Modulation Of Presynaptic Cholinergic Mechanisms By Volatile Anaesthetic Agents

A Thesis submitted to The University of Leicester for the degree of Doctor of Medicine.

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Summary

The effects of volatile anaesthetic agents on three aspects of presynaptic acetylcholine (ACh) metabolism were investigated in vitro. Choline uptake and choline acetyltransferase (ChAT) activity were studied using rat cortical synaptosomes and ACh release was investigated employing rat cortical slices. The effect of volatile anaesthetic agents on neurotransmitter systems and the importance of ACh as a central neurotransmitter involved in the regulation of the conscious state is discussed. The evidence suggesting that central ACh function may be altered by anaesthesia is reviewed.

Total choline uptake was inhibited non-competitively by all three volatile anaesthetic agents studied and the IC$_{50}$ was 1.38 % for halothane, which is close to the ED$_{50}$ for unconsciousness (minimum alveolar concentration, MAC). If present in vivo this may contribute to the process of anaesthesia. The Michaelis constant (K$_{m}$) of ChAT was reduced significantly by enflurane but was unaffected by halothane and isoflurane. If present in vivo, an enflurane-induced reduction in K$_{m}$ of ChAT could lead to facilitation of ACh synthesis and contribute to the convulsant properties of enflurane. ACh release was measured using a modified chemiluminescent assay. ACh release was inhibited by halothane with an IC$_{50}$ of 0.38 %, approximately one third of MAC. This is the most potent anaesthetic effect on neurotransmitter release reported in vitro to date. The results of this study demonstrate the in vitro sensitivity of presynaptic cholinergic metabolism to clinically relevant doses of anaesthetic agents and suggests that the cholinergic system may be a target for their action.
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Parts of this thesis have already been presented to meetings and published as abstracts and as full papers as detailed below, these full papers are included after the bibliography.

Abstracts


Full Publications


Declaration

This thesis was written and composed by myself and all books and papers quoted in this thesis were consulted by me personally. The work has not been submitted for a degree of another university.

The research was conducted while I was a British Journal of Anaesthesia Research Fellow and a clinical lecturer (Honorary Senior Registrar) in The University Department of Anaesthesia at The Leicester Royal Infirmary between 1991 and 1994.
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List of Abbreviations

μl  microlitre
5-HT  5-hydroxytryptamine
a.c.  alternating current
Acetyl-CoA  Acetyl coenzyme A
ACH  Acetylcholine
AChE  Acetylcholinesterase
Atm  Atmosphere
ATP  Adenosine triphosphate
ATPase  Adenosine triphosphatase
Ca²⁺  Calcium
cDNA  Copy deoxyribonucleic acid
ChAT  Choline acetyltransferase
CNS  Central nervous system
CO₂  Carbon dioxide
CSF  Cerebrospinal fluid
DNA  Deoxyribonucleic acid
DOPA  Dihydroxyphenylalanine
DPM  Disintegration per minute
ECF  Extracellular fluid
ED₅₀  Median effective dose
EDTA  Ethylenediaminetetraacetic acid
EEG  Electroencephalogram
EPSP  Excitatory postsynaptic potentials
g  gram
GABA  Gamma-aminobutyrate
GAD  Glutamic acid decarboxylase
HC-3  Hemicholinium-3
IC50  Median inhibitory dose
IPSP  Inhibitory postsynaptic potentials
K+   Potassium
M    Molar
MAC  Minimum alveolar concentration
mg   miligram
min  minute
ml   mililitre
mM  Milimolar
MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA Messenger ribonucleic acid
Na+  Sodium
NMDA N-methyl-D-aspartate
NMS  N-methyl-scopolamine
O2   Oxygen
pg   picogram
PVP  Polyvinylpyrrolidine
REM  Rapid eye movement
SD   Standard deviation
SEM  Standard error of the mean
THA  Tetrahydroaminacrine
TRH  Thyrotrophin releasing hormone
VIP  Vasoactive intestinal polypeptide
1. The Effects of Anaesthetics on Neurotransmitter Systems

1.1 Introduction

Despite being in common use for almost 150 years, there is still no firm agreement on how general anaesthetic agents bring about anaesthesia. While the molecular basis of the action of most anaesthetic agents is unknown, it is a commonly held view that general anaesthetic drugs have a more pronounced effect on synaptic mechanisms in the central nervous system than on the propagation of electrical signals along axons (Larrabee & Posternak, 1952). A more contentious issue relates to whether anaesthetic agents act generally, producing a range of metabolic alterations which together result in anaesthesia or at, as yet, unidentified specific sites in neuronal mechanisms (Franks & Lieb, 1994). The possibility also exists, that a single agent may achieve different effects, such as unconsciousness and muscle relaxation by different mechanisms (Kissin, 1993). However, the intriguing aspect of anaesthetic mechanisms is how a wide spectrum of unrelated compounds produce unconsciousness and then recovery so predictably, rapidly and so safely.

Information is passed from cell to cell in the central nervous system (CNS) by neurotransmitters. These transmitter substances are released in the synaptic cleft between adjacent cells and, depending on the particular synapse, can interact with presynaptic and/or postsynaptic receptors. The post synaptic actions of transmitter substances are mediated classically via excitatory postsynaptic potentials (EPSP) or inhibitory postsynaptic potentials (IPSP) and the size of these can be modulated by the regulation
of transmitter release from the nerve terminal by the interaction of neurotransmitter substances at receptors in the presynaptic membrane.

The diversity of different transmitter substances and the complex interplay between transmitter systems results in a large number of potential sites of action of anaesthetic agents (figure 1.1). A recent review of mechanisms of general anaesthesia (Franks & Lieb, 1994), shows how most of the research effort has focused on ion channels and receptors. However, presynaptic events involving neurotransmitter uptake, synthesis and release have been poorly covered and by-passed for more fashionable topics. In this introductory chapter the current evidence concerning the effects of various classes of general anaesthetic agents on neurotransmitter systems will be considered together with the question of the specificity of anaesthetic action at the molecular level.
Figure 1.1
Diagrammatic representation of a presynaptic nerve terminal showing potential sites of anaesthetic drug interaction.

1 = precursor uptake, 2 = presynaptic receptors, 3 = released transmitter reuptake, 4 = transmitter release, 5 = transmitter synthesis, 6 = presynaptic calcium entry.
1.1.1 Experimental Systems

Although there have been a number of in vivo studies (Arai et al, 1990, Becquet et al, 1988, Ngai et al, 1978, O'Neill et al, 1984, Osbourne et al, 1990, Segal et al, 1990), by far the majority of studies on the action of anaesthetic agents on transmitter systems have been carried out on in vitro preparations. Three major experimental paradigms, namely synaptosomes, brain slices and cells in culture have been employed in in vitro studies. Each of these systems has limitations which must be considered when interpreting observations, and caution must be exercised in extrapolating conclusions from these studies to the clinical situation.

Synaptosomes are pinched off nerve endings that are formed when brain tissue is homogenised. The synaptosome fraction is separated from particulate material containing mitochondria and other cell debris by centrifugation in density gradients of either sucrose (Gray & Whittaker, 1962), Ficoll (Booth & Clark, 1978) or Percoll (Dunkley et al, 1986). A detailed description of the synaptosome preparation is given in section 3.2.

Brain slice preparations have also been used extensively to study neurotransmitter metabolism. This is a more complex preparation as some functional connections are maintained. Possibly the simplest in vitro preparations are neuronal and neuronal-like cells in culture, which permit the study of intact, isolated cell types. Again the absence of functional tissue organisation cannot be ignored and the relevance of observations made on non-neuronal cells, such as chromaffin cells, and cells which may be only partially differentiated must be considered.
1.2 Neurotransmitter Classification

Neurotransmitter systems have been subdivided according to their classification into (1) amino acids, (2) other classical transmitters and (3) neuropeptide systems (McMahon & Nicholls, 1991). In this chapter the effects of anaesthetics on the Group 1 transmitters, including aspartate, glutamate, gamma-aminobutyrate (GABA) and glycine and the Group 2 transmitters, including the catecholamines, dopamine, noradrenaline, and adrenaline, 5-hydroxytryptamine (5-HT) will be reviewed. Little work has been directed to the effects of anaesthetic agents on Group 3 transmitter systems. Acetylcholine (ACh) is considered separately in chapter 2, as it is the focus of the experimental work included in the thesis.

1.2.1 Group I

Amino Acid Transmitters

1.2.1.1 Excitatory Transmitters - Glutamate and Aspartate

Glutamate and aspartate are both believed to act as excitatory neurotransmitters in the mammalian brain (Fagg & Foster, 1983). In particular, neurotransmission mediated by glutamate has been implicated with learning and memory, neurodegenerative disorders, epilepsy and the effects of hypoxia, ischaemia and possibly other neurological and psychiatric states (Choi & Rothman, 1990, Croucher et al, 1982).

Biosynthesis of glutamate can occur via two pathways, either from the deamination of glutamine or from the tricarboxylic acid cycle (Horton,
1989) (figure 1.2). It would appear that most of the glutamate released following depolarising stimuli is derived originally from glutamine (Horton, 1989). Following release into the synapse, signal transmission by the excitatory amino acids is mediated at the postsynaptic membrane by receptors for these neurotransmitters. These are classified into three classes, N-methyl-D-aspartate (NMDA), quisqualate and kainate, depending on their pharmacology (Watkins & Evans, 1981). The NMDA receptor subclass is of particular importance to the anaesthetist as the anaesthetic ketamine is a non-competitive antagonist at this receptor (Yamamura et al, 1990). Post synaptic events are terminated largely by the uptake of glutamate and aspartate into neighbouring glial cells. In view of their wide distribution and suggested roles in the central nervous system, the excitatory amino acid transmitter systems constitute an important potential target for anaesthetic action.

1.2.1.2 Release of Excitatory Neurotransmitters

In early brain slice studies, barbiturates were shown to reduce the release of aspartate from electrically stimulated rat lateral olfactory tract slices in a dose-dependent manner (Collins, 1980). Similarly, a depression of potassium-stimulated aspartate release has also been observed in rat brain slices in the presence of pentobarbitone, methohexitone and thiopentone (Arai et al, 1990, Minchin, 1981, Waller & Richter, 1980). However, a biphasic pattern of release in the presence of anaesthetic agents was observed in a rat thalamic slice preparation in which low concentrations of thiopentone (10^{-6}M) and methohexitone (10^{-5}M)
Figure 1.2
Diagrammatic representation of a presynaptic nerve terminal showing the relationship between the metabolism of excitatory amino acid neurotransmitters and GABA. A = Aspartate glutamate co-transporter, B = aspartate aminotransferase, C = glutaminase, D = glutamic acid dehydrogenase, GAD = glutamic acid decarboxylase.
enhanced release and higher doses depressed release of D-aspartate and GABA (Kendall & Minchin, 1982). The higher concentration of thiopentone was within the clinical range but that of methohexitone was higher than clinical levels. The mechanism to explain the biphasic response to barbiturates in excitatory amino acid release remains to be resolved but may involve indirect effects on GABA transmission (Kendall & Minchin, 1982). Depolarisation-induced calcium uptake in the nerve terminal, which triggers transmitter release, has been shown to be inhibited by thiopentone and pentobarbitone (Blaustein & Ector, 1975). Hence, alteration in calcium handling by the nerve terminal may provide an explanation for the action of barbiturates on excitatory amino acid transmission.

The steroid anaesthetic, hydroxydione, is the only other agent to be shown to inhibit potassium-stimulated aspartate efflux (Minchin, 1981). Other anaesthetic agents including althesin (a combination of alphaxalone and alphadolone), ketamine, urethane and halothane have been reported to have no effect or to enhance the release of aspartate from rat brain slice preparation (Arai et al, 1990, Kendall & Minchin, 1982, Minchin, 1981). At clinical doses, halothane (1 – 2%) had no significant effect upon the efflux of $[^3]H$-aspartate from rat brain slices (Arai et al, 1990), although an increased aspartate efflux has been observed at higher concentrations of halothane (4 – 8%) which are considerably above the clinical dose range. By contrast, a study investigating glutamate release from mouse synaptosomes (Hirose et al, 1992) demonstrated an increase in release when the preparation was exposed to clinical concentrations of halothane and enflurane; the increase in release being greater for enflurane than for halothane at equivalent concentrations. Interestingly, the excitatory amino acids are implicated in the pathogenesis of some types of epilepsy and
(Croucher et al., 1982) it is possible that the enhanced release of glutamate in the presence of enflurane could contribute to the EEG abnormalities seen with this agent (Steen & Michenfelder, 1979).

In vivo measurements of excitatory amino acid release, by monitoring brain ascorbate levels voltammetrically, has shown that anaesthesia with chloral hydrate reduces the release of excitatory neurotransmitters and that transmitter release recovers as the animal recovers (O’Neill et al., 1984). These observations provide in vivo evidence for the interruption of excitatory neurotransmitter release by an anaesthetic agent during the process of anaesthesia.

From the limited evidence available, it is possible to conclude that some anaesthetics may act on synaptic transmission mediated by the excitatory amino acids (Collins, 1980, Hirose et al., 1992, Malgouris et al., 1989, Minchin, 1981, O’Neill et al., 1984, Richter & Waller, 1977). Perhaps as important, other agents would appear not to be implicated (Arai et al., 1990, Minchin, 1981). Together these results suggest a possible selectivity of action of some anaesthetic agents for the excitatory amino acid release mechanism. The absence of a "general" effect on these transmitter mechanisms is also observed for the volatile agents as while halothane, and particularly enflurane, increased transmitter release, diethylether had no effect in the same study (Hirose et al., 1992); further suggesting a selectivity of action of anaesthetic agents on the excitatory transmitter systems.

Recently, a new compound, riluzole (54274 RP), has been found to have a possible anaesthetic use and pharmacologically this compound appears to exert its effects by interrupting glutamate neurotransmission (Chéramy et al., 1986). Riluzole has been shown to reduce the presynaptic
release of glutamate (Chéramy et al, 1986) and also to affect some postsynaptic properties (Romo et al, 1980). In one study, riluzole induced loss of righting reflex in rats and in lower doses it also reduced the MAC for halothane (Malgouris et al, 1989). The inability of riluzole to bind at glutamate receptors (Watkins et al, 1990) suggests that the hypnotic state induced by this agent is different from that produced by ketamine, which is known to be an NMDA receptor antagonist (Yamamura et al, 1990). Interestingly, riluzole protects against glutamate induced neural injury following ischaemia (McMahon & Nicholls, 1991) and this may also provide a further possible use for this novel compound in neurosurgical intensive care. Further investigation of the anaesthetic mechanisms possibly mediated by altered excitatory amino acid transmission is certain to follow the initial observations on riluzole action.

1.2.1.3 Excitatory Amino Acid Uptake

When glutamate is released from nerve endings its actions are terminated by uptake into surrounding glial cells and neurons. In addition, glutamate uptake occurs in the nerve terminal where it is recycled to glutamine (Horton, 1989). This carrier also transports aspartate into the nerve terminal.

Uptake of aspartate was not affected by thiopentone, methohexitone, ketamine or urethane in a rat brain slice preparation (Minchin, 1981). Similar findings were found in rat thalamic slices using anaesthetic doses of thiopentone, methohexitone, urethane and ketamine (Kendall & Minchin, 1982). Pentobarbitone, in the same study, did reduce slightly the uptake of aspartate but the authors concluded that this effect
was unlikely to contribute to the mechanism of action of the barbiturates.

At present there is little evidence to link the excitatory amino acid neurotransmitters to anaesthetic mechanisms, despite their possible relevance to consciousness. More work in this area is clearly required to clarify the significance of anaesthetic effects reported to date (Arai et al, 1990, Hirose et al, 1992, Malgouris et al, 1989).

1.2.1.4 Inhibitory Transmitters

1.2.1.5 GABA

GABA is formed from the irreversible decarboxylation of glutamate catalysed by the enzyme glutamic acid decarboxylase (GAD) (figure 1.2). Following release into the synapse, GABA transmission is terminated by uptake of the transmitter by surrounding glial cells and nerve terminals and this process, similar to that for glutamate, limits the activity of GABA in the synapse.

GABA is the major inhibitory neurotransmitter in the central nervous system (Horton, 1989). By acting on both pre- and postsynaptic receptors it causes an increase in chloride ion conductance which results in hyperpolarisation of the nerve membrane. The barbiturate and steroid anaesthetic agents increase this GABA-stimulated chloride conductance resulting in an enhanced hyperpolarisation whereas the volatile anaesthetics have a weaker effect on the GABA receptor/Cl⁻ channel complex (Moody et al, 1988).
1.2.1.6 Catabolism of GABA

In a study using rat brain slices, it was found that halothane (3 %) had no effect on either the uptake or the release of GABA (Cheng & Brunner, 1981a). However, the catabolism of GABA in the preparation was reduced. This could lead indirectly to increased GABA levels in the synapse. Similar effects of halothane on GABA catabolism have also been observed in synaptosomes prepared from rat brain (Cheng & Brunner, 1981b). In addition, clinical concentrations (1 MAC) of chloroform, enflurane and diethylether all affect GABA catabolism significantly (Cheng & Brunner, 1981b). Of the barbiturates studied, only thiopentone inhibited GABA catabolism at clinical concentrations, pentobarbitone and phenobarbitone only having effects at very high doses (Cheng & Brunner, 1981b). The differing actions that have been described, namely a reduction in GABA catabolism by the volatile anaesthetic agents and an interaction with the GABA receptor/Cl⁻ channel by the barbiturates illustrates the possibility that different classes of anaesthetic agents may achieve the same physiological effect via differing pharmacological mechanisms.

1.2.1.7 GABA Uptake

No effect was found on the uptake of GABA into rat brain slices in the presence of thiopentone, methohexitone, althesin, ketamine halothane or urethane (Minchin, 1981). Similarly, pentobarbitone had no effect in rabbit hippocampal slices (Jessell & Richards, 1977) nor thiopentone, methohexitone, urethane and ketamine in rat thalamic slices (Kendall &
It would appear that the uptake process for GABA does not play a significant role in the anaesthetic action of these compounds.

### 1.2.1.8 GABA Release

Potassium-stimulated GABA release from rat brain slices has been shown to be reduced by the intravenous barbiturates, hydroxydione and pentobarbitone (Minchin, 1981). However, in the rat olfactory cortex preparation there appeared to be an increase in the release of GABA in the presence of pentobarbitone (Collins, 1980). In a third study, a biphasic response was observed in the rat thalamus slice preparation, in which low concentrations of thiopentone and methohexitone produced enhanced release and higher concentrations reduced release (Kendall & Minchin, 1982). The contrasting effects of barbiturates on GABA release from different brain regions probably reflect the complexity of action of these agents such that it is the balance of the effect on excitatory and inhibitory transmission that results in net excitatory or inhibitory synaptic events.

Results obtained from slice preparations may be complicated by secondary cellular crosstalk and the synaptosome preparation may be more useful for distinguishing primary effects at the level of the GABA releasing terminal.

The conflicting results concerning the effects of anaesthetic agents on the release of GABA from different areas of the brain in vitro (Collins, 1980, Kendall & Minchin, 1982, Minchin, 1981, Waller & Richter, 1980), necessitate caution in the extrapolation of observations to the whole animal. In one study the effect of halothane on the levels of GABA release in an in vivo preparation has been investigated (Osbourne et al, 1990). A microdialysis technique was used in which catheters were placed into the
extracellular fluid (ECF) surrounding the brain and the levels of neurotransmitters measured. A reduction in GABA content of the ECF was observed in the halothane anaesthetised animals. Although, it was not possible to tell if this reduction was due to a decrease in release or an increase in uptake but this technique did indicate an alteration in transmitter homeostasis in the intact animal.

In summary, the present evidence points to a number of potential sites of action of anaesthetic agents involving the GABA transmitter system but as with other transmitters further work is still required.

1.2.1.9 Glycine

Glycine is the major inhibitory neurotransmitter in the spinal cord and medulla and is also implicated widely in other brain regions. This transmitter acts on a receptor-operated chloride channel complex similar to the GABA receptor/Cl⁻ complex (Kruk & Pycock, 1991). The effects of anaesthetics on glycine transmission have not been studied extensively but one study on rat brain slices has indicated that pentobarbitone has no effect on the potassium-stimulated release of glycine (Waller & Richter, 1980). Further study is necessary to investigate the possible role of glycine transmission in anaesthetic mechanisms.
1.2.2 Group 2

Catecholamines and 5-Hydroxytryptamine

1.2.2.1 Noradrenaline and Adrenaline

The characteristics of release of noradrenaline from peripheral nervous tissue have been well documented owing to the availability of the chromaffin cell preparation derived from bovine adrenal medullary glands. This cultured cell preparation has been used as a model to investigate the action of anaesthetics on the process of neurosecretion (Akeson & Deamer, 1989, Pocock & Richards, 1987, Pocock & Richards, 1988). While contributing to the understanding of anaesthetic mechanisms and, in particular, to their effect on stimulus-secretion coupling it should be remembered that observations from this experimental system are only a model of the mechanisms in the CNS.

The barbiturates, at clinical concentrations, have been shown to reduce both the potassium- and carbachol-stimulated release of noradrenaline from chromaffin cell preparations (Pocock & Richards, 1987), and from potassium-stimulated synaptosomes (Haycock et al, 1977). When the volatile anaesthetic agents halothane, enflurane, methoxyflurane and isoflurane were studied a similar reduction in adrenaline and noradrenaline release was seen when carbachol stimulation was employed. However, in contrast to the barbiturates, much higher doses of the volatile anaesthetic agents were needed to inhibit the release following potassium-stimulation. Again, evidence from this experimental system indicates a possible selectivity for different classes of anaesthetic
agents. However, this apparent selectivity could simply reflect the ability of different anaesthetic agents to reduce calcium levels in the cells (Pocock & Richards, 1987 and 1988) (see 1.3). In contrast to observations on chromaffin cells, reduced potassium-evoked release of noradrenaline has been observed in rat brain slice preparations in the presence of volatile anaesthetic agents at clinical doses (Bazil & Minneman, 1989a and 1989b) (figure 1.3). When applied at equipotent concentrations for producing loss of righting reflex in rats, three volatile anaesthetics, halothane, enflurane and methoxyflurane were found to reduce potassium-stimulated noradrenaline release by 30% (Bazil & Minneman, 1989a).

