibit [^{125}I]- α -LTX binding to neuronal membranes. Thus it is unlikely that venom action simply requires surface protein redistribution and association.

Grasso *et al.* (1980) reported that venom action on PC12 cells was associated with the entry of Ca^{+2} via a mechanism separate from voltage activated Ca^{+2} channels sensitive to verapamil. Though these authors found that Mg^{+2} could not substitute for Ca^{+2} in the venom induced

transmitter release, others have reported that Mg^{+2} supports venom stimulated transmitter release (see section 1.4.2). One report has noted that there is a requirement for some divalent cation for venom to have an effect but not necessarily Ca^{+2} (Meldolesi *et al* , 1983). However, the toxin induced entry of Ca^{+2} and depolarization of brain synaptosomes have also been reported by Nicholls *et al* (1982), who found that the plasma membrane was depolarized by α -LTX in the presence or absence of Ca^{+2} . Furthermore, they noted that the mitochondrial membrane was slightly depolarized as well, probably due to the large Ca^{+2} influx. The channel blockers, tetrodotoxin and verapamil, had little or no effect on depolarization and Ca^{+2} entry. Thus it would appear that venom can act to open a channel or to form one. Finkelstein *et al.* (1976) found that α -LTX could increase the permeability of an artificial membrane, consistent with the view that the toxin can form its own channel. The venom seems to have formed a non-specific cation channel (mono and divalent cations could cross) having a large conductance. Watanabe *et al.* (1986) also reported similar findings in PC12 cells, but the cation channel formed by the toxin was found to be different from the known cation channels of PC12 cells as well as from that channel described by Finkelstein *et al.* (1976). Scheer *et al.* (1985) review the findings of toxin channel formation and the ionic currents.

At this time, channel formation appears to be the most promising line of inquiry as to the mechanism of action of α -LTX and BWSV on vertebrate systems. The channel cannot explain all that is known because such cations as Mg^{+2} which are known not to support transmitter release by other secretagogues can function for the toxin. Thus it would seem that toxin, in addition to forming or opening a channel, must make the terminal release mechanism sensitive to ions that normally do not activate it. There is one report that found that α -LTX induced the turnover of phosphoinositides in PC12 cells. Obviously the toxin induced changes will probably be many, but which is the critical event remains unknown. Other toxins, such as β -bungarotoxin, which can inhibit α -LTX action (Tzeng and Tian, 1984), could be useful in the elucidation of a mechanism of action for BWSV.

STATEMENT OF THE PROBLEM

As mentioned in the introduction, AH5183 can block the stimulated release of ACh from the superior cervical ganglion in a rate dependent fashion. Furthermore, the amount of ACh that is released by nerve impulses, in the presence of the drug, is not dependent on the rate of stimulation but represents about 14% of the initial ganglial ACh content. These results were interpreted to indicate that the ACh present in vesicles could be released by nerve impulses but, while AH5183 is present, once the vesicles empty of their store of transmitter they are prevented from refilling with new ACh. Because the amount of ACh released in the presence of AH5183 appears to constitute that portion of transmitter stores referred to as the readily releasable pool, the initial studies attempted to establish whether this constituted the entire vesicular ACh content of the ganglion. The venom of the black widow spider was chosen as a secretagogue because of its ability to induce the exocytosis of vesicular contents. It was the intention of these studies to better understand what role vesicular ACh plays in the release of transmitter by nerve impulses.

During the course of these initial experiments it was discovered that the venom could release prepackaged and newly synthesized ACh from AH5183-blocked ganglia. Thus, studies were undertaken to attempt to understand what vesicular compartments might be involved in the initial release of transmitter and what compartments were not directly involved in the release process but functioned more like the storage pool of ACh described by Birks and MacIntosh (1961). The first objective of these studies was to try and identify which of the intracellular pools of ACh that might not be affected by AH5183 exposure during stimulation were mobilized to release by the venom. Because of the release of newly synthesized ACh by black widow spider venom from the ganglia, our second objective was to investigate if one of the occluded compartments of ACh could incorporate newly synthesized ACh in the presence of the drug, suggesting a difference in the sensitivity of the ACh uptake system by different vesicular fractions. For these experiments the hippocampal formation of the rat brain was used due to the ease with which this

tissue lends itself to subcellular fractionation, unlike the ganglia used in the initial studies.

The final objective of the studies described in this thesis was to investigate how the different subcellular ACh compartments recovered from exposure to AH5183. It was of further interest that such a study could elucidate the possible role of the different ACh compartments to the release of transmitter. These studies looked at the recovery of ACh release from the effects of AH5183, and the associated changes taking place in the subcellular compartments of ACh in rat hippocampal slices.

MATERIALS AND METHODS

2.1 IN SITU PERFUSION OF THE CAT SUPERIOR CERVICAL GANGLIA.

Cats of either sex, weighing between 1.8 and 3.5 kilograms, were used in experiments where perfusion of the superior cervical ganglion (SCG) was carried out. The animals were obtained from the McIntyre Medical Animal Resource Center on the day of the experiment. Cats were not conditioned to handling or to the anaesthesia box prior to their use.

2.1.1 Induction and maintenance of anaesthesia.

Cats were placed in a clear perspex box with inner dimensions of 25 cm x 26 cm x 47 cm (width x height x length). The box contained two valves, opposite each other, through which gas could be passed from one side of the box to the other. In order to sedate them, cats were first exposed to a gaseous mixture containing 2 parts nitrous oxide to 1 part oxygen at a flow rate of 300 cc/min for about 15 min. Anaesthesia was then first induced using this same gaseous mixture plus 2% halothane for at least another 15 min. Anaesthesia was then maintained by the administration of 80 mg/kg of α -chloralose (Sigma Chemical Co., St. Louis, MO, USA), dissolved in warm physiological saline (0.8 mg/ml of α -chloralose), through a cannulated femoral vein. Occasionally further anaesthesia was needed; on these occasions sodium pentobarbital was administered through the cannulated femoral vein. The animal's core temperature was checked using a rectal thermometer and their core temperature maintained by use of an electrically heated blanket. When necessary, animals were ventilated.

2.1.2 Preparation of the superior cervical ganglia for perfusion.

The preparation of the SCG for *in situ* perfusion was done according to the method of Kibjakow (1933) as described by Collier and Kwok (1982). A ventral incision was made from the jaw to the upper projection of the sternum and the common carotid artery exposed. The trachea

was then intubated near the base of the incision for the purpose of ventilating the cat if necessary. Vessels entering and leaving the pharyngeal lymph gland were ligated and the gland removed so as to have an unobstructed view of the internal carotid artery. The SCG, including the nodose ganglia, and its vasculature were then carefully isolated from surrounding tissue. The common carotid artery was prepared for cannulation so that the SCG could be perfused through its own vascular bed. The internal carotid artery past the SCG was ligated and the prevertebral vein prepared for the collection of the perfusate. In instances where the internal jugular vein was large, this was prepared for the collection of the perfusate instead of the prevertebral vein. Thus, the SCG of the cat was prepared in such a way that the only circulation leading to the ganglion was via the common carotid artery and the only circulation leaving the ganglion was via the prevertebral vein or the internal jugular.

A length of about 4 cm of the preganglionic nerve was separated from the vagus and surrounding tissue so that the ganglia could be stimulated. Stimulation was delivered via a platinum electrode connected to a Grass stimulator capable of delivering square pulses of depolarizing current. Frequency of stimulation varied depending on the experiment (see results for details of frequency) but the pulse was always set to a 0.5 msec duration and an 8 V potential. In experiments where 20 Hz were used, the electrode was moved along the nerve towards the ganglia every 15 min. To check that stimulation was being effective in releasing ACh, the nictitating membrane was attached to a force transducer and the force of retraction recorded.

Media used for the perfusion were placed in 125 ml buchner funnels which had thin polyethylene tubing attached at the bottom so as to deliver the solution to the ganglia. The tubing of the various flasks were joined at a glass cannula which was then introduced into the carotid artery near the ganglia (about 2-3 cm away). A pressure of 50 to 110 mm of mercury was used as the driving force for the perfusion medium. The pressure was established using a gaseous mixture of 95% O₂ and 5% CO₂ and a large gas reservoir was used to minimize pressure

changes. In this way the medium could be pushed through the tubing and vasculature and at the same time the media remained oxygenated. The standard perfusion medium was a Krebs medium consisting of (mM): NaCl 120, KCl 4.6, CaCl_2 2.5, KH_2PO_4 1.2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, dextrose 9.9, and NaHCO_3 25. This medium was saturated with 95% O_2 and 5% CO_2 so as to give a final pH of 7.4 at 37 °C. When the release of ACh was measured, the medium also contained 15 μM eserine to inhibit acetylcholinesterase. Usually Ch was also present in the media at a concentration of 10 μM , but where changes were made they are outlined in the results of the specific experiments. Certain experiments required the use of drugs or toxins and their concentrations are given where appropriate. In order to warm up the perfusion medium prior to perfusing the ganglia, the polyethylene tubes were passed through the esophagus of the cat. Perfusion rates varied from 0.2-0.4 ml/min.

2.2. DETERMINATIONS INVOLVING BLACK WIDOW SPIDER VENOM.

The venom from the black widow spider, *Latrodectus hesperus*, was obtained from Sigma Chemical Co. in brown glass vials containing 100 venom sacs which had been homogenized, filtered, and lyophilized. The contents of the vial was resuspended in 2 ml of a solution containing 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) as a buffer at pH 7.4 and the venom solution aliquoted into 400 μl plastic vials so that 5 sac equivalents were present in each vial. The vials were then frozen and kept in a -80 °C freezer. As needed, one vial was removed from the freezer just prior to its use to minimize degradation of the toxin.

2.2.1. Determination of the quantity of α -LTX in the black widow spider venom.

Latrodectus hesperus is a new world black widow spider whose venom is known to be less potent on vertebrate synapses and neuromuscular junctions than that of the European black widow spider (McCrone, 1964).

It now appears that the differences in venom potency are due to varying amounts of the same toxin, α -LTX, present in the venom mixture (McCrone and Netzloff, 1965). Because the experiments done on the cat SCG were done with the venom and not purified α -LTX it was important to attempt to quantify the concentration of this active venom component. By separating the component proteins of the venom by polyacrylamide gel electrophoresis (PAGE) and then running a densitometric scan of the stained gel, it should be possible to estimate the percentage of α -LTX (molecular weight of approximately 130,000 daltons) in our venom. All PAGE methods were done according to Smith (1984).

One vial of the black widow spider venom (BWSV) was prepared for separation on PAGE by adding one volume (100 μ l) of a solution containing 0.16 M sodium dodecyl sulfate (SDS), 2.2 M glycerol, 0.12 M 2-amino-2-(hydroxymethyl)-propane-1,3-diol (Tris base), 10% v/v β -mercaptoethanol, and 0.01% w/v bromophenol blue. The resulting mixture was then placed in boiling water for 2 min. The sample was then stored in a -20 °C freezer. The original BWSV solution (prior to preparation for PAGE) was assayed for protein and the concentration of the protein found to be $12.1 \pm 0.9 \mu\text{g}/\mu\text{l}$. An electrophoresis molecular weight standards solution was obtained from Bio-Rad Laboratories (Richmond, CA; USA) and consisted of 40 $\mu\text{g}/\mu\text{l}$ of the following proteins (molecular weight in brackets is in daltons); myosin (200,000), β -galactosidase (116,250), phosphorylase B (97,400), bovine serum albumin (66,200), ovalbumin (45,000).

In order to separate the proteins of BWSV a 10 cm X 14 cm (height x width) slab gel made of 7% acrylamide and 2.5% bis acrylamide, as the cross-linker, was prepared. A 1 cm x 14 cm stacking gel, made from 3.6% acrylamide and 1.3% bis acrylamide, was polymerized with wells over the separating gel. Twenty-five μ l of the molecular weight standards were placed on lanes 5 and 13. Samples of the BWSV solution were placed in lanes 7, 9, and 11 with 10, 20, and 30 μ l of the solution used, respectively. The samples were electrophoretically moved through the stacking gel with a current of 14 mA and then through the separating gel with a current of 10 mA. The buffer used in the reservoir for the

electrophoretic procedure contained 0.19 M glycine, 0.025 M Tris base, and 0.1% w/v SDS at a pH of 8.3.

The gel was removed and stained with a solution containing 0.1% w/v coomassie brilliant blue R250, 50% v/v methanol, and 10% v/v glacial acetic acid. Staining of the gel was accomplished by immersing it in the staining solution for about 2 hr with gentle agitation. The gel was then destained to remove uncoupled dye with a solution containing 10% v/v methanol and glacial acetic acid. This was accomplished by immersing the stained gel in the destaining solution for approximately 24 hr with gentle agitation and several changes of the solution. The band pattern obtained from the molecular weight standards and BWSV solutions are shown in figure 1M panel A.

In order to run the densitometric scan, the gel was immersed in distilled water for 24 hr with gentle agitation and several changes in the water so as to remove the acetic acid which can damage the optical instrument. The densitometric scan of the lane containing 10 μ l of BWSV is shown in figure 1M panel B. This and other scans (10 scans from 3 gels) indicated that α -LTX comprised approximately $18.8 \pm 5.2\%$ of the total protein in the BWSV solution.

These determinations yield an approximate concentration of α -LTX of 43 nM in the medium used to perfuse the superior cervical ganglia.

2.2.2. Determination of the amount of anti- α -LTX antiserum needed to neutralize the black widow spider venom.

To understand whether the venom of the black widow spider causes release of ACh due to its content of α -LTX it was necessary to investigate whether a polyclonal antiserum raised against purified α -LTX could interfere with the venom's secretagogue action. The antiserum was the kind gift of Dr. Heimo Scheer and was that characterized by Valtora *et al.* (1984).

Two male Sprague-Dawley rats weighing between 150 and 175 grams were obtained through the McIntyre Medical Animal Resource Center from

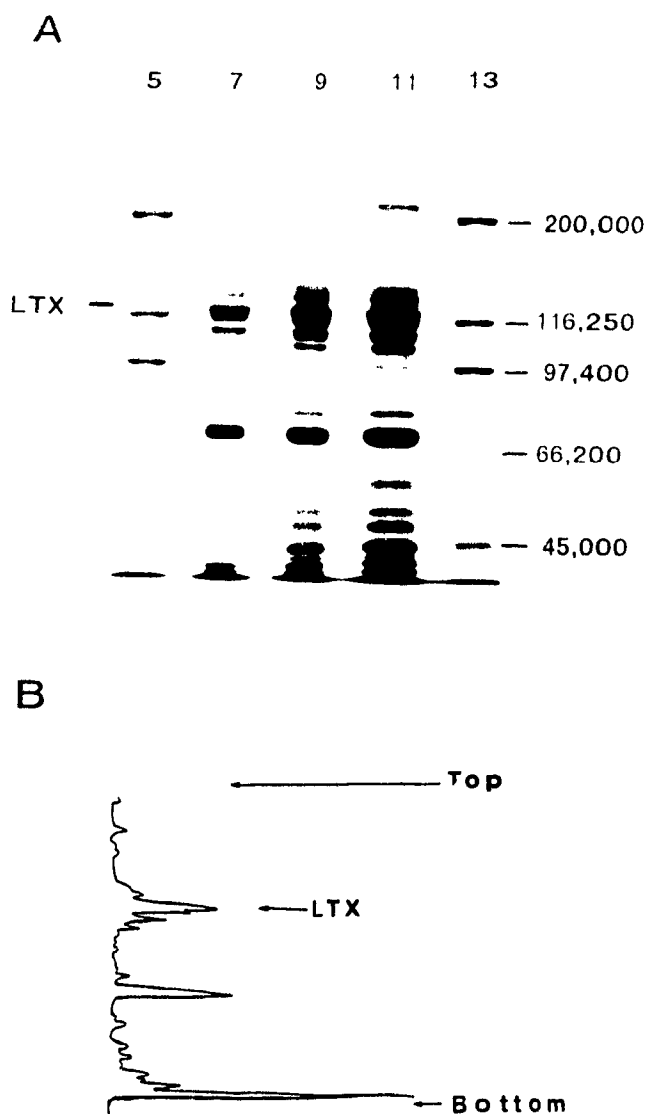


Figure 1M. Gel electrophoresis and densitometric scan of BWSV. The venom from 5 sac equivalents was used to determine protein concentration and prepared for PAGE. Samples were run on a 7% gel at 10 mA. 25 μ l of molecular weight standards (myosin (200,000), β -galactosidase (116,250), phosphorylase B (97,400), bovine serum albumin (66,200), ovalbumin (45,000) (molecular weight in daltons)) were run on lanes 5 and 13. The venom samples were run on lanes 7, 9, and 11 with 10 μ l, 20 μ l, and 30 μ l, respectively. Panel A shows the stained gel with the location of α -LTX (130,000 daltons). Panel B is a tracing of a densitometric scan of lane 7 and the α -LTX peak is marked.

Charles River Canada Inc. (St. Constant, Quebec). Rats were stunned by a sharp blow to the neck, decapitated by guillotine, and the brain removed and placed in ice-cold Krebs medium (see section 2.1). The striata were then quickly dissected, weighed, and placed in a 15 ml Douce homogenizer (Wheaton, Thomas Scientific) previously cooled on ice. Nine volumes of a 0.32 M sucrose solution containing 5 mM HEPES at pH=7.4 was then added to the homogenizer and the tissue homogenized with 7 up and down strokes using the A pestle which gives a clearance of 0.14 mm. The resulting homogenate was transferred to two ice-cold 1.5 ml eppendorf centrifuge tubes and spun for 10 min at 1000 x g in a refrigerated Sorvall RC-5B centrifuge (4 °C) using an SS-34 rotor with adapters. The supernatants were removed and transferred to another set of ice-cold eppendorf tubes and spun for 15 min at 17000 x g. The supernatants were discarded and the P₂ pellets (crude synaptosomal pellet) washed once with 0.5 ml of ice-cold sucrose solution by gently resuspending the pellets using a pasteur pipette and re-centrifuged as in the previous step. The supernatants were discarded and the pellets resuspended in 0.5 ml of Krebs medium supplemented with 5 mM HEPES, 6×10^{-4} M N-methyl-N-2-propynylbenzylamine (Pargyline) (Sigma Chemical), 0.1 mg/ml of ascorbic acid, and 1 µCi/ml of ³H-dopamine (³H-DA) (New England Nuclear-17.4 Ci/mmmole) (K-H medium) using very gentle strokes of the homogenizer. The P₂ suspensions were then allowed to stand for 30 min at 30 °C. The suspensions were then spun as before, the supernatants discarded, and the pellets washed with ³H-DA free K-H medium. The pellets were resuspended in 2 ml of Krebs medium containing 5 mM HEPES and 10^{-6} M desmethyl-imipramine (Sigma) (K-D medium) using a Dounce homogenizer.

A serial dilution of the venom, ranging from 0.1 to 0.001 sac equivalents per 10 µl, was then prepared using the K-D medium. Two hundred µl aliquotes of one of the P₂ suspensions and 10 µl of the serial dilutions were placed in 1.5 ml eppendorf tubes and allowed to incubate for 15 min at 30 °C. The suspensions were then spun in a fixed speed eppendorf centrifuge (Brinkman model 5412), placed in a refrigerator set at 4 °C, for 5 min. The pellets were washed gently with ice-cold K-D medium, lysed in H₂O, and prepared for liquid scintil-

lation spectrophotometry by transferring the lysate to a counting vial and adding 5 ml of Aquasol (New England Nuclear). Samples were analyzed using an LKB-Wallac liquid scintillation counter (Model 1211) with a standard ^3H window setting giving approximately 54% counting efficiency. The solid curve (*) in figure 2M illustrates the dose-response curve of this experiment.

As the dose of venom was increased there was a concomitant decrease in the ^3H -DA content of the synaptosomes. The decrease in ^3H -DA content appeared to be fairly linear from a dose of 0.01 sac equivalents to 0.1 sac equivalents. A dose of 0.04 sac equivalents, which caused a 75% decrease in the ^3H -DA content, was used to titrate the anti- α -LTX antiserum.

In order to titrate the amount of antiserum needed to neutralize the venom, a serial dilution of the antiserum, ranging from 2 μl to 0.002 μl per 10 μl , was prepared using K-D medium. A dose of 0.04 sac equivalents per 10 μl was then added to each antiserum concentration and the mixture allowed to stand on ice for 1.5 hr. Two hundred μl aliquotes of the second P_2 suspension were then added to eppendorf tubes along with 20 μl of the venom-antiserum mixtures. The procedures outlined above for the venom dose-response were then repeated. The dashed curve (o) in figure 2M shows the venom neutralization by varying amounts of antiserum.

These experiments indicate that 0.15 μl of antiserum is needed to neutralize 0.04 sac equivalents of venom, with respect to ^3H -DA. Thus, 10 μl of antiserum were necessary to neutralize the 2.5 sac equivalents of venom needed to do the experiments described in section 3.1.2.

2.3. PREPARATION OF RAT BRAIN HIPPOCAMPAL SLICES.

All rats used in experiments described in this thesis were male, Sprague-Dawley rats weighing between 150 and 225 grams. These animals were obtained through the McIntyre Medical Animal Resource Center from Charles River Canada Inc. and were always used 1 to 14 days after their arrival to the center.

Antiserum Neutralization of Venom

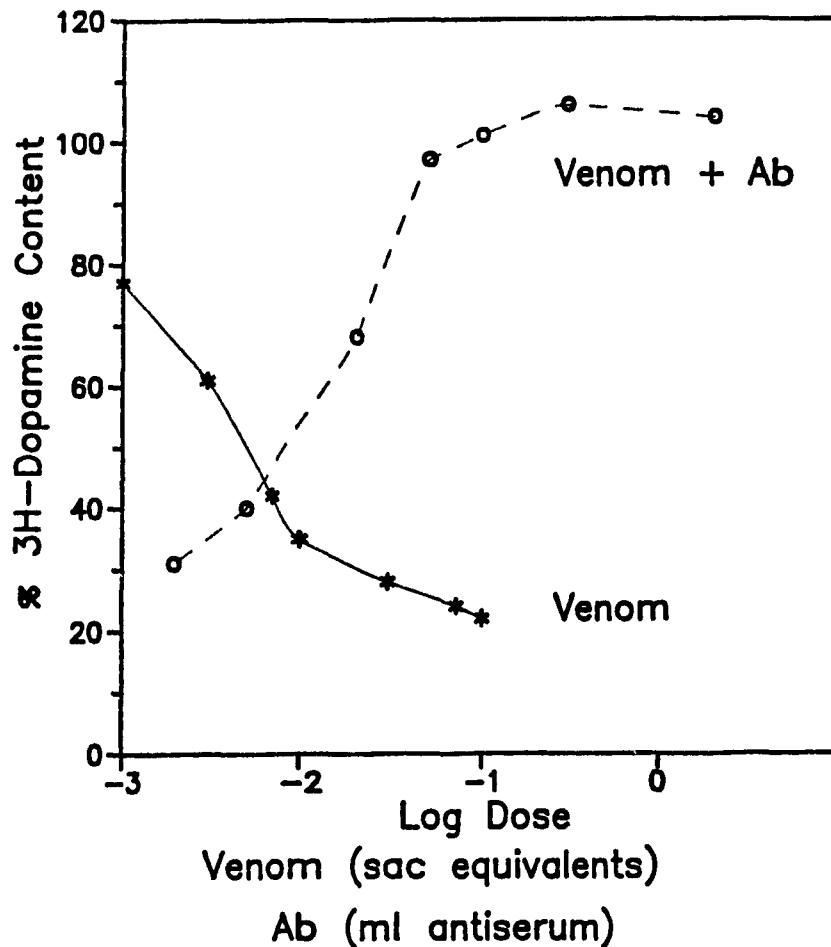


Figure 2M. Quantification of antiserum needed to neutralize BWSV activity in striatal synaptosomes. Synaptosomes from rat striata were prepared and prelabeled with ^3H -dopamine as described in the materials and methods. Synaptosomes were exposed to various doses of BWSV for 15 min and subsequently they were pelleted. The pellet was lysed and the amount of ^3H -dopamine remaining in the pellet measured (*). The equivalent of 0.04 venom sacs was then incubated with various dilutions of antiserum, raised against α -LTX, and synaptosomes were then incubated with this mixture for 15 min. The pelleted synaptosomes were then measured for the retained ^3H -dopamine (o). Each point represents the mean of duplicates. The figure represents one of two experiments.

Rats were stunned by a sharp blow to the neck and quickly decapitated by use of a guillotine. The brains were then quickly removed from the cranium and placed into 60 ml of an ice-cold Krebs medium consisting of (mM): NaCl 120, KCl 4.6, CaCl_2 2.5, KH_2PO_4 1.2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, dextrose 9.9, and NaHCO_3 25. This medium was saturated with 95% O_2 and 5% CO_2 . The medium was supplemented with 10 μM Ch and, in certain instances (see results section), 15 μM eserine. The brains were placed in a petri dish containing a piece of absorbent paper (Whatman chromatographic paper) wetted with ice-cold Krebs. A cold scalpel blade (#11) was used to separate the two cerebral hemispheres so that the hippocampal formations could be dissected out. The removed hippocampi were transferred to 1.5 ml eppendorf tubes which were pre-filled with ice-cold Krebs medium. All steps outlined above were carried out over crushed ice.

Dissected hippocampi were transferred on to a dry piece of absorbent paper and were cleaned and trimmed there. The trimmed hippocampi were then placed on a piece of weighing paper and weighed with a Mettler analytical balance capable of weighing 0.0001 grams (Mettler AE-160). Individual hippocampal formations usually weighed between 35 and 45 mg (wet tissue weight). The weighed hippocampi were then cut into 0.3 mm slices using a McIlwain tissue chopper and transferred to 1.5 ml eppendorf tubes containing 1 ml of oxygenated Krebs medium. The slices were dispersed by gentle swirling of the tubes, placed into a water bath set to 37 °C, and continuously shaken.

In all experiments that utilized slices, the slices were allowed to incubate for 45 min before any treatment was performed. To change the incubation medium, slices were spun down on a fixed speed eppendorf centrifuge (Model 3200) for 3 seconds and the medium removed with a pasteur pipette. New medium was added using a 1 ml Gilson adjustable pipette (Pipetman p1000) in such a way that the slices were gently resuspended. Following the initial incubation period all changes in medium were done with medium that was continuously bubbled with a mixture of 95:5% O_2 : CO_2 and pre-equilibrated to 37 °C.

When slices were K^+ stimulated the Krebs medium contained (mM): NaCl 90, KCl 34.6, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, dextrose 9.9, and $NaHCO_3$ 25. The medium was supplemented with 10 μM choline and, where released ACh was measured, it also contained 15 μM eserine. The medium was continuously bubbled with 95:5% $O_2:CO_2$ so as to give a pH of 7.4 at 37 °C.

2.4. PREPARATION OF THE P_2 PELLET AND SUBCELLULAR FRACTIONS FROM HIPPOCAMPAL SLICES.

Much of the work presented in this dissertation deals with the isolation of the crude synaptosomal pellet (P_2) and a variety of subcellular fractions that can be separated from it. In all experiments, described in the results, where these fractions were used the P_2 pellet was isolated from the hippocampal (HIPP) slices used in the experiment. The methods used for the isolation of all fractions arising from slices were basically those described by Ricny and Collier (1986).

2.4 1. Preparation of the P_2 pellet from hippocampal slices.

At the end of the appropriate incubation period HIPP slices were resuspended in their eppendorf tubes with 1 ml of an ice-cold saline solution containing 154 mM NaCl, 5 mM HEPES, and 1 mM [ethylenedis(oxyethylenenitrilo)]tetraacetic acid (EGTA). In cases where both the P_3 and S_3 fractions or where total tissue were to be examined for their ACh content this saline solution also contained 15 μM eserine so as to preserve the cytoplasmic (S_3) fraction's ACh. The tubes containing the slices were placed on ice for between 2 and 5 min. When all slices used in an experiment were thus resuspended they were rapidly centrifuged in a bench top micro-centrifuge for 3 sec and the saline solution was removed.

The slices were resuspended with 1 ml of an ice-cold 0.32 M sucrose solution containing 5 mM HEPES and 1 mM EGTA; in cases where

the total or cytoplasmic ACh content was to be measured this solution also contained 15 μ M eserine. The slices from each eppendorf tube were then transferred to separate douce type smooth glass homogenizers (Konte), that had been kept on ice 1 hr prior to the end of the experiment, using a pasteur pipette. One milliliter of the same solution was then added to the eppendorf tube to recover any remaining slices and these were then transferred to the appropriate homogenizer. The slices were homogenized with the A pestle, which gives a clearance of 0.0035 inches, using 7 up and down hand-strokes of the pestle. The contents of the homogenizer were transferred to pre-chilled pyrex 15 ml centrifuge tubes by use of a pasteur pipette. The homogenizer was then washed with an additional 500 or 1000 μ l of the sucrose solution by passing the pestle up and down once through the length of the tube. The contents were transferred as before.

The pre-chilled tubes were spun in a Sorvall high speed centrifuge, with the temperature set to between 4 and 8 degrees, at 1,000 x g for 10 min using an SS-34 rotor. The resulting supernatant (S_1) was transferred to a second set of pre-chilled, thick-walled pyrex centrifuge tubes by use of a pasteur pipette and the first pellet (P_1) discarded. The S_1 was then subjected to a second centrifugation of 15 min at 10,000 x g. The resulting supernatant (S_2) was discarded by aspiration and the pellet (P_2) saved. This pellet, P_2 , was the crude synaptosomal pellet referred to as synaptosomes in the rest of the thesis. Figure 3M outlines the procedures described above for the isolation of the P_2 pellet.

In order to determine the pattern of recovery for ACh using these procedures a set of HIPPP slices were processed using the procedures described above and the ACh content of each fraction was extracted and assayed as described in sections 2.7 and 2.8 of the materials and methods.

As shown in table 1M, the first separation step resulted in about 66% of the total ACh remaining in the S_1 fraction. Since most of the cholinergic input to the hippocampal formation is in the form of nerve terminals it is not too surprising that most of the ACh should not have

P₂ Pellet Preparation

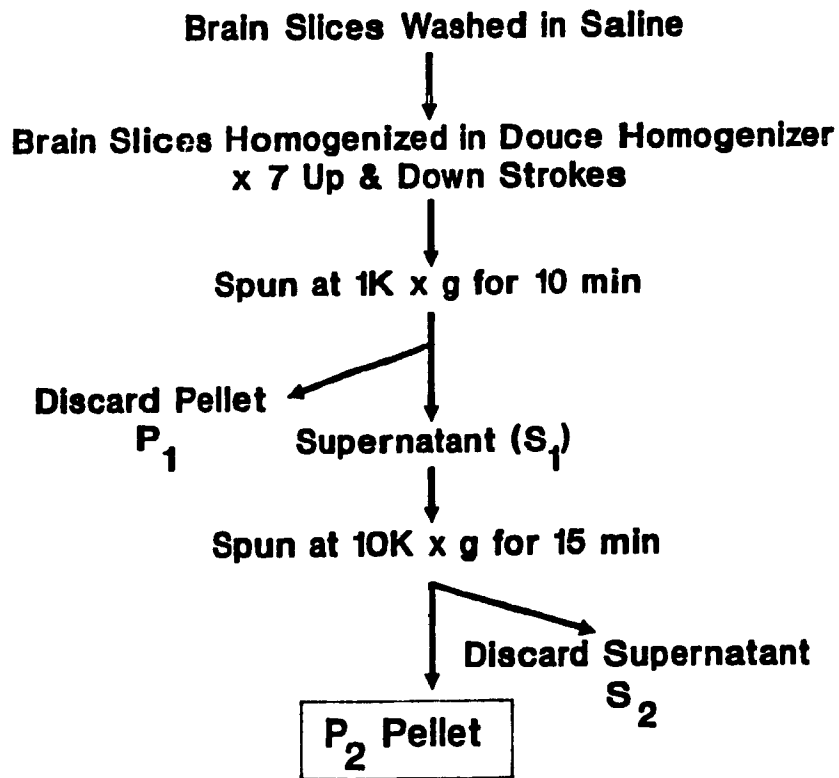


Figure 3M. Scheme describing the isolation of the P₂ pellet from hippocampal slices. For details see description of method in the materials and methods.

Table 1M. ACh Recovery in Synaptosomal Pellet Isolation.

	Total	P1	S1	P2	S2
ACh content (pmoles/g wet weight)	14187 +/- 610	4269 +/- 124	9319 +/- 509	3563 +/- 326	3841 +/- 150
Percent recovery from total	100	30.1 +/- 0.1	65.7 +/- 3.6	25.1 +/- 2.3	27.1 +/- 1.1
Percent recovery of each separation	100	31.6 +/- 1.2 ^A	68.4 +/- 1.2 ^A	47.7 +/- 2.0 ^B	52.3 +/- 2.0 ^B

A = % of P1+S1

B = % of P2+S2

Table 1M. Hippocampal slices were prepared as described in the materials and methods and were incubated in a normal Krebs medium containing 15 μ M for 45 min. Slices were then washed in a physiological saline and homogenized in 0.32 M sucrose as described in section 2.4.1 of the materials and methods. At each step of the separation, aliquotes were removed from each fraction and assayed for ACh. The tabulated results represent the mean $\pm$ s.e.m. of 4 determinations.

pelleted with the large cellular debris containing dense structures such as nuclei. The 30% of tissue ACh that remained in the P_1 pellet was slightly higher than that reported by Hebb and Whittaker (1958) and by Whittaker (1959), using a similar method, but these authors washed the P_1 pellet twice in order to recover more of the nerve endings. Of the 66% of tissue ACh left in S_1 about half was recovered with the crude synaptosomal pellet (P_2) and the other half in the resulting supernatant (S_2). Thus these procedures yield about a 25% recovery of the original slice's ACh content in the synaptosomal pellet. The differences in the recovery of ACh using the method of Ricny and Collier (1988) versus that used by Whittaker and co-workers might in part be due to differences in the species of animals (rat vs. guinea-pig and rabbit) or to the tissue used (hippocampus vs brain).

2.4.2. Preparation of the P_3 and S_3 fractions from hippocampal slices.

In order to separate the cytoplasmic compartment of ACh from ACh that is occluded in some membranous structure, the P_2 pellet obtained following the above procedures was lysed using a buffered aqueous solution containing 5 mM HEPES, 1 mM EGTA, and 15 μ M eserine. Lysing of the P_2 pellet was achieved by resuspending the pellet in 1 to 1.5 ml of the aqueous solution by using a pasteur pipette. The pellet and solution were gently drawn up and down with a pasteur pipette until a homogenous milky solution was achieved with no obvious lumps. The resulting mixture was allowed to remain on ice for 15 min to ensure that lysing had gone to completion and the slurry then transferred to ultracentrifuge tubes capable of holding 10 ml. The lysed P_2 pellet was spun at 100,000 x g for 1 hr. in a Beckman T-40 rotor. The centrifugation pelleted all membranous structures leaving the free or cytoplasmic compartments in the supernatant. Figure 4M illustrates the basic procedure used for the isolation of the P_3 and S_3 fractions. Following the centrifugation step, the supernatant (S_3) was separated from the pellet with a pasteur pipette by slow aspiration so that liquid clinging to the wettable walls

Cellular Fractionation

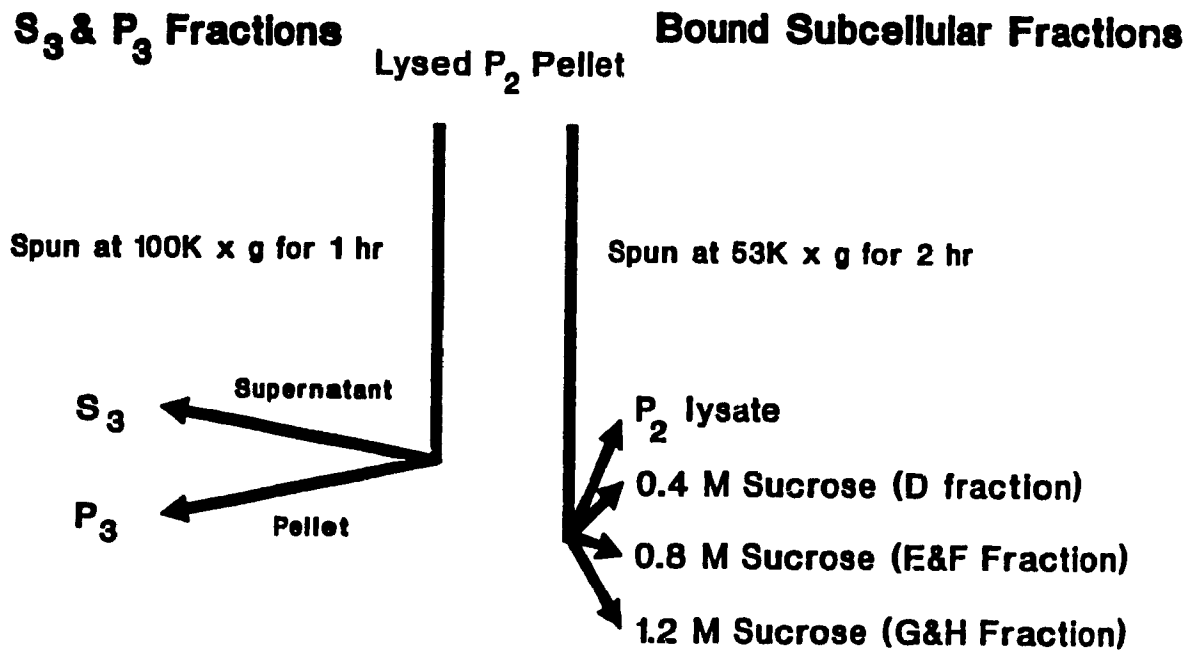


Figure 4M. Scheme describing the isolation of the subcellular compartments from the P₂ pellet. The left side describes the isolation of the cytosolic and occluded compartments (S₃ and P₃) and the right describes the isolation of the subcellular occluded fractions. For details see description of method in the materials and methods.

could be recovered. The supernatant was then placed in a 1.5 ml eppendorf tube and immediately placed in a -20 °C freezer until the sample was ready for ACh extraction. The pellet's (P₃) ACh was then initially extracted using 800 µl of a 10% solution of trichloroacetic acid (TCA) in double distilled water. The acid-pellet mixture was then either processed after an hour or left overnight in a -20 °C freezer for processing the following morning. All extractions of ACh and the assays are described in sections 2.7 and 2.8 of the materials and methods.

Since all subcellular fractions are corrected in this thesis for the amount of isolated synaptosomal protein, it was necessary to measure the total protein present in the P₂ pellet. This was done by removing 50 µl of the lysed P₂ pellet just before the high speed centrifugation step. The resulting samples were then assayed for protein content according to the method of Lowry *et al.* (1951) as described in section 2.9 of the materials and methods. Typically the synaptosomal preparations used in experiments dealing with the cytosolic and occluded fractions of ACh gave about 2.44 ± 0.20% (mean ± standard deviation) of the tissue wet weight as protein. This estimate was based on 96 randomly picked measures of synaptosomal protein from experiments where P₃ and S₃ fractions were investigated.

2.4.3. Preparation of the bound subcellular compartments of ACh from hippocampal slices.

The P₃ fraction is a mixture of several compartments that can be further subdivided by buoyant density centrifugation (Whittaker *et al.*, 1964). These compartments of ACh appear to have functionally different properties (Zimmermann and Whittaker, 1977; Suszkiw *et al.*, 1978; von Schwarzenfeld, 1979; Giompres *et al.*, 1981 (a & b)) and so further fractionation of the bound subcellular compartments of ACh was undertaken in order to study the behavior of these various components.

The P₂ pellet used in these experiments was obtained as described above and was lysed by 1 ml of a similar aqueous solution as that mentioned in the above section except that no eserine was present. The

pellet was resuspended in the solution by use of a pasteur pipette and was allowed to stand on ice for 15 min so as to ensure the lysis of the synaptosomes. While the synaptosomes were lysing, three discontinuous sucrose layers were prepared in polyallomer centrifuge tubes having a 4 ml capacity. Each layer contained 700 μ l of the appropriate sucrose solution and the density gradient was established by layering the lightest layer first followed by the next densest and so on. The three layers were of 0.4, 0.8, and 1.2 M sucrose made with an aqueous solution containing 5 mM HEPES as a buffering agent and 1 mM EGTA as a calcium chelating agent. The interfaces of each layer were then marked with a dark marker on the tubes so that following centrifugation they could more easily be identified. At the end of the 15 min of lysis for the P_2 pellet, 50 μ l of the resulting slurry was removed for estimation of synaptosomal protein and the rest (950 μ l) carefully layered on top of the sucrose gradient. The gradients were then centrifuged in a Beckman SW-60 swinging bucket rotor for 2 hr at 53,000 x g. The method described here is outlined in figure 4M. At the end of the centrifugation the different layers were aspirated by a 5 ml syringe fitted with a narrow gage stainless steel needle. All collected layers were placed in 1.5 ml eppendorf type tubes. The sucrose layers had their ACh initially extracted by adding 233 μ l of a 40% TCA aqueous solution so as to give a final TCA concentration of 10%. The capped tubes were then vortexed and all fractions were placed in a -20 °C freezer overnight. The ACh was extracted from each fraction as described in section 2.7 of the materials and methods.

Although the sucrose layers remain distinct with these procedures, diffusion of soluble materials is possible between the layers and it is possible that the transit of the P_3 particulates affect the rate of diffusion. Radioactively labeled ACh was used to test the extent to which soluble ACh, that is non-occluded ACh, can diffuse from the top layer to the lower sucrose layers. To 1 ml of either lysing solution or a lysed P_2 pellet was added 2 nmoles of [^{3} H]-ACh with a specific activity of 764 dpm/pmole. These were then layered on discontinuous sucrose gradients, prepared as mentioned above, centrifuged, and the layers isolated and

counted by liquid scintillation spectrophotometry to determine their content of [^3H]-ACh. Table 2M tabulates the results of 2 trials and indicates that considerable amounts of ACh diffuse from the top layer to the first sucrose layer. The diffusion of ACh found in the top layer to the first sucrose layer was not greatly affected by the presence of particulates but the further diffusion of small amounts of soluble ACh to the other layers was increased. Because the S_3 fraction contains more than three times the amount of ACh found in the P_3 fraction, as a whole, it was necessary that no eserine be present in the lysing medium. Thus, cytoplasmic ACh was not preserved and diffusion of ACh from the S_3 fraction to the first sucrose layer did not interfere with our estimates of that layer's ACh content. Under these conditions the soluble component layer (top layer) usually contained between 4 and 5 pmoles/mg synaptosomal protein of ACh.

2.5. ENZYME CHARACTERIZATION OF THE TOTAL TISSUE, P_2 PELLET, AND SUBCELLULAR FRACTIONS FROM HIPPOCAMPAL SLICES.

The total tissue and the fractions that were derived from it were characterized enzymatically by measuring their choline acetyltransferase (ChAT), acetylcholinesterase (AChE), lactate dehydrogenase (LDH), and succinic dehydrogenase (SDH) contents. The enzymes ChAT and AChE were chosen because they are reasonable markers of cholinergic neurons, ChAT being more specific than AChE. LDH was chosen as a marker enzyme because of its well known compartmentation in the cytoplasm (Johnson and Whittaker, 1963) and SDH was chosen as a mitochondrial enzyme marker (Aldridge and Johnson, 1959; Johnson, 1960).

Sprague-Dawley, male rats weighing between 150-300 gm were used. HIPP slices were prepared from brains as described in section 2.3 and the resulting slices were placed in an un-eserinized, standard Krebs medium for 45 min at 37 °C. The slices were then treated as described in section 2.4.1 of the materials and methods. The resulting P_2 pellet was lysed and either the P_3 and S_3 fractions prepared according to the

Table 2M. The diffusion of ACh from the soluble compartment to the bound subcellular fractions.

Layered Fraction	Top Layer	0.4 M Sucrose	0.8 M Sucrose	1.2 M Sucrose
Lysing Solution Alone	91.3%	8.5%	0.1%	0.0%
Lysed P ₂ Pellet	90.0%	9.4%	0.2%	0.1%

Table 2M. Synaptosomes from rat cortex were prepared as described in the materials and methods, section 2.4.1. The synaptosomes were lysed using an aqueous solution containing 5 mM HEPES, 1 mM EGTA, and 15 μ M eserine. Two nmoles of [³H]-ACh were added to 1 ml of lysate and to 1 ml of lysing solution. These were layered on a discontinuous sucrose gradient and centrifuged as described in the materials and methods section 2.4.3. Each cell contains the mean percent recovery of two determinations for each layer.

method described in section 2.4.2 or the bound-subcellular fractions were prepared as described in section 2.4.3. The only difference in the treatment of the fractions was that, prior to measuring the different enzyme activities, Triton X-100 was added so as to give a final concentration of 1% Triton X-100. This concentration of detergent did not affect any of the assays and it was used so as to ensure that no enzyme would be trapped in a membranous inclusion and thus go unmeasured. All enzyme activities were normalized to either total tissue protein, in the case of the enzyme activities measured in whole hippocampal formations, or to synaptosomal protein, for the P₂ fraction and all subcellular fractions.

ChAT activity was measured according to the method of Fonnum (1969b) as described by Tucek (1978). Briefly, 15 μ l of a buffer containing 200 mM NaCl, 40 mM sodium phosphate (buffered to pH=7.4), and 0.5% Triton X-100 was added to 20 μ l of the sample. This mixture was then combined with 15 μ l of a buffer yielding a final concentration of 300 mM NaCl, 0.2 mM eserine sulfate, 0.5 mg/ml albumin, 12.5 mM choline, and 0.25 mM acetylcoenzyme A (with 0.05 μ Ci of ³H-acetylcoenzyme A as a tracer; Amersham). The mixture was incubated for 15 min at 38 °C and the reaction stopped by adding 350 μ l of a 10 mM sodium phosphate buffer containing 0.2 mM ACh. The newly synthesized ACh was separated from the unreacted acetylcoenzyme A by adding 400 μ l of heptanone, containing 10 mg/ml of tetraphenyl boron (see section 2.7), agitating the liquids so as to aid the extraction by the tetraphenyl boron and centrifuging the tubes so as to separate the organic from the aqueous layer. A 300 μ l aliquot of the organic phase was taken and the [³H]-ACh content determined by liquid scintillation spectrometry (LKB).

The AChE activity of the various samples was measured according to the method of Ellman (1961). Briefly, 10 to 50 μ l of sample were added to 50 μ l of a 10 mM solution of dithiobisnitrobenzoic acid and to 1.42 to 1.38 ml of a 0.1 M sodium phosphate buffer of pH=8.0. The reaction was started by the addition of 20 μ l of acetylthiocholine iodide at a concentration of 75 mM or the addition of phosphate buffer to the blank or zeroing cuvette so as to give a final volume of 2.5 ml in each

cuvette. The absorbance was followed at 412 nm and recorded every minute for a total reaction time of 5 min. The slope of the change in absorbance versus time was estimated using linear regression and this estimate was used to calculate the rate of the reaction.

LDH activity was estimated using the method described by Eichner (1982). In this procedure we measured the rate at which LDH could reduce pyruvate using reduced β -nicotinamide adenine dinucleotide (NADH) as the reducing agent. Briefly, 10 to 100 μ l of sample were added to 10 μ l of the sodium salt of pyruvate (33 mg/ml; Sigma Chemical Co.), 100 μ l of NADH (3.55 mg/ml; Sigma Chemical Co.), and from 2.88 to 2.79 ml of a 0.1 M sodium phosphate buffer at pH=7.6, so that the final reaction volume was 3 ml. The blank cuvette contained everything but the NADH. The reaction was started by the addition of the NADH and the reaction was followed by monitoring the disappearance of NADH at 340 nm. The reaction was allowed to proceed for 5 min and readings were taken every minute. The rate of decrease in absorbance over time was estimated from the slope of the regression line calculated from the readings.

The activity of the mitochondrial enzyme, SDH, was measured using the method of Bonner (1955) as described by Maeno *et al.* (1971). In this method 50 to 400 μ l of sample were added to 820 to 470 μ l of a sodium phosphate buffer to give a final concentration of 100 mM sodium phosphate at a pH=7.2. To this was added 20 μ l of 500 mM potassium cyanide, 10 μ l of 14 mM 2,6-dichloroindophenol (Sigma Chemical Co.), and 100 μ l of 260 mM sodium succinate (Sigma Chemical Co.). The blank cuvette did not contain the substrate, succinate, but otherwise was the same as the samples. The rate of reaction was monitored by the decrease in the absorbance of the 2,6-dichloroindophenol at 600 nm and the reaction was allowed to proceed for 10 min with the absorbance noted every 2 min. The reaction rate was estimated from the slope of the linear regression carried out on the measures taken.

For all enzymes, except ChAT, several concentrations of the samples were taken from each sample in order to ensure that increasing amounts of protein led to a linear increase in the measured activities. This was found to be so. In the case of ChAT, in one trial, doubling

the concentration of protein led to a doubling in the rate of measured ChAT activity. Table 3M tabulates the results obtained. In the case of the subcellular bound fractions (top of gradient, D, E&F, and G&H fractions) the activity of the enzymes was corrected, based on the data given in table 2M, for diffusion from the soluble fraction through the different sucrose layers.

The cholinergic marker enzyme, ChAT, is known to co-purify with ACh (Hebb and Whittaker, 1958) as brain tissue is fractionated to render a synaptosomal fraction, the P_2 pellet. The present results indicate that there is an increase in enzyme activity when the P_2 fraction is prepared and that about 60% of the activity found in the P_2 pellet is associated with the particulate fraction of the P_3 pellet. Part of the reason that more ChAT activity is associated with the P_3 fraction may be that ChAT is known to stick to membranes when low ionic strength media are used (Fonnum, 1967) and the lysing media used in these experiments has a low ionic content. The distribution of ChAT activity in the bound-subcellular fractions very closely parallels the protein concentration of each fraction. Thus, the D fraction, which contains about 6% of the protein found in the G&H fraction, contains about 1/11 the ChAT activity of the heavier fraction and the E&F fraction, containing about 1/5 the protein of the denser fraction, contains about 1/4 the enzyme activity of the G&H fraction.

AChE activity was found to be the same, relative to protein concentration, in total tissue and in the P_2 pellet. About 90% of the enzyme activity was associated with the occluded, P_3 fraction, similar to that reported by Whittaker *et al.* (1964). The greatest AChE activity was found in the G&H fraction but the increase in activity over that of the other two fractions was much less than the increase in protein, thus suggesting that AChE is not bound to proteins or membranes in a random fashion.

The activity of the cytoplasmic marker, LDH, was mainly associated with the soluble fraction (S_3 ; 66%) but 34% of the P_2 fraction's activity remained in the occluded fraction (P_3). Whittaker and co-workers found almost all the activity associated with the soluble fraction.

**Table 3M. Enzyme Characterization of
Subcellular Fractions**

FRACTION	ChAT ^A nmoles/hr/mg protein	AChE ^B nmoles/min/mg protein	LDH ^C nmoles/min/mg protein	SDH ^D nmoles/min/mg protein
Total tissue	16.1 +/- 1.6 (3)	133.1 +/- 16.8 (7)	742.7 +/- 17.1 (6)	18.0 +/- 1.4 (5)
P ₂	27.5 +/- 1.9 (5)	126.4 +/- 11.8 (10)	622.1 +/- 61.0 (10)	9.5 +/- 0.9 (9)
S ₂	9.3 +/- 2.2 (3)	8.3 +/- 0.8 (6)	480.5 +/- 97.7 (9)	1.4 +/- 0.4 (6)
P ₃	13.2 +/- 1.8 (3)	102.9 +/- 15.3 (6)	248.0 +/- 12.8 (10)	4.8 +/- 0.2 (7)
Top of Sucrose Gradient (S ₂)	11.7 (2)	11.3 +/- 0.3 (3)	467.3 +/- 15.6 (3)	2.7 +/- 0.2 (3)
D	1.3 (2)	4.0 +/- 0.5 (3)	50.3 +/- 3.9 (3)	Not Detectable (3)
E & F	3.3 (2)	32.2 +/- 1.0 (3)	67.8 +/- 3.5 (3)	Not Detectable (3)
G & H	14.5 (2)	73.4 +/- 3.7 (3)	230.1 +/- 13.5 (3)	2.4 +/- 0.8 (3)

A = Choline Acetyltransferase

B = Acetylcholinesterase

C = Lactose Dehydrogenase

D = Succinate Dehydrogenase

Table 3M. Rat hippocampal slices were prepared and incubated for 45 min in normal Krebs medium as described in the materials and methods. Slices were then washed in physiological saline and homogenized in 0.32 M sucrose. The P₂ pellet and the various subcellular fractions were isolated as described in the materials and methods. Samples were taken from each fraction and the enzyme activity assayed according to the methods described in section 2.6. Each estimate is the mean $\pm$ s.e.m. of the number of determinations noted in parenthesis below the estimate.

They reported finding 74% of the LDH activity in the top portion of their gradients, but since they did not correct their recovery for diffusion from the lysed P₂ pellet layer to the top sucrose layer the activity in this soluble component layer must be greater than the 74% reported. LDH activity in the bound-subcellular compartments was found to be highest in the G&H fraction with the D fraction and the E&F fraction containing about the same level of LDH activity. Fonnum (1967) reported that LDH bound to membrane constituents in low ionic media, such as that used in these fractionation procedures, thus, it is likely that at least some of this LDH is the result of such binding.

SDH is an enzyme that is tightly bound to the inner membrane of mitochondria and is not easily made soluble. Measurements of SDH activity in the various fractions showed a halving of activity in the P₂ pellet as compared to total tissue. Most of the SDH activity of the P₂ fraction was associated with the P₃ pellet (78%). There was no detectable activity associated with the D fraction or the E&F fraction, while the G&H fraction contained 1/2 of the activity associated with the P₃ pellet. Whittaker and co-workers reported finding most of the activity in the pellet from the sucrose gradient (not assayed in experiments reported here) and in the densest sucrose fraction (H by Whittaker *et al.*, 1964's nomenclature). These findings indicate that homogenization likely leads to the disruption of some mitochondria and that the G&H fraction contains most of the mitochondria.

2.6. EXTRACTION PROCEDURES FOR ACh, [³H]-ACh, [³H]-DEHCh, AND [³H]-ADEHCh.

The extraction of Ch and its various analogues (ACh, ADEHCh, and DEHCh) from aqueous solutions was done according to the method of Fonnum (1969a) as described by Welner and Collier (1984). This method involved the complexing of the cations from the aqueous solution (from 200 to 500 μ l of sample) with tetraphenylboron (TPB) (Sigma Chemical Co., St. Louis, MO, USA) dissolved in either 3-heptanone (Eastman Kodak, Rochester, NY, USA) or butyronitrile (Sigma Chemical Co.) (10 mg/ml

[TPB], 500 μ l). The biphasic mixture of sample and butyronitrile was agitated for 4 min to ensure maximal extraction and the tubes spun for 2 min on a fixed speed eppendorf centrifuge so as to separate the layers. This extracted the Ch and analogues into the organic layer from which 400 μ l were removed and 200 μ l of a silver nitrate solution was then added (20 mg/ml). The tubes were again shaken for 4 min and spun for 1 min. The silver complexed with the TPB to form an insoluble salt and released the Ch and analogues back into an aqueous environment. The organic layer was aspirated and 175 μ l of the silver containing solution was then transferred to an eppendorf tube containing 20 μ l of a 1 M magnesium chloride solution. The tubes were shaken for 2 min and spun for 2 min so as to pellet the precipitate. This step caused the precipitation of any remaining silver by chloride and thus, left the cations, such as Ch and its analogues, with chloride as the counter ion. 170 μ l of the resulting solution were placed in ethanol washed, borosilicate, glass tubes with dimensions of 6 x 50 mm and the liquid removed by applying a high vacuum while the samples were centrifuged. In cases where [^{3} H]-ACh or [^{3} H]-ADEHCh were to be assayed, the 170 μ l of sample were placed in 1.5 ml eppendorf tubes and dried by vacuum as described. The dried pellets were then stored at -20 °C until sampled.

When Ch or its analogues were to be extracted from tissues or from subcellular fractions the method of MacIntosh and Perry (1950) was used. Choline and its analogues were first extracted using an aqueous solution of 10% TCA. This precipitated the proteins and allowed Ch and its analogues to leach out of the bound compartments or cells. The TCA was then removed by mixing the aqueous mixture with either 7 volumes of water saturated ethyl ether 4 times or with 10 volumes of ethyl ether 3 times. The ethyl ether was suctioned off each time and following the removal of the final ether volume any residual ether was removed by passing air over the samples until the ether could no longer be detected by olfaction. The aqueous solutions containing Ch and its analogues were then extracted using the method described in the previous paragraph.

2.7. DETERMINATION OF ACh, [^3H]-ACh, AND [^3H]-AChECh.

ACh was assayed according to the radio-enzymatic method of Goldberg and McCaman (1973). In the first reaction of this method, choline was phosphorylated using choline kinase (EC 2.7.1.32, ATP:choline phosphotransferase)(Sigma Chemical Co.) and adenosine triphosphate (ATP) to form phosphorylcholine. This was accomplished by dissolving the extract, obtained from the extraction method described above, in a solution containing 46 mM glycyl glycine as a buffer at pH=8.0, 0.75 mM ATP, 11.2 mM MgCl_2 , 0.75 mg/ml of dithiothreitol (Sigma Chemical Co.), and 5 milliunits of choline kinase and allowing the mixture to react for 45 min at 30 °C. After this step the ACh was hydrolyzed using 150 milliunits of acetylcholinesterase (EC 3.1.1.7, ACh hydrolase, type V-S)(Sigma Chemical Co.) and the resulting Ch phosphorylated by the mixture described above, except that approximately 0.5 μCi of [^{32}P]-ATP was added so that some of the phosphorylcholine formed from the ACh would be labeled. This second reaction was allowed to go to completion by reacting the mixture for 45 min at 30 °C.

The [^{32}P]-phosphorylcholine was separated from unreacted [^{32}P]-ATP by use of anion-exchange chromatography. This separation was carried out using 50 mM NaOH to both stop the second reaction and to ensure that the [^{32}P]-ATP carried its maximal negative charge (pH=12). The solution was passed through a pre-pH equilibrated (pH=12.0), 2.5-3.0 cm long column of Amberlite (Sigma Chemical Co.) using 2.8 ml of the 50 mM NaOH to elute the [^{32}P]-phosphorylcholine from the column. The effluent was collected and the ^{32}P content quantified by use of liquid scintillation spectrometry (LKB). Either Aquasol-2 (New England Nuclear) or Scintiverse-II (Fisher Scientific Co.) were used as the fluores, both giving approximately 98-99% counting efficiency for ^{32}P when the ^{32}P window setting was used.

The determinations of the ACh in samples was based on the parallel extraction and assay of standard amounts of ACh dissolved in the appropriate medium or water. When the tissue ACh content, or that contained in fractions was determined part of the sample was extracted

separately and it was exposed to AChE before the addition of [^{32}P]-ATP. Samples thus treated served as blanks for the estimates of ACh contents. A set of programs, described in section 2.11 and given in appendix A, were then used to calculate the unknowns based on the linear parameters obtained from the ACh standards.

A similar method was used to determine the amounts of [^3H]-ACh and [^3H]-ADEHCh in samples. The [^3H]-Ch or [^3H]-DEHCh were first phosphorylated using the same reaction mixture of choline kinase and ATP as described above. The reaction was allowed to go to completion at 30 °C for 60 min and then ice cold water was added to make the total volume 300 μl . To separate the [^3H]-phosphorylcholine or the [^3H]-phosphoryl-diethylhomocholine from the [^3H]-ACh or [^3H]-ADEHCh, 300 μl of a TPB containing butyronitrile solution was added to the tube. This extracted the unphosphorylated forms into the organic phase and left the phosphorylated forms in the aqueous layer. An aliquot of each layer was then removed and placed in a liquid scintillation vial with 5 ml of either Aquasol-2 or Scintiverse-II. These fluors and the ^3H window on the counter gave a counting efficiency for ^3H of between 27-36%. Standard amounts of [^3H]-ACh and [^3H]-Ch or [^3H]-ADEHCh and [^3H]-DEHCh were used to determine the extraction efficiency so that the dpm could be corrected for loss. A [^3H]- H_2O standard (New England Nuclear) was used to correct the cpm readings for quench and counting efficiency. See section 2.11 of the materials and methods for a description of the calculations used to correct the cpm readings to dpm and to correct for spillover.