It has been suggested that a mechanism of anaesthetic agents may be to increase the ionic permeability of synaptic vesicles and thereby lead to a decrease in the proton gradients that are essential for synaptic vesicles to accumulate transmitters (Akeson & Deamer, 1989). This "Pump Leak" hypothesis has been investigated for noradrenaline in the chromaffin cell system. Halothane, diethyl ether, butanol and ethanol were found to cause only a small insignificant loss of noradrenaline from chromaffin granules (Akeson & Deamer, 1989), inconsistent with the "Pump Leak" hypothesis. By inference, it seems unlikely that depletion of noradrenaline or other neurotransmitters from secretory vesicles could lead to a loss of efficiency of synaptic transmission and subsequent anaesthetic state. However, taken together all in vitro observations on noradrenaline and adrenaline release suggest a susceptibility of stimulated release mechanisms to modification by anaesthetic agents.

Complimentary studies have been performed in vivo. By manipulation of central noradrenaline levels it has been shown that the MAC of halothane increases with raised levels and decreases with reduced
Figure 1.3

$[^{3}\text{H}]$Noradrenalin (NA) release from rat slice preparation in the absence (C) and presence of 1.25% halothane (Hal). Release stimulated with buffer containing potassium 28 mmol litre$^{-1}$. Diagram also demonstrates the smaller release in a calcium free buffer. □ = control release; ■ potassium-stimulated release. Each point is the mean (SEM) of at least three experiments (reproduced from Bazil & Minneman, 1989b, with permission).
levels suggesting that reduced noradrenaline transmission may be an important contributory factor to the induction of the anaesthetic state (Miller et al, 1968). Thus, observations from both in vitro and in vivo investigations indicate that central noradrenaline metabolism is worthy of further study in unravelling the actions of anaesthetic drugs.

1.2.2.2 Dopamine

Dopamine has a better understood and more localised central action than noradrenaline. As a result Parkinson's disease and schizophrenia can both be treated using drugs that manipulate central dopamine metabolism. It is noteworthy that the drug amphetamine, which leads to a state of arousal, stimulates dopamine release centrally. By inference, therefore, the dopaminergic system is a potential target for anaesthetic action.

Levels of dopamine in the CNS can be manipulated by either increasing levels of the dopamine precursor, L-Dopa (dihydroxyphenylalanine), to increase dopamine levels or by depleting dopamine with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), which is an analogue of pethidine that selectively kills central dopaminergic neurons by inhibiting respiratory chain enzymes via a metabolic product. When mice were subjected to these two treatments L-Dopa decreased the MAC of halothane and MPTP treatment increased the MAC for halothane (Segal et al, 1990). Furthermore, when the dopamine levels of MPTP-treated mice were repleted with L-Dopa, the MAC of halothane was restored to control levels.

In a second complex study, employing an in vivo microdialysis technique, the effect of halothane anaesthesia on dopamine secretion was
investigated (Osbourne et al., 1990). Halothane was found to increase basal dopamine levels when rats were anaesthetised. Results from both these studies conflict with expectations arising from the effects of amphetamine on dopamine metabolism. *In vivo* and *in vitro* studies are urgently required to characterise the effects of anaesthetic agents on dopamine metabolism in more detail. In view of the number of patients with an alteration in their central dopamine pharmacology an enhanced understanding of anaesthetic mechanisms in the dopaminergic system are likely to have important clinical implications.

### 1.2.2.3 5-Hydroxytryptamine

A precise functional role for the transmitter, 5-HT, has yet to be fully established. However, 5-HT has been implicated as an important transmitter in the feeding, sleep, mood, behaviour and cardiovascular control pathways (Althaus et al., 1985).

5-HT is synthesised from the amino acid L-tryptophan in a two step pathway. The first, rate limiting step, involves the conversion of L-tryptophan to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase. This is followed by decarboxylation of 5-hydroxytryptophan to 5-HT catalysed by L-aromatic acid decarboxylase. The precursor, L-tryptophan, is actively transported into the nerve terminal and this may be a further limiting factor in the synthesis of 5-HT. Following stimulated release into the synapse the postsynaptic response is terminated by removal of 5-HT from the cleft by a high-affinity active transport mechanism.

To date only two aspects of 5-HT metabolism have been investigated in the presence of anaesthetic agents, 5-HT uptake and
release. There have been no studies on the effects of anaesthetic agents on the uptake of L-tryptophan, the precursor, or on the rate limiting enzyme tryptophan hydroxylase which may represent important control points in this transmitter system.

1.2.2.4 5-Hydroxytryptamine Uptake

In a series of experiments using the rat synaptosome preparation, ketamine, enflurane, halothane and isoflurane (Mantz et al, 1992, Martin et al, 1988a, 1988b, 1990a, 1990b) inhibited the uptake of 5-HT into the synaptosomal fraction. It is possible that such inhibition of uptake may increase the effect of the transmitter at its post-synaptic receptor site as the synaptic activity of 5-HT is terminated by an active re-uptake mechanism. 5-HT is believed to exert its influence on analgesic mechanisms by activating inhibitory mechanisms in the dorsal horn of the spinal cord, hence, a decrease in re-uptake may be contribute to the anaesthetic effects of these drugs. In contrast to the above, during the same series of experiments, pentobarbitone and etomidate were found to have little effect on the activity of the 5-HT uptake process again suggesting selectivity for anaesthetic agents on different neuronal activities.

To further investigate the selectivity of action of anaesthetic agents on the 5-HT uptake mechanism competition binding studies have been carried out using $[^3H]$paroxetine, which binds competitively to the substrate recognition site of the 5-HT transporter (Martin et al, 1990a). Ketamine brought about a concentration-dependent inhibition of specific $[^3H]$paroxetine binding whereas halothane had no effect. Interestingly, ketamine and halothane were found to induce a similar level of inhibition
of 5-HT uptake at clinically relevant concentrations of anaesthetic agents. Thus, while ketamine may bring about inhibition of 5-HT uptake by direct interaction with the active site of the transport protein, the inhibition of uptake by halothane must be mediated, at the very least, by a distinct site on the transporter protein or possibly by mechanisms located elsewhere in the membrane.

Ethanol in sufficient quantities causes general anaesthesia in humans (Alexi & Azmitia, 1991). When an equivalent dose of ethanol was applied in vitro to a synaptosome preparation the uptake of 5-HT was stimulated (Alexi & Azmitia, 1991), in contrast to other anaesthetic agents studied above (Martin et al., 1988a, 1988a, 1990a, 1990b). These contrasting effects on the 5-HT transmitter uptake mechanism do not offer a simple explanation for the possible role of this process in anaesthesia.

1.2.2.5 5-Hydroxytryptamine Release

To date, the effect of pentobarbitone only on 5-HT release from rat midbrain slices has been studied. A small decrease (29%) in 5-HT release was reported although this was not significantly different to untreated control samples (Waller & Richter, 1980).

1.2.2.6 5-Hydroxytryptamine and Other Transmitters

The complex interaction between neurotransmitter systems was well documented in an in vivo study (Becquet et al., 1988) in which a push-pull cannula technique was employed to study the influence of acetylcholine on 5-HT release in the cat caudate nucleus. In the control group there was a
ACh-dependent reduction in the release of 5-HT, which was not present in the halothane anaesthetised group. Further experiments suggested that the reduction in 5-HT release seen with the application of ACh may be due to stimulation of GABA interneurons. This work suggests that 5-HT levels could vary in response to anaesthetic agents due to indirect effects on other transmitter systems, and demonstrates again the complex interconnections between neurotransmitter systems that are seen in whole animal preparations which cannot be studied easily with *in vitro* systems.

### 1.2.3 Other Transmitters

A number of other transmitter systems, in particular those classified as Group 3, including neuropeptides such as substance P, the opioids and thyrotrophin releasing hormone (TRH), have received little or no attention with respect to the effects of anaesthetics. At present 40 neuroactive peptides have been identified but not all satisfy the criteria for neurotransmitters (Kruk & Pycock, 1991). There remains, therefore, a large amount of work before questions regarding selectivity of anaesthetic action can be answered fully.

### 1.3 The Role of Calcium in Neurotransmitter release: The effect of Anaesthetic Drugs

Influx of extracellular calcium is essential for the coupling of electrical excitation to neurotransmitter release (Cohen & Van der Kloof, 1985). Two phases of depolarization induced calcium entry into the nerve terminal have been characterised, a fast phase and a slower plateau phase.
The release of different neurotransmitters may rely on different phases of the calcium current (McMahon & Nicholls, 1991). As calcium is crucial to neurotransmitter release this aspect of stimulus-secretion coupling may be a potential site of action of anaesthetic agents and may present a mechanism by which some selectivity of anaesthetic action could occur.

Depolarisation-induced calcium uptake into synaptosomes has been shown to be reduced in the presence of a wide range of anaesthetic agents (Blaustein & Ector, 1975, Daniell & Harris, 1988, Harris & Bruno, 1985). Furthermore, in bovine chromaffin cells anaesthetic induced reduction in calcium influx has been linked to a decrease in evoked noradrenaline release in the presence of pentobarbitone, halothane (figure 1.4), isoflurane, enflurane and methoxyflurane (Pocock & Richards, 1987, Pocock & Richards, 1988, Yashima et al, 1986). When noradrenaline release from chromaffin cells was stimulated with carbachol or potassium, carbachol evoked release appeared to be more susceptible to the action of clinical doses of volatile anaesthetic agents than the potassium evoked release. Inhibition of potassium-stimulated release was only affected at supraclinical doses of anaesthetic agents. These results are most likely to be explained by the level of calcium in the cell achieved following the two stimulation protocols. When stimulated with potassium the influx of calcium was much greater than when stimulation was with carbachol, thus halothane induced reduction in intracellular calcium levels to threshold levels for transmitter release could be more easily achieved under carbachol stimulation. These results are consistent with an important role for calcium homeostasis in anaesthetic induced modulation of neurotransmitter release.
Figure 1.4
Effects of halothane on secretion of catecholamines (○) and influx of $^{45}\text{Ca}$ (●) evoked by carbachol in cultured bovine adrenal medulla cells. Values are mean (SEM) from 10 separate experiments, expressed as a percentage of carbachol-evoked responses in the absence of halothane. * P < 0.05, ** P < 0.01. (reproduced from Yashima et al, 1986, with permission.)
The possibility that reductions in calcium influx in the presence of anaesthetic agents may be linked to the membrane disordering properties of these drugs has been further investigated (Daniell & Harris, 1988, Harris & Bruno, 1985). No simple relationship between the ability of anaesthetic agents to reduce calcium influx and disorder membrane structure was shown. A better correlation was found between the effect on sodium influx and membrane perturbation (Harris & Bruno, 1985).

1.4 Direct Versus Indirect Actions of Anaesthetic Agents on Presynaptic Transmitter Mechanisms.

So far in this discussion consideration of anaesthetic effects at the level of the presynapse has focused on specific aspects of synaptic transmission mediated by a variety of transmitter agents. A variety of effects have been reported some of which may be compatible with the onset of anaesthesia. An important question that remains is how the anaesthetic agents exert their effects on such a range of biochemical systems.

From a pharmacological perspective most general anaesthetic agents would appear to be essentially non-specific in their actions at the level of transmitter metabolism and release. However, as can be seen from the above discussion not all anaesthetics produce the same response in the assay systems exploited, indicating some degree of possible selectivity of action. Indeed, it is possible that certain anaesthetic agents will be found to exert their primary effects in a specific manner at the level of discrete proteinaceous acceptors, e.g. ketamine acts at the NMDA receptor (Mimura et al, 1992) and at the active site of the 5-HT transporter (Martin
et al, 1988), the barbiturates act at the GABA receptor/Cl⁻ complex (Segal et al, 1990) and isoflurane produces stereoselective activation of a potassium channel subtype (Franks & Lieb, 1991). However, when agents such as the volatile anaesthetic agents are considered, a wide spectrum of effects in different transmitter systems is plainly observed involving not only presynaptic but also post-synaptic events. In these cases anaesthetic effects may reflect the susceptibility of discrete cellular functions to non-specific, anaesthetic mediated changes in their local environment rather than specific interactions with proteinaceous acceptors. Hence, anaesthesia may be the net result of a combination of a large number of non-specific toxicological effects on central nervous system function that any particular agent exerts. If so, the "physiological mechanism" of anaesthesia may well be different for each agent.

At the present time there have been insufficient studies to allow any definitive statements to be made concerning the relevance of the action of anaesthetic agents on presynaptic transmitter release and reuptake mechanisms to the overall mechanisms of general anaesthesia. In many cases our knowledge of the effects of anaesthetic agents on discrete presynaptic activities depends on just one or two studies. Often these have been carried out on more than one experimental system and under different experimental conditions such that a true comparison of results is not possible. More systematic studies of the actions of general anaesthetic agents on individual presynaptic activities in defined experimental systems and under comparable conditions are urgently required. Furthermore, these should include both in vitro and in vivo studies to allow primary and secondary effects of anaesthetic agents to be dissected. While in vitro studies may allow effects on specific functions to be studied more easily
the importance of in vivo studies must be stressed to permit the
discrimination of statistically significant changes measured in vitro which
have no physiological relevance in vivo. Since a universal action of general
anaesthetic agents cannot be ruled out, one aim of these studies should be
to seek to identify central activities that are universally altered by general
anaesthetic agents in a manner consistent with the induction of the
anaesthetic state. The likelihood is, however, that physiological
mechanisms of anaesthesia will prove to be much more complex.

The feature that unifies all general anaesthetic agents is their
relatively high hydrophobicity. Indeed, lipid solubility is the only property
of all anaesthetic agents that appears to correlate with their anaesthetic
potency (Miller, 1985). Hence, anaesthesia may depend solely on the
ability of any agent to disrupt local hydrophobic environments such that
central mechanisms in the control of consciousness become disrupted.
Selectivity of agents may, therefore, be related to their ability to penetrate
hydrophobic domains of different central functional structures (Evers et al,
1987). It is likely, that many of the effects of anaesthetic drugs will be
mediated via their disordered effects in the lipid bilayer of the cell
membrane (Miller, 1985). Where anaesthetic effects on membrane
functions are shown it will be important to investigate the possible
relationship of these changes with alterations in the membrane
environment.

It is of interest that when individual central activities are considered
the anaesthetic agents appear not to act in concert but to exert some
selectivity. For example, the barbiturates clearly exert different effects on
excitatory amino acid transmitter release than the volatile anaesthetics
Further study of presynaptic transmitter systems may allow agents to be grouped functionally which, in turn, may simplify the investigation of their mechanisms.
2. Acetylcholine and Central Neurotransmission

2.1 Introduction

This chapter outlines the functional importance of acetylcholine (ACh) as a central transmitter. The historical development of the 'reticular activating system' will be discussed, and the overlap between natural sleep states and anaesthesia. The evidence that has accumulated implicating central cholinergic pathways in the mechanisms of unconsciousness will be reviewed. A brief account of the *in vitro* studies on ACh and anaesthesia will be given as more detail will appear in the individual chapters.

2.1.1 Historical Perspective

Loewi (1923) had discovered that a substance released from the vagus nerve slowed a perfused frog heart. Later this substance was found to be ACh (Loewi & Navratil, 1926). The actions of ACh at the neuromuscular junction were first demonstrated by Dale *et al* (1934), by collecting ACh in the perfusion fluid around a motor nerve fibre. It was not until Eccles *et al* (1953) demonstrated that interneurones in the spinal cord released ACh, that the presence of ACh as a transmitter in the mammalian central nervous system was established. The central distribution and function of ACh have been more extensively understood in the last 10 years, although physiologists and pharmacologists had suspected since the 1940's and 1950's that cortical
arousal and sleep patterns were cholinergically generated (Richter & Crossland, 1949, Tobias *et al*, 1946).

### 2.1.2 Neuroanatomy

Early anatomical experiments performed by Bremer (1935, 1937), demonstrated that transecting the brain of an experimental animal led to differing states of vigilance. A bulbospinal transection (*encepalé isolé* preparation) left an animal that alternated between sleep and wakefulness. A mid-brain transection (*cerveau isolé* preparation) rendered the animal comatose. This suggested that an area of the mid-brain was essential for alternating the vigilant state of the animal between sleep and wakefulness.

Momizi and Magoun (1949) conducted further physiological experiments, which suggested that the reticular core of the brain stem was essential for electroencephalograph (EEG) activation. These investigators invented the concept 'reticular activating system' from their EEG studies but no anatomical pathways were mapped and they did not know which transmitters were involved in EEG activation.

Shute and Lewis (1967), were the first to investigate the distribution of central cholinergic neurones by assuming that neurones that contained acetylcholinesterase (AChE) would be cholinergic. By a combination of AChE histochemistry and specific brain lesions they mapped cholinergic pathways in the albino rat. They proposed that the pathways that they found were ascending, and that they arose from the reticular and tegmental nuclei of the brainstem and fore-brain. They concluded in the summary of their paper
that the pathways that they had discovered were probably identical with the neurophysiologists concept of 'the reticular activating system'.

In the 1980's more specific tools were developed to identify the cholinergic pathways, employing monoclonal and polyclonal antibodies against choline acetyltransferase (ChAT), the synthetic enzyme for ACh (Eckenstein & Thoenen, 1982). The technique, referred to as ChAT immunocytochemistry, revealed that there are no cholinergic interneurones in the cortex in all species except the rat. The technique confirmed the earlier AChE histochemistry (Shute & Lewis, 1967) that there are two extrinsic sources of ACh in the cortex. These are cholinergic neurones in the basal forebrain and in the mesopontine tegmentum. The basal forebrain projection provides an extensive input to the cerebral cortex supplying all brain regions.

The most recent technique employed to identify cholinergic neurones in the brain is in situ hybridisation (Butcher et al, 1993). The method relies on the identification of brain regions that demonstrate messenger ribonucleic acid (mRNA) encoding ChAT. This is done in combination with ChAT immunohistochemistry and has provided the most comprehensive cartography of central cholinergic neurones. There are two basic types of neurone, local circuit neurones which are located totally within the neural structure that contains them and projection neurones that interconnect central structures. Figure 2.1 illustrates the main cholinergic projection neurones, the basal forebrain cholinergic complex and the pontomesencephalotegmental cholinergic complex. The in situ hybridisation data reveals less cholinergic tissue than the ChAT immunohistochemistry, but this was probably due to false positive results.
Figure 2.1
Schematic representation in the horizontal plane of the major cholinergic systems in the mammalian brain. Central cholinergic neurones are arranged into two groups.
a) Local circuit cells, examples are the interneurones of the caudate-putamen nucleus, nucleus accumbens, olfactory tubercle and Islands of Calleja complex.
b) Projection neurones, there are two major sub constellations.
1. Basal Forebrain Cholinergic Complex composed of neurones in the medial septal nucleus (ms), diagonal band nuclei (td), substantia inominata (si), magnocellular preoptic field (poma) and nucleus basalis (bas) and projecting to the nonstriatal telencephalon.
2. Pontomesencephalotegmental cholinergic complex composed of neurones in the pendunculopontine (tpp) and laterodorsal (dltn) tegmental nuclei and projecting ascendingly to the thalamus and other diencephalic loci and descendingly to the pontine and medullary reticular formations (Rt), deep cerebellar (DeC) and vestibular (Ve) nuclei and cranial nerve nuclei.

Extra abbreviations
amyg, amygdala; ant cg, anterior cingulate cortex; CrN, dorsal cranial nerve nuclei; diencep, diencephalon; DR, dorsal raphe nucleus; ento, entorhinal cortex; PN; pontine nuclei; pr, perirhinal cortex; parietal, parietal cortex; SN, substantia nigra; Sp5, spinal nucleus of cranial nerve 5; temporal, temporal cortex; vislat, lateral visual cortex; visual med, medial visual cortex.

(From Butcher et al, 1993, reproduced with permission)
stemming from cross reactivity of antibodies with proteins other than ChAT (Butcher et al, 1993).

In summary the anatomical evidence has revealed that one cholinergic cell group within the mesopontine tegmentum makes ascending projections into the forebrain and the other major cholinergic cell group within the basal forebrain projects to the cortical mantle.

2.1.3 Neurophysiology

The functional role of the central cholinergic system has been investigated in parallel with the anatomical studies mentioned above. The pioneering physiological experiments of Moruzzi and Magoun (1949) developed the concept that cortical activation led to desynchronisation of the EEG. It was not until complimentary anatomical studies by Shute and Lewis (1967) that the substrate for the ascending reticular activating system was found to be ACh, although MacIntosh and Oborin (1953) had demonstrated that the cortical release of ACh was related to the electrical activity of the cortex.

Electrophysiological stimulation of the mesencephalic reticular formation leads to desynchronisation of the EEG (Kanai and Szerb, 1965) and also was accompanied by large increases in the release of ACh from the cerebral cortex. Collier and Mitchell (1967) correlated positively the level of arousal of freely moving animals with cortical release of ACh (figure 2.3). They collected the ACh with cortical cups and assayed the collected transmitter using a leech bioassay. Szerb (1967) showed that a lesion
undercutting the cortex reduced the amount of ACh measured at the surface and concluded that the cholinergic fibres with projections to the cortex originated in deeper structures. In the same study it was concluded that the projection neurones responsible for EEG activation and the increased cortical ACh release originated in the mesopontine tegmentum. These early experiments demonstrated that cholinergic neurones were part of the reticular core of the brain and were essential for determining the state of vigilance of the animal.

2.1.4 Natural Sleep and Acetylcholine

The understanding of the physiology of sleep has developed in parallel with the advances made in cholinergic neuroanatomy and physiology. Interestingly in the same year that Eccles et al (1953) identified ACh as a central transmitter, Aserensky and Kleitman (1953) described rapid eye movement (REM) sleep. It was later realised that the pontine reticular formation controlled the generation of REM sleep (Jouvet, 1962). This area is part of the 'ascending reticular activating system' (Moruzzi and Magoun, 1949) and also includes the mesopontine tegmentum which contains mainly cholinergic neurones.

There is no firm agreement on which transmitters are involved in the control of the sleep/wakefulness cycle but evidence has accumulated that the cholinergic system plays an important regulatory role in the generation of rapid eye movement sleep (REM). Monoaminergic neurones in the brainstem cease firing during REM sleep but brainstem cholinergic neurones show
increased firing during the period just before the transition from slow wave sleep to REM sleep (Steriade et al., 1990). Animal studies support the theory that REM sleep is generated by cholinergic neurones located in the pontine reticular formation and REM sleep is inhibited by noradrenaline and 5-HT neurones located in the locus ceruleus and raphe nuclei (Hobson et al., 1986). There are three states of vigilance, wakefulness, REM sleep and non-REM sleep (slow-wave sleep).

Microinjection of cholinergic agonists such as carbachol (Silberman et al., 1980) or bethanechol (Hobson et al., 1983) promoted REM sleep. Similarly, increasing the local concentration of endogenous ACh by microinjection of the AChE inhibitor, neostigmine, enhanced REM sleep in cats (Baghdoyan et al., 1984). Another AChE inhibitor, physostigmine, injected intravenously in a single dose of 0.5 mg in human volunteers caused arousal when injected during a period of REM sleep and promoted the generation of REM sleep when injected during a period of non-REM sleep (Sitaram et al., 1976). Tetrahydroaminacrine (THA, tacrine), a reversible AChE inhibitor, previously employed in anaesthesia to prolong the action of suxamethonium, also shortens REM latency (Riemann et al., 1994). In a complimentary investigation Hazra (1970) showed that when the choline uptake inhibitor, HC-3, was injected into the 4th ventricle of the cat slow wave sleep was facilitated and REM sleep was virtually abolished. In human volunteers, the muscarinic agonist SDZ 210-086 was found to shorten REM latency, increase the percentage sleep time as REM and increase the total duration of REM sleep (Hohagen et al., 1993). Evidence from animal studies that the M1 and M2 muscarinic receptors may control different functions of
REM sleep (Velazquez-Moctezuma et al., 1989), however SDZ 210-086 lacks selectivity for the M1 or M2 receptor subtypes.

Since cholinergic agonists promote REM sleep and arousal, it is not surprising that scopolamine, a muscarinic antagonist, delays the onset of REM sleep and reduces the duration (Sagales et al., 1969). Atropine blocks the increase of REM sleep caused by carbachol (Baghdoyan et al., 1989), and the chronic administration of scopolamine in the rat reduced REM sleep (Shiromani & Fishbein, 1986). Thus, the evidence for cholinergic involvement in REM sleep and arousal mechanisms is extensive. In a recent editorial, Lydic and Biebuyck (1994) have suggested that natural sleep states and anaesthesia may share common mechanisms. Parallel studies conducted by physiologists during the last 30 years have clearly demonstrated that cholinergic mechanisms are important in sleep and arousal. In the following section the evidence linking anaesthesia to cholinergic processes is reviewed and the theory expounded by Lydic and Biebuyck (1994) that biological sleep and anaesthesia may share common mechanisms through the central cholinergic system is further examined.
2.2 Evidence Linking Anaesthesia to Central Cholinergic Transmission.

There is pharmacological and experimental evidence linking disruption of central ACh transmission to anaesthesia. In physiology experiments, conducted in the mid 1940's and through to the 1960's investigators commented on the release of ACh as anaesthesia was deepened in experimental animals. Macintosh and Oborin (1953) found that the output of ACh collected from the surface of the brain decreased as anaesthesia was deepened. They also noticed that as the amount of transmitter decreased the spontaneous electrical activity of the cortex was also reduced proportionally.

Tobias et al (1946) had reported that the total ACh content of rat brain was increased following barbiturate anaesthesia. The observations for barbiturates were also confirmed in the frog. Elliot et al (1950) extended these investigations by investigating the effect of diethyl ether and barbiturates on total ACh content in rats and cats (figure 2.2). Diethyl ether also increased the total ACh content of the brains of the animals and another important observation made during the studies was the greater increase seen in ACh levels as anaesthesia was deepened (figure 2.3). The authors concluded that the anaesthetics inhibited brain activity and this was probably achieved by either a decrease in the liberation of ACh or indirectly by a decrease in the rate of destruction of ACh by cholinesterase. These investigators could not directly prove that it was the release mechanism that was inhibited but their interpretation of their data is still relevant to studies carried out much later, when ACh turnover rate was reportedly decreased by
Figure 2.2
Total acetylcholine content of rat's brains at various times after equal intraperitoneal injections of Nembutal (barbiturate). Each curve represents results with a group of rats treated at the same time. As anaesthesia is lightened the total amount of acetylcholine decreases, eventually falling to the lowest level when the animal is awake.
(From Elliot et al, 1950, reproduced with permission).
halothane anaesthesia *in vivo* (Ngai *et al*, 1978). These investigators
concluded that the increase in ACh concentrations and decrease in turnover
rate may be caused by decreases in transmitter release. However, all these
conclusions were speculative and there was no experimental data to back up
the theory of reduced transmitter release, stimulation of the synthesis of ACh
or inhibition of AChE.

Mitchell (1963), demonstrated that as a sheep recovered from
anaesthesia with cyclopropane, diethyl ether or pentobarbitone there was a
steady increase in the ACh output. In these experiments perspex cups were
placed over the surface of the cortex and were irrigated using Ringer's
solution. To assay ACh physostigmine was added to the Ringer's and the
solution subsequently removed by suction from the perspex cups and assayed
for the stimulation of contraction of either the frog rectus abdominis muscle or
on the leech muscle preparation. During these investigations when the depth
of anaesthesia was altered, the EEG was recorded. Output of ACh depended
on the depth of anaesthesia and this was positively correlated with a decrease
in the spontaneous electrical activity of the cortex. Mitchell agreed with the
previous workers, Elliot *et al* (1950), Richter and Crossland (1949) and
Tobias *et al* (1946), that ACh concentrations increase in the brain during
anaesthesia and this was likely to be caused by a decrease in the amount of
transmitter liberated.

A development in cholinergic physiology was the demonstration by
Kanai and Szerb (1965) that cholinergic neurones participated in the EEG
arousal response. An increase in ACh output of 6-fold was achieved by direct
electrical stimulation of the reticular formation. They also showed that, as
halothane anaesthesia was deepened from 1.0 to 1.5 % halothane, the amount of ACh collected decreased. This study was a limited dose response investigation of the effect of halothane on cortical ACh release, although these workers were investigating the cholinergic basis of the arousal response and could not quantify which aspect of ACh metabolism was affected by halothane. Kanai and Szerb (1965) had successfully demonstrated that EEG activation and ACh were linked, however, they did not realise that they had demonstrated a sensitivity of ACh release to the depth of halothane anaesthesia.

The next study to link arousal and anaesthetic mechanisms was the finding that periods of activity, sleeping and anaesthesia were linked to cortical ACh release (figure 2.3). Collier and Mitchell (1967) found that the activity and conscious state of the animal was related to the output of ACh. They commented in this paper that 'the ACh output was much higher than when the animals were anaesthetised, supporting observations that anaesthesia depresses ACh release in the cortex'.

To summarise these early physiological investigations, it is apparent that while studying the cholinergic mechanisms of arousal and cerebral EEG activity a consistent depression of cortical ACh activity by anaesthetics was noted. The depression was caused by both barbiturates and inhalational agents and appeared to be dose-dependent. The object of these studies was not related to the determination of anaesthetic mechanisms and the inclusion of anaesthesia in their protocols was often incidental to the main study. As a result several of the authors did not comment upon their findings related to anaesthetic agents.
There are many aspects of ACh metabolism that could interact with anaesthetic agents. Precursor uptake, the activity of enzymes involved in ACh synthesis, AChE inhibition, ACh release and post synaptic interaction of ACh with cholinergic receptors may all be influenced. The experimental work quoted in the previous section emphasises gross changes in either ACh measured at the surface of the brain or on total brain ACh concentrations.

Indirect evidence implicating cholinergic processes in arousal has accumulated from the use of cholinergic antagonists in anaesthetic practice. The evidence that has emerged from the administration of muscarinic antagonists like atropine and scopolamine during the perioperative period and the treatment of their adverse affects with cholinesterase inhibitors will be reviewed.
Figure 2.3

Acetylcholine release from the visual cortex of a free-moving rabbit during anaesthesia and consciousness. Legend on lower abscissa indicates state of animal as follows: A, anaesthetised after cannula implantation; B, recovered from anaesthesia and continuously active, exploring cage, eating and drinking; C, quiet and moving only occasionally; D, active; E, quiet; F, anaesthetised after Nembutal (30 mg/kg i. v.) at arrow; G, dead after lethal dose of Nembutal at arrow.

(From Collier and Mitchell, 1967, reproduced with permission)
2.3 Indirect Pharmacological Evidence Linking Cholinergic Transmission and Anaesthesia.

2.3.1 The Anticholinergic Syndrome

The anticholinergic syndrome consists of severe drowsiness, hallucinations and ataxia (Longo, 1966). This syndrome was described following the administration of scopolamine but was subsequently reported following the intraoperative use of atropine (Smith et al, 1979). By the time the clinical condition had been described, it had been noticed that physostigmine could reverse the adverse features. Although anticholinergic drugs produce somnolence, they do not produce unconsciousness consistently. It is likely that they interact with either pre or post synaptic cholinergic receptors, resulting in a reduction in ACh output or blockade of the post synaptic response. The first recorded use of physostigmine to treat anticholinergic poisoning was by Kleinwachter (1864), when he reported the use of extract of calabar against atropine poisoning. Physostigmine is a AChE inhibitor derived from the calabar bean, the seed of Physotigma venenosum. It is a tertiary amine which can readily cross the blood brain barrier, in contrast to neostigmine, the AChE inhibitor used to reverse neuromuscular block, which is a quarternary compound and cannot enter the brain.

There is both in vivo (Bertorelli & Consol, 1990) and in vitro evidence (Gibson & Peterson, 1981) that central ACh release decreases with ageing. It is perhaps not surprising that the central anticholinergic syndrome is more prevalent in the elderly, especially when scopolamine premedication was used.
(Holzgrafe et al., 1973). It is tempting to speculate that the mechanisms of unconsciousness produced by anticholinergic drugs and anaesthetic drugs have common pathways. The MAC, an estimate of the ED$_{50}$ of an agent, is reduced in the elderly and the likelihood of developing adverse central effects to anticholinergics is also increased. Alzheimer's disease may also be a result of decreased central cholinergic transmission (Dunnett & Fibiger, 1993) and it is interesting that these patients are particularly vulnerable to developing the central anticholinergic syndrome (Tune & Bylsma, 1991).

However, even before the deleterious effects of anticholinergic agents were described, there was evidence that the intraoperative administration of AChE inhibitors improved the recovery from general anaesthesia. (McCaul & Robinson, 1962). One agent, that was employed to lengthen the neuromuscular block produced by suxamethonium, was THA (tacrine). THA is the hydrogenated form of the antiseptic aminacrine and was developed during and just after the second world war. During the early clinical trials of THA, McCaul and Robinson (1962) noted that the recovery from anaesthesia was rapid and they speculated that this was due to medullary stimulation. Another interesting feature of these early studies was that THA appeared to be an antagonist to opiate induced narcosis. This effect was not quantified, but the investigators noted that the patients who received THA required more analgesia than the control group.

Physostigmine has also been employed to improve the rapidity of post operative recovery. This was initially a follow up from the discovery that physostigmine reversed the sedation following the administration of anticholinergic agents. Hill et al (1977) examined the effectiveness of
Physostigmine on the recovery from general anaesthesia. The motivation for the study was the new fashion for day case surgery with the need for a rapid recovery. Patients received only a barbiturate induction and maintenance of anaesthesia with 50% nitrous oxide and halothane. When they arrived in the recovery room they received either 2 mg of physostigmine intravenously or saline. The patients were assessed for 3 hours in the recovery room and at all time points the physostigmine group were more responsive than the control group. There were minimal cholinergic side effects reported in this study. The assumption was that physostigmine increases the ACh concentration at synapses, by inhibition of AChE, and leads to cortical activation. In this study, anaesthesia was for a minimum of 45 minutes, and although physostigmine improved recovery characteristics there was no evidence that a reduction in synaptic ACh content had caused unconsciousness.

Physostigmine has been employed to stimulate respiration following the administration of opioids without affecting analgesia. Weinstock et al (1980) reversed morphine induced respiratory depression in conscious rabbits without affecting the quality of analgesia. In the same series of experiments physostigmine reversed morphine induced respiratory depression in dogs anaesthetised with ketamine, again with no influence on analgesia. The animal experiments were followed by a study to investigate the effect of physostigmine on morphine induced post operative pain and somnolence (Weinstock et al, 1982). A small intravenous dose of physostigmine, 1 mg per patient, abolished the somnolent effect of morphine, restored the respiratory rate to pre-drug values and may have increased the quality of analgesia.
The problem with the intravenous administration of physostigmine is the short half life of the drug. The effect of the drug commences within 5 minutes but the effect only lasts for 40 to 60 minutes. It appears that the pharmacokinetic limitations of physostigmine have restricted its use, combined with the development of improved perioperative drugs and analgesic techniques. Somnolence caused by a variety of central nervous system depressants, benzodiazepines, opioids, phenothiazines, has been reversed with physostigmine (Nilsson & Himberg, 1982, Bidwai et al, 1979, Bourke et al, 1984). Taken together these observations suggest that there may be a final common pathway for the development of unconsciousness via a depression of central cholinergic transmission with agents acting indirectly via other transmitters or directly on these cholinergic processes.

A group of anaesthetists working in Sweden developed the beneficial effects of physostigmine further by employing the drug in neurosurgery to combat over sedation and to even provide an alternative source of analgesia. Petersson et al (1986), first showed that a 2 mg bolus of physostigmine produced analgesia comparable to 50 mg of pethidine. The study was double blind and included a placebo group. The analgesic effect of physostigmine was for 30 minutes only, after which time its effect on analgesia decreased to that of placebo. The proposed advantage of physostigmine in the immediate perioperative period was its effect on producing an alert, pain free patient, in contrast to an alert patient in pain achieved with naloxone. This technique proved useful in assessing neurosurgical patients in the immediate postoperative period, however, the duration of action was short. Further investigations revealed that using physostigmine by various routes it was only
possible to increase the duration of effect to approximately 90 minutes (Hartvig et al, 1986). This group tried a constant two-rate infusion of physostigmine but they concluded that this offered no advantage over the single intravenous bolus injection. There has been little work on the proposed mechanisms for the effect of physostigmine on the side effects of opioids. There has certainly been little enthusiasm for the use of this particular AChE inhibitor in the postoperative period, although with the development of more specific inhibitors, with better pharmacokinetic profiles a use for this class of drug may be found.

2.3.2 *In Vitro* Evidence Linking Acetylcholine and Anaesthesia

Acetylcholine is synthesised in the nerve terminal from choline and acetyl coenzyme A (acetyl-CoA) in a reaction catalysed by ChAT. Following release, the ACh signal in the synapse is terminated by hydrolysis by AChE and the choline is taken up by the presynaptic cell for resynthesis of ACh. The rate limiting step in the synthesis of ACh is probably the uptake of choline into the nerve terminal, making this stage a candidate for the action of anaesthetic agents (Jope, 1979).

2.3.3 Acetylcholine Metabolism

The effect of halothane on the uptake of $[^3]$H methyl choline has been investigated in rat brain synaptosomes (Johnson & Hartzell, 1985). A 40%
reduction in the uptake of the precursor was demonstrated at halothane levels of 3 %, which corresponds to over twice the MAC value for the adult rat (see 3.3.1). The inhibition of choline uptake reported in this study was non-competitive and was observed at a supra clinical dose of halothane. As choline uptake is rate limiting (Jope, 1979) for the biosynthesis of ACh, the inhibition of precursor uptake mediated by the volatile anaesthetics could contribute to the state of anaesthesia.

ACh turnover *in vivo* in rat brain regions has been measured under halothane, enflurane and ketamine anaesthesia (Ngai *et al*, 1978). The concentrations of acetylcholine and choline in various brain regions did not alter during anaesthesia with any agent. However, halothane did decrease the turnover rate for acetylcholine by 10 % at 0.7-1.0 % inspired concentrations in both cortical and sub cortical structures. Interestingly, enflurane did not show any decrease in acetylcholine turnover in sub cortical structures and it is possible that this may be associated with the seizure like activity that develops under enflurane anaesthesia (Modica *et al*, 1990).

### 2.3.4 Acetylcholine Release

Few studies of the effects of anaesthetic agents on ACh release have been performed. In rat slice preparations potassium-stimulated ACh release has been shown to be decreased in the presence of pentobarbitone (Richter & Waller, 1977, Richter & Werling, 1979, Waller & Richter, 1980) and methohexitone (Richter & Werling, 1979). Similarly, halothane, at a concentration of 3.0 %, has been shown to reduce, by approximately 50%,
potassium-stimulated release of acetylcholine from a rat brain synaptosome preparation (Johnson & Hartzell, 1985). A reduction in acetylcholine release has also been demonstrated with 1 and 2 % halothane in the cat superior cervical ganglion preparation (Bosnjak et al, 1988). However, conflicting results have been obtained in other studies. Halothane, methoxyflurane, and enflurane at concentrations of 1.25%, 0.2% and 3% respectively were reported to have no effect on ACh release from a rat brain slice preparation (figure 2.4) (Bazil & Minneman, 1989a and 1989b) and in an isolated cat superior cervical ganglion preparation chloral hydrate was found to either decrease potassium-stimulated release (Richter & Waller, 1979) or to have no effect (Matthews & Quilliam, 1964).

In the above studies total ACh release was measured in synaptosomes (Johnson & Hartzell, 1985), while release of tritiated ACh was monitored in the brain slice studies (Bazil & Minneman, 1989a and 1989b). Differential labelling of intracellular pools of ACh in the nerve terminal (Webster, 1989) could underlie some of the differences in observations (Bazil & Minneman, 1989a and 1989b, Johnson & Hartzell, 1985). In addition, rat brain slices are known to be more sensitive to potassium-depolarisation than synaptosomes (Schulz & MacDonald, 1981) and this could also account for differences in action of anaesthetics between the preparations. Comparisons between these studies may be further complicated by the different doses of anaesthetic agents applied in the various studies (Bazil & Minneman, 1989a, Johnson & Hartzell, 1985).

In summary there is anatomical, physiological and pharmacological evidence implicating central cholinergic pathways in the genesis of natural
sleep and anaesthesia. This evidence has been accumulating since the mid 1940's but is complicated by the complex interactions between different transmitters and neural pathways in the intact animal. In the present thesis the pre-synaptic effects of volatile agents on cholinergic function has been investigated in isolated neural preparations.
Figure 2.4

$[^3]$H acetylcholine release from rat slice preparation in the absence (C) and presence of 1.25 % halothane (Hal). Release stimulated with potassium 16 mmol litre$^{-1}$. Figure also shows smaller release in a calcium free buffer.

□ = control release; ■ = stimulated release. Each point is the mean (SEM) of 1-4 experiments, each in triplicate or quadruplicate. (Reproduced from Bazil and Minneman, 1989b, with permission).
3. Materials and Methods

3.1 Introduction

3.1.1 The Synaptosome Preparation

Nerve terminals contain neurotransmitter packaged in vesicles, or in the general cytoplasm that is released when the terminal is depolarised. The arrival of an action potential is the physiological event that precedes release of the transmitter. The neurone also contains membrane transport proteins which may terminate the action of the transmitter by reuptake, enzymes for the degradation of transmitter and receptors and ion channels. The nerve terminal is connected to the nerve cell body by filamentous axons but if isolated, the terminal can exist as a separate entity. For the past 30 years neurochemists have employed the principal of nerve terminal isolation to study biochemical processes in the nerve terminal. This is achieved by the synaptosome preparation.

3.1.2 Historical Perspective

Gray and Whittaker (1962) described the isolation of nerve endings from guinea pig brain homogenate. They were attempting to isolate nerve fragments that contained synaptic vesicles, as they believed that this would prove a useful model for the study of neurotransmitter release. Figure 3.1 is taken from Gray and Whittaker (1962), and illustrates the method for the
Figure 3.1
Flow diagram summarising the preparation of subcellular fractions on sucrose gradients. Reproduced from Gray and Whittaker (1962) with permission.
preparation of the nerve terminal particles, subsequently called synaptosomes.
The mechanism of synaptosome formation is unknown, but Gray and Whittaker (1962) speculated that during homogenisation of the tissue the nerve endings are torn from the axons and the membrane then reseals at its point of rupture to form a continuous structure, analogous to the formation of microsomes from endoplasmic reticulum. Mitochondria were identified in some of the synaptosomes and Gray and Whittaker (1962) also commented on some limitations of the sucrose gradient method. They noticed that delicate subcellular structures like nucleoli and neurofilaments were not present in the preparations. Since the original description of synaptosomes other methods have appeared which improve on the original method.

The main limitations of the sucrose method are as follows.

1. The relatively long preparation time, 4 to 5 hours.

2. The reduction in metabolic activity due to the long preparation time.

3. The contamination of the preparation with other subcellular elements. Up to 50 % of the material present may not be synaptosomes.

4. The synaptosomes may be damaged by the hyperosmolarity of the sucrose solutions used. This problem is worse if continuous sucrose gradients are used. These gradients improve the yield of the synaptosomes but require more hypertonic sucrose solutions resulting in greater damage to the synaptosomes.
5. Many workers chose to use just the P2 fraction from the Gray and Whittaker protocol, as this only takes 45 minutes to prepare. However, this crude mitochondrial pellet is contaminated with myelin and extrasynaptosomal mitochondria, both of which can interfere with presynaptic phenomena.

3.1.2 Improvements to The Sucrose Gradient Method

Booth and Clark (1978) reported the use of discontinuous Ficoll/sucrose gradients for the preparation of synaptosomes. Ficoll is a synthetic polysaccharide which, when used in discontinuous gradients, has the advantage of a lower osmolality than equivalent mono and disaccharide solutions. This technique was an improvement on the original sucrose method as the preparation time was shortened to 2 hours and there was less contamination of the preparation with free mitochondria. However, polysaccharides have higher viscosity than disaccharides which leads to poorer resolution of the bands and longer centrifugation times. The polysaccharides cannot be removed from the preparation by dialysis or ultrafiltration and can only be removed by diluting the gradient fractions and centrifugation.

Dunkley et al (1986) described a method for the preparation of synaptosomes that used discontinuous Percoll/ sucrose gradients. Percoll is a colloidal silica that is coated in polyvinylpyrrolidine (PVP). Thus Percoll, is a colloidal suspension and not a true solution. The coating is essential as colloidal silica particles are toxic to cells and are unstable in solution. The
PVP coated particles are 15-30 nm in diameter and Percoll has to be filtered prior to use in order to remove unbound PVP. The main advantage of Percoll over sucrose and Ficoll is its low viscosity which allows for more rapid centrifugation. Isotonicity can also be maintained with Percoll/sucrose mixtures. The first description by Dunkley et al (1986) applied the P2 fraction to the discontinuous Percoll gradients. Dunkley et al, (1988), modified the original method to apply the S1 fraction to the Percoll gradients. The advantages of this modification are that, it requires only 15 minutes centrifugation time, no ultracentrifuge is needed and no re-suspension steps are required (figure 3.2).