2.8. PROTEIN DETERMINATION.

All protein determinations were carried out according to the method of Lowry *et al.* (1951) as described by Waterborg and Matthews (1984). All determinations were made from a set of known concentrations of bovine serum albumin fraction V (Sigma Chemical Co.) ranging in concentrations from 10 $\mu\text{g/ml}$ to 500 $\mu\text{g/ml}$ in 500 μl . All samples were diluted to 500 μl with water. From the starting 500 μl of sample or

standard, 100 μ l were placed in a glass test tube along with 100 μ l of 2 N NaOH, the mixture was vortexed and the proteins hydrolyzed by heating for 10 min at 100 °C with a marble on the mouth of the test tube so as to minimize evaporation. When the protein mixtures reached room temperature 1 ml of a solution containing 20 g/l of Na_2CO_3 , 0.1 g/l of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 0.2 g/l of sodium potassium tartrate was added. The resulting mixtures were vortexed and allowed to stand for 10 min at room temperature. Each test tube then received 0.1 ml of a 1 N Folin reagent (BDH Inc., Toronto, Ont., Canada) and immediately vortexed. The reaction with the Folin reagent was allowed to proceed for 30 min and the absorbance of all tubes was then read at 750 nm. The samples were never allowed to be with the Folin reagent more than 60 min before reading the absorbance because the color produced by the reaction began to fade after this time.

2.9. SYNTHESIS OF DIETHYLHOMOCHOLINE, [^3H]-DIETHYLHOMOCHOLINE AND [^3H]-ACETYLDIETHYLHOMOCHOLINE.

The synthesis of both tritiated and unlabeled DEHCh was done according to the method described by Barker and Mittag (1975). The tertiary amine, 3-diethylamino-1-propanol (Aldrich Chemical, Milwaukee, WI, USA) was reacted with either methyl iodide, to produce the unlabeled analogue, or with [^3H]-methyl iodide (8 Ci/mmol; Amersham, Arlington Heights, IL, USA) to give the ^3H form of DEHCh. The unlabeled compound formed a visible precipitate which was washed with anhydrous ethyl ether and recrystallized from ethanol. The crystals were then stored in a dessicator or sealed in ampoules. The ^3H form of the analogue was purified by cation-exchange chromatography as outlined by Barker and Mittag (1975), except that 1 M NH_4HCO_3 was used to displace the analogue. The collected fractions were then lyophilized to remove the NH_4HCO_3 . The salt is known to decompose into NH_3 , H_2O , and CO_2 under conditions of low atmospheric pressure and since all three products are gasses they are removed by the lyophilization. Purity of the unlabeled and labeled DEHCh was checked by thin layer chromatography (TLC) as

described by Welner and Collier (1984) and the products were found to produce two spots, one corresponding to the DEHCh and the other to the counter ion, which was either iodide in the case of the unlabeled analogue or bicarbonate in the case of the labeled one.

The [^3H]-ADEHCh was synthesized using the [^3H]-DEHCh synthesized by the methods described above as a precursor. About 2 μCi of [^3H]-DEHCh was placed in a 10 ml sealable ampule and approximately 7 ml of acetylchloride (Fisher Scientific; Montreal, Quebec) was added to this and then the ampule sealed while held in a beaker of ice so that the acetylchloride would not heat up and release large amounts of vapors. The sealed ampule was placed in a boiling water bath and the two reagents were allowed to react for a period of 30 min. The ampule was then cooled, opened, and the unreacted acetylchloride driven off by passing a stream of air into the ampule. TLC analysis of the product revealed one radioactive spot which migrated at a slower rate than the unacetylated [^3H]-DEHCh.

2.10. CALCULATIONS, STATISTICS, AND MATERIALS.

All calculations of the primary data were carried out using a series of Basic programs written by me. These programs allowed for data entry and basic calculations, including some simple statistics. All statistical procedures found in these programs were adapted from a statistics text by William Scheffler (1969). These programs are given in their entirety in appendix A of this thesis and a short description of the basic principles used for the computations is given below.

ACh, Ch, and protein assays were done using a series of standards to calculate the unknown values from samples. The standards were analyzed using standard linear regression methods which can estimate the y-intercept based on an equal weighing of all standard concentration used as in the case of the ACh and Ch determinations, or the best fit line going through the origin could be calculated as in the case of the protein determinations. The statistical significance of each standard curve was calculated and all curve parameters given. Unknowns were cal-

culated using these linear parameters and corrections were made for sample size and for the volumes used in the assays.

[³H]-ACh and [³H]-ADEHCh were estimated based on counting efficiencies and on the recovery and spill-over into the [³H]-Ch and [³H]-DEHCh layers. Counting efficiency was determined by use of a [³H]-H₂O standard available from New England Nuclear and recovery was determined by the use of known amounts of [³H]-ACh (available from New England Nuclear) and [³H]-ADEHCh (synthesis described in section 2.10) as extraction standards. To correct for spill over between the organic phase, which contains mainly the acetylated compounds, and the aqueous layer, which contains the phosphorylated compounds, both [³H]-ACh and [³H]-Ch were used as standards for the determination of [³H]-ACh contents of samples or [³H]-ADEHCh and [³H]-DEHCh were used for the determination of [³H]-ADEHCh. Based on the extraction of the standards, a Crammer 2X2 matrix was used to correct for spill-over after the cpm readings were corrected to dpm.

All other procedures carried out by the programs involved simple corrections for protein, liquid scintillation spectrophotometric quench, specific activity determinations, and file manipulations. The programs were also able to calculate means and standard errors of the means as well as performing simple t-test evaluations.

For the analysis of the results by different statistical tests and procedures, the commercially available program package, CSS (Complete Statistical System, 1987, by Statsoft Inc., Tulsa, OK, U.S.A.) was used. This program was purchased by the Department of Pharmacology and Therapeutics and was available for use in its computer facilities.

All materials whose source was not mentioned in the appropriate sections were of reagent grade and came from Fisher Scientific Company (Montreal, PQ, Canada). AH5183 was either the kind gift of Dr. S. Parsons, Department of Chemistry, University of California at Santa Barbara, or it was kindly synthesized from its immediate precursor by Dr. T. H. Chang's laboratory, Department of Chemistry, McGill University. The purified toxin, α-LTX, of BWSV was prepared and kindly provided by Dr. H. Scheer, Department of Pharmacology, University of Montreal.

RESULTS

3.1 THE EFFECT OF AH5183 AND BLACK WIDOW SPIDER VENOM ON THE RELEASE OF ACETYLCHOLINE FROM THE CAT SUPERIOR CERVICAL GANGLIA.

The compound 2-(4-phenylpiperidino)cyclohexanol (AH5183) is known to block the active uptake of acetylcholine (ACh) by vesicles (Anderson *et al.*, 1983) and to affect the stimulated release of this neurotransmitter (Gandiha and Marshall, 1973; Melega and Howard, 1984; Carroll, 1985; Otero *et al.*, 1985; Collier *et al.*, 1986; Jope and Johnson, 1986; Michaelson *et al.*, 1986; Ricny and Collier, 1986; Suszkiw and Toth, 1986). The present experiments were designed to test whether the drug's effect on ACh release from the cat superior cervical ganglion (SCG) is due to a depletion of vesicular ACh. The approach taken to answer this question was to test if the venom from the black widow spider (BWSV) released ACh following the block induced by AH5183; since BWSV causes the release of vesicular ACh, if AH5183 blocks release by depleting the vesicles of transmitter, the BWSV should cause no further release of ACh. Three points were addressed: first, the effects of AH5183 on the stimulated release of ACh from the ganglion had to be confirmed; second, it was necessary to determine whether BWSV induces vesicular release of ACh from the ganglion; third, the effect of the venom on the release of ACh from an AH5183-blocked ganglion could then be tested.

3.1.1 The release of acetylcholine from ganglia in the presence and absence of AH5183.

The cat SCG was prepared for *in situ* perfusion with a Krebs solution as described in the materials and methods. Ganglia were allowed to recover from this procedure by perfusing them for 15 min before collection of the perfusate; after the recovery period, they were left at rest for 5 min and then electrically stimulated for 30 min at 5 Hz via the preganglionic nerve (see materials and methods). Samples were collected every 5 minutes and their ACh content was measured. In experiments where AH5183 was added to the perfusion medium, it was present at a concentration of 10 μ M. These ganglia were perfused with the drug-

containing medium 15 min before the collection of the first sample and throughout the experiment.

As shown by figure 1R, under control conditions electrical stimulation of the preganglionic nerve was able to maintain the release of ACh at 6-8 times the basal level. However, in the presence of AH5183 this release increased only during the first 5 min of stimulation and then quickly waned to basal levels. In these experiments, release of ACh was lower at all times when the drug was present. This dramatic effect of AH5183 on the release of ACh by nerve stimulation is in agreement with the report of Collier *et al.* (1986).

In those experiments where AH5183 was present, the total ACh released by ganglia in response to nerve stimulation was 263 ± 57 (mean $\pm$ s.e.m.) pmoles. This amount of ACh represents about 14% percent of the ganglia's initial ACh content, as determined by assaying the untreated, contralateral ganglia's ACh content (1836 ± 264 pmoles).

3.1.2 The vesicular release of acetylcholine by black widow spider venom.

The first experiments determined if the BWSV available to us could induce the release of ACh from the cat SCG. Ganglia were prepared for *in situ* perfusion and were perfused for 15 min before the collection of samples. To ensure that the ganglia were capable of releasing ACh in response to nerve impulses they were left at rest for 5 min, then stimulated preganglionically at 5 Hz for 10 min, and subsequently returned to rest for another 10 min. After this, perfusion was with a medium containing 0.125 sac equivalents of BWSV per ml of medium for 60 min with samples being collected every 5 min during the entire experiment.

Figure 2R shows that BWSV was able to release ACh from the SCG. The response to BWSV was quite variable in terms of the rate of ACh release and its time course, but the BWSV was always able to elicit the release of transmitter in the five experiments that were done. The time to peak release ranged from 30 to 55 min and, at the peak of ACh release, release ranged from 25 to 54 pmoles/min.

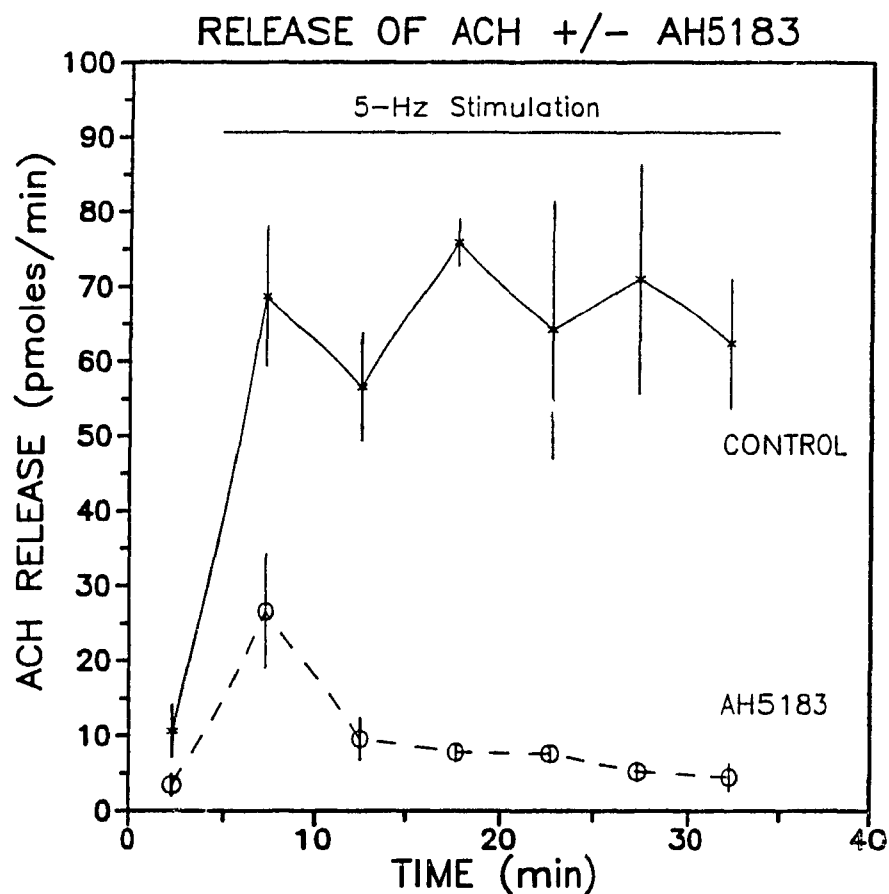


Figure 1R Release of ACh ± AH5183. Ganglia were perfused in situ with either a normal Krebs medium or with a Krebs medium containing 10 μ M AH5183. Both media contained 10 μ M choline and 15 μ M eserine. The preganglionic nerve was stimulated at 5 Hz for the period of time indicated by the horizontal line. The control represents the mean $\pm$ s.e.m. of four ganglia and that for the AH5183 illustrates the results of five ganglia.

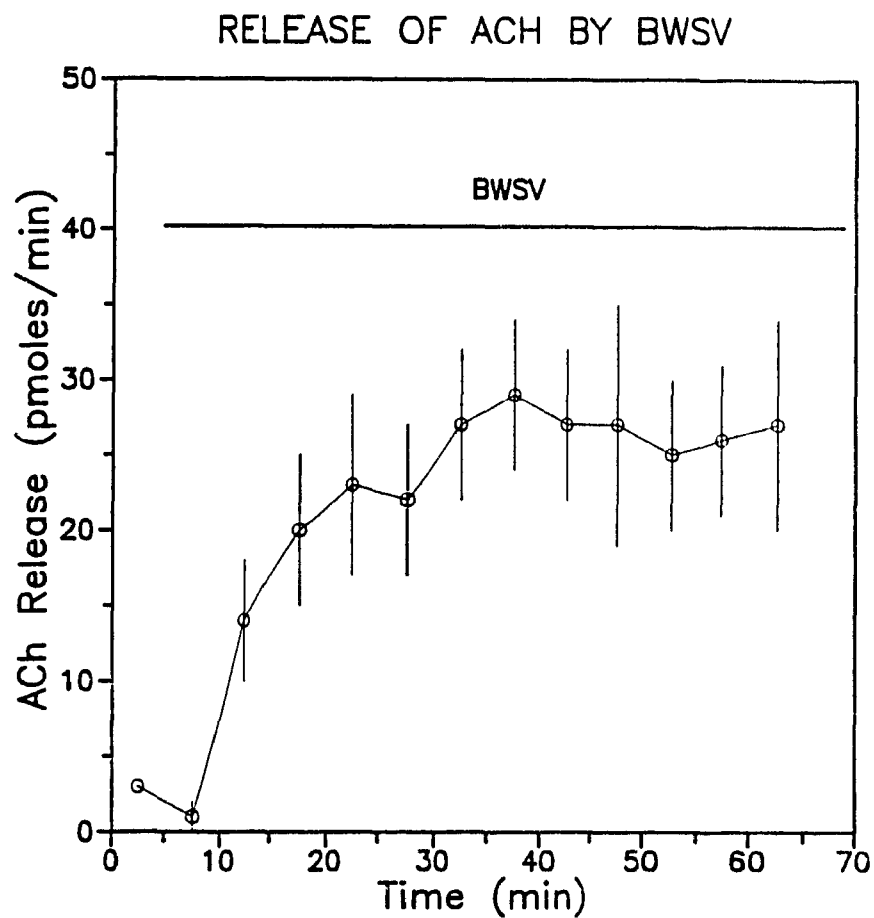


Figure 2R. Release of ACh by BWSV. Ganglia prepared for in situ perfusion were treated with a medium containing 0.125 sac equivalents of BWSV per ml of medium for the time indicated by the horizontal bar and samples collected every 5 min. The medium also contained 10 μ M choline and 15 μ M eserine. The plot illustrates the result of five experiments with each point representing the mean $\pm$ s.e.m.

BWSV causes the release of ACh at the neuromuscular junction by inducing vesicular exocytosis (Clark *et al.*, 1970, 1972; Frontali *et al.*, 1976; Tzeng *et al.*, 1978). This effect of the venom can be attributed to one component, α -latrotoxin (α -LTX) (Frontali *et al.*, 1976; Grasso, 1976; Tzeng and Siekevitz, 1978), a protein with no known enzymatic activity. To test whether α -LTX or some other component of the venom was responsible for the release of ACh evoked by the BWSV, the polyclonal antibody raised against α -LTX and described by Valtora *et al.* (1984) was used.

Ganglia were prepared for *in situ* perfusion. Following a 15 min recovery period, samples were collected every 5 min. An initial 5 min sample was collected at rest, the ganglia were then stimulated at 5 Hz via the preganglionic nerve for another 5 min and returned to resting conditions for a subsequent 5 min. A medium containing a mixture of BWSV (0.125 sac equivalents/ml) and anti- α -LTX antiserum (see materials and methods for the determination of the quantity of antisera needed to neutralize venom), that had been allowed to react with each other for 2 hr prior to the start of the experiment, was then perfused through the ganglia for 30 min. This mixture was washed out of the ganglia by a 10 min perfusion with a normal Krebs medium and, again, the preganglionic nerve was stimulated at 5 Hz for 5 min to ensure that release via nerve impulses remained unaffected. The ganglia were allowed to return to rest for 5 min, following which, a medium containing 0.125 sac equivalents of BWSV per ml of medium was perfused through the ganglia for 30 min to ensure that the venom used in the first perfusion was indeed active.

Figure 3R illustrates the results of one of three experiments. Stimulation at 5 Hz caused the release of ACh well above the basal level. During perfusion with the venom-antiserum mixture there was no release of ACh above that of basal rate. The treatment of the ganglia with this mixture did not affect the ability of electrical stimulation to increase the release of ACh, and subsequent perfusion with venom alone evoked a large release of the neurotransmitter. These results demonstrate that the antiserum could inhibit venom action, presumably

RELEASE OF ACh BY BWSV +/- ANTI-LTX AB

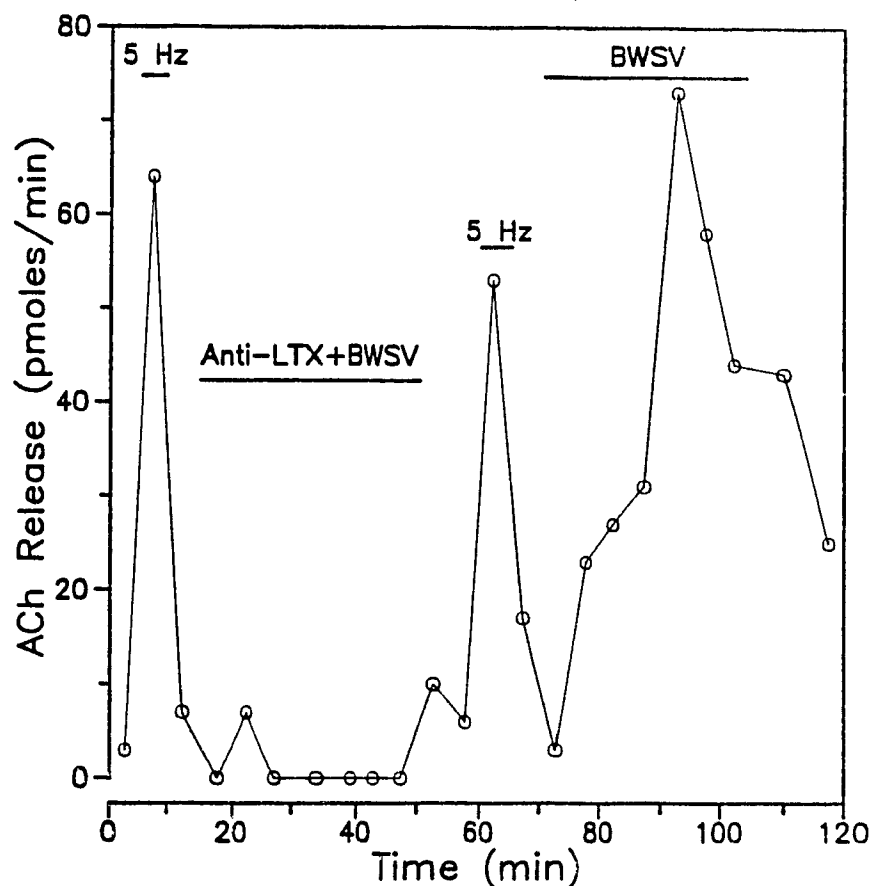


Figure 3R. Release of ACh by BWSV ± anti- α -LTX antiserum. Ganglia were perfused with a normal Krebs solution containing 10 μ M choline and 15 μ M eserine and received 5 min of preganglionic nerve stimulation at 5 Hz where indicated on the figure. Following the first test stimulation a medium containing a pre-mixed solution of BWSV and anti- α -LTX antiserum was perfused for 30 min. This medium gave a final concentration of 0.125 sac equivalents of BWSV per ml of medium. Subsequently, the solution was washed out with the normal Krebs medium and a second test stimulation delivered via the nerve. The medium was then changed to one containing BWSV, at the same concentration as before, but with no antiserum. The figure represents one of three experiments which gave similar results.

indicating that the release of ACh in response to the venom was due to α -LTX.

To eliminate the possibility that the protective effect of the anti- α -LTX antiserum described above was due to serum and not the antibody, the following experiment was done. A ganglion was prepared as described above and the experiment of figure 3R was repeated except that the BWSV was incubated with naive serum for 2 hr instead of the antiserum raised against α -LTX. Figure 4R shows that the BWSV-serum mixture was able to release ACh from the ganglion.

Even though α -LTX is known to cause vesicles to fuse to the plasma membrane and thereby cause the release of ACh, it is important to investigate the possibility that the venom might also induce a change in the plasma membrane and so cause the release of some cytoplasmic constituents. This point is crucial to the interpretation of results pertaining to the source of released ACh because ACh is synthesized and present in the cytoplasm of nerve endings as well as in the vesicles. Experiments designed to test the possibility that molecules in the size range of ACh might be released from the cytoplasmic compartment due to the action of BWSV used the choline analogue diethylhomocholine (DEHCh).

The compound, DEHCh, can be taken into cholinergic nerve terminals and once inside it is acetylated to acetyldiethylhomocholine (ADEHCh). The ADEHCh is generated in the cytoplasm of the nerve terminals, but it is not readily transported into vesicles; thus, it is a good marker of the cytoplasmic compartment (Welner and Collier, 1984, 1985). Because of this selective localization within the nerve terminal, measures of its efflux in the presence of the BWSV should address the possibility of venom-induced cytoplasmic release.

Ganglia were perfused for 1 hr with a medium containing 5 μ M [^{3} H]-DEHCh and 5 μ M choline while the preganglionic nerve was stimulated at 20 Hz in order to load the ganglia with the analogue and to stimulate the generation of [^{3} H]-ADEHCh. The medium was changed to normal Krebs for 10 minutes to wash out any remaining extracellular [^{3} H]-DEHCh. From this point on, samples were collected every 5 min and, following one more 5 min collection period at rest, the perfused medium was changed to

RELEASE OF ACh BY BWSV IN THE PRESENCE OF SERUM

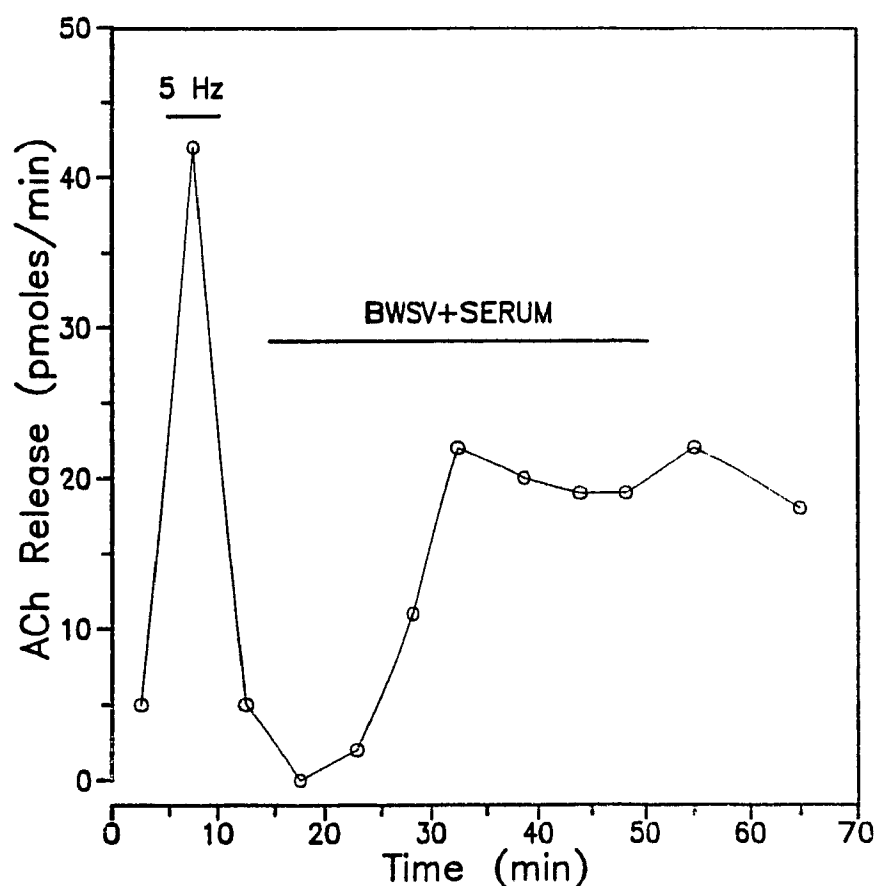


Figure 4R. The release of ACh by BWSV in the presence of naive serum. A ganglion was perfused with a normal Krebs medium containing 10 μ M choline and 15 μ M eserine for 15 min during which a 5 min test stimulation at 5 Hz was delivered via the preganglionic nerve as indicated in the figure. The medium was then changed to one containing a pre-mixed combination of BWSV and naive serum so as to give a final concentration of 0.125 sac equivalents of venom per ml of medium

one containing BWSV. As seen in figure 5R, the venom elicited the release of ACh well above basal levels, but during this period the efflux of [^3H]-ADEHCh decayed with no increases in its rate of efflux occurring even at the peak of ACh release. This result indicates that BWSV did not change the membrane so as to alter the release of cytoplasmic ADEHCh; thus it seems reasonable to presume that the efflux of cytoplasmic ACh did not increase either.

The above results on the actions of BWSV at the cat SCG indicate that the active component of the venom is the well characterized protein α -LTX and that released ACh is from a vesicular source, not a cytoplasmic one. Thus, it was possible to test whether the block of impulse evoked release of ACh brought about by AH5183 at the SCG was the result of an exhausted vesicular source of transmitter.

3.1.3 The release of acetylcholine by black widow spider venom in the presence of AH5183.

To test whether BWSV could induce the release of ACh from the SCG after blockade by AH5183, ganglia were prepared for in situ perfusion. During a 15 min recovery period ganglia were perfused with a Krebs medium containing 10 μM AH5183 and following this period samples were collected every 5 min. One sample was taken at rest and subsequently the preganglionic nerve was stimulated for 30 min at 5 Hz to evoke the release of ACh available for release in the presence of AH5183. The ganglia were then perfused for 60 min with a medium containing 0.125 sac equivalents of BWSV per ml of medium in the continued presence of AH5183.

The results of 3 of 5 experiments are illustrated in figure 6R. As described above, AH5183 blocked the nerve impulse-stimulated release of ACh; however, when BWSV was perfused there was a large release of ACh. This result indicates that a vesicular source of ACh still resided in the ganglia even though release evoked by nerve impulses had been blocked by the presence of AH5183. The variability in the response was more evident under these conditions than with BWSV alone. The 3 panels

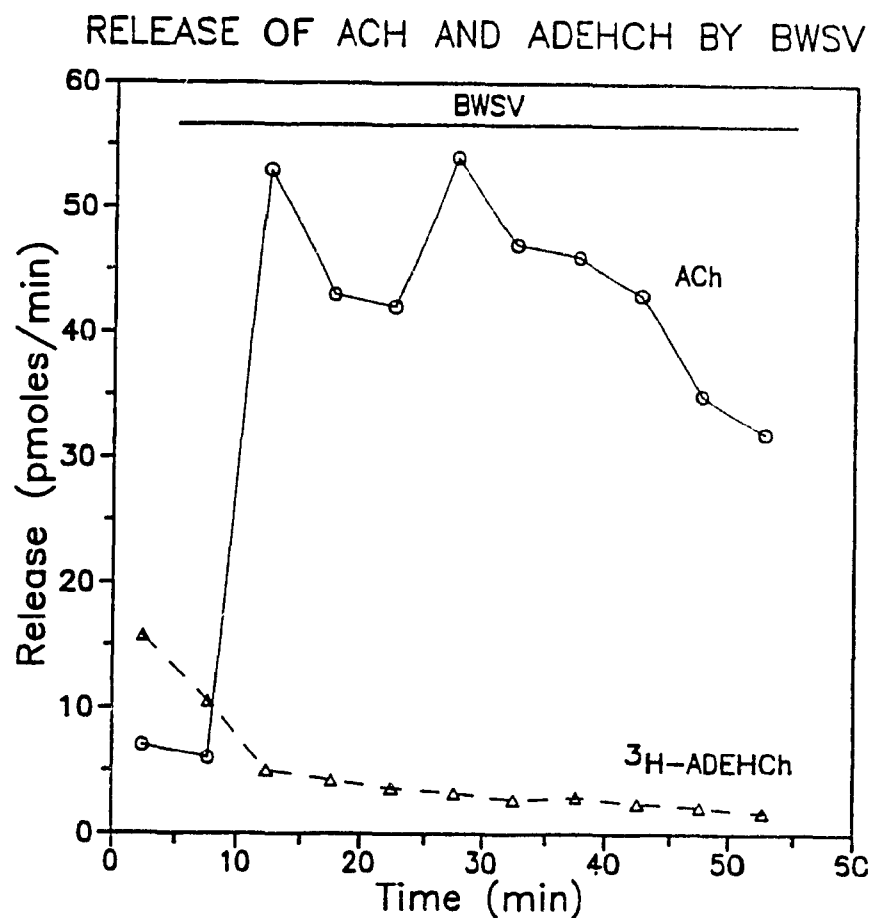


Figure 5R The release of ACh and [³H]-ADEHCh by BWSV. Ganglia were prepared for in situ perfusion and were first perfused with a Krebs solution of 5 μ M [³H]-DEHCh, 5 μ M choline, and eserine for 60 min while the preganglionic nerve was stimulated at 20 Hz. The perfusion medium was changed to normal Krebs for 15 min and then to a medium containing 0.125 sac equivalents of BWSV per ml of medium. The figure represents one of three experiments.

ACh RELEASE BY BWSV IN THE PRESENCE OF AH5183

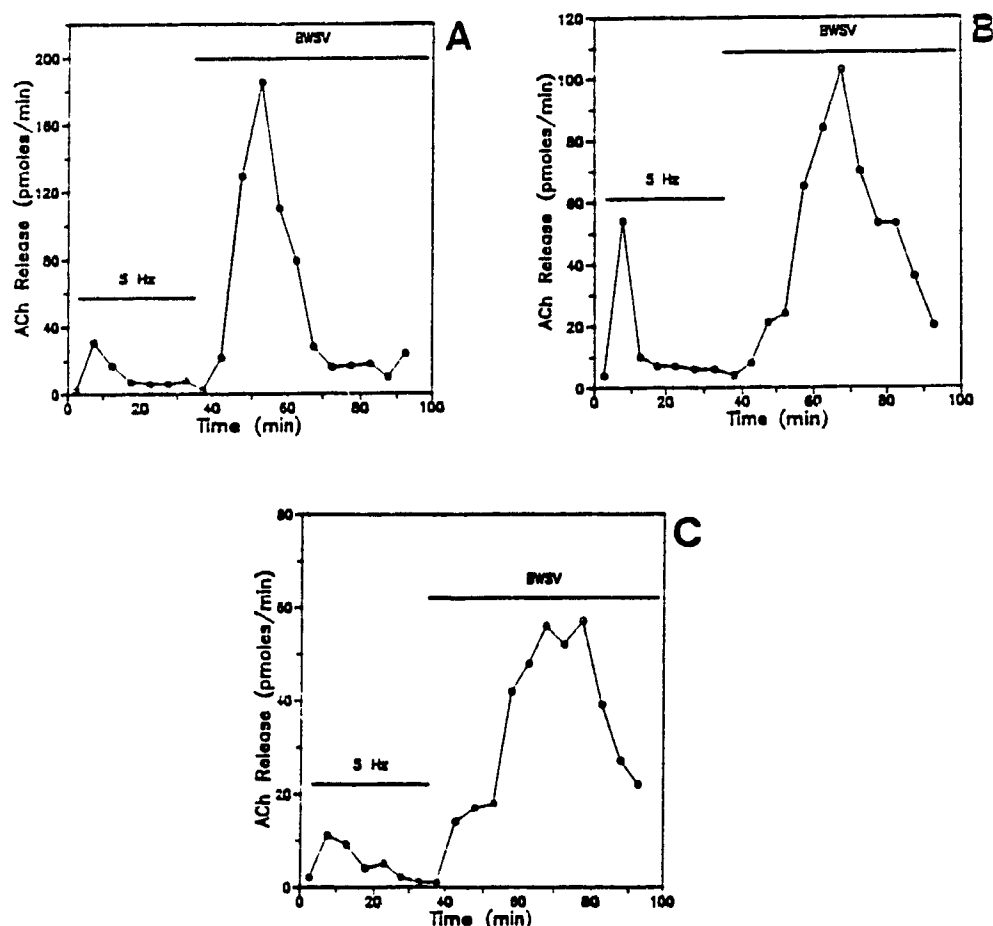


Figure 6R The release of ACh by BWSV in the presence of AH5183 following an AH5183 induced block. Ganglia were perfused with a Krebs solution containing 10 μ M AH5183, 10 μ M choline and 15 μ M eserine while the preganglionic nerve was stimulated for 30 min at 5Hz. Following this, the ganglia were perfused with a similar medium containing BWSV (0.125 sac equivalents of BWSV/ml of medium). Panel A illustrates an early peak in ACh release induced by BWSV, panel B illustrates a late peak of release, and panel C demonstrates a sustained peak of ACh release. This figure illustrates three of five experiments (see table 2R for summary of results of all experiments).

of figure 6R illustrate an early peak release response (A), a late peak (B), and a more sustained peak (C). It is obvious from these findings that not only was the time to peak release variable, but so also was the peak rate of release. These parameters ranged from 20-55 min for the time to peak ACh release and from 44-185 pmoles/min of ACh for the peak rates of release. However, the important feature is that the venom released appreciable amounts of ACh in all the experiments (see table 1R). Calculation of the total amount of ACh liberated by the venom under control conditions and those described here, shows that the quantity is greater after treatment with AH5183 (figure 7R). This difference just fails statistical significance ($p = .09$) due to the large variability of the response under both experimental conditions.

If AH5183 inhibits vesicular uptake of ACh it was expected that the venom induced release of transmitter should be less in an AH5183-blocked ganglion than in a control ganglion and yet the release of ACh from the drug-exposed ganglion was at least equal to the control, if indeed not more; this raises an interesting question: does part of the ACh released by the venom represent only prepackaged ACh or is some of it newly synthesized neurotransmitter? This question could be addressed because, under the conditions of this experiment, there is no inhibition of ACh synthesis by AH5183, at least during the period of preganglionic nerve stimulation (Collier *et al.*, 1986).

Ganglia were prepared for perfusion and treated in the same way as the above experiments with the exception that [^3H]-Ch (10 μM , 790 dpm/pmole) was added to the medium during the 30 min period of preganglionic nerve stimulation in the presence of the drug and that a 10 min washout period for the [^3H]-Ch was given following the stimulation. Figure 8R illustrates the results of two of five experiments. The circles and line show the release of total ACh in response to BWSV and the bars represent the release of [^3H]-ACh. These results indicate that the ACh released by the venom represents not only preformed ACh but that some of the ACh synthesized in the presence of AH5183 is also released. Again, the pattern of release is variable and the two experiments in figure 8R demonstrate the occurrence of an early peak and a late one

Table 1R. Release of ACh from AH5183 and BWSV-treated ganglia.

Experiment #	1	2	3	4	5
ACh release before venom (pmoles/min)	2	5	3	6	6
ACh release at peak venom effect (pmoles/min)	57	185	44	103	47
Time to peak ACh release (min)	45	20	45	35	55
Total ACh released by venom in 60 min (nmoles)	1.96	3.12	1.61	2.82	1.11
Total ACh released by nerve impulses in the presence of AH5183 (pmoles)	144	160	225	449	335

Table 1R The release of ACh from AH5183-treated ganglia exposed to BWSV is given. Ganglia were perfused with a medium containing 10 μ M AH5183 and choline for 30 min while their preganglionic nerves were stimulated at 5 Hz. The perfusion medium was then changed to one containing BWSV and the effluent collected every 5 min for a total of 60 min. The ACh released was determined for each collection period.

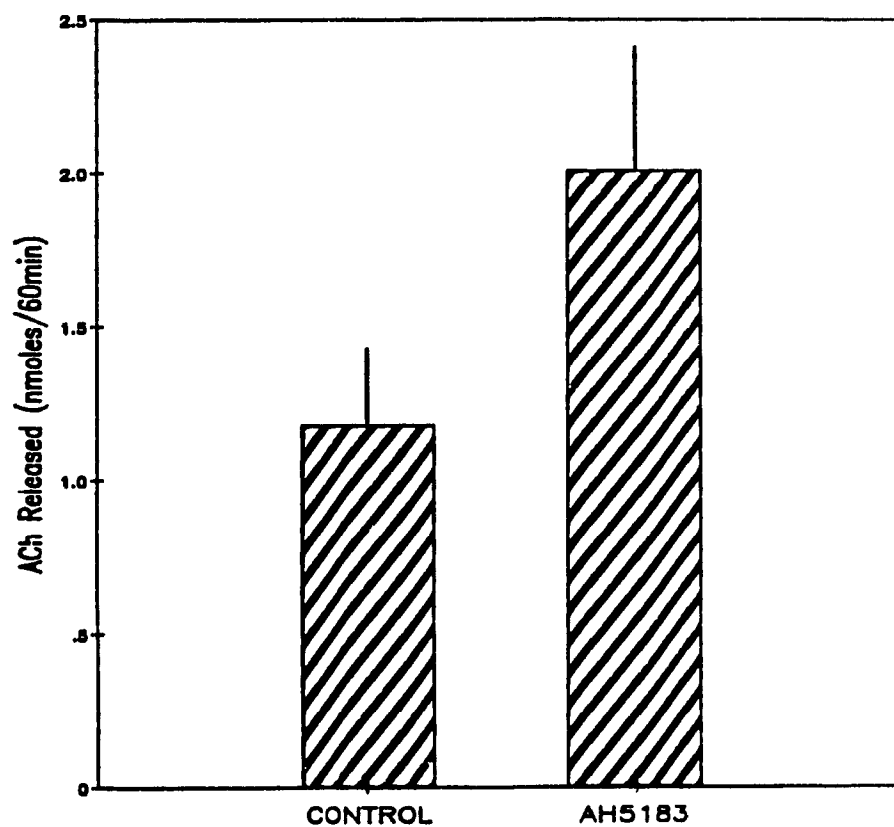


Figure 7R. Total release of ACh by BWSV $\pm$ AH5183. The total amounts of ACh released by ganglia in response to BWSV from the experiments illustrated in figures 2R and 6R. Each bar represents the mean $\pm$ s.e.m. of five experiments.

RELEASE OF ACh AND ^3H -ACh BY BWSV

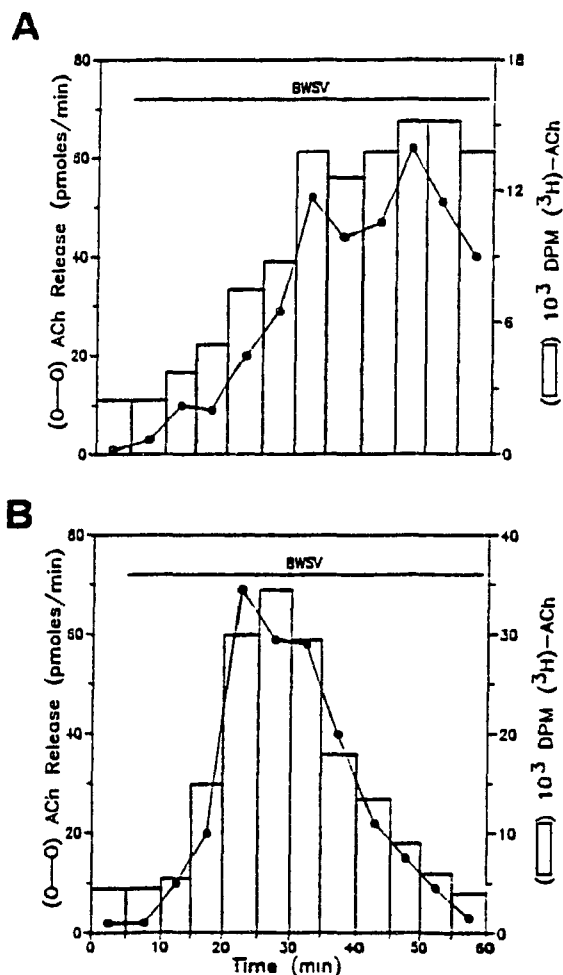


Figure 8R The release of ACh and ^3H -ACh by BWSV. Ganglia were perfused with a medium containing 10 μM AH5183, 10 μM ^3H -choline and 15 μM eserine and the preganglionic nerve stimulated at 5 Hz for 30 min. The ^3H -choline was washed out by a 10 minute perfusion with normal Krebs. Following this period a similar medium with no ^3H but 10 μM choline, 15 μM eserine, and BWSV (0.125 sac equivalents of BWSV/ml of medium) was perfused. The circles and lines represent the release of total ACh and the bars represent the release of ^3H -ACh. Panel A, shows the results of an experiment in which there was a late and sustained peak of ACh release and panel B shows the results of one where there was an early, sharp peak in activity. The figure illustrates two of five experiments (see table 2R for a summary of all results).

Regardless of the pattern of release of ACh both total and [^3H]-ACh are released with the same temporal pattern, suggesting that the two forms of ACh are present in the same compartment with respect to the actions of BWSV. This can be seen in table 2R, where the parameters of time to peak release, peak release of ACh and [^3H]-ACh, and basal release of ACh and [^3H]-ACh prior to venom action, are shown for all five experiments, the peak release of [^3H]-ACh occurs within one collection period of that of ACh.

SUMMARY AND DISCUSSION OF SECTION 3.1

These results suggest the existence of at least two vesicular ACh compartments that are pharmacologically different with regards to AH5183 action. An AH5183-sensitive compartment appears to be directly involved in the nerve impulse directed release of ACh while an AH5183-insensitive compartment appears not to release its ACh into the synaptic cleft in response to this stimulus. A model of the terminal based on the behavior of these compartments is illustrated in figure 9R. This model is similar to the one proposed by Birks and MacIntosh (1961) but includes the possible site of action of AH5183 with respect to the release process.

To further investigate the possible existence and characteristics of these two pools of vesicular ACh requires the measurement of ACh in a variety of subcellular compartments. The cat SCG does not readily lend itself to subcellular fractionation because of the large amount of connective tissue. Therefore, to pursue this problem the hippocampus of the rat brain (HIPPO) was used. This tissue appeared reasonable for such experiments for the following reasons: first, most of its cholinergic input is in the form of nerve terminals and not interneurons which make it similar to the SCG with respect to its cholinergic input, second, it is a tissue that lends itself readily to homogenization and the isolation of subcellular compartments; and third, techniques for the incubation and manipulation of this tissue are well characterized.

Table 2R. Release of ACh and [^3H]ACh from AH5183 and BWSV-treated ganglia.

Experiment #	1	2	3	4	5
ACh release before venom (pmol/min)	2	5	3	1	2
ACh release at peak venom effect (pmol/min)	87	57	50	68	62
Time to peak effect on total ACh release (min)	25	50	45	25	50
[^3H]ACh release before venom (dpm/min)	3,010	2,710	2,402	4,501	2,526
[^3H]ACh release at peak venom effect (dpm/min)	12,003	8,131	8,008	34,509	15,154
Time to peak effect on [^3H]ACh release (min)	25	45	40	30	50

Table 2R. The release of total ACh and [^3H]-ACh from AH5183-treated ganglia exposed to BWSV is given. Ganglia were perfused with a medium containing 10 μM AH5183 and 10 μM [^3H]-Ch for 30 min while their preganglionic nerves were stimulated at 5 Hz. The perfusion medium was changed to one free of [^3H]-Ch and the stimulation stopped. After a 10 min washout of the residual [^3H]-Ch the perfusate was changed to one containing BWSV and the effluent collected every 5 min for a total of 60 min. Total ACh and [^3H]-ACh were determined for each collection period.

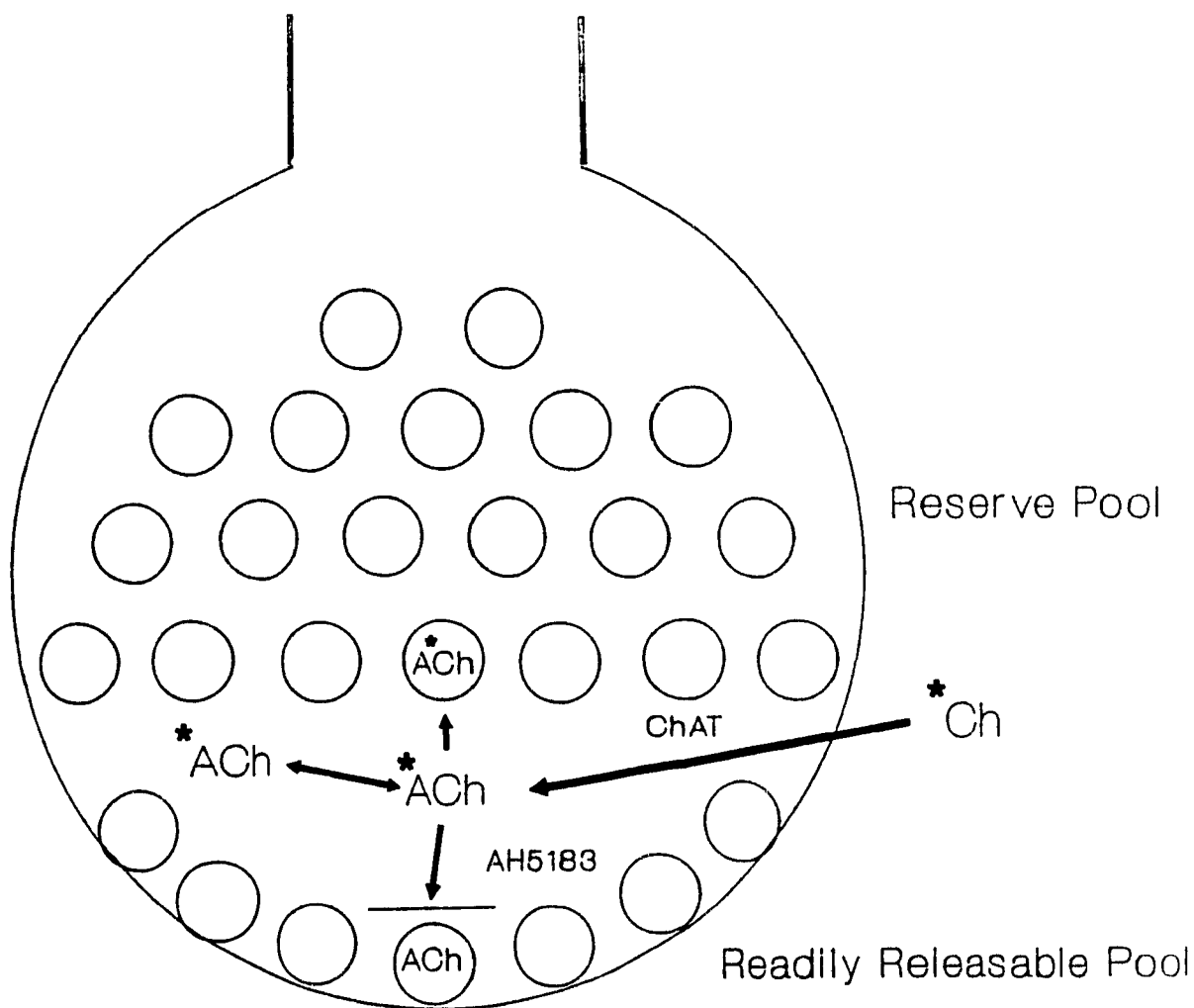


Figure 9R Model of the cholinergic terminal The model illustrated above is based on the results of section 3 1. It points out the site of action of AH5183 as being on the readily releasable pool, it also contains a reserve pool which is not affected by AH5183. The terminology for the vesicular pools of ACh are those of Birks and MacIntosh, 1961.

3.2 THE READILY RELEASABLE POOL OF ACETYLCHOLINE IN HIPPOCAMPAL BRAIN SLICES AND SYNAPTOSOMES.

The term, readily releasable pool, refers to that compartment of ACh which first releases its store of neurotransmitter upon stimulation. As discussed by Birks and MacIntosh (1961), using a kinetic model of ACh release, in the cat SCG this compartment accounts for about 14% of the total ACh of the ganglia; as well, Collier *et al.* (1986), and the results of section 3.1.1, also found this compartment to be 14% in the cat SCG by measuring the total amount of ACh released by nerve impulses in the presence of the drug AH5183.

Kinetic studies on the release of ACh from HIPP slices by nerve impulses are difficult to do. Therefore, AH5183 was used to estimate the size of the readily releasable pool in the hippocampal formation. The size of this compartment was estimated by measuring the decrease in the ACh content of HIPP slices, and of the synaptosomal fraction prepared from them, when the slices were stimulated with 35 mM K^+ in the presence of AH5183 and HC-3. The rationale was that a decrease in ACh content would result from its being liberated, while HC-3 would block further ACh synthesis. The Krebs medium in these experiments did not contain eserine because this lead to a differential increase in the ACh content of HIPP slices and the synaptosomal fraction derived from them (table 3R). The estimate of the readily releasable pool would thus be skewed disproportionately between slices and synaptosomes derived from them.

HIPP slices were prepared for incubation as described in the materials and methods. Slices were given a 45 min recovery period in a Krebs solution that contained 10 μ M choline. Control slices were incubated for another 10 min in this medium and then, for the remaining 50 min of the experiment, the medium was changed to one that also contained 10 μ M HC-3; the incubation medium was changed every 10 min. In the case of the AH5183-exposed slices, following the recovery period, they were incubated with a Krebs medium that contained 10 μ M AH5183 and 10 μ M choline for 10 min. The slices were then incubated for a further 10 min

Table 3R. Effects of eserine on ACh content.

Tissue Fraction	ACh Content	% Change
	* =nmoles/g tissue weight ** =pmoles/mg synaptosomal protein	
Total tissue (-eserine)	43.8 +/- 03.4 *	----
Total tissue (+eserine)	96.0 +/- 07.1 *	120 +/- 8
P ₂ (-eserine)	1360 +/- 40 **	----
P ₂ (+eserine)	2060 +/- 120 **	51 +/- 4

Table 3R The effect of eserine on the hippocampal slice's and synaptosomal fraction's content of ACh is shown. Hippocampal slices were prepared for incubation as described in the materials and methods and were then given a 45 min recovery period in which the slices were incubated in a 10 μ M choline containing Krebs medium. During this recovery period half of the slices were also exposed to 15 μ M eserine. The slices were then washed in an eserinated physiological saline (15 μ M eserine) at 0 °C and homogenized in 0.32 M sucrose containing 15 μ M eserine, 5 mM HEPES, and 1mM EGTA at 0 °C. Part of the homogenate was used to determine the total ACh content of the slices and the rest was processed to isolate the P₂ pellet as described in the materials and methods. The ACh content of the slices and the P₂ pellet was determined. Each entry represents the mean $\pm$ s.e.m from 3 hippocampal formations.

with a similar medium containing 10 μ M HC-3. Drug-exposed slices were then stimulated by a 35 mM K^+ Krebs medium containing AH5183, choline, and HC-3 at 10 μ M concentrations for the remaining 50 min. Again, the medium was changed every 10 min. In order to determine the size of the change in the ACh content following the recovery period and every 10 min interval afterwards, HIPPP slices were homogenized in a sucrose medium containing 15 μ M eserine, their total content of ACh measured, the synaptosomal fraction isolated as described in the materials and methods, and total synaptosomal ACh content determined. The top panel of figure 10R outlines the experiment. At all time points, one hippocampal formation served as the source of the control slices and its contralateral partner that of the drug exposed slices.

The two lower panels of figure 10R show the results of the measures of the ACh content of both the tissue and the synaptosomal fraction. Tissue exposed to AH5183 contained less ACh than did those not exposed to the drug. The decrease in slice ACh content amounted to 19.8 ± 2.2 and that of the synaptosomal ACh 26.7 ± 0.5 . Thus, the readily releasable pool of ACh in the hippocampal formation appears to be about 20% if determined by measuring total tissue ACh content and about 27% if determined by measuring the synaptosomal fraction's ACh content.

SUMMARY AND DISCUSSION OF SECTION 3.2

The experiments of section 3.2 attempt to quantify the size of the readily releasable pool of ACh in HIPPP slices. The results from this section indicate that estimation of the size of this compartment is somewhat dependent on whether one measures the decrease in total tissue ACh content or the decrease in the synaptosomal fraction. Because the synaptosomal fraction contains the nerve endings, the measurements from this fraction (27%) may represent the best estimate of the readily releasable pool.

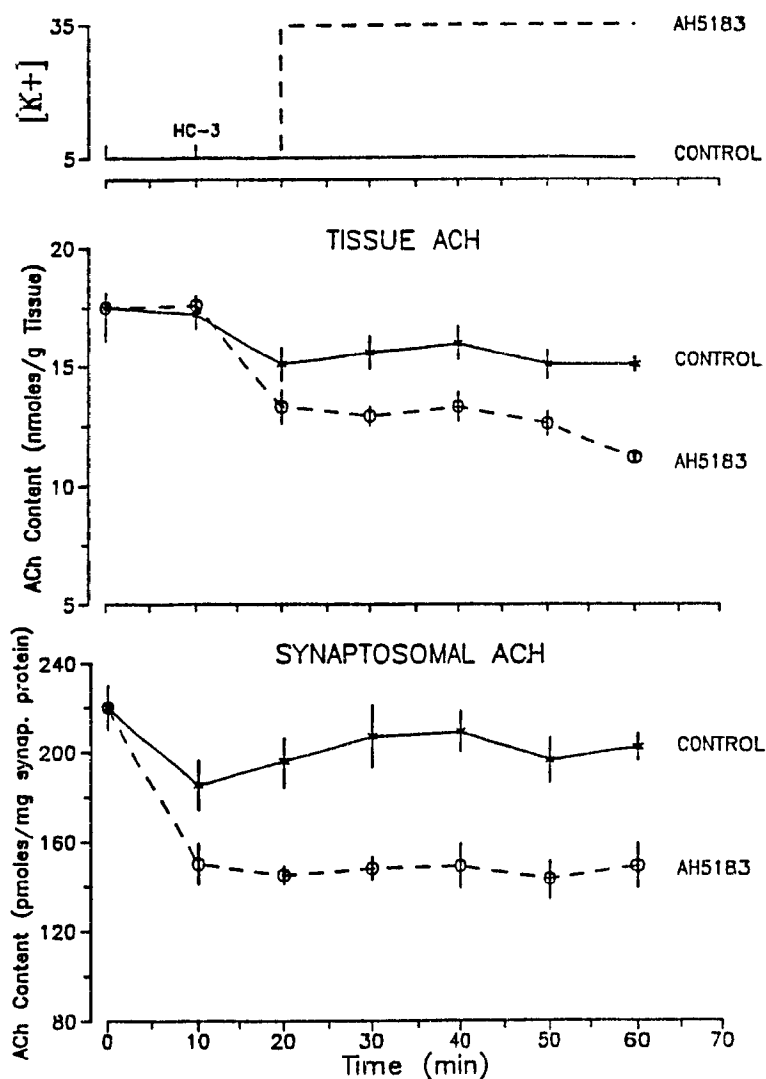


Figure 10R. The readily releasable pool of ACh in the hippocampus of the rat. Hippocampal slices were given a 45 min recovery period. They were incubated with a Krebs medium containing 10 μ M choline and no eserine. AH5183 treated slices were then exposed to a 10 μ M concentration of the drug for the duration of the experiment. The upper panel indicates where HC-3 (10 μ M) was added for the duration of the experiment. AH5183-exposed slices were stimulated with a Krebs medium containing 35 mM K^+ , HC-3, and AH5183 as marked in the upper panel. Incubation of the slices was stopped at ten minute intervals and the tissue and synaptosomal ACh determined as described in the materials and methods. The middle panel illustrates the change in the total tissue ACh content and the lower panel that of the synaptosomal ACh. The solid line represent the control slices and the dashed line the AH5183-treated ones. Each point is the mean $\pm$ s.e.m. of 10 determinations.

3.3 THE EFFECT OF AH5183 AND α -LTX ON THE RELEASE AND STORAGE OF ACETYLCHOLINE FROM RAT HIPPOCAMPAL BRAIN SLICES

The response of the hippocampus of the rat to AH5183 and to α -LTX, the active component of BWSV, was studied next in an attempt to characterize the various pools of ACh discussed in section 3.1. The prediction was that, in the presence of AH5183, high K^+ would release the 20-30% of hippocampal ACh identified as "readily releasable" by the experiments of section 3.2 and that α -LTX might release the residual ACh; measures of subcellular fractions containing ACh would then test whether the two compartments, the AH5183 sensitive and insensitive compartments, could be separated biochemically. Towards this end, we designed experiments to answer the following four points: first, what is the release profile of ACh in the presence and absence of AH5183 and does α -LTX cause the release of ACh following stimulation in the presence of AH5183?; second, what is the effect of these treatments on the tissue content of ACh and on its distribution between cytoplasmic and bound compartments in the synaptosome?; third, what is the effect of these treatments on the occluded pools of ACh?; and fourth, what is the effect of the α -LTX on the efflux rate of [^3H]-ACh?

3.3.1 The release of acetylcholine from hippocampal slices in the presence and absence of AH5183 $\pm$ α -LTX.

It was first necessary to determine how AH5183 affects the release of ACh from the HIPP in response to a high K^+ Krebs medium and what α -LTX would do to the release of ACh from control and AH5183-exposed slices. Slices of the HIPP were prepared for incubation as described in the materials and methods. Following the recovery period (45 min), control slices were left in normal Krebs for 30 min with three changes of the medium and were then exposed to a Krebs solution containing 35 mM K^+ (high K^+) for another 30 min; samples were collected every 10 min with each change in the bathing medium. Slices used in AH5183 experiments were exposed to a 10 μ M solution of the drug following the first

rest period and all subsequent incubations of these slices contained the drug. Following the 30 min incubations at rest, AH5183-treated slices were incubated for 30 min in a high K^+ Krebs medium. Subsequent to the high K^+ stimulation both sets of slices, drug-free and drug-exposed, were returned to a normal Krebs medium for 10 min following which they received a medium also containing hemicholinium-3 (HC-3) at a 10 μ M concentration. The HC-3 was added at this point so that further entry of choline by the high affinity uptake system would not occur and, thus, neither would further synthesis of ACh, making changes in the different intracellular pools of ACh more evident. Along with the HC-3, half of the HIPP received a 3 nM concentration of α -LTX so that the venom effect could be compared to the unexposed slices of both groups. Figure 11R outlines the procedures followed for both control and AH5183 treated slices.