When the viability of the subcellular fractions was assessed by the uptake of $^{32}$P$_i$, by the uptake of $[^3]$Hnoradrenaline and by measuring the depolarisation-stimulated increase in $[^3]$Hnoradrenaline release (Harrison et al, 1988), subcellular fraction 4 was found to be most enriched in synaptosomes. This was the most homogeneous fraction and synaptosomes were the most viable as judged by the criteria set out above. Fraction 3 also contains synaptosomes and could be combined with fraction 4 but such combination increases contamination by membranous material. The S1 and S2 fractions also contain synaptosomes but these are less viable than those from S3 and S4.
Figure 3.2
Flow diagram summarising the preparation of synaptosomes on Percoll gradients using the $S_1$ fraction.
3.2 Methods

3.2.1 Preparation of Cortical Synaptosomes From Discontinuous Sucrose Gradients

Synaptosomes were prepared from the cerebral cortex of female Wistar rats (200-250g) using a modification of the method of Gray and Whittaker (1962) that was described by Postma and Catterall (1984). Rats were killed humanely by a Schedule 1 method under the Animals (Scientific Procedures) Act 1986, the head removed post mortem and the cortex dissected. All subsequent procedures were performed at 4°C. The cortex was washed with ice cold 0.32 M sucrose (10 ml. g⁻¹ tissue) and then homogenised in 0.32 M sucrose (10 ml. g⁻¹) by 12 strokes in a motor driven Potter homogeniser (Teflon glass). The homogenate was centrifuged at 5,000 x g for 10 min. The supernatant was retained and the pellet re-homogenised in 0.32 M sucrose (10 ml. g⁻¹ of original tissue) and centrifuged at 5,000 x g for 10 min. The second pellet was discarded and the two supernatants were combined and centrifuged at 20,000 x g for 60 min. The supernatant was discarded and the pellet re-suspended in 0.32 M sucrose 5 mM potassium phosphate (pH 7.4) at an approximate protein concentration of 1 mg ml⁻¹. The suspension was layered onto discontinuous sucrose gradients consisting of 0.6 M, 0.8 M, 1.0 M and 1.2 M sucrose, in 5 mM potassium phosphate (pH 7.4). The 0.4 M layer in the Postma and Catterall method was omitted as it did not alter the separation of synaptosomes in the gradients obtained (figure 3.3). Centrifugation of gradients was for 105 min at 100,000 x g at 4°C.
Figure 3.3
Photograph of sucrose gradients following centrifugation at 100,000 X g for 105 minutes. The 0.4 M sucrose layer was omitted from the method described by Postma and Catterall (1984).
Synaptosomes were recovered from the 1.0/1.2 M interface, resuspended in 0.32 M sucrose, 5 mM potassium phosphate (pH 7.4) at 0.4 mg. ml⁻¹ protein and centrifuged at 150,000 x g for 30 min. The supernatant was discarded and the pellet taken up in 0.32 M sucrose, 5 mM potassium phosphate to a final protein concentration of between 5-12 mg ml⁻¹. Protein concentration was measured by the method of Lowry et al (1951) using bovine serum albumin as standard. Figure 3.4 illustrates the method that was employed for the preparation of the synaptosomes on discontinuous sucrose gradients.

The sucrose gradient method took approximately 6 hours to obtain the synaptosome preparation. For the experiments on ChAT, this did not hinder the work as the preparation could be frozen at -70°C and used for the ChAT assay later. However, for the choline uptake experiments the synaptosomes did not survive freezing and had to be used on the day of the experiment. They were used within 10 minutes of completion of the preparation.

There were two main problems with the sucrose method. The first was the need to use an ultracentrifuge and the second was the low yield of synaptosomes obtained. To overcome these problems the synaptosomes were prepared using discontinuous Percoll/sucrose gradients, using a bench top centrifuge and the whole preparation time was reduced to one hour.
Figure 3.4
Preparation of synaptosomes on discontinuous sucrose gradients.
3.2.2 Percoll Gradients

Synaptosomes were prepared from the cortex of female Wistar rats (200-250g) using the method described by Dunkley et al (1988). One gram of rat cerebral cortex was homogenised in 10 ml of 0.32 M sucrose by 12 strokes in a motor driven Potter homogeniser (Teflon glass). The homogenate was sedimented at 5,000 x g for 10 min. The supernatant was retained and made up to a total volume of 14 ml with ice cold 0.32 M sucrose. Two millilitres of the suspension were layered over freshly prepared 8 ml Percoll gradients, comprising 3%, 10%, 15%, and 23% Percoll(v/v) in 0.32 M sucrose, in a 10 ml polycarbonate tube (figure 3.5). Gradients were centrifuged in a fixed angle rotor for 10 minutes at 20,000 x g. The fractions at the 23%/15% and 15%/10% interfaces were collected and washed by centrifugation with ice cold Krebs' buffer three times at 20,000 x g for 10 minutes before use in experiments. Protein concentration was measured by the method of Lowry et al (1951) using bovine serum albumin as standard.

Electron micrographs of synaptosomes prepared on sucrose and Percoll gradients are shown in figure 3.6.
Figure 3.5
Photograph of Percoll gradients following centrifugation at 20,000 X g for 10 minutes.
Figure 3.6

Electron micrographs of synaptosomes prepared on sucrose gradients X 104,000 (top) and on Percoll gradients X 150,000 (bottom).
3.2.3 Preparation of Cortical Slices

ACh release experiments were performed using rat cortical slices. Synaptosomes had been used initially but were found unsuitable for the release experiments. A full explanation of the rationale for using the cortical slice preparation is to be found in section 6.6.

Female Wistar rats were killed humanely by a Schedule 1 method under the Animals (Scientific Procedures) Act 1986, the head removed post mortem and the dissected brain placed in 10 ml of ice cold Krebs buffer pH 7.4 (composition in mM: NaCl 115, KCl 4.7, MgCl₂ 1.2, NaHCO₃ 25, glucose 8.8, Hepes 20). The cortex was dissected and cut into 350 x 350 µM slices with a McIlwain tissue chopper. The slices were then warmed to 37°C by washing three times with oxygenated Krebs buffer at 37°C.

For release experiments the suspension of slices was split into two equal aliquots. One was incubated for 10 minutes with oxygenated Krebs buffer and the other with oxygenated Krebs buffer that contained 65 µM phospholine, an organophosphorus anticholinesterase. Both sets of slices were washed subsequently with 10 ml of oxygenated Krebs three times to remove excess phospholine prior to the release experiments.

Prior to the release experiments the slices were allowed to sediment by standing in a 20 ml tube and excess buffer was removed from the surface of the slices. A modified Eppendorf multipipette, with a Gilson yellow pipette tip glued to the end, was used to aliquot the slices to the test tubes containing control (low potassium) or release (high potassium) buffer. The slices were allowed to settle in the barrel of the pipette for one minute before they were
added to the test tubes. Delivery of the slices was volume controlled, by adjusting the setting of the multipipette, volumes from 50 μL to 250 μL of packed slices was added to the experimental tubes.

At the completion of the incubation phase of the experiments the slices were centrifuged at 5,000 x g for 10 minutes, the supernatant removed and kept for analysis and the test tubes were weighed. All the experimental tubes were weighed at the start of the experiment and by subtracting the final weight, the wet weight of the slices was used as a measure of the protein content of the individual sample.

3.2.4 Delivery of Anaesthetic Gases To Experimental Samples

Anaesthetics were delivered to experimental samples via a manifold of 10 outlets, which were modified intravenous ramps and intravenous extension tubes (figure 3.7). The flow of gas was controlled by a rotameter block and each outlet was calibrated using a bubble flow meter. When the flow of gas from the rotameter was 1.5 litres per minute, each outlet delivered 60 ml min⁻¹ of air, which was measured with the bubble flow meter. Anaesthetics were delivered from calibrated vaporisers (Cyprane TEC 3) which had recently been calibrated using a laser refractometer (Index Instruments) by General Anaesthetic Services, Keighly, UK.

The amount of volatile anaesthetic delivered was regularly checked using a Capnomac monitor placed in the gas delivery system distal to the vaporiser (McPeak et al, 1988).
For the choline uptake and ChAT experiments control samples and samples exposed to anaesthetics were performed sequentially. In order to avoid time-dependent bias in these results control and anaesthetic samples were alternated so that there would be no effect from prolonged incubation at 4°C.

For the release experiments the circuit was modified in order to do control and anaesthetic experiments simultaneously. A flow splitter was placed in the circuit distal to the rotameter block, with one limb going to the vaporiser and the other acting as the control limb. Both gas flows were humidified using cold water humidification and connected to separate sides of the manifold of intravenous extension tubing. The flows in this system were also calibrated using a bubble flow meter and were 50 ml min⁻¹ for a rotameter setting of 2.0 litres.

For the release experiments, using rat cortical slices, the control carrier gas was 95%O₂/5%CO₂ to ensure adequate oxygenation of the preparation. For the choline uptake and ChAT experiments, when rat cortical synaptosomes were used the carrier gas was medical air as described previously by Johnson & Hartzell (1985).
Figure 3.7
Diagrammatic representation of apparatus used to deliver anaesthetic gases to experimental samples.
3.2.5 Determination of Dissolved Halothane Concentration
By Gas Chromatography

The aqueous concentration of halothane in buffer and the time to reach
equilibrium of 10 minutes was confirmed by n-heptane extraction and
measurement in a Perkin-Elmer 8410 gas chromatograph (Rutledge et al.,
1963). The column used was a 30 meter megabore DB-17 (Fisons), and the
detection method was flame ionisation. The injector, detector and oven
temperature were 120°C, 100°C and 90°C respectively. The helium carrier
gas flow rate was 20 ml min⁻¹, measured with a bubble flow meter. The
integrator used was a Perkin-Elmer 8410.

The halothane equilibration time was investigated utilising the gas
delivery system described above and by administering halothane from a
Cyprane TEC 3 vaporiser to 300μl of Krebs buffer. This volume of buffer
was chosen as the release experiments were conducted with this volume.
Halothane concentration was assayed at 5 min intervals up to 20 min. At the
end of the time period an equal volume of n-heptane was added to extract the
dissolved halothane from the aqueous Krebs buffer. 1.5μL portions of the
organic layer were then injected onto the column. Halothane concentration
was measured as the integrated peak height, the time to equilibration is shown
in figure 3.8.

Known halothane standards were made up in sealed vials at 4°C to
prevent evaporation. A standard curve was measured along with extracted
unknown samples. The standard curve obtained is shown in figure 3.9. The
Figure 3.8
Time to equilibration for halothane delivered to 300 μL buffer. Numbers represent dose of halothane delivered from TEC 3 vaporiser. Each point is a single determination.
Figure 3.9
Halothane standard curve. Each point is a single determination, $r = 0.99$, broken line represents 95% confidence intervals.
results obtained delivering halothane from 0.5 to 5.0 % on the vaporiser, to 300µL Krebs buffer are summarised in table 3.1.

The ACh release experiments were conducted with 300µL samples in the test tubes for equilibration. The ChAT experiments contained 500µL of buffer and the choline uptake experiments had 1 ml of buffer for equilibration. A limited number of experiments were performed using 1 ml buffer and the time to equilibration was found to be the same as for 300µl.

For the dose-response portion of the ACh release experiments the delivered halothane concentration had to be as low as 0.1 %. The Cyprane TEC 3 vaporiser was calibrated in 0.1 % graduations by laser refractometry by General Anaesthetic Services (Keighly, U.K.). Another standard curve was constructed using lower concentrations of halothane (figure 3.10).

Table 3.2 shows the results of halothane concentrations measured by gas n-heptane extraction and gas chromatography for the low concentrations of agent. There is a good correlation between the delivered % of halothane and the equivalent aqueous concentration, and the time to equilibrium is less than 10 minutes at all concentrations. For all experiments buffers were pre-equilibrated with carrier gas containing anaesthetic for a minimum of 10 minutes before any experiment was started.
Table 3.1

Aqueous halothane concentrations and equivalent Atm % values for 300 μL Krebs

<table>
<thead>
<tr>
<th>Delivered Atm %</th>
<th>Aqueous (halothane) μM</th>
<th>Equivalent Atm %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>120.9 (23)</td>
<td>0.43 (0.08)</td>
</tr>
<tr>
<td>1.0</td>
<td>220.0 (28.7)</td>
<td>0.79 (0.10)</td>
</tr>
<tr>
<td>2.0</td>
<td>437.2 (11.2)</td>
<td>1.56 (0.04)</td>
</tr>
<tr>
<td>3.0</td>
<td>618.9 (68.2)</td>
<td>2.21 (0.24)</td>
</tr>
<tr>
<td>4.0</td>
<td>932.3 (104.7)</td>
<td>3.33 (0.37)</td>
</tr>
<tr>
<td>5.0</td>
<td>1193.0 (41.4)</td>
<td>4.26 (0.15)</td>
</tr>
</tbody>
</table>

All data are Mean (SD), n = 5 for all concentrations. Equivalent Atm % is calculated assuming 1 Atm % at equilibrium with aqueous gives 280 μM (Franks & Lieb, 1993).

**Correlation:** Delivered Atm % versus Equivalent Atm %

\[ r = 0.99 \]
Figure 3.10

Halothane standard curve for low vaporiser settings of halothane, 0.1 to 0.5 \% . Each point is the mean of 2 determinations. Broken line represents 95 \% confidence intervals, $r = 0.99$. 
### Table 3.2

**Aqueous halothane concentrations and equivalent Atm % values for 300μl Krebs.**

<table>
<thead>
<tr>
<th>Delivered Atm %</th>
<th>Aqueous (halothane) μM</th>
<th>Equivalent Atm %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>16.3 (5.4)</td>
<td>0.058 (0.02)</td>
</tr>
<tr>
<td>0.2</td>
<td>51.2 (9.5)</td>
<td>0.18 (0.03)</td>
</tr>
<tr>
<td>0.5</td>
<td>112.8 (29.7)</td>
<td>0.40 (0.10)</td>
</tr>
</tbody>
</table>

All data are mean (SD), 0.1 % n = 4, 0.2 % n = 5, 0.5 % n = 5. Equivalent Atm % is calculated assuming 1 Atm % at equilibrium with aqueous phase gives 280 μM (Franks & Lieb, 1993).

**Correlation:**
- Delivered Atm % versus Equivalent Atm %
- \( r = 0.91 \)
3.3 Discussion

3.3.1 Doses of Anaesthetics Delivered

Initially the effect of halothane on ChAT activity was examined (chapter 5). The dose of halothane chosen for the experiments was 3 %, so that direct comparisons could be made with the earlier observations of Johnson and Hartzell (1985). It is useful to compare the administered dose of anaesthetic to potency, as this allows comparison of effect when another agent is delivered to the particular assay system. In animals, different end points have been used in order to compare the ED\textsubscript{50}’s of the agents in common use today. Mazze \textit{et al} (1985) determined MAC (minimum alveolar concentration) by clamping a 6-inch haemostat to the first ratchet position on the mid-potion of the tail. If the animal made a purposeful movement within 1 minute, it was considered to have moved. The value that was obtained for halothane in adult non pregnant female rats was 1.03 %. Thus 3 % halothane is equal to approximately 3 rat MAC. White \textit{et al} (1974) had also used the tail clamp technique to determine MAC, but had applied a clip to the distal third of the tail for one minute, and not in the middle. The value obtained for halothane in 8 month old rats was 1.00 %, but in rats aged 3 months the value was 1.24 %. The animals employed in the studies conducted in this thesis were usually 3 to 4 months old, so the rat MAC of 3 % halothane was equivalent to 2.4 rat MAC.

For enflurane the picture is simpler as only one study (Mazze \textit{et al}, 1985) has given a value for rat MAC which is 2.2 %.
For isoflurane Mazze et al (1985) give a value for rat MAC of 1.46 %.
White et al (1974), again using slightly different methodology to Mazze et al (1985), gave rat MAC for isoflurane as 1.45 % in the young rats (3.5 months old) and 1.28 % in rats aged 8 months old.

The literature is, therefore, unresolved regarding comparisons of equipotent doses of anaesthetic agents. In this thesis the data from White et al (1974) has been taken as standard, but the different MAC values published may affect interpretation of the data. The doses employed are given in the relevant chapters, together with interpretation based on the conflicting MAC values for rats.
4. Choline Uptake

4.1 Introduction

Choline is a quaternary ammonium compound that is distributed widely in animals and is necessary for the normal function of the mammalian organism. It is a precursor for the biosynthesis of phospholipids, an essential component of membranes, and is a precursor for the biosynthesis of ACh. There is no recognised deficiency syndrome in humans, although Zeisel et al (1991) suggested that choline was an essential nutrient in humans when excess methionine and folate were not present in the diet, as experimental subjects fed a diet deficient in choline developed liver abnormalities.

The plasma concentration of choline in man is 10 μM and following a meal containing choline this can rise to 20 μM (Bligh, 1952). There is a specific carrier mechanism in the blood brain barrier that transports choline into the brain (Zeisel, 1981). At normal plasma concentrations of choline (< 10 μM) the transport mechanism is unsaturated and choline is transported at a rate proportional to the plasma concentration; thus there is a linear relationship between the plasma level of choline and the movement of choline between the plasma and the brain (Klein et al, 1990). During, and immediately following, a meal choline is taken up from the blood into the brain and into glial cells where it is phosphorylated. Between meals choline is released from the stores and returns to the blood via the cerebrospinal fluid (CSF). The concentration of choline in the CSF is 2 μM in man and it is likely that this is close to the level of the interstitial fluid in the brain (Aquilonius et
Choline is transported from the CSF into the plasma by carriers located in the choroid plexus and these keep the CSF concentration of choline lower than in the plasma.

Recently, attention has been focused on the possible beneficial effects of dietary choline, or its main source lecithin, in the treatment of neurodegenerative disorders (Iversen, 1993). The systemic administration of choline can raise brain ACh concentration (Cohen & Wurtman, 1976), but there is little evidence that ACh synthesis is limited by dietary choline availability. However, choline has been used successfully to treat some neurological disorders. For example, tardive dyskinesia, a movement disorder, has been treated successfully using oral choline (Davis et al., 1975), although an improvement in symptoms was only noticed when 16 g of choline a day was administered. This large dose was accompanied by cholinergic side effects like excessive salivation and sweating. A choline enriched diet has been shown to improve memory in old mice (Bartus et al., 1980), although enrichment of the diet with choline or its precursor, lecithin, has proved unsuccessful in the management of human degenerative disorders such as Alzheimer's Disease (Iversen, 1993). It has been concluded that the control mechanisms for choline uptake must occur distal to the blood brain barrier.

The biosynthesis of ACh in the brain is determined by the availability of the precursors acetyl-CoA and choline. Acetyl-CoA is produced from pyruvate via oxidative metabolism. Choline, for the biosynthesis of ACh, gains access to cholinergic neurones via a high affinity choline uptake transporter. The choline is supplied to the neurones from the extracellular fluid surrounding them and is derived from three sources; release from
choline containing phospholipids, hydrolysis of released ACh by the enzyme AChE and from plasma.

4.1.1 Neuronal Choline Uptake

The processes that control the synthesis of ACh are the availability of the precursors and the activity of the synthetic enzyme, ChAT (5.1). Sufficient acetyl-CoA for the synthesis of ACh is provided as long as oxidative metabolism is maintained. Choline is exchanged between the cytoplasm of the neurone and the extracellular fluid via two transport mechanisms. A low affinity carrier system is common to all nerve cells and is the mechanism by which choline for the synthesis of phospholipids is introduced into the cell (Kuhar & Murrin, 1978). The Michaelis constant (K_M) of the low-affinity carrier is >30 μM. This choline transport system is sodium-independent and is widely distributed in neuronal tissues.

A second, high affinity, sodium-dependent mechanism is located specifically in cholinergic neurones. The K_M of the high-affinity carrier is <5 μM and choline transport is both sodium and chloride dependent and is highly sensitive to competitive inhibition by HC-3. This sodium-dependent transport of choline is believed to be rate limiting for the synthesis of ACh (Jope 1979).

A unique property of the choline transport system is that its activity is coupled to neuronal activity. Changes in transporter V_max reflect the activity of cholinergic neurones in vitro and in vivo. Murrin and Kuhar (1976) observed that potassium-depolarisation of synaptosomes stimulated the rate of choline uptake. The rate of choline uptake is also related inversely to the
ACh content in synaptosomes (Jenden et al., 1976) and brain slices (Antonelli et al., 1981). It appears from these observations that a decrease in the ACh content of the nerve terminals stimulates the transport of choline into the nerve terminals. Although the precise control of ACh levels has not yet been elucidated, the availability of the precursor, choline, which may be rate limiting for ACh synthesis, may be an important factor. Inhibition of choline transport by relevant doses of volatile anaesthetic agents may contribute to reduced evoked ACh release and thereby reduced cholinergic excitability during anaesthesia.

Two series of experiments were performed to investigate the sensitivity of choline transport to the volatile anaesthetics, enflurane, halothane and isoflurane.

The first series of experiments examined the effects of equipotent doses of the three commonly employed volatile agents, enflurane, halothane and isoflurane on total choline uptake into synaptosomes prepared on sucrose gradients. The dose administered was at the top end of the clinical range, 2.4 rat MAC.

The second series of experiments investigated the dose-response relationship of halothane on total choline transport and the effect of 2.4 rat MAC halothane on low-affinity choline uptake in synaptosomes prepared using the Percoll gradient method.
4.2 Materials and Methods

4.2.1 Synaptosome Preparation

Synaptosomes were prepared according to the method of Gray and Whittaker (1962) and Dunkley et al (1988) (3.2)

4.2.2 [Methyl-\(^3\)H] Choline Uptake Assay

All assays were performed within 10 minutes of preparation of the synaptosome suspensions. The synaptosomal suspension was diluted with salt medium, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1.2 mM MgCl\(_2\), 20 mM Hepes and 100 \(\mu\)M sodium ethylenediaminetetraacetate (EDTA) to a final protein concentration of 0.62 mg ml\(^{-1}\), this was to ensure a final assay concentration of 0.5 mg ml\(^{-1}\) when the reaction mixture was added to the synaptosomes.