Figure 12R. shows the results of these experiments. Panel A shows that 35 mM K^+ increased the release of ACh from drug-free slices by 6-10 times that seen under basal conditions, with a plateau in the release rate occurring in the second 10 min incubation with high K^+ . Upon returning to a normal Krebs medium (5 mM K^+) the rate of release decreased to pre-stimulation levels when slices were not exposed to the toxin. However, when α -LTX was included in the final incubation, it stimulated the release of ACh by a factor of approximately 4 versus the toxin-free slices. Panel B illustrates the results obtained with the AH5183-treated slices. In the presence of AH5183 high K^+ stimulated release of ACh was markedly attenuated, although, unlike the cat SCG, the slices did not show a peak of activity and then a subsequent decrease to basal levels of release when stimulated in the presence of the drug. This could be a peculiarity of the tissue or of high K^+ stimulation. Nevertheless, release was severely affected by the drug with 35 mM K^+ only doubling the release of transmitter when AH5183 was present. Switching the medium back to one containing 5 mM K^+ brought the release of ACh a little below the pre-stimulation rate. Exposure of the drug-treated slices to α -LTX caused a four fold increase in the release of ACh over basal rates. In fact, the release of transmitter

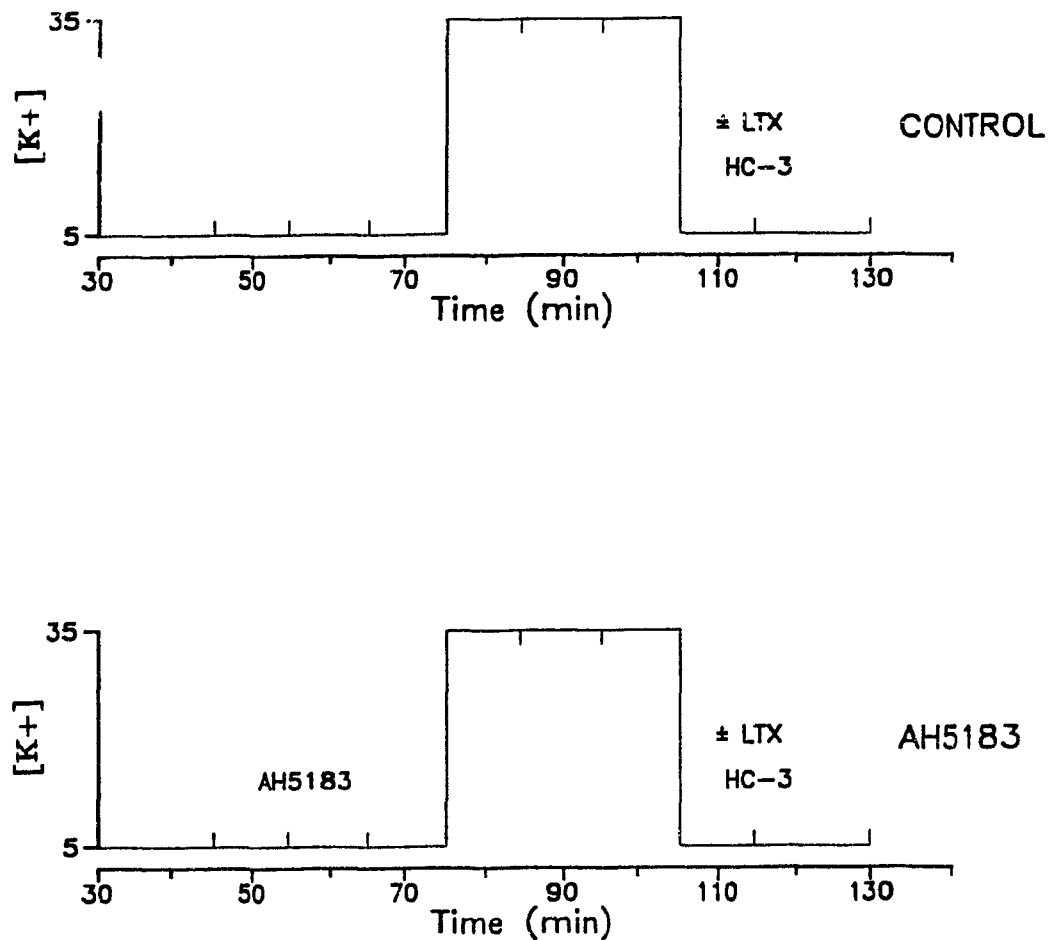


Figure 11R. Outline of the incubation procedure used in the hippocampal slices. Control slices were incubated in a Krebs medium containing 10 μ M choline and 15 μ M eserine. Stimulation by high K^+ was achieved by raising the K^+ concentration to 35 mM while decreasing the Na^+ concentration by the same amount. These slices were incubated in a medium containing 10 μ M HC-3 during the last incubation period or received the HC-3 and 3 nM α -LTX. AH5183 slices were treated in the exact same way except that 10 μ M AH5183 was present in the incubation medium starting where indicated in the lower illustration and was present for the duration of the experiment. The tick marks on the line indicate a change in the incubation media.

RELEASE OF ACh BY α -LTX FROM HIPPOCAMPAL SLICES

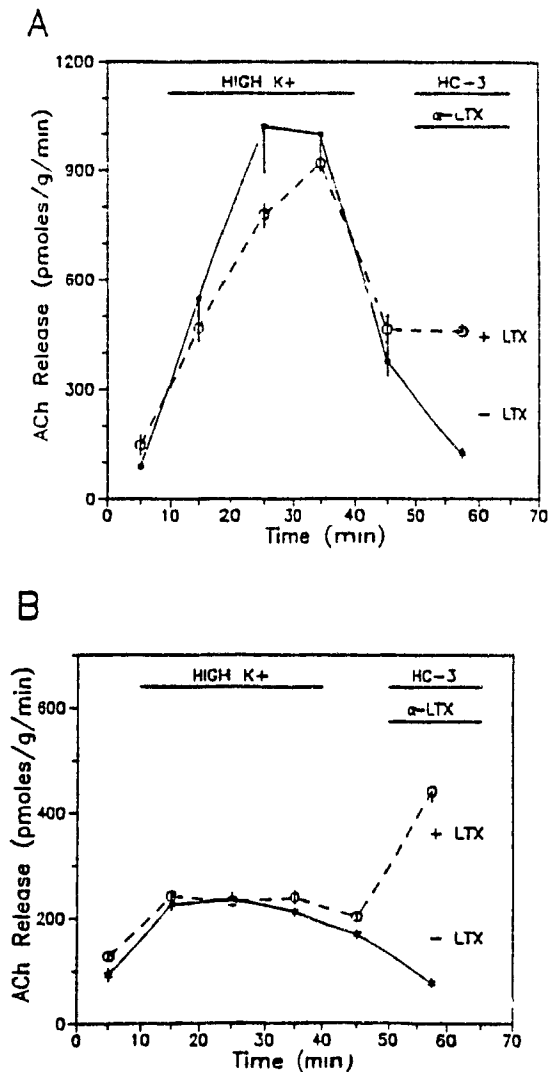


Figure 12R. Release of ACh by high K^+ and α -LTX from hippocampal slices in the presence and absence of AH5183. Slices were treated as described in figure 11R. Slices were stimulated with a 35 mM K^+ containing Krebs medium where indicated. HC-3 refers to where the hemicholinium-3 was added at a concentration of 10 μ M and in the toxin-exposed slices α -LTX refers to the exposure of slices to 3 nM toxin. The solid line represents the response of the toxin-free slices and the dashed line to the toxin-exposed ones. A) Slices not exposed to AH5183. B) Slices exposed to 10 μ M AH5183. Each point is the mean $\pm$ s.e.m. of 12 determinations.

induced by the toxin was the same whether AH5183 was present (448 ± 18 pmoles) or not (462 ± 16 pmoles). Furthermore, the toxin induced release of ACh, in the presence of AH5183, was twice that brought about by high K^+ in the same slices.

These results indicate that AH5183 inhibits ACh release by high K^+ and that, like the cat SCG, toxin is not prevented from releasing the transmitter even after the "readily releasable" pool of ACh has supposedly been emptied.

3.3.2 The tissue acetylcholine content and the subcellular acetylcholine distribution from synaptosomes of hippocampal slices exposed to AH5183 and α -LTX.

Because it was important to the interpretation of the results that no net new synthesis take place on exposure to the toxin, the total amounts of ACh, content plus release, were calculated. If HC-3 blocked any further new synthesis of ACh that might be taking place in slices exposed to α -LTX, then any increase in ACh release must be compensated by a decrease in tissue ACh content. After slices were incubated using the protocols illustrated in figure 11R they were homogenized as described in the materials and methods and a portion of the homogenate used for the determination of total tissue ACh. Table 4R shows the results of these measures and those obtained from the release data. Slices not exposed to AH5183 released about 2.7 nmoles of ACh per gram of tissue when α -LTX was present and their ACh content decreased by approximately 4.9 nmoles/g. When the total amount of ACh was summed it was obvious that no net synthesis of ACh took place when the slices were exposed to the toxin in the presence of HC-3 (64.7 vs. 62.4 nmoles/g, control vs. control + α -LTX). In slices exposed to AH5183, treatment with the toxin increased release by 6.6 nmoles/g while decreasing the content of the slices by 7.3 nmoles/g. Both of these changes were significant but there was no statistically significant change in the summed total ACh (45.5 vs. 44.8 nmoles/g, AH5183 vs. AH5183 + α -LTX).

Table 4R. Effects of AH5183 and α -LTX on ACh release and tissue content.

EXPERIMENT	CONTROL	CONTROL+LTX	AH5183	AH5183+LTX
Release (nmoles/g)	32.1 +/- 3.5	34.8 +/- 1.2	10.5 +/- 0.4	17.1 +/- 0.4*
Content (nmoles/g)	32.5 +/- 2.1	27.6 +/- 1.6	35.0 +/- 2.2	27.7 +/- 2.0*
Total (nmoles/g)	64.7 +/- 5.2	62.4 +/- 2.1	45.5 +/- 2.0	44.8 +/- 2.0

Table 4R The total release and tissue content of ACh from hippocampal slices exposed to AH5183 and α -LTX. Hippocampal slices were prepared for incubation and treated as outlined in figure 11R. The ACh released at each collection period was summed and the ACh content of the slices determined as described in the materials and methods. Total ACh was calculated by adding the released ACh and the ACh content for every set of hippocampal slices. Each entry represents the mean $\pm$ s.e.m. for 12 determinations. (*) indicates a $p < 0.05$ for values obtained in the presence or absence of α -LTX (cont versus cont + α -LTX; AH5183 versus AH5183 + α -LTX) by unpaired student t-test.

Since exposure of the slices to α -LTX in the presence of HC-3 did not lead to a net increased synthesis of ACh, it was expected that changes in the levels of ACh found in the various subcellular fractions would reflect mobilization of the ACh from these pools by the toxin. To investigate where the ACh released by α -LTX came from, the homogenates of slices, treated as described in figure 11R, were centrifuged to yield a P_2 pellet (synaptosomal pellet) as described in the materials and methods. The pellet was lysed and then centrifuged so as to separate the free compartments of synaptosomes (cytosolic, S_3) from the bound compartments (occluded, P_3) (see materials and methods). The ACh of both fractions was then extracted and measured. Figure 13R shows the results of these measures. In control slices (panel A, figure 13R) both the S_3 and P_3 fraction's content of ACh was lowered by the toxin. The P_3 fraction showed a decrease of 32 pmoles/mg synaptosomal protein (pmoles/mg syn prot) (a 46% drop) and the S_3 fraction a decrease of 55 pmoles/mg syn prot (a 26% drop); both changes were statistically significant. Similarly, when slices were exposed to AH5183, the toxin effected a decrease in both the free and occluded fractions (panel B, figure 13R). The P_3 fraction's ACh content decreased by 9 pmoles/mg syn prot (a 28% drop) and the S_3 fraction's ACh content was decreased by 237 pmoles/mg syn prot (a 56% drop). Unlike the drug-free slices, in AH5183-exposed slices only the change in the S_3 fraction was found to be statistically significant.

It is interesting to note that in the absence of α -LTX, AH5183 caused a decrease in the occluded compartment's ACh content (a decrease of 37 pmoles/mg syn prot) and an increase in the free compartment's ACh (an increase of 98 pmoles/mg syn prot). This finding is in agreement with previously published work (Otero *et al.*, 1985; Ricny and Collier, 1986; Suszkiw and Toth, 1986) and with the notion that the drug inhibits vesicular uptake of cytoplasmic ACh.

3.3.3 The acetylcholine content of the bound subcellular compartments from hippocampal slices exposed to AH5183 and α -LTX.

The ability of α -LTX to decrease the ACh content of the P_3 fraction was significant in control slices, whereas in drug-exposed slices the effect was small. Because the P_3 fraction is an amalgamation of a variety of subcellular bound compartments, further separation of these compartments was attempted so as to investigate whether the α -LTX mobilization of ACh was different in drug-free slices and slices exposed to AH5183. Experiments were therefore designed to look specifically at these subcellular pools. HIPP slices were prepared as described above and the same experiments outlined in figure 11R were done except that eserine was not present in the incubation medium. Slices were homogenized, the synaptosomal pellet isolated and lysed, and the resulting lysate layered on a discontinuous sucrose gradient and centrifuged as described in the materials and methods. The layers were then separated and each layer's ACh was extracted and assayed. The cellular constituents of the three fractions, D, E&F, and G&H, are described in the materials and methods.

Figure 14R shows the results of the ACh contents obtained from these experiments. In slices not exposed to AH5183, toxin did not have the expected large change (panel A, fig 14R). The D fraction was the most affected by the toxin with a significant decrease of 8 pmoles/mg syn prot of ACh while the G&H and the E&F fractions showed no significant change. When slices were exposed to AH5183 the toxin caused a decrease in all three fractions' content of ACh (panel B, fig 14R) and this drop in ACh content was similar for all the fractions, about 8 pmoles/mg syn prot (D, 8 pmoles; E&F, 7 pmoles; G&H, 9 pmoles), all the toxin induced decreases in the subcellular-bound fractions of the drug-exposed slices were statistically significant.

It is interesting that α -LTX decreased the content of all fractions from the AH5183-treated slices, showing no selectivity for one over another, whereas in the toxin-exposed control slices the toxin only decreased the D fraction's ACh content. In both control and AH5183-treated slices the ACh content of the D fraction was lowered by the same amount.

ACH CONTENT OF THE P₃ & S₃ FRACTIONS OF HIPPOCAMPAL SLICES +/- LTX

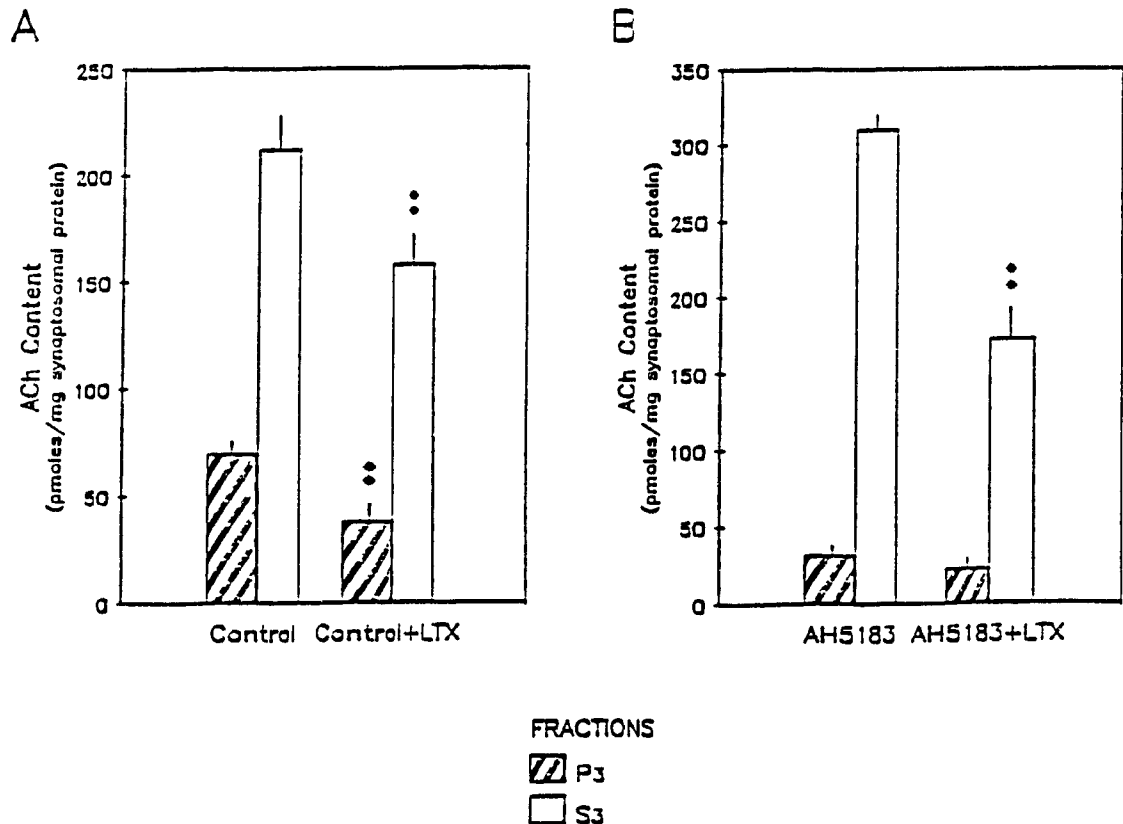


Figure 13R. The ACh content of the P₃ and S₃ fractions from slices treated with or without α -LTX. Synaptosomes were isolated from the homogenates of slices incubated according to the protocols outlined in figure 11R. The crude synaptosomal pellets were then lysed with a weakly buffered aqueous solution of 5 mM HEPES, 1 mM EGTA and 15 μ M eserine so as to separate the intrasynaptosomal cytosolic (S₃) and occluded (P₃) compartments of ACh (see materials and methods). A) Results of AH5183-free slices. B) Results of AH5183-exposed slices. Each bar represents the mean $\pm$ s.e.m. of 12 determinations. Statistical significance was calculated using the student t-test for unpaired data. (*) signifies a p < 0.01. The comparison made is between control and control + α -LTX and between AH5183 and AH5183 + α -LTX.

ACH CONTENT OF THE BOUND SUBCELLULAR POOLS OF SLICES +/- LTX

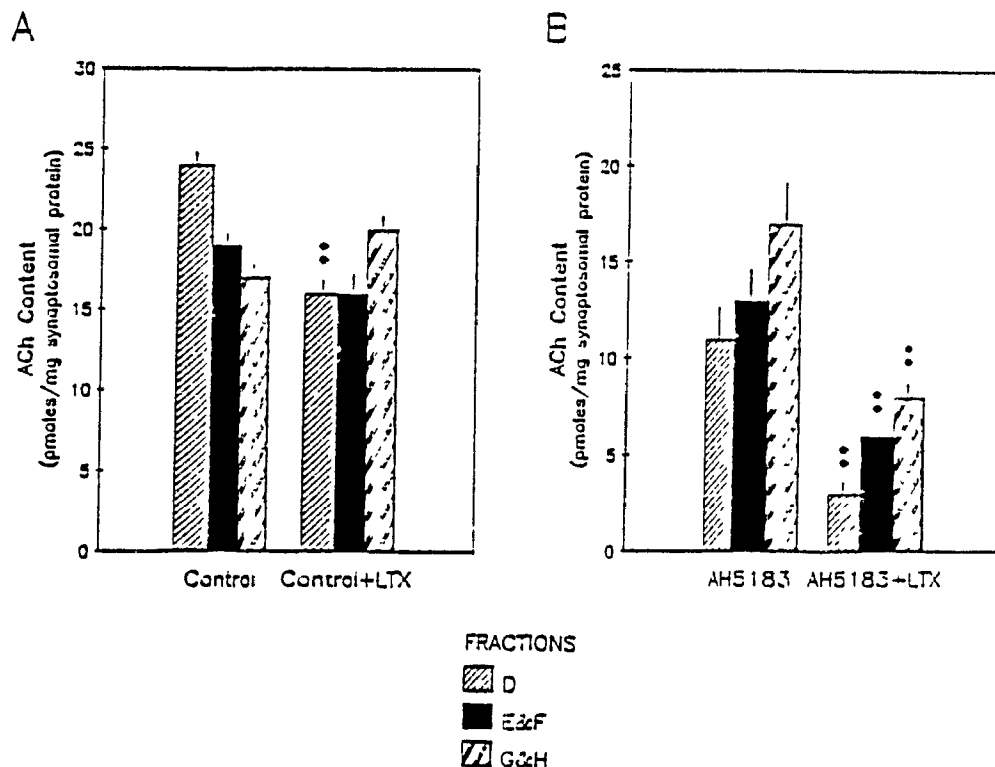


Figure 14R. The ACh content of the bound-subcellular compartments of slices treated with or without α -LTX. Slices were incubated following the protocol outlined in figure 11R except that eserine was not present in the different media. Slices were homogenized in a 0.32 M sucrose solution containing 5 mM HEPES and 1 mM EGTA. The synaptosomal pellet was then lysed using a 5 mM HEPES and 1 mM EGTA solution. The lysate was then layered and fractionated on a discontinuous sucrose gradient. For the contents of the D, E&F, and G&H fractions see the materials and methods. A) Results from AH5183-free slices. B) Results from AH5183-exposed slices. Each bar represents the mean $\pm$ s.e.m. of 12 determinations except for the Control slices where only 6 determinations are graphed. Statistical significance was calculated using the student t-test for unpaired data. (*) signifies a $p < 0.01$. The comparison is between control and control + α -LTX and between AH5183 and AH5183 + α -LTX.

3.3.4. The release of acetyldiethylhomocholine by α -LTX from hippocampal slices.

The experiments described in section 3.3.2 indicated that α -LTX not only affected the occluded pools of ACh (P_3) but that the cytosolic fraction's ACh (S_3) was also affected and that the largest absolute change was associated with the cytosolic fraction. These experiments can not distinguish between 2 possibilities that could explain the observed effect on the S_3 fraction; one, that ACh from the cytoplasm was released via a limited recycling of vesicles (exocytosis) and two, that the membrane was altered in a way that led to the direct liberation of ACh through the membrane. It was therefore important to investigate the possibility that the toxin alters the membrane of HIPP slices in a fashion that could lead to a change in its permeability to molecules like ACh. Therefore, experiments using the compound DEHCh (see section 3.1.2) were designed to test the possibility of an altered membrane permeability. HIPP slices were prepared for incubation as previously described, and following a 50 min recovery period, with one change of the incubation media, they were exposed for 20 min to a 5 mM K^+ Krebs medium containing 10 μ M AH5183, 50 μ M [^{3} H]-DEHCh, and no choline. The medium was then changed to one of similar composition except that the K^+ concentration was raised to 35 mM with an appropriate molar decrease of the Na^+ concentration for 20 min with one change of the medium after 10 min. This was done so as to load the slices with the labeled DEHCh. The slices were then incubated for 10 min in a high K^+ medium containing 10 μ M AH5183 and 10 μ M choline, but no [^{3} H]-DEHCh. This was followed by four 5 min incubations in a normal K^+ Krebs medium containing AH5183 and choline. The last high K^+ incubation and the basal incubations were needed to wash out the unincorporated [^{3} H]-DEHCh. α -LTX was then added to half of the HIPP slices at a 3 nM concentration for 15 min while the other half was given another basal incubation of 15 min duration.

Figure 15R shows the toxin induced release of both ACh and [^{3} H]-ADEHCh in these experiments. As illustrated in the figure (panel A), the release of ACh increased by about 5 fold when the slices were ex-

posed to α -LTX (67 vs. 322 pmoles/min/g wet tissue weight (- vs + toxin)). This increase in the release of ACh is in the same range as reported in section 3.3.1. Also, the α -LTX caused a statistically significant increase in the rate of [^{3} H]-ACh release from the HIPP slices (panel B), from 11689 to 15011 dpm/g tissue weight/min.

These results indicate that the α -LTX is not just causing the release of neurotransmitter from the vesicular compartments, but that under these conditions, there is a change in the HIPP slices' plasma membrane that allows for the release of cytosolic components in the size range of ACh, presumably not via exocytosis.

SUMMARY AND DISCUSSION OF SECTION 3.3

The results of these experiments demonstrate that α -LTX is able to release ACh from HIPP slices whether they are blocked by AH5183 or maintained in a drug free medium. Furthermore, the toxin is able to elicit the release of ACh from both the occluded (P_3) and cytosolic (S_3) fractions. Unlike the results of section 3.1.2 however, the release of ACh appears not to be wholly the result of vesicular exocytosis. It is therefore likely that at least part of the released ACh comes from the cytoplasmic compartment directly by efflux through the plasma membrane. That α -LTX has an effect on the bound-subcellular compartments is evident from the results shown in section 3.2.3, but what proportion of the released ACh is made up from this component cannot be ascertained.

3.4 THE EFFECT OF AH5183 ON THE STORAGE AND TURNOVER OF ACETYLCHOLINE FROM HIPPOCAMPAL SLICES.

It is not clear whether AH5183 affects the turnover of ACh in the various occluded intracellular compartments as a result of stimulation. The results obtained in section 3.1.4 raise the possibility that some vesicles may be insensitive to the drug's inhibition of ACh vesicular uptake under stimulating conditions, but no direct evidence is available.

RELEASE OF ACh AND ADEHCh BY α -LTX

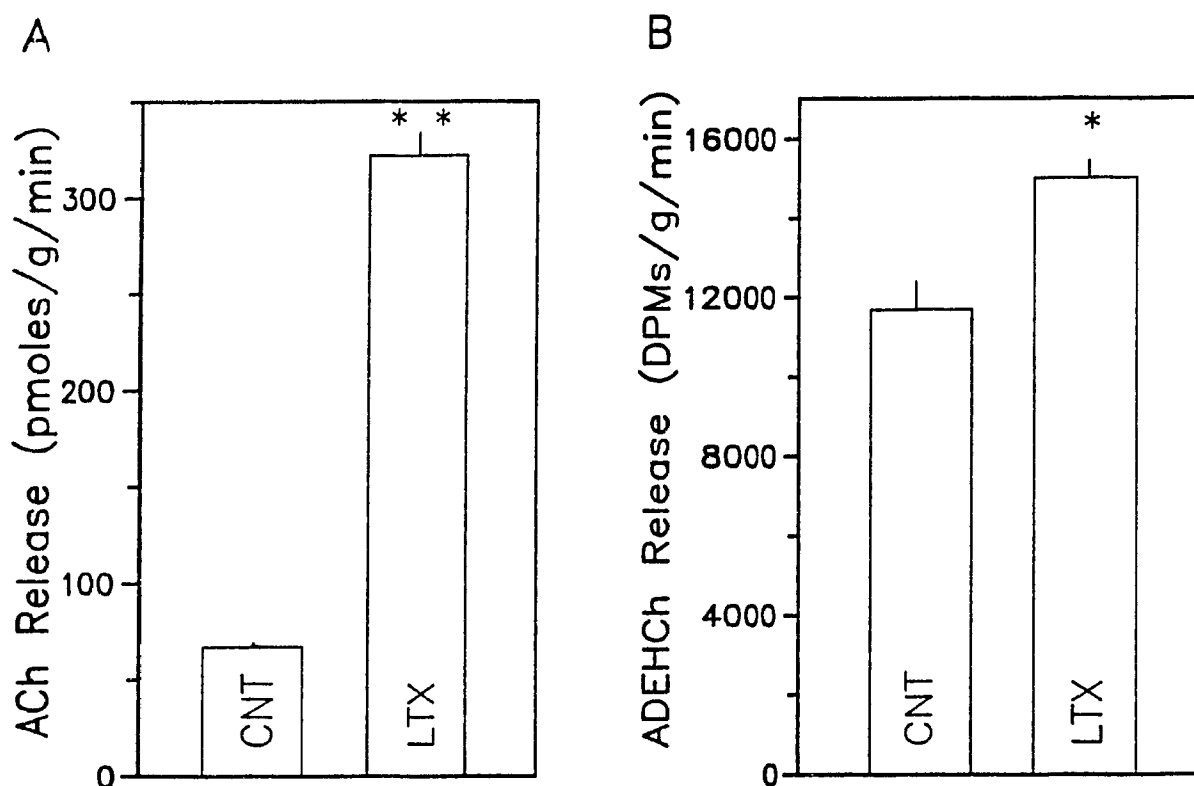


Figure 15R. The release of ACh and ADEHCh by α -LTX. Slices were incubated as outlined in the text. All media used contained 15 μ M eserine. A) illustration of the release of ACh from slices following the addition of 3 nM α -LTX (LTX) or when left at rest (CNT). B) illustration of the release of [^{3} H]-ADEHCh during the exposure to 3 nM α -LTX (LTX) or when slices were left at rest (CNT). Each bar represents the mean $\pm$ s.e.m. for 3 experiments. (***) signifies a $p < 0.01$ and (*) signifies a $p < 0.05$ by the use of a student t-test for unpaired data.

able. Measuring the turnover of ACh in HIPP slices in the presence of AH5183 requires that a comparison be made between slices left at rest and stimulated slices because the drug is known to only weakly inhibit the passive vesicular uptake of ACh (Anderson et al., 1983). Experiments were designed to test if AH5183 affects the turnover of ACh in any of the occluded compartments.

3.4.1 The effect of AH5183 on the release of acetylcholine and its turnover in the cytoplasmic and occluded compartments of rested and stimulated hippocampal slices.

The turnover in the ACh of any compartment can be estimated by the use of [^3H]-Ch. This is done by allowing the tissue to take up the [^3H]-Ch and convert it to [^3H]-ACh. As the preformed ACh leaves a compartment some of the [^3H]-ACh may enter and thereby increase the specific activity of the ACh in that compartment. This increase in specific activity is therefore a reflection of the rate at which ACh is turning over in that compartment.

HIPP slices were prepared for incubation as previously described (materials and methods). Control slices were given a 55 min recovery period in a normal Krebs medium with one change of the incubation medium after 45 min. AH5183-exposed slices were given a 45 min recovery period and were then exposed to 10 μM AH5183, 10 min prior to the collection of the first sample and for the duration of the experiment. All slices were left at rest for a further 20 min. Half of the slices in the control and drug exposed groups were then stimulated by exposure to a Krebs medium containing 35 mM K^+ and 10 μM [^3H]-Ch (440 dpm/pmole) for 20 min (with a change of medium every 10 min), the other half were left at rest but received the [^3H]-Ch. All slices were then left at rest for 40 min in a medium containing the [^3H]-Ch with the medium changed every 20 min. Figure 16R outlines the procedure used in the incubation of these slices.

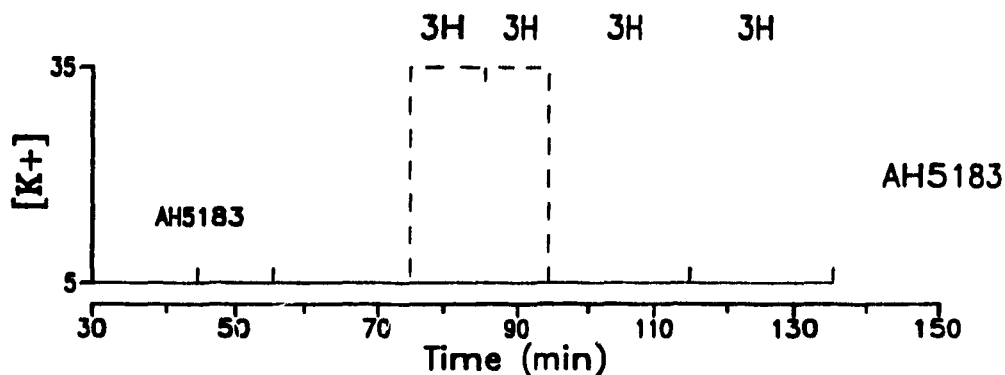
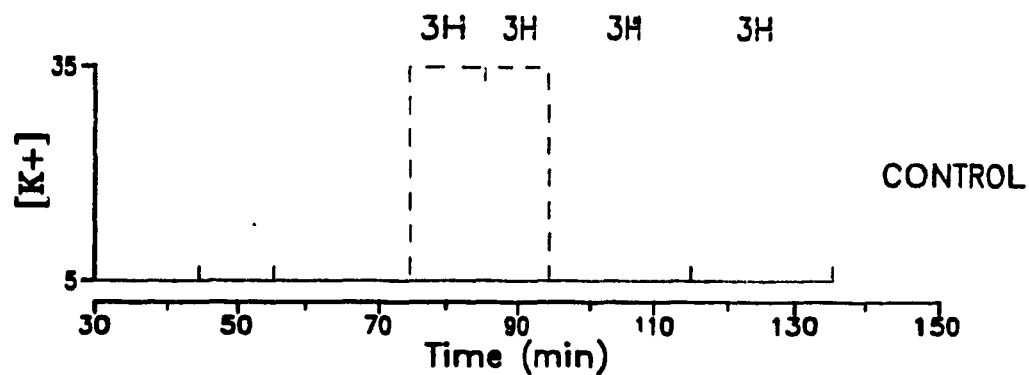


Figure 16R. Experimental protocol to test the turnover of ACh in the subcellular compartments of AH5183 exposed slices. Slices were prepared for incubation as described in the materials and methods. Slices were exposed to a Krebs medium containing 10 μ M choline and 15 μ M eserine and following a 45 min recovery period AH5183 treated slices received 10 μ M AH5183 for the duration of the experiment. Stimulation of the slices was achieved by raising the K^+ concentration to 35 mM but only half of the slices from each group were stimulated. 3H refers to the incubations where slices were exposed to [^{3} H]-Ch. The tick marks refer to times when the incubation medium was changed and the dashed line refers to the protocol followed for those slices which were stimulated.

The stimulated release of ACh from control and drug exposed slices is shown in figure 17R. As reported above, in section 3.3.1, high K^+ increased the release of ACh by 5-8 fold in control slices while the release of ACh from slices left at rest did not change much (≈ 110 pmoles/mg tissue weight)(panel A). Panel B of figure 17R again shows that AH5183 attenuated the release of ACh by 35 mM K^+ . Slices left at rest showed little change in their basal release of ACh (≈ 100 pmoles/g tissue weight).

If an AH5183-insensitive compartment exists in the occluded pools of ACh, one would expect stimulation, a process that increases the release and turnover of ACh in terminals, to increase the specific activity of ACh in this fraction over that seen with slices left at rest. On the other hand, if AH5183 completely blocks the uptake of ACh by all occluded compartments one might expect that stimulation would not alter the turnover of ACh in this fraction since no [^3H]-ACh would enter these compartments even when the release of ACh from these compartments is brought about by high K^+ . Thus, the first clue to the existence of an AH5183-insensitive vesicular compartment in this tissue would be an increased turnover in the ACh of the occluded fraction, P_3 , when slices are stimulated.

HIPP slices were prepared for incubation as described in the materials and methods and the protocols outlined in figure 16R followed. The P_3 and S_3 fractions were prepared and the ACh content of the fractions measured; at the same time parallel samples were taken from the different fractions for the determination of the amounts of [^3H]-ACh because, in order to estimate the turnover of ACh, the amount of [^3H]-ACh in each fraction must be measured. If one now uses both the determinations of the ACh content and the determinations of the [^3H]-ACh content for the different fractions, one can obtain the specific activity of the ACh present in each fraction. Changes in the specific activity of the ACh in any given compartment can then be used as an estimate of the turnover of the ACh in that compartment.

Figure 18R illustrates the results of stimulation on the ACh and [^3H]-ACh content and the ACh specific activity (SA) of the P_3 and S_3

ACH RELEASE FROM HIPPOCAMPAL SLICES

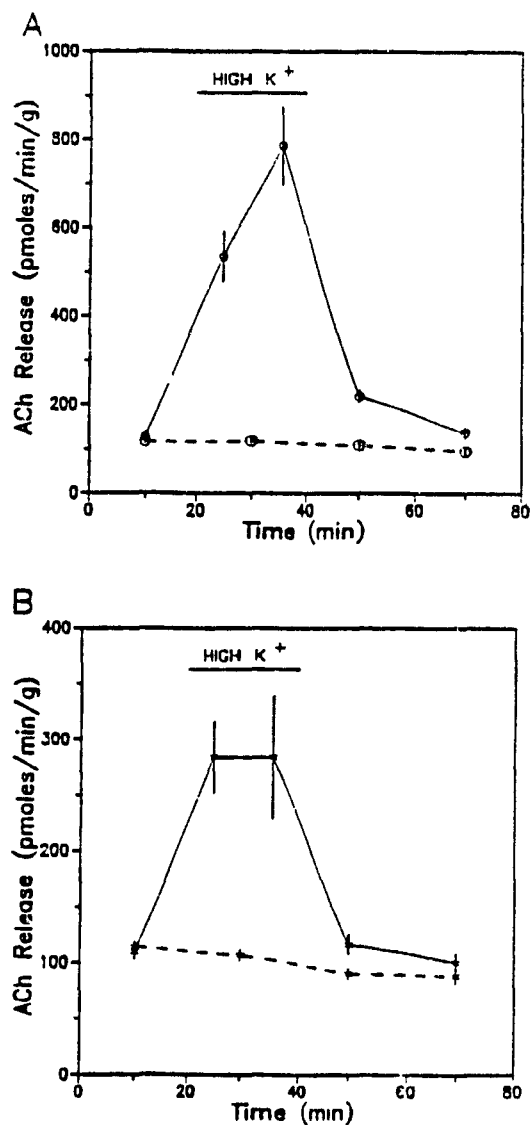


Figure 17R. The release of ACh from hippocampal slices $\pm$ AH5183. Slices were prepared for incubation as previously mentioned and the protocol outlined in figure 16R was followed. Slices were stimulated with 35 mM K⁺ for 20 min as indicated by the bar in both panels. The solid line represents the release of ACh from stimulated slices, and the dashed line that from slices left at rest. Panel A illustrates the results from drug-free slices. Panel B illustrates the results from AH5183-exposed slices. Each point represents the mean of 10 determinations $\pm$ s.e.m.

EFFECT OF STIMULATION ON ACH TURNOVER IN THE P₃ & S₃ FRACTIONS OF CONTROL HIPPOCAMPAL SLICES

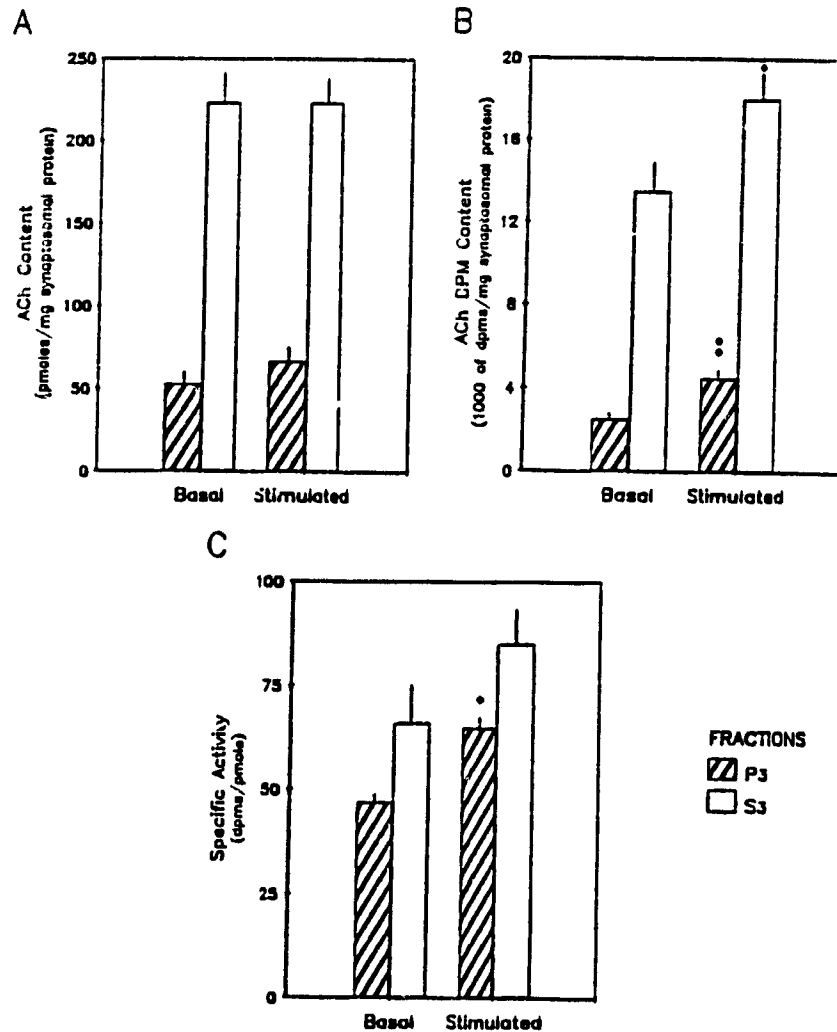


Figure 18R. The effect of stimulation on the turnover of ACh in the P₃ and S₃ fractions of control slices. HIPP slices were prepared for incubation as described in the materials and methods and the protocols outlined in figure 16R were followed. The slices were then homogenized with a 0.32 M sucrose solution containing 5 mM HEPES, 1 mM EGTA, and 15 μ M eserine. The synaptosomes were isolated and then lysed with a weakly buffered aqueous solution containing HEPES, EGTA, and eserine at the same concentrations as for the homogenizing buffer. Both P₃ and S₃ fractions were then isolated and their ACh and [³H]-ACh measured. A) Effect of stimulation on the ACh contents. B) Effect of stimulation on the [³H]-ACh content. C) Effect of stimulation on the specific activity of ACh. Each bar represents the mean $\pm$ s.e.m. of 10 determinations. Statistical significance was determined by the student t-test for unpaired data. (*) signifies $p < 0.05$ and (**) $p < 0.01$. Comparison is between basal and stimulated conditions.

fractions from control slices. Stimulation of control slices by K^+ had no effect on the S_3 ACh content and only slightly increased the P_3 fraction's ACh content as compared to unstimulated slices (panel A). However, stimulation did significantly increase the amount of [^3H]-ACh accumulated by these fractions; as shown in panel B, stimulation raised the [^3H]-ACh of the P_3 fraction by 85% and that of the S_3 fraction by 32%. Stimulation also increased the SA of ACh for both fractions but only the increase seen in the P_3 fraction was found to be significant (panel C). These results indicate that in the absence of AH5183, stimulation mainly increased the turnover of ACh in the occluded fractions and although the ACh turnover of the cytosolic fraction was also increased this change was not significant.

When HIPP slices were exposed to AH5183 and the protocol outlined in figure 16R followed, the results were slightly different, as shown in figure 19R. Panel A illustrates the changes in the ACh content of the occluded and cytosolic fractions that occurred with stimulation. The P_3 fraction was unaltered by stimulation but the S_3 fraction's ACh content was significantly increased. These results were consistent with the notion that AH5183 blocks vesicular ACh uptake, thereby causing newly synthesized ACh to accumulate in the cytoplasmic compartment. When slices were stimulated in the presence of AH5183, both the P_3 and S_3 [^3H]-ACh content were significantly higher (panel B) (80% higher for the P_3 fraction and 30% for the S_3 fraction). Panel C shows that stimulation had no effect on the SA of the ACh found in the cytosolic fraction. While stimulation did increase the ACh SA of the P_3 fraction by 42%, this increase just failed statistical significance ($p=0.07$) due to the variability obtained from the stimulated slice's P_3 fraction.

On the basis of these results one might conclude that no vesicular fraction is insensitive to the blocking actions of AH5183 because the observed increase in the SA of the ACh in the P_3 fraction was not significant, but the variation from experiment to experiment may explain why this difference did not yield statistical significance. One possible approach that may decrease the variation is to calculate the ratio of the SA of ACh between the P_3 and S_3 fractions. If the exchange of

EFFECT OF STIMULATION ON ACh TURNOVER IN THE P₃ & S₃ FRACTIONS OF AH5183-EXPOSED SLICES

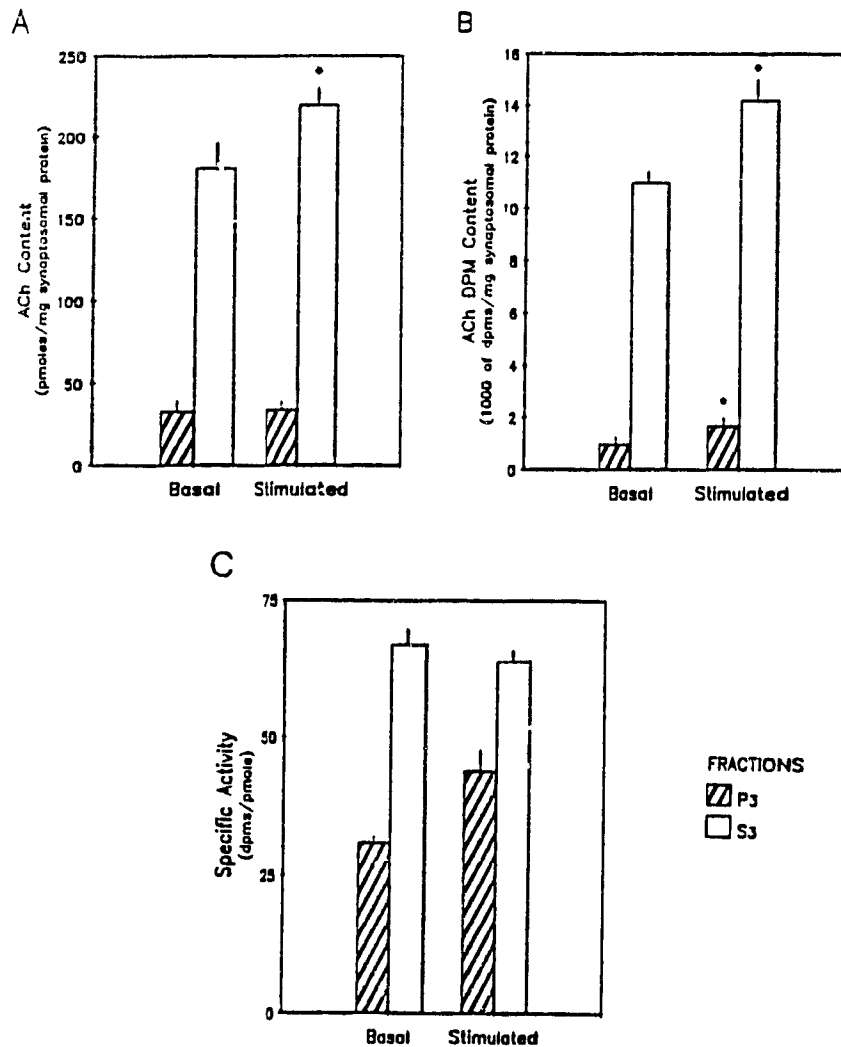


Figure 19R The effect of stimulation on the turnover of ACh in the P₃ and S₃ fractions of slices exposed to AH5183. HIPP slices were prepared and incubated as described in figure 16R and subcellular isolations were done as described in figure 18R. A) Effect of stimulation on the ACh contents. B) Effect of stimulation on the [³H]-ACh content. C) Effect of stimulation on the specific activity of ACh. Each bar represents the mean ± s.e.m. of 10 determinations for the unstimulated slices and 9 determinations for the stimulated ones. Statistical significance was determined by the student t-test for unpaired data. (*) signifies p<0.05 Comparison is between basal and stimulated conditions.

ACh between the cytoplasmic compartment, where the ACh is synthesized, and the occluded compartments are completely free then the ratio of the specific activity of ACh in the P_3 and S_3 fractions should be 1. As this ratio approaches 1 it signifies that little or no inhibition occurs in the transport of ACh from one compartment to the other. Figure 20R shows the results of these calculations. Panel A, shows that stimulation in control slices did not alter the exchange of ACh between these two compartments such that, as the SA of the cytoplasmic ACh increased so did that of the occluded fractions and by the same amount as under resting conditions. Panel B illustrates that under resting conditions AH5183 decreased this ratio by about 30%, as compared to the control, unstimulated slices, and that stimulation of the drug-exposed slices brought the ratio back to control levels. However, this increase in the ratio again just failed statistical significance ($p=0.06$).

The qualitative aspects of the above results suggest that stimulation of slices in the presence of AH5183 does increase the turnover of ACh in the occluded fractions but quantitatively the changes are not large enough for the experimental variation. If there is an AH5183-insensitive vesicular pool, this pool may constitute a small fraction of the different pools of ACh to be found in the P_3 fraction. Therefore, experiments were designed to investigate how the different occluded pools of ACh, found in the P_3 fraction, responded to stimulation in the presence of AH5183.

3.4.2 The effect of AH5183 on the acetylcholine content and turnover of the subcellular-bound compartments of stimulated and rested hippocampal slices.

Rat HIPPO slices were prepared for incubation as previously described and the protocols outlined in figure 16R were followed with the only difference being that no eserine was present in the Krebs media that were used. In the case of these experiments the [^3H]-Ch was raised to 1100 dpm/pmol but the choline concentration stayed at 10 μM . After the experiment, slices were homogenized and the P_2 fraction isolated.

SPECIFIC ACTIVITY P_3/S_3 RATIO FROM HIPPOCAMPAL SLICES

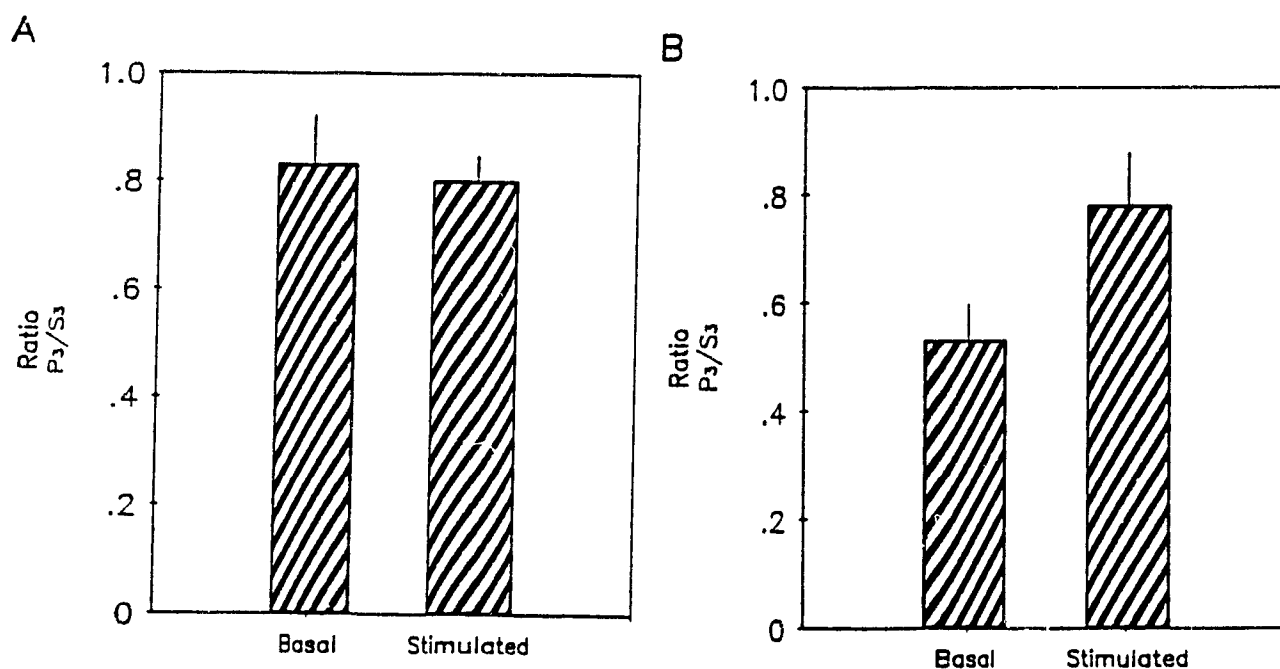


Figure 20R. The ratio of the specific activities of ACh from the P_3 and S_3 fractions of hippocampal slices. The measures of specific activity for the ACh found in fractions P_3 and S_3 were used to derive the ratio P_3/S_3 . A) Ratios of control slices. B) Ratios of AH5183-exposed slices. The bars represent the mean $\pm$ s e m for 10 determinations except in the case of the drug-exposed, stimulated slices where only 9 determinations were done.

This synaptosomal fraction was then lysed using a weakly buffered solution of 5 mM HEPES and 1 mM EGTA and layered on a discontinuous sucrose gradient containing 0.4 M, 0.8 M, and 1.2 M sucrose so as to separate the occluded compartments of the P_2 fraction. Three fractions were obtained, the D, E&F, and G&H fractions, whose contents are described in the materials and methods. These fractions were then extracted for the determinations of ACh and [^3H]-ACh.

Figure 21R shows the results of stimulation on the ACh turnover in the various occluded compartments from control HIPP slice. In the absence of AH5183, stimulation had little effect on the content of ACh in the subcellular-bound fractions (panel A). Stimulation did, however, significantly increase the amount of [^3H]-ACh accumulated by all the fractions with the D fraction showing the largest gain (178%)(panel B). Thus, the SA of ACh was also increased in all fractions with stimulation and again the D fraction showed the largest increase (208%)(panel C).

These results indicate that stimulation increased the turnover of ACh in all of the fractions, because while the content of ACh did not change much, the specific activities did. This implies that a greater proportion of the ACh present in these fractions came from the incorporation of newly synthesized, and therefore ^3H -labeled, ACh. The ACh turnover of the D fraction was smallest in unstimulated slices but stimulation increased the turnover of ACh in all fractions to about the same level.

Figure 22R, panel A, illustrates the changes in the ACh content of these fractions in drug-exposed slices. When the drug was present, K^+ stimulation caused a small but significant decrease in the D and E&F fractions. Although total ACh content decreased for the various occluded pools, stimulation did lead to a significant increase in the accumulation of [^3H]-ACh in the E&F and G&H fractions (panel B) by 80% and 100% respectively. The D fraction of drug-exposed slices left unstimulated did not contain measurable amounts of [^3H]-ACh and that from stimulated tissues was not significantly greater than 0. Panel C, shows the results of stimulation on the SA of ACh from drug-exposed slices. Stimulation led to a significant increase in only the E&F and G&H frac-

EFFECT OF STIMULATION ON ACh TURNOVER IN THE SUBCELLULAR-BOUND FRACTIONS OF CONTROL SLICES

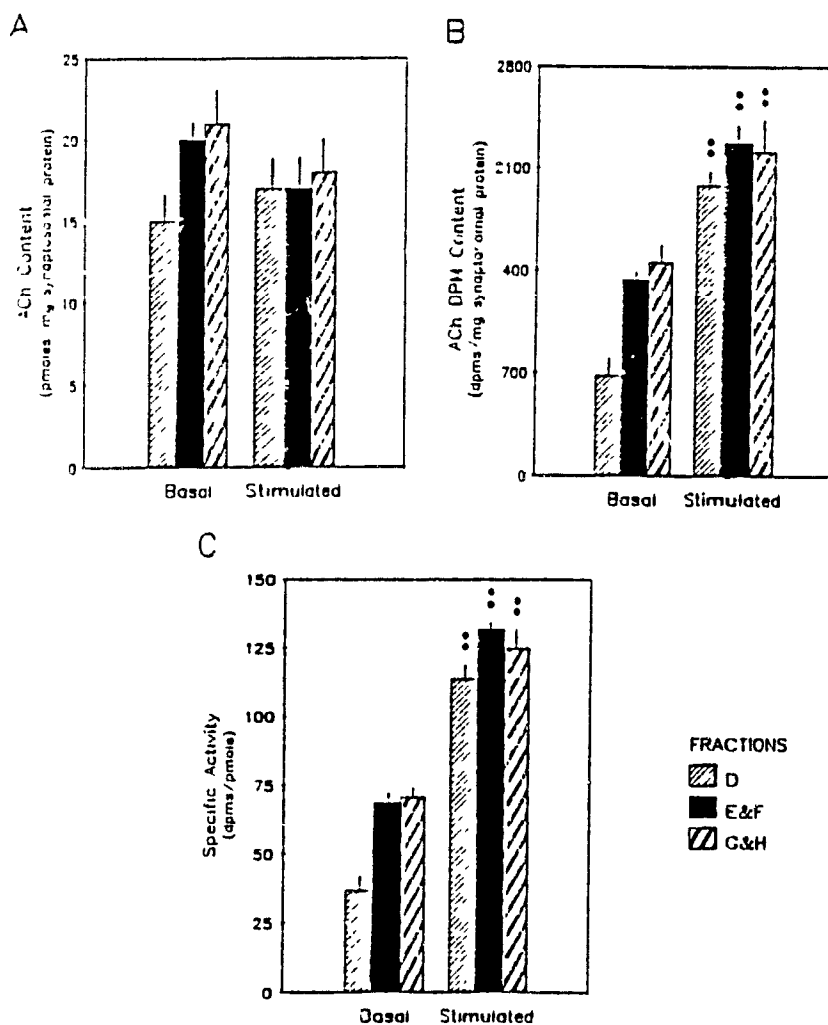


Figure 21R The effect of stimulation on the turnover of ACh in the bound-subcellular fractions of control slices. HIPP slices were prepared for incubation as described in the materials and methods and the protocols outlined in figure 16R followed. The slices were then homogenized with a 0.32 M sucrose solution containing 5 mM HEPES, and 1 mM EGTA. The synaptosomes were isolated and then lysed with a weakly buffered aqueous solution containing HEPES and EGTA at the same concentrations as in the homogenizing buffer. The lysate was then layered on a discontinuous sucrose gradient and the subcellular-bound fractions, D, E&F, and G&H isolated and their ACh and [^{3} H]-ACh content determined. A) Effect of stimulation on the ACh contents. B) Effect of stimulation on the [^{3} H]-ACh content. C) Effect of stimulation on the specific activity of ACh. Each bar represents the mean $\pm$ s.e.m. of 12 determinations. Statistical significance was determined by the student t-test for unpaired data. (*) signifies $p < 0.01$. Comparison is between basal and stimulated conditions.

EFFECT OF STIMULATION ON ACh TURNOVER IN THE SUBCELLULAR-BOUND FRACTIONS OF AH5183-TREATED SLICES

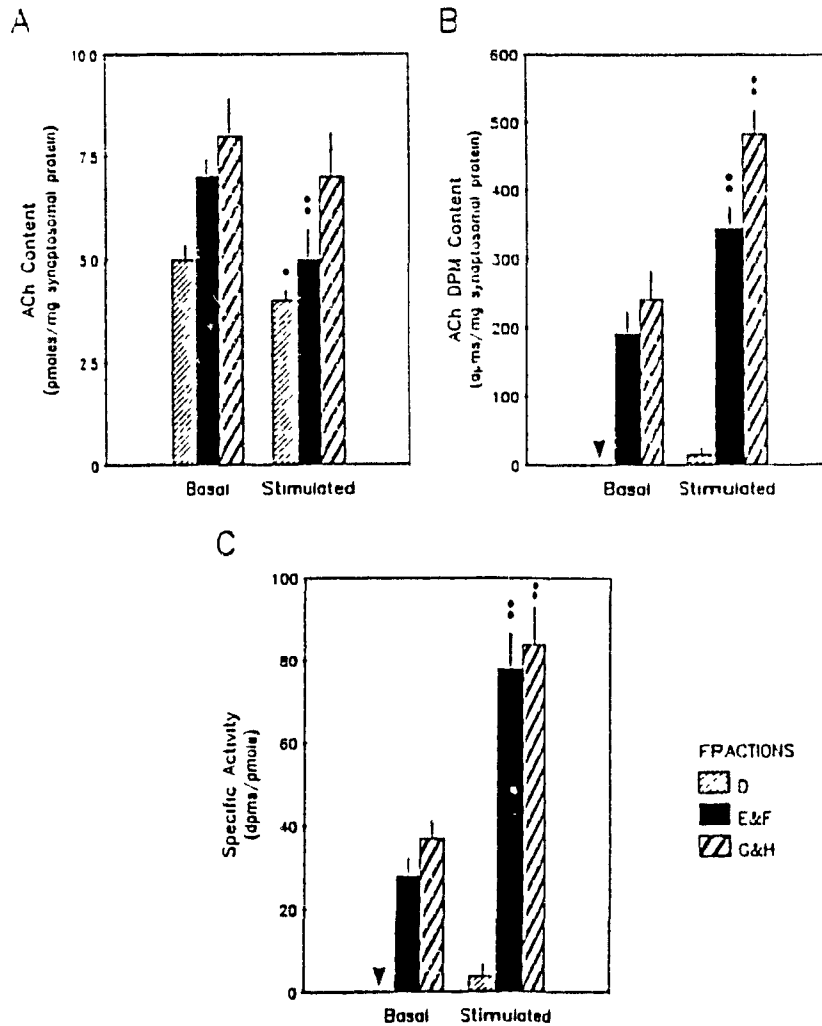


Figure 22R. The effect of stimulation on the turnover of ACh from the bound-subcellular compartments of slices exposed to AH5183. HIPP slices were prepared, incubated, and their bound-subcellular compartments isolated as described in figure 21R. A) Effect of stimulation on the ACh contents. B) Effect of stimulation on the [^{3} H]-ACh content. C) Effect of stimulation on the specific activity of ACh. Each bar represents the mean $\pm$ s.e.m. of 12 determinations. Statistical significance was determined by the student t-test for unpaired data. (*) signifies $p < 0.05$ and (**) $p < 0.01$. Comparison is between basal and stimulated conditions. (▼) Indicates no [^{3} H]-ACh detected.

tions with changes amounting to 178% and 127% respectively. As expected from the [^3H]-ACh accumulation results, the D fraction's ACh SA was 0 in unstimulated slices and negligible in stimulated slices.

SUMMARY AND DISCUSSION OF SECTION 3.4

Although stimulation of drug-exposed slices increased the turnover of the ACh in two of the subcellular-bound pools, the total ACh content of all the subcellular-bound pools were lower in the presence of the drug. This finding was again consistent with the observed inhibitory effect of AH5183 on the active vesicular uptake of ACh. However, these findings do suggest that the two compartments (E&F and G&H), whose ACh turned over to a greater extent when slices were stimulated in the presence of AH5183, are at least less sensitive to the drug's inhibitory effects than the D fraction, whose ACh appeared not to turnover in the drug's presence.

As mentioned above, AH5183 is known to only weakly block the passive ACh transport into vesicles. If stimulation raises the level of [^3H]-ACh then could not this increased turnover of ACh in these compartments just be the result of passive ACh exchange? This alternative is unlikely because stimulation in the presence of the drug only increased the turnover of ACh in two of the compartments and not all three.