One millilitre of the diluted synaptosomal preparation was placed in a series of polycarbonate test tubes and exposed to air or air containing anaesthetic agent for 10 minutes in a water bath at 37°C. Following equilibration with gases, 0.25 ml of a reaction mixture containing choline chloride (final assay concentration ranging from 2 to 15 \(\mu\)M), neostigmine 100 \(\mu\)M, sodium pyruvate 5 mM and 50 \(\mu\)Ci of [methyl-\(^3\)H] choline chloride (1.0 mCi ml\(^{-1}\)) (Amersham International plc U.K.) was added.

A 300 \(\mu\)L sample was withdrawn and added to 1.0 ml of ice cold salt medium containing 200 \(\mu\)M choline chloride and centrifuged for 5 minutes in
a refrigerated microfuge. The pellet was washed, resuspended and recentrifuged twice before being resuspended in 0.25 ml of distilled water, and allowed to lyse for 4 hours before addition of 4 ml of Optiphase X (LKB Pharmacia) and scintillation counting. Radioactivity in these samples was used to establish baseline at the start of the assay. Assay mixtures were exposed to air or air containing anaesthetic agents for 45 minutes for the sucrose gradient prepared synaptosomal suspension. At the end of the incubation period two 300μL samples were withdrawn from each assay, centrifuged, resuspended and washed twice before being lysed in 0.25 ml distilled water and scintillation counting.

For the synaptosomes prepared using the Percoll method (Dunkley et al, 1988) the assay was similar to that described for the sucrose gradient synaptosomes except that the sampling time for the Percoll synaptosomes was 15 minutes.

4.2.3 Low-Affinity Choline Uptake

The low-affinity choline uptake assay was performed by replacing the sodium chloride in the salt medium with an equimolar concentration of lithium chloride. The assay was then followed as for total choline uptake. These experiments were conducted using 5 μM choline as the substrate. Parallel experiments were also conducted measuring total choline uptake simultaneously on the same synaptosome preparation. Low-affinity uptake was only investigated in synaptosomes prepared by the Percoll method.
4.2.4 Anaesthetic Apparatus

Volatile anaesthetic agents were delivered to the synaptosome preparation as described in 3.2.4.

All experiments were conducted with a paired control that was exposed to humidified air. The following concentrations of volatile agents were employed in these initial experiments: enflurane 5.5 %, halothane 3.0 % and isoflurane 3.5 %.

For the dose-response experiments the following halothane concentrations were delivered; 1, 2, 3, 5 and 10 %. The aqueous concentration of halothane in buffer and the time to reach equilibrium of 10 minutes was confirmed by n-heptane extraction and measurement in a Perkin-Elmer 8410 gas chromatograph (Rutledge et al, 1963) as described in 3.2.5.

For the low-affinity choline uptake experiments 3 % halothane was delivered to the synaptosomes.

4.2.5 Kinetic Parameters

Kinetic parameters, maximal velocity ($V_{\text{max}}$) and $K_m$ were calculated for choline uptake in each experiment using Pharm/PCS computer software (Tallarida & Murray, 1986). Results were compared using paired analysis of variance and Student's paired t-test. A value of $p<0.05$ was considered statistically significant. The dose-response curve was analysed statistically by
analysis of variance and an IC$_{50}$ value was obtained by computer assisted curve fitting (non-linear regression) using Graphpad (V2.0).

4.3 Results

4.3.1 Linearity

At a saturating substrate concentration (15 μM choline chloride), total $^3$H choline uptake was linear for 45 minutes in the synaptosome suspension prepared on sucrose gradients (figure 4.1). For all subsequent experiments the sampling time was 45 minutes. $^3$H choline uptake was linear for 20 minutes in the synaptosome suspension prepared on Percoll gradients (figure 4.2). For $^3$H choline uptake the sampling time was 15 minutes.

4.3.2 Volatile Anaesthetics and Choline Uptake

To compare the effects of equipotent doses of enflurane, halothane and isoflurane on choline uptake, synaptosomes prepared on sucrose gradients were used. The kinetic variables observed in the control experiments, where air only was delivered, $V_{\text{max}}$ was 1.01 pmoles mg$^{-1}$ min$^{-1}$ (SEM 0.08, n = 24) and the Michaelis constant, $K_m$, was 3.27 μM (SEM 0.26, n = 24). Figure 4.3 shows a representative air control experiment.
Figure 4.1
Total $^3$H choline uptake at saturating substrate concentration, (15 μM choline chloride), for synaptosomes prepared on sucrose gradients. Points are the mean of 2 determinations.
Figure 4.2
Total $^3$H choline uptake at saturating substrate concentration (15 μM choline chloride) for synaptosomes prepared on Percoll gradients. Points are single determinations.
A significant reduction in $V_{\text{max}}$ was observed for total choline uptake in the presence of 3% halothane (figure 4.4). The change in kinetic variables is illustrated in a double reciprocal transformation (Lineweaver-Burk) (figure 4.5). There was no significant change in $K_m$ in the presence of 3% halothane.

Similarly, for 5.5% enflurane (figure 4.6) and 3.5% isoflurane (figure 4.7), there were significant reductions in $V_{\text{max}}$, with no change in $K_m$. In 11 experiments the reduction in $V_{\text{max}}$ for enflurane and isoflurane was 24 and 23% respectively, an equipotent dose of halothane caused a 38% reduction in $V_{\text{max}}$ (12 experiments) (table 4.1).
Figure 4.3
Representative air control experiment. Velocity of total choline uptake plotted against substrate concentration.
Figure 4.4
Representative experiment showing the effect of 3% halothane on total $[^3H]$ choline uptake. $\nabla$ = air control, $\bullet$ = 3% halothane. Points are the mean of 2 determinations.
Figure 4.5
Lineweaver-Burk analysis of total choline uptake into rat cortical synaptosomes. This representative experiment shows the effect of 3% halothane on synaptosomes prepared on sucrose gradients. There is a significant reduction in the $V_{\text{max}}$, with no significant change in the $K_m$. ▼ = control, • = 3% halothane. Points are means of 2 determinations.
Figure 4.6
Lineweaver-Burk analysis of total choline uptake into rat cortical synaptosomes. This representative experiment shows the effect of 5.5 % enflurane. o = control, ● = 5.5 % enflurane. Points are means of 2 determinations.
Figure 4.7
Lineweaver-Burk analysis of total choline uptake into rat cortical synaptosomes. This representative experiment shows the effect of 3.5% isoflurane. • = control, △ = 3.5% isoflurane. Points are mean of 2 determinations.
Table 4.1

Vmax and Km Determined For Total $[^3H]$ Choline Uptake Into Synaptosomes Prepared Using Sucrose Gradients in the Absence and Presence of Volatile Anaesthetics.

<table>
<thead>
<tr>
<th></th>
<th>Vmax pmoles mg$^{-1}$ min$^{-1}$</th>
<th>Km $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air Agent</td>
<td>Air Agent</td>
</tr>
<tr>
<td>Enflurane</td>
<td>0.97 (0.10)* 0.74 (0.07)</td>
<td>3.17 (0.36)</td>
</tr>
<tr>
<td>Halothane</td>
<td>1.09 (0.12)** 0.68 (0.07)</td>
<td>3.27 (0.36)</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>0.92 (0.09)$\infty$ 0.71 (0.08)</td>
<td>3.33 (0.38)</td>
</tr>
</tbody>
</table>

Mean ± (SEM)

n = 11 for enflurane. *p < 0.05
n = 12 for halothane. **p < 0.05
n = 11 for isoflurane. $\infty$ p < 0.05.
4.3.3 Low-Affinity Choline Uptake

Low-affinity choline uptake was approximately 25% of the total at a substrate concentration of 5 μM and was not altered significantly in the presence of 3% halothane (figure 4.8).

4.3.4 Dose-Response

The total choline uptake rate was significantly higher in the Percoll prepared synaptosomes (figure 4.9). The mean $V_{max}$ for this preparation in air was $2.46 \pm 0.26$ pmoles mg$^{-1}$ min$^{-1}$ (SEM, n = 14) which was two and a half fold higher than sucrose gradient prepared synaptosomes. The $K_m$ of $4.82 \pm 0.60$ μM (SEM, n = 14) for Percoll synaptosomes was also significantly higher than determined in sucrose gradient synaptosomes.

At each concentration of halothane tested from 1% up to 10%, there was a significant reduction in the total choline uptake. Maximal inhibition was observed at 3% halothane, above which no further inhibition of uptake was observed. The IC$_{50}$ for halothane-induced inhibition of total choline uptake was $1.38 \% \pm 0.32 \%$ (figure 4.10).
Figure 4.8

The contribution of low-affinity uptake to total choline uptake (synaptosomes prepared on Percoll gradients). 3% halothane had no effect upon low affinity uptake compared to control (p > 0.80). Values represent means ± SEM (n = 4).
Figure 4.9
Representative air control experiment for synaptosomes prepared on Percoll gradients. A) Velocity of choline uptake plotted against substrate concentration, B) data as Lineweaver-Burk plot.
Figure 4.10

Effect of halothane on synaptosomal total choline uptake, (synaptosomes prepared on Percoll gradients). There were 10 determinations for 1, 2 and 3 % halothane, 5 for 5 % and 3 for 10 % halothane. Control uptake values were as quoted in results section. Points represent means ± SEM.
4.4 Discussion

The cholinergic basal forebrain is implicated in the maintenance of sleep and arousal (2.1.4). There is recent and historical evidence that volatile anaesthetic agents interfere with cholinergic transmission (2.2).

Choline uptake is believed to be the rate limiting step for ACh synthesis (Jope 1979) and the levels of ACh in brain tissue and the rates of synthesis are reduced when the transport of choline into the cells is inhibited by HC-3 (Guyenet et al, 1973), the specific competitive antagonist of high-affinity choline uptake. This uptake mechanism represents a possible sensitive point in ACh metabolism at which volatile anaesthetic agents may exert their action. To investigate the sensitivity of choline uptake to volatile anaesthetics, the alteration in [methyl-\(^3\)H]choline uptake in rat cortical synaptosomes was investigated in the absence and presence of either enflurane, halothane or isoflurane. Initially, these agents were studied at a supra-anaesthetic concentration equivalent to 2.4 times the rat minimum alveolar concentration (2.4 rat MAC) using synaptosomes prepared on sucrose gradients. In the presence of each agent \(V_{\text{max}}\) for choline uptake was reduced significantly with no significant change in \(K_{m}\) indicative of non-competitive inhibition of uptake.

The interpretation of the data was complicated by the different values that have been obtained for MAC in the rat. There was probably no real difference between the agents studied as there has been no detailed examination of the anaesthetic potencies of all three agents in the rat.
In this study, the rats were approximately 3 months old and the value of MAC for halothane applied was 1.24 % (White et al., 1974). Using this value equilibration of samples with 3 % halothane equated to 2.4 rat MAC. This dose of halothane inhibited choline uptake by 38 % for sucrose synaptosomes and 32 % for Percoll synaptosomes. For isoflurane 3.5 % delivered also equated to 2.4 rat MAC (White et al., 1974), but this apparently equipotent dose inhibited choline uptake in the sucrose synaptosomes by 24 %. For enflurane the calculated MAC value is 2.2 % (Mazze et al., 1985), although the methodology used to determine MAC was slightly different to that used by White et al. (1974). Thus the dose of enflurane delivered was 2.5 rat MAC, which inhibited choline uptake by 23 %. However, different values for the MAC of halothane have been determined, 0.88 % (Waizer et al., 1973), 1.03 % (Mazze et al., 1985), 1.17 % (Vitez et al., 1974), which would make the dose of halothane delivered initially in this study, 3.4, 2.9, and 2.6 rat MAC respectively. To make direct comparisons between the effects at defined MACs of agents may lead to misleading interpretation of the data because of the different methods of determining MAC. It is likely that the greater effect of halothane observed in this study is due to the incomplete potency studies that have been conducted, and not to a unique property of halothane.

Investigation of the dose-response relationship for halothane was performed on synaptosomes prepared from Percoll gradients. Both preparations showed similar reductions in $V_{\text{max}}$ for choline uptake when exposed to 3 % halothane, 38 % for synaptosomes prepared on sucrose gradients and 32 % for synaptosomes prepared on Percoll gradients. The
Percoll gradient method was employed for the dose-response part of the study for three main reasons. First, the preparation time is considerably shortened, 2 hours compared to 6 for the sucrose gradient method. Second, the Percoll gradient method allows the separation of viable from non-viable synaptosomes. Third, the method allows the preparation to remain isotonic throughout preparation (Harrison et al, 1988). These factors are responsible for the improved uptake rates seen with the Percoll synaptosomes and are explained in detail in 3.1.2.

The dose response for halothane revealed an increasing inhibition of choline uptake, with increasing concentrations of halothane, up to a maximum inhibition of 32% at 3% halothane, above which there was no further inhibition (figure 4.10). The concentration of halothane producing a half-maximal inhibition of the anaesthetic-sensitive component of choline uptake of 1.5% was close to the rat MAC value for halothane of 1.24% (White et al, 1974) which would be consistent with a role for choline uptake in the process of anaesthesia. Low affinity, sodium independent uptake contributed 25% of choline uptake to the total choline uptake measured and was consistent with previous studies (Mrak & North, 1988, Richardson, 1986). This component of choline uptake was insensitive to 3% halothane (figure 4.8).

Little work has been directed to the study of the alteration of presynaptic ACh metabolism by volatile anaesthetic agents. In contrast to the above results, a previous study reported a "competitive-like" inhibition of choline uptake by halothane at 3% partial pressure (Johnson & Hartzell, 1985) in rat cortical synaptosomes. In these experiments on a small number of samples, $K_m$ was reported to increase in the presence of halothane with no
change in $V_{\text{max}}$. As part of this thesis the published data was reanalysed (Instat v2.02 Graphpad Software). It was found that the differences reported were not significant statistically ($p = 0.09$). As choline and the volatile anaesthetic agents are unrelated structurally, the statistically significant non-competitive inhibition indicated by our study is a more likely mechanism.

The effect of halothane on choline uptake has also been observed in rat cortical slices. Bazil et al (1987) reported that a 1 MAC dose of halothane had no significant effect on choline uptake. However, the 1 MAC dose did reduce the $V_{\text{max}}$ for choline uptake in this preparation by 19%. Perhaps a fuller dose response investigation would have revealed a similar inhibition to that reported from this study.

A significant decrease in ACh release from the pontine reticular formation of the cat in vivo in the presence of 1 MAC halothane has been shown recently (Keifer et al, 1994). In addition, inhibition of depolarisation-evoked release of ACh from rat cortical synaptosomes (Johnson & Hartzell, 1985) and cortical slices (this study) by halothane has been reported. The contribution of the inhibition of choline uptake to these decreases in presynaptic ACh release is not known. The inhibition in ACh release may result from reduced availability of ACh due to inhibition of the choline uptake mechanism. It is known that competitive inhibition of choline uptake by hemicholinium-3 leads to a reduction in brain ACh levels (Guyenet et al, 1973) and, similarly, the non-competitive inhibition demonstrated by volatile agents is likely to result in reduced ACh concentrations. There is also likely to be a direct inhibition of the ACh release process. These possibilities remain to be investigated.
Choline uptake in rat brain synaptosomes is also sensitive to ethanol, being inhibited in a non-competitive manner (Mrak & North, 1988) with similar changes in $V_{\text{max}}$, but not $K_m$, produced by volatile agents in this study. At ethanol concentrations of 50 mM, a concentration that in the human would bring on advanced inebriation, choline uptake was reduced by 15%. Interestingly, in the same study uptake of the inhibitory neurotransmitter GABA was unaffected by ethanol. Similar insensitivity to volatile anaesthetic agents has been reported for GABA uptake (Cheng & Brunner, 1981b). By contrast, 5-HT uptake is reduced in the presence of halothane, although this has been shown to be due to be an indirect interaction with the transporter (Martin et al, 1990a). El-Maghrabi and Eckenhoff (1993) demonstrated an inhibition of dopamine uptake into rat brain synaptosomes by halothane, although the IC$_{50}$ was 0.72 mM, which approximates to 2.5 rat MAC. It is unlikely that an effect occurring at such a supraclinical concentration of agent contributes to the process of anaesthesia, unless only a small percentage reduction is required for unconsciousness.

The amino acid sequences of transporters for a number of neurotransmitters and transmitter metabolites have been deduced recently including those for choline (Maysaer et al, 1992), GABA (Guastella et al, 1990) and 5-HT (Blakely et al, 1991). This family of transporters share structural similarities, with 12 proposed transmembrane domains, and are likely to share functional mechanisms (Wonnall & Williams, 1994). The choline transporter cloned by Maysaer et al (1992) was derived from rat spinal cord and was found to contain 635 amino acids, it shared 37-49% identity with other known transporters. The high-affinity uptake systems share
functional similarities and they are all dependent on sodium for activity. There are areas in the transporters where the amino-acid sequences are well conserved but the N and C terminals are variable in sequence and length and are not essential for transport (Worrall & Williams, 1994). Although similar in structure, and possibly function, the response of these transporters to the presence of volatile anaesthetic agents and ethanol differs. The IC50 for inhibition of transport by halothane is 0.38 mM for choline (this investigation), 0.72 mM for dopamine (El-Maghrabi & Eckenhoff, 1993) and 1.0 mM for 5-HT (Martin et al, 1990b). Such differential sensitivity could underlie apparent selectivity of agents for individual transmitter systems.

There has been limited work on the transport mechanisms of the family of transmitter transporters, although it is hoped that molecular biological studies will reveal eventually more information on which amino acid residues are involved in the transport process (Worrall & Williams, 1994). However, the change in the capacity of the choline transporter with neuronal activity, reflected in the $V_{\text{max}}$ of the transporter, may be caused either by an increase in the number of functional transporters or in turnover rate of existing carriers (Murrin & Kuhar, 1976). Choline transport activity may be modified by specific interaction of ethanol and anaesthetic agents with the target protein. Alternatively, susceptibility of choline transport to these agents could reflect more generalised changes in membrane properties such as increased fluidity (Appadu et al, 1993). Which mechanism is responsible for the modification of choline transport remains to be elucidated.

Investigations have also revealed that lipids may have a regulatory role in choline uptake. Boksa et al (1988) observed that arachidonic acid blocked
synaptosomal choline uptake and subsequent ACh synthesis, although it had no effect on the release of ACh. Pre treatment of rat synaptic membranes with phospholipase A2 was also found to enhance the affinity and the capacity of [3H] HC-3 binding (Yamada et al., 1988). The enhancement of [3H] HC-3 binding in the presence of phospholipase A2 was calcium dependent and so could be modified by depolarisation and calcium influx. It was proposed that following depolarisation arachidonic acid and calcium dependent activation of phospholipase A2 have opposing roles to control choline transport (Salterelli et al., 1990). Calmodulin inhibitors blocked the increase in [3H] HC-3 binding and choline uptake, and it has been suggested that the unmasking of new transporters may be mediated via calmodulin (Yamada et al., 1991). One possibility for the action of the volatile agents is by modification of the calmodulin-dependent choline uptake mechanism, although there is no direct evidence to support this possibility.

Further investigations have concentrated on the possible role of second messengers in the control of choline uptake. It is possible that the alteration in activity of choline transport measured in this study is reflecting responses to changes in cellular signalling mechanisms (Lambert, 1993). In insect species, choline uptake activity has been found to be elevated by activation of protein kinases A and C (Knipper et al., 1992). Specifically Knipper et al. (1992) concluded that activation of protein kinase A increased the total number of [3H] HC-3 binding sites by increasing the total number of carriers, whereas activation of protein kinase C prevented the down regulation of the transporters already present.
Stimulation of protein kinase C by volatile anaesthetic agents (Slater et al, 1993) would be inconsistent with anaesthetic induced regulation of choline transport via protein kinase C as choline uptake was reduced by volatile agents. Phosphorylation of the transport protein, therefore, is unlikely to be involved in the reduction in choline uptake observed in this study.

The choline uptake mechanism has the lowest IC50 amongst candidate transmitter transporters studied so far, which indicates a more likely role for choline uptake in the anaesthetic process than other transporters. This reinforces the view that general anaesthetic targets are likely to be on specific protein targets and not a generalised effect on the membrane environment (Franks & Lieb, 1994). Further studies on the nature of the transport mechanism are needed before the mechanism of anaesthetic interaction is understood.

The results of this study show that the activity of the choline transporter in cortical synaptosomes is inhibited in the presence of volatile anaesthetic agents. The level of inhibition produced by clinically relevant doses of anaesthetics was similar in two synaptosome preparations and was also comparable to the inhibition achieved in rat synaptosomes with doses of alcohol that would cause unconsciousness in vivo. Such inhibition may underlie the decreases in release of ACh observed in vivo from the surface of brains of anaesthetised animals (Kanai & Szerb, 1965, Keifer et al, 1994). Whether inhibition of choline uptake is the prime cause of the reduction in ACh release or is a consequence of the reduction in release remains to be determined. Salehmoghaddam and Collier (1976) demonstrated that the stores of ACh in rat cortical slices lasted for 10 minutes following potassium-
depolarisation. Thus it is unlikely that the halothane induced reduction in choline uptake is the principal cause of the reduction in ACh release that has been shown both in vitro and in vivo.

To conclude, the high-affinity choline transporter is the most sensitive of this family of proteins to volatile agents. The anaesthetic-induced reduction in precursor uptake may contribute to the maintenance of anaesthesia but is unlikely to be a direct cause of the loss of consciousness.
5. Choline Acetyltransferase

5.1 Introduction

Choline acetyltransferase (ChAT) is the enzyme that catalyses acetylcholine biosynthesis which involves the transfer of an acetyl group from acetyl-CoA to choline. ChAT appears to operate in cholinergic neurones well below its $K_m$ and so is not believed to be the rate-limiting step for ACh biosynthesis (Rylett & Schmidt 1993). It is essential for the synthesis of ACh, although it is assumed that it has no regulatory role in the maintenance of the levels of ACh or activation of the synthesis. However, Collier et al (1993) found that when colchicine was applied to the cervical sympathetic trunk of the cat, there was a decrease in ACh content which was associated with a similar decrease in ChAT activity. This could indicate that ChAT does have some role in the regulation of ACh levels.