3.5 THE RECOVERY OF ACETYLCHOLINE RELEASE AND OF THE SUBCELLULAR COMPARTMENTS FROM THE BLOCKING ACTIONS OF AH5183.

The above results are compatible with the view that vesicles are differentially sensitive to the blocking action of AH5183, but they do not indicate which vesicle pool is responsible for the stimulated release of ACh, that is to say, the readily-releasable pool. However, this differential sensitivity may be used to glean some information about the possible roles that these vesicle pools play in the release

process. Because the blocking action of AH5183 is reversible, it should be possible to study the time course of recovery for ACh release and for the ACh content of the subcellular fractions. A not too extravagant expectation is that if release is associated with some compartment, then both release and compartment should show similar recovery kinetics. Since the vesicular compartments appear to be differentially sensitive to the drug's blocking action, they may recover with different enough kinetics to distinguish whether one of them plays a role in the direct release of ACh. Thus, experiments were designed to investigate the rate at which ACh release from HIPP slices recovers from AH5183 and the associated changes in the subcellular compartments' ACh content.

3.5.1 Recovery time course of the release of acetylcholine and [³H]-acetylcholine from AH5183 blockade

Investigation of the recovery of ACh release from AH5183-treated slices requires that the state of the tissue, with regards to ACh release, be ascertained at given intervals of time following the removal of the drug and that the source of the released transmitter be discernible. The release of transmitter can be estimated by simply measuring the evoked release of ACh in response to a secretagogue, such as K⁺. To discern the source of the released ACh is more difficult, but by labeling the newly synthesized ACh and measuring its S.A. in the subcellular fractions, the source of the transmitter may become evident.

HIPP slices were prepared for incubation as previously described in the materials and methods. All slices were allowed a 45 min recovery period in a normal Krebs medium containing 10 μ M choline and 15 μ M eserine. For control studies, the medium was replaced with fresh medium and the slices incubated for another 15 min. In experiments where AH5183 was used, the medium was replaced with one containing 10 μ M AH5183 and the slices incubated in this medium for 15 min. Thus all slices were given a 60 min resting period prior to the collection of the first samples.

Control slices had the medium replaced and were then incubated for 10 min in a normal Krebs medium followed by two 10 min incubations in a Krebs medium containing 35 mM K^+ . Following the K^+ stimulation, the slices were returned to a normal Krebs medium containing 2 μ Ci/ml of [^3H]-Ch (0.2 Ci/mmol) for one to five further 10 min incubations. Some HIPP slices were collected after each 10 min incubation to assess the subcellular stores of ACh and [^3H]-ACh. Each group of collected slices constituted all of the slices from one hippocampal formation. The slices from the contralateral hippocampal formations were treated in an identical way except that following the appropriate recovery period(s), their medium was changed to a 35 mM K^+ Krebs medium containing no Ch or [^3H]-Ch. They were incubated for 5 min in this medium and the collected medium was used to measure the ACh released in response to the K^+ stimulus. A summary of the experiment is shown in the upper panel of figure 23R.

The slices used in the AH5183 experiments were treated in the same way as the controls except that 10 μ M AH5183 was present during the first collected sample and the two 10 min stimulation incubations that followed. The drug was then removed from the medium and both AH5183-treated and control slices were incubated using the same protocol. All time points following the removal of the drug are referred to as recovery times. The incubation protocol used for the AH5183 experiments is outlined in the bottom panel of figure 23R.

The ACh content of all collected incubation media was measured; the 5 min test stimulus samples were also processed to determine the [^3H]-ACh content so that the specific activity of the released transmitter could be calculated. At the conclusion of each experiment, slices were homogenized and their total, as well as cytosolic (S_3) and occluded (P_3) subcellular ACh and [^3H]-ACh contents measured (see materials and methods). The experiments in this section were randomized with respect to the various recovery times and with respect to controls vs. AH5183-treatment.

The release of ACh by control HIPP slices is shown in figure 24R, panel A. Under these conditions, high K^+ increased the rate of ACh

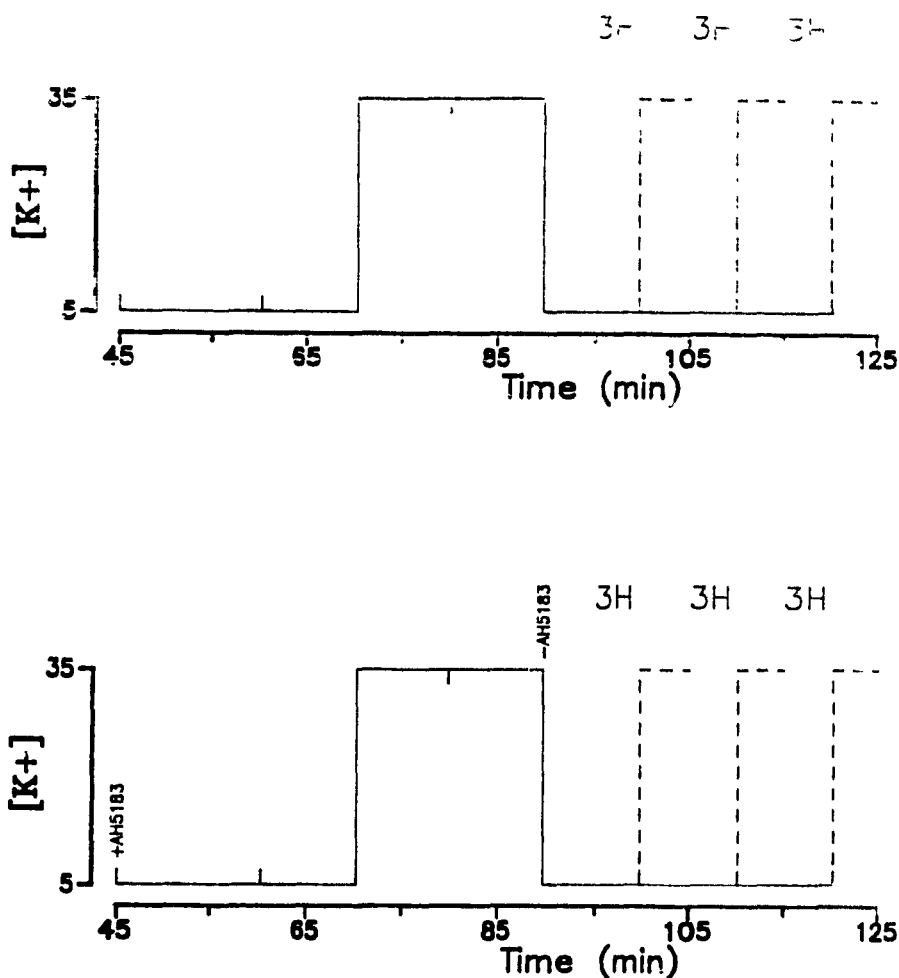
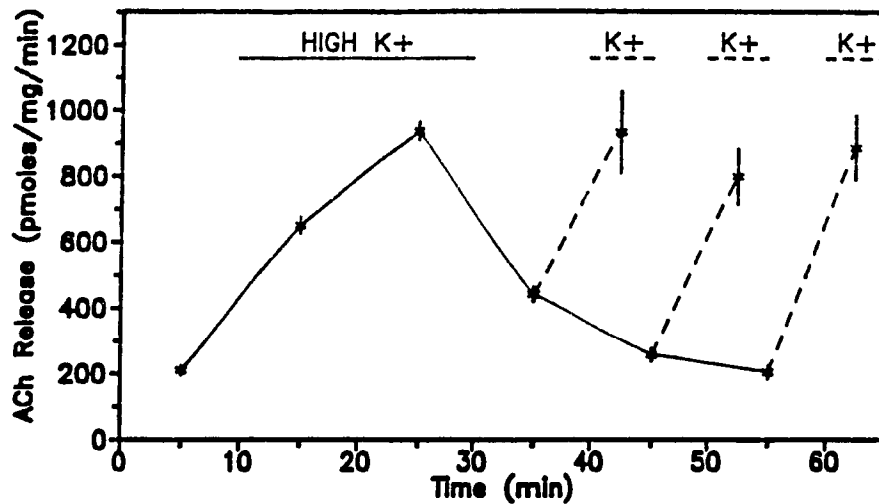


Figure 23R Experimental protocol to determine the recovery kinetics of ACh release from AH5183-treated HIPP slices. HIPP slices were prepared for incubation according to the procedure described in the materials and methods. All slices were given a 45 min recovery period in normal Krebs medium containing 15 μ M eserine and 10 μ M choline. Control slices then had their medium replaced with the same medium for a further 15 min while slices receiving AH5183 were exposed at this time to a similar medium containing 10 μ M AH5183. The medium of each was accordingly replaced with fresh medium and the first basal collection taken after 10 min. All slices were then stimulated with a Krebs medium containing 35 mM K^+ for two 10 min periods. At the end of this incubation slices were returned to a 5 mM K^+ medium and in the case of AH5183-exposed slices the drug was removed from the medium. The new medium for all slices contained 2 μ Ci/ml of $[^3H]$ -Ch. Some slices were incubated from 10 to 30 min in this medium with the medium replaced every 10 min. The contralateral HIPP slices were stimulated for 5 min, following each recovery time, by using a 35 mM K^+ Krebs medium containing no choline. The top panel outlines the procedure for the control slices and the bottom panel that for the drug-treated slices. The 3H on the figure indicates incubations containing $[^3H]$ -Ch and the dashed lines the time periods when slices were stimulated with high K^+ for 5 min.

A



B

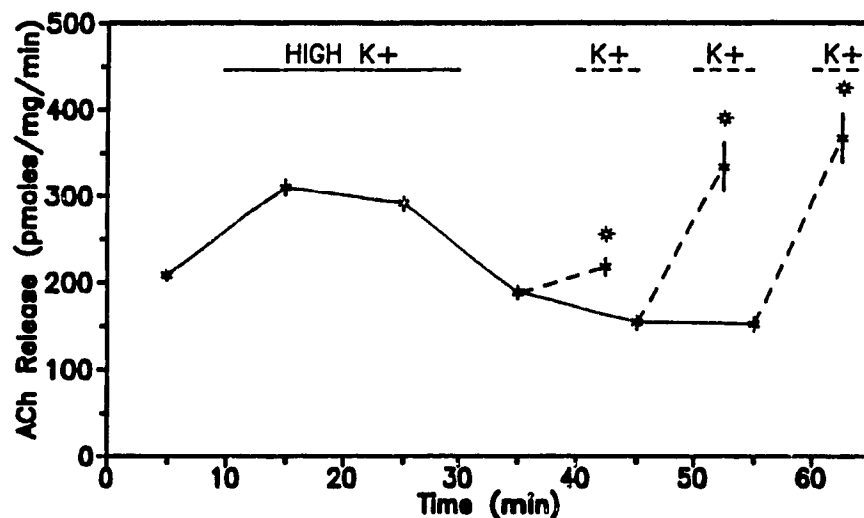


Figure 24R. The recovery of ACh release from HIPP slices following the removal of AH5183. HIPP slices were prepared according to the procedure described in the materials and methods and were then incubated as outlined in figure 23R. A) The release profile of ACh from control slices. B) The release profile of ACh from AH5183-treated slices. The solid bar marked high K^+ denotes the period when slices were exposed to 35 mM K^+ and the dashed bars labeled K^+ denote those 5 min incubations of the contralateral HIPP slices with 35 mM K^+ medium. The dashed lines denote the ability of the slices to release ACh in response to the K^+ stimulations at specific times following the initial high K^+ 20 min stimulation. Each point represents the mean $\pm$ s.e.m. of at least 8 determinations. (*) denotes a $p < 0.05$ between the control and AH5183-exposed slices as determined by the Tukey post-hoc test and ANOVA.

release by 3 to 5 fold over that of the basal rate. Upon returning the slices to resting conditions, ACh release returned to pre-stimulus levels by the third recovery time point (30 min). As illustrated by the dashed lines in fig 24R panel A, during the subsequent 3 high K^+ exposures the slices released ACh at the same rate as the maximal rate seen during the initial 20 min of high K^+ exposure. After 30 min the rate of ACh release returned to that found in the initial 10 min of stimulation by K^+ .

When slices were exposed to AH5183 there was only about a 50% increase over basal levels in the ACh released by K^+ (figure 24R. panel B) and unlike control slices the second 10 min K^+ stimulation did not increase the release of ACh above the first stimulatory incubation. After washing out AH5183 and returning the slices to a normal Krebs medium, the basal release of ACh returned to pre-stimulation levels. The first K^+ test stimulation following drug removal had very little effect on the release of ACh. In fact, this incubation in high K^+ only caused ACh release to increase by half the amount seen with the first K^+ stimulation when the drug was present. The next two test stimulations with K^+ yielded a release of ACh that was slightly higher than the maximal release seen in the presence of the drug. These two stimulations released approximately the same amount of ACh (approximately 360 pmoles/g/min) and showed a slow time dependent recovery from AH5183 blockade.

The S.A. of released ACh in these experiments can provide an estimate of the turnover of the ACh in the releasable pool. In AH5183-treated slices, as inhibition is removed due to drug washout, the size of the releasable pool of ACh should grow, and one might expect that as labeled transmitter enters this pool the S.A. of released ACh should begin to approach control levels. Thus, the origin of released transmitter might be identified by the rate at which the S.A. of ACh in the various cellular fractions recover following drug removal.

The [^3H]-ACh released by the test K^+ stimuli was measured and the S.A. of released ACh calculated. Figure 25R illustrates the results of these measures. In control slices, the S.A. of released transmitter did

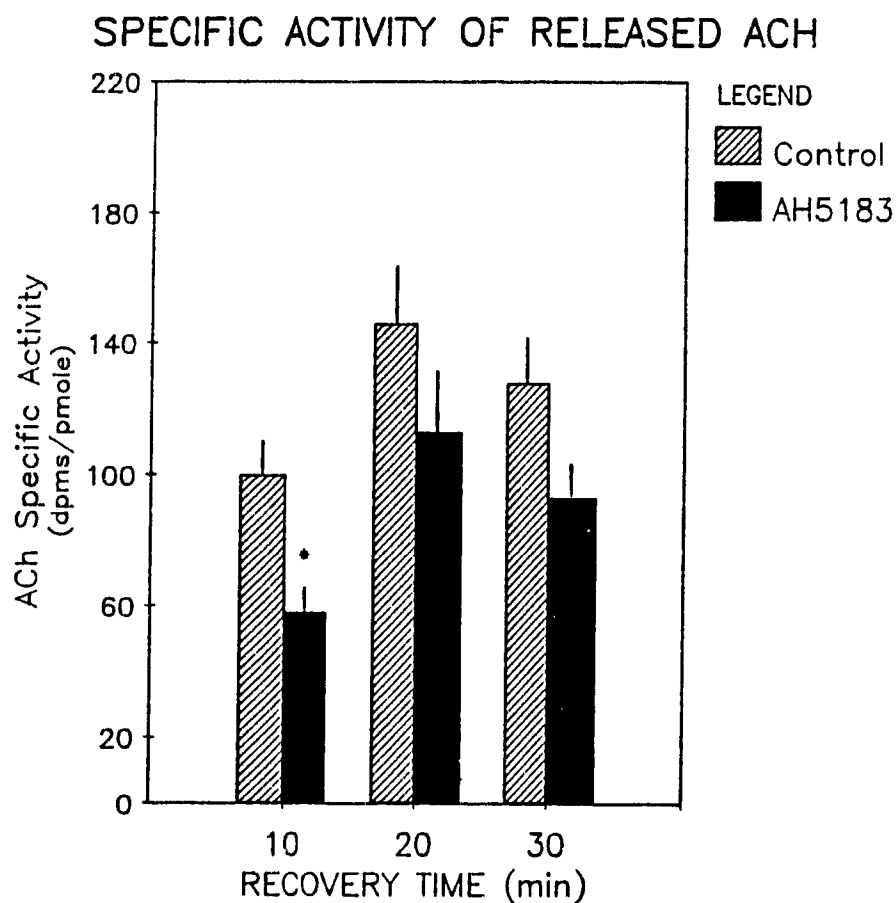


Figure 25R. The specific activity of released ACh from AH5183-treated HIPP slices following the removal of the drug, AH5183. Slices for this experiment are the same as those described in figure 24R. The ACh and [^3H]-ACh content of the test 5 min K^+ stimuli were determined and the S.A. of the ACh calculated. Each bar represents the mean $\pm$ s.e.m. of 8 determinations. The statistical significance between means was determined by ANOVA and the Tukey post-hoc test. (*) signifies $p < 0.05$ between the control and AH5183-treated slices.

not rise in a linear manner, as might be expected from the increase in ^3H incorporation seen with time (see table 5R), but peaked 20 min into the recovery phase. The S.A. of released ACh from AH5183-treated slices showed a similar pattern of release with the peak of ACh S.A. occurring at the 20 min point of the recovery period. The pattern of the S.A. of released ACh was the same in control and drug-exposed slices, but the S.A. of ACh was always less in the AH5183-treated tissues. Inspection of the data by ANOVA indicated that, in control slices, there was no significant effect of time on the S.A. of released ACh, while in the AH5183-treated slices, the ACh released 20 min following drug washout had a significantly higher S.A. than that of the 10 min time point. Only the ACh S.A. of the first release period was significantly lower in AH5183-treated slices than in controls.

It is not immediately apparent why the S.A. of released ACh does not increase with time but tends to drop after reaching a maximum at 20 min of the recovery period. These results suggest that either preformed and stored ACh can be mobilized without complete mixing with other, newer pools of ACh, or that the source of the Ch used in the synthesis of releasable ACh need not come directly from an extracellular source and may indeed change with time even under resting conditions. In the case of the drug-exposed slices, this point is especially interesting because one would expect that as inhibition from AH5183 is decreased, the refilling pool of ACh would obtain increasing amounts of newly synthesized, and presumably tritiated, ACh. Thus, it appears that the source of releasable ACh may vary with time, even under conditions plentiful in extracellular Ch.

3.5.2. Total tissue acetylcholine content and acetylcholine specific activity.

In an experiment using double labeling techniques to investigate the releasability of ACh synthesized in the presence of AH5183 as compared to that synthesized during the recovery from drug blockade, Cabeza and Collier (1988) reported that 40 min after removal of the drug the

isotopic ratio of the ACh released from the cat SCG was the same as that found in the ganglia. Thus, measures of total tissue ACh and [^3H]-ACh accumulation were made to test whether the S.A. of the released ACh was similar to that found in the tissue. If this were so, then one would expect the S.A. of accumulated ACh to also reach a peak at about the 20 min time point of the recovery period, implying that the use of, and perhaps need for, extracellular Ch in the synthesis of new ACh varied with time.

Both control and drug-exposed HIPPI slices treated in a similar fashion to the slices described in the experiments of section 3.5.1, except that they were not stimulated with high K^+ during the recovery phase, were homogenized in 0.32 M sucrose containing 5 mM HEPES, 1 mM EGTA, and 15 μM eserine at the end of the experiment. This allowed a comparison between the S.A. of released ACh and the S.A. of the ACh content present in the tissue prior to stimulation to be made. A portion of the homogenate was then used to extract and quantify the ACh and [^3H]-ACh content (see materials and methods for the details of the procedures used). The total ACh content of both control and AH5183-treated slices are shown in figure 26R.

There were no statistically significant changes in the total ACh content of control slices with time; the percent change between the lowest and the highest contents of ACh was only 17%. The AH5183-exposed slices also showed little change in their ACh content following the removal of AH5183 and as in the controls there was only a 17% difference between the highest and the lowest ACh contents. Though the content of AH5183-exposed slices was always lower the differences were not statistically significant. The changes in the ACh content of HIPPI slices used in these experiments were small and may represent a normal variance in the measurement of ACh content.

The determinations of the S.A. of ACh in the slices of both control and drug-exposed slices are shown in figure 27R. The control slices increased their ACh S.A. at a rate of 18.9 dpm/pmole/10 min such that the highest obtained S.A. occurred at the 30 min time period. In the case of the AH5183-treated slices, there was also a continuous in

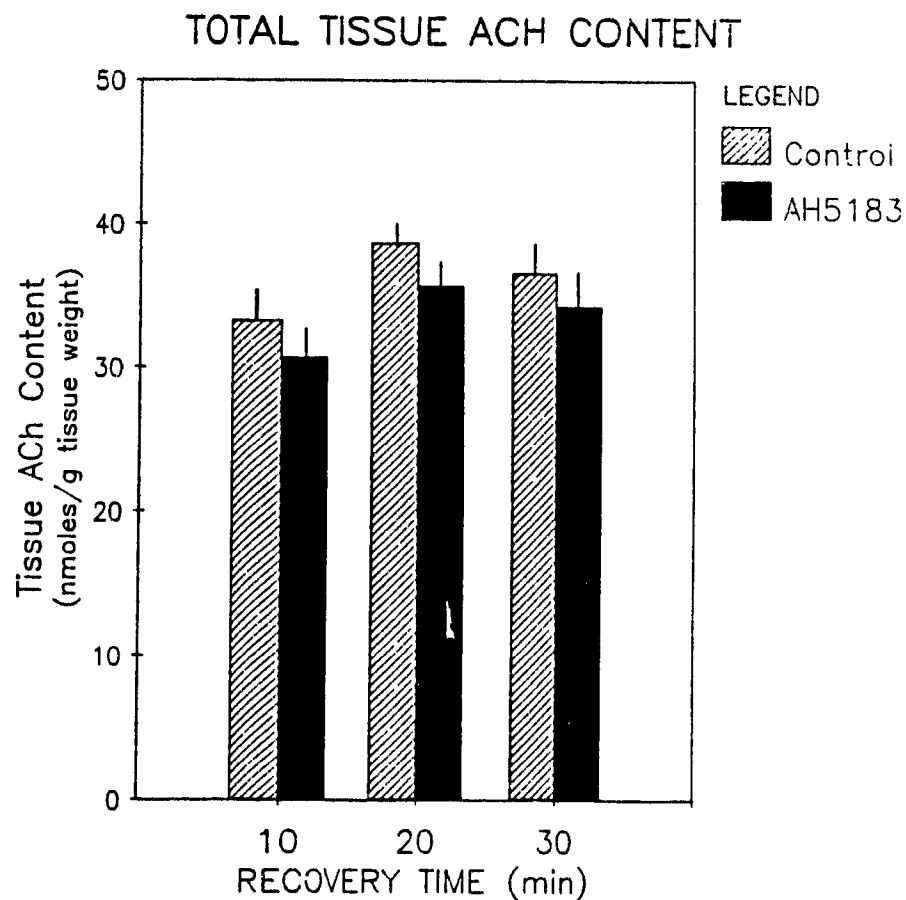


Figure 26R. The effect of drug removal on total tissue ACh content from AH5183-treated slices. Slices from the experiments outlined in figure 23R were homogenized in a 0.32 M sucrose solution containing 15 μ M eserine, 5 mM HEPES, and 1 mM EGTA. A portion of the homogenate was used to estimate the total tissue ACh. Only experiments where the slices were not tested for their ability to release ACh during the recovery period were used. Each bar represents the mean $\pm$ s.e.m. of 8 determinations.

SPECIFIC ACTIVITY OF ACh IN TOTAL TISSUE

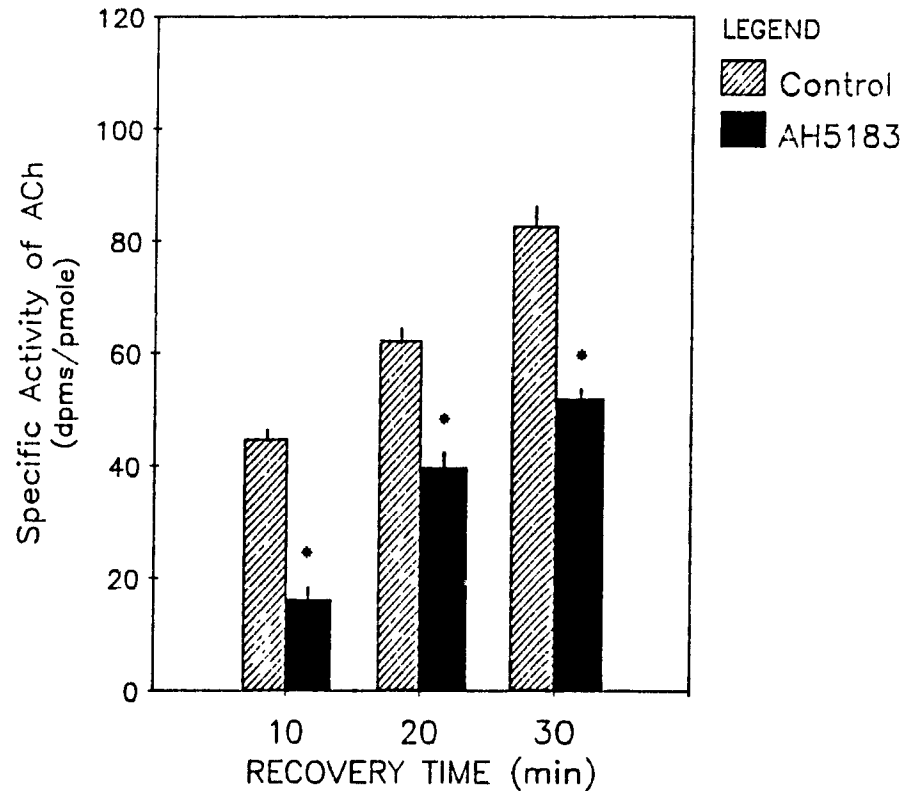


Figure 27R. Effect of AH5183 removal on the specific activity of ACh in the total tissue from AH5183-exposed slices. The [^3H]-ACh content of slices used in the experiments outlined in figure 23R was measured. Only slices not tested for their ability to release ACh are illustrated here. The S.A. of the ACh content was calculated. Each bar represents the mean $\pm$ s.e.m. of 8 determinations (*) signifies a $p < 0.05$ between controls and AH5183-exposed slices as determined by ANOVA and Tukey post-hoc test.

crease in the ACh S.A. with a rate of 17.9 dpm/pmole/10 min. Analysis of the data by ANOVA indicated that the effect of time on the increasing S.A. of ACh was significant for both the control and drugged slices. When the S.A. of ACh for the control slices was compared to that of the AH5183-exposed slices by ANOVA it was found that the control's S.A. was significantly higher at all times, though the rate at which the ACh S.A. increased was similar.

It is interesting that both control and AH5183-treated slices accumulated [^3H]-ACh at a similar rate while the overall level of the ACh S.A. was lower for the drug-exposed tissues. This could be due either to a difference in how much [^3H]-Ch is accumulated in the first 10 min of drug washout, assuming that ACh synthesis follows choline uptake, or that the ACh turnover is higher during the first 10 min in control slices than in drug-exposed slices. To ascertain whether the entry of [^3H]-Ch is the same in control and AH5183-treated slices, during the recovery period, the accumulation of ^3H was measured for both the total tissue and the synaptosomal fraction.

Table 5R tabulates these measures. The accumulation of ^3H by control and AH5183-exposed slices and by their synaptosomal fractions were found to be linear. Control slices did accumulate more ^3H than their drug-exposed counterparts during the first recovery period, but this difference was not found to be statistically significant. Linear regression of the data showed that controls accumulated ^3H at a slightly slower rate than AH5183-treated slices (2×10^6 dpm/10 min/g tissue vs 2.9×10^6 dpm/10 min/g tissue, respectively). In the case of synaptosomal fractions, control tissues also accumulated more ^3H than those exposed to AH5183 but in these fractions the difference was found to be statistically significant by ANOVA for the 10 and 20 min time periods. Again, the rate of ^3H incorporation proved to be higher in the fractions from drug-exposed slices than from the controls (2.0×10^4 dpm/10 min/mg synaptosomal protein vs 1.6×10^4 dpm/10 min/mg synaptosomal protein, respectively). Thus the difference in the S.A. of ACh between control and AH5183-exposed slices during the 10 min time point of the recovery period was the result of more [^3H]-Ch being transported by control

Table 5R. Accumulation of ^3H in
Tissue and Synaptosomes

Recovery Time	Control Tissue ^A	Control Synaptosomes ^B	AH5183 Tissue ^A	AH5183 Synaptosomes ^B
10 minutes	4.71 +/- 0.12	34.4 +/- 1.3	3.40 +/- 0.30	24.4 +/- 0.8*
20 minutes	6.83 +/- 0.21	53.3 +/- 1.0	6.97 +/- 0.21	44.9 +/- 1.2*
30 minutes	8.62 +/- 0.17	66.3 +/- 1.2	9.19 +/- 1.78	65.0 +/- 3.7

A= 10⁶ DPMS/g Tissue Weight

B= 10⁷ DPMS/mg synaptosomal protein

*= p<0.05 as compared to control (Tukey post-hoc test)

Table 5R The accumulation, with time, of ^3H by both the slices and the synaptosomal fraction of control and AH5183-treated HIPP slices is shown. HIPP slices were incubated following the protocol outlined in figure 23R. The slices were washed in a physiological saline and then homogenized in a 0.32 M sucrose solution. A portion of the homogenate was used to determine total ACh content and ^3H accumulation. The homogenate was then used to isolate the crude synaptosomal pellet (P_2) as described in the materials and methods. The pellet was lysed using a weakly buffered aqueous solution and a portion of the lysate used to estimate the synaptosomal ^3H accumulation. Only slices not used to test for release of ACh during the recovery period were used. The means $\pm$ S.E.M. of 8 determinations are shown. (*) denotes a p<0.05 in the difference between controls and AH5183-treated slices according to the Tukey post-hoc test.

slices during this period and thus a greater turnover in the control slices' ACh.

In both control slices and in those treated with AH5183 the S.A. of released ACh was about 2 to 3 fold higher than the S.A. of the slices' ACh content (see figures 25R and 27R). Furthermore, the S.A. pattern of accumulated ACh from both control and drug-treated slices does not resemble the S.A. pattern of released ACh. These findings suggest that measures of tissue ACh and [^{3} H]-ACh accumulation may not reflect the recovery kinetics from AH5183 exposure of the releasable pool of ACh and that compartmentation is a complex phenomenon. A better estimate of the behavior of the ACh releasable pool might be found by investigating the behavior of the cytoplasmic and occluded pools of ACh present in synaptosomes from HIPP slices exposed to AH5183.

3.5.3. Recovery time course of the acetylcholine and [^{3} H]-acetylcholine contents from the cytoplasmic and occluded fractions of AH5183-treated hippocampal slices

The readily-releasable pool of ACh only constitutes about 20% of total tissue ACh (section 3.2) and changes in this compartment might not be reflected in the bulk changes observed in the above section. By isolating synaptosomes from AH5183-treated slices and subdividing the ACh into cytoplasmic (S_3) and occluded (P_3) compartments one might be able to associate one of these compartments with the source of released ACh found in section 3.5.1. These experiments should also allow us to study some of the dynamics between the cytoplasmic pool of ACh and that of the occluded compartments as the latter begin to recover from AH5183 blockade.

The experiments outlined in section 3.5.1 were designed to investigate the recovery kinetics from AH5183-blockade of both ACh release and of the subcellular compartment's ACh content. HIPP slices of both control and AH5183-exposed slices were homogenized and processed so as to isolate a crude synaptosomal fraction (P_2 pellet). Only slices that were not tested for their ability to release ACh in response to a test

stimulus with high K^+ were used. The P_2 pellet was then lysed at 0-4 °C for 15 min with an aqueous solution containing 5 mM HEPES, 1 mM EGTA, and 15 μ M eserine. The lysed pellet was then centrifuged at a high speed so as to separate the cytoplasmic compartment (S_3) from the occluded compartments (P_3). The ACh and [^3H]-ACh was then extracted and measured and the S.A. of the transmitter calculated.

Figure 28R shows the results obtained from these measures in control slices. Panel A contains the measures made on the ACh content. The ACh content of the P_3 fraction was unchanged throughout the 30 min of the recovery period, while that of the S_3 fraction rose somewhat, although not significantly, during the 30 min following exposure to high K^+ . The accumulation of [^3H]-ACh by the P_3 fraction increased significantly between 10 and 20 min of the recovery period but then did not change (panel B). [^3H]-ACh accumulation by the S_3 fraction increased in a linear and statistically significant fashion throughout the recovery period at a rate of 8×10^3 dpm/10 min/mg synaptosomal protein. The general pattern of [^3H]-ACh and ACh accumulation for both the P_3 and S_3 fraction was similar, with the highest accumulations occurring at the 20 min time point for the P_3 fraction and the 30 min time point for the S_3 fraction. Measurements of ACh and [^3H]-ACh content were used to calculate the S.A. of the ACh content of each fraction and the results are illustrated in panel C of figure 28R. The S.A. of the ACh present in the P_3 and S_3 fractions increased during the recovery period but the S_3 fraction's ACh S.A. grew at a rate about twice as fast as that of the P_3 fraction's ACh S.A.

The pattern of changes seen in the ACh and [^3H]-ACh contents obtained from slices treated with AH5183 are illustrated in figure 29R. Following the removal of the drug, the ACh content of the P_3 fraction did not change in the first two time periods but did increase slightly by the 30 min time point (panel A); this increase at the 30 min time point was not found to be significant by ANOVA. Furthermore, the ACh content of the P_3 fraction of drug-exposed slices was found to be significantly less than control values at all times (ANOVA). The amount of ACh in the S_3 fraction, following the removal of AH5183, was similar at

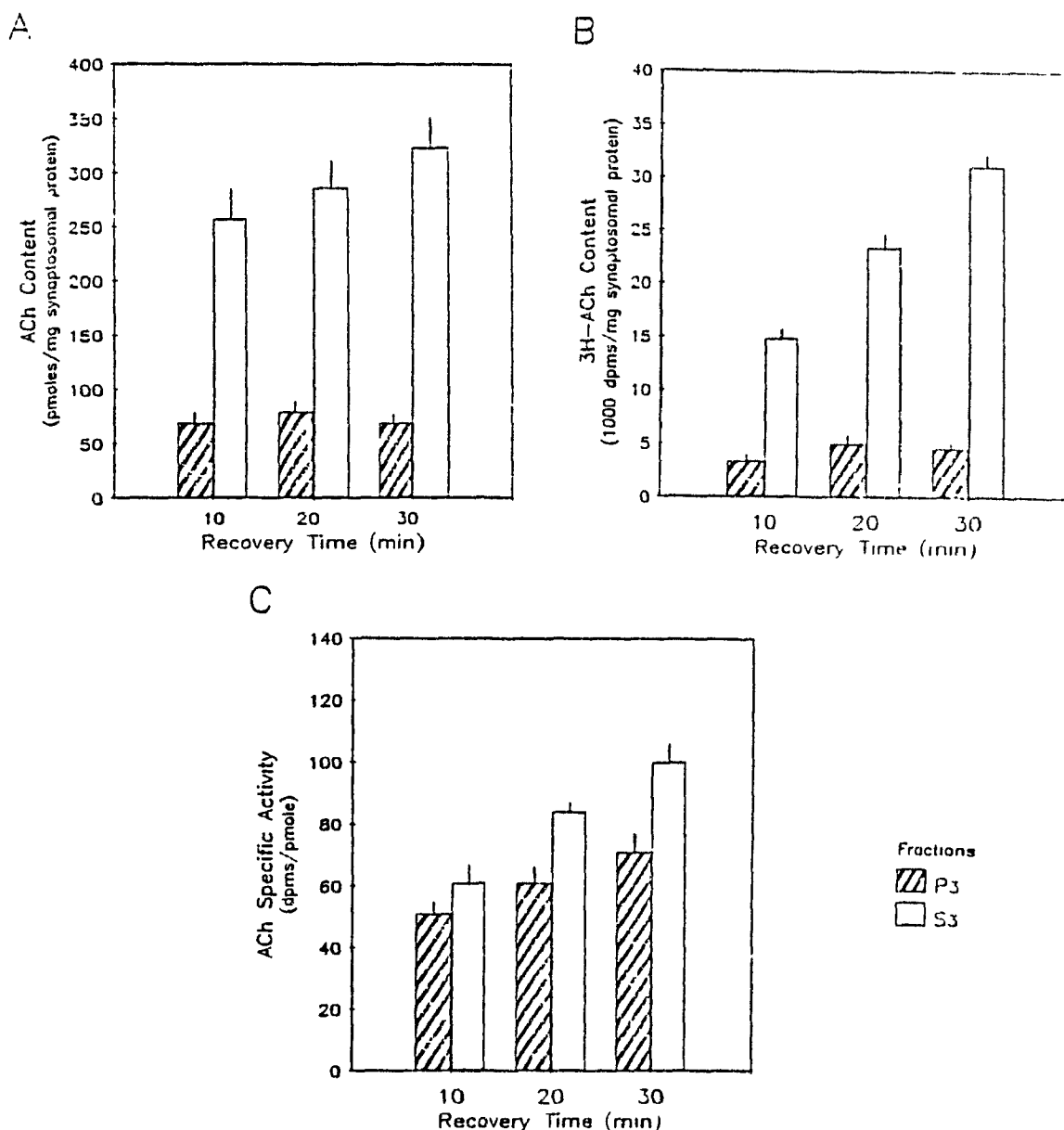


Figure 28R. The effect of time on the ACh and [³H]-ACh content from the cytoplasmic and occluded pools of control slices. Slices were incubated following the protocol outlined on the top panel of figure 23R and the slices homogenized at the end of the experiment. The P₂ pellet was isolated, lysed, and the cytoplasmic fraction (S₃) separated from the occluded fraction (P₃) by centrifugation. Only slices not stimulated during the recovery phase of the experiment were used. Panel A illustrates the changes in ACh content of fractions seen with time. Panel B illustrates the changes in the [³H]-ACh content of fractions with time. Panel C contains the specific activity of the ACh content from the fractions with time. Each bar represents the mean $\pm$ s.e.m. of 8 determinations.

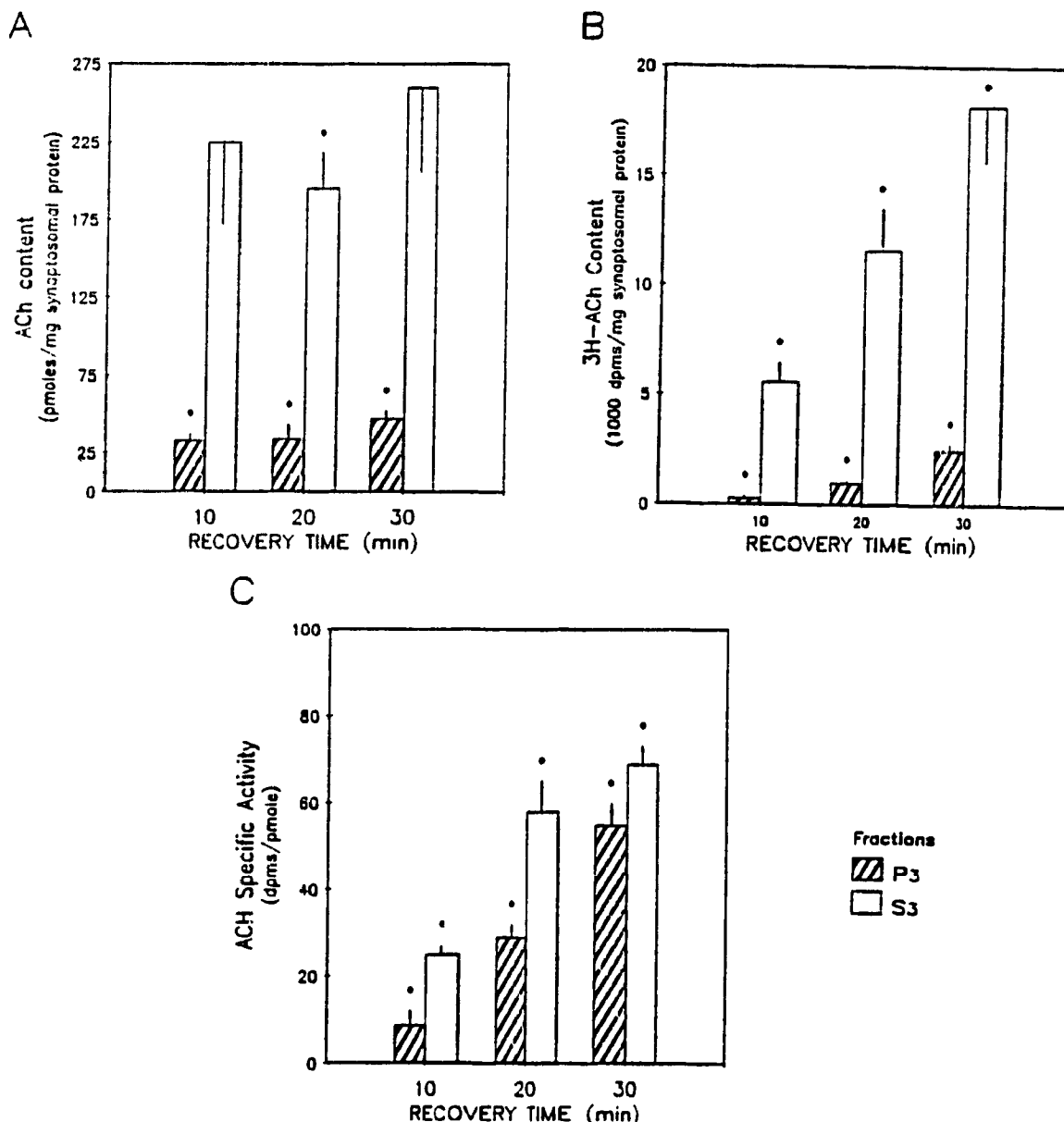


Figure 29R. The effect of time following the removal of AH5183 on the ACh and [³H]-ACh content from the cytoplasmic and occluded pools of drugged slices. Slices were incubated following the protocol outlined on the bottom panel of figure 23R and the slices homogenized at the end of the experiment. The P₂ pellet was isolated, lysed, and the cytoplasmic fraction (S₃) separated from the occluded fraction (P₃) by centrifugation. Only slices not stimulated during the recovery phase of the experiment were used. Panel A illustrates the changes in ACh content of fractions seen with time. Panel B illustrates the changes in the [³H]-ACh content of fractions with time. Panel C contains the specific activity of the ACh content from the fractions with time. Each bar represents the mean $\pm$ s.e.m. of 8 determinations. (*) denotes a $p < 0.05$ between control and AH5183-treated slices as determined by ANOVA and Tukey post-hoc test.

all time points; the differences were not statistically significant by ANOVA, but the decrease in ACh content at the 20 min time point was significant when compared to its control. [^3H]-ACh accumulation by both fractions is given in panel B of figure 29R. The P_3 fraction increased its accumulation of [^3H]-ACh in a linear fashion for the duration of the recovery period (1.1×10^4 dpm/10 min/mg synaptosomal protein) as did the S_3 fraction (6.3×10^4 dpm/10 min/mg synaptosomal protein), but the accumulation by the S_3 fraction was about 6 fold higher than that of the P_3 fraction. The accumulation of the [^3H]-ACh was found to be significantly less for both fractions at all times when compared to their appropriate controls. A similar pattern to that seen in controls was found when the S.A. of the ACh content of these fractions were calculated (panel C, figure 29R). The S.A. of the ACh content of both fractions increased linearly during the recovery period, but unlike their controls the rate of increase in the ACh's S.A. was the same for both fractions (21 dpm/pmole and 22 dpm/pmole for the P_3 and S_3 fractions, respectively). As with the [^3H]-ACh accumulation, the S.A. of the ACh content for both fractions was found to be statistically less than the levels achieved by their controls (ANOVA).

Neither the changes in the ACh content of either fractions, nor the changes in the S.A. of the ACh content resemble the observed changes in the release profiles obtained from slices exposed to AH5183 (see figures 24R, B and figure 29R). The S.A. of the ACh content of both fractions from these slices rose in a linear fashion and were significantly smaller than control values at all times, yet only the S.A. of released ACh for the first recovery period was significantly less than that of controls. Furthermore, the S.A. of the ACh content of both fractions was found to be significantly smaller than the S.A. of released ACh at all times.

The changes seen in the P_3 fraction are a mean of the changes taking place in several subcellular-bound pools of ACh. To see if any given component of the P_3 fraction has kinetics that match that of released ACh requires that the fraction be further subfractionated. It should be noted here that the major finding of section 3.4 is that one

part of the P₃ fraction (the D fraction) appears to be more sensitive to the blocking action of AH5183 than the others, and this difference might be helpful in the identification of a subfraction of the occluded pools of ACh as the source of released ACh.

3.5.4. Recovery of the acetylcholine and [³H]-acetylcholine content of the subcellular bound pools from AH5183-treated slices.

The purpose of the following experiments was two fold. First it was of interest to investigate the recovery of the various subcellular-bound fractions of ACh from AH5183-blockade because if there is a differential sensitivity to the blocking action of the drug by these fractions one might expect that the least sensitive of these should recover first. These experiments should, therefore, relate to the results obtained in experiments described in section 3.4. Secondly, it was important to evaluate if any changes observed in these fractions, following the removal of the drug, correlated with the changes in the release of ACh. Thus, the experiments described in this section were designed to investigate the way in which the subcellular-bound fractions recover with time from the actions of AH5183. The ability of these various fractions to accumulate ACh and [³H]-ACh were studied in HIPP slices.

Slices from the hippocampal formations of rats were prepared for incubation as described in the materials and methods. Slices used for both the control and AH5183 treatment were placed in a normal Krebs medium containing 10 μ M Ch (no eserine was used since only the subcellular stores of ACh were measured) for 45 min. Control slices had their medium replaced with fresh Krebs and were incubated a further 25 min with one change in the medium after 15 min. The slices were then stimulated with a 35 mM K⁺ Krebs medium containing 10 μ M Ch for 20 min with one change of the medium after 10 min. In slices exposed to AH5183, the medium, similar to that of controls except that it contained 10 μ M AH5183, was changed and slices incubated for 25 min with one change in the medium after 15 min. AH5183-treated slices were then stimulated using a 35 mM K⁺ Krebs medium containing the same concentration of Ch

and the drug as the previous medium. Slices were incubated for 20 min in this high K^+ medium with one change in the medium made after 10 min. Following the exposure to high K^+ , both control and AH5183-exposed slices were returned to normal Krebs medium containing 10 μ M Ch and 5 μ Ci/ml of [^{3} H]-Ch (0.5 Ci/mole). Slices were incubated in this medium from 10 to 30 min with the medium being changed every 10 min. Figure 30R outlines the incubation protocol used for control slices, top panel, and for slices exposed to AH5183, bottom panel.

At the end of this "recovery period", varying from 10 to 30 min, both control and drug-exposed slices were washed with a physiological saline solution, homogenized with a 0.32 M sucrose solution, and the P_2 pellet isolated. The P_2 pellet was lysed using a solution containing 5mM HEPES and 1mM EGTA and left on ice for 15 min. The lysed pellet was then layered on a three layered discontinuous sucrose gradient and centrifuged so as to separate the various fractions by buoyant density. At the conclusion of the centrifugation the layers were separated and the ACh and [^{3} H]-ACh contents extracted and assayed. A detailed description of these procedures is provided in the materials and methods.

The changes in the subcellular bound fractions of control slices are illustrated in panel A of figure 31R. Relative to the 10 min values, there is an increase in the ACh content of all fractions at the 20 min time point, but not at the 30 min time point (panel A). It is interesting that although changes in the ACh content of all fractions were found, with time, no change was noted in the tissue's ability to release the transmitter (see figure 24R). The [^{3} H]-ACh contents of the various fractions are shown in panel B of figure 31R. With the exception of the E&F fraction, all fractions' [^{3} H]-ACh content was found to be highest at the 20 min time point. The increase in the [^{3} H]-ACh accumulation by all fractions at 20 min was found to be significantly larger than at other times, with the exception of the E&F fraction where the small increase detected at 30 min was not found to be different from the accumulation of [^{3} H]-ACh occurring 20 min into the recovery period. Specific activities for the various fractions of ACh

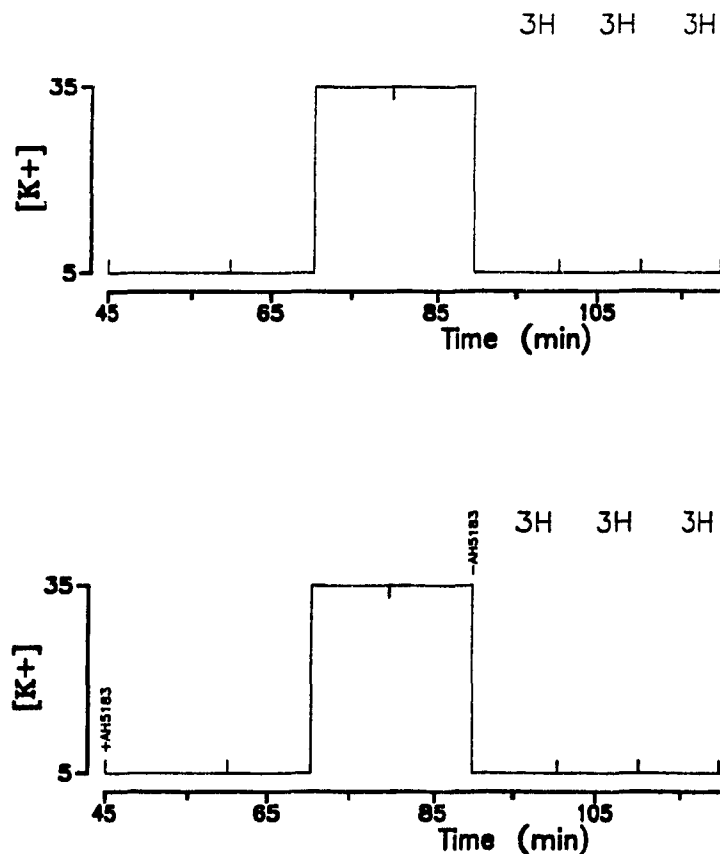


Figure 30R. Experimental protocol to determine the recovery kinetics of the ACh content of the subcellular occluded fractions from AH5183-treated HIPP slices. HIPP slices were prepared for incubation according to the procedure described in the materials and methods. All slices were given a 45 min recovery period in normal Krebs medium containing 10 μ M choline. Control slices then had their medium replaced with the same medium for a further 25 min while slices receiving AH5183 were exposed at this time to a similar medium containing 10 μ M AH5183. The medium of each was accordingly replaced with fresh medium after 15 min. All slices were then stimulated with a Krebs medium containing 35 mM K^+ for two 10 min periods. At the end of this incubation slices were returned to a 5 mM K^+ medium and in the case of AH5183-exposed slices the drug was removed from the medium. The new medium for all slices contained 5 μ Ci/ml of [^{3} H]-Ch. Slices were incubated from 10 to 30 min in this medium with the medium replaced every 10 min. The top panel outlines the procedure for the control slices and the bottom panel that for the drug-treated slices. The 3H on the figure indicates incubations containing [^{3} H]-Ch.

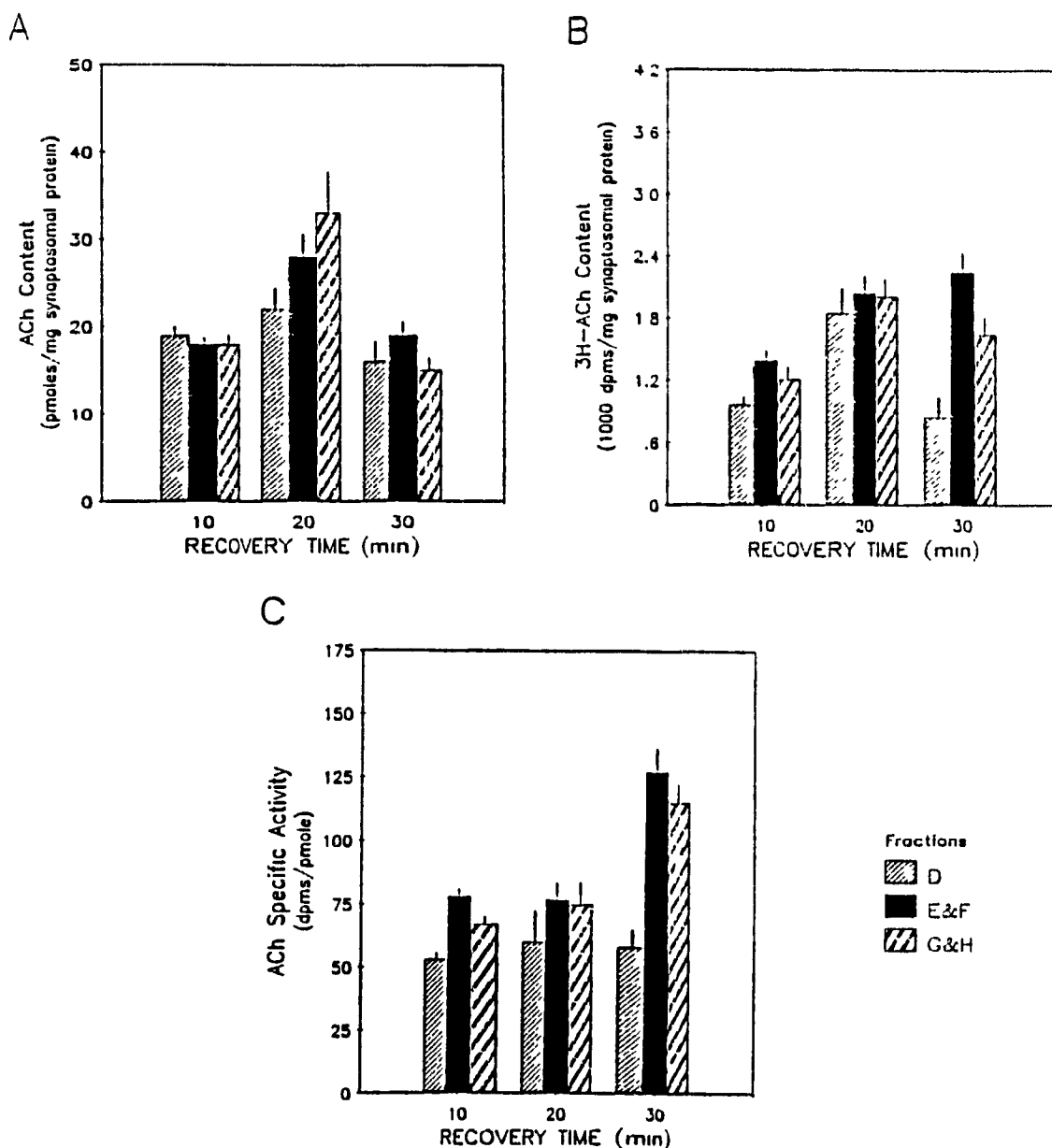


Figure 31R. The effect of time on the ACh and [³H]-ACh content of the subcellular bound pools from control slices. Slices were incubated following the procedure outlined in figure 30R, top panel. At the end of the experiment slices were homogenized and the P₂ pellet isolated. The P₂ pellet was lysed and layered on top of a discontinuous sucrose gradient and the fractions separated by buoyant density. The layers were separated and extracted and assayed for their content of ACh and [³H]-ACh. Panel A illustrates the effect of time on the ACh content from the various fractions. Panel B shows the accumulation of [³H]-ACh seen with time for the three fractions. Panel C graphs the calculated ACh S.A. of the various fractions with time. Each bar represents the mean ± s.e.m. of 12 determinations.

were calculated, based on the above measurements, and the results are illustrated in the figure 31R, panel C. The S.A. of ACh in all fractions was stable for the first 20 min of the recovery phase but then increased significantly by the final time point in the E&F and G&H fractions (the D fraction showed no change in the ACh S.A. during the recovery phase).

Figure 32R, panel A, shows the changes in the ACh content of the various occluded compartments following the removal of AH5183. The D fraction ACh content increased throughout the 30 min at a rate of 4.8 pmoles/mg synaptosomal protein/10 min. Both the E&F fraction and the G&H fraction increased their contents of ACh in a linear fashion over the 30 min of the recovery period at a rate of 8 and 7.8 pmoles/mg synaptosomal protein/10 min respectively. When a test of parallelism was done on the rate of ACh accumulation between the D and G&H fraction, it was found that the rates differed significantly from one another. The content of ACh for the three fractions was significantly less than that of controls for all the time points except for the 30 min time point; at this time the ACh content for the D fraction and the E&F fraction were not different from controls but the G&H fraction's content of ACh was significantly higher than the controls. The [^3H]-ACh content of the various fractions following the removal of AH5183 is shown in panel B of figure 32R. Both the E&F fraction and the G&H fraction, increased their accumulation of [^3H]-ACh following the removal of AH5183. Curiously, the D fraction showed no accumulation of [^3H]-ACh during the whole of the recovery period. All the compartments derived from AH5183-treated slices accumulated significantly less [^3H]-ACh than did the controls. The S.A. of the different fractions was estimated and the results of these calculations are shown in panel C of figure 32R. Since no appreciable [^3H]-ACh was accumulated by the D fraction, the S.A. of its ACh remained at or very near 0 for the entire recovery period. The E&F fraction's ACh S.A. reached a plateau from 20 to 30 min of the recovery period while the G&H fraction's ACh S.A. reached a peak in its ACh S.A. at 20 min following the removal of AH5183. As noted in figure 32R, the S.A. of all the fractions' ACh contents were significantly less

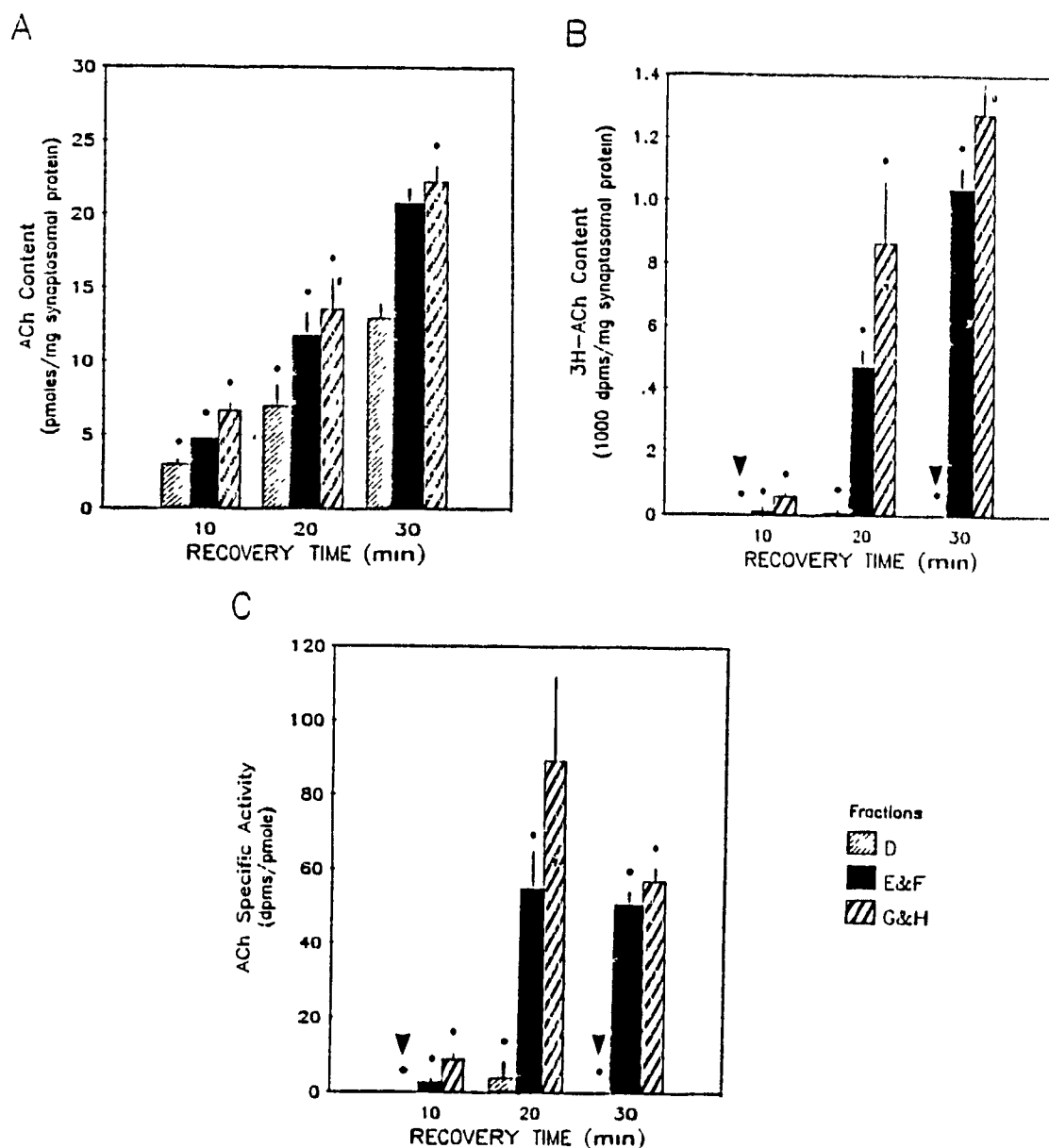


Figure 32R. The effect of time following the washout of AH5183 on the ACh and [^3H]-ACh content of the subcellular bound pools from AH5183-treated slices. Slices were incubated following the procedure outlined in figure 30R, bottom panel. At the end of the experiment slices were homogenized and the P_2 pellet isolated. The P_2 pellet was lysed and layered on top of a discontinuous sucrose gradient and the fractions separated by buoyant density. The layers were separated and extracted and assayed for their content of ACh and [^3H]-ACh. Panel A illustrates the effect of time following the removal of AH5183 on the ACh content from the various fractions. Panel B shows the accumulation of [^3H]-ACh seen with time for the three fractions. Panel C graphs the calculated ACh S.A. of the various fractions after drug removal. Each bar represents the mean $\pm$ s.e.m. of 12 determinations. The statistical significance in the difference of the means between control and AH5183-treated slices was determined by ANOVA and Tukey post-hoc test. (*) denotes a $p < 0.05$. (▼) Indicates that no [^3H]-ACh was detected.

than controls with the exception of the G&H fraction, whose ACh S.A. was not different from controls 20 min into the recovery period. The S.A. of ACh for the various fractions was always lower than that of released ACh (see figure 25R). It should be remembered that the S.A. of the [^3H]-Ch used in these experiments was 2.5 times than that used in the release experiments.

SUMMARY AND DISCUSSION OF SECTION 3.5

Slices recovering from AH5183 induced blockade of ACh release increase their release of ACh 20 to 30 min following the removal of the drug, but this recovery only reaches about 42% of the amounts seen in the controls. Interestingly the S.A. of released ACh does not increase linearly or reach a plateau but instead peaks 20 min into the recovery phase for both control and AH5183-treated slices. The pattern of ACh release and its S.A. could not be correlated to that of the total tissue ACh nor to that of the cytoplasmic or bound fractions of synaptosomal ACh. In general, the S.A. of the ACh found in the tissue increased with time as did the S.A.s of the ACh content of both the cytoplasmic (S_3) and occluded (P_3) fractions of synaptosomes taken from HIPP slices exposed to AH5183. Thus, it appears that the source of the releasable ACh from slices recovering from AH5183-blockade cannot be associated with the larger, and conglomerate, pools of ACh.

Fractionation of the bound subcellular compartments was undertaken with the expectation that more specific quantification of these ACh pools would uncover a relationship between released ACh and the state of the ACh in one or more of the subcellular compartments. The changes in the ACh S.A. of the E&F and G&H fractions from drugged slices resembled the pattern of released ACh S.A. more closely than the changes seen in the fractions obtained from control slices. However, the ACh content's S.A. for all fractions of both control and AH5183-treated slices were less than the S.A. of released ACh. Table 6R summarizes the S.A. data for released ACh and for that of the transmitter stores of various tissue fractions.

Table 6R. Summary of the specific activity of released ACh and that of various tissue fractions.

Recovery times	10 min	20 min	30 min
Released ACh	29 +/- 4	56 +/- 9	46 +/- 5
Total Tissue ACh	8 +/- 1	20 +/- 1	26 +/- 1
S ₁ Fraction	12 +/- 1	29 +/- 3	34 +/- 2
P ₁ Fraction	4 +/- 2	14 +/- 1	28 +/- 2
D Fraction	0 +/- 0	1 +/- 1	0 +/- 0
E&F Fraction	1 +/- 0	11 +/- 2	10 +/- 1
G&H Fraction	2 +/- 0	18 +/- 5	11 +/- 1

The specific activities have been normalized by dividing the dpm/pmol specific activity for each sample by the nCi of [³H]-Chol of incubation medium

Table 6R. Summary of the specific activity of released ACh and that of various tissue fractions. HIPP slices were treated as described in figures 23R and 30R and the specific activity of released ACh as well as the specific activity of the tissue and fractions were calculated. Because experiments where the occluded compartments of ACh were investigated utilized [³H]-Ch that was 2.5 times more radioactive than that of experiments where the recovery of ACh release was ascertained, the values presented above are normalized. All values are from AH5183 exposed slices. The values given are the mean $\pm$ s.e.m. for from 8 to 12 determinations.

All fractions from drug-exposed slices recovered their ACh content to some extent, upon removal of the drug, but the source of the accumulated ACh appears to differ greatly between the D fraction and that of the other two fractions (E&F fraction and G&H fraction). The D fraction's increase in ACh did not involve [³H]-ACh, thus indicating that the source of either the ACh or the Ch used in its synthesis were from different compartments than that of the other two fractions.

DISCUSSION

4.1. INHIBITION OF ACETYLCHOLINE RELEASE BY AH5183 IS NOT ACCOMPANIED BY A DEPLETION OF VESICULAR ACETYLCHOLINE IN THE SUPERIOR CERVICAL GANGLION.

The main effect of 2-(4-phenylpiperidino)cyclohexanol (AH5183) is to inhibit the active uptake of acetylcholine (ACh) by vesicles (Anderson *et al.*, 1983) while not emptying them of their prepackaged transmitter stores (Anderson *et al.*, 1986). Results presented here and those reported by Collier *et al.* (1986) demonstrate that in the presence of 10 μ M AH5183 nerve impulses can cause the release of 14% of the initial ACh content of the cat superior cervical ganglion (SCG). Thus, it appears that approximately 14% of the SCG's ACh content is present in a vesicular compartment which is capable of directly releasing its transmitter in response to nerve impulses since it seems unlikely that much vesicular refilling occurs in the presence of AH5183. These findings agree remarkably well with those of Birks and MacIntosh (1961) who found, by analyzing the kinetics of ACh release from the SCG, that 14-15% of the initial ACh content was present in a form that was readily available for release by nerve impulses while the rest of the releasable ACh seemed to belong to a second component which required mobilization to a releasable form.

If one accepts that vesicular ACh is the source of ACh released by nerve impulses (Suszkiw *et al.*, 1978; von Schwarzenfeld, 1979; Carroll and Aspry, 1980; Giompres *et al.*, 1981(b); Michaelson and Burstein, 1985), then it would appear that at least 14% of the ACh content of the cat SCG is to be found in a vesicular compartment. If this were to represent the totality of vesicular ACh, the cat SCG contains much less of its transmitter stores in an occluded compartment than does the *Torpedo* electric plaques, 30% (Giompres *et al.*, 1981b), rat cortex, 50% (Salehmoghaddam and Collier, 1976), bovine SCG, 25% (Wilson and Cooper, 1972), or rat hippocampus, 25% (results presented here). These estimates of the occluded compartment of the respective tissues should be considered underestimates because of the likely loss of ACh from vesicular stores during their preparation.

The venom of the black widow spider (BWSV) was used to test whether the blockade of stimulated ACh release caused by AH5183 is due to the exhaustion of vesicular transmitter stores. This venom is known to cause vesicle exocytosis and to eventually lead to the disappearance of vesicle profiles from nerve terminals (Clark *et al.*, 1970, 1972, Frontali *et al.*, 1976; Tzeng *et al.*, 1978). By using the choline (Ch) analogue, diethylhomocholine (DEHCh), which upon acetylation is mainly located in the cytoplasm due to its poor accumulation by vesicles (Welner and Collier, 1984, 1985), it was confirmed that the venom does not lead to the release of cytoplasmic components in the size range of ACh from ganglia. Thus if a vesicular population of transmitter stores still exists in the ganglion one would expect that BWSV will cause this ACh to be released. The results demonstrate that BWSV, in the presence of AH5183, can release substantial amounts of ACh when nerve impulses can no longer elicit the release of ACh above basal levels.

These results suggest that there is a population of vesicles which is involved in the nerve impulse stimulated release of ACh and a second vesicular population which is not mobilized by nerve impulses in AH5183's presence but is by BWSV. Given that in the presence of AH5183, approximately 14% of ACh stores can be released by nerve impulses, and since this is in quantitative agreement with Birks and MacIntosh's (1961) readily-releasable pool of ACh, it suggests that this transmitter pool is prepackaged and only requires exocytosis. Further release of ACh appears to require the mobilization of either newly synthesized ACh or ACh from another compartment into this vesicular pool via the cytoplasm. This concept is in agreement with studies at the neuromuscular junction where the miniature end-plate potentials (m.e.p.p.) were measured under conditions where ACh synthesis was blocked by hemicholinium-3 (HC-3) (Elmqvist and Quastel, 1965), a high-affinity Ch uptake blocker, or where the Ch analogue, monoethylcholine (MECh), was used (Large and Rang, 1978). Elmqvist and Quastel (1965) reported that the size of m.e.p.p.s measured in hemidiaphragms decreased in a graded fashion when the preparation was bathed with HC-3 and the nerve terminals stimulated by high K^+ . The drop in m.e.p.p. size was gradual,

thus suggesting that there was a rapid re-shuffling of existing ACh between the source of released transmitter and the rest of the terminal. Large and Rang (1978) used the Ch analogue, MECh, because it can be taken into nerve terminals, acetylated, and then the acetylated compound can act as a false transmitter giving a slightly shorter nicotinic receptor open time. As the acetylmonoethylcholine (AMECh) replaces the ACh one could expect two kinds of results, one where decay constants of the measured m.e.p.p.s show a very broad distribution (bi-modal), suggesting that some m.e.p.p.s consist of ACh and some m.e.p.p.s consist of AMECh, and two, where the variance in m.e.p.p. time constants of decay increases but stays below the theoretical variance expected by a mixture of ACh m.e.p.p.s and AMECh m.e.p.p.s, suggesting that each m.e.p.p. has a mixture of ACh and AMECh. They found the second type of result and thus concluded, like Elmqvist and Quastel, that mixing was occurring.

Collier *et al.* (1986) first observed that preganglionic stimulation of the SCG in the presence of AH5183 lead to an increase in the ACh content of the ganglion by about 50%. Because this increase could be observed even in the absence of an acetylcholinesterase (AChE) inhibitor they suggested that the increased transmitter content had to be in a compartment that was protected from the action of AChE. If this extra store of ACh is located in a cytoplasmic compartment, one would expect that releasing part of the vesicular ACh stores, by stimulating the preganglionic nerve of the SCG while in the presence of AH5183, would lead to BWSV releasing less ACh from these ganglia than from those which have not been exposed to the drug. This was not the case, as BWSV released the same or more ACh from AH5183-blocked ganglia than from controls. One possible interpretation is that part of the increase in ACh content seen with stimulation in the presence of AH5183 is present in a vesicular compartment.

Because this increase in ACh content is seen only when AH5183-exposed ganglia are stimulated, AH5183 alone does not stimulate transmitter synthesis but rather seems not to interfere in the normal synthesis of ACh that is triggered by the stimulus (Collier *et al.*, 1986); therefore, it is possible that some of the ACh synthesized in the

presence of AH5183 may be located in a vesicular compartment. To test this possibility, ganglia were perfused with [^3H]-Ch during the period of stimulation and AH5183 exposure so as to label the ACh synthesized during this period. BWSV released [^3H]-ACh and did so with the same temporal pattern as that of total ACh. Thus, even though AH5183 is known to inhibit the active uptake of ACh by vesicles, a vesicular compartment appears to be capable of accumulating newly synthesized ACh in the continued presence of the drug. It is unlikely that this newly synthesized ACh is packaged into empty vesicles, because the temporal pattern of [^3H]-ACh release matched that of total ACh. Rather it would seem that there is an equilibration between cytoplasmic and vesicular ACh and [^3H]-ACh during stimulation, and/or that the vesicle's content of ACh increases by the additional transport of [^3H]-ACh. Reports by Collier *et al.* (1986) and Cabeza and Collier (1988) suggest that both processes may be taking place, first because a good way to protect the increased content of ACh, seen with AH5183 exposure, from AChE would be to store it in vesicles and, because in SCG recovering from AH5183 blockade, it was found that ACh synthesized in the presence of AH5183 is as releasable, by a short stimulus, as is the ACh synthesized following the washout of the drug (neither was preferentially released).

The model of a cholinergic terminal shown in figure 1D combines these observations and formed the basis for all subsequent investigations presented here. In this working model, the readily releasable pool of ACh stores is represented as a compartment consisting of vesicles located next to or near the release sites of the plasma membrane. This compartment is completely inhibited from accumulating new ACh when 10 μM AH5183 is present. These vesicles are those which participate in nerve impulse stimulated release of transmitter and constitute, in the cat SCG, 14% of the initial ACh stores. A second vesicular population is depicted which is not involved in the nerve impulse stimulated release of transmitter into the synaptic cleft, but appears to contribute ACh for release via a cytoplasmic route to the readily releasable compartment, and therefore most likely serves as a reserve pool of ACh. Furthermore, this reserve vesicular compartment is

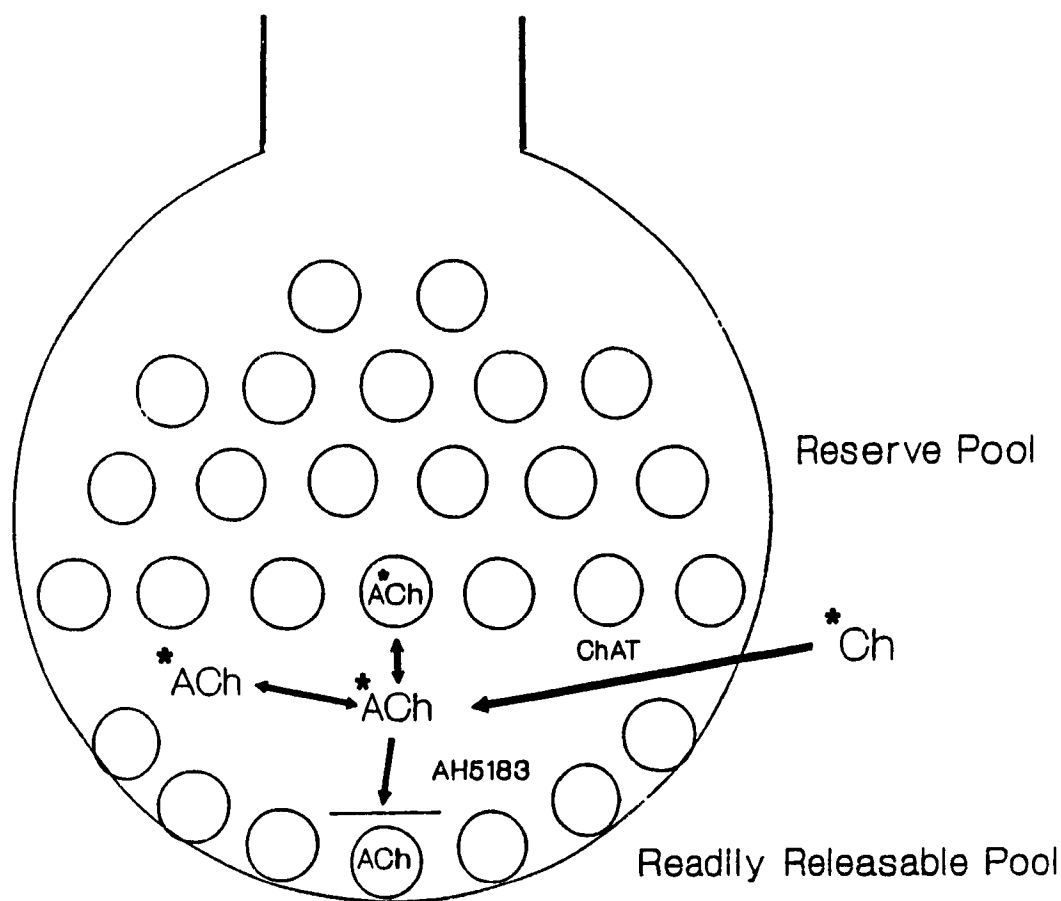


Figure 1D. Model of the cholinergic terminal determined from experiments where the effects of AH5183 and BWSV were tested in the cat SCG. The readily-releasable pool is that compartment which is inhibited from accumulating ACh by AH5183 and which constitutes 14% of the initial ACh content of the ganglion. The reserve pool of vesicles are those thought to be mobilized by BWSV to release their ACh content after AH5183 has blocked ACh release by nerve impulses. Since the venom released ACh contains transmitter synthesized in the presence of AH5183, these vesicles are illustrate as being insensitive to AH5183.

capable of accumulating ACh that is synthesized in the presence of AH5183. It is this second pool of vesicular ACh which is expected to be the source of ACh released by BWSV from AH5183-blocked ganglia.

In order to pursue the various predictions that this model of the cholinergic terminal contains, it was necessary to directly measure the various ACh compartments under a variety of conditions. For this, slices of the hippocampal formation of rat brain were used. This model offers some advantages over the SCG in that it is a tissue that can easily be homogenized and various subcellular fractions isolated from it, and at the same time it is similar to the SCG in that most of its cholinergic input is in the form of nerve terminals and not interneurons (reviewed by Kasa, 1986).

4.2 THE READILY-RELEASABLE POOL OF ACETYLCHOLINE IN HIPPOCAMPAL SLICES IS ESTIMATED TO BE ABOUT 20-27% OF THE ACETYLCHOLINE CONTENT.

AH5183 can decrease the stimulated release of ACh from a variety of cholinergic tissues and by a variety of stimuli (Marshall, 1970; Gandhi and Marshall, 1973; Carroll, 1985; Otero *et al.*, 1985; Jope and Johnson, 1986; Michaelson *et al.*, 1986; Collier *et al.*, 1986; Ricny and Collier, 1986; Suszkiw and Toth, 1986). In hippocampal (HIPPO) slices 10 μ M AH5183, greatly decreased the release of ACh by 35 mM K^+ , such that stimulated release was only 2 fold higher than the basal rate instead of the 4-7 fold increase seen in the drug's absence. Whereas in drug-exposed ganglia, the initial first 2 min of stimulated ACh release was normal (Collier *et al.*, 1986) and then subsequently decreased to basal levels, in the drug-exposed HIPPO slices, high K^+ stimulated release of ACh was greatly attenuated but transmitter release remained the same after 30 min of stimulation and did not return to basal levels until the stimulus was withdrawn. One likely explanation for this is that the difference is due to the secretagogue, K^+ versus electrical stimulation of the nerve, and not to tissue differences. Collier and Katz (1971) noted that high K^+ was capable of releasing surplus ACh from the cat SCG, while electrical stimulation of the preganglionic nerve was

not. Surplus ACh is a pool of ACh which forms when AChE inhibitors, such as eserine, are used and is not only not released by physiological stimuli (Birks and Macintosh, 1961), but also appears to have a different turnover from the rest of the ACh stores (Collier and Katz, 1971). Thus it is likely that even after the readily-releasable pool of ACh is exhausted, due to the presence of AH5183, the continued release of ACh by high K^+ is the result of surplus ACh release.

Due to the difference in the release of ACh between AH5183-treated HIPPI slices and the cat SCG it is unlikely that a reliable estimate of the readily-releasable ACh pool can be made by measuring released ACh and comparing this to the initial ACh content of the slices. The size of this ACh compartment was instead estimated by measuring the decrease in the ACh content seen in AH5183-treated slices exposed to high K^+ and this measure was compared to the ACh content of unstimulated, not drug-exposed tissue. Because stimulation and exposure to AH5183 may lead to an increase in the ACh content of HIPPI slices, as with the cat SCG, the high-affinity Ch uptake blocker, HC-3, was used to prevent the synthesis of ACh during stimulation (Birks and Macintosh, 1961; Matthews, 1966; Collier and MacIntosh, 1969). When total tissue ACh was used for the estimate, this compartment was found to be 20% of the ACh content but if synaptosomal ACh was used, the estimate of the readily-releasable ACh pool was 27%. The ACh content of AH5183-treated slices did not decrease until the slices were exposed to both AH5183 and HC-3, just prior to the period of high K^+ stimulation, and a similar but smaller decrease was also seen in control slices upon exposure to HC-3. In contrast, when the synaptosomal ACh content from these slices was measured, the decrease in content of both drug-treated and control tissue was found to occur just prior to HC-3 exposure. Surprisingly, exposure to high K^+ only caused a further small decrease in the ACh content of slices and had no effect on the ACh content of synaptosomes derived from these slices. These results suggest that even when slices were incubated in normal Krebs medium, there was a rapid turnover of readily-releasable ACh. This behavior of AH5183-treated slices might be explained by the

observation that upon exposing cortical synaptosomes to AH5183 there was a transient increase in the basal release of ACh (Otero *et al.*, 1985)

4.3. ACETYLCHOLINE RELEASED BY α -LTX FROM AH5183-BLOCKED HIPPOCAMPAL SLICES IS MAINLY ASSOCIATED WITH CYTOPLASMIC ACETYLCHOLINE

Hippocampal slices were exposed to AH5183, stimulated with 35 mM K^+ , and were exposed to α -Latrotoxin (α -LTX), the active component of BWSV (Grasso, 1976; Tzeng and Siekevitz, 1978; Knipper *et al.*, 1986), to test whether slices responded in the same way as did the cat SCG. AH5183-treated slices subsequently exposed to the toxin released more ACh than high K^+ and did so to the same extent as controls. Thus, as in the cat SCG, toxin was capable of releasing ACh that otherwise appeared unreleasable by other secretagogues when AH5183 was present. Since the working model of the cholinergic terminal suggests that the source of this toxin released transmitter might be that population of vesicles which serve as a reserve pool of ACh it was important to directly determine from whence this released transmitter came.

To identify the source of this released ACh, slices were also treated with HC-3 to stop the synthesis of any new ACh which could thus contribute transmitter to one or more compartments. Table 4R demonstrates that no increase in synthesis was seen in tissues exposed to α -LTX under these conditions. That is, the increase in ACh release seen with toxin exposure was quantitatively matched by a decrease in the ACh content of these slices. Isolation of the cytoplasmic (S_3) and occluded (P_3) compartments of ACh from synaptosomes, derived from slices used in the release experiments, indicates that most of the loss of ACh content seen with toxin exposure came from the cytosolic fraction and not the occluded fractions, opposite to what one might have anticipated. There was a decrease in the occluded compartment's content of ACh, with toxin treatment, but the decrease was small, and in AH5183-treated tissues, the difference seen with exposure to α -LTX was not significant. These changes were not the result of either exposure to AH5183 or HC-3, for recent preliminary experiments using only toxin, in previously un-

stimulated slices from rat hippocampal formations, gave similar results, with most of the ACh changes being seen in the S_3 fraction (results not shown here). Though some indirect measures of the source of α -LTX released ACh have been previously reported (Gorio *et al.*, 1978), this is the first report of the direct measurement of changes in the ACh content of the various subcellular compartments seen as a result of toxin treatment.

If the vesicular compartment not mobilized to release ACh by K^+ , in the presence of AH5183, can accumulate ACh from the cytoplasm, as our working model predicts, then it is possible that this compartment shuttles ACh from the cytoplasm to the terminal's exterior in response to α -LTX. Though α -LTX has been shown, in many cholinergic tissues, to cause vesicular exhaustion (Clark *et al.*, 1972; Frontali *et al.*, 1976; Tzeng *et al.*, 1978) not all studies report the loss of vesicular profiles from the terminals (Baba and Cooper, 1986). Furthermore, even in preparations where the toxin eventually caused the loss of intraterminal vesicles, there appeared to be some recycling taking place (Fesce *et al.*, 1986). This limited recycling was seen when toxin was given in a medium containing Ca^{+2} , as was the case in these experiments. Only when no Ca^{+2} was present and the major divalent cation was Mg^{+2} did there appear to be no vesicle recycling with exposure to toxin (Hurlbut *et al.*, 1990).

When the Ch analogue, DEHCh, was used to test whether the toxin could release cytoplasmic constituents in the size range of ACh, it was found that α -LTX did increase the rate of [^3H]-ADEHCh release. The increase in the release of the analogue was about 27% over the efflux of this compound from slices not exposed to the toxin. Though the compound, ADEHCh, is mainly found in the cytoplasm, some can accumulate in the occluded compartments (Welner and Collier, 1984). Because the rate of [^3H]-ADEHCh was a single time determination, unlike the similar experiment done in the SCG, an estimate of the temporal pattern of release of this compound by α -LTX was not possible and, thus, one cannot know if the increased release of analogue occurs late in the 15 min exposure or whether the change occurs much earlier. Since α -LTX depolarizes the

membrane, it is possible that the driving force for the leakage of a cation increased. Nonetheless, it is unlikely that most of the ACh released by α -LTX came from the cytoplasmic leakage of transmitter since the change in ACh release seen with α -LTX was about 388% above that seen in slices left at rest, while the increased efflux of the analogue was considerably less.

Examination of the various occluded compartments that make up the P₃ pellet showed that, in AH5183-exposed slices, all occluded compartments lost ACh to about the same extent. In control slices, only the fraction containing the monodispersed vesicles (D fraction) was significantly affected by toxin treatment. If α -LTX causes vesicles to fuse to the plasma membrane of terminals and in the presence of Ca^{+2} allows for some vesicle recycling, then perhaps a longer exposure of control slices to α -LTX could be required before a significant decrease in the ACh content of the vesicular fraction associated with plasma membrane (fraction G&H in these experiments) could occur.

This body of results do not directly implicate one vesicular compartment as an important contributor to ACh release, in AH5183-treated slices exposed to α -LTX. However, if a vesicular compartment was capable of shuttling ACh from the cytoplasm to the exterior of the terminal, it must be capable of loading ACh while in the continued presence of AH5183. Such a vesicular compartment is also implicated from the results obtained in the cat SCG. Thus, experiments were designed to directly test the ability of various subcellular compartments to accumulate ACh while in the presence of 10 μM AH5183.

4.4. TWO OCCLUDED FRACTIONS ARE CAPABLE OF ACCUMULATING [³H]-ACETYLCHOLINE IN THE PRESENCE OF AH5183.

Previous studies using precursors of ACh have found that the occluded, subcellular fractions of tissues containing cholinergic terminals could accumulate ACh synthesized in the presence of AH5183 (Otero *et al.*, 1985; Jope and Johnson, 1986; Suszkiw and Manalis, 1987). These

studies were done in such a way that no conclusion could be reached concerning whether an occluded compartment actively accumulated this newly synthesized ACh, because the studies were only done in conditions where stimulation was used. Work of Anderson *et al.* (1983) has demonstrated that AH5183 is much less potent at inhibiting the passive uptake of ACh by vesicles (IC_{50} around 20 μ M versus 40 nM for active uptake) and so it is difficult to know if this ACh accumulation is the result of passive or active processes. Experiments where AH5183-treated HIPP slices were left at rest or were stimulated in the presence of [^{3} H]-Ch were done to distinguish between passive and active ACh accumulation by the occluded compartments since the passive process accumulates ACh to a much lower extent than the active one and does not increase the accumulation of [^{3} H]-ACh with time (Anderson *et al.*, 1986).

Stimulation of drug-exposed slices did not lead to a decrease in the ACh content of the P_3 fraction but did increase the ACh content of the S_3 fraction. The fact that stimulation did not decrease the ACh content of the P_3 fraction by any sizable amount was not too surprising, given that in experiments where the ACh readily-releasable pool of HIPP slices was estimated, it was found that this fraction of ACh was quickly lost by the synaptosomal fraction even in the absence of stimulation (see section 3.2). These estimates of the readily-releasable pool were done in the presence of HC-3, which can block the new synthesis of ACh. Thus, if the content of the synaptosomal fraction did not decrease with stimulation, it suggests that that portion of ACh which can be released by stimulation with high K^+ , when eserine is absent from the medium, must already have been lost prior to the stimulation. The results from the present experiments further suggest that the release of ACh from slices, treated with AH5183, by high K^+ may indeed all be surplus ACh. Because experiments where the release of ACh was measured, were done in the presence of eserine and this was shown to increase the stores of ACh (see section 3.2), as has been noted in the SCG (Birks and MacIntosh, 1961; Collier and Katz, 1971), it was likely that surplus ACh was formed. This ACh was not releasable by nerve impulses in the SCG but it could be mobilized by high K^+ (Collier and Katz, 1971). Thus, it is

possible that in the absence of eserine no ACh would be released by K^+ from those slices exposed to AH5183.

In these experiments only the P_3 fraction of AH5183-exposed stimulated slices increased the specific activity (S.A.) of its ACh over that of AH5183-exposed slices left at rest. Furthermore, the S.A. ratio of the P_3/S_3 fractions from stimulated slices was similar to that of their controls. This suggests that stimulation increased the mixing of ACh between occluded and cytoplasmic compartments. When the occluded fractions were subdivided and analyzed for the effects of stimulation on their content of ACh and [^3H]-ACh, only the E&F and G&H fractions were seen to increase the S.A. of their ACh content significantly, while the D fraction's ACh S.A. remained almost 0. The enzymatic activity profiles of the different fractions showed that the G&H fraction contained a high amount of lactate dehydrogenase (LDH) activity, an enzyme known to be located in the cytoplasm. The possibility thus existed that the high S.A. of the ACh in this fraction was due to entrapment of cytoplasmic ACh and not to vesicular ACh. This possibility seems unlikely given that the S.A. of the ACh of both the E&F and G&H fractions increased by about two fold while that of the cytoplasmic fraction (S_3) remained unchanged. Given that LDH has been reported to stick to membranes under low ionic conditions (Fonnum, 1967), it seems more likely that the measured LDH of these occluded fractions was the result of adsorption.

Though the working model's prediction that a vesicular compartment should exist which is capable of accumulating ACh during AH5183-exposure is confirmed by these experiments, it is obvious that none of the occluded compartments was insensitive to the actions of AH5183. Even though two of the occluded fractions actively accumulated [^3H]-ACh in the presence of AH5183, their overall content of ACh was significantly decreased by drug exposure whether the slices were stimulated or left at rest. In contrast, Ricny and Collier (1986) found that in striatal slices exposed to AH5183 only the ACh content of the D fraction was significantly affected while that of the E&F fraction and the G&H fraction were found not to be different from controls. The reason for this dif-

ference in results is not at present clear. Because the monodispersed vesicle fraction (D fraction) did not accumulate any significant amount of [^3H]-ACh, it is obvious that at least part of the ACh occluded compartments is completely inhibited by AH5183.

The model depicted in figure 2D incorporates the results obtained from experiments described in section 3.4 and discussed above. In this model the major change is that two types of reserve vesicles are predicted to exist; one type which can actively accumulate [^3H]-ACh and is associated with the denser occluded compartments (E&F and/or G&H fraction) and a second type which is unable to accumulate any [^3H]-ACh in the presence of the drug and is associated with the lightest occluded fraction, fraction D. It has been noted in a variety of studies that this fraction, D, is the occluded fraction with the slowest incorporation of newly synthesized ACh (Barker *et al.*, 1972; Zimmermann and Whitaker, 1977; Suszkiw *et al.*, 1978; von Schwarzenfeld, 1979; Agoston *et al.*, 1985), but under drug-free conditions this fraction nonetheless incorporates significant amounts of [^3H]-ACh. Thus, these compartments can be distinguished by their ability to take up [^3H]-ACh in the presence of AH5183. Though the idea that a drug insensitive, or at least less sensitive, occluded compartment was predicted by the results obtained from the cat SCG, it is surprising that *in vitro* and in PC12 cells this drug is such a potent inhibitor of active vesicular ACh uptake (Anderson *et al.*, 1983; Melega and Howard, 1984) by all vesicular fractions measured, while in these mammalian preparations there should exist fractions which can actively accumulate newly synthesized ACh while the drug is present. Though it is not at all clear at this time why these apparent discrepancies exist, the possible existence of an endogenous compound that may modulate the AH5183 binding site has been suggested to exist (Norenberg and Parsons, 1989).

4.5. RECOVERY OF ACETYLCHOLINE AND [^3H]-ACETYLCHOLINE RELEASE FROM AH5183 BLOCKADE IS NOT TEMPORALLY RELATED TO THE RECOVERY OF THE SUBCELLULAR FRACTION'S ACETYLCHOLINE AND [^3H]-ACETYLCHOLINE CONTENT.

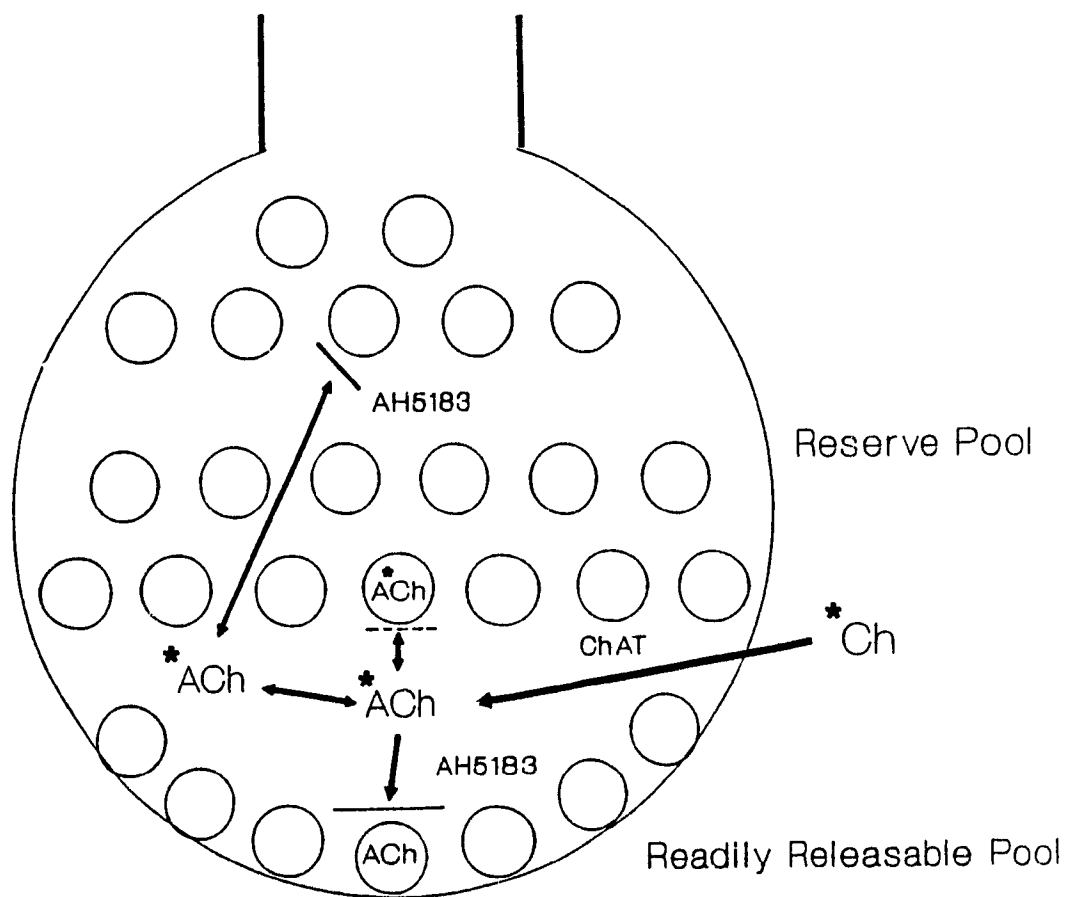


Figure 2D. Model of the cholinergic terminal derived from experiments done on hippocampal slices to determine the existence of an occluded fraction capable of accumulating newly synthesized ACh in the presence of AH5183. Model is the same as that depicted in figure 1D except that a second vesicular component of the reserve pool is hypothesized to exist. This second component does not accumulate newly synthesized ACh in the presence of AH5183 and is therefore depicted as being blocked by the drug.

Results on the HIPPP slices discussed so far do not allow us to identify what subcellular fraction would contain the readily-releasable pool of ACh, or whether, in the absence of AH5183, any of the measured fractions can participate in some way in the stimulus evoked release of transmitter directly. The kinetics of ACh release and changes in the ACh content of the various subcellular fractions following the wash out of AH5183 were investigated to test whether the subcellular fractions recover with different kinetics and to see if any of these fractions recover with similar kinetics to the release of ACh. If the difference in the effect of AH5183 between fraction D and fractions E&F and G&H is due to a differential sensitivity to AH5183 it was expected that they would show different recovery kinetics.

Slices previously exposed to AH5183 recovered the ability to release ACh to about 50% of controls thirty minutes following the wash out of the drug. This slow recovery from the effects of AH5183 has previously been described (Ricny and Collier, 1986; Cabeza and Collier, 1988). The largest change in the release of ACh following drug removal was found to occur between 10 and 20 min with little additional recovery being found at 30 min. Given that slices were continuously exposed to [^3H]-Ch during the recovery period, it was surprising that in both controls and AH5183-exposed slices the highest S.A. of released ACh was achieved at 20 min and not at the last time point investigated (30 min). Furthermore, although the release of ACh and [^3H]-ACh in drug-exposed slices was significantly less than controls, the S.A. of released ACh was similar between these slices at 20 and 30 min, though this measure was always less for slices exposed to AH5183. This suggests that the source of ACh for the packaging of released ACh is similar in slices after the removal of AH5183 and in controls, though drug-exposed slices appear to show slower kinetics.

Cabeza and Collier (1988) noted in the cat SCG that following the removal of AH5183, ACh released by nerve impulses contained ACh synthesized during drug exposure and ACh synthesized during the wash out of AH5183 in the same ratio as that present in the ganglion. Results presented here with HIPPP slices showed that the S.A. of tissue ACh in-

creased continuously during the recovery period and that the difference in the S.A. of the ACh content between control slices and slices exposed to AH5183 was larger than that seen in the released ACh. Thus, changes in tissue ACh content and S.A. did not reflect the changes seen in released ACh.

When the occluded and cytoplasmic compartments of ACh from slices treated with AH5183 were measured for recovery of ACh content following drug wash out, it was found that the P_3 fraction increased its content of ACh with time, while the S_3 fraction did not change consistently during these recovery times. The changes in the S.A. of the ACh found in these compartments reflected that seen in whole slices; with increased time there was a continuous increase in the ACh S.A. of both fractions. Thus, the changes in ACh S.A. for neither compartment resembled the changes seen in released ACh; as well, the S.A. of ACh found in these fractions was significantly lower than the S.A. of released ACh.

Suszkiw and O'Leary (1982) have also reported that the S.A. of released ACh is higher than that of the ACh found in the cytoplasmic or occluded fractions. However, when the P_3 fraction was further subfractionated, a highly labeled fraction was found whose ACh S.A. matches that seen in released ACh (Suszkiw *et al.*, 1978; von Schwarzenfeld, 1979). Further subfractionation of the occluded compartments from slices exposed to AH5183 was undertaken to test whether one of these fractions recovered with similar kinetics to those of ACh release and to investigate the prediction made by the model shown in figure 2D, that one compartment (fraction D) should recover with slower kinetics than the other occluded compartments.

All subcellular fractions increased their content of ACh in a linear fashion following the removal of AH5183. The rate of ACh accumulation for the E&F and G&H fractions (about 8 pmoles/mg synaptosomal protein/10 min) was about 1.6 times greater than the rate seen in the D fraction (5 pmoles/mg synaptosomal protein/10 min), the difference being statistically significant. Surprisingly, while the E&F and G&H fraction's accumulation of total ACh, following removal of the drug, was

due to an accumulation of both ACh and [^3H]-ACh, that of the D fraction was the result of ACh only, with no significant amounts of [^3H]-ACh being accumulated at any point of the recovery period. Thus, the increase in the ACh content of the D fraction resulted from the accumulation of either a preformed pool of ACh, one made prior to [^3H]-Ch exposure, or by a pool of ACh synthesized from non-external Ch, probably Ch containing lipids. In either case, this result suggests that some compartmentalization occurs in the cytoplasm. Since these results were not seen in the absence of AH5183, it is possible that the drug accentuated a small difference normally existing in the cytoplasm. This reasoning might explain why differences in the labeling of the ACh content of the various occluded fractions was seen even when AH5183 is not present (Suszkiw *et al.*, 1978; Von Schwarzenfeld, 1979; Agoston *et al.*, 1986).

The finding that fraction D recovered significantly slower than the other two occluded compartment subfractions is consistent with the model proposed in figure 2D; however, given that fraction D failed to accumulate [^3H]-ACh even during the recovery from AH5183, indicates that there need not be a difference in the AH5183 sensitivity between this fraction and those of fractions E&F and G&H. The possibility exists that this fraction, D, simply is the slowest to refill. The results do not support the idea that vesicles from a denser fraction become lighter by the accumulation of ACh and H_2O and therefore migrate from the denser to the lightest fraction of the occluded pool (Giompres *et al.*, 1981b). If this was the case one would expect to see [^3H]-ACh accumulated by this fraction.

The changes in S.A. of the ACh content of the various fractions of the occluded compartments indicate that only the G&H fraction had qualitatively similar changes in its ACh S.A. to those seen in the released ACh. However, the ACh S.A. of all fractions was less than that seen in the released transmitter. (It is important to note here that the [^3H]-Ch used in experiments where the various occluded compartments were investigated had a S.A. 2.5 times that of the experiments used to determine the release). It is, of course, possible that a component of

the G&H fraction contributes directly to the release of transmitter, but given that at 30 min the ACh content of this fraction was larger than that of the controls it must be a small portion because the total release of ACh at this time was still substantially less than that of controls. Furthermore, as with the experiments discussed in section 4.3, although the G&H fraction contains a reasonable amount of LFD, it is unlikely that much cytoplasmic ACh is entrapped in this fraction since the S.A. of the ACh found in this occluded fraction decreased at 30 min while that of the cytoplasm increased.

Results of the recovery from AH5183 blockade presented here do not identify a specific compartment as the source of released ACh. Of the fractions investigated in these experiments the most likely to play this role is the G&H fraction of the occluded compartments. However, since the results also suggest that cytoplasmic compartments may exist, it would be unreasonable to expect that the bulk characteristics of the cytoplasm would resemble those of a small compartment playing a role in the direct release of transmitter. It is known that vesicles associated with the heavier occluded fraction (in these experiments the G&H fraction) are more osmotically sensitive (Barker *et al.*, 1972; Suszkiw *et al.*, 1978) and, thus, during the isolation of the subcellular fractions it is possible that part of these vesicles are lysed (Barker *et al.*, 1972). If this is the case, then it is expected that the S₃ fraction contains both cytoplasmic ACh and some of the occluded ACh (for discussion of the "vesicular" verses the "cytoplasmic" hypothesis of release please refer to the introduction section 1.5).

The model illustrated in figure 3D incorporates the results from experiments investigating the recovery kinetics from AH5183-exposure. Unlike the model of figure 2D, the D fraction associated vesicles, though recovering more slowly, are here illustrated as being equally sensitive to AH5183 as the other vesicles forming the reserve pool of transmitter. The important observation included in this model is that this group of vesicles recovers its ACh content from a pool of ACh which in 30 min does not incorporate labeled ACh. If the vesicles of the reserve pool contribute to ACh release by releasing ACh into the

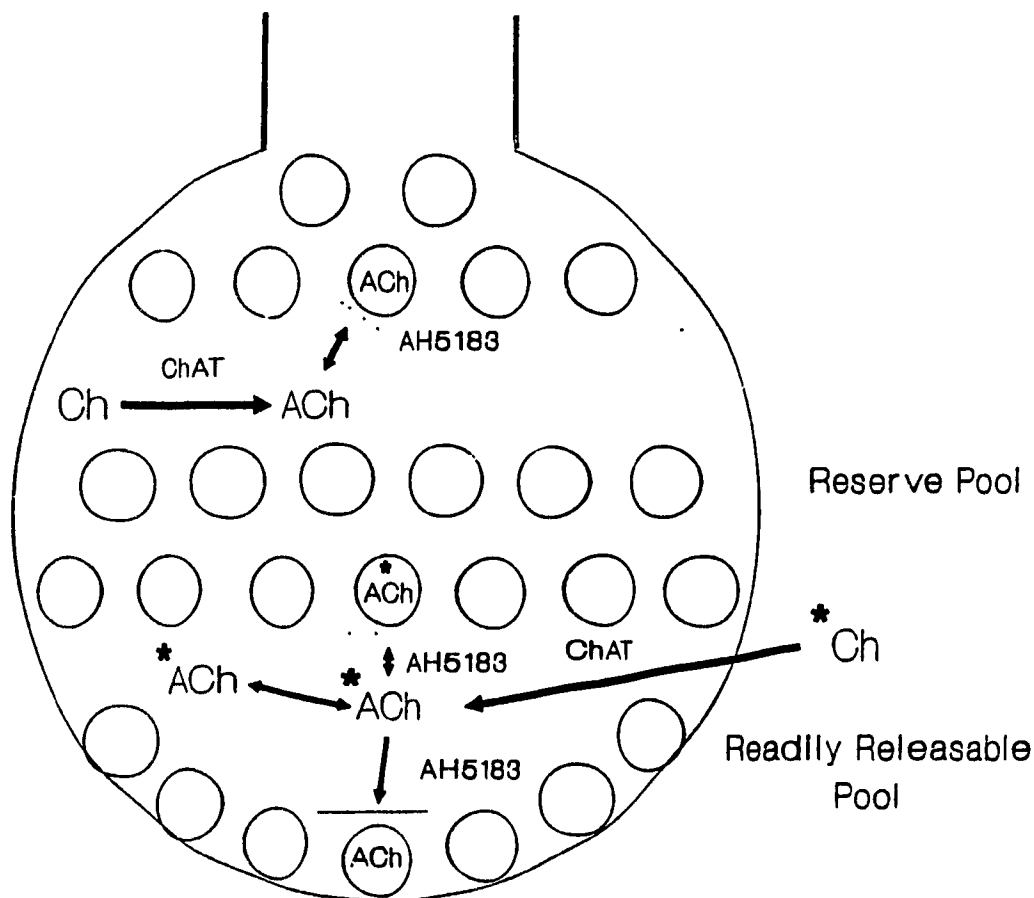


Figure 3D. Model of the cholinergic terminal derived from experiments done in hippocampal slices to determine the recovery of slices from the actions of AH5183. The model is similar to that illustrated in figure 2D but in this model all vesicular compartments are shown to be sensitive to the blocking actions of AH5183. The second vesicular component of the reserve pool is shown here as being equally sensitive to AH5183 as the rest of the vesicular components of the reserve pool. A separate cytoplasmic compartment containing ACh synthesized from an internal pool of unlabeled Ch is illustrated here. Double arrows leading from cytoplasmic stores of ACh to vesicles of the reserve pool of transmitter stores indicate an ability to exchange ACh with the cytoplasm in the continued presence of AH5183.

cytoplasm so that mobilization to a more readily-releasable state is possible, then, in the presence of AH5183, they may be able to keep this function while being partially inhibited from re-accumulating lost transmitter. This could explain why all occluded fractions show a decrease in their content when exposed to 10 μ M AH5183. Thus, double-headed arrows are added to the model to illustrate this possibility.

4.6 THE CHOLINERGIC NERVE TERMINAL

The experimental work comprising this thesis suggests that compartmentalization of ACh in the terminal is more complex than previously believed. These conclusions are all based on AH5183 having one main presynaptic action, that of inhibiting the active uptake of ACh by vesicles. Evidence from *Torpedo* electric organ, the cat SCG, and the neuromuscular junction suggests that AH5183 does not interfere with vesicle exocytosis or mobilization. In the *Torpedo* study, when AH5183 was added after labeling of transmitter pools, no drug effect was noted on the release of prepackaged ACh (Suszkiw and Manalis, 1987; Michaelson and Burstein, 1985; Michaelson *et al.*, 1986). When mammalian neuromuscular preparations were blocked by nerve stimulation at 1 Hz in the presence of AH5183, a shift to 0.1 Hz in the rate of stimulation lead to a transient increase in muscle contraction (though ACh release was not measured here, the results are consistent with a transient increase in ACh release)(Marshall, 1970a). If the cat SCG was exposed to 4-aminopyridine (4-AP) after the onset of transmitter release blockade by AH5183, there was a small and transient increase in ACh release (Collier *et al.*, 1986) and in the neuromuscular junction, blocked by AH5183, addition of 4-AP caused a transient increase in muscle contraction strength when the nerve was continuously stimulated at 1 Hz (Estrella *et al.*, 1988). AH5183 does have other presynaptic effects, it can competitively block Ch uptake, function as an anesthetic, block alpha receptors, block nicotinic receptors, and act as an inhibitor of AChE, but at the drug concentrations used in these experiments these effects were either not seen or were insignificant (see introduction for

details). Thus far, it remains a reasonable assumption that the major action of AH5183 at the cholinergic terminal is to block vesicular ACh uptake.

The observation, at the neuromuscular junction, that simply by shifting the stimulation rate from 1 Hz to 0.1 Hz leads to a transient recovery from AH5183-blockade suggests another complication to how terminal transmitter pools may contribute to release. In the electric organ of *Torpedo*, it has been previously noted that different compartments of ACh can be differentially released (Luz, et al., 1985). Fox (1990) has noted, using electron microscopy, that stimulation of the electric organ of *Torpedo* at 10 Hz preferentially caused the disappearance of synaptic vesicles in the size range of 68 nm, with little or no change in those vesicles having a diameter of around 90 nm, while stimulation at 0.1 Hz preferentially depleted the larger vesicles without affecting the smaller vesicle population. If specific vesicles can be mobilized by specific rates of stimulation then Marshall's (1970a) observations may be easily explained. In hemidiaphragms exposed to AH5183, 1 Hz stimulation is expected to deplete one component of a readily-releasable pool while sparing that component which releases at slower rates of stimulation. Upon changing the stimulation to 0.1 Hz a second component of the readily-releasable pool is stimulated to release its content of transmitter until it too is exhausted, thus producing only a temporary alleviation of the AH5183-induced blockade. The measure of muscle contraction is indirect, but recent studies suggest that even in the cat SCG, the readily-releasable pool of ACh may contain two components. In cat SCG perfused with AH5183 (10 μ M) and stimulated by preganglionic nerve stimulation to produce a blockade in the release of ACh, after the release of about 15% of the initial transmitter content, exposure to tityustoxin, in the absence of further electrical stimulation but in the presence of AH5183, lead to the release of a further 11% of the initial ACh content before the blockade of transmitter release was re-established (M. Prado, personal communication). Furthermore, cat SCG exposed to AH5183 and tityustoxin, with no preganglionic stimulation, released 16% of ganglial transmitter stores. Tityustoxin, is a scorpion

toxin known to cause the phosphorylation of synapsin I (Ribeiro and Gomez, 1986), to depend on Na^+ voltage channels for its action (Gomez et al., 1973, 1975), and is believed to act on transmitter release by a vesicular mechanism.

Figure 4D represents a speculative model of the cholinergic nerve terminal and how its ACh compartments may be involved in release. First, the model hypothesizes the existence of at least two vesicular components which together form the sum total of the readily-releasable pool, in that they are hypothesized to be ready to release their transmitter content when the appropriate stimulus is received. There is no reason to believe that each component will only respond to a specific stimulus or specific set of conditions; if only the probability of release between components varies with different stimuli, one can foresee that under specific circumstances released transmitter would preferentially come from one of the components. The reserve pool of ACh can be thought to consist of both cytoplasmic ACh and occluded ACh. The reserve, vesicular pool could make ACh available to the readily-releasable compartment via the cytoplasm. Vesicle components of the reserve pool seem to be located farther away from sites of Ch uptake and appear to be in a cytoplasmic region where ACh synthesized from external sources of Ch and Ch liberated from lipids mix, thus making their source of ACh dependent on the synthetic rate of each component. When the terminal is stimulated, transmitter stores from the readily-releasable pool are released to the outside of the terminal, while occluded transmitter components of the reserve pool could liberate their ACh into the cytoplasm so as to contribute ACh for release. At the end of an impulse, Ch uptake is increased and ACh synthesis replenishes the lost transmitter. During this period, all vesicles involved in the storage and release of transmitter are expected to refill with ACh. Since it is expected that vesicles forming the readily-releasable compartment will be near the plasma membrane, where Ch uptake is taking place, they should accumulate more of the newly synthesized ACh. Vesicles of the reserve pool, on the other hand, are likely to not be near the membrane and will refill with the ACh nearest to them, likely

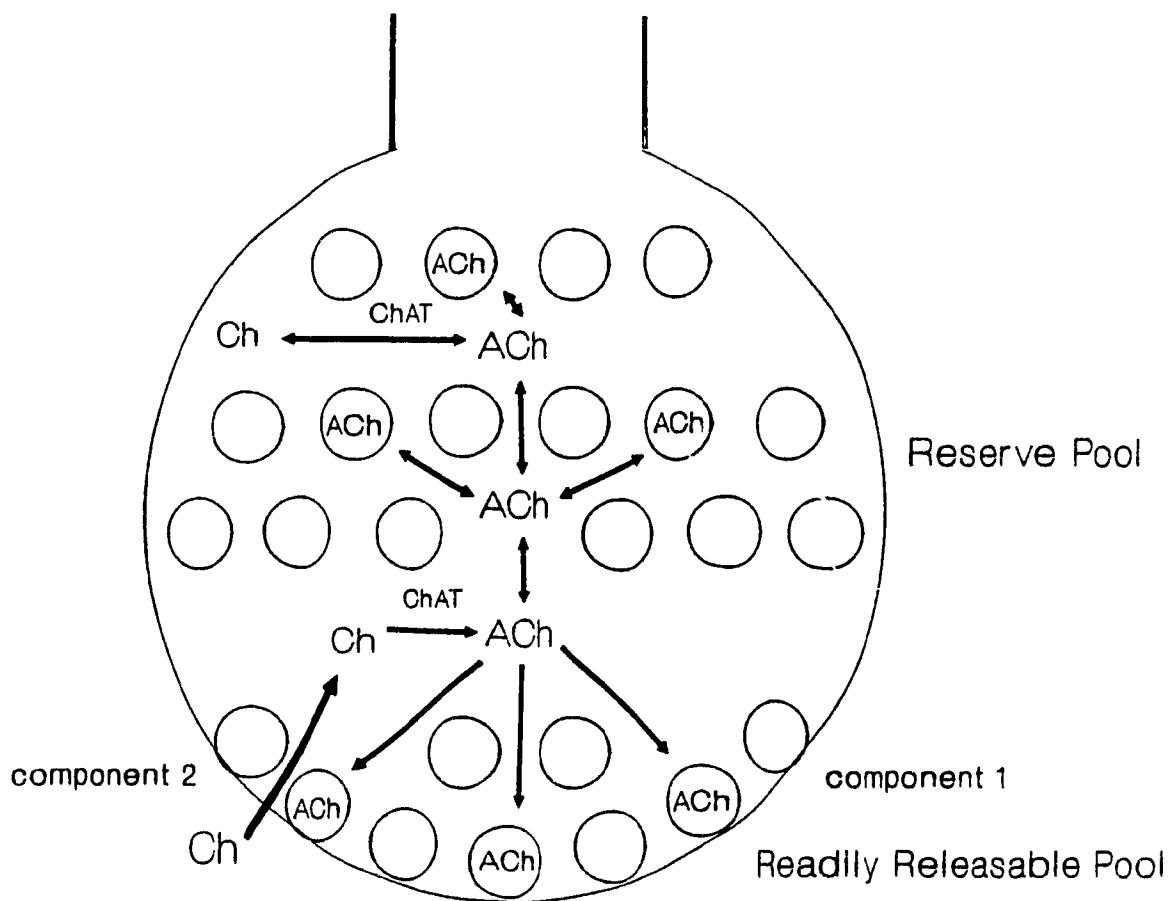


Figure 4D. Model of the cholinergic terminal hypothesizing the inter-relationship of the ACh compartments. The readily-releasable pool of ACh is illustrated as being composed of two components, each releasing its contents in response to different conditions. The vesicular component of the reserve pool is shown to derive its ACh from two sources, one produced by the synthesis of ACh from transported Ch and another produced by the synthesis of ACh from lipid Ch. Double arrows indicate the movement of ACh in either direction.

to be part of the older stores of transmitter. In this way newly synthesized ACh is preferentially released and rapid mixing of internal ACh stores can take place (see section 1.2).

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APPENDIX A

THESE ARE THE LATEST VERSIONS OF BASIC PROGRAMS USED IN CALCULATIONS

PROGRAM A.1 MAIN MENU PROGRAM

```
10 'THIS PROGRAM IS CALLED BRANCH.BAS
20 ' BY RAFAEL CABEZA JUNE-1987
30 '#####
40 'THIS PROGRAM IS THE MAIN BRANCHING PROGRAM TO ALL THE AVAILABLE
    CHOICES
50 '*****
60 CLS
70 BEEP
80 ON ERROR GOTO 800
90 PRGLK=1
100 '*****
110 'THIS PORTION SETS UP THE MENU BLOCK, CHOICES AND INCLUDES THE USER'S
120 ' NAME
130 '*****
140 'VARIABLES NUM=SPACES IN NAME, NAM$=NAME, SP=NUMBER THAT CENTERS BOX
    FOR USER'S NAME,
150 ' CHB=USER'S CHOICE OF OPTIONS, PRCH=PROGRAM SUB-ROUTINE
160 ' CHANNELING, SPCH=PROGRAM CHAINING VARIABLE
170 '*****
180 OPEN "PERS.PFL" FOR INPUT AS #1 'ENTERS DISKET USER'S NAME
190 INPUT #1, NUM,NAM$,CLRSC
200 CLOSE
210 IF CLRSC=2 THEN COLOR 0,3:GOTO 230
220 COLOR 0,7
230 CLS
240 COLOR 7,0
250 FOR N=20 TO 22
260 LOCATE N,1:PRINT STRING$(80,32)
270 NEXT
280 SP=(80-NUM)/2-3:SP=CINT(SP) 'CENTERS BOX FOR NAME
290 '*****
300 ' THE HEADINGS AND APPROPRIATE BOXES
310 '*****
320 LOCATE 2,SP:PRINT STRING$(NUM+6,32)
330 LOCATE 3,7:PRINT STRING$(15,32):LOCATE 3,60:PRINT STRING$(15,32)
340 LOCATE 5,2:PRINT STRING$(25,32):LOCATE 8,2:PRINT STRING$(27,32)
350 LOCATE 11,2:PRINT STRING$(20,32):LOCATE 14,2:PRINT STRING$(20,32)
360 LOCATE 17,2:PRINT STRING$(21,32)
370 LOCATE 2,SP+3:PRINT NAM$
```

```

380 LOCATE 3,9:PRINT DATE$:LOCATE 3,62:PRINT TIME$
390 LOCATE 5,3:PRINT "BRAIN SLICE PREPARATION".LOCATE 8,3:PRINT
    "PERFUSIONS & SUPERFUSIONS"
400 LOCATE 11,3:PRINT"LOWRY CALCULATIONS":LOCATE 14,3:PRINT"ENZYME
    CALCULATIONS "
410 LOCATE 17,3:PRINT "FILES MANIPULATIONS"
420 COLOR 0,7
430 '*****
440 ' LISTING OF CHOICES WITH THE DATE AND TIME
450 '*****
460 IF CLRSC=2 THEN COLOR 0,3
470 LOCATE 3,2:PRINT "DATE":LOCATE 3,55 PRINT "TIME"
480 LOCATE 5,35:PRINT "1. ACH RELEASE & CONTENT"
490 LOCATE 6,35:PRINT "2 RADIO-ISOTOPES"
500 LOCATE 8,35:PRINT "3. ACH RELEASE & CONTENT"
510 LOCATE 9,35 PRINT "4 RADIO-ISOTOPES"
520 LOCATE 11,35:PRINT "5. TISSUE AND FRACTION PROTEIN"
530 LOCATE 14,35:PRINT "6. ACHE FROM TISSUE & LIQUIDS "
535 LOCATE 15,35:PRINT "7. CHAT ASSAYS "
540 LOCATE 17,35:PRINT "8. TO FILE MENU "
550 COLOR 7,0
560 '*****
570 'THIS SECTION ALLOWS USER TO ENTER CHOICE BUT STOPS USER FROM USING A
580 ' NUMBER THAT IS TOO LARGE
590 '*****
600 LOCATE 22,2:PRINT "ENTER THE NUMBER OF YOUR CHOICE (RETURN TO GO
    BACK TO SYSTEM) "
605 LOCATE 22,70:CHB$=INPUT$(1):CH=VAL(CHB$)
610 IF CH>8 THEN BEEP:LOCATE 22,1:PRINT STRING$(80,32) LOCATE 21,2:PRINT
    "YOUR CHOICE MUST BE BETWEEN 1 AND 8 !".GOTO 600
620 '*****
630 'THIS SECTION BRANCHES TO THE APPROPRIATE PROGRAM AND ALSO INITIALIZES
640 ' BOTH THE PROGRAM CHAINING (SPCH) AND SUB PROGRAM CHAIN (PRCH)
    SO THAT
650 ' THE PROPER SEQUENCE OF PROGRAMS WILL BE CALLED UP
660 '*****
670 IF CH=0 THEN SYSTEM
680 IF CH=1 THEN PRCH=1:SPCH=1:COMMON CLRSC,PRCH,SPCH:CHAIN "STDCUR BAS"
690 IF CH=2 THEN PRCH=1:SPCH=2:COMMON CLRSC,PRCH,SPCH:CHAIN "RADIO BAS"
700 IF CH=3 THEN PRCH=2:SPCH=1:COMMON CLRSC,PRCH,SPCH:CHAIN "STDCUR BAS"
710 IF CH=4 THEN PRCH=2:SPCH=2:COMMON CLRSC,PRCH,SPCH:CHAIN "RADIO BAS"
720 IF CH=5 THEN PRCH=3:SPCH=1:COMMON CLRSC,PRCH,SPCH:CHAIN "STDCUR BAS"
730 IF CH=6 THEN PRCH=4:SPCH=1:COMMON CLRSC,PRCH,SPCH:CHAIN "ACHE BAS"

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735 IF CH=7 THEN CLS:COMMON CLRSC:CHAIN "CHAT.BAS"
740 IF CH=8 THEN CLS:COMMON CLRSC:CHAIN "FLMN.BAS"
750 END
760 *****
770 'THIS LETS YOU START FROM THE VERY BEGINNING IF YOU COMMITTED AN
780 ' UNFORSEEN ERROR
790 '*****
800 CLS
810 PRINT "YOU HAVE COMMITTED A TERMINAL ERROR!!!!!!"
820 PRINT "YOU WILL HAVE TO START OVER AGAIN BECAUSE OF THIS ERROR !!!!!"
830 PRINT "THE ERROR CODE IS("ERR") AND IT OCCURED IN LINE NUMBER
      ("ERL")  "
840 INPUT "PRESS RETURN WHEN YOU ARE READY ",RTN
850 RUN"BRANCH

```

PROGRAM A 2. STANDARD CURVE PROGRAM.