ChAT has a widespread distribution within the central nervous system and its presence has been utilised in order to map cholinergic pathways (Butcher et al, 1993). Acetylcholinesterase (AChE) was originally used as a marker of cholinergic neurons but AChE is found in chinoceptive neurones as well as ACh transmitting neurones. Eckenstein and Thoenen (1982) developed antibodies to ChAT which were used histochemically to investigate cholinergic neuroanatomy. The cellular presence of ChAT indicates that a neurone is cholinergic, synthesises and utilises ACh as a neurotransmitter. The levels of both ChAT and ACh appear to be similar in
cholinergic tissue suggesting that the sole function of ChAT is the production of ACh. However, there are a number of non-neuronal sources of ChAT, for example in the primate placenta and in spermatozoa (Salvaterra 1989). The function of this non-neuronal ChAT is unknown at present.

More recently, questions have been raised concerning the accuracy of ChAT and AChE immunohistochemistry. Oh et al (1992) described the use of in situ hybridisation histochemistry to identify cholinergic neurons. There is generally good agreement between areas identified to contain mRNA encoding ChAT and ChAT immunoreactivity, although there are exceptions which are probably caused by cross reactivity of ChAT antibodies with proteins other than ChAT.

ChAT has a diffuse distribution throughout cholinergic neurones and is present in the cell bodies, axons and synaptic terminals (Salvaterra, 1989). The precise intracellular location of ChAT is still not known. Within the nerve terminals ChAT is believed to be located in the cytoplasm, designated cChAT, but 10-20% of the enzyme is membrane bound (Salvaterra 1989). This membrane bound ChAT (mChAT) may form part of a presynaptic membrane bound complex that is associated with the sodium-dependent high affinity choline transporter (Jope 1979). The mChAT appears to be biochemically different from cChAT but the functional role of the membrane bound enzyme is still unclear (Benishin & Carroll, 1983). ChAT protein may also be located on the extracellular synaptic membrane surface, as, antibodies to ChAT can be used to purify cholinergic synaptosomes (Docherty et al, 1987), although this may be an artefact of the synaptosome preparation procedure.
Despite the lack of evidence for a role for ChAT in the regulation of 
ACh synthesis numerous studies have been conducted looking at the short 
and long term regulation of ChAT (Salvaterra 1989). Rossier et al (1977) 
discovered that ChAT from rat brain is activated by anions, specifically 
chloride. Up to a 60 fold increase in the V_{\text{max}} of the enzyme was reported at 
low ionic strength. The activity of ChAT can be modulated by some 
hormones and growth factors including thyroid hormone and nerve growth 
factor but it is the short term regulation of ChAT activity that is likely to be 
susceptible to the action of volatile anaesthetic agents.

There is also some evidence that ChAT may be modified by 
phosphorylation (Lapchak and Collier, 1988). The gene sequence of the rat 
brain ChAT cDNA revealed four serine and threonine residues which may 
serve as potential phosphorylation sites (Brice et al 1989) and may regulate 
the activity of the enzyme. It has been confirmed that cChAT exists as a 
phosphoprotein in rat hippocampal synaptosomes and the level of the enzyme 
was dependent on cytosolic calcium (Schmidt and Rylett 1990). Thus there 
are potential targets for the action of the volatile anaesthetic agents to interact 
with ChAT \textit{in vivo}. One potential interaction site is with mChAT, but at 
present there is no identified physiological role for this part of the enzyme 
fraction and there is no evidence that it displays any different functional role 
from the cytoplasmic enzyme (Tucek 1990).

There have been few studies on the interaction of drugs with ChAT. 
Volatile anaesthetic agents may act by altering membrane function (Miller, 
1985) and so one would predict that an enzyme that is located mainly in the 
cytosol would be unaffected by volatile anaesthetic agents. However, either
the membrane bound ChAT or other second messengers may influence the activity of ChAT and so the activity of the enzyme may be affected indirectly by general anaesthetics.

In this study the effects of the halogenated anaesthetics enflurane, halothane and isoflurane on ChAT activity in rat cortical synaptosomes were investigated.
5.2 Methods

5.2.1 Preparation of Synaptosomes

Rat cortical synaptosomes were prepared using discontinuous sucrose gradients (Gray and Whittaker, 1962) as described in 3.2.1.

5.2.2 Principles of The Choline Acetyltransferase Assay

A radiochemical assay method was used for the determination of ChAT activity as described by Fonnum (1975). This method separates labelled ACh formed during the assay from labelled substrate, acetyl-CoA, by liquid-cation exchange using sodium tetraphenylboron (Kalignost, Koch Light Chemicals). When a reaction mixture containing kalignost and both ACh and acetyl-CoA is mixed with a toluene containing scintillation fluid, the ACh is extracted into the toluene phase while the acetyl-CoA is left in the aqueous phase. The toluene phase separates from the aqueous phase and a clear junction is visible between the two phases. The upper, toluene phase, containing ACh is easily removed and placed in scintillation vials prior to liquid scintillation spectroscopy.

5.2.3 Choline Acetyltransferase Assay

Synaptosomes were suspended in an incubation medium containing 300 mM NaCl, 20 mM EDTA, 1 mM neostigmine, 0.5 % Triton X-100, 50
mM sodium phosphate (pH 7.4), to a protein concentration of 0.5 mg ml\(^{-1}\). A substrate mixture (0.5 ml) containing choline bromide at concentrations ranging from 0.2 mM to 4.0 mM, 2 mM acetyl-CoA and 0.5 µCi \(^3\)H acetyl-CoA (NEN Research Products) in the incubation medium but without the Triton X-100 was placed in a 10 ml polycarbonate test tube at 37 °C in a water bath and exposed to air or air plus anaesthetic agent to equilibrate the solutions.

After allowing 10 minutes for equilibration, 0.5 ml of the synaptosomal suspension (0.5 mg ml\(^{-1}\)) was added to the substrate mixture and incubated at 37 °C. Immediately after the addition of the synaptosomal suspension, a 100 µL aliquot was withdrawn and added to 2 ml of acetonitrile which contained Kalignost at a concentration of 5 mg ml\(^{-1}\) in glass test tubes. Following the addition of the synaptosomal suspension 10 ml of scintillation cocktail, Optiscint T (LKB Pharmacia), was added to the sample and with the glass stopper firmly placed on the test tube it was gently inverted for one minute. It is during this procedure that the ACh is extracted into the toluene phase. Over vigorous shaking of the mixture at this stage would have resulted in stable mixtures and poor separation of ACh from acetyl-CoA. This initial 100 µL sample was used as time zero control for background subtraction from the test samples.

After 60 minutes of incubation in the water bath 300 µL samples were withdrawn in duplicate and added to 2 ml of acetonitrile with Kalignost (5 mg ml\(^{-1}\)). For both the time zero and 60 minute samples the organic and aqueous layers were allowed to separate for 60 minutes. The upper organic layer was pipetted in two 3.5 ml aliquots into separate scintillation inserts, thus each
sample was counted in two portions. This was done so that the lower aqueous layer was not disturbed during the pipetting procedure. At the end of each experiment a 50 μL sample was withdrawn from each assay tube and placed in a scintillation insert with 4 ml of Optiphase (LKB Pharmacia). These samples acted as totals for later calculation of kinetic parameters. Protein concentration of each preparation was measured according to the method of Lowry et al (1951) using bovine serum albumin as standard.

5.2.3.1 Assay Linearity

Before any experiments involving anaesthetics were performed the linearity of the assay was established. The assay conditions were the same as those in the method above but a saturating substrate concentration was employed of 30 mM choline bromide and 100 μL samples were withdrawn in duplicate. Samples were taken at time zero, 15, 30, 45, 60, 75, 90 and 120 minutes.

5.2.4 Anaesthetic Apparatus

Air and air containing anaesthetic agents were delivered to the synaptosome preparation as described in 3.2.4. Initially halothane was delivered at 3 % concentration. This was to directly compare the results with an earlier study by Johnson and Hartzell (1985) that had also used 3 % halothane, which corresponds to 4 human MAC. Enflurane and isoflurane
were then delivered at equipotent human MAC concentrations, which are 6.5 % and 4.5 % respectively.

5.2.5 Kinetic Parameters

Michaelis-Menten kinetic parameters, $V_{\text{max}}$ and $K_m$ were calculated for ChAT in each experiment using Pharm/PCS computer software (Tallarida & Murray, 1986).

5.2.6 Statistical Analysis

Results were compared using analysis of variance, Student's paired t-test and Wilcoxon’s signed rank test. A value of $p<0.05$ was considered statistically significant.
5.3 Results

5.3.1 Linearity

Under the conditions employed ACh synthesis was linear over 120 minutes \( r = 0.99 \) (figure 5.1).

5.3.2 Results From Anaesthetic Experiments

There were twelve experiments conducted in each group, each had its own paired control value. Figure 5.2 shows a representative control experiment demonstrating the substrate dependence of ChAT activity.

The \( V_{\text{max}} \) and \( K_m \) for ChAT were determined for synaptosomes exposed to air only or air containing anaesthetic agents. The results of the initial experiments performed for anaesthetic concentrations of 4 human MAC are shown in table 5.1.

Enflurane at 6.5 % reduced significantly the \( K_m \) for choline determined for ChAT while the \( V_{\text{max}} \) did not alter significantly. A representative experiment is shown in figure 5.3. The effect of enflurane was not clearly seen in plots of substrate dependence, but became more apparent when plotted in the Lineweaver-Burk, double reciprocal, form (figure 5.4).

By contrast, at equivalent human anaesthetic doses halothane, 3 % and isoflurane 4.5 % did not produce any significant effect on \( V_{\text{max}} \) or \( K_m \) of the synaptosomal ChAT (table 5.1). A representative experiment from the
Figure 5.1
ChAT assay linearity, (r = 0.99).
Figure 5.2
A representative air control experiment demonstrating the substrate dependence of ChAT activity.
Table 5.1

Effect of Volatile Anaesthetic Agents on Choline Acetyltransferase Activity

<table>
<thead>
<tr>
<th></th>
<th>Vmax nmoles mg⁻¹ min⁻¹</th>
<th>Km mM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Air</strong></td>
<td><strong>Agent</strong></td>
<td><strong>Air</strong></td>
</tr>
<tr>
<td>Enflurane</td>
<td>0.84 (0.11)</td>
<td>0.80 (0.06)</td>
</tr>
<tr>
<td>Halothane</td>
<td>0.80 (0.04)</td>
<td>0.84 (0.05)</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>0.80 (0.04)</td>
<td>0.78 (0.08)</td>
</tr>
</tbody>
</table>

Results show mean ± (SEM). N = 12 for each agent.
*p = 0.012
Figure 5.3
A representative experiment of ChAT activity in the absence (\(\nabla\)) and presence of 6.5% enflurane (\(\nabla\)).
Figure 5.4
The same data as Figure 3 plotted in the double reciprocal, Lineweaver-Burk form. (V) = air control, (▼) = 6.5% enflurane.
Figure 5.5
A representative Lineweaver-Burk plot of ChAT activity demonstrating no significant change in $V_{\text{max}}$ or $K_m$ by halothane. (o) = air, (●) = 3 % halothane.
Figure 5.6
A representative Lineweaver-Burk plot of ChAT activity demonstrating no significant change in $V_{\text{max}}$ or $K_m$ by isoflurane. (□) = air, (■) = 4.5 % isoflurane.
halothane and isoflurane experiments is shown in figures 5.5 and 5.6 respectively.

The dose response relationship for the reduction in $K_m$ by enflurane was investigated at 1.1 % enflurane, 2.2 % enflurane and 4.4 % enflurane. Enflurane at 2.2 % and 4.4 % reduced significantly the $K_m$ determined for ChAT ($p=0.043$) by 13 and 26 % respectively. The $K_m$ for ChAT was also reduced by enflurane 1.1 %, but this change was not statistically significant. The relationship between the concentration of enflurane and the reduction in $K_m$ for ChAT is shown in figure 5.7. The $V_{max}$ did not alter significantly at any of the concentrations of enflurane studied (figure 5.8).
**Figure 5.7**

Effect of enflurane on ChAT activity. % change in $K_m$ with increasing dose of enflurane. Values are means ± SEM. There were 5 determinations for each point up to 4.4 % enflurane and 12 determinations for 6.5 % enflurane. *difference from control is statistically significant (p<0.05).
Figure 5.8
Effect of enflurane on ChAT activity. % change from control in $V_{\text{max}}$ with increasing dose of enflurane. There were 5 determinations up to 4.4 % enflurane and 12 for 6.5 % enflurane.
5.4 Discussion

This study has demonstrated that enflurane, at concentrations of 6.5 % lowered the $K_m$ of ChAT without altering the $V_{\text{max}}$, while halothane, 3 %, and isoflurane, 4.5 %, had no effect on either variable. These concentrations of anaesthetics correspond to approximately four human MAC. However, these doses corresponded to 3 rat MAC (Mazze et al., 1985). A subsequent dose response analysis of the enflurane induced effect on $K_m$ showed a progressive reduction from a concentration of 1.1 % (0.5 rat MAC) up to 6.5 % (3 rat MAC), although the reduction at 0.5 MAC was not statistically significant.

The control values for ChAT activity compare favourably to those published by other workers. In this study the $V_{\text{max}}$ obtained in control experiments was 0.8 nmoles mg$^{-1}$ min$^{-1}$. Lapchak and Collier (1988) reported a value of 0.4 nmoles mg$^{-1}$ min$^{-1}$ for ChAT activity from rat hippocampal synaptosomes. Schimdt and Rylett (1993), also using the rat hippocampal synaptosome preparation, observed a value for ChAT activity of 0.17 nmoles mg$^{-1}$ min$^{-1}$. Williams and Rylett (1990), working with cortical synaptosomes, found the ChAT activity to be 0.58 nmoles mg$^{-1}$ min$^{-1}$, a value similar to that obtained in this study with the same synaptosome preparation.

For this study on ChAT a synaptosome preparation from rat cortex was used. The preparation was prepared by homogenisation of the tissue which results in shearing of nerve endings and then resealing. The synaptosomes contain the necessary machinery for neurotransmitter uptake, synthesis and
release (McMahon & Nicholls 1991). However, there are limitations with the preparation which may influence the interpretation of this study.

When synaptosomes are produced from the rat cortex a heterogeneous vesicle population is produced that contains synaptosomes from nerve endings secreting many different transmitters. This may lead to non-physiological communication between presynaptic mechanisms when one particular transmitter system is being investigated. It is generally agreed that a cell that contains ChAT is cholinergic (Salvaterra 1989), although there is evidence that some brain regions possess intrinsic as well as extrinsic cholinergic synaptic inputs. This has developed because of the identification of ChAT positive areas in the cortex using immunohistochemistry (Eckenstein & Baughman, 1982). The role of these intrinsic ChAT positive neurones is unknown, but the majority of them also contain the neuroactive substance vasoactive intestinal polypeptide (VIP) (Eckenstein & Baughman, 1984). When a synaptosome preparation is made there is no way of distinguishing the extrinsic from the intrinsic ChAT positive cells. The assay employed in these studies measures total ChAT activity in the synaptosome preparation and does not differentiate between the different possible sources of ChAT. In the heterogeneous vesicle population there are also likely to be indirect effects on the cholinergic synaptosomes from other transmitter systems that are present.

VIP is known to increase ACh synthesis in rat hippocampal slices (Lapchak & Collier 1988). ChAT activity was increased by VIP in a hippocampal homogenate with a 30% increase in ACh synthesis when the homogenates were exposed to $10^{-7}$ M VIP. Interestingly, this stimulation was
not observed in a soluble enzyme preparation, although in trying to identify if second messenger systems could be responsible for the stimulation in the whole homogenate preparation the stimulation was not associated with either protein kinase C or adenylate cyclase activation. The simplest explanation for this anomaly, is that VIP had a direct effect on G-proteins. The only treatment that abolished the increase in ACh production caused by the application of VIP was the removal of extracellular calcium from the preparation. The dual presence of ACh and VIP in 80 % of the intrinsic cholinergic neurones in the rat cortex (Eckenstein & Baughman 1984) implies that any modification of ChAT activity may be caused either by a direct effect on ChAT or a secondary effect due to the influence of VIP.

Docherty et al (1987) have tried to overcome the problem of the heterogeneous population of transmitters that are obtained with synaptosome preparations by purifying sub-populations of synaptosomes. Cholinergic synaptosomes can be purified by using antiserum that recognises ChAT, which is expressed on the outer membrane of synaptosomes. GABAergic synaptosomes can also be purified using antiserum that recognises glutamate decarboxylase (Docherty et al, 1987). By purification of specific populations of synaptosomes the problem of other transmitters interfering in pre or post synaptic events should be reduced. However, Docherty et al (1987) found that when the cholinergic synaptosomes were exposed to chemical depolarising agents glutamate and aspartate were also released. Thus, although the preparation was purer than the whole synaptosome preparation several transmitters were released which again could lead to non-
physiological communication between synaptosomes and possibly obscure any drug effects.

During this investigation the synaptosome preparation was lysed with detergent, Triton X-100, so that access was gained to the intracellular ChAT. There is evidence that ChAT solubilised by detergents, such as employed in this study, may not be representative of the form of the enzyme that is present in vivo (Collier et al., 1993). There are also conflicting reports of the relative contributions to acetylcholine synthesis from the membrane bound form of the enzyme. A recent investigation revealed that the basal synthesis of Ach was not dependent upon membrane bound ChAT in rat hippocampal synaptosomes (Schimdt & Rylett 1993). Although a detergent was employed in this study it is unlikely that all the cellular components were removed from the preparation and so contributions to ChAT activity could have come from the cytosolic and the membrane bound components. To summarise, the heterogeneous nature of the synaptosome preparation, together with knowledge that more than one transmitter may be present in cholinergic neurones makes the results on one enzyme system difficult to interpret.

There is a lack of drugs that specifically affect ChAT (Collier et al., 1993), and few studies have been conducted into the possible interaction of volatile anaesthetic agents and ChAT. Ethanol, in concentrations which caused moderate to severe intoxication in the rat, had no effect on ChAT activity in the rat cortical slice preparation (Kalant et al., 1967).

A study by Johnson and Hartzell (1985) indicated that halothane had no effect on ChAT activity up to a concentration of 4 %, which corresponds to 3.9 rat MAC. This study also found no effect with 3 % halothane (3.0 rat
MAC). The radiochemical assay for ChAT, described by Fonnum (1975) was also employed in this investigation. The observations from this study on the effect of halothane on ChAT agree with the findings of Johnson and Hartzell (1985), and no further concentrations of halothane were tested as any effect on the kinetics of the enzyme from a lower concentration of halothane were unlikely. It was important to confirm the earlier results of Johnson and Hartzell (1985) prior to extending the investigation by examining enflurane and isoflurane.

The results from this investigation suggest a selective effect of enflurane on ChAT. Isoflurane, a structural isomer of enflurane, showed no effect on ChAT at an equipotent concentration. It seems unlikely that the reduction in $K_m$ could be a simple chemical concentration effect of the anaesthetic agents in solution as isoflurane at 4.5% and halothane at 3% showed no effect, whereas statistically significant effects of enflurane on the kinetics of ChAT were observed down to a concentration of 2.2%.

All clinical anaesthetists are aware that enflurane behaves differently to halothane and isoflurane with respect to the excitatory phenomena it produces on the electroencephalogram (Modica et al 1990). Enflurane has been implicated in the development of EEG abnormalities and in the genesis of seizures (Niejadlik, 1975, Neigh, 1971). The appearance of these abnormalities in the presence of enflurane is related to the dose of agent received and is particularly evident at high inspired enflurane concentrations (Neigh, 1971). The effect is also enhanced by hypocarbia and some of the excitatory phenomena are reversed by restoring normocarbia (Neigh, 1971).
Evidence has accumulated that one possible mechanism for the genesis of seizures is cholinergic and involves alterations in central acetylcholine metabolism. There are strains of mice and rats that are susceptible to seizures which display abnormalities in central cholinergic processes. A seizure prone strain of mice displays a 40 to 60 % higher total brain acetylcholine level than non-seizure prone strains (Naruse et al, 1960). Interestingly, the rate of synthesis of ACh observed in vitro was higher than in the control strains. Similar evidence is available for seizure susceptible strains of rats (Woolley et al, 1963). The release of ACh from the cerebral cortex of rats, anaesthetised with urethane, and injected with various stimulant drugs changed in parallel with the electrical activity of the brain observed with EEG monitoring (Hemsworth & Neal 1968). The acetylcholine content of rat brains was increased during status epilepticus induced with pilocarpine (Jope et al, 1987). Acetylcholine levels increased approximately 500 % in the cortex during status epilepticus. During the same study the seizure activity was inhibited by the prior application of atropine to the rats. Synaptosomes were also prepared from the experimental animals and those prepared from the cortex and hippocampus of rats with epilepsy had elevated levels of acetylcholine. This extra acetylcholine was present in a releasable compartment as stimulated release from cortical slices was significantly increased from seized rats.

There is pharmacological evidence linking cholinergic processes to seizures. Cholinesterase inhibitors promote seizures in experimental animals that can be blocked by the application of atropine (Maynert et al, 1975). A link between enflurane seizure activity and central cholinergic processes
comes from two in vivo studies (Moorthy, 1980, Ngai et al, 1978). Mongrel dogs were anaesthetised with 3.5 % enflurane, to induce EEG spike activity, and then given scopolamine. The anticholinergic agent significantly reduced the spike activity (Moorthy, 1980) which suggests that the abnormal EEG patterns were caused by acetylcholine. Acetylcholine turnover has also been measured under enflurane anaesthesia in rats (Ngai et al, 1978). The turnover was reduced in cortical but not sub-cortical areas in the brain, whereas halothane reduced turnover in both areas. The enflurane induced hyperactivity of the brain could be reflected in the turnover rates of acetylcholine from the sub-cortical structures. These two complimentary studies imply a role for acetylcholine in the genesis of the EEG abnormalities observed with enflurane.

If the enflurane induced reduction in the $K_m$ of ChAT occurs in vivo it could contribute to a facilitation of ACh synthesis in susceptible brain regions and lead to the excitatory phenomena. The lack of effect on the kinetics of ChAT by the structural isomer of enflurane, isoflurane or of the unrelated compound halothane, which do not cause EEG abnormalities, is consistent with the above suggestion.