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10 'THIS PROGRAM IS CALLED STDCUR.BAS
20 '   BY KAFTEL CATEZA           JUNE-1987
30 '*****
40 'THIS PROGRAM CALCULATES REGRESSION LINES FOR ACH AND
    LOWRY CALCULATIONS
50 'IT USES THE LINEAR REGRESSION METHOD OUTLINED IN WILLIAM SCHEFLER,
60 ' STATISTICS FOR THE BIOLOGICAL SCIENCES, ADDISON-WESLEY,
    1969, PP.139-162
70 '*****
80 'VARIABLES--- EFLN$=FILE NAME OF EXPERIMENT, STDNM=# OF STANDARD
    CURVES TO BE ENTERED, STNM$()-NAME OF EACH STANDARD CURVE,
90 'STCP()-# OF CONCENTN. POINTS FOR EACH CURVE,
100 'STCPT()-# OF TRIALS PER [] POINT, STC(,)= EACH [] POINT,
110 'STCPTR=EACH TRIAL CPM OR ABSORBANCE FOR EACH CONCENTRATION PT.
120 'STCPM(,)=MEAN CPM OR ABSORBANCE FOR EACH [] POINT, BLK()-BLANK VALUE
130 'FOR EACH CURVE, CC()-CORRELATION COEFFICIENT FOR EACH CURVE,
140 'TVAL()-T-VALUE FOR EACH CURVE, SLP()-SLOPE FOR EACH CURVE,
150 'CBK()-Y-INTERCEPT FOR EACH CURVE AS CHOSEN BY USER
160 '*****
170 ON ERROR GOTO 2660
180 PRGLK-2
190 CLS
200 BEEP

```

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210 '*****
220 'T-TABLE TAKEN FROM SCHEFLER IBID, P 214
230 '*****
240 'VARIABLES FOR THE T-TABLE ARE FI(N),NI(N),NINI(N)
250 DIM FI(15),NI(15),NINI(15)
260 '*****
270 'THESE ARE THE DATA ARRAYS FOR THE T-SIG TABLE
280 '*****
290 ' THIS IS THE 95% CONFIDENCE LIMIT ARRAY
300 DATA 12.71, 4.30, 3.18, 2.78, 2.57, 2.45, 2.36, 2.31, 2.61, 2.23,
      2.20, 2.18, 2.16, 2.14, 2.13
310 '*****
320 'THIS IS THE 99% CONFIDENCE LIMIT ARRAY
330 DATA 63.66, 9.92, 5.84, 4.60, 4.03, 3.71, 3.50, 3.36, 3.25, 3.17,
      3.11, 3.06, 3.01, 2.98, 2.95
340 '*****
350 'THIS IS THE 99.9% CONFIDENCE LIMIT ARRAY
360 DATA 636.62, 31.60, 12.94, 8.61, 6.86, 5.96, 5.40, 5.04, 4.78, 4.50,
      4.44, 4.32, 4.22, 4.14, 4.07
370 '*****
380 'THIS SET OF LOOPS IS WHAT READS THE TABLE INTO THE DATA ARRAY
390 '*****
400 FOR N=1 TO 15
410     READ FI(N)
420 NEXT
430 FOR N=1 TO 15
440     READ NI(N)
450 NEXT
460 FOR N=1 TO 15
470     READ NINI(N)
480 NEXT
490 '*****
500 'ENTERS FILE NAME AND CHECKS ITS LENGTH AND APPROPRIATENESS AS A FILE
      NAME
510 '*****
520 INPUT "WHAT IS THE NAME OF YOUR EXPERIMENT
      (8 OR LESS CHARACTERS) ";EFLN$
530 IF LEN(EFLN$)>8 THEN PRINT "THE NAME IS TOO LONG ENTER IT
      AGAIN!";GOTO 520
531 '*****
532 '*****
533 CLS:LOCATE 10,10.INPUT "DO YOU WISH TO ENTER STANDARDS FROM A
      TEMPLATE (1=Y,2=N)? ",TMP1

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```

541 IF TMP1=1 THEN GOSUB 3000
543 CLS
550 '*****
560 'ENTERS STANDARD CURVE NUMBER <MAX # IS 5> AND NAMES AS WELL AS
    THE # OF POINTS & TRIALS
570 '*****
580 LOCATE 10,10:INPUT "HOW MANY STANDARD CURVES DO YOU
    WISH TO ENTER ",STDNM
590 IF STDNM>5 THEN CLS LOCATE 13,10:PRINT "YOU CANNOT ENTER MORE
    THAN 5 STANDARD CURVES AT ONCE !!":GOTO 530
600 CLS
610 FOR N=1 TO STDNM
620     LOCATE 1+(N-1)*4,5:PRINT "WHAT IS YOUR (",N") STANDARD
        CURVE CALLED "
630     COLOR 15,0
640     LOCATE 1+(N-1)*4,50:INPUT "---",STNM$(N)
650     LOCATE 2+(N-1)*4,5 INPUT "HOW MANY CONCENTRATION POINTS DOES THIS
        CURVE HAVE ---",STCPT(N)
660     LOCATE 3+(N-1)*4,5:INPUT "HOW MANY TRIALS FOR EACH CONCENTRATION
        POINT ---",STCPT(N)
670     COLOR 7,0
680 NEXT
690 CLS
700 '*****
710 'PRINTS BLOCK WITH NAME OF STANDARD CURVE
720 '*****
730 FOR N=1 TO STDNM
735     IF CLRSC=2 THEN CLS:COLOR 0,2:GOTO 750
740     CLS:COLOR 0,7
750     FOR N1=1 TO 3
760         LOCATE 1+N1,10:PRINT STRING$(60,32)
770     NEXT N1
780     COLOR 7,0:LOCATE 3,34:PRINT STRING$(12,32)
790     LOCATE 3,36:PRINT STNM$(N)
800     GOSUB 840
810     CLS
820 NEXT N
830 GOTO 1020
840 '*****
850 'ENTERS THE DATA FOR EACH STANDARD CURVE
860 '*****
870 LOCATE 6,1:PRINT "CONCENTRATION"
880 FOR SN1=1 TO STCPT(N)

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890     LOCATE 6,20+(SN1-1)*15:PRINT "TRIAL ("SN1")"
900 NEXT SN1
910 FOR SN2=1 TO STCP(N)
915     IF CLRSC=2 THEN COLOR 2,0
920     LOCATE 6+SN2,1:INPUT "",STC(N,SN2)           'CONCENTRATION N P
930     FOR SN3=1 TO STCPT(N)
940         LOCATE 6+SN2,22+(SN3-1)*15:INPUT "",STCPTR
950         TCPMCP=TCPMCP+STCPTR
960     NEXT SN3
970     STCPM(N,SN2)=TCPMCP/STCPT(N)                 'MEAN CIM OR ABS.
980     IF STC(N,SN2)=0 THEN BLK(N)=STCPM(N,SN2)     'INITIALIZES THE BLK
990     TCPMCP=0
1000 NEXT SN2
1005 COLOR 7,0
1010 RETURN 810
1020 '*****
1030 'ALLOWS ENTERED VALUES TO BE CORRECTED
1040 '*****
1050 LOCATE 1,15:INPUT "DO YOU WISH TO MAKE CHANGES TO ENTERED VALUES
      (1=Y,2=N)",LPCRT
1060 IF LPCRT=1 THEN GOSUB 2280
1061 CLS
1062 LOCATE 10,10:INPUT "DO YOU WISH TO MAKE A TEMPLATE OF CURVES
      (1=Y,2=N)? ",TMP2
1064 IF TMP2=1 THEN GOSUB 4000
1065 CLS
1070 '*****
1080 'CALCULATES THE REGRESSION CURVE FROM THE DATA ENTERED FOR EACH
1090 'CURVE THE CALCULATION FOR THE BEST FIT SLOPE GOING THROUGH THE
1100 'ORIGIN WAS TAKEN FROM TALLARIDA & MURRAY, MANUAL OF PHARMACOLOGIC
      CALCULATIONS, P 8
1110 '*****
1120 FOR N1=1 TO STDNM
1130     FOR N2=1 TO STCP(N1)
1140         SX=SX+STC(N1,N2)                        'SUM OF X
1150         SY=SY+(STCPM(N1,N2) - BLK(N1))           'SUM OF Y
1160         XY=STC(N1,N2)*(STCPM(N1,N2) - BLK(N1)):SXY=SXY+XY 'SUM OF X Y
1170         XS=STC(N1,N2)^2:SXS=SXS+XS               'SUM OF X^2
1180         YS=(STCPM(N1,N2) - BLK(N1))^2:SYS=SYS+YS   'SUM OF Y^2
1190     NEXT N2
1200     SSX=SX^2                                     '(SUM OF X)^2
1210     SSY=SY^2                                     '(SUM OF Y)^2
1220     NUMER=SXY - ((SX*SY)/STCP(N1))

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1230     DN1=SXS-(SSX/STCP(N1))
1240     DN2=SYS-(SSY/STCP(N1))
1250     R=NUMER/(DN1*DN2)^.5           'CORRELATION COEFF.
1260     R=INT((R*100000!)+.5)/100000!
1270     IF R> 999999 THEN T=700:GOTO 1290
1280     T=R/((1-(R*R))/(STCP(N1)-2))^.5   'T-VALUE
1290     M=NUMER/DN1                     'REGRESSION SLOPE
1300     YINT=(SY/STCP(N1))-(M*(SX/STCP(N1))) 'Y-INTERCEPT
1310     YINT=YINT+BLK(N1)               'CORRECTED Y-INTERCEPT
1320     MBLK=SXY/SXS                   'SLOPE THROUGH ORIGIN
1330     IF PRCH=1 OR PRCH=2 GOTO 1350
1340     IF PRCH=3 GOTO 1530
1350     '*****
1360     'THIS PRINTS THE RESULT OF THE STANDARD CURVES ONE AT A TIME FOR
1370     'ACH AND RADIO-ISOTOPE TYPE OF CALCULATIONS
1380     '*****
1385     IF CLRSC=2 THEN COLOR 2,0
1390     PRINT "THE STANDARD CURVE IS "STNM$(N1)
1400     PRINT
1410     PRINT "THE CORRELATION COEFFICIENT IS -----" "R
1420     PRINT "THE T-VALUE IS-----" "T
1430     PRINT "THE SLOPE IS -----" "M
1440     PRINT "THE Y-INTERCEPT IS -----" "YINT
1450     PRINT "YOUR BLANK VALUE IS -----" "BLK(N1)
1460     PRINT "THE BEST SLOPE GOING THROUGH THE BLANK IS -----
           "MBLK
1470     PRINT
1475     COLOR 7,0
1480     GOTO 1630
1490     '*****
1500     'THIS PRINTS THE RESULT OF THE STANDARD CURVES ONE AT A TIME FOR
1510     'LOWRY TYPE CALCULATIONS
1520     '*****
1525     IF CLRSC=2 THEN COLOR 2,0
1530     PRINT "THE STANDARD CURVE IS "STNM$(N1)
1540     PRINT
1550     PRINT "THE CORRELATION COEFFICIENT IS -----" "R
1560     PRINT "THE T-VALUE IS-----" "T
1570     PRINT "THE SLOPE IS -----" "MBLK
1580     PRINT "THE Y-INTERCEPT IS -----" "YINT
1590     PRINT
1595     COLOR 7,0
1600     '*****

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```

1610 'THIS DETERMINES THE SIGNIFICANCE LEVEL OF EACH STANDARD CURVE
1620 '*****
1630 N=STCP(N1)
1640 IF T>=NINI(N-2) THEN PRINT "SIGNIFICANT TO THE 99.9% CONFIDENCE
      LIMIT!":SGF(N1)=99.9:GOTO 1690
1650 IF T>=NI(N-2) THEN PRINT "SIGNIFICANT TO THE 99% CONFIDENCE
      LIMIT!":SGF(N1)=99:GOTO 1690
1660 IF T>=FI(N-2) THEN PRINT "SIGNIFICANT TO THE 95% CONFIDENCE
      LIMIT!":SGF(N1)=95:GOTO 1690
1670 IF T<FI(N-2) THEN BEEP:PRINT "NOT SIGNIFICANT TO THE
      95% CONFIDENCE LIMIT!":SGF(N1)=0:GOTO 1690
1680 IF PRCH=3 THEN SPC=1:PRINT :INPUT "PRESS RETURN TO
      CONTINUE ",STPP:GOTO 1710
1690 IF PRCH=3 THEN SPC=1:PRINT :INPUT "PRESS RETURN TO
      CONTINUE ",STPP:GOTO 1710
1700 PRINT :INPUT "WOULD YOU LIKE THE Y-INTERCEPT AT YOUR BLANK VALUE
      (1=Y,2=N,3=OTHER) ";SPC
1710 IF SPC=1 THEN YINT=BLK(N1):M=MBLK
1720 IF SPC=3 THEN INPUT "WHAT IS YOUR DESIRED BLANK VALUE ";YINT
1730 SX=0:SY=0:SXY=0:SXS=0:SYS=0
1740 CLS
1750 '*****
1760 'THIS INITIALIZES THE CORRELATION COEFFICIENT, THE T-VALUE,
      THE SLOPE,
1770 'AND THE Y-INTERCEPT FOR EACH CURVE ACCORDING TO THE
      USER'S CHOICE
1780 '*****
1790 CC(N1)=R:TVAL(N1)=T:SLP(N1)=M:CBK(N1)=YINT
1800 NEXT N1
1810 INPUT "WOULD YOU LIKE THE STANDARD CURVE(S) PRINTED (1=Y,2=N) ";PRT
1820 IF PRT=2 GOTO 2100
1830 '*****
1840 'THIS PRINTS OUT THE STANDARD CURVE INFORMATION ON THE PRINTER
1850 '*****
1860 LPRINT STRING$(50,42)
1870 LPRINT STRING$(15,42)+"          "EFLN$          "+STRING$(15,42)
1880 LPRINT STRING$(50,42)
1890 LPRINT
1900 FOR N=1 TO STDNM
1910 LPRINT STRING$(15,42)+"          "+STNM$(N)+"          "+STRING$(15,42)
1920 LPRINT
1930 IF PRCH=1 OR PRCH=2 THEN LPRINT "CONCENTRATION",,"CPMS"
1940 IF PRCH=3 THEN LPRINT "CONCENTRATION",,"ABSORBANCE"

```

```

1950     FOR NN=1 TO STCP(N)
1960         LPRINT STC(N,NN) , ,STCPM(N,NN)
1970     NEXT NN
1980     LPRINT
1990     LPRINT "THE CORRELATION COEFFICIENT IS" "CC(N)
2000     LPRINT "THE T VALUE IS" "TVAL(N)
2010     LPRINT "THE SLOPE IS" "SLP(N)
2020     LPRINT "THE Y-INTERCEPT IS" "CBK(N)
2030     LPRINT "THE LINE IS SIGNIFICANT TO" "SGF(N)"%
           C.L."
2040     LPRINT :LPRINT
2050 NEXT N
2060 '*****
2070 'THIS ENTERS THE STANDARD CURVE INFORMATION INTO A PROGRAM FILE FOR
2080 'LATER USE BY OTHER PROGRAMS. PFL=PROGRAM FILE
2090 '*****
2100 OPEN "DATAFL1.PFL" FOR OUTPUT AS #1
2110 WRITE #1, STDNM
2120 FOR N=1 TO STDNM
2130     WRITE #1, STNM$(N)
2140 NEXT
2150 FOR N=1 TO STDNM
2160     WRITE #1,SLP(N),CBK(N)
2170 NEXT
2180 CLOSE
2190 '*****
2200 'CHAINS TO APPROPRIATE PROGRAMS ACCORDING TO CHOICE ENTERED WITH
2210 ' BRANCH.BAS AND DOES THIS BY THE USE OF PRCH & SPCH
2220 '*****
2222 CLS:INPUT "DO YOU WISH TO DO ANOTHER SET OF CURVES
           (1-Y,2-N,3-EXIT)? ",DOAG
2224 IF DOAG=1 THEN CLS:GOTO 520
2226 IF DOAG=3 THEN RUN"BRANCH.BAS
2230 COMMON EFLN$,PRCH,SPCH,CLRSC
2240 IF PRCH=1 OR PRCH=2 THEN CHAIN "DATENTRY.BAS"
2250 IF PRCH=3 THEN CHAIN "LOWRY.BAS"
2260 END
2270 '*****
2280 'THIS IS THE LOOP THAT CORRECTS MISS-ENTERED CURVE DATA
2290 '*****
2300 CLS
2310 FOR N=1 TO STDNM
2320     PRINT N,STNM$(N)

```

```

2330 NEXT
2340 PRINT :INPUT "WHICH STANDARD CURVE DO YOU WISH TO CORRECT
      (#)    ",STCC
2350 CLS:PRINT " #","[STD]","MEAN READING":PRINT
2360 FOR N=1 TO STCP(STCC)
2370     PRINT N,STC(STCC,N),STCPM(STCC,N)
2380 NEXT
2390 PRINT
2400 INPUT "DO YOU WISH TO CHANGE ANY ONE OF THESE LINES
      (1=Y,RETURN=N) ";ERET
2410 IF ERET<>1 THEN CLS:GOTO 2620
2420 INPUT "HOW MANY LINES DO YOU WISH TO CHANGE ";LNFC
2430 INPUT "WRITE DOWN THE # OF THE LINES YOU WISH TO CHANGE
      AND PRESS RETURN";ERET
2440 CLS
2450 FOR N=1 TO LNFC
2460     PRINT "WHAT IS THE ("N") LINE YOU WISH TO CHANGE
      ":INPUT "N= ",LNFCN
2470     CLS
2480     PRINT "THE [STD] IS          "STC(STCC,LNFCN):PRINT :INPUT "WHAT
      IS THE CORRECT [STD], (IF SAME THEN RE-ENTER) ",STCNEW
2490     STC(STCC,LNFCN)=STCNEW
2500     PRINT :PRINT "THE MEAN READING IS          "STCPM(STCC,LNFCN):
      PRINT :INPUT "DO YOU WISH TO CORRECT THIS
      (Y=RETURN,N=2) ";NWCPM
2510     IF NWCPM>0 THEN GOTO 2600
2520     CLS:PRINT "THE [STD] = "STC(STCC,LNFCN)
2530     FOR N1=1 TO STCPT(STCC)
2540         PRINT "READING ("N1") ":INPUT "          NEW
      READING = ",STCPTR
2550         TCPMCP=TCPMCP+STCPTR
2560     NEXT N1
2570     STCPM(STCC,LNFCN)=TCPMCP/STCPT(STCC)          'MEAN CPM OR ABS
2580     IF STC(STCC,LNFCN)=0 THEN BLK(STCC)=STCPM(STCC,LNFCN)
      'REINIT. BLK.
2590     TCPMCP=0
2600     CLS
2610 NEXT N
2620 INPUT "DO YOU WISH TO MAKE ANY MORE CHANGES (Y=1, N=RETURN) ",FCHLP
2630 IF FCHLP=0 THEN CLS:GOTO 2650
2640 GOTO 2300
2650 RETURN 1061
2660 '*****

```

```

2670 'THIS IS THE ERROR TRAPPING ROUTINE WHICH CHAINS TO ERRTRP.BAS
2680 '*****
2690 EN=ERR
2695 IF ERR=25 THEN CHAIN "ERRTRP.BAS",10,ALL
2700 IF ERR=24 OR ERR=27 THEN CHAIN "ERRTRP.BAS",10,ALL
2710 IF ERR=61 OR ERR=67 THEN CHAIN "ERRTRP.BAS",10,ALL
2720 IF ERR=64 OR ERR=52 THEN CHAIN "ERRTRP.BAS",10,ALL
2730 IF ERR=70 OR ERR=71 THEN CHAIN "ERRTRP.BAS",10,ALL
2740 GOTO 9090
3000 '*****
3001 '*****
3002 CLS:FILES "\ACH\STD TMP\*.TPL":LOCATE 15,10:INPUT "WHICH FILE DO YOU
WANT TO USE (NAME- NO .TPL)? ",TMFLN$
3003 OPEN "\ACH\STD TMP\"+TMFLN$+".TFL" FOR INPUT AS #3
3004 INPUT #3, STDNM
3005 FOR N1=1 TO STDNM
3006     INPUT #3, STNM$(N1),STCP(N1),STCPT(N1)
3007     FOR N2=1 TO STCP(N1)
3008         INPUT #3, STC(N1,N2),STCPM(N1,N2)
3009     NEXT N2
3010 NEXT N1
3011 CLOSE #3
3012 FOR N=1 TO STDNM
3019     IF CLRSC=2 THEN CLS:COLOR 0,2:GOTO 3030
3020     CLS:COLOR 0,7
3030     FOR N1=1 TO 3
3040         LOCATE N1,10:PRINT STRING$(60,32)
3050     NEXT N1
3060     COLOR 7,0:LOCATE 2,34:PRINT STRING$(12,32)
3063     LOCATE 5,1:PRINT "CONCENTRATION"
3065     LOCATE 5,30:PRINT "MEAN VALUE"
3080     LOCATE 2,36:PRINT STNM$(N)
3090     FOR N2=1 TO STCP(N)
3100         LOCATE 6+N2,1:PRINT STC(N,N2)
3110         LOCATE 6+N2,30:PRINT STCPM(N,N2)
3113         IF STC(N,N2)=0 THEN BLK(N)=STCPM(N,N2)
3120     NEXT N2
3130     LOCATE 23,10:INPUT "PRESS RETURN TO CONTINUE ",RT
3140 NEXT N
3142 CLS
3150 RETURN 1040
4000 '*****
4001 '*****

```

```

4010 CLS:LOCATE 10,10:INPUT "WHAT DO YOU WISH TO CALL THIS SET OF
      CURVES? ",TMFLN$
4020 OPEN "\ACH\STD TMP\"+"TMFLN$+".TPL" FOR OUTPUT AS #3
4030 WRITE #3, STDNM
4040 FOR N1=1 TO STDNM
4050     WRITE #3, STNM$(N1),STCP(N1),STCPT(N1)
4060     FOR N2=1 TO STCP(N1)
4070         WRITE #3, STC(N1,N2),STCPM(N1,N2)
4080     NEXT N2
4090 NEXT N1
4100 CLOSE #3
4110 RETURN 1065
9000 '*****
9010 'THIS RETURNS YOU TO AN APPROPRIATE LINE IN THE PROGRAM AFTER AN
9020 ' ANTICIPATED ERROR
9030 '*****
9040 ON ERROR GOTO 2270
9045 IF EN=25 THEN GOTO 1860
9050 IF EN=24 OR EN=27 THEN GOTO 1860
9060 IF EN=61 OR EN=67 THEN GOTO 540
9070 IF EN=64 OR EN=52 THEN GOTO 540
9080 IF EN=70 OR EN=71 THEN GOTO 540
9090 CLS
9100 PRINT "YOU HAVE COMMITTED A TERMINAL ERROR!!!!!!"
9110 PRINT "YOU WILL HAVE TO START OVER AGAIN BECAUSE OF THIS
      ERROR !!!!!"
9120 PRINT "THE ERROR CODE IS("ERR") AND IT OCCURED IN LINE
      NUMBER ("ERL") ."
9130 INPUT "PRESS RETURN WHEN YOU ARE READY ",RTN
9140 RUN"BRANCH

```

PROGRAM A.3 DATA ENTRY PROGRAM

```

10 'THIS PROGRAM IS CALLED DATENTRY.BAS
20 '     BY RAFAEL CABEZA     JUNE-1987
30 '#####
40 'THIS PROGRAM IS INTENDED TO HELP YOU ENTER YOUR DATA SO THAT ACH
50 'PROGRAMS MAY USE IT IN FURTHER CALCULATIONS
60 '*****
70 'VARIABLES== EFLN$=EXPERIMENT FILE NAME, TMP$=NAME OF DATA TEMPLATE,

```



```

      NM$( )=NAME OF SAMPLE,
80 'SC( )=# OF THE STANDARD CURVE TO USE WITH SAMPLE,
      MW$( )=MG WET WEIGHT FOR SAMPLE, SVL( )=SAMPLED VOLUME
90 'FROM SAMPLE. TVL( )=TOTAL VOLUME OF SAMPLE, TM( )=TIME OF
      COLLECTION FOR SAMPLE, MFT( )=MULTIPLICATION FACTOR FOR THE SAMPLE,
      NN=# OF SAMPLES ENTERED, LN=# OF LINES OF DATA
100 'YOU WISH TO CHANGE, LNN=# OF THE LINE YOU WISH CHANGED,
      CNM$( )=CORRECTED
110 'SAMPLE NAME, CSC=COR.SAMP.STAND.CURVE, CMW$( )=COR.SAMP.MG WET WT.,
      CSVL=COR. SAMPL.SAMPLED VOL., CTVL=COR.SAMP.TOTAL VOL.,
      CTM=COR.SAMP. TIME, CMFT=COR.SAMP.MULT.FACT.,
120 'TPNM$( )=TEMPLATE NAME (BEING FORMED), FCT( )=CORRECTION MULTIPLICATION
      FACTOR INCORPORATING -SVL,TVL,TM,MW$,MFT-,
130 'DENT2=# OF TRIALS FOR EACH SAMPLE (CPM),
      CPMS( )=MEAN CPMS FOR EACH SAMPLE
140 '*****
150 CLS
160 PRGLK=3
163 KEY 7,CHR$(13)+CHR$(13)+CHR$(13)+CHR$(13)+CHR$(13)+CHR$(13)+CHR$(13)
165 KEY 8,CHR$(13)+CHR$(13)+CHR$(13)+CHR$(13)+CHR$(13)+CHR$(13)
170 ON ERROR GOTO 3060
180 DIM NM$(75),SC(75),MW$(75),SVL(75),TVL(75),TM(75),MFT(75),
      FCT(75),CPMS(75)
190 '*****
200 'PRINTS OUT THE NAMES AND NUMBERS OF THE STANDARD CURVES ENTERED BY
210 'THE PREVIOUS PROGRAM STDCUR.BAS
220 '*****
230 PRINT "PRINT "YOUR STANDARD CURVE(S) IS (ARE) AS FOLLOWS."
240 OPEN "DATAFL1.PFL" FOR INPUT AS #1
250 INPUT #1, STDCN
260 FOR N=1 TO STDCN
270     INPUT #1, STDNM$(N)
280     PRINT N,STDNM$(N)
290 NEXT
300 CLOSE
310 '*****
320 'PRINTS THE NAME OF THE EXPERIMENT FILE AND LETS YOU ENTER A NEW ONE
330 '*****
340 LOCATE 10,10:PRINT "THE NAME OF THIS FILE IS",EFLN$( )
350 LOCATE 12,10:INPUT "IS THIS THE EXPERIMENT FILE NAME (1=Y,2=N) ";FLN$( )
360 IF FLN$( )=2 THEN LOCATE 10,1:PRINT STRING$(80,32):LOCATE 10,10:INPUT
      "WHAT IS THE EXPERIMENT FILE NAME ";EFLN$( )
370 IF LEN(EFLN$( ))>8 THEN LOCATE 15,10:PRINT "THE NAME IS TOO

```

```

        LONG!!":FLN=2:GOTO 360
380 '*****
390 'LETS YOU INPUT THE SAMPLE INFORMATION FROM A TEMPLATE
400 '*****
410 CLS:LOCATE 10,10:INPUT "DO YOU WISH TO ENTER SAMPLE DATA FROM A FILE
      (1=YES,2=NO) ";DIMP
420 IF DIMP=1 THEN GOSUB 2730
430 '*****
440 'SETS UP BLOCK WITH FILE NAME AND HEADINGS FOR ENTRIES IF CHOICE IS
450 'FOR PERFUSIONS mg WET WEIGHT IS SKIPPED.
460 '*****
465 IF CLRSC=2 THEN CLS:COLOR 0,5:GOTO 480
470 CLS:COLOR 0,7
480 FOR N=1 TO 3
490     LOCATE N,10:PRINT STRING$(60,32)
500 NEXT
502 IF PRCH=2 THEN LOCATE 25,20:PRINT "F8=ENTER SAME AS PREVIOUS
      SAMPLE";:GOTO 510
503 LOCATE 25,20:PRINT "F7=ENTER SAME AS PREVIOUS SAMPLE";
510 COLOR 7,0:LOCATE 2,33:PRINT STRING$(14,32)
520 COLOR 15,0:LOCATE 2,35:PRINT EFLN$
530 COLOR 7,0
540 LOCATE 5,20:PRINT "STD."
550 IF PRCH=2 GOTO 570
560 LOCATE 5,29:PRINT "mg"
570 LOCATE 6,37:PRINT "VOL."
580 LOCATE 5,46:PRINT "TOTL"
590 LOCATE 5,56:PRINT "TIME"
600 LOCATE 5,63:PRINT "MULT."
610 LOCATE 6,12:PRINT "NAME"
620 LOCATE 6,19:PRINT "CURVE"
630 IF PRCH=2 GOTO 650
640 LOCATE 6,27:PRINT "WT WGT"
650 LOCATE 5,37:PRINT "SAMPLED"
660 LOCATE 6,46:PRINT "VOL."
670 LOCATE 6,55:PRINT "(min)"
680 LOCATE 6,63:PRINT "FACTOR"
690 '*****
700 'ENTERS THE DATA FOR EACH SAMPLE AND ALLOWS FOR ENTRY DOWN THE SCREEN
710 'IN ORDER
720 '*****
730 FOR N=1 TO 16
735     IF CLRSC=2 THEN COLOR 5,0

```

```

740      MFT(0)=1                                'INITIALIZES MFT
750      NN=NN+1
760      LOCATE N+7,12:INPUT "",NM$(NN)
770      IF NM$(NN)="" THEN NN=NN-1:GOTO 1150
780      LOCATE N+7,20:INPUT "",SC(NN)
790      IF SC(NN)=0 THEN SC(NN)=SC(NN-1):LOCATE N+7,19:PRINT SC(NN)
800      IF PRCH=2 GOTO 830
810      LOCATE N+7,28:INPUT "",MW(NN)
820      IF MW(NN)=0 THEN MW(NN)=MW(NN-1):LOCATE N+7,27:PRINT MW(NN)
830      LOCATE N+7,37:INPUT "",SVL(NN)
840      IF SVL(NN)=0 THEN SVL(NN)=SVL(NN-1):LOCATE N+7,36:PRINT SVL(NN)
850      LOCATE N+7,47:INPUT "",TVL(NN)
860      IF TVL(NN)=0 THEN TVL(NN)=TVL(NN-1):LOCATE N+7,46:PRINT TVL(NN)
870      LOCATE N+7,57:INPUT "",TM(NN)
880      IF TM(NN)=0 THEN TM(NN)=TM(NN-1):LOCATE N+7,56:PRINT TM(NN)
890      LOCATE N+7,65:INPUT "",MFT(NN)
900      IF MFT(NN)=0 THEN MFT(NN)=MFT(NN-1):LOCATE N+7,64:PRINT MFT(NN)
910 NEXT
920 '*****
930 'THIS ALLOWS FOR CONTINUED ENTRY OF DATA WITH PROPER SCROLLING OF
    SCREEN
940 '*****
950 NN=NN+1
960 INPUT "          ",NM$(NN)
970 IF NM$(NN)="" THEN NN=NN-1:GOTO 1150
980 LOCATE 23,20:INPUT "",SC(NN)
990 IF SC(NN)=0 THEN SC(NN)=SC(NN-1):LOCATE 23,19:PRINT SC(NN)
1000 IF PRCH=2 GOTO 1030
1010 LOCATE 23,28:INPUT "",MW(NN)
1020 IF MW(NN)=0 THEN MW(NN)=MW(NN-1):LOCATE 23,27:PRINT MW(NN)
1030 LOCATE 23,37:INPUT "",SVL(NN)
1040 IF SVL(NN)=0 THEN SVL(NN)=SVL(NN-1):LOCATE 23,36:PRINT SVL(NN)
1050 LOCATE 23,47:INPUT "",TVL(NN)
1060 IF TVL(NN)=0 THEN TVL(NN)=TVL(NN-1):LOCATE 23,46:PRINT TVL(NN)
1070 LOCATE 23,57:INPUT "",TM(NN)
1080 IF TM(NN)=0 THEN TM(NN)=TM(NN-1):LOCATE 23,56:PRINT TM(NN)
1090 LOCATE 23,65:INPUT "",MFT(NN)
1100 IF MFT(NN)=0 THEN MFT(NN)=MFT(NN-1):LOCATE 23,64:PRINT MFT(NN)
1110 GOTO 950
1120 '*****
1130 'ALLOWS YOU TO CONTINUE ENTERING DATA IF YOU MISTAKENLY ENTERED ""
    FOR NM$
1140 '*****

```

```

1150 CLS
1155 COLOR 7,0
1160 INPUT "ARE YOU THROUGH ENTERING DATA (1=YES, 2=NO) ",DENT
1170 IF DENT=2 GOTO 460
1180 '*****
1190 'ALLOWS YOU TO MAKE ANY CHANGES TO ENTERED DATA IN CASE OF ERROR
1200 '*****
1210 PRINT :PRINT :INPUT "DO YOU WISH TO MAKE ANY CHANGES TO THE DATA YOU
      ENTERED ABOVE (1=YES, 2=NO)";DENT1
1220 IF DENT1=1 THEN GOSUB 1890
1230 '*****
1240 'ALLOWS YOU TO FORM A TEMPLATE FROM ENTERED DATA
1250 '*****
1260 CLS
1270 INPUT "DO YOU WISH TO MAKE THIS DATA INTO A TEMPLATE
      (1=YES,2=NO)";DENT3
1280 IF DENT3=1 THEN GOSUB 1760
1290 '*****
1300 'CALCULATES A COMPOSITE MULTIPLICATION FACTOR--SEE VARIABLES FOR
      FCT()
1310 '*****
1320 FOR N=1 TO NN
1330     IF PRCH=2 THEN FCT(N)=(TVL(N)*MFT(N))/(SVL(N)*TM(N)) : GOTO 1350
1340     FCT(N)=(TVL(N)*MFT(N))/(MWV(N)*SVL(N)*TM(N))
1350 NEXT N
1360 CLS
1370 '*****
1380 'ENTERS CPMS FOR EACH SAMPLE ACCORDING TO NUMBER OF TRIALS USER
      PERFORMED
1390 '*****
1400 INPUT "HOW MANY DETERMINATIONS OF EACH SAMPLE DID YOU DO
      (SINGLE=1,DOUBLE=2,ETC)";DENT2
1410 CLS
1420 FOR N=1 TO NN
1430     PRINT NM$(N)
1440     FOR DT=1 TO DENT2
1445         COLOR 7,0
1450         PRINT "          WHAT IS THE ("DT") CPMS FOR THIS SAMPLE?"
1455         IF CLRSC=2 THEN COLOR 5,0
1460         INPUT "                                CPMS=",CPM1
1470         TCPM=CPM+TCPM
1480     NEXT
1490     CPMS(N)=TCPM/DENT2

```

```

1500      TCPM=0
1505      COLOR 7,0
1510 NEXT
1520 '*****
1530 'ALLOWS YOU TO CHECK YOUR CPM ENTRIES FOR ERROR
1540 '*****
1550 CLS
1560 INPUT "DO YOU WISH TO CHECK YOUR MEAN CPMS FOR YOUR SAMPLES
      (1=YES,2=NO)";DENT4
1570 IF DENT4=1 THEN GOSUB 2360
1580 '*****
1590 'ENTERS DATA FROM EACH SAMPLE INTO A FILE FOR FURTHER CALCULATIONS
1600 'BY ACHCALCU.BAS
1610 '*****
1620 OPEN "DATAFL2.PFL" FOR OUTPUT AS #1
1630 WRITE #1, EFLN$
1640 FOR N=1 TO NN
1650     IF PRCH=2 THEN WRITE #1, NM$(N),SC(N),TM(N),CPMS(N),FCT(N):GOTO
      1670
1660     WRITE #1, NM$(N),SC(N),MWV(N),TM(N),CPMS(N),FCT(N)
1670 NEXT N
1680 CLOSE
1690 IF SPCH=2 THEN GOSUB 2920
1700 '*****
1710 'CHAINS TO ACHCALCU.BAS FOR PROCESSING OF ENTERED DATA
1720 '*****
1730 COMMON PRCH,SPCH,EFLN$,CLRSC
1740 CHAIN "ACHCALCU BAS"
1750 END
1760 '*****
1770 'FORMS A TEMPLATE FROM ENTERED DATA ACCORDING TO DATA TYPE
1780 '*****
1790 CLS INPUT "WHAT DO YOU WISH TO CALL THIS TEMPLATE ";TPNM$
1800 IF LEN(TPNM$)>8 THEN BEEP:INPUT "THE NAME IS TOO LONG! ENTER A NAME
      WITH EIGHT OR LESS CHARACTERS ",TPNM$
1810 IF PRCH=2 THEN OPEN "\ACH\TMP2\"+TPNM$+".TPL" FOR OUTPUT AS #1
1820 IF PRCH=1 THEN OPEN "\ACH\TMP1\"+TPNM$+".TPL" FOR OUTPUT AS #1
1830 FOR N=1 TO NN
1840     IF PRCH=2 THEN WRITE #1, NM$(N),SC(N),SVL(N),TVL(N),
      TM(N),MFT(N):GOTO 1860
1850     WRITE #1, NM$(N),SC(N),MWV(N),SVL(N),TVL(N),TM(N),MFT(N)
1860 NEXT N
1870 CLOSE

```

```

1880 RETURN 1290
1890 '*****
1900 'ALLOWS YOU TO CHANGE DATA YOU HAVE ENTERED IN CASE OF AN ERROR
1910 '*****
1915 IF CLRSC=2 THEN COLOR 4,0
1920 IF PRCH=2 THEN CLS:PRINT "      NAME      ST.CR.      SAMP VOL      TOTL VOL

      TIME      MULT.FACT.":GOTO 1940
1930 CLS:PRINT "      NAME      ST.CR.      MG.WT.WT      SAMP.VOL.      TOTL VOL
      TIME      MULT.FACT."
1940 RRD=NN/20:RRD=(INT(RRD))+1
1950 N=1
1960 IF N>RRD GOTO 2350
1970     FOR N2=1 TO 20
1980         CNT=CNT+1
1990         IF PRCH=2 THEN PRINT CNT;"      ";NM$(CNT);"      ",
                SC(CNT);"      ";SVL(CNT),"      ",
                TVL(CNT);"      ";TM(CNT);"      ";MFT(CNT):GOTO 2010
2000         PRINT CNT;"      ";NM$(CNT);"      ";SC(CNT);"      ";
                MWV(CNT);"      ";SVL(CNT);"      ";TVL(CNT);"      ";
                TM(CNT);"      ";MFT(CNT)
2010         IF CNT=NN GOTO 2030
2020     NEXT
2025     COLOR 7,0
2030     INPUT "DO YOU WISH TO CHANGE ONE OF THESE (1=YES,RETURN=NO)",RTN
2040     IF RTN=0 GOTO 2340
2050     INPUT "HOW MANY LINES DO YOU WISH TO CHANGE (# LINES)":LN
2060     PRINT "NOTE DOWN THE LINE NUMBERS LOCATED ON THE LEFT"
2070     INPUT "PRESS RETURN WHEN READY",PS.CLS
2080     FOR CTL=1 TO LN
2090         PRINT "WHICH IS THE (';CTL;") LINE NUMBER"
                INPUT "LINE =",LNN
2100         PRINT NM$(LNN):INPUT "ENTER NEW NAME OR RETURN FOR
                NO CHANGE ",CNM$
2110         IF CNM$="" THEN CNM$=NM$(LNN)
2120         NM$(LNN)=CNM$
2130         PRINT SC(LNN):INPUT "ENTER NEW STANDARD CURVE NUMBER
                OR RETURN FOR SAME ",CSC
2140         IF CSC=0 THEN CSC=SC(LNN)
2150         SC(LNN)=CSC
2160         IF PRCH=2 GOTO 2200
2170         PRINT MWV(LNN):INPUT "ENTER NEW mg WET WEIGHT OR RETURN
                FOR SAME ",CMWV

```

```

2180      IF CMWW=0 THEN CMWW=MWW(LNN)
2190      MWW(LNN)=CMWW
2200      PRINT SVL(LNN):INPUT "ENTER NEW SAMPLE VOLUME OR RETURN
          FOR SAME ",CSV
2210      IF CSV=0 THEN CSV=SVL(LNN)
2220      SVL(LNN)=CSV
2230      PRINT TVL(LNN):INPUT "ENTER NEW TOTAL VOLUME OR RETURN
          FOR SAME ",CTV
2240      IF CTV=0 THEN CTV=TVL(LNN)
2250      TVL(LNN)=CTV
2260      PRINT TM(LNN):INPUT "ENTER NEW TIME OR RETURN FOR
          SAME ",CTM
2270      IF CTM=0 THEN CTM=TM(LNN)
2280      TM(LNN)=CTM
2290      PRINT MFT(LNN):INPUT "ENTER NEW MULTIPLICATION FACTOR
          OR RETURN FOR SAME ",CMFT
2300      IF CMFT=0 THEN CMFT=MFT(LNN)
2310      MFT(LNN)=CMFT
2320      CLS
2330      NEXT
2340      IF CNT=NN GOTO 2350
2345      N=N+1:GOTO 1960
2350      RETURN 1260
2360      '*****
2370      'DISPLAYS MEAN CPMS FOR EACH SAMPLE AND ASKS IF YOU WISH TO
          CHANGE ANY
2380      '*****
2390      CNT=0
2400      CN=NN/20:CN=(INT(CN))+1
2410      N1=1
2420      IF N1>CN GOTO 2530
2430      CLS
2435      IF CLRSC=2 THEN COLOR 4,0
2440      PRINT "LINE #","SAMPLE","MEAN CPMS"
2450      FOR N=1 TO 20
2460          CNT=CNT+1
2470          PRINT CNT,NM$(CNT),CPMS(CNT)
2480          IF CNT=NN GOTO 2500
2490      NEXT
2495      COLOR 7,0
2500      INPUT "DO YOU WISH TO CHANGE ANY OF THESE
          (1=YES, RETURN=NO) ";CHN
2510      IF CHN=1 THEN GOSUB 2570

```

```

2520 IF CNT=NN GOTO 2530
2525 N1=N1+1.GOTO 2420
2530 RETURN 1580
2540 '*****
2550 'LETS YOU RE-ENTER THE CPM READINGS FOR EACH SAMPLE YOU INDICATE
2560 '*****
2570 INPUT "HOW MANY LINES DO YOU WISH TO CHANGE ";LN
2580 INPUT "NOTE DOWN THE LINE NUMBER(S) ON THE LEFT AND THEN
      PRESS RETURN ",PS
2590 CLS
2600 FOR LCH=1 TO LN
2610     CLS
2620     INPUT "WHAT LINE NUMBER DO YOU WISH TO CHANGE ",LNN
2630     TCPM=0
2640     FOR NSM=1 TO DENT2
2650         PRINT NM$(LNN)
2660         PRINT "          THE (";NSM;") CPMS ARE "
2670         INPUT "                                CPMS= ",CPM
2680         TCPM=CPM+TCPM
2690     NEXT
2700     CPMS(LNN)=TCPM/DENT2
2710 NEXT
2720 RETURN 2520
2730 '*****
2740 'DATA ENTRY FROM A PREFORMED TEMPLATE ACCORDING TO DATA TYPE
2750 '*****
2760 IF PRCH=2 THEN CLS:FILES "\ACH\TMP2\*.TPL":GOTO 2780
2770 CLS:FILES "\ACH\TMP1\*.TPL"
2780 INPUT "WHICH TEMPLATE DO YOU WISH TO USE
      (NAME WITHOUT - .TPL) ";TMP$
2790 IF PRCH=2 THEN OPEN "\ACH\TMP2\"+TMP$+".TPL" FOR INPUT AS #1.
      GOTO 2810
2800 OPEN "\ACH\TMP1\"+TMP$+".TPL" FOR INPUT AS #1
2810 N=0
2820 WHILE NOT EOF(1)
2830     N=N+1
2840     IF PRCH=2 THEN INPUT #1, NM$(N),SC(N),SVL(N),
      TVL(N),TM(N),MFT(N)
2850     IF PRCH=2 THEN PRINT N"  "NM$(N)"  "SC(N)"  "SVL(N)"  "IVL(N)
      "  "TM(N)"  "MFT(N):GOTO 2880
2860     INPUT #1, NM$(N),SC(N),MWW(N),SVL(N),TVL(N),TM(N),MFT(N)
2870     PRINT N"  "NM$(N)"  "SC(N)"  "MWW(N)"  "SVL(N)"  "TVL(N)
      "  "TM(N)"  "MFT(N)

```



```

2880 WEND
2890 NN=N
2900 CLOSE
2910 RETURN 1210
2920 '*****
2930 'ENTERS DATA FOR LATER USE BY RADIO-L2.BAS IF RADIO-ISOTOPE WORK WAS
2940 ' INDICATED WITH BRANCH.BAS
2950 '*****
2960 OPEN "\RAD\"+EFLN$ FOR OUTPUT AS #2
2970 FOR N=1 TO NN
2980     IF PRCH=2 THEN WRITE #2, NM$(N),TM(N):GOTO 3000
2990     WRITE #2, NM$(N),TM(N),MW(N)
3000 NEXT
3010 CLOSE
3020 RETURN 1700
3030 '*****
3040 'THIS IS THE ERROR TRAPPING ROUTINE.  THIS CHAINS TO THE
      ERRTRAP.BAS
3050 '*****
3060 EN=ERR
3070 IF ERR=24 OR ERR=27 THEN CHAIN "ERRTRP.BAS",10,ALL
3080 IF ERR=61 OR ERR=67 THEN CHAIN "ERRTRP.BAS",10,ALL
3090 IF ERR=64 OR ERR=52 THEN CHAIN "ERRTRP.BAS",10,ALL
3100 IF ERR=70 OR ERR=71 THEN CHAIN "ERRTRP.BAS",10,ALL
3110 GOTO 9090
9000 '*****
9010 'SENDS CONTROL BACK TO APPROPRIATE LINE IN PROGRAM AFTER ANTICIPATED
9020 'ERROR HAS BEEN CORRECTED BY ERRTRP.BAS
9030 '*****
9040 ON ERROR GOTO 3030
9050 IF EN=24 OR EN=27 GOTO 10
9060 IF EN=61 OR EN=67 GOTO 2960
9070 IF EN=64 OR EN=52 GOTO 2960
9080 IF EN=70 OR EN=71 GOTO 2960
9090 CLS
9100 PRINT "YOU HAVE COMMITTED A TERMINAL ERROR!!!!!!!"
9110 PRINT "YOU WILL HAVE TO START FROM THE BEGINNING BECAUSE OF
      THIS!!!!!"
9120 PRINT "THE ERROR CODE IS("ERR") AND IT OCCURED IN LINE NUMBER
      ("ERL") ."
9130 INPUT "PRESS RETURN WHEN YOU ARE READY ",RTN:RUN"BRANCH

```

PROGRAM A.4. LOWRY PROTEIN CALCULATION PROGRAM.

```
10 'THIS PROGRAM IS CALLED LOWRY.BAS
20 ' BY RAFAEL CABEZA                JUNE-1987
30 '#####
40 'THIS PROGRAM TAKES THE STANDARD CURVE FROM STDCUR.BAS AND CALCULATES
50 'PROTEIN AMOUNTS & TOTAL SAMPLE PROTEIN
60 '*****
70 'VARIABLES---NAM$()-NAME OF STANDARD CURVE,
    SLP()-SLOPE OF STAND.CURV., BLK()-STAND. CURVE BLANK,
    EFLN$-EXPERIMENT FILE NAME, TMP$-NAME OF TEMP.
80 'TO BE USED, SPNM$()-SAMPLE NAME, STDC()-SAMPLE STANDARD CURVE TO USE,
90 'SVOL()-SAMPLED VOLUME, TVOL()-TOTAL SAMPLE VOLUME,
    MLFT()-SAMPLE MULT.FACT., TRL=# OF TRIALS,
    ABC()-MEAN ABSORBANCE FOR SAMPLE, UGPT()-ug IN SAMPLES VL
100 'TUGPT()-TOTAL ug PROTEIN, UGPUL()-ug/VOL [CONC ]
110 '*****
120 ON ERROR GOTO 1/40
130 PRGLK=5
135 KEY 9, "END"+CHR$(13)
136 KEY 10, CHR$(13)+CHR$(13)+CHR$(13)+CHR$(13)+CHR$(13)
140 CLS
150 BEEP
160 '*****
170 DIM SPNM$(50),STDC(50),SVOL(50),TVOL(50),MLFT(50),FCT(50),ABC(80),
    UGPT(50),TUGPT(50),UGPVL(50)
180 '*****
190 'INFORMS USER OF THE STANDARD CURVES TO BE USED AND THE NAME OF THE
200 'EXPERIMENTAL FILE AND ALLOWS FOR A CHANGE IN FILE NAME
210 '*****
220 PRINT " YOUR STANDARD CURVE(S) IS(ARE) CALLED : " :PRINT
230 OPEN "DATAFL1.PFL" FOR INPUT AS #1
240 INPUT #1, NUM
250 FOR N=1 TO NUM
260     INPUT #1, NAM$(N)
270     PRINT N, NAM$(N)
280 NEXT
290 FOR N=1 TO NUM
300     INPUT #1, SLP(N),BLK(N)
310 NEXT
320 CLOSE
330 PRINT :PRINT "THE NAME OF YOUR FILE IS ---"EFLN$:PRINT
```

```

340 INPUT "IS THIS THE CORRECT NAME (1=Y,2=N) ";CNM
350 IF CNM=2 THEN CLS:LOCATE 10,10:INPUT "ENTER THE CORRECT FILE
      NAME ";EFLN$
360 IF LEN(EFLN$)>8 THEN BEEP:CLS:PRINT "THE NAME IS TOO LONG!!":INPUT
      "ENTER NAME WITH 8 OR LESS CHARACTERS ",EFLN$
370 '*****
380 'SETS BLOCK WITH FILE NAME AND PREPARES SCREEN FOR DATA ENTRY
390 '*****
400 CLS
405 IF CLRSC=2 THEN COLOR 0,3:GOTO 420
410 COLOR 0,7
420 FOR N=1 TO 3
430     LOCATE N,1:PRINT STRING$(80,32)
440 NEXT
450 COLOR 7,0
460 V=LEN(EFLN$)
470 CNTD=(80*(V+2))/2:CNTD=INT(CNTD)
480 LOCATE 2,CNTD:PRINT STRING$(V+2,32)
490 LOCATE 2,CNTD+1:PRINT EFLN$
500 '*****
510 'PRINTS HEADINGS FOR DATA ENTRY
520 '*****
530 LOCATE 5,2:PRINT "SAMPLE":LOCATE 6,2:PRINT "NAME"
540 LOCATE 5,18:PRINT "STAND.":LOCATE 6,18:PRINT "CURVE"
550 LOCATE 5,34:PRINT "SAMPLED":LOCATE 6,34:PRINT "VOLUME"
560 LOCATE 5,51:PRINT "TOTAL":LOCATE 6,51:PRINT "VOLUME"
570 LOCATE 5,67:PRINT "MULT.":LOCATE 6,67:PRINT "FACT."
575 LOCATE 25,10:PRINT "F9='END' ";
576 LOCATE 25,30:PRINT "F10=SAME AS PREVIOUS SAMPLE";
580 IF RET=2 GOTO 830
590 LOCATE 10,10:INPUT "DO YOU WISH TO ENTER SAMPLE DATA FROM A TEMPLATE
      (1=Y,2=N) ";TMP
600 IF TMP=1 THEN GOSUB 2030
610 LOCATE 10,1:PRINT STRING$(80,32)
615 IF CLRSC=2 THEN COLOR 3,0
620 '*****
630 'ENTERS DATA ON SAMPLES. IF SAME DATA AS PREVIOUS SAMPLE
      ONLY A RETURN IS
640 'NEEDED AND PREVIOUS VALUE IS ASSIGNED TO VARIABLE
650 '*****
660 FOR N=1 TO 17
670     LOCATE N+6,4:INPUT "",SPNM$(N)
680     IF SPNM$(N)="END" OR SPNM$(N)="" GOTO 990

```

```

690     LOCATE N+6,20:INPUT "",STDC(N)
700     IF STDC(N)=0 THEN STDC(N)=STDC(N-1):LOCATE N+6,19:PRINT STDC(N)
710     LOCATE N+6,36:INPUT "",SVOL(N)
720     IF SVOL(N)=0 THEN SVOL(N)=SVOL(N-1):LOCATE N+6,35:PRINT SVOL(N)
730     LOCATE N+6,53:INPUT "",TVOL(N)
740     IF TVOL(N)=0 THEN TVOL(N)=TVOL(N-1):LOCATE N+6,52:PRINT TVOL(N)
750     LOCATE N+6,69:INPUT "",MLFT(N)
760     IF MLFT(N)=0 THEN MLFT(N)=MLFT(N-1):LOCATE N+6,68:PRINT MLFT(N)
770 NEXT
780 '*****
790 'ALLOWS FOR ORDERLY SCROLLING OF THE SCREEN WHEN DATA ENTRY ON SCREEN
800 ' LINE 22 HAS BEEN REACHED
810 '*****
820 N=17
830 N=N+1
840 LOCATE 23,4:INPUT "",SPNM$(N)
850 IF SPNM$(N)="END" OR SPNM$(N)="" GOTO 990
860 LOCATE 23,20:INPUT "",STDC(N)
870 IF STDC(N)=0 THEN STDC(N)=STDC(N-1):LOCATE 23,19:PRINT STDC(N)
880 LOCATE 23,36:INPUT "",SVOL(N)
890 IF SVOL(N)=0 THEN SVOL(N)=SVOL(N-1):LOCATE 23,35:PRINT SVOL(N)
900 LOCATE 23,53:INPUT "",TVOL(N)
910 IF TVOL(N)=0 THEN TVOL(N)=TVOL(N-1):LOCATE 23,52:PRINT TVOL(N)
920 LOCATE 23,69:INPUT "",MLFT(N)
930 IF MLFT(N)=0 THEN MLFT(N)=MLFT(N-1):LOCATE 23,68:PRINT MLFT(N)
940 PRINT
950 GOTO 830
960 '*****
970 'ALLOWS DATA ENTRY TO CONTINUE IN CASE OF ERROR (HITTING RETURN
    BY MIST.)
980 '*****
990 CLS
995 COLOR 7,0
1000 LOCATE 10,10:INPUT "ARE YOU DONE ENTERING SAMPLE DATA
    (1=Y,2=N) ";RET
1010 IF RET=2 THEN N=N-1:GOTO 370
1020 TTL=N-1
1030 CLS
1040 LOCATE 10,10:INPUT "DO YOU WISH TO MAKE THIS DATA INTO A TEMPLATE
    (1=Y,2=N) ";RETT
1050 IF RETT=1 THEN GOSUB 1830
1060 CLS
1070 '*****

```

```

1080 'ENTERS THE ABSORBANCE READINGS FOR THE # OF TRIALS PERFORMED
      FOR EACH
1090 'CONCENTRATION POINT OF THE UNKNOWN AND DETERMINES
      THE MEAN ABSORBANCE
1100 '*****
1110 LOCATE 10,5:INPUT "HOW MANY TRIALS PER SAMPLE CONCENTRATION POINT
      DID YOU DO (5 OR LESS) ";TRL
1120 CLS
1130 PRINT "SAMPLE"
1140 FOR N=1 TO TRL
1150     LOCATE 1,N*12:PRINT "TRIAL("N")"
1160 NEXT
1170 FOR N=1 TO 21
1180     IF N>TRL GOTO 1390
1190     LOCATE N+1,1:PRINT SPNM$(N)
1200     FOR N1=1 TO TRL
1210         LOCATE N+1,(N1*12)+2:INPUT "",ABSR
1220         TABS=TABS+ABSR
1230     NEXT N1
1240     ABC(N)=TABS/TRL                                'MEAN ABSORBANCE
1250     TABS=0
1260 NEXT N
1270 N=21
1280 N=N+1
1290 IF N>TRL GOTO 1390
1300 LOCATE 23,1:PRINT SPNM$(N)
1310 FOR N1=1 TO TRL
1320     LOCATE 23,(N1*12)+2:INPUT "",ABSR
1330     TABS=TABS+ABSR
1340 NEXT
1350 ABC(N)=INT(((TABS/TRL)*1000)+.5)/1000
1360 TABS=0
1370 PRINT
1380 GOTO 1280
1390 '*****
1400 'CALCULATES THE PROTEIN AMOUNTS FROM THE ABSORBANCE AS WELL AS THE
1410 ' TOTAL PROTEIN AND PROTEIN/VOLUME
1420 '*****
1430 CLS
1440 FOR N=1 TO TTL
1445     FCT=(TVOL(N)/SVOL(N))*MLFT(N)
1450     UGPT(N)=INT(((ABC(N)/SLP(STD(N)))*10)+.5)/10 'ug
      IN SAMPLED VOL.

```

```

1460     TUGPT(N)=INT(((UGPT(N)*FCT)*10)+.5)/10      'TOTAL ug PROTEIN
1470     UGPVL(N)=INT(((TUGPT(N)/(TVOL(N)*MLFT(N)))*100+.5)/100
           'ug/vol
1480 NEXT
1490 '*****
1500 'PRINTS OUT THE CALCULATED AMOUNTS OF PROTEIN ON THE SCREEN
           AND TO THE
1510 'PRINTER AS WELL AS FORMING A FILE IN THE LOWRY SUBDIRECTORY OF B:
1520 '*****
1530 LPRINT STRING$(65,42)
1540 LPRINT :LPRINT "SAMPLE","ABS","SAMPLE(ug)","TOTAL(ug)","ug/ul"
1550 OPEN "B:\LOWRY\"+EFLN$ FOR OUTPUT AS #1
1560 WRITE #1,5,"SAMPLE","ABSOR.","ug SAMPLED","TOTAL ug","ug/ul"
1570 FOR N=1 TO TTL
1580     LPRINT SPNM$(N),ABC(N),UGPT(N),TUGPT(N),UGPVL(N)
1590     PRINT SPNM$(N),ABC(N),UGPT(N),TUGPT(N),UGPVL(N)
1600     WRITE #1,SPNM$(N),ABC(N),UGPT(N),TUGPT(N),UGPVL(N)
1610 NEXT
1615 LPRINT CHR$(12)
1620 CLOSE
1630 '*****
1640 'BRANCHES PROGRAM BACK TO BRANCH.BAS OR FURTHER IF S3P3 CHOICE MADE
1650 '*****
1660 IF SPCH=2 THEN GOSUB 1690
1670 RUN "BRANCH.BAS"
1680 END
1690 '*****
1700 'BRANCHES TO CORRECT.BAS FOR FURTHER COMPILATION OF PROTEIN DATA
1710 '*****
1720 COMMON SPCH,PRCH
1730 CHAIN "CORRECT.BAS"
1740 '*****
1750 'THIS IS THE ERROR TRAPPING SUB-ROUTINE WHICH BRANCHES TO ERRTRP.BAS
1760 '*****
1770 EN=ERR
1780 IF ERR=24 OR ERR=27 THEN CHAIN "ERRTRP.BAS",10,ALL
1790 IF ERR=61 OR ERR=67 THEN CHAIN "ERRTRP.BAS",10,ALL
1800 IF ERR=64 OR ERR=52 THEN CHAIN "ERRTRP.BAS",10,ALL
1810 IF ERR=70 OR ERR=71 THEN CHAIN "ERRTRP.BAS",10,ALL
1820 ON ERROR GOTO 9100
1830 '*****
1840 'FORMS THE TEMPLATE OF SAMPLE DATA ENTERED ABOVE IN A FILE UNDER
1850 ' THE SUBDIRECTORY \LOWRY

```

```

1860 '*****
1870 LOCATE 15,5:INPUT "WHAT DO YOU WISH TO CALL THIS TEMPLATE
      (8 CHAFACTERS OR LESS) ";TMP$
1880 CLS.COLOR 0,7
1890 FOR N=1 TO 3
1900     LOCATE N+10,10:PRINT STRING$(60,32)
1910 NEXT
1920 COLOR 15,0:LOCATE 9,31:PRINT "ENTERING TEMPLATE"
1930 V=LEN(TMP$):CNTD=(80-(V+2))/2:CNTD=INT(CNTD)
1940 COLOR 7,0:LOCATE 12,CNTD:PRINT STRING$(V+2,32)
1950 COLOR 31,0:LOCATE 12,CNTD+1:PRINT TMP$
1960 COLOR 7,0
1970 OPEN "\ACH\LOWRY\"+TMP$+".TPL" FOR OUTPUT AS #1
1980 FOR N=1 TO TTL
1990     WRITE #1, SPNM$(N),STDC(N),SVOL(N),TVOL(N),MLFT(N)
2000 NEXT
2010 CLOSE
2020 RETURN 1060
2030 '*****
2040 ' ENTERS DATA FROM A SAMPLE DATA TEMPLATE IN THE SUBDIRECTORY LOWRY
2050 '*****
2060 LOCATE 10,1:PRINT STRING$(80,32)
2070 LOCATE 10,1:FILES "\ACH\LOWRY\*.TPL":PRINT:INPUT "WHICH TEMPLATE DO
      YOU WISH TO USE (NAME WITHOUT THE .TPL) ";TMP$
2080 FOR N=1 TO 13
2090     LOCATE N+9,1:PRINT STRING$(80,32)
2100 NEXT
2110 OPEN "\ACH\LOWRY\"+TMP$+".TPL" FOR INPUT AS #1
2120 N=0
2130 WHILE NOT EOF(1)
2140     N=N+1
2150     INPUT #1, SPNM$(N),STDC(N),SVOL(N),TVOL(N),MLFT(N)
2151     IF N>17 THEN GOSUB 2250
2152     GOSUB 2200
2160 WEND
2170 CLOSE
2180 TTL=N
2190 RETURN 830
2200 '*****
2203 LOCATE N+6,4:PRINT SPNM$(N):LOCATE N+6,19:PRINT STDC(N)
2206 LOCATE N+6,35:PRINT SVOL(N):LOCATE N+6,52:PRINT TVOL(N)
2209 LOCATE N+6,68:PRINT MLFT(N);
2210 RETURN 2160

```

```

2250 LOCATE 24,4:PRINT SPNM$(N):LOCATE 23,19:PRINT STDC(N)
2253 LOCATE 23,35:PRINT SVOL(N):LOCATE 23,52:PRINT TVOL(N)
2256 LOCATE 23,68:PRINT MLFT(N);
2259 RETURN 2160
9000 '*****
9010 'THIS CHANNELS BACK INTO THE PROGRAM AT THE APPROPRIATE LINE AFTER
9020 ' AN ERROR HAS BEEN COMMITTED. THESE ARE THE LINES CHAINED TO FROM
9030 ' ERRTRP.BAS AFTER THE ANTICIPATED ERROR WAS COMMITTED
9040 '*****
9050 ON ERROR GOTO 1740
9060 IF EN=24 OR EN=27 GOTO 1530
9070 IF EN=61 OR EN=67 GOTO 1550
9080 IF EN=64 OR EN=52 GOTO 1550
9090 IF EN=70 OR EN=71 GOTO 1550
9100 CLS:BEEP
9110 PRINT "YOU HAVE COMMITTED A TERMINAL ERROR!!!!!!!!!!!!!!"
9120 PRINT "YOU WILL HAVE TO START AGAIN BECAUSE OF THIS!!!!"
9130 PRINT :PRINT "THE ERROR CODE IS "ERR" AND THE LINE ON WHICH
      IT OCCURED IS "ERL
9140 PRINT "NOTE BOTH OF THESE AND CHECK THE BASIC MANUAL"
9150 INPUT "WHEN READY PRESS RETURN ",RTN
9160 RUN "BRANCH

```

PROGRAM A.5. ACETYLCHOLINE CALCULATION PROGRAM

```

10 ' THIS PROGRAM IS CALLED ACHCALCU.BAS
20 '   BY RAFAEL CABEZA           JUNE-1987
30 ' #####
40 ' THIS PROGRAM DOES CALCULATIONS FOR ACH DATA ENTERED WITH
      DATENTRY.BAS AND
50 ' USES THE STANDARD CURVES ENTERED WITH STDCUR.BAS
60 ' *****
70 ' VARIABLES== NM$( )=SAMPLE NAME, SC( )=STANDARD CURVE # FOR A GIVEN
      SAMPLE,
80 ' MW( )=SAMPLE mg WET WEIGHT, TM( )=SAMPLE TIME, CPM( )=MEAN SAMPLE
      CPMS,
90 ' FCT( )=SAMPLE CORRECTION MULTIPLICATION FACTOR (/mg/min), ACH( )=ACH IN
100 ' SAMPLED ALIQUOTE, ACHPTW( )=ACH/min/mg (PRCH=1) OR ACH/min (PRCH=2),
110 ' ACHPW( )=ACH/mg (1) OR TOTAL ACH (2), ACHPT( )=ACH/min, TACH( )=TOTAL
      ACH (1), EFLN$=FILE NAME, PRCH=SUB-PROGRAM CHANNELING VARIABLE,
      SPCH=PROGRAM

```



```

120 'CHANNELING VARIABLE, TTL-TOTAL # OF DATA ENTRIES, NUM=# OF STANDARD
    CURVES, NAM$()-STANDARD CURVE NAMES, SLP()-STANDARD CURVE SLOPES,
    CBK()-Y-INTER.
130 'FOR EACH STANDARD CURVE
140 ' *****
150 ON ERROR GOTO 1210
160 PRGLK=4
170 DIM NM$(80),SC(80),MWW(80),TM(80),CPM(80),FCT(80),ACH(80),
    ACHPTW(80),ACHPW(80),ACHPT(80),TACH(80)
180 CLS:BEEP
190 ' *****
200 'ENTERS SAMPLE DATA FROM DATAFL2.PFL- CREATED BY DATENTRY.BAS
210 'ALSO CREATES A BOX WITH NAME OF FILE IN IT
220 ' *****
230 OPEN "DATAFL2.PFL" FOR INPUT AS #1
240     INPUT #1, EFLN$
245 IF CLRSC=2 THEN COLOR 0,3:GOTO 260
250 COLOR 0,7
260 FOR N=1 TO 3
270     LOCATE N+9,10:PRINT STRING$(60,32)
280 NEXT
290 COLOR 7,0
300 LOCATE 11,35:PRINT STRING$(10,32)
310 LOCATE 11,36:PRINT EFLN$
312 COLOR 31,0:LOCATE 8,35:PRINT "WORKING ON":COLOR 7,0
320 N=0
330 WHILE NOT EOF(1)
340     N=N+1
350     IF PRCH=2 THEN INPUT #1, NM$(N),SC(N),TM(N),CPM(N),FCT(N):
        GOTO 370
360     INPUT #1, NM$(N),SC(N),MWW(N),TM(N),CPM(N),FCT(N)
370 WEND
380 TTL=N
390 CLOSE
400 ' *****
410 'ENTERS STANDARD CURVE DATA FROM DATAFL1.PFL - CREATED BY STDCUR.BAS
420 ' *****
430 OPEN "DATAFL1.PFL" FOR INPUT AS #1
440 INPUT #1, NUM
450 FOR N=1 TO NUM
460     INPUT #1, NAM$(N)
470 NEXT
480 FOR N=1 TO NUM

```

```

490     INPUT #1, SLP(N),CBK(N)
500 NEXT
510 CLOSE
520 '*****
530 'CALCULATES ACH AMOUNTS FROM INFORMATION ENTERED FROM THE
    PROGRAM FILES
540 '*****
550 FOR N=1 TO TTL
560     ACH(N)=(CPM(N)-CBK(SC(N)))/SLP(SC(N))           'ACH SAMPLED
565     IF ACH(N)<0 THEN ACH(N)=0
570     ACHPTW(N)=ACH(N)*FCT(N)                         'ACH/TIME/WET WEIGHT
580     ACHPTW(N)=INT(ACHPTW(N)*1000+.5)/1000
590     ACHPW(N)=ACH(N)*FCT(N)*TM(N)                   'ACH/WET WEIGHT
600     ACHPW(N)=INT(ACHPW(N)*100+.5)/100
610     IF PRCH=2 GOTO 670
620     ACHPT(N)=ACH(N)*FCT(N)*MWW(N)                 'ACH/TIME
630     ACHPT(N)=INT(ACHPT(N)*100+.5)/100
640     TACH(N)=ACH(N)*FCT(N)*MWW(N)*TM(N)            'TOTAL ACH IN SAMPLE
650     TACH(N)=INT(TACH(N)*10+.5)/10
660     ACH(N)=INT(ACH(N)*10+.5)/10
670 NEXT
680 '*****
690 'PRINTS CALCULATED DATA TO SCREEN AND PRINTER FOR PRCH=1 CHOICES
700 '*****
710 IF PRCH=2 THEN GOTO 840
720 CLS
730 LPRINT STRING$(80,42)
740 PRINT "SAMPLE    TIME    mg WEIGHT", "SAMP. ACH", "
    TOTAL ACH", "ACH/min/mg"
750 LPRINT "SAMPLE    TIME    mg WEIGHT", "SAMP. ACH", "TOTAL ACH",
    "ACH/min/mg"
760 FOR N=1 TO TTL
770     PRINT " " "NM$(N)"      "TM(N)"      "MWW(N),ACH(N),TACH(N),ACHPTW(N)"
780     LPRINT " " "NM$(N)"      "TM(N)"      "
    MWW(N),ACH(N),TACH(N),ACHPTW(N)"
790 NEXT
795 LPRINT CHR$(12)
800 GOTO 920
810 '*****
820 'PRINTS CALCULATED DATA TO SCREEN AND PRINTER FOR PRCH=2 CHOICES
830 '*****
840 CLS
850 LPRINT STRING$(80,42)

```

```

860 PRINT "SAMPLE   TIME","SAMP. ACH","TOTAL ACH","ACH/min"
870 LPRINT "SAMPLE   TIME","SAMP. ACH","TOTAL ACH","ACH/min"
880 FOR N=1 TO TTL
890     PRINT " "NM$(N)"      "TM(N),ACH(N),ACHPW(N),ACHPTW(N)
900     LPRINT " "NM$(N)"      "TM(N),ACH(N),ACHPW(N),ACHPTW(N)
910 NEXT
915 LPRINT CHR$(12)
920 '*****
930 'ENTERS CALCULATED DATA INTO THE STORAGE DISKETT IN SUBDIRECTORY
    CALLED
940 '\ACH\ FOR PRCH=2,SPCH=1
950 '*****
960 CLS
970 IF SPCH=2 THEN OPEN "B:\ACH\"+EFLN$ FOR OUTPUT AS #1:GOTO 990
980 OPEN "B:\ACH\"+EFLN$ FOR OUTPUT AS #1
990 IF PRCH=2 THEN WRITE #1,5,"SAMPLE","TIME","SAMP.ACH","TOTAL ACH","
    ACH/min":GOTO 1010
1000 WRITE #1,6,"SAMPLE","TIME","mg WET WT.","SAMP.ACH","TOTAL ACH","
    ACH/min/mg"
1010 FOR N=1 TO TTL
1020     IF PRCH=2 THEN WRITE #1,NM$(N),TM(N),ACH(N),ACHPW(N),ACHPTW(N):
        GOTO 1040
1030     WRITE #1,NM$(N),TM(N),MW(N),ACH(N),TACH(N),ACHPTW(N)
1040 NEXT
1050 CLOSE
1060 '*****
1070 'BRANCHES TO THE APPROPRIATE PROGRAM ACCORDING TO THE CHOICE ENTERED
    IN
1080 'BRANCH.BAS
1090 '*****
1100 IF SPCH=1 THEN CLEAR:RUN "BRANCH
1110 IF SPCH=2 GOTO 1130
1120 END
1130 '*****
1140 'CHAINS TO RADIO-L1.BAS IF RADIO-ISOTOPE DATA IS TO BE ENTERED
1150 '*****
1160 CLS
1170 INPUT "DO YOU WISH TO CONTINUE WITH THE RADIO-ISOTOPE CALCULATIONS
    OR WOULD YOU LIKE TO RETURN TO THESE LATER (1=Y,2=N) ",RESP
1180 IF RESP=2 THEN RUN"BRANCH
1190 COMMON PRCH,SPCH,EFLN$
1200 CHAIN "RADIO-L1.BAS"
1210 '*****

```

```

1220 'THIS IS THE ERROR TRAPPING ROUTINE WHICH CHAINS TO ERRTRP.BAS
1230 '*****
1240 EN=ERR
1250 IF ERR=24 OR ERR=27 THEN CHAIN "ERRTRP.BAS",10,ALL
1260 IF ERR=61 OR ERR=67 THEN CHAIN "ERRTRP.BAS",10,ALL
1270 IF ERR=64 OR ERR=52 THEN CHAIN "ERRTRP.BAS",10,ALL
1280 IF ERR=70 OR ERR=71 THEN CHAIN "ERRTRP.BAS",10,ALL
1290 GOTO 9080
9000 '*****
9010 'THIS RE-DIRECTS CONTROL TO AN APPROPRIATE LINE IN THE PROGRAM
      FOLLOWING
9020 'A PREDICTABLE ERROR
9030 '*****
9040 ON ERROR GOTO 1210
9050 IF EN=24 OR EN=27 GOTO 680
9060 IF EN=61 OR EN=67 GOTO 920
9070 IF EN=70 OR EN=71 GOTO 920
9080 PRINT "YOU HAVE COMMITTED A TERMINAL ERROR!!!!!!"
9090 PRINT "YOU WILL HAVE TO START AGAIN BECAUSE OF THIS!!!"
9100 PRINT "THE ERROR CODE IS ("ERR") AND IT OCCURED IN LINE NUMBER
      ("ERL") ."
9110 INPUT "WHEN YOU ARE READY PRESS RETURN ",RTN:RUN"BRANCH

```

PROGRAM A.6. RADIO-LABEL CALCULATIONS PROGRAM. 2ND MENU PROGRAM

```

10 'THIS PROGRAM IS CALLED RADIO.BAS
20 ' BY RAFAEL CABEZA          JUNE-1987
30 '#####
40 'THIS PROGRAM ALLOWS YOU TO GO TO THE RADIO-ISOTOPE TYPE OF PROGRAMS
50 'DIRECTLY IF YOU HAVE ALREADY CALCULATED TOTAL ACH AMOUNTS
60 '*****
70 CLS:BEEP
80 ON ERROR GOTO 400
90 '*****
100 'THIS SETS UP THE BOX AND CHOICES
110 '*****
115 IF CLRSC=2 THEN COLOR 0,9:GOTO 130
120 COLOR 0,7
130 FOR N=1 TO 10
140     LOCATE N,1:PRINT STRING$(80,32)
150 NEXT

```

```

160 COLOR 7,0
170 FOR N=2 TO 9
180     LOCATE N,4:PRINT STRING$(74,32)
190 NEXT
200 LOCATE 4,20:PRINT "THESE ARE YOUR CHOICES:"
210 LOCATE 6,20:PRINT "1. SINGLE LABEL CALCULATIONS "
220 LOCATE 7,20:PRINT "2. DOUBLE LABEL CALCULATIONS "
225 LOCATE 8,20:PRINT "3. QUENCH CORRECTIONS ONLY"
240 '*****
250 'ALLOWS YOU TO ENTER 1, 2 or 3 DEPENDING ON THE NEED AND CHAINs TO
    THE
260 'APPROPRIATE PROGRAM
270 '*****
280 LOCATE 15,20:PRINT "WHICH IS YOUR CHOICE  ( ) ?"
290 LOCATE 15,43:CHB$=INPUT$(1):CH=VAL(CHB$)
300 IF CH>3 OR CH=0 THEN BEEP:LOCATE 20,20:PRINT "THE CHOICE MUST BE 1,2
    OR 3" GOTO 280
310 IF CH=1 THEN CLS:COMMON PRCH,SPCH,CLRSC:CHAIN "RADIO-1.BAS"
320 IF CH=2 THEN CLS:COMMON PRCH,SPCH,CLRSC:CHAIN "RAD-2-1.BAS"
325 IF CH=3 THEN CLS:COMMON PRCH,SPCH,CLRSC:CHAIN "QUENCH.BAS"
360 END
370 '*****
380 'IN CASE OF AN UNFORSEEN ERROR THIS ALLOWS YOU TO START FROM THE
    BEGINNING
390 '*****
400 CLS
410 PRINT "YOU HAVE COMMITTED A TERMINAL ERROR!!!!!!!"
420 PRINT "YOU WILL HAVE TO START OVER AGAIN BECAUSE OF THIS ERROR !!!!!:"
430 PRINT "THE ERROR CODE IS("ERR") AND IT OCCURED IN LINE NUMBER
    ("ERL") ."
440 INPUT "PRESS RETURN WHEN YOU ARE READY ",RTN
450 RUN"BRANCH

```

PROGRAM A.7. SINGLE LABEL CALCULATION PROGRAM PART 1.

```

10 'THIS IS CALLED RADIO-1.BAS
20 'BY RAFAEL CABEZA
22 '*****
25 'THIS PROGRAM CALCULATES MACHINE EFFICIENCY AND QUENCHING FOR THE
    SYSTEM
26 'AND THE DIFFERENT FRACTIONS AS WELL AS THE EXTRACTION STANDARDS

```

```

27 ' #####
28 ' VARIABLES FOR THIS PROGRAM ARE FLN$=FILE NAME, TC14=C14 STD DPM,
    TH3=H3
29 ' STD DPMS, C14ST=C14 CPM, H3ST=H3 CPM, C14=3 C14 EFF., H3=3 H3 EFF ,
    QST=QNCH
30 ' STD DPM, QSTD()-QNCH CPM, QBK=QNCH BLK, QP=3 QNCH, CHQSTR()-CH STD QNCH
    STD DPM,
31 ' QBK=BLK, CHQP=3 QNCH OF CH STD, CHST()-CH STD CPM, CHP=CH STD
    DPM, ACHQSTR()-
32 ' ACH STD QNCH STD CPM, ACHTR()-ACH STD CPM, ACHQP=ACH STD 3 QNCH,
    ACHP=ACH STD
33 ' DPM, AQSD=ACETYL QNCH STD, AQ=3 ACETYL QNCH, CHASTR=ACETYL CH CPM,
    CHADPM=
34 ' CH DPM IN ACETYL FR, ACASTR=ACETYL ACH STD CPM, AADPM=ACH DPM IN ACETYL
    FR,
35 ' CQSD=UNALT QNCH STD, CQ=3 UNALT QNCH, CHCSTR=UNALT CH CPM, CHCDPM=CH DPM
    IN
36 ' UNALT FR, ACCSTR=UNALT ACH CPM, ACDPM=ACH DPM IN UNALT FR
37 ' *****
38 ' INPUTS FILE NAME
39 ' *****
40 CLS:BEEP
41 IF CLRSC=2 THEN COLOR 4,0
42 LOCATE 10,10:INPUT "WHAT IS THE EXPERIMENT NAME ---> ", FLN$
43 COLOR 7,0
44 CLS
45 ' *****
46 ' CALCULATES MACHINE EFFICIENCY
47 ' *****
48 IF CLRSC=2 THEN COLOR 0,1:GOTO 110
49 COLOR 0,7
50 FOR N=1 TO 3
51     LOCATE N,20:PRINT STRING$(40,32)
52 NEXT
53 COLOR 7,0
54 LOCATE 2,30:PRINT STRING$(20,32)
55 IF CLRSC=2 THEN COLOR 3,0
56 LOCATE 2,31:PRINT "MACHINE EFFICIENCY"
57 LOCATE 5,10:INPUT "WHAT ARE THE TOTAL DPMS FOR 14C ---> ", TC14
58 LOCATE 7,10:INPUT "WHAT ARE THE TOTAL DPMS FOR 3H ---> ", TH3
59 LOCATE 6,10:INPUT "WHAT IS THE READING FOR 14C STANDARD ----->
    ", C14ST
60 LOCATE 8,10:INPUT "WHAT IS THE READING FOR 3H STANDARD ----->

```

```

        ",H3ST
220 COLOR 7,0
230 FOR N=1 TO 4
240     LOCATE N+4,10:PRINT STRINGS(70,32)
250 NEXT
260 C14=C14ST/TC14:H3=H3ST/TH3
270 '*****
271 'CALCULATES SYSTEM EFFICIENCY
272 '*****
280 LOCATE 2,30:PRINT STRING$(20,32)
290 IF CLRSC=2 THEN COLOR 2,0
300 LOCATE 2,31:PRINT "SYSTEM QUENCHING "
310 LOCATE 5,10:INPUT "WHAT ARE THE TOTAL DPMS FOR YOUR QUENCH ==>
        ",QST
320 LOCATE 5,10:PRINT STRING$(70,32)
330 LOCATE 5,10:INPUT "HOW MANY TRIALS FOR THE QUENCH STANDARD (1,2,ETC)
        ",QSTM
340 LOCATE 5,10:PRINT STRING$(70,32)
350 LOCATE 7,5:PRINT "QUENCH ST"
360 FOR N=1 TO QSTM
370     LOCATE 7,15*N:PRINT "TRIAL("N")"
380 NEXT
390 FOR N=1 TO QSTM
400     LOCATE 8,15*N:INPUT "",QSTD(N)
410     QS=QS+QSTD(N)
420 NEXT
430 LOCATE 10,10:INPUT "WHAT IS THE BLANK READING FOR THESE ",QBK
440 QP=((QS/QSTM)-QBK)/QST
445 QS=0
450 FOR N=5 TO 10
460     LOCATE N,5:PRINT STRING$(75,32)
470 NEXT
471 '*****
472 'CALCULATES THE DPMS USED FOR THE ACH AND CH EXTRACTION STDS
473 '*****
480 LOCATE 2,29:PRINT STRING$(22,32)
490 LOCATE 2,31:PRINT "EXTRACTION STANDARDS"
510 LOCATE 5,10:PRINT STRING$(70,32)
520 LOCATE 5,10:INPUT "HOW MANY TRIALS PER EXTRACTION STANDARD (1,2,ETC)
        ",QSTN
530 LOCATE 5,10:PRINT STRING$(70,32)
540 LOCATE 7,5:PRINT "QUENCH ST"
550 FOR N=1 TO QSTN

```

```

560     LOCATE 7,15*N:PRINT "TRIAL("N")"
570 NEXT
580 FOR N=1 TO QSTN
590     LOCATE 8,15*N:INPUT "",CHQSTR(N)
600     CHQS=CHQS+CHQSTR(N)
610 NEXT
620 LOCATE 10,10:INPUT "WHAT IS THE BLANK READING FOR THESE ",QBK
630 CHQP=((CHQS/QSTN)-QBK)/QST
635 CHQS=0
640 LOCATE 12,5:PRINT "3H-CH ST"
650 FOR N=1 TO QSTN
660     LOCATE 12,15*N:PRINT "TRIAL("N")"
670 NEXT
680 FOR N=1 TO QSTN
690     LOCATE 13,15*N:INPUT "",CHTR(N)
700     CHS=CHS+CHTR(N)
710 NEXT
720 LOCATE 15,10:INPUT "WHAT IS THE BLANK READING FOR THESE ",CBK
730 CHP=((CHS/QSTN)-CBK)/CHQP
735 CHS=0
740 '*****
750 FOR N=5 TO 16
760     LOCATE N,5:PRINT STRING$(75,32)
770 NEXT
780 LOCATE 7,5:PRINT "QUENCH ST"
790 FOR N=1 TO QSTN
800     LOCATE 7,15*N:PRINT "TRIAL("N")"
810 NEXT
820 FOR N=1 TO QSTN
830     LOCATE 8,15*N:INPUT "",ACHQSTR(N)
840     ACHQS=ACHQS+ACHQSTR(N)
850 NEXT
860 LOCATE 10,10:INPUT "WHAT IS THE BLANK READING FOR THESE ",QBK
870 ACHQP=((ACHQS/QSTN)-QBK)/QST
875 ACHQS=0
880 LOCATE 12,5:PRINT "3H-ACH ST"
890 FOR N=1 TO QSTN
900     LOCATE 12,15*N:PRINT "TRIAL("N")"
910 NEXT
920 FOR N=1 TO QSTN
930     LOCATE 13,15*N:INPUT "",ACHTR(N)
940     ACHS=ACHS+ACHTR(N)
950 NEXT

```



```

960 LOCATE 15,10:INPUT "WHAT IS THE BLANK READING FOR THESE ",ACBK
970 ACHP=((ACHS/QSTN)-ACBK)/ACHQP
975 ACHS=0
980 CHP=INT(CHP+.5):ACHP=INT(ACHP+.5)
990 '*****
1000 CLS
1010 LPRINT STRING$(75,42)
1020 LPRINT STRING$(33,42)+FLN$+STRING$(33,42)
1030 LPRINT STRING$(75,42)
1040 LPRINT
1050 C14=CINT(C14*1000)/1000:H3=CINT(H3*1000)/1000
1060 QP=CINT(QP*1000)/1000:CHQP=CINT(CHQP*1000)/1000:
      ACHQP=CINT(ACHQP*1000)/1000
1070 LPRINT "THE MACHINE EFFICIENCY IS AS FOLLOWS"
1080 LPRINT "CARBON 14 ----->"C14
1090 LPRINT "TRITIUM ----->"H3
1100 LPRINT :LPRINT :LPRINT "THE SYSTEM EFFICIENCY
      IS ----->"QP
1110 LPRINT :LPRINT :LPRINT "YOUR 3H-CH DPMS ARE -----> "CHP
1120 LPRINT "YOUR 3H-ACH DPMS ARE -----> "ACHP
1130 LPRINT :LPRINT STRING$(75,42)
1140 '*****
1150 IF CLRSC=2 THEN COLOR 0,1:GOTO 1170
1160 COLOR 0,7
1170 FOR N=1 TO 3
1180     LOCATE N,20:PRINT STRING$(40,32)
1190 NEXT
1200 COLOR 7,0
1210 IF CLRSC=2 THEN COLOR 3,0
1220 LOCATE 2,30:PRINT STRING$(20,32)
1230 LOCATE 2,31:PRINT "ACETYLATED FRACTN"
1240 LOCATE 5,10:INPUT "HOW MANY TRIALS FOR THE ACETYLATED STD (1,2,ETC)
      ",QSTN
1250 LOCATE 5,10:PRINT STRING$(70,32)
1260 LOCATE 7,5:PRINT "QUENCH ST"
1270 FOR N=1 TO QSTN
1280     LOCATE 7,15*N:PRINT "TRIAL("N")"
1290 NEXT
1300 FOR N=1 TO QSTN
1310     LOCATE 8,15*N:INPUT "",AQSD(N)
1320     AQ=AQ+AQSD(N)
1330 NEXT
1340 LOCATE 10,10:INPUT "WHAT IS THE BLANK READING FOR THESE ",ABK

```

```

1350 AQ=((AQ/QSTN)-ABK)/QST
1360 FOR N=5 TO 10
1370     LOCATE N,5:PRINT STRING$(75,32)
1380 NEXT
1390 LOCATE 7,5:PRINT "3H-CH ST"
1400 FOR N=1 TO QSTN
1410     LOCATE 7,15*N:PRINT "TRIAL("N")"
1420 NEXT
1430 FOR N=1 TO QSTN
1440     LOCATE 8,15*N:INPUT "",CHAS1R(N)
1450     CHAS=CHAS+CHASTR(N)
1460 NEXT
1470 CHADPM=((CHAS/QSTN)-ABK)/AQ
1475 CHAS=0
1480 LOCATE 12,5:PRINT "3H-ACH ST"
1490 FOR N=1 TO QSTN
1500     LOCATE 12,15*N:PRINT "TRIAL("N")"
1510 NEXT
1520 FOR N=1 TO QSTN
1530     LOCATE 13,15*N:INPUT "",ACASTR(N)
1540     AAS=AAS+ACASTR(N)
1550 NEXT
1560 AADPM=((AAS/QSTN)-ABK)/AQ
1565 AAS=0
1570 CAP=CINT((CHADPM/CHP)*100)/100
1580 AAP=CINT((AADPM/ACHP)*100)/100
1590 '*****
1600 LPRINT "*****ACETYLATED FRACTION*****"
1610 LPRINT:LPRINT "% QUENCH OF FRACTION -----> "AQ
1620 LPRINT "BLANK READING IS -----> "ABK
1630 LPRINT:LPRINT "% 3H-CH RECOVERED -----> "CAP
1640 LPRINT "% 3H-ACH RECOVERED -----> "AAP
1650 LPRINT:LPRINT STRING$(70,42)
1660 '*****
1670 FOR N=5 TO 13
1680     LOCATE N,1:PRINT STRING$(80,32)
1690 NEXT
1700 COLOR 0,7
1710 IF CLRSC=2 THEN COLOR 0,1
1720 FOR N=1 TO 3
1730     LOCATE N,20:PRINT STRING$(40,32)
1740 NEXT
1750 COLOR 7,0

```

```

1760 IF CLRSC=2 THEN COLOR 1,0
1770 LOCATE 2,30:PRINT STRING$(20,32)
1780 LOCATE 2,31:PRINT "UNALTERED FRACTN"
1790 LOCATE 5,10:INPUT "HOW MANY TRIALS FOR THE ACETYLATED STD (1,2,ETC)
      ",QSTM
1800 LOCATE 5,10:PRINT STRING$(70,32)
1810 LOCATE 7,5:PRINT "QUENCH ST"
1820 FOR N=1 TO QSTM
1830     LOCATE 7,15*N:PRINT "TRIAL("N")"
1840 NEXT
1850 FOR N=1 TO QSTM
1860     LOCATE 8,15*N:INPUT "",CQSD(N)
1870     CQ=CQ+CQSD(N)
1880 NEXT
1890 LOCATE 10,10:INPUT "WHAT IS THE BLANK READING FOR THESE ",CBK
1900 CQ-((CQ/QSTM)-CBK)/QST
1910 FOR N=5 TO 10
1920     LOCATE N,5:PRINT STRING$(75,32)
1930 NEXT
1940 LOCATE 7,5:PRINT "3H-CH ST"
1950 FOR N=1 TO QSTN
1960     LOCATE 7,15*N:PRINT "TRIAL("N")"
1970 NEXT
1980 FOR N=1 TO QSTN
1990     LOCATE 8,15*N:INPUT "",CHCSTR(N)
2000     CHCS=CHCS+CHCSTR(N)
2010 NEXT
2020 CHCDPM-((CHCS/QSTN)-CBK)/CQ
2025 CHCS=0
2030 LOCATE 12,5:PRINT "3H-ACH ST"
2040 FOR N=1 TO QSTN
2050     LOCATE 12,15*N:PRINT "TRIAL("N")"
2060 NEXT
2070 FOR N=1 TO QSTN
2080     LOCATE 13,15*N:INPUT "",ACCSTR(N)
2090     ACS=ACS+ACCSTR(N)
2100 NEXT
2110 ACDPM-((ACS/QSTN)-CBK)/CQ
2115 ACS=0
2120 CCP=CINT((CHCDPM/CHP)*100)/100
2130 ACP=CINT((ACDPM/ACHP)*100)/100
2140 '*****
2150 LPRINT "*****UNALTERED FRACTION*****"

```

```

2160 LPRINT:LPRINT "% QUENCH OF FRACTION -----> "CQ
2170 LPRINT "BLANK READING IS -----> "CBK
2180 LPRINT :LPRINT "% 3H-CH RECOVERED -----> "CCP
2190 LPRINT "% 3H-ACH RECOVERED -----> "ACP
2200 LPRINT :LPRINT STRING$(70,42):LPRINT
2210 '*****
2220 OPEN "RAD1.PFL" FOR OUTPUT AS #1
2230 WRITE #1, FLN$
2240 WRITE #1, ABK,AQ,CAP,AAP
2250 WRITE #1, CBK,CQ,CCP,ACP
2260 CLOSE
2270 COMMON CLRSC:CHAIN "RADIO-2
2280 END

```

PROGRAM A.8. SINGLE LABEL CALCULATION PROGRAM PART 2

```

10 'THIS PROGRAM IS CALLED RADIO-2.BAS
20 ' BY RJC
30 '*****
40 CLS:BEEP
50 DIM SMP$(35),MF(35),ASF(35),USF(35),TH(35),TC(35),C(35),H(35)
60 OPEN "RAD1.PFL" FOR INPUT AS #1
70 INPUT #1, FIL$
80 CLOSE
90 COLOR 0,7
95 IF CLRSC=2 THEN COLOR 0,3
100 FOR N=1 TO 3
110     LOCATE N,15:PRINT STRING$(50,32)
120 NEXT
130 COLOR 7,0
140 LOCATE 2,33:PRINT STRING$(14,32)
150 LOCATE 2,36:PRINT FIL$
160 LOCATE 10,10:INPUT "HOW MANY TRIALS PER SAMPLE DID YOU DO ";NM
170 LOCATE 10,1:PRINT STRING$(80,32)
175 IF CLRSC=2 THEN COLOR 3,0
180 LOCATE 5,1:PRINT "SAMPLE":LOCATE 5,10:PRINT "MLT.FCT."
190 LOCATE 5,30:PRINT "ACETYLATED":LOCATE 5,60:PRINT "UNALTERED"
200 F=(30/NM)
210 FOR N=1 TO NM
220     LOCATE 6,(F*(N-1))+20:PRINT "("N")"
230     LOCATE 6,(F*(N-1))+50:PRINT "("N")"

```

```

240 NEXT
250 N1=0:TASF=0:TUSF=0
260 FOR N=1 TO 17
270     N1=N1+1
280     LOCATE 6+N,1:INPUT "",SMP$(N1)
290     IF SMP$(N1)="" THEN N1=N1-1:GOTO 630
300     LOCATE 6+N,10:INPUT "",MF(N1)
310     IF MF(N1)=0 THEN MF(N1)=MF(N1-1):LOCATE 6+N,9:PRINT MF(N1)
320     FOR NN=1 TO NM
330         LOCATE 6+N,(F*(NN-1))+20:INPUT "",AS(NN)
340         TASF=TASF+AS(NN)
350     NEXT NN
360     ASF(N1)=INT(TASF/NM+.5)
370     TASF=0
380     FOR NN=1 TO NM
390         LOCATE 6+N,(F*(NN-1))+50:INPUT "",US(NN)
400         TUSF=TUSF+US(NN)
410     NEXT NN
420     USF(N1)=INT(TUSF/NM+.5)
430     TUSF=0
440 NEXT N
450 N1=N1+1
460 LOCATE 24,1:INPUT "",SMP$(N1)
470 IF SMP$(N1)="" THEN N1=N1-1:GOTO 630
480 LOCATE 23,10:INPUT "",MF(N1)
490 IF MF(N1)=0 THEN MF(N1)=MF(N1-1):LOCATE 23,9:PRINT MF(N1)
500 FOR NN=1 TO NM
510     LOCATE 23,(F*(NN-1))+20:INPUT "",AS(NN)
520     TASF=TASF+AS(NN)
530 NEXT NN
540 ASF(N1)=INT(TASF/NM+.5)
550 TASF=0
560 FOR NN=1 TO NM
570     LOCATE 23,(F*(NN-1))+50:INPUT "",US(NN)
580     TUSF=TUSF+US(NN)
590 NEXT NN
600 USF(N1)=INT(TUSF/NM+.5)
610 TUSF=0
620 GOTO 450
630 CLS:LOCATE 10,10:INPUT "ARE YOU DONE ENTERING DATA (1=Y,2=N) ";RET
640 IF RET=2 THEN CLS:GOSUB 10000
650 CLS
660 COLOR 0,7

```

```

665 IF CLRSC=2 THEN COLOR 0,5
670 FOR N=1 TO 5
680     LOCATE 9+N,15:PRINT STRING$(50,32)
690 NEXT
700 COLOR 7,0:LOCATE 12,35:PRINT STRING$(10,32):LOCATE 12,38:PRINT FIL$
710 LOCATE 8,35:PRINT "WORKING ON"
720 OPEN "RAD1.PFL" FOR INPUT AS #1
730 INPUT #1, FLN$
740 INPUT #1, ABK,AQ,CH,HH
750 INPUT #1, CBK,CQ,CC,HC
760 CLOSE
770 OPEN "B:\RADIO\"+FLN$ FOR OUTPUT AS #1
775 WRITE #1, 5,"SAMPLE","DPM-ACH","DPM-CH","TL-DPM-ACH","TL-DPM-CH"
780 FOR N=1 TO N1
790     TH(N)=INT(((ASF(N)-ABK)/AQ)+.5)
800     TC(N)=INT(((USF(N)-CBK)/CQ)+.5)
810     M=(CH*HC)-(HH*CC)
820     C(N)=((TH(N)*HC)-(TC(N)*HH))/M
830     H(N)=((TC(N)*CH)-(TH(N)*CC))/M
832     IF C(N)<0 THEN C(N)=0
833     IF H(N)<0 THEN H(N)=0
834     IF C(N)=0 THEN H(N)=TH(N)
835     IF H(N)=0 THEN C(N)=TC(N)
840     C(N)=INT((C(N)*MF(N))+.5):H(N)=INT((H(N)*MF(N))+.5)
850     WRITE #1, SMP$(N),TH(N),TC(N),H(N),C(N)
860 NEXT
870 CLOSE
880 CLS:LOCATE 10,25:PRINT "*****PRINTING FILE "FLN$*****"
890 LPRINT :LPRINT "SAMPLE","ACH-DPM","TL/ACH","CH-DPM","TL/CH"
900 FOR N=1 TO N1
910     LPRINT SMP$(N),TH(N),H(N),TC(N),C(N)
920 NEXT
930 LPRINT CHR$(12)
935 RUN "BRANCH
940 END
10000 LOCATE 24,1:PRINT SMP$(N1):LOCATE 23,10:PRINT MF(N1)
10010 FOR NN=1 TO NM
10020     LOCATE 23,(F*(NN-1))+20:PRINT AS(NN)
10030     LOCATE 23,(F*(NN-1))+50:PRINT US(NN)
10040 NEXT
10050 RETURN 450

```

PROGRAM A 9. SAMPLE QUENCH CALCULATION PROGRAM

```
10 'THIS PROGRAM IS CALLED QUENCH.BAS
20 ' BY RJC
30 '*****
40 DIM SMP$(50),MF(50),RD(50),DPM(50)
50 CLS:BEEP
60 IF CLRSC=2 THEN COLOR 5,2
70 CLS
80 LOCATE 10,10:INPUT "WHAT IS THE NAME OF THIS FILE ";FIL$
90 CLS:LOCATE 10,10:INPUT "HOW MANY DETERMINATIONS DID YOU DO
    (1,2,ETC) ";NM
100 COLOR 7,0
110 CLS
120 COLOR 0,7
130 IF CLRSC=2 THEN COLOR 0,2
140 FOR N=1 TO 3
150     LOCATE N,15:PRINT STRING$(50,32)
160 NEXT
170 COLOR 7,0
180 LOCATE 2,35:PRINT STRING$(10,32)
190 LOCATE 2,35:PRINT " "+FIL$
200 IF CLRSC=2 THEN COLOR 2,0
210 LOCATE 5,5:INPUT "HOW MANY DPMS IN THE QUENCH STANDARD ==> ",QNST
220 T=(60/NM)
230 FOR N=1 TO NM
240     LOCATE 6,T*N+5:PRINT "READING ("N")"
250     LOCATE 7,T*N+5:INPUT "",QRD
260     TQRD=TQRD+QRD
270 NEXT
280 QUPT=TQRD/NM
290 LOCATE 9,10:INPUT "WHAT IS THE BLANK READING FOR THESE ";BLK
300 FOR N=5 TO 9
310     LOCATE N,1:PRINT STRING$(80,32)
320 NEXT
330 QCF=(QUPT-BLK)/QNST
340 LOCATE 10,1:INPUT "HOW MANY DETERMINATIONS FOR EACH SAMPLE
    (1,2,ETC.) ";NM
350 LOCATE 10,1:PRINT STRING$(80,32)
360 LOCATE 5,1:PRINT "SAMPLE":LOCATE 5,10:PRINT "M.F."
370 FOR N=1 TO NM
380     LOCATE 5,(60/NM)*N:PRINT "TRIAL ("N")"
```

```

390 NEXT
400 N=0
410 FOR NN=1 TO 18
420     N=N+1
430     LOCATE NN+5,1:INPUT "",SMP$(N)
440     IF SMP$(N)="" GOTO 660
450     LOCATE NN+5,10:INPUT "",MF(N)
460     IF MF(N)=0 THEN MF(N)=MF(N-1):LOCATE NN+5,9:PRINT MF(N)
470     FOR N1=1 TO NM
480         LOCATE NN+5,(60/NM)*N1:INPUT "",TRL
490         TTRL=TTRL+TRL
500     NEXT N1
510     RD(N)=TTRL/NM
520     TTRL=0
530 NEXT NN
540 N=N+1
550 LOCATE 24,1:INPUT "",SMP$(N)
560 IF SMP$(N)="" GOTO 660
570 LOCATE 23,10:INPUT "",MF(N)
580 IF MF(N)=0 THEN MF(N)=MF(N-1):LOCATE 23,9:PRINT MF(N)
590 FOR N1=1 TO NM
600     LOCATE 23,(60/NM)*N1:INPUT "",TRL
610     TTRL=TTRL+TRL
620 NEXT
630 RD(N)=TTRL/NM
640 TTRL=0
650 GOTO 540
660 CLS
670 N=N-1:T=N
680 COLOR 31,0:LOCATE 10,10:PRINT "WORKING ON FILE -----> "FIL$
690 FOR N=1 TO T
700     DPM(N)=(RD(N)-BLK)*MF(N)/QCF
710     DPM(N)=INT(DPM(N)+.5)
720 NEXT
730 OPEN "B:\RADIO\"+FIL$+".Q" FOR OUTPUT AS #1
740 LPRINT STRING$(60,42):LPRINT STRING$(25,42)+FIL$+STRING$(25,42)
750 LPRINT :LPRINT "THE QUENCH FOR THESE SAMPLES IS -----> "QCF
760 LPRINT :LPRINT "SAMPLE","CPMS","DPMS"
770 WRITE #1, 3,"SAMPLE","CPM","DPM"
780 FOR N=1 TO T
790     LPRINT SMP$(N),RD(N),DPM(N)
800     WRITE #1, SMP$(N),RD(N),DPM(N)
810 NEXT

```



```

820 CLOSE
830 LPRINT CHR$(12)
840 RUN "BRANCH
850 END

```

PROGRAM A.10. ACETYLCHOLINESTERASE CALCULATIONS PROGRAM.

```

10 'THIS PROGRAM IS CALLED ACHE.BAS
20 ' BY RAFAEL CABEZA OCTOBER, 1987
30 '*****
40 'IT IS INTENDED TO CARRY OUT ACETYLCHOLINE-ESTERASE DETERMINATIONS
50 '*****
60 DIM SMP$(30),RD(30,5),M(30),VS(30),CO(30),R(30)
70 BEEP
80 CLS
90 LOCATE 10,10:INPUT "WHAT IS THE NAME OF YOUR FILE
    (8 CHARACTERS OR LESS) ",EFLN$
100 IF LEN(EFLN$)>8 THEN BEEP:CLS:LOCATE 1,3:PRINT "THE NAME YOU HAVE
    CHOSEN IS TOO LONG! IT HAS TO BE LESS THAN 8 CHARACTERS":GOTO 90
110 '*****
120 CLS
130 LNFN=LEN(EFLN$)+4
140 BLKS=(80-LNFN)/2:COLOR 0,7
150 FOR N=1 TO 3
160 LOCATE N,1:PRINT STRING$(80,32)
170 NEXT
180 COLOR 7,0
190 LOCATE 2,BLKS:PRINT STRING$(LNFN,32)
200 LOCATE 2,BLKS+2:PRINT EFLN$
210 LOCATE 5,2:PRINT "SAMPLE NAME"
220 LOCATE 5,22:PRINT "ABS. #1"
230 LOCATE 5,35:PRINT "ABS. #2"
240 LOCATE 5,48:PRINT "ABS. #3"
250 LOCATE 5,61:PRINT "ABS. #4"
260 LOCATE 5,74:PRINT "ABS. #5"
270 '*****
280 FOR N=1 TO 18
290 C=C+1
300 LOCATE 5+N,3:INPUT "",SMP$(C)
310 IF SMP$(C)="" THEN GOTO 570
320 LOCATE 5+N,22:INPUT "",RD(C,1)

```

```

330     IF RD(C,1)=0 THEN RD(C,1)=RD(C-1,1):LOCATE 5+N,21:PRINT RD(C,1)
340     LOCATE 5+N,35:INPUT "",RD(C,2)
350     IF RD(C,2)=0 THEN RD(C,2)=RD(C-1,2):LOCATE 5+N,34:PRINT RD(C,2)
360     LOCATE 5+N,48:INPUT "",RD(C,3)
370     IF RD(C,3)=0 THEN RD(C,3)=RD(C-1,3):LOCATE 5+N,47:PRINT RD(C,3)
380     LOCATE 5+N,61:INPUT "",RD(C,4)
390     IF RD(C,4)=0 THEN RD(C,4)=RD(C-1,4):LOCATE 5+N,60:PRINT RD(C,4)
400     LOCATE 5+N,74:INPUT "",RD(C,5)
410     IF RD(C,5)=0 THEN RD(C,5)=RD(C-1,5):LOCATE 5+N,73:PRINT RD(C,5)
420 NEXT
430 C=C+1
440 LOCATE 24,3:INPUT "",SMP$(C)
450 IF SMP$(C)="" THEN GOTO 570
460 LOCATE 23,22:INPUT "",RD(C,1)
470 IF RD(C,1)=0 THEN RD(C,1)=RD(C-1,1):LOCATE 23,21:PRINT RD(C,1)
480 LOCATE 23,35:INPUT "",RD(C,2)
490 IF RD(C,2)=0 THEN RD(C,2)=RD(C-1,2):LOCATE 23,34:PRINT RD(C,2)
500 LOCATE 23,48:INPUT "",RD(C,3)
510 IF RD(C,3)=0 THEN RD(C,3)=RD(C-1,3):LOCATE 23,47:PRINT RD(C,3)
520 LOCATE 23,61:INPUT "",RD(C,4)
530 IF RD(C,4)=0 THEN RD(C,4)=RD(C-1,4):LOCATE 23,60:PRINT RD(C,4)
540 LOCATE 23,74:INPUT "",RD(C,5)
550 IF RD(C,5)=0 THEN RD(C,5)=RD(C-1,5):LOCATE 23,73:PRINT RD(C,5)
560 GOTO 430
570 CLS:LOCATE 10,10:INPUT "ARE YOU DONE ENTERING READINGS
      (Y=1,N=2) ",DT
580 IF DT=2 THEN C=C-1:GOTO 120
590 C=C-1
600 '*****
610 CLS
620 LPRINT STRING$(70,42):LPRINT STRING$(BLKS-5,42)+" "+EFLN$+" "+
      STRING$(BLKS-5,42):LPRINT STRING$(70,42)
630 LPRINT "SAMP NAME",,"ABS/MIN","SIGNIFICANT?"
640 PRINT "SAMP NM",,"SLOPE","SIGNIFICANT?"
650 FOR N=1 TO C
660     FOR N1=1 TO 5
670         SX=SX+N1                                'SUM OF X
680         SY=SY+RD(N,N1)                          'SUM OF Y
690         XY=N1*RD(N,N1):SXY=SXY+XY               'SUM OF XY
700         XS=N1^2:SXS=SXS+XS                      'SUM OF X^2
710         YS=RD(N,N1)^2:SYS=SYS+YS                'SUM OF Y^2
720     NEXT N1
730     SSX=SSX+N1^2                                '(SUM OF X)^2

```

```

740      SSY=SY^2                                '(SUM OF Y)^2
750      NUMER=SXY-((SX*SY)/5)
760      DN1=SXS-(SSX/5)
770      DN2=SYS-(SSY/5)
780      R=NUMER/(DN1*DN2)^.5                    'CORR. COEFF.
790      IF R>.99999 THEN T=700:GOTO 810
800      T=R/((1-(R*R))/3)^.5                    'T-VALUE
810      RSLO=SXY/SXS                            'REGR. SLOPE THRU 0,0
820      RSLO=INT((RSLO*1000)+.5)/1000
830      M(N)=RSLO
840      IF T<3.18 THEN BEEP:LPRINT SMP$(N),M(N),"N.S."
850      IF T<3.18 THEN BEEP:PRINT SMP$(N),M(N),"N.S.":GOTO 880
860      LPRINT SMP$(N),M(N),"* p<.05 *"
870      PRINT SMP$(N),M(N),"* p<.05 *"
880      SY=0:SX=0:SXY=0:SXS=0:SYS=0:SSX=0:SSY=0
890 NEXT
900 PRINT :INPUT "PRESS RETURN WHEN YOU ARE READY TO CONTINUE ",R
910 LPRINT :CLS
920 '*****
930 LOCATE 2,1:INPUT "WHAT IS THE VALUE OF THE EXTINCTION
      COEFFICIENT =",EXCF
940 LOCATE 7,20:PRINT "1. ABS * ml/mmole      "
950 LOCATE 8,20:PRINT "2. ABS * ml/umole      "
960 LOCATE 9,20:PRINT "3  ABS * ml/nmole      "
970 LOCATE 10,20:PRINT "4. OTHER              "
980 LOCATE 15,10:INPUT "HOW HAVE YOU EXPRESSED THE
      EXTINCTION COEFF. ";EPC
990 IF EPC<>4 THEN GOTO 1020
1000 CLS:INPUT "WHAT UNITS WILL THE FINAL RESULT BE IN =",FUNITS$
1010 '*****
1020 CLS:PRINT "WHICH EQUATION ARE YOU USING "
1030 LOCATE 10,10:PRINT " 1.  R= "+CHR$(127)+"ABS * Vt/"+CHR$(238)+
      " * Vs * Co"
1040 LOCATE 11,10:PRINT "2.  R= "+CHR$(127)+"ABS * Vt/"+CHR$(238)+"* Vs "
1050 LOCATE 15,10:INPUT " CHOICE = ",CHP
1060 IF CHP>2 THEN BEEP:LOCATE 22,10:PRINT "YOUR CHOICE HAS TO
      BE 1 OR 2 !":GOTO 1050
1070 CLS
1080 INPUT "WHAT IS THE TOTAL REACTION VOLUME Vt = ",VT
1090 CLS
1100 '*****
1110 COLOR 0,7
1120 FOR N=1 TO 3

```

```

1130     LOCATE N,1:PRINT STRING$(80,32)
1140 NEXT
1150 COLOR 7,0
1160 LOCATE 2,BLKS:PRINT STRING$(LNFN,32)
1170 LOCATE 2,BLKS+2:PRINT EFLN$
1180 LOCATE 5,2:PRINT "SAMPLE NAME"
1190 LOCATE 5,20:PRINT "SAMPLE VOLUME"
1200 IF CHP=1 THEN LOCATE 5,45:PRINT "PROTEIN [ ]"
1210 FOR N=1 TO 17
1220     CC=CC+1
1230     IF CC>C GOTO 1370
1240     LOCATE 6+N,3:PRINT SMP$(CC)
1250     LOCATE 6+N,21:INPUT "",VS(CC)
1260     IF VS(CC)=0 THEN VS(CC)=VS(CC-1):LOCATE 6+N,20:PRINT VS(CC-1)
1270     IF CHP=1 THEN LOCATE 6+N,46:INPUT "",CO(CC)
1280     IF CHP=1 AND CO(CC)=0 THEN CO(CC)=CO(CC-1):LOCATE 6+N,45
        PRINT CO(CC)
1290 NEXT
1300 CC=CC+1
1310 IF CC>C GOTO 1370
1320 LOCATE 24,3:PRINT SMP$(CC)
1330 LOCATE 23,21:INPUT "",VS(CC)
1340 IF VS(CC)=0 THEN VS(CC)=VS(CC-1):LOCATE 23,20:PRINT VS(CC-1)
1350 IF CHP=1 THEN LOCATE 23,46:INPUT "",CO(CC)
1360 IF CHP=1 AND CO(CC)=0 THEN CO(CC)=CO(CC-1):LOCATE 23,45:PRINT CO(CC)
1365 GOTO 1300
1370 CLS
1380 '*****
1390 FOR N=1 TO C
1400     IF CHP=1 THEN R(N)=(M(N)*VT)/(EXCF*VS(N)*CO(N))
1410     IF CHP=2 THEN R(N)=(M(N)*VT)/(EXCF*VS(N))
1420     R(N)=INT((R(N)*1000)+.5)/1000
1430 NEXT
1440 PRINT "SAMP. NM.", "SAMP. VOL.", "RATE"
1450 IF EPC=1 AND CHP=1 THEN LPRINT "SAMP. NM.", "
        SAMPLE VOL", "mmole/min/mg":GOTO 1530
1460 IF EPC=1 AND CHP=2 THEN LPRINT "SAMP. NM.", "SAMPLE VOL", "
        mmole/min/ml":GOTO 1540
1470 IF EPC=2 AND CHP=1 THEN LPRINT "SAMP. NM.", "SAMPLE VOL", "
        umole/min/mg":GOTO 1550
1480 IF EPC=2 AND CHP=2 THEN LPRINT "SAMP. NM.", "SAMPLE VOL", "
        umole/min/ml":GOTO 1560
1490 IF EPC=3 AND CHP=1 THEN LPRINT "SAMP. NM.", "SAMPLE VOL", "

```

```

nmole/min/mg":GOTO 1570
1500 IF EPC=3 AND CHP=2 THEN LPRINT "SAMP. NM.", "SAMPLE VOL", "
nmole/min/ml":GOTO 1580
1510 IF EPC=4 THEN LPRINT "SAMP. NM.", "SAMPLE VOL", FUNITS$:GOTO 1590
1520 OPEN "B:\ACHE\"+EFLN$ FOR OUTPUT AS #1:WRITE #1, 3, "SAMP. NM.", "
SAMPLE VOL", "RATE":GOTO 1600
1530 OPEN "B:\ACHE\"+EFLN$ FOR OUTPUT AS #1:WRITE #1, 3, "SAMP. NM.", "
SAMPLE VOL", "mmole/min/mg":GOTO 1600
1540 OPEN "B:\ACHE\"+EFLN$ FOR OUTPUT AS #1:WRITE #1, 3, "SAMP. NM.", "
SAMPLE VOL", "nmole/min/ml":GOTO 1600
1550 OPEN "B:\ACHE\"+EFLN$ FOR OUTPUT AS #1:WRITE #1, 3, "SAMP. NM.", "
SAMPLE VOL", "umole/min/mg":GOTO 1600
1560 OPEN "B:\ACHE\"+EFLN$ FOR OUTPUT AS #1:WRITE #1, 3, "SAMP. NM.", "
SAMPLE VOL", "umole/min/ml":GOTO 1600
1570 OPEN "B:\ACHE\"+EFLN$ FOR OUTPUT AS #1:WRITE #1, 3, "SAMP. NM.", "
SAMPLE VOL", "nmole/min/mg":GOTO 1600
1580 OPEN "B:\ACHE\"+EFLN$ FOR OUTPUT AS #1:WRITE #1, 3, "SAMP. NM.", "
SAMPLE VOL", "nmole/min/ml":GOTO 1600
1590 OPEN "B:\ACHE\"+EFLN$ FOR OUTPUT AS #1:WRITE #1, 3, "SAMP. NM.", "
SAMPLE VOL", FUNITS$:GOTO 1600
1600 FOR N=1 TO C
1610 PRINT SMP$(N), VS(N), R(N)
1620 LPRINT SMP$(N), VS(N), R(N)
1630 WRITE #1, SMP$(N), VS(N), M(N), R(N)
1640 NEXT
1650 CLOSE
1660 RUN"BRANCH
1670 END

```

PROGRAM A 11 CHOLINE ACETYLTRANSFERASE CALCULATION PROGRAM.

```

10 'THIS PROGRAM IS CALLED CHAT.BAS
20 'BY RJC JULY, 1989
30 '*****
40 '*****
50 CLS:BEEP
60 INPUT "HOW MANY SAMPLES DO YOU HAVE TO ENTER ";S
70 S=S+1
80 DIM SMP$(S), TM(S), MF(S), CPM(S), LNN(S), ACH(S), TDPMS(S)
90 LOCATE 10,10:INPUT "WHAT IS THE NAME OF YOUR FILE ";FLNMS$
100 A=LEN(FLNMS$)

```

```

110 CLS
120 IF CLRSC=2 THEN COLOR 0,4:GOTO 140
130 COLOR 0,7
140 FOR N=1 TO 3
150     LOCATE N,1:PRINT STRING$(80,32)
160 NEXT
170 COLOR 7,0
180 AA=(80-(A+4))/2:AA=CINT(AA)
190 LOCATE 2,AA:PRINT STRING$(A+4,32)
200 LOCATE 2,AA+2:PRINT FLNM$
210 '*****
220 '*****
230 LOCATE 10,10:INPUT "WHAT ARE THE DPMS OF YOUR QUENCH STANDARD ",DPMS
240 LOCATE 10,1:PRINT STRING$(80,32)
250 LOCATE 10,10:INPUT "HOW MANY TRIALS FOR THE QUENCH DID YOU DO ",QTL
260 LOCATE 10,1:PRINT STRING$(80,32)
270 LOCATE 5,7:PRINT "QUENCH STANDARD CPMS"
280 FOR N=1 TO QTL
290     LOCATE 6,N*15:PRINT "CPM("N")"
300     LOCATE 7,N*15:INPUT "",RD
310     TRD=RD+TRD
320 NEXT N
330 MRD=TRD/QTL
340 LOCATE 9,10:INPUT "WHAT IS THE BLANK READING ";BLK
350 MRD=MRD-BLK
360 QNC=INT(((MRD/DPMS)*1000)/.5)/1000
370 FOR N=1 TO 5
380     LOCATE N+4,1:PRINT STRING$(80,32)
390 NEXT
400 '*****
410 '*****
420 LOCATE 10,10:INPUT "WHAT IS THE SPECIFIC ACTIVITY OF AcCOA
      (Ci/mmmole) ";SPACT
430 SPDPM=SPACT*2.2*10^12
440 LOCATE 10,1:PRINT STRING$(80,32)
450 LOCATE 10,10:INPUT "HOW MANY TRIALS PER SAMPLE DID YOU DO
      (1,2,..4) ";TRL
460 LOCATE 10,1:PRINT STRING$(80,32)
470 '*****
480 '*****
490 LOCATE 5,1:PRINT "SAMPLE":LOCATE 5,10:PRINT "TIME(hr)":
      LOCATE 5,22:PRINT "M.F."
500 FOR N=1 TO TRL

```

```

510     LOCATE 5,20+(N*12):PRINT "CPM("N")"
520 NEXT
530 '*****
540 '*****
550 FOR N=1 TO 13
560     LOCATE 5+N,1:INPUT "",SMP$(N)
570     IF SMP$(N)="" OR SMP$(N)="END" GOTO 810
580     LOCATE 5+N,10:INPUT "",TM(N)
590     IF TM(N)=0 THEN TM(N)=TM(N-1):LOCATE N+5,9:PRINT TM(N)
600     LOCATE N+5,22:INPUT "",MF(N)
610     IF MF(N)=0 THEN MF(N)=MF(N-1):LOCATE N+5,21:PRINT MF(N)
620     FOR NN=1 TO TRL
630         LOCATE N+5,20+(NN*12):INPUT "",CPM
640         TCPM=TCPM+CPM
650     NEXT NN
660     CPM(N)=TCPM/TRL:TCPM=0
670 NEXT N
680 LOCATE 24,1:INPUT "",SMP$(N)
690 IF SMP$(N)="" OR SMP$(N)="END" GOTO 810
700 LOCATE 23,10:INPUT "",TM(N)
710 IF TM(N)=0 THEN TM(N)=TM(N-1):LOCATE 23,9:PRINT TM(N)
720 LOCATE 23,22:INPUT "",MF(N)
730 IF MF(N)=0 THEN MF(N)=MF(N-1):LOCATE 23,21:PRINT MF(N)
740 FOR NN=1 TO TRL
750     LOCATE 23,20+(NN*12):INPUT "",CPM
760     TCPM=TCPM+CPM
770 NEXT NN
780 CPM(N)=TCPM/TRL:TCPM=0
790 N=N+1
800 GOTO 680
810 '*****
820 '*****
830 CNT=N-1
840 CLS
850 LOCATE 10,10:INPUT "DO YOU WISH TO MAKE CORRECTIONS TO THIS FILE
      (1-Y,2-N) ";CRCT
860 IF CRCT=1 THEN CLS:GOSUB 1420
870 '*****
880 '*****
890 CLS
900 IF CLRSC=2 THEN COLOR 2,0
910 LOCATE 10,10:INPUT "WHAT IS YOUR BLANK VALUE READING FOR YOUR
      SAMPLES ",BLK

```

```

920 CLS
930 LOCATE 10,10:PRINT "PLEASE WAIT, PROCESSING DATA!"
940 FOR N=1 TO CNT
950     TDPMS(N)=((CPH(N)-BLK)/QNC)*(MF(N)/TM(N))
960     TDPMS(N)=INT(TDPMS(N)+.5)
970     ACH(N)=(TDPMS(N)/SPDPM)*10^6           'MMOLES/HR
980     ACH(N)=INT((ACH(N)*1000)+.5)/1000
990 NEXT N
1000 '*****
1010 '*****
1020 CLS
1030 LOCATE 10,1:PRINT SPACE$(33)+STRING$(15,219)
1040 LOCATE 11,1:PRINT SPACE$(33)+CHR$(221)+"PRINTING FILE"+CHR$(222)
1050 LOCATE 12,1:PRINT SPACE$(33)+STRING$(15,219)
1060 LPRINT STRING$(70,42)
1070 LPRINT STRING$(AA-5,42)+" "+FLNM$+" "+STRING$(AA-5,42)
1080 LPRINT STRING$(70,42)
1090 LPRINT :LPRINT
1100 LPRINT "THE QUENCH FOR THIS EXPERIMENT IS -----> "QNC
1110 LPRINT "THE SPECIFIC ACTIVITY FOR ACETYLCoA WAS -----> "
      SPACT" Ci/mmole"
1120 LPRINT "THE SAMPLE BLANK READING WAS -----> "BLK
1130 LPRINT STRING$(70,42)
1140 LPRINT
1150 LPRINT "SAMPLE", "TIME", "TOTAL DPM", "ACH"
1160 LPRINT , "(hr)", "(/hr)", "nmoles/hr"
1170 LPRINT
1180 FOR N=1 TO CNT
1190     LPRINT SMP$(N), TM(N), TDPMS(N), ACH(N)
1200 NEXT N
1210 LPRINT CHR$(12)
1220 '*****
1230 '*****
1240 CLS
1250 LOCATE 10,1:PRINT SPACE$(33)+STRING$(15,219)
1260 LOCATE 11,1:PRINT SPACE$(33)+CHR$(221)+"STORING FILE"+CHR$(222)
1270 LOCATE 12,1:PRINT SPACE$(33)+STRING$(15,219)
1280 OPEN "B:\CHAT\"+FLNM$ FOR OUTPUT AS #1
1290 WRITE #1, 4, "SAMPLE", "TIME (hr)", "TOTAL DPM/hr", "ACH nmoles/hr"
1300 FOR N=1 TO CNT
1310     WRITE #1, SMP$(N), TM(N), TDPMS(N), ACH(N)
1320 NEXT N
1330 CLOSE

```



```

1340 '*****
1350 '*****
1360 CLS
1370 IF CLRSC=2 THEN COLOR 6,0
1380 LOCATE 10,10:INPUT "WOULD YOU LIKE TO ENTER ANOTHER FILE
      (1-Y,2-N) ";ANF
1390 IF ANF=1 THEN CLEAR:GOTO 50
1400 RUN"BRANCH.BAS"
1410 END
1420 '*****
1430 '*****
1440 LOP=1
1450 PRINT "N", "SAMPLE", "TIME", "MF", "MEAN CPMS"
1460 FOR N=1 TO 18
1470     NN=N+((LOP-1)*18)
1480     IF NN>CNT GOTO 1510
1490     PRINT NN,SMP$(NN),TM(NN),MF(NN),CPM(NN)
1500 NEXT N
1510 LOCATE 20,5:INPUT "DO YOU WISH TO CHANGE ONE OF THESE LINES
      (1-Y,2-N) ";CHG
1520 IF CHG=2 THEN LOP=LOP+1:CLS:IF NN>CNT THEN RETURN 850
1530 IF CHG=2 GOTO 1450
1540 LOCATE 21,10:INPUT "HOW MANY LINES DO YOU WISH TO CHANGE ";LNCG
1550 FOR N=1 TO LNCG
1560     LOCATE 22,20:PRINT "LINE ("N") YOU WISH TO CHANGE = "
1570     LOCATE 22,54:INPUT "",LNN(N)
1580     LOCATE 22,1:PRINT STRING$(80,32)
1590 NEXT N
1600 IF CLRSC=2 THEN COLOR 3,0
1610 FOR N=1 TO LNCG
1620     CLS
1630     PRINT "LINE #="LNN(N),SMP$(LNN(N))
1640     INPUT "TYPE NEW NAME OR IF SAME PRESS RETURN ",NMP$
1650     IF NMP$<>" " THEN SMP$(LNN(N))=NMP$
1660     PRINT TM(LNN(N)):INPUT "TYPE NEW TIME OR IF SAME PRESS
      RETURN ",CTM
1670     IF CTM<>0 THEN TM(LNN(N))=CTM
1680     PRINT MF(LNN(N)):INPUT "TYPE NEW MULTIPLICATION FACTOR OR IF
      SAME PRESS RETURN ",CMF
1690     IF CMF<>0 THEN MF(LNN(N))=CMF
1700     PRINT CPM(LNN(N)):INPUT "IS THIS THE CORRECT MEAN
      (1-Y,2-N) ";CRM
1710     IF CRM=1 GOTO 1780

```

```

1720      FOR N1=1 TO TRL
1730          INPUT "CPM READING - ",NCPM
1740          TNCPM=TNCPM+NCPM
1750      NEXT N1
1760      CPM(LNN(N))-TNCPM/TRL
1770      TNCPM=0
1780 NEXT N
1790 CLS
1800 IF NN>CNT THEN RETURN 850
1810 LOP=LOP+1
1820 GOTO 1450
1830 '*****
1840 '*****

```

PROGRAM A.12. ERROR TRAPPING ROUTINE FOR SOME PROGRAMS

```

10 'THIS PROGRAM IS CALLED ERRTRP.BAS
20 ' BY RAFAEL CABEZA      JUNE-1987
30 '*****
40 'THIS PROGRAM IS AN ERROR TRAPPING ROUTINE FOR ALL PROGRAMS OF
50 ' THE LAB UTILITY DISK
60 '*****
65 IF EN=25 GOTO 120
70 IF EN=24 OR EN=27 THEN GOTO 120
80 IF EN=61 OR EN=67 THEN GOTO 270
90 IF EN=64 OR EN=52 THEN GOTO 490
100 IF EN=70 OR EN=71 THEN GOTO 650
110 GOTO 870
120 '*****
130 ' THIS ERROR TRAP IS FOR PRINTER PROBLEMS LIKE OFF LINE & PAPER OUT
140 '*****
150 CLS
160 FOR SND=1 TO 5
170     SOUND SND*37,SND*7
175     IF CLRSC=2 THEN COLOR 0,SND
180     LOCATE SND*3,40:PRINT "ERROR"
190 NEXT
200 CLS
210 COLOR 0,7
220 LOCATE 15,15:PRINT "THE PRINTER IS EITHER OFF LINE OR HAS NO PAPER!!"
230 LOCATE 16,15:PRINT "      WHEN YOU ARE READY PRESS RETURN!"