The cholinergic theory of enflurane induced seizures fits in neatly with the evidence from this study and from the two in vivo investigations but there is evidence that other neurotransmitter systems may be influenced by convulsant ethers. Enflurane is an ether which has both anaesthetic and convulsant properties. Hexafluorodiethylether (Indoklon), is a convulsant ether and Richter et al (1977) showed that it had different effects on the crab neuromuscular junction to a purely anaesthetic ether methoxyflurane.
Hexafluorodiethylether selectively blocked inhibitory transmission (GABA) over excitatory transmission (glutamate). The opposite effect was observed for methoxyflurane. Hirose et al (1992) also reported an increase in the release of the excitatory transmitter, glutamate, in rat cortical synaptosomes exposed to 0.75 mM enflurane at 32 C and they concluded that this increase could be responsible for the excitatory phenomena observed with this agent.

In summary it has been shown the activity of ChAT is susceptible to kinetic modification in the presence of concentrations of enflurane towards the top of the clinically relevant range. It is not possible to ascertain from this study if this is a direct interaction or an effect secondary to cellular debris in the preparation. The extrapolation of this finding into whole animals is hindered by the possible interaction of anaesthetics with other transmitter systems. Further areas of enquiry would be to determine if other convulsant ethers such as hexafluorodiethylether and its structural isomer iso-hexafluorodiethylether, which is purely anaesthetic (Koblin et al, 1981) displayed similar kinetic effects on ChAT and on other transmitter systems in mammalian preparations. If the findings were confirmed then the effect of volatile anaesthetic agents on the kinetics of ChAT could serve as a screening procedure for newly synthesised agents.

The importance of the findings in this study may also contribute to the debate on where anaesthetic agents act. The theory that has stood the test of time is the relationship between anaesthetic potency and lipid solubility described by Meyer (1899) and Overton (1901) in the early part of the twentieth century. However, protein targets have become more plausible targets for volatile anaesthetic action. Halsey and Smith (1970) and White
and Dundas (1970) reported inhibition of the light emitted from luminous bacteria by volatile anaesthetic agents. The IC$_{50}$'s for the anaesthetic induced inhibition of light production corresponded to those for MAC in mammals and Halsey and Smith (1970) commented that the mechanism of general anaesthesia may be more specific than originally supposed. Complimentary investigations by Franks and Lieb (1984, 1985), demonstrated the interaction of anaesthetics and alcohols on a purified enzyme, firefly luciferase. Again the IC$_{50}$'s for inhibiting enzyme action were close to those for anaesthetising mammals and were competitive.

Another enzyme that has been investigated recently is plasma membrane bound calcium - ATPase on the human erythrocyte. Kosh-Kosicka and Roszczynska (1993) revealed that halothane, enflurane and isoflurane all inhibited the enzyme in a dose dependent fashion with IC$_{50}$'s within the clinical range. They could not conclude if the inhibition was specific but there was an impressive relationship between the effect and the potency of the agents studies. Slater et al, (1993) demonstrated an inhibition of protein kinase C, an important enzyme in signal transduction, that regulates synaptic function by the phosphorylation of membrane proteins. The potency of the effect observed with different agents varied according to their lipid solubility, but the experiments were done with a lipid free assay, which suggests that a hydrophobic area of the protein may be involved as the target. The IC$_{50}$ for halothane was , however, 0.8 mM which is almost three times the MAC value for halothane in the rat. Many other potential protein targets have been described in the last twenty years and the case for proteins versus lipids was vigorously defended in a recent review by Franks and Lieb (1994). The
difference between these studies and the present findings is that a possible mechanism for a side effect of an anaesthetic may rely on an interaction with a protein. It would seem probable that it would be easier to explain specific adverse effects of agents on interactions with proteins as opposed to non-specific interactions with lipids.
6. Development of a Chemiluminescent Method to Measure Acetylcholine

6.1 Introduction

Acetylcholine is synthesised in cholinergic nerve terminals from its precursors choline and acetyl-CoA through a reaction catalysed by choline acetyltransferase. ACh is unique amongst the classical neurotransmitters, because its action at the synapse is terminated by enzymatic hydrolysis by the enzyme acetylcholinesterase. This is one of the fastest enzymes known (Quinn et al, 1992) and it is present both on the presynaptic nerve terminal and on the target cell, either nerve or muscle. The proportion of AChE from pre and post synaptic sources varies in different types of synapse.

The presence of AChE poses a problem when the release of ACh is measured from neural tissue. ACh is hydrolysed almost instantaneously at cholinergic synapses which prevents the direct measurement of the transmitter in its usual synaptic environment. Fifty percent of the choline liberated by the breakdown of ACh is taken up again into the nerve terminal by the high affinity choline transport mechanism. The strategy that has developed in the various assay systems for ACh, is to inhibit the AChE and so prevent breakdown of ACh. This solution is not ideal because AChE probably has other neuronal functions apart from catalysing the hydrolysis of ACh such as a role in cellular function and neural development (Whittaker, 1993).
In summary the measurement of ACh is complicated by the rapid breakdown of the transmitter, reliance on inhibition of AChE and the small quantity of transmitter present (Scremin & Jenden, 1993).

Methods employed previously to measure ACh in isolated neural preparations are reviewed below along with development of an existing assay to measure ACh.

6.2 Acetylcholine Assays

6.2.1 Bioassay

The observation that a substance released upon stimulation of the vagus nerve slowed the heart lead to the discovery of ACh as a neurotransmitter (Loewi, 1923). The original methods for measuring ACh developed from its biological effects on isolated muscle preparations or on physiological effects in animal preparations. With the bioassays it is assumed, that as the tissue extract behaves in a similar manner to ACh, it must contain ACh itself. At the end of the measurements, it must be demonstrated that the extracts become inactive on treatment with alkali and that the action of the extracts is reduced to the same degree as ACh standards when applied to curarized or atropinized muscle.
6.2.1.1 Leech Assay

Szerb (1961) described the estimation of ACh, from samples obtained by cannulae leading from cortical cups, on a narrow longitudinal strip of the oral part of the dorsal muscle of the leech. The whole of the body is sensitive to ACh but the dorsal wall gives the best response. The contraction of the muscle deflected an incident beam of light onto a mirror, which reflected the light into a photo cell, the current was amplified in an a.c. amplifier and recorded by a pen recorder. This assay was used extensively by physiologists working on samples generated from cortical cup experiments on intact animals. However, the lower limit of detection was 25-100 pg of ACh in 50 µL of sample, which is approximately 2000 pmoles. It is not sensitive enough to detect ACh released from in vitro rat brain preparations. The other drawback of this method is the time taken to analyse each sample, as in some preparations the muscle takes 30 minutes to relax. The preparation lasts for 4 to 5 hours, which allows only 10 samples to be analysed in a slow preparation.

6.2.1.2 Frog Rectus Assay

The frog rectus abdominis preparation (Chang & Gaddum, 1933) behaves like the leech muscle, but the time taken for full relaxation is usually less than 8 minutes. Although this method is quicker than the leech muscle it is ten fold less sensitive.
6.2.1.3 Cat Blood Pressure Assay

This procedure involves removing the gastrointestinal tract of the animal and cannulating the trachea, femoral vein and carotid artery and measuring the blood pressure response to injected ACh (Brown & Feldberg, 1936). First the sensitivity of the cat to a test dose of ACh is tested, as approximately 1 in 10 cats are unsuitable. The blood pressure should fall, and test samples and standards can be alternated every 3 minutes until the blood pressure no longer recovers. The animal usually survives for 4 hours but before the samples have all been tested it must be demonstrated that the depressor effect of extracts is destroyed by alkalinizing them and that the depressor effect cannot be elicited in the atropinized cat. The sensitivity of this assay is similar to the leech but is much quicker, however the preparation of the animal is time consuming and may be unsuccessful for 10% of cats. There are also humanitarian concerns in using an animal to assay experimental samples when other simpler models may give the same results.

6.2.2 Radiochemical Assays

In this assay system the tissue under investigation is loaded with radioactively labelled choline (\[^{14}\text{C}\] or \[^{3}\text{H}\]), the precursor for ACh. This assumes that the labelled precursor is taken up into appropriate cells in the preparation and is released from these cells upon stimulation. The labelled precursor may be taken up into neural cells which do not contain the release mechanism for the specific transmitter under investigation. Once access is
gained to the interior of the cell the labelled precursor may not equilibrate
with the endogenous transmitter and it may be released from an inappropriate
pool. There is evidence that labelled transmitter may not exchange with
endogenous transmitter and for ACh the labelled transmitter may be released
preferentially upon depolarisation (Richter & Marchbanks, 1971).

Another potential problem is that labelled precursor and transmitter
cannot be distinguished by radioactive counting methods and hence there is
uncertainty concerning the identity of released radioactivity. This is a
particular problem with ACh as it is broken down rapidly by AChE. Another
source of inaccuracy could occur owing to the large release of choline from
the surface of rat brains following decapitation (Dross & Kewitz, 1972). This
particular phenomenon occurs also following homogenisation of brain tissue
and may lead to large amounts of choline being labelled on the surface of the
tissue and in the bathing medium during incubation with labelled precursor.
Studies with ACh and labelled precursors must ensure that there is first
inhibition of AChE and, secondly, that it is possible to separate labelled
choline from labelled ACh. These factors make the measurement of ACh
using exogenous precursors complicated and laborious.

6.2.3 Radioenzymatic Assay

This assay involves the removal of endogenous choline by
phosphorylation with choline kinase. This step removes endogenous choline
and is accomplished before the estimation of ACh. Once the choline is
removed, endogenous ACh is hydrolysed by eel AChE, and then
phosphorylated with $[^{32}\text{P}]{\text{ATP}}$. The labelled product, $[^{32}\text{P}]{\text{phosphorylcholine}}$ is then separated from the labelled substrate by precipitation of the ATP and by ion exchange chromatography (Goldberg & McCaman, 1973).

This assay can detect picomole amounts of ACh but has several drawbacks. It is a three stage procedure, the enzymatic removal of endogenous choline, hydrolysis and phosphorylation of endogenous ACh and then ion exchange chromatography. The aim for the measurement of ACh from the synaptosome and cortical slice preparations in this thesis was to avoid any method that employed radiolabelled precursors or products.

6.2.4 Chemiluminescent Assay

In 1981 Israel and Lesbats described a chemiluminescent assay for ACh (figure 6.1). Chemiluminescence occurs when a molecule is raised to an excited state as a result of a chemical reaction. The decay of the excited molecule to the ground state results in the emission of light. The oxidation of choline produces oxygen, betaine and hydrogen peroxide. The hydrogen peroxide reacts with luminol (5-amino-2,3-dihydro-1,4-phthalazine) to produce aminophthalic acid and light, the reaction being catalysed by microperoxidase. The assay for acetylcholine requires that acetylcholine is first hydrolysed by AChE (Israel & Lesbats, 1981).

Previous workers had used this assay for the determination of ACh from both synaptosome preparations (Johnson & Hartzell, 1985, Willoughby et al, 1986) and rat brain slice preparations (Alberch et al, 1985). The
The chemiluminescent assay has the major advantage of measuring endogenous ACh, which was the principal aim of this thesis.

During our initial experiments, using the published method for the chemiluminescent detection of ACh (Israel & Lesbats, 1981), a number of problems occurred that related either to the reagents that were employed or the synaptosome preparation. Before the addition of the final enzyme, AChE (figure 6.1), the reaction mixture is added to the sample so that endogenous choline is removed. Once this peak has returned to the baseline, AChE is added to measure the amount of ACh in the sample. However, difficulties with the chemiluminescent assay were encountered, which are now described in order to introduce the steps that were taken to overcome the problems of the assay.

The acetylcholinesterase from *Electrophorus electricus* contained choline despite prior electrophoresis of the enzyme. The choline in the enzyme would lead to an overestimation of the ACh signal when added to the assay. Modifications to the electrophoretic procedure were introduced to remove the choline contamination of the AChE.

Secondly, there was a large release of choline from the synaptosome preparation which may enter the assay and could lead to overestimation of the ACh release.

Thirdly, Israel and Lesbats (1982), in a modification of their original assay for application to samples from mammalian tissue, advocated the use of potassium iodate in order to overcome unidentified substances present in the tissue which inhibited exogenous AChE. These 'inhibitors' of the added AChE would lead to an underestimation of the ACh present in the sample.
Fourthly, endogenous AChE present in the synaptosome preparation may cause an underestimation of the ACh in the sample if it was not inhibited completely prior to the release experiments. Phospholine, an irreversible organophosphorus anticholinesterase has been used previously to inhibit AChE activity present in the synaptosome preparation (Willoughby et al, 1978).

Inhibition of endogenous AChE could be employed to assay ACh indirectly, if all the potential problems described above hinder the chemiluminescent assay of ACh, by assaying choline and omitting AChE from the assay. Acetylcholine in phospholine treated synaptosome samples is not hydrolysed and the choline signal would consist of choline released from the synaptosomal membranes. In the samples in standard Krebs, as soon as the ACh is released, it is hydrolysed to choline and acetate by AChE present in the preparation. Therefore, the total choline signal represents choline from the hydrolysis of ACh and choline from the synaptosomal membranes. The contribution to the choline pool from the hydrolysis of ACh could be used to give an indirect measure of the amount of ACh present if parallel experiments in the absence and presence of an AChE inhibitor were performed. The choline signal from the phospholine sample would consist only of the choline leak from the membranes and not a contribution from ACh, as the AChE was inhibited by the phospholine. By determining the choline signal in both preparations, the difference between the total signal in the control preparation and the choline signal in the phospholine preparation would be the choline signal from the hydrolysis of ACh. This would give an indirect assessment of the ACh released from the preparation (figure 6.13).
acetylcholinesterase
1) Acetylcholine $\rightarrow$ acetate + choline

choline oxidase
2) Choline $\rightarrow$ betaine $+$ H$_2$O$_2$

microperoxidase
3) H$_2$O$_2$ $+$ luminol $\rightarrow$ aminophthalic acid $+$ light

Figure 6.1
The chemiluminescent assay for acetylcholine.
6.3 Materials and Methods

6.3.1 Purification of Acetylcholinesterase

Initial experiments with the chemiluminescent assay revealed that AChE contained choline contaminants. Therefore, purification of AChE was required prior to use in the chemiluminescent assay in order to try and remove the choline. The AChE was purified as instructed in BioOrbit application note 401.

6.3.1.1 Initial Purification of Acetylcholinesterase (Sigma C2888)

Five hundred units of AChE was chromatographed on a 10 ml column containing Sephadex G 50. The column was eluted with 10 mM Tris-HCl buffer and sixteen 1.0 ml aliquots were collected in a Gilson fraction collector. The flow rate through the column was set at 90 µL min⁻¹ controlled by a peristaltic pump.

6.3.1.2 Dialysis of Acetylcholinesterase (Sigma C2888)

The AChE, that had been chromatographed on the Sephadex G 50 column was dialysed. A 400 µL portion of the AChE recovered from the Sephadex column was dialysed overnight in Tris-HCl buffer pH 7.4 at 4°C. Three hundred microlitres were recovered and the dialysed enzyme was
analysed for its choline content using the chemiluminescent assay described above.

6.3.1.3 Purification Acetylcholinesterase (Boehringer 101885)

The AChE (Boehringer 101885) was chromatographed on a Sephadex G 50 column equilibrated with 0.1 M phosphate buffer pH 7.0 at 4° C. The flow rate was 90 μL min⁻¹ and 9 fractions were collected.

In order to reduce choline contaminants further fraction 2 (highest protein content) was split into two 250 μL aliquots and the following chromatography performed. Two 6.5 ml Sephadex columns were prepared, both were connected to a peristaltic pump and calibrated to deliver 44μL min⁻¹ to the Gilson fraction collector. The first sample was equilibrated with 10 mM Tris buffer pH 7.4 and nine 1 ml fractions were collected from the column.

To disrupt ionic bonds between the AChE and choline, the second sample was adjusted to contain 0.5 M KCl before chromatography as above. Nine 1 ml fractions were collected.

At the end of the chromatography procedures the protein content of each fraction was determined (Lowry et al, 1951).

Following each purification procedure, the fractions with the highest protein content were analysed individually for their choline content. An aliquot of each fraction was added to the assay also, along with a standard quantity of ACh to investigate the ability of the enzyme to hydrolyse ACh.
6.3.2 Hardware

A Perkin-Elmer Model LS-50 luminescence spectrophotometer fitted with the total emission accessory was used for the quantitative bioluminescent determination of ACh and choline. The LS-50 was interfaced with an Epson-386 personal computer so that a graphic representation of the reaction could be seen on the computer monitor (figure 6.2).

6.3.3 Measurement of Acetylcholine and Choline Standards

The initial conditions for the assay were as follows (BioOrbit application note 401).

The following reagents were used for the assay;

1. Sixty-seven mM glycine-NaOH buffer, pH 8.6.

2. Choline Oxidase, Sigma C5896 (Sigma Chemical Company, Poole, U.K.). A solution of 30 units/ml was prepared in 10 mM Tris-HCl buffer pH 7.4. This was aliquoted into 500 µL samples and frozen at -70°C until required for the assay.

3. Microperoxidase, Sigma M6756. A stock solution of 5.0 mg/ml was prepared in 10 mM Tris-HCl buffer pH 7.4. This was diluted 1:10 and
Figure 6.2
Print out from computer interfaced with Perkin-Elmer LS 50 spectrophotometer. Time drive set for 300 seconds. Signal is peak minus background. Figure shown represents signal from choline standard (1000 picomoles)
 aliquoted into 500 µL samples and frozen at -70°C until required for the assay.

4. Luminol (Sigma A8511). 17.72 mg was dissolved in a few drops of 1.0 M NaOH. This was made up to 10 ml with 0.2 M Tris-HCl buffer, pH 8.6 to a final concentration of 10 mM. Prior to use in the assay this was diluted 1:20 with 0.2 M Tris-HCl buffer, pH 8.6.

5. AChE from Electrophorus electricus (Boehringer 101885). A solution of 4 units/ml was made up in Tris-HCl buffer pH 7.4. Choline contaminants were removed from the enzyme as described above.

6. ACh Standards (acetylcholine chloride, Sigma A6625). A 10 mM stock solution was prepared and serial dilutions were made ranging from 1 in 100 to 1 in 1,600 in order to construct a standard curve prior to the measurement of samples.

7. Choline Standards (choline chloride, Sigma C1879). A 10 mM stock solution was prepared and serial dilutions prepared as for the ACh standards. A standard curve was drawn prior to the analysis of experimental samples.

The BioOrbit application note 401 describes the following assay conditions with the reagents that have been described above;

- 960 µL Glycine buffer
- 20 µL choline oxidase
20 μL microperoxidase
20 μL luminol, 0.5 mM

This assay mixture was prepared in a cuvette and the background light intensity recorded. For estimation of ACh in experimental samples, supernatants were treated with 10 μl of 0.5 % (w/v) potassium iodate before the sample was analysed.

To avoid overestimation of the ACh signal, the assay was conducted in two stages. The first stage contained all the reaction mixture, except for the AChE, and measured the choline from the preparation. When this signal had reduced back to baseline AChE (20 μL) was added and any signal obtained was derived from ACh in the sample (figure 6.2).

To measure choline in experimental samples the AChE step was omitted from the assay (figure 6.1).
6.3.4 Measurement of Choline Released From Synaptosomes

Synaptosomes were prepared as described in 3.2.2. They were suspended at a protein concentration of 1 mg ml\(^{-1}\) in Krebs buffer, at 37 °C in a water bath and 200 µl samples were withdrawn at 0, 5, 10, 15, 30 and 60 minutes. The samples were centrifuged in a microfuge at 13,000 rpm for 5 minutes and the resultant supernatants analysed for their choline content using the chemiluminescent assay described above (figure 6.1).

6.3.5 Measurement of Released Acetylcholine From Synaptosomes

6.3.5.1 Inhibition of Endogenous Acetylcholinesterase

Before commencing experiments to measure the amount of ACh released from synaptosomes, investigations were conducted to determine the optimum concentration of phospholine for inhibition of endogenous AChE. Phospholine is an organophosphorus anticholinesterase that is still used clinically for the treatment of glaucoma. The ophthalmological preparation of phospholine (Cusi, UK) was employed in the experiments.

Synaptosomes were prepared on Percoll gradients as described in 3.2.2. Phospholine was added to Krebs buffer to final concentrations varying from 0 to 500 µM. Synaptosomes were incubated with the phospholine Krebs
medium for ten minutes, centrifuged at 5,000 x g for 5 minutes and then washed three times with Krebs buffer that contained no phospholine.

First, AChE activity was investigated by assaying the ability of a 10 µL aliquot of synaptosome suspension (1 mg ml\(^{-1}\)) to hydrolyse a known quantity of ACh (1000 pmoles).

Secondly, 300 µL synaptosomal suspension (1 mg ml\(^{-1}\)) incubated in phospholine Krebs buffer (0 to 500 µM) was placed in a water bath at 37 °C for 30 minutes. The suspension was centrifuged in a microfuge at 13,000 rpm for one minute. The supernatants were removed and a known amount of ACh standard (670 pmoles) was added to them to investigate any inhibition of the exogenous AChE in the ACh assay.

6.3.5.2 Release of Acetylcholine From Synaptosomes

Rat cortical synaptosomes were prepared on Percoll gradients as described in 3.2.2. The F3 and F4 synaptosome bands were combined to increase the yield of synaptosomal protein from the preparation (Harrison et al, 1988).

Synaptosomes were incubated with Krebs buffer that contained 65 µM phospholine for 10 minutes and then washed and resuspended three times to remove any excess phospholine.

Phospholine treated synaptosomes were suspended in Krebs buffer or Krebs buffer modified to contain 46 mM K\(^+\) at a protein concentration of 1 mg ml\(^{-1}\), total assay volume was 1.5 ml. At time zero a 500 µl sample was removed, centrifuged at 13,000 rpm in a microfuge for use as background
release of ACh. The synaptosomal suspensions were incubated in a water bath at 37 °C and after 30 minutes were removed and centrifuged as the time zero samples. Supernatants were removed from the synaptosomal pellets and kept on ice until analysis later for ACh.
6.4 Measurement of Acetylcholine

The synaptosome preparation was divided into two aliquots, one portion was suspended in Krebs buffer and was used to measure the total choline signal from the preparation. The total choline signal would comprise that from the hydrolysis of ACh and also the choline released from the preparation. The other portion of the preparation was incubated for 10 minutes in Krebs buffer that contained 65 μM phospholine. Following this incubation, excess phospholine was removed by washing and centrifuging the synaptosomal suspension at 5,000 x g for 10 minutes in standard Krebs buffer three times. The control sample was also washed and centrifuged three times to ensure that similar manipulation of the preparation took place.