```

```

240 COLOR 7,0
250 INPUT "",ETRP
260 GOTO 870
270 '*****
280 ' THIS ERROR TRAP IS FOR FULL DISKETTE OR TOO MANY FILES
290 '*****
300 CLS
310 SCREEN 2
320 FOR DSKT=1 TO 8
330     TTT=DSKT*70
340     LINE (TTT,0)-(TTT,150)
350     LOCATE 23,20+(DSKT*6):PRINT "ERROR"
360     SOUND 170,10
370 NEXT
380 SCREEN 0
390 CLS
395 IF CLRSC=2 THEN COLOR 0,3:GOTO 410
400 COLOR 0,7
410 LOCATE 15,15:PRINT " THE DISKETTE YOU ARE WORKING WITH IS
      EITHER FULL "
420 LOCATE 16,15:PRINT "      OR YOU HAVE TOO MANY
      FILES      "
430 LOCATE 17,15:PRINT "      "
440 LOCATE 18,15:PRINT " PLACE A NEW DISKETTE WITH THE SAME
      SUBDIRECTORIES "
450 LOCATE 19,15:PRINT "      IN B: AND THEN PRESS RETURN      "
460 COLOR 7,0
470 INPUT " ",ETRP
480 GOTO 870
490 '*****
500 ' THIS ERROR TRAP IS FOR IMPROPER FILE NAMES
510 '*****
520 CLS
530 SCREEN 2
540 FOR FLNM=1 TO 8
550     CIRCLE (320,100),FLNM*21
560     LOCATE 23,12+(FLNM*6):PRINT "ERROR"
570     SOUND 70*FLNM,10
580 NEXT
590 SCREEN 0
600 CLS
605 IF CLRSC=2 THEN COLOR 0,4
610 LOCATE 15,15:PRINT " THE FILE NAME YOU CHOSE CANNOT BE USED "

```

```

620 LOCATE 16,15:PRINT "      THE NAME ";EFLN$;" IS NOT PROPER "
630 LOCATE 17,15:INPUT " PLEASE ENTER A PROPER NAME ",EFLN$
635 COLOR 7,0
640 GOTO 870
650 '*****
660 ' THIS ERROR TRAP IS FOR OPEN DISK DRIVES AND WRITE PROTECTS
670 '*****
680 CLS
690 FOR DRVDR=1 TO 8
700     LOCATE 15,10:PRINT "ERROR !! ERROR !! ERROR !!"
710     SOUND 140,5
720     BEEP
730     CLS
740 NEXT
745 IF CLRSC=2 THEN COLOR 0,2:GOTO 760
750 COLOR 0,7
760 LOCATE 15,15:PRINT "
770 LOCATE 16,15:PRINT "      THE B: DISK DRIVE IS OPEN OR YOU HAVE A
780 LOCATE 17,15:PRINT "      WRITE PROTECTOR ON YOUR DISKETTE
790 LOCATE 18,15:PRINT "      PLEASE
800 LOCATE 19,15:PRINT "      DON'T PLAY GAMES AND CHECK THE DRIVE
810 LOCATE 20,15:INPUT "      WHEN YOU ARE READY JUST PRESS
      RETURN      ",ETRP
820 COLOR 7,0
830 '*****
840 ' THIS CHAINS BACK TO THE PROGRAM WHERE THE ERROR OCCURED AND TO THE
850 ' LINE WHERE IT HAPPENED
860 '*****
870 CLS
880 IF PRGLK=1 THEN CHAIN "BRANCH.BAS",9000,ALL
890 IF PRGLK=2 THEN CHAIN "STDCUR.BAS",9000,ALL
900 IF PRGLK=3 THEN CHAIN "DATENTRY.BAS",9000,ALL
910 IF PRGLK=4 THEN CHAIN "ACHCALCU.BAS",9000,ALL
920 IF PRGLK=5 THEN CHAIN "LOWRY.BAS",9000,ALL
930 IF PRGLK=6 THEN CHAIN "RADIO-L1.BAS",9000,ALL
940 IF PRGLK=7 THEN CHAIN "RADIO-L3.BAS",9000,ALL
950 IF PRGLK=8 THEN CHAIN "ACHE.BAS",9000,ALL
960 END

```

PROGRAM A.13. FILE MANAGEMENT MENU PROGRAM.

```

10 'THIS PROGRAM IS CALLED FLMN.BAS
20 ' RJC
30 '*****
40 CLS:BEEP
50 COLOR 0,7
60 IF CLRSC=2 THEN COLOR 0,5
70 FOR N=1 TO 10
80     LOCATE N,1:PRINT STRING$(80,32)
90 NEXT
100 COLOR 7,0
110 FOR N=2 TO 9
120     LOCATE N,3:PRINT STRING$(75,32)
130 NEXT
140 LOCATE 3,31:PRINT "FILE MANIPULATIONS"
150 LOCATE 5,23:PRINT "0. RETURN TO MAIN MENU"
160 LOCATE 6,23:PRINT "1. CORRECT VALUES FOR mg PROTEIN"
170 LOCATE 7,23:PRINT "2. CALCULATE SPECIFIC ACTIVITY"
180 LOCATE 8,23:PRINT "3. SPECIAL FILE MENU "
190 LOCATE 15,25:PRINT "WHICH TASK DO YOU WISH TO DO ( ) ?"
200 LOCATE 15,55:CH$=INPUT$(1):CHC=VAL(CH$)
210 IF CHC>3 THEN BEEP:LOCATE 17,25:PRINT "YOUR CHOICE MUST BE ONE OF THE
      ABOVE !" GOTO 190
220 CLOSE
230 COMMON CLRSC
240 IF CHC=0 THEN CHAIN"BRANCH.BAS"
250 IF CHC=1 THEN CHAIN"FIL-LWR.BAS"
260 IF CHC=2 THEN CHAIN"FIL-SA.BAS"
270 IF CHC=3 THEN RUN "SPECTBL.BAS"
280 END

```

PROGRAM A.14. PROTEIN CORRECTION CALCULATION PROGRAM: FROM FILES.

```

10 'THIS PROGRAM IS CALLED FIL-LWR.BAS
20 ' BY RJC
30 '*****
40 '*****
50 BEEP:CLS
60 DIM SMP1$(80),SMP2$(80),VL1(80),VL2(80),VL3(80),RST(80),CLC(80)
70 CNT1=0:CNT2=0
80 COLOR 0,7
90 IF CLRSC=2 THEN COLOR 5,3

```

```

100 CLS
110 LOCATE 9,10:PRINT "1. ACH FILES TO CORRECT FOR mg PROTEIN"
120 LOCATE 10,10:PRINT "2. RADIO-ISOTOPE FILES TO CORRECT FOR mg PROTEIN"
130 LOCATE 11,10:PRINT "3. QUENCH CORRECTIONS TO CORRECT FOR mg PROTEIN"
132 LOCATE 12,10:PRINT "4. ACHE ACTIVITY UNITS TO CORRECT FOR mg PROTEIN"
134 LOCATE 13,10:PRINT "5. CHAT ACTIVITY UNITS TO CORRECT FOR mg PROTEIN"
140 LOCATE 16,10:INPUT "WHICH TYPE OF FILE DO YOU WISH TO CORRECT ";TP
150 IF TP=1 THEN CLS:FILES "B:\ACH\"
160 IF TP=2 THEN CLS:FILES "B:\RADIO\*."
170 IF TP=3 THEN CLS:FILES "B:\RADIO\*.Q"
172 IF TP=4 THEN CLS:FILES "B:\ACHE\"
174 IF TP=5 THEN CLS:FILES "B:\CHAT\"
180 LOCATE 18,10:INPUT "WHAT FILE DO YOU WISH TO WORK ON ";FL1$
190 CLS:FILES "B:\LOWRY\"
200 LOCATE 18,10:INPUT "WHAT IS THE NAME OF THE LOWRY FILE
      (IF SAME PRESS RETURN) ";FL2$
210 IF FL2$="" THEN FL2$=FL1$
220 CLS
230 '*****
231 '*****
240 IF TP=1 THEN OPEN "B:\ACH\"+FL1$ FOR INPUT AS #1
250 IF TP=2 THEN OPEN "B:\RADIO\"+FL1$ FOR INPUT AS #1
260 IF TP=3 THEN OPEN "B:\RADIO\"+FL1$+".Q" FOR INPUT AS #1
262 IF TP=4 THEN OPEN "B:\ACHE\"+FL1$ FOR INPUT AS #1
264 IF TP=5 THEN OPEN "B:\CHAT\"+FL1$ FOR INPUT AS #1
270 OPEN "B:\LOWRY\"+FL2$ FOR INPUT AS #2
280 INPUT #1, NM1:INPUT #2, NM2
290 FOR N=1 TO NM1
300     INPUT #1,NM$(N)
310     PRINT N,,NM$(N)
320 NEXT
330 LOCATE 15,10:INPUT "WHICH COLUMN HAS YOUR DATA (2,3,ETC) ";DT1
340 CLS
350 FOR N=1 TO NM2
360     INPUT #2,NM2$(N)
370     PRINT N,,NM2$(N)
380 NEXT
390 LOCATE 15,10:INPUT "WHICH COLUMN HAS YOUR mg PROTEIN (2,3,ETC) ";DT2
400 '*****
401 '*****
410 WHILE NOT EOF (1)
420     CNT1=CNT1+1
430     INPUT #1, SMP1$(CNT1)

```

```

440     FOR N=2 TO NM1
450         INPUT #1, VL
460         IF N=DT1 THEN VL1(CNT1)=VL
470     NEXT
480 WEND
490 WHILE NOT EOF (2)
500     CNT2=CNT2+1
510     INPUT #2, SMP2$(CNT2)
520     FOR N=2 TO NM2
530         INPUT #2, VL
540         IF N=DT2 THEN VL2(CNT2)=VL
550     NEXT
560 WEND
570 CLOSE
580 '*****
590 CLS
600 LOCATE 15,1:INPUT "IS THERE A ONE TO ONE CORRESPONDENCE IN YOUR
        DATA FILES (1-Y,2-N) ";CRSP
610 IF CRSP=1 GOTO 850
620 '*****
630 CLS
640 FOR N=1 TO CNT2
650     IF N>60 THEN LOCATE N-60,60:PRINT N"      "SMP2$(N):GOTO 690
660     IF N>40 THEN LOCATE N-40,40:PRINT N"      "SMP2$(N):GOTO 690
670     IF N>20 THEN LOCATE N-20,20:PRINT N"      "SMP2$(N):GOTO 690
680     LOCATE N,1:PRINT N"      "SMP2$(N)
690 NEXT N
700 LOCATE 22,1:INPUT "WHICH SAMPLES CONTAIN YOUR PROTEIN DATA
        (STARTING N, ENDING N) ";STR,STP
710 CLS
720 FOR N=1 TO CNT1
730     IF N>60 THEN LOCATE N-60,60:PRINT N"      "SMP1$(N):GOTO 770
740     IF N>40 THEN LOCATE N-40,40:PRINT N"      "SMP1$(N):GOTO 770
750     IF N>20 THEN LOCATE N-20,20:PRINT N"      "SMP1$(N):GOTO 770
760     LOCATE N,1:PRINT N"      "SMP1$(N)
770 NEXT
780 LOCATE 22,1:INPUT "WHICH SAMPLES CONTAIN YOUR DATA FOR CORRECTION
        (STARTING N, ENDING N) ";SR,SP
790 CLS
800 '*****
810 LOCATE 10,1:PRINT "THE LEFT MOST CHARACTER WILL BE USED TO
        IDENTIFY APPROPRIATE PAIRS !!"
820 LOCATE 12,1:INPUT "IS THIS O.K. (1-Y,2-N) ";OK

```

```

830 IF OK=2 THEN CLS:LOCATE 10,1:INPUT "THE PROGRAM WILL NOT RUN PROPERLY.
      (PRESS RETURN) ",UN:RUN"BRANCH
840 CLS
850 '*****
860 COLOR 7,0
870 CLS
880 COLOR 0,7
890 IF CLRSC=2 THEN COLOR 0,3
900 FOR N=1 TO 5
910     LOCATE 9+N,20:PRINT STRING$(40,32)
920 NEXT
930 COLOR 7,0
940 IF CLRSC=2 THEN COLOR 5,0
950 LOCATE 12,34:PRINT STRING$(12,32)
960 LOCATE 12,36:PRINT FL1$
970 LOCATE 8,27:PRINT "WORKING ON THE CORRECTIONS"
980 IF CRSP=1 THEN GOSUB 1350
990 IF CRSP=2 THEN GOSUB 1430
1000 '*****
1010 CLS
1020 PRINT "SAMPLE",NM$(DT1),NM2$(DT2),NM$(DT1)+"/mg PROTEIN"
1030 FOR N=SR TO SP
1040     PRINT SMP1$(N),VL1(N),VL3(N),RST(N)
1050 NEXT
1060 '*****
1070 CLS
1080 IF TP=1 THEN LOCATE 10,10:PRINT
      "*****WRITING FILE "+FL1$+".ACH*****"
1090 IF TP=2 THEN LOCATE 10,10:PRINT
      "*****WRITING FILE "+FL1$+".RAD*****"
1100 IF TP=3 THEN LOCATE 10,10:PRINT "*****WRITING FILE "+FL1$+".Q*****"
1102 IF TP=4 THEN LOCATE 10,10:PRINT
      "*****WRITING FILE "+FL1$+".CHE*****"
1104 IF TP=5 THEN LOCATE 10,10:PRINT
      "*****WRITING FILE "+FL1$+".CAT*****"
1110 IF TP=1 THEN OPEN "B:\FILE\PROT\"+FL1$+".ACH" FOR OUTPUT AS #1
1120 IF TP=2 THEN OPEN "B:\FILE\PROT\"+FL1$+".RAD" FOR OUTPUT AS #1
1130 IF TP=3 THEN OPEN "B:\FILE\PROT\"+FL1$+".Q" FOR OUTPUT AS #1
1132 IF TP=4 THEN OPEN "B:\FILE\PROT\"+FL1$+".CHE" FOR OUTPUT AS #1
1134 IF TP=5 THEN OPEN "B:\FILE\PROT\"+FL1$+".CAT" FOR OUTPUT AS #1
1140 WRITE #1, 4,"SAMPLE",NM$(DT1),NM2$(DT2),NM$(DT1)+"/mg PROTEIN"
1150 FOR N=SR TO SP
1160     WRITE #1, SMP1$(N),VL1(N),VL3(N),RST(N)

```



```

1170 NEXT
1180 CLOSE
1190 '*****
1200 CLS
1210 IF TP=1 THEN LOCATE 10,10:PRINT
      "*****PRINTING FILE  "+FL1$+".ACH*****"
1220 IF TP=2 THEN LOCATE 10,10:PRINT
      "*****PRINTING FILE  "+FL1$+".RAD*****"
1230 IF TP=3 THEN LOCATE 10,10:PRINT
      "*****PRINTING FILE  "+FL1$+".Q*****"
1232 IF TP=4 THEN LOCATE 10,10:PRINT
      "*****PRINTING FILE  "+FL1$+".CHE*****"
1234 IF TP=5 THEN LOCATE 10,10:PRINT
      "*****PRINTING FILE  "+FL1$+".CAT*****"
1240 LPRINT STRING$(60,42):LPRINT STRING$(20,42)+FL1$+STRING$(20,42):
      LPRINT STRING$(60,42):LPRINT
1250 LPRINT "SAMPLE",NM$(DT1),NM2$(DT2),NM$(DT1)+"/mg PROTEIN"
1260 FOR N=SR TO SP
1270     LPRINT SMP1$(N),VL1(N),VL3(N),RST(N)
1280 NEXT
1290 LPRINT CHR$(12)
1300 '*****
1310 CLS:LOCATE 10,10:INPUT "WOULD YOU LIKE TO DO ANOTHER SET
      (1=Y,2=N) ";ANT
1320 IF ANT=1 THEN ERASE SMP1$,SMP2$,VL1,VL2,VL3,RST,CLC :GOTO 50
1330 COMMON CLRSC:CHAIN "FLMN.BAS"
1340 END
1350 '*****
1360 FOR N=1 TO CNT1
1370     FOR NN=1 TO CNT2
1380         IF SMP1$(N)=SMP2$(NN) THEN
              RST(N)=VL1(N)/(VL2(NN)/1000):VL3(NN)=VL2(NN)
1390     NEXT
1400     RST(N)=INT((RST(N)+.5)*100)/100
1410 NEXT
1415 SR=1:SP=CNT1
1420 RETURN 1000
1430 '*****
1440 FOR N=STR TO STP
1450     SP$=LEFT$(SMP2$(N),2)
1460     FOR NN=SR TO SP
1470         IF CLC(NN)=1 GOTO 1500
1480         SM$=LEFT$(SMP1$(NN),2)

```

```

1490      IF SP$=SM$ THEN RST(NN)=VL1(NN)/(VL2(N)/1000):
          VL3(NN)=VL2(N):CLC(NN)=1:
          RST(NN)=INT((RST(NN)+.5)*100)/100
1500      NEXT
1510 NEXT
1520 RETURN 1000
1530 '*****

```

PROGRAM A.15. SPECIFIC ACITIVITY CALCULATION PROGRAM FROM FILES

```

10 'THIS PROGRAM IS CALLED FIL-SA.BAS
20 ' BY RJC
30 '*****
31 DIM SMP$(2,70),VL1(70),VL2(70),SPACT(70)
35 CNT=0:CNA=0
50 COLOR 0,7
60 IF CLRSC=2 THEN COLOR 0,2
70 CLS:BEEP
80 FILES "B:\FILE\PROT\*.RAD"
90 INPUT "WHAT IS THE NAME OF THE RADIO-LABEL DATA FILE
    (NAME WITHOUT EXTENSION) ";FIL1$
100 IF CLRSC=2 THEN COLOR 0,3
110 CLS
120 FILES "B:\FILE\PROT\*.ACH"
130 INPUT "WHAT IS THE NAME OF THE ACH DATA FILE
    (IF SAME PRESS RETURN) ";FIL2$
140 IF FIL2$="" THEN FIL2$=FIL1$
150 COLOR 7,0
160 IF CLRSC=2 THEN COLOR 3,0
170 CLS
180 '*****
190 OPEN "B:\FILE\PROT\"+FIL1$+".RAD" FOR INPUT AS #1
200 OPEN "B:\FILE\PROT\"+FIL2$+".ACH" FOR INPUT AS #2
210 INPUT #1, T1:INPUT #2, T2
220 FOR N=1 TO T1
230     INPUT #1, TIT$
240 NEXT
250 FOR N=1 TO T2
260     INPUT #2, TIT$
270 NEXT
280 WHILE NOT EOF(1)

```

```

290      CNT=CNT+1
300      INPUT #1, SMP$(1,CNT),P,V,VL1(CNT)
310 WEND
320 WHILE NOT EOF(2)
330      CNA=CNA+1
340      INPUT #2, SMP$(2,CNA),P,V,VL2(CNA)
350 WEND
360 IF CNT<>CNA THEN BEEP:INPUT "FILE SIZES DO NOT MATCH SO
      COMPARISON NOT POSSIBLE!                                DO YOU WISH TO
      TRY AGAIN (1-Y 2-N) ";FMM
370 IF FMM=1 GOTO 35
380 IF FMM=2 THEN RUN"BRANCH
390 CLOSE
400 '*****
401 OPEN "B:\FILE\SP-ACT\"+FIL1$ FOR OUTPUT AS #1
402 WRITE #1, 4,"SAMPLE","DPMS","ACH","SP. ACT."
405 PRINT "SAMPLE","DPMS","ACH","SP. ACT."
406 LPRINT STRING$(50,42)
407 LPRINT STRING$(20,42)+FIL1$+STRING$(20,42)
408 LPRINT STRING$(50,42):LPRINT
409 LPRINT "SAMPLE","DPMS","ACH","SP. ACT."
410 FOR N=1 TO CNT
420     FOR NN=1 TO CNA
430         IF SMP$(1,N)=SMP$(2,NN) THEN GOSUB 1000
440     NEXT NN
450 NEXT N
460 LPRINT CHR$(12)
470 CLOSE
500 '*****
510 CLS
520 LOCATE 10,10:INPUT "DO YOU WISH TO DO ANOTHER SET (1-Y,2-N) ";G
530 IF G=1 GOTO 35
540 IF G=2 THEN COMMON CLRSC:CHAIN"FLMN
550 BEEP:GOTO 510
900 END
1000 SPACT(N)=VL1(N)/VL2(NN)
1010 SPACT(N)=INT(SPACT(N)*10+.5)/10
1011 PRINT SMP$(1,N),VL1(N),VL2(NN),SPACT(N)
1012 WRITE #1, SMP$(1,N),VL1(N),VL2(NN),SPACT(N)
1013 LPRINT SMP$(1,N),VL1(N),VL2(NN),SPACT(N)
1020 RETURN 440

```

PROGRAM A.16. FILE MANIPULATION SUB MENU PROGRAM.

```
10 'THIS PROGRAM IS CALLED SPECTBL.BAS
20 ' BY RJC
30 ' *****
40 CLS
45 LOCATE 10,10:INPUT "PLACE SPECIAL FILE DISKETTE IN B ",TTTT
47 CLS
50 LOCATE 1,1:PRINT STRING$(80,219)
60 FOR N=2 TO 11
70     LOCATE N,1:PRINT CHR$(219);SPACE$(78),CHR$(219)
80 NEXT
90 LOCATE 12,1:PRINT STRING$(80,219)
100 ' *****
110 LOCATE 4,15:PRINT "0. RETURN TO MAIN MENU "
120 LOCATE 6,15:PRINT "2. SELECTION OF DATA FROM NORMAL DATA FILES "
130 LOCATE 7,15:PRINT "3. DATA ENTRY FOR READINGS "
140 LOCATE 8,15:PRINT "4. CALCULATION OF MEANS  $\pm$  S.E.M. FROM
    SPECIAL FILES "
150 LOCATE 9,15:PRINT "5. GET T-VALUES FROM TWO MEANS  $\pm$  SEMS"
160 LOCATE 5,15:PRINT "1. PREPARATION OF DATA FOR GRAPHER FILES"
170 LOCATE 10,15:PRINT "6. PREPARATION OF DATA FOR CSS FILES"
180 ' *****
190 LOCATE 14,30:PRINT "WHICH DO YOU WISH TO DO ( )"
200 LOCATE 25,30:PRINT "*** MEANS PROGRAM NOT YET AVAILABLE ",
210 LOCATE 14,55:CHS=INPUT$(1):CHC=VAL(CH$)
220 IF CHC>6 THEN BEEP:LOCATE 17,20:INPUT "          YOU MADE A MISTAKE
    (PRESS RETURN TO CONTINUE)",CNT:GOTO 190
230 CLS
240 ' *****
250 IF CHC=0 THEN RUN "BRANCH.BAS"
260 IF CHC=2 THEN RUN "DATAS-FL.BAS"
270 IF CHC=3 THEN RUN "ENTRY-FL.BAS"
280 IF CHC=4 THEN RUN "MEAN-FL.BAS"
290 IF CHC=5 THEN RUN "TT.BAS"
300 IF CHC=1 THEN RUN "GRAPHTBL.BAS"
310 IF CHC=6 THEN RUN "STAT-FL.BAS"
320 END
```

PROGRAM A.17. DATA SELECTION PROGRAM: FROM FILES

```

10 'THIS PROGRAM IS CALLED DATAS-FILES
20 ' BY RJC
30 '*****
35 LOCATE 10,10:INPUT "PLACE YOUR SERIAL DATA FILE DISKETTE IN A: ",U
40 CLEAR
50 DIM A(70),B(50),C(50),NM$(70)
60 BEEP:CLS
70 '*****
90 CLS
100 FILES "A:\"
110 LOCATE 17,10:INPUT "WHAT SUBDIRECTORY DO YOU WISH TO USE? ",DIRC1$
120 CLS
130 FILES "A:\"+DIRC1$+"\"
140 LOCATE 17,10:INPUT "IS THERE A SUBDIRECTORY HERE YOU WISH TO USE
      (1=Y,2=N)? ",YSD
150 IF YSD=1 THEN LOCATE 19,10:INPUT "WHAT SUBDIRECTORY IS TO BE
      USED? ",DIRC2$:CLS:FILES "A: "-DIRC1$+"\"+DIRC2$+"\":YSD=1
160 LOCATE 17,10:PRINT STRING$(70,32)
170 LOCATE 17,10:INPUT "WHAT FILE DO YOU WISH TO WORK ON
      (FULL NAME.EXT)? ",FLNM$
180 CLS
190 '*****
200 CLS
210 IF YSD=1 THEN OPEN "A:\"+DIRC1$+" "-DIRC2$+"\"+FLNM$ FOR
      INPUT AS #1:GOTO 230
220 OPEN "A:\"+DIRC1$+"\"+FLNM$ FOR INPUT AS #1
230 INPUT #1, NMB
240 FOR N1=1 TO NMB
250     INPUT #1, CLNM$(N1)
260     PRINT N1,CLNM$(N1)
270 NEXT
280 LOCATE 17,10:INPUT "WHAT IS THE COLUMN # YOU WISH TO USE
      (NOT 1)? ",CN
290 '*****
300 CLS
310 LOCATE 10,10:PRINT STRING$(60,177)
320 LOCATE 11,10:PRINT CHR$(177)+SPACES(58)+CHR$(177)
330 LOCATE 12,10:PRINT CHR$(177)+SPACES(21)+
      "RETRIVING FILE"+SPACES(22)+CHR$(177)
340 LOCATE 13,10:PRINT CHR$(177)+SPACES(58)+CHR$(177)
350 LOCATE 14,10:PRINT STRING$(60,177)
355 CNTR=0

```

```

360 WHILE NOT EOF(1)
370     CNTR=CNTR+1
380     INPUT #1, NM$(CNTR)
390     FOR N1=2 TO NMB
400         INPUT #1, Z
410         IF N1=CN THEN A(CNTR)=Z
420     NEXT
430 WEND
440 CLOSE
450 '*****
460 CLS
470 FOR N1=1 TO CNTR
480     IF N1>60 THEN LOCATE N1-60,60:PRINT N1"  "NM$(N1):GOTO 520
490     IF N1>40 THEN LOCATE N1-40,40:PRINT N1"  "NM$(N1):GOTO 520
500     IF N1>20 THEN LOCATE N1-20,20:PRINT N1"  "NM$(N1):GOTO 520
510     IF N1>0 THEN LOCATE N1,1:PRINT N1"  "NM$(N1)
520 NEXT
530 LOCATE 22,2:INPUT "DO YOU WISH TO SEPARATE THE FILE INTO
      SUBSETS (1=Y,2=N)? ",SBST
540 IF SBST=2 GOTO 690
550 '*****
560 LOCATE 22,1:PRINT STRING$(80,32)
570 LOCATE 22,2:INPUT "HOW MANY SUBSETS DO YOU WISH TO MAKE OF THE
      FILE (<9)? ",SBSTS
580 LOCATE 22,1:PRINT STRING$(80,32)
590 FOR N1=1 TO SBSTS
600     LOCATE 22,1:PRINT "WHAT ARE THE #'S CONTAINING THE
      SUBSET "N1" (ST,END)"
610     LOCATE 22,65:INPUT "",B(N1),C(N1)
620     LOCATE 22,1:PRINT STRING$(80,32)
630     Y$=STR$(B(N1)):X$=STR$(C(N1)):X$=RIGHT$(X$,LEN(X$)-1)
      Y$=RIGHT$(Y$,LEN(Y$)-1)
640     LOCATE 25,(N1*10)-9:PRINT "("Y$","X$)";
650 NEXT
660 LOCATE 22,1:PRINT STRING$(80,32)
670 LOCATE 22,5:INPUT "ARE THESE THE PAIRS YOU WANT LISTED
      BELOW (1=Y,2=N)? ",PR
680 IF PR=2 THEN GOSUB 800
690 '*****
700 CLS
710 LOCATE 15,10:INPUT "WHAT DO YOU WISH TO CALL THE NEW FILE
      (NO EXT)? ",NFLNM$
720 IF SBST=2 THEN GOSUB 950

```

```

730 IF SBST=1 THEN GOSUB 1090
740 '*****
750 CLS
760 LOCATE 10,10:INPUT "DO YOU WISH TO WORK ON ANOTHER FILE
      (1=Y,2=N)? ",ANFL
770 IF ANFL=1 THEN INPUT "SAME DIRECTORY (1=Y,2=N) ";DRSM:
      IF DRSM=1 GOTO 120
775 IF DRSM=2 GOTO 40
777 CLS:INPUT "PLACE PROGRAM DISKETTE IN A: ";TTTT
780 RUN "SPETBL.BAS"
790 END
800 '*****
810 LOCATE 22,1:PRINT STRING$(80,32)
820 LOCATE 22,5:INPUT "HOW MANY PAIRS DO YOU WISH TO CHANGE? ",CPR
830 LOCATE 22,1:PRINT STRING$(80,32)
840 FOR N1=1 TO CPR
850   LOCATE 22,5:PRINT "WHICH IS THE ("N1") PAIR YOU WISH TO CHANGE?"
860   LOCATE 22,65:INPUT "",PRTC
870   LOCATE 25,(PRTC*10)-9:PRINT "          ";
880   LOCATE 22,1:PRINT STRING$(80,32)
890   LOCATE 22,10:INPUT "WHAT IS THE CORRECT PAIR
      (ST,END)? ",B(PRTC),C(PRTC)
900   LOCATE 22,1:PRINT STRING$(80,32)
910   Y$=STR$(B(PRTC))+X$=STR$(C(PRTC)).X$=RIGHT$(X$,LEN(X$)-1):
      Y$=RIGHT$(Y$,LEN(Y$)-1)
920   LOCATE 25,(PRTC*10)-9:PRINT "("Y$","X$)";
930 NEXT
940 RETURN 660
950 '*****
960 CLS
970 LOCATE 17,10:INPUT "WHAT EXTENSION DO YOU WANT
      (<4 CHARACTERS)? ",NEXTN$
980 CLS
990 LOCATE 10,10:PRINT STRING$(60,178):LOCATE 11,10:PRINT
      CHR$(178)+SPACE$(58)+CHR$(178)
1000 LOCATE 12,10:PRINT CHR$(178)+SPACE$(20)+"ENTERING NEW FILE"+
      SPACE$(21)+CHR$(178)
1010 LOCATE 14,10:PRINT STRING$(60,178):LOCATE 13,10:PRINT CHR$(178)+
      SPACE$(58)+CHR$(178)
1020 OPEN "B:\SPECIAL\"+NFLNM$+"."+NEXTN$ FOR OUTPUT AS #1
1030 WRITE #1, CNTR,CLNM$(1),CLNM$(CN)
1040 FOR N1=1 TO CNTR
1050   K=LEN(NM$(N1)):NM$(N1)=RIGHT$(NM$(N1),K-2)

```



```

30 '*****
40 ON KEY (10) GOSUB 750
50 KEY 9, "END"+CHR$(13)
60 KEY 8, "0"+CHR$(13)
70 DIM SMP$(15,30),RLS(15,30)
80 CLS:BEEP
90 '*****
100 LOCATE 10,10:INPUT "HOW MANY TRIALS DO YOU WISH TO ENTER
      (8 OR <) ",TRL#
110 MP=1
120 KEY (10) ON
130 IF MP>TRL# GOTO 420
140 CLS:COLOR 0,7
150 LOCATE 25,35:PRINT "F9-'END'";
160 LOCATE 25,55:PRINT "F10-IN CASE OF ERROR";
170 FOR N=1 TO 3
180     LOCATE N,1:PRINT STRING$(80,32)
190 NEXT
200 COLOR 7,0
210 LOCATE 2,10:PRINT STRING$(3,32)
220 LOCATE 2,30:PRINT STRING$(6,32):LOCATE 2,60:PRINT STRING$(7,32)
230 LOCATE 2,11:PRINT "N":LOCATE 2,31:PRINT "NAME":LOCATE 2,61:
      PRINT "VALUE"
240 '*****
250 LOCATE 25,5:PRINT "TRIAL-"MP" OF "TRL#;
260 FOR N=5 TO 23
270     NN=NN+1
280     LOCATE N,10:PRINT NN
290     LOCATE N,31:INPUT "",SMP$(MP,NN)
300     IF SMP$(MP,NN)="-END" GOTO 390
310     LOCATE N,61:INPUT "",RLS(MP,NN)
320 NEXT
330 NN=NN+1
340 LOCATE 24,10:PRINT NN
350 LOCATE 23,31:INPUT "",SMP$(MP,NN)
360 IF SMP$(MP,NN)="-END" GOTO 390
370 LOCATE 23,61:INPUT "",RLS(MP,NN)
380 GOTO 330
390 CNT(MP)=NN-1:NN=0:MP=MP+1
400 CLS
410 GOTO 130
420 '*****
430 COLOR 0,7

```

```

440 FOR N=1 TO 3
450     LOCATE N,20:PRINT STRING$(40,32)
460 NEXT
470 COLOR 7,0
480 LOCATE 2,34:PRINT STRING$(12,32):LOCATE 2,35:PRINT "FILE NAMES"
490 LOCATE 5,10:INPUT "WHAT IS THIS GROUP'S FILE NAME";FLNM$
500 FOR N=1 TO TRL#
510     LOCATE N+7,5:PRINT "TRIAL ("N")"
520     LOCATE N+7,25:INPUT "WHAT IS THE EXTENSION (3 OR <)";EXT$(N)
530 NEXT
540 CLS
560 LOCATE 6,10:INPUT "WHAT DO YOU WISH TO NAME THE FIRST COLUMN? "
    ,CLNM1$
570 LOCATE 7,10:INPUT "WHAT DO YOU WISH TO NAME THE SECOND COLUMN? "
    ,CLNM2$
575 FOR N=1 TO TRL#
580     LOCATE N+5,1:PRINT STRING$(79,32)
590     LOCATE N+6,1:PRINT STRING$(79,32)
600     OPEN "B:\SPECIAL\"+FLNM$+"."+EXT$(N) FOR OUTPUT AS #1
610     LOCATE N+5,10:PRINT "WRITING FILE ("FLNM$"."EXT$(N)") "
620     WRITE #1, CNT(N),CLNM1$,CLNM2$
630     FOR NN=1 TO CNT(N)
640         WRITE #1, SMP$(N,NN),RLS(N,NN)
650     NEXT NN
660     CLOSE #1
670 NEXT N
680 '*****
690 CLS
700 LOCATE 10,10:INPUT "DO YOU WISH TO DO ANOTHER SET (1=Y,2=N)";STN
710 IF STN=1 THEN CLEAR:GOTO 30
720 RUN"SPETBL.BAS"
730 END
740 '*****
750 LOCATE 25,1:PRINT STRING$(78,32);
760 LOCATE 25,5:PRINT "ENTER N IN BOX          F8 TO QUIT CORRERCTIONS",
770 COLOR 0,15
780 FOR TST=1 TO 3
790     LOCATE 8+TST,1:PRINT STRING$(7,32)
800 NEXT
810 LOCATE 10,2:INPUT "N=",CRN$
820 COLOR 7,0
830 VL=VAL(CRN$):IF VL>0 GOTO 910
840 FOR TST=1 TO 3

```

```

850     LOCATE 8+TST,1:PRINT STRING$(7,32)
860 NEXT
870 LOCATE 25,1:PRINT STRING$(78,32);
880 LOCATE 25,5:PRINT "TRIAL="MP;:LOCATE 25,35:PRINT "F9-'END' ";
890 LOCATE 25,55:PRINT "F10-IN CASE OF ERROR";
900 RETURN
910 IF NN<=19 GOTO 940
920 IF NN>19 GOTO 980
930 '*****
940 TMPN=VL+4
950 LOCATE TMPN,31:PRINT STRING$(10,32):LOCATE TMPN,31:INPUT ""
      ,SMP$(MP,VL)
960 LOCATE TMPN,61:PRINT STRING$(10,32):LOCATE TMPN,61:INPUT ""
      ,RLS(MP,VL)
970 GOTO 770
980 '*****
990 CRCNT=NN-19:TMPN=(VL+4)-CRCNT
1000 LOCATE TMPN,31:PRINT STRING$(10,32):LOCATE TMPN,31:INPUT ""
      ,SMP$(MP,VL)
1010 LOCATE TMPN,61:PRINT STRING$(10,32):LOCATE TMPN,61:INPUT ""
      ,RLS(MP,VL)
1020 GOTO 770
1      0      3      0
'*****

```

PROGRAM A.19. CALCULATION OF MEANS FOR CALCULATED DATA PROGRAM:
FROM FILES.

```

10 'THIS PROGRAM IS CALLED MEAN-FL.BAS
20 ' BY RJC
30 '*****
40 CLEAR
50 BEEP:CLS
60 DIM NM$(10,15,30),VL(10,15,30),EXTN(10,15),NMB(10,15),
      M(30),SDEV(30),SEM(30),TNB(30)
70 '*****
80 FILES "B:\SPECIAL\*.*"
90 LOCATE 17,10:INPUT "HOW MANY SETS OF FILES DO YOU WISH TO USE ",FLSTS
100 IF FLSTS>10 THEN BEEP:GOTO 90
110 LOCATE 17,10:PRINT STRING$(70,32)
120 FOR N=1 TO FLSTS

```

```

130 LOCATE 17,1:PRINT "N="N:LOCATE 17,10:INPUT "WHAT IS THIS SET
      (FIRST PART OF NAME ONLY) ",FL$(N)
140 LOCATE 18,5:INPUT "HOW MANY FILES FROM THIS SET DO YOU WISH
      TO USE ",FLST(N)
150 LOCATE 18,5:PRINT STRING$(75,32)
160 FOR N2=1 TO FLST(N)
170     LOCATE 19,5:PRINT "WHICH IS THE ("N2") EXTENSION (NO )"
      LOCATE 19,50:INPUT "":EXTN$(N,N2)
180     LOCATE 19,5:PRINT STRING$(75,32)
190 NEXT N2
200 LOCATE 17,1:PRINT STRING$(80,32)
210 NEXT N
220 '*****
230 CLS
240 LOCATE 10,10:PRINT STRING$(60,8)
250 LOCATE 11,10:PRINT CHR$(8)+SPACE$(58)+CHR$(8)
260 LOCATE 12,10:PRINT CHR$(8)+SPACE$(23)+
      "PLEASE WAIT"+SPACE$(23)+CHR$(8)
270 LOCATE 13,10:PRINT CHR$(8)+SPACE$(58)+CHR$(8)
280 LOCATE 14,10:PRINT STRING$(60,8)
290 '*****
300 FOR N1=1 TO FLSTS
310     FOR N2=1 TO FLST(N1)
320         OPEN "B:\SPECIAL\"+FL$(N1)+". "+EXTN$(N1,N2) FOR INPUT AS #1
330         INPUT #1, NMB(N1,N2),N$,M$
340         IF NMB(N1,N2)>TP THEN TP=NMB(N1,N2)
350         WHILE NOT EOF(1)
360             N3=N3+1
370             INPUT #1, NM$(N1,N2,N3),VL(N1,N2,N3)
380         WEND
390         N3=0
400         CLOSE
410     NEXT N2
420 NEXT N1
430 '*****
440 FOR N1=1 TO FLSTS
450     FOR N2=1 TO FLST(N1)
460         IF NMB(N1,N2)=TP THEN S=N1:E=N2:GOTO 490
470     NEXT N2
480 NEXT N1
490 '*****
500 CLS
510 LOCATE 10,10:PRINT STRING$(60,215)

```

```

520 LOCATE 11,10:PRINT CHR$(215)+SPACE$(58)+CHR$(215)
530 LOCATE 12,10:PRINT CHR$(215)+SPACE$(18)+"WORKING ON MEANS & SEM"+
      SPACE$(18)+CHR$(215)
540 LOCATE 13,10:PRINT CHR$(215)+SPACE$(58)+CHR$(215)
550 LOCATE 14,10:PRINT STRING$(60,215)
560 '*****
570 FOR N1=1 TO NMB(S,E)
580   FOR N2=1 TO FLSTS
590     FOR N3=1 TO FLST(N2)
600       FOR N4=1 TO NMB(N2,N3)
610         IF NM$(S,E,N1)=NM$(N2,N3,N4) THEN GOSUB 1050
620       NEXT N4
630     NEXT N3
640   NEXT N2
650   GOSUB 1120
660 BEEP
670 NEXT N1
680 '*****
690 CLS
700 LOCATE 20,10:INPUT "WHAT DO YOU WISH TO CALL THE FILE --",MFLNM$
710 LOCATE 10,10:PRINT STRING$(60,232)
720 LOCATE 11,10:PRINT CHR$(232)+SPACE$(58)+CHR$(232)
730 LOCATE 12,10:PRINT CHR$(232)+SPACE$(16)+"STORING FILE OF MEANS & SEM"
      +SPACE$(15)+CHR$(232)
740 LOCATE 13,10:PRINT CHR$(232)+SPACE$(58)+CHR$(232)
750 LOCATE 14,10:PRINT STRING$(60,232)
760 OPEN "B \SPECIAL\MEAN\"+MFLNM$+" MST" FOR OUTPUT AS #2
770 WRITE #2, 5,N$, "MEAN", "N", "STD-DEV", "SEM"
780 FOR N1=1 TO NMB(S,E)
790   WRITE #2, NMS(S,E,N1),M(N1),TNB(N1),SDEV(N1),SEM(N1)
800 NEXT N1
810 CLOSE
820 '*****
830 CLS
840 LOCATE 10,10:PRINT STRING$(60,233)
850 LOCATE 11,10:PRINT CHR$(233)+SPACE$(58)+CHR$(233)
860 LOCATE 12,10:PRINT CHR$(233)+SPACE$(15)+"PRINTING FILE OF
      MEANS & SEM"+SPACE$(15)+CHR$(233)
870 LOCATE 13,10:PRINT CHR$(233)+SPACE$(58)+CHR$(233)
880 LOCATE 14,10:PRINT STRING$(60,233)
890 LPRINT STRING$(80,233)
900 TSP=LEN(MFLNM$):SP=INT((80-TSP)/2)
910 LPRINT SPACE$(SP)+MFLNM$

```

```

920 LPRINT STRING$(80,233)
930 LPRINT N$, "  MEAN", " N", "  STD-DEV", "  SEM"
940 LPRINT ,M$
950 FOR N1=1 TO NMB(S,E)
960   LPRINT  NM$(S,E,N1),M(N1),TNB(N1),SDEV(N1),SEM(N1)
970 NEXT N1
980 LPRINT
990 '*****
1000 CLS
1010 LOCATE 10,10:INPUT "DO YOU WISH TO DO ANOTHER SET (1=Y,2=N) ",QST
1020 IF QST=1 GOTO 30
1030 RUN"SPETBL.BAS"
1040 END
1050 '*****
1060 TN=TN+1
1070 SX=SX+VL(N2,N3,N4)
1080 SXS=SXS+(VL(N2,N3,N4)^2)
1090 N4=NMB(N2,N3)
1100 RETURN 620
1110 '*****
1120 SSX=SX^2
1130 TNB(N1)=TN
1140 M(N1)=SX/TN
1150 NUMER=SXS-(SSX/TN)
1160 DENOM=TN-1
1170 SDEV(N1)=(NUMER/DENOM)^.5
1180 SEM(N1)=SDEV(N1)/(TN^.5)
1190 SX=0:SXS=0:SSX=0:TN=0
1200 RETURN 660

```

PROGRAM A.20. T-TEST FROM TWO MEANS WITH STANDARD ERRORS PROGRAM
FOR MANUAL ENTRY

```

10 ' this is called ttest.bas
20 'RAFAEL CABEZA
30 CLS
40 PRINT "THIS TTEST IS FOR UNPAIRED DATA"
50 PRINT :PRINT :INPUT "WHAT IS THE FIRST MEAN, SEM, N ";X1,SEM1,N1
60 PRINT :INPUT "WHAT IS THE SECOND MEAN, SEM, N ";X2,SEM2,N2
70 CLS
80 SX1=(SEM1*(N1^2-N1)^.5)^2

```

```

90 SX2=(SEM2*(N2^2-N2)^.5)^2
100 S1=(SX1+SX2)/(N1+N2-2)
110 SS1=((S1/N1)+(S1/N2))^.5
120 T=(X1-X2)/SS1
130 DF=N1+N2-2
140 LOCATE 10,10:PRINT "THE T-VALUE IS" "T
150 LOCATE 11,10:PRINT "THE DEGREES OF FREEDOM ARE" "DF
160 LOCATE 15,10:INPUT "WOULD YOU LIKE TO DO ANOTHER SET (1=Y,2=N) ";RET
170 IF RET=1 THEN CLEAR:GOTO 30
180 IF RET=2 THEN RUN"SPETBL.BAS
190 END

```

PROGRAM A.21. FORMAT OF DATA FOR THE STATISTICAL PROGRAM "CSS":
FROM FILES.

```

10 'THIS PROGRAM IS CALLED STAT-FL.BAS
20 'BY RJC 6,89
30 '*****
40 CLS
50 INPUT "PLACE FILE DISKETTE IN A: ",STTT
60 CLS
70 '*****
80 CLEAR
90 CLS
100 DIM A$(12,15,10), VL1(12,15,10), FL$(12), FLNB(12), ET$(12,15),
    VL$(12,15), NB(12,15), VA$(10)
110 '*****
120 FILES "A:\ "
130 LOCATE 21,10:INPUT "WHAT IS THE SUBDIRECTORY YOU WISH TO USE ";SBD1$
140 CLS
150 FILES "A:\ "+SBD1$+"\ "
160 LOCATE 21,10:INPUT "HOW MANY SETS OF FILES DO YOU WISH TO COMBINE ";Z
170 LOCATE 21,1:PRINT STRING$(80,32)
180 FOR N=1 TO Z
190 LOCATE 21,10:PRINT "WHAT IS THE ("N") FILE SET "
200 LOCATE 21,40:INPUT "";FL$(N)
210 LOCATE 22,10:INPUT "HOW MANY FILES IN THIS SET ";FLNB(N)
220 LOCATE 22,1:PRINT STRING$(80,32)
230 FOR NN=1 TO FLNB(N)
240 LOCATE 22,10:PRINT "WHAT IS THE ("NN") EXTENSION "
250 LOCATE 22,40:INPUT "";ET$(N,NN)

```

```

260         LOCATE 22,1:PRINT STRING$(80,32)
270     NEXT
280     LOCATE 21,1:PRINT STRING$(80,32)
290 NEXT
300 '*****
310 CLS
320 LOCATE 8,20:PRINT STRING$(40,219)
330 LOCATE 9,20:PRINT CHR$(221):LOCATE 9,59:PRINT CHR$(222)
340 LOCATE 10,20:PRINT CHR$(221):LOCATE 10,59:PRINT CHR$(222)
350 LOCATE 11,20:PRINT CHR$(221):LOCATE 11,59:PRINT CHR$(222)
360 LOCATE 12,20:PRINT STRING$(40,219)
370 LOCATE 10,32:PRINT "RETRIEVING FILES"
380 '*****
390 FOR N=1 TO Z
400     FOR NN=1 TO FLNB(N)
410         OPEN "A:\"+SBD1$+"\"+FL$(N)+". "+ET$(N,NN) FOR INPUT AS #1
420         INPUT #1, NB(N,NN),B$,VL$(N,NN)
430         FOR NNN=1 TO NB(N,NN)
440             INPUT #1, A$(N,NN,NNN),VL1(N,NN,NNN)
450         NEXT NNN
460         CLOSE
470     NEXT NN
480 NEXT N
490 '*****
500 CLS
510 LOCATE 10,10:INPUT "WHAT DO YOU WISH TO CALL THIS FILE
      (NO EXT) ";STFL$
520 CLS
530 FOR N=1 TO Z
540     FOR NN=1 TO FLNB(N)
550         LOCATE 5,5:PRINT "WHAT CODE SHOULD THE
              FILE= "FL$(N)". "ET$(N,NN)" -GET"
560         LOCATE 6,6:INPUT "-----> ",CD(N,NN)
570         CLS
580     NEXT NN
590 NEXT N
600 '*****
610 FOR N=1 TO Z
620     FOR NN=1 TO FLNB(N)
630         IF NB(N,NN)<>NB(1,1) THEN BEEP:GOSUB 1000
640     NEXT NN
650 NEXT N
660 '*****

```



```

670 FOR N=1 TO NB(1,1)
680     PRINT A$(1,1,N):INPUT "WHAT SHOULD THIS VARIABLE BE
        CALLED--> ",VA$(N)
690 NEXT N
700 CLS
710 '*****
720 LOCATE 8,20:PRINT STRING$(40,219)
730 LOCATE 9,20:PRINT CHR$(221):LOCATE 9,59:PRINT CHR$(222)
740 LOCATE 10,20:PRINT CHR$(221):LOCATE 10,59:PRINT CHR$(222)
750 LOCATE 11,20:PRINT CHR$(221):LOCATE 11,59:PRINT CHR$(222)
760 LOCATE 12,20:PRINT STRING$(40,219)
770 LOCATE 10,32:PRINT "WRITING TO FILE"
780 OPEN "B:\SPECIAL\USER\"+STFL$+".STT" FOR OUTPUT AS #1
790 FOR N=1 TO Z
800     FOR NN=1 TO FLNB(N)
810         IF NB(1,1)=1 THEN WRITE #1,CD(N,NN),VL1(N,NN,1)
820         IF NB(1,1)=2 THEN WRITE #1,CD(N,NN),VL1(N,NN,1),VL1(N,NN,2)
830         IF NB(1,1)=3 THEN WRITE #1,CD(N,NN),VL1(N,NN,1),
            VL1(N,NN,2),VL1(N,NN,3)
840         IF NB(1,1)=4 THEN WRITE #1,CD(N,NN),VL1(N,NN,1),
            VL1(N,NN,2),VL1(N,NN,3),VL1(N,NN,4)
850         CNTR=CNTR+1
860     NEXT NN
870 NEXT N
880 IF NB(1,1)=1 THEN WRITE #1,CNTR,"TREATMENT",VA$(1)
890 IF NB(1,1)=2 THEN WRITE #1,CNTR,"TREATMENT",VA$(1),VA$(2)
900 IF NB(1,1)=3 THEN WRITE #1,CNTR,"TREATMENT",VA$(1),VA$(2),VA$(3)
910 IF NB(1,1)=4 THEN WRITE #1,CNTR,"TREATMENT",
    VA$(1),VA$(2),VA$(3),VA$(4)
920 CLOSE
930 '*****
940 CLS
950 INPUT "DO YOU WISH TO WORK ON ANOTHER SET (1=Y,2=N) ";RET
960 IF RET=1 GOTO 70
970 INPUT "PLACE PROGRAM FILE IN A: ";T
980 RUN "SPETBL.BAS"
990 END
1000 '*****
1010 CLS
1020 PRINT "THE FILES ARE NOT HOMOGENEOUS----->TROUBLE"
1030 PRINT :BEEP
1040 INPUT "DO YOU WISH TO TRY ANOTHER SET OF FILES (1=Y,2=N) ";TRB
1050 IF TRB=1 THEN RETURN 70

```

1060 RETURN 980

PROGRAM A.22. MENU PROGRAM FOR THE FORMAT OF DATA FOR THE
GRAPHING PROGRAM "GPAPER"

```
10 'THIS PROGRAM IS CALLED GRAPHTBL.BAS
20 ' BY RJC
30 '*****
40 CLEAR:CLS:BEEP
50 LOCATE 1,10:PRINT STRING$(60,219)
60 LOCATE 2,10:PRINT CHR$(219)+SPACE$(58)+CHR$(219)
70 LOCATE 3,10:PRINT CHR$(219)+SPACE$(10)+"0. RETURN TO SPECIAL MENU"+
    SPACE$(23)+CHR$(219)
80 LOCATE 4,10:PRINT CHR$(219)+SPACE$(10)+"1. PREPARE DATA FOR LINE
    GRAPH "+SPACE$(17)+CHR$(219)
90 LOCATE 5,10:PRINT CHR$(219)+SPACE$(10)+"2. PREPARE DATA FOR BAR
    GRAPH"+SPACE$(19)+CHR$(219)
100 LOCATE 6,10:PRINT CHR$(219)+SPACE$(10)+"3. PREPARE DATA FOR BAR GRAPH
    (REAL X-AXIS)+"SPACE$(5)+CHR$(219)
110 LOCATE 7,10:PRINT CHR$(219)+SPACE$(58)+CHR$(219)
120 LOCATE 8,10:PRINT STRING$(60,219)
130 '*****
140 LOCATE 15,10:PRINT "WHAT IS THE JOB YOU WISH TO DO ( )"
150 LOCATE 15,42:INPUT "",CHC
160 IF CHC>3 THEN BEEP:GOTO 140
170 '*****
180 IF CHC=0 THEN RUN"SPETBL.BAS
190 IF CHC=1 THEN RUN"GRAPH-FL.BAS
200 IF CHC=2 THEN RUN"BAR1-FL.BAS
210 IF CHC=3 THEN RUN"BAR2-FL.BAS
220 END
```

APPENDIX B

F Values and Degrees of Freedom for ANOVA for Experiments of Section 3.5

Dependent Variable	Treatment Effects	Time Effects	Treatment X Time Effects
Released ACh	96.7 * 1 **	0.4 2	2.0 2
Released ³ H-ACh S.A.	11.6 1	7.5 2	0.1 2
Tissue ³ H-ACh S.A.	212.4 1	49.1 2	13.0 2
P ₃ ACh	39.7 1	0.6 2	1.5 2
P ₃ ³ H-ACh	83.7 1	0.6 2	1.5 2
P ₃ ³ H-ACh S.A.	63.8 1	26.3 2	4.2 2
S ₃ ACh	9.0 1	2.7 2	0.7 2
S _c ³ H-ACh	80.6 1	44.2 2	0.8 2
S ₃ ³ H-ACh S.A.	58.6 1	35.4 2	0.6 2

F Values and Degrees of Freedom for ANOVA for Experiments of Section 3.5 (Continuation)

Dependent Variable	Treatment Effects	Time Effects	Treatment X Time Effects
D Fraction	77.1 *	3.7	10.9
ACh	1 **	2	2
D Fraction	308.7	21.0	20.3
³ H-ACh	1	2	2
D Fraction	120.4	0.4	0.1
³ H-ACh S.A.	1	2	2
E&F Fraction	56.9	21.1	21.5
ACh	1	2	2
E&F Fraction	419.1	65.0	2.4
³ H-ACh	1	2	2
E&F Fraction	114.6	27.2	10.8
³ H-ACh S.A.	1	2	2
G&H Fraction	17.4	10.8	17.5
ACh	1	2	2
G&H Fraction	94.5	35.6	8.0
³ H-ACh	1	2	2
G&H Fraction	12.1	10.0	6.2
³ H-ACh S.A.	1	2	2

* = F values

** = Degrees of Freedom