Background release experiments were performed on synaptosomal suspensions (1 mg ml⁻¹) for untreated and phospholine treated samples with Krebs buffer containing 4.7 mM potassium, and stimulated release was performed using Krebs modified to contain 46 mM potassium. The total assay volume was 1000 μL. The test preparations were exposed to air via the anaesthetic manifold (3.2.4) in a water bath at 37 °C. After 45 minutes the samples were centrifuged and supernatants withdrawn and kept on ice until analysis.

The choline present in the supernatants was measured using the chemiluminescent assay (figure 6.1). Acetylcholine was calculated as the difference in choline signals between the control samples and the phospholine treated samples.
6.5 Results

6.5.1 Purification of Acetylcholinesterase (Sigma C 2888)

The elution curve for the purification of the enzyme is shown in figure 6.3. However, when 10- or 20- μL samples of the AChE were added to the assay without ACh present choline was still in the AChE. 20 μL gave a choline signal of 59 ± 18 (n = 6, mean ± SD), equivalent to 700 pmoles of choline.

This level of choline in the AChE would over estimate the amount of ACh present in subsequent experimental samples and standards.

Overnight dialysis of AChE did not remove the choline from the AChE.

6.5.2 Purification of Acetylcholinesterase (Boehringer 101885)

Initial chromatography of the enzyme did not remove the choline contaminants. Figure 6.4 shows the protein content of each fraction with the level of choline in each sample and the ability of each fraction to hydrolyse a known ACh standard (1,700 pmoles).

Further chromatography of the fraction with the highest protein content did not remove the choline contaminants (figure 6.5). However, the fraction exposed to 0.5 M KCl, had the choline contaminants removed successfully, without affecting the activity of the enzyme (figure 6.6).
Figure 6.3
Elution curve from chromatography of AChE (Sigma C2888).
Figure 6.4
AChE activity and choline contamination of AChE (Sigma C2888).
■ = protein content of fraction (μg ml⁻¹).
● = peak height in response to addition of 1,700 pmoles ACh to assay.
Δ = peak height when sample of AChE added to assay without ACh standard (choline contamination of AChE).
Figure 6.5
AChE activity and choline contamination of AChE (Boehringer 101885) following further chromatography.

\( \nabla \) = protein concentration of fraction (\( \mu g \text{ ml}^{-1} \)).

\( \nabla \) = peak height in response to addition of 1,300 pmoles ACh.

\( \bullet \) = peak height when sample of AChE added to assay without ACh standard (choline contamination of AChE).
Figure 6.6
AChE activity and choline contamination of AChE (Boehringer 101885) following chromatography in 0.5 M KCl.

▼ = protein concentration of fraction (μg ml⁻¹).
▽ = peak height in response to 1,300 pmoles ACh.
• = peak height when sample of AChE added to assay without ACh standard (choline contamination of AChE).
6.5.3 Inhibition of Endogenous Acetylcholinesterase

6.5.3.1 Acetylcholinesterase Activity of Synaptosomes

A 10 μL sample of synaptosomal suspension (1 mg ml⁻¹) hydrolysed a 1000 pmole sample of ACh standard to give a peak height of 62. Further 10 μL samples of synaptosomal suspension incubated in phospholine contained no AChE activity (figure 6.7). The phospholine did not inhibit endogenous AChE, added to the assay (figure 6.7).

ACh standards made up in supernatants from synaptosomal suspensions prepared in varying concentrations of phospholine did not inhibit exogenous AChE when the synaptosomes were only suspended in the buffer for 5 minutes. However, when ACh standards were prepared in supernatants that had been incubated with synaptosomes in a water bath at 37 °C for 30 minutes the activity of exogenous AChE was reduced (figure 6.8).
Figure 6.7

Phospholine inhibition of endogenous AChE in synaptosomal tissue.

\( o \) = peak height obtained when 10 \( \mu l \) of synaptosomal protein (1 mg ml\(^{-1}\)) added to 1000 pmoles ACh.

\( \bullet \) = peak height obtained following addition of exogenous AChE (Boehringer 101885).
Figure 6.8

Inhibition of chemiluminescent assay by endogenous inhibitors. Reduction in peak height when ACh standard (600 pmoles) was assayed in the presence of buffer incubated with synaptosomes with and without different phospholine concentrations.

A = Standard in de-ionised water (n = 4, ± SD)
B = Standard prepared in buffer from control synaptosomes (n = 4, ± SD)
C = Standard prepared in supernatant from synaptosomes incubated with 25 μM phospholine (mean of 2 observations)
D = as C, but in synaptosomes incubated with 500 μM phospholine (mean of 2 observations)
6.5.4 Measurement of Choline and Acetylcholine From Synaptosomes

6.5.4.1 Choline

A choline standard curve was constructed before analysis of experimental samples (figure 6.9). The release of choline from the synaptosome preparation was linear for 60 minutes in Krebs buffer and in Krebs buffer modified to contain 46 mM K⁺ (figure 6.10).

Choline release from synaptosomes incubated in Krebs buffer was 42.6 pmoles mg⁻¹ min⁻¹ (SD = 9.2, n = 4). Choline release from potassium stimulated synaptosomes was 42.8 pmoles mg⁻¹ min⁻¹ (SD = 10.0, n = 4). There was no significant difference in choline release from the synaptosome preparation between the control and potassium-stimulated samples.

6.5.4.2 Acetylcholine

ACh standard curve was constructed before the experimental samples were analysed (figure 6.11).

No ACh was detected in synaptosomes incubated in either Krebs buffer or Krebs buffer modified to contain 46 mM K⁺ (8 separate synaptosome preparations). The addition of potassium iodate (Israel & Lesbats, 1982) to the samples before analysis did not improve the ACh signal.
**Figure 6.9**

A representative choline standard curve, broken lines represent 95% confidence intervals. Correlation coefficient $r = 0.99$. 
Figure 6.10
Choline release from synaptosome preparation.
• = release from synaptosomes incubated in Krebs buffer.
∇ = release from synaptosomes incubated in modified buffer (46 mM K⁺)
All points are means of 2 determinations.
Figure 6.11
A representative ACh standard curve, broken lines represent 95% confidence intervals. Correlation coefficient $r = 0.98$. 
6.5.5 Indirect Measurement of Acetylcholine

Preliminary experiments demonstrated that there was a difference between the choline signal derived from phospholine treated synaptosomes and untreated synaptosomes. However, there was no increase in ACh release when synaptosomes were stimulated with 46 mM K+ buffer compared to non-stimulated release (figure 6.12). Acetylcholine release was approximately 5 pmoles mg⁻¹ min⁻¹ in the stimulated and basal release groups (figure 6.12).
Figure 6.12
Indirect estimation of ACh from synaptosome preparation.
Open bars represent synaptosomes incubated in Krebs buffer
Filled bars represent synaptosomes incubated in Krebs buffer containing 65 μM phospholine.
A and B basal release, C and D potassium stimulated release.
The difference between the open and filled bars for each buffer represents choline derived from the hydrolysis of ACh. Values given are means ± SEM, n = 6 for each group.
Modifications to Chemiluminescent assay for acetylcholine

A. Minus Phospholine

\[ \text{ACh} \rightarrow \text{AChE} \rightarrow \text{Choline leak} \]

B. Plus Phospholine

\[ \text{ACh} \rightarrow \text{phospholine} \rightarrow \text{AChE} \rightarrow \text{Choline leak} \]

\[ \text{Choline} \rightarrow \text{Betaine} + \text{H}_2\text{O}_2 \]

\[ \text{H}_2\text{O}_2 + \text{Luminol} \rightarrow \text{Aminophthalic Acid} + \text{Light} \]

\[ \text{AChE} = \text{endogenous acetylcholinesterase} \]

\[ \text{ACh} = A - B \]

Figure 6.13

Summary of the modifications to the chemiluminescent assay for the measurement of acetylcholine.
6.6 Discussion

Application of the full chemiluminescent assay to the rat synaptosome preparation proved unsuccessful. However, by omission of the first step in the three enzyme reaction, an indirect method for the measurement of ACh was developed. The initial aim, to use an assay that did not use radio-labelled precursors was achieved and the new assay also yielded additional information as the release of choline from the synaptosome preparation was measured also.

The initial problem was the purification of endogenous AChE that was added to the assay. Choline, present in the enzyme, was not removed by standard chromatography. The contaminating choline was successfully removed by adding a high concentration of potassium to the chromatography buffer. Although this procedure removed the choline from the enzyme, the AChE step was abandoned from the assay due to another complication, the presence of endogenous substances released from the synaptosome preparation which inhibited the action of the added AChE.

The chemiluminescent assay for ACh was described originally in torpedo electric organ synaptosomes (Israel & Lesbats, 1981). However, when the assay was applied to mammalian tissue (mouse brain slices), inhibitors were released from the preparation which interfered with the added AChE (Israel & Lesbats, 1982). In order to overcome this inhibition of endogenously added AChE, Israel and Lesbats (1982), added an oxidant to the sample which was successful in relieving the inhibition of the mouse brain substances. The oxidant did not overcome the inhibition of AChE in the rat
brain synaptosomes used in this thesis. Experiments performed to assess the optimal concentration of phospholine demonstrated that it was not excess phospholine that caused the inhibition (figures 6.7 & 6.8).

Previous investigators had measured ACh release from rat cortical synaptosomes using the chemiluminescent assay (Johnson & Hartzell, 1985, Willoughby et al, 1986). In both these studies the full chemiluminescent assay was employed and ACh release rates of 20 pmoles mg<sup>-1</sup> min<sup>-1</sup> and 5 pmoles mg<sup>-1</sup> min<sup>-1</sup> were measured by Johnson and Hartzell (1985) and Willoughby et al (1986), respectively. Both these studies employed synaptosomes prepared on Ficoll gradients (Booth & Clark, 1978) but it is unlikely that the gradients employed for the synaptosome preparation could account for the inability of this study to overcome the inhibitors produced by the tissue. A personal communication from Israel and Lesbats revealed that the identity of the inhibitors is still unknown. The presence of the unidentified inhibitors was an insurmountable problem in this work which necessitated the modification of the assay.

Although the full assay was not successful in the synaptosomes used in this study, the indirect assay measured ACh release rate of 5 pmoles mg<sup>-1</sup> min<sup>-1</sup>, similar to the rate measured by Willoughby et al (1986) employing the full three enzyme assay. However, the rate of release from synaptosomes incubated in Krebs buffer was almost the same as the rate of release from potassium stimulated samples. The synaptosomes appeared to be unresponsive to potassium depolarisation. Willoughby et al (1986) found similar unresponsiveness to potassium depolarisation in synaptosomes with the ACh release rate increasing by only 30% above basal release. Jope
(1981) reported that ACh release was only increased by 20 to 50% following potassium depolarisation in synaptosomes. In this study there was no difference between the stimulated and spontaneous release of ACh. Although, Jope (1981) justifies the study of spontaneous release, as it may modulate the activity of cholinergic neurones, synaptosomes are a poor model for the study of calcium dependent potassium stimulated ACh release.

The modified assay also yielded information regarding the release of choline from the synaptosome preparation. Choline is released from the surface of the brains of experimental animals as soon as the brain is removed from the animal (Dross & Kewitz, 1972). The released choline is not derived from the post mortem disintegration of the tissue but by persisting biochemical reactions in the absence of blood flow. The choline efflux also occurs in brain homogenates, although the rate is slower than in intact brain (Dross & Kewitz, 1972). The rate of choline release is related to the time elapsing between death and removal and fixing of the tissue (Dross & Kewitz, 1972) which may lead to variation in the rates of choline release. The rate of choline release from the synaptosomes in this study of 42 pmoles mg⁻¹ min⁻¹ is similar to the choline release rates of 24 pmoles mg⁻¹ min⁻¹ and 40 pmoles mg⁻¹ min⁻¹ reported by Dross and Kewitz (1972) and Willoughby et al (1986) respectively.

In summary, the modified chemiluminescent assay developed for this study does require that the preparation is split into two portions in order to separate the choline release from the tissue and the choline derived from the hydrolysis of ACh. However, the advantages of the method are the omission of the first enzyme, AChE. This enzyme was difficult to purify and if the
purification was not thorough, the choline still present would cause an overestimation of the ACh signal. Secondly, the presence of unidentified substances released from the preparation inhibited the signal derived from the hydrolysis of ACh by AChE and would lead to an under estimation of the amount of ACh present. These complications are removed by the omission of AChE.

The results from the initial investigations into the development of the assay confirmed that the synaptosome preparation was unsuitable for studying stimulated ACh release due to the insensitivity to potassium depolarisation. However, the rat cortical slice preparation is sensitive to potassium depolarisation and ACh release is increased several fold compared to spontaneous release (Salemgoghaddam & Collier, 1976). Further investigations were undertaken using the rat cortical slice preparation employing the modified chemiluminescent assay and are discussed in chapter 7.
7. The Release of Acetylcholine From A Rat Cortical Slice Preparation; Modulation of Release by Halothane

7.1 Introduction

Depolarisation of the pre-synapse releases transmitters and leads to chemical interaction with pre and post synaptic receptors. The conversion of electrical signals into chemical signals by the release of neurotransmitters is crucial to normal neuronal function. The synaptosome preparation is not suited to the study of potassium stimulated ACh release because 60 to 70 % of released transmitter is derived from spontaneous release (Jope, 1981). The rat cortical slice preparation is suited to the study of ACh release as stimulated release is several times greater than spontaneous release (Salemooghaddam & Collier, 1976). It follows that anaesthetic agents, which cause a temporary alteration in conscious state, may interfere with neurotransmitters that are closely linked to the development of sleep/wake states. The evidence implicating ACh as a transmitter target for volatile anaesthetic agents is reviewed in 2.2. In summary in vivo studies could only imply that release was affected and could not differentiate between a possible decrease in ACh release or an increase in ACh breakdown. There are few studies on the effects of anaesthetic agents on ACh release in vitro. A decrease in potassium evoked release was observed in rat synaptosomes, although supraclinical doses of halothane were administered (Johnson & Hartzell, 1985). Bazil and Minneman (1989b) reported no effect of halothane
on potassium evoked ACh release from the rat cortical slice preparation. Interpretation of any data on ACh release must be scrutinised due to deficiencies in the measurement of ACh and the interference due to released choline when labelled choline is employed in the assay for ACh.

In this study ACh release has been investigated in the rat cortical slice preparation using a modification of the chemiluminescent assay (Israel & Lesbats, 1981). The method avoids the use of radio labelled precursors and because the assay also measures choline release from the preparation the data obtained may explain important differences between this study and those of previous investigators.

7.2 Materials and Methods

7.2.1 Cortical Slice Preparation

The rat cortical slices were prepared as described in 3.2.3. For release experiments the suspension of slices was split into two equal aliquots. One was incubated for 10 minutes with oxygenated Krebs buffer and the other with oxygenated Krebs buffer that contained 65 µM phospholine. Both sets of slices were washed subsequently with 10 ml of oxygenated Krebs buffer three times to remove excess phospholine prior to the release experiments.
7.2.2 Experimental Protocol

In all experiments the release of choline and ACh from cortical slices was measured in Krebs buffer at 37°C containing 2.0 mM CaCl₂ (unstimulated basal release) or the same buffer adjusted to contain 46 mM KCl (potassium-stimulated release) and 79 mM NaCl. None of the buffers used during the preparation of the slices contained added calcium. The time from the preparation of the slices to the start of the experiments was approximately 15 minutes.

Following equilibration of buffers with the carrier gas or carrier gas containing halothane for 10 minutes, 50 µL of packed slices were added to 100 µL of appropriate buffer and centrifuged immediately in a refrigerated microfuge for 5 minutes. Supernatants were removed and frozen at -70°C until analysis. These samples were employed to determine the background signal at time zero. For T>0 minute incubations 100 µL of packed slices were added to 200 µL of either Krebs buffer or release buffer pre-equilibrated with the appropriate agent and the tubes were agitated in a water bath at 37°C with carrier gas or carrier gas containing halothane blown over the surface of the sample. At 30 minutes the tubes were centrifuged at 5,000 x g for 5 minutes and supernatants removed and frozen as above for analysis later.

The wet weight of the slices from each incubation was determined by weighing experimental tubes before the experiments and following removal of the supernatants. Release of ACh was expressed as pmoles mg wet weight of slices per minute.
7.2.2 Experimental Protocol

In all experiments the release of choline and ACh from cortical slices was measured in Krebs buffer at 37°C containing 2.0 mM CaCl₂ (unstimulated basal release) or the same buffer adjusted to contain 46 mM KCl (potassium-stimulated release) and 79 mM NaCl. None of the buffers used during the preparation of the slices contained added calcium. The time from the preparation of the slices to the start of the experiments was approximately 15 minutes.

Following equilibration of buffers with the carrier gas or carrier gas containing halothane for 10 minutes, 50 µL of packed slices were added to 100 µL of appropriate buffer and centrifuged immediately in a refrigerated microfuge for 5 minutes. Supernatants were removed and frozen at -70°C until analysis. These samples were employed to determine the background signal at time zero. For T>0 minute incubations 100 µL of packed slices were added to 200 µL of either Krebs buffer or release buffer pre-equilibrated with the appropriate agent and the tubes were agitated in a water bath at 37°C with carrier gas or carrier gas containing halothane blown over the surface of the sample. At 30 minutes the tubes were centrifuged at 5,000 x g for 5 minutes and supernatants removed and frozen as above for analysis later.

The wet weight of the slices from each incubation was determined by weighing experimental tubes before the experiments and following removal of the supernatants. Release of ACh was expressed as pmoles mg wet weight of slices per minute.
7.2.3 Assay of Acetylcholine

ACh was measured using a modification of a coupled chemiluminescent assay and as described in 6.4 (Israel & Lesbats, 1981, BioOrbit, 1990).

The amount of ACh released was determined by subtraction of the value for released choline from phospholine-treated slices from the value determined for released choline from untreated slices, in which endogenous AChE activity remained.

7.2.4 Radio-Receptor Binding Assay For Acetylcholine

The modified chemiluminescent assay previously described, measured the amount of ACh indirectly. In order to confirm the presence of ACh in experimental samples a radio-receptor assay was used to assay the presence of ACh in supernatants from 9 experimental samples. These 9 samples were analysed by both methods. The displacement of N-methyl-scopolamine NMS from rat cortical membranes was quantified by standard amounts of ACh with liquid scintillation counting (Appadu & Lambert, 1994).

7.2.4.1 Preparation of Cortical Membranes

A female Wistar rat was killed by a blow to the head and the cortex was removed post-mortem. The tissue was cut and homogenised with a glass/Teflon homogeniser in 10 ml of buffer containing 10 mM HEPES and
10 mM EDTA. The homogenate centrifuged and washed in 10 ml of buffer five times at 20,000 × g for 10 minutes. The protein concentration was measured using the method of Lowry et al (1951). The membranes were frozen in aliquots at -70°C for future use.

7.2.4.2 Radio-Receptor Assay

Binding of NMS was performed at 37°C for 30 minutes in 500μL volumes using approximately 100μg of protein and 10 nCi of NMS. Bound and free radioactivity were separated by vacuum filtration on to Whatman GF/B filters using a Brandell cell harvester. Non-specific binding was defined in the presence of 1 μM atropine.

Three different standards were analysed on three separate assays to assess the inter-assay variability and one standard was also introduced into an assay 12 times to assess the intra-assay variability.

7.2.5 Anaesthetic Apparatus

Halothane was delivered via a manifold of 10 outlets, each delivering 60 ml min⁻¹ of humidified carrier gas (95 % O₂ / 5 % CO₂) as described in 3.2.4.
7.2.6 Statistical Analysis

Results were compared using paired analysis of variance and Student's paired t-test. A values of $p<0.05$ was considered statistically significant. The dose response curves were analysed statistically by analysis of variance and an IC$_{50}$ value was obtained by computer assisted curve fitting (non-linear regression model) using GraphPad (V2.0).
7.3 Results

7.3.1 Linearity

Choline release from the rat cortical slice preparation, either untreated or treated with phospholine, was linear over 30 minutes under basal release conditions (4.7 mM K+) (figure 7.1) and on potassium-stimulation (46 mM K+) (figure 7.2).

7.3.2 Calcium Dependence

Levels of choline released in unstimulated basal samples after 30 minutes, measured in the presence or absence of calcium, were essentially indistinguishable (figure 7.3) and the same as that measured in the presence of phospholine indicating that choline release from the slice preparation under basal conditions was independent of calcium. No ACh release was observed in the absence of calcium in the unstimulated basal samples (figure 7.3).

Levels of choline released from potassium stimulated untreated slices after 30 minutes were significantly different between the group containing calcium in the buffer and those without calcium (figure 7.4). In the phospholine treated slices there was no significant difference between the choline released in the presence and absence of calcium (figure 7.4).

In these initial experiments, after correction for choline release, ACh release was estimated to be linear over 30 minutes and to be higher from the potassium-stimulated slices compared to release from slices treated with low
potassium buffer (4.7 mM). The release of ACh was calcium dependent for unstimulated and potassium stimulated samples (figures 7.3 & 7.4).

7.3.3 Experimental Results

In the second series of experiments there was an approximate eightfold increase in ACh release from the potassium-stimulated slices compared to unstimulated preparations. This is represented by the difference between the choline measured without and with treatment with phospholine in figure 7.5. ACh release from the unstimulated and potassium-stimulated slices was 0.23 pmoles mg⁻¹ min⁻¹ (SEM 0.04 n = 33) and 1.98 pmoles mg⁻¹ min⁻¹ (SEM 0.15 n = 33), respectively. The increased rate of ACh release from the potassium-stimulated slices was accompanied by a significant fall in the rate of choline released such that the levels of total choline release observed from unstimulated and potassium-stimulated samples in the absence of phospholine achieved a similar apparent rate (figure 7.5).

Halothane caused a significant reduction in potassium-stimulated ACh release at all concentrations of halothane tested (figure 7.6). Maximal inhibition of ACh release was observed at 3 % halothane, above which concentration no further inhibition occurred. The IC₅₀ for the inhibition of potassium-stimulated ACh release in rat cortical slices was 0.38 % halothane. This equated to 0.3 MAC for the rat (White et al, 1974).

There was a trend for an increase in ACh release from unstimulated samples at all concentrations of agent tested but this did not reach statistical significance (table 7.1).
Halothane had no effect on choline release from the potassium-stimulated cortical slice preparation. However, the release of choline from the unstimulated samples was decreased significantly in the presence of 3 and 5% halothane (figure 7.7).
Figure 7.1
Time dependence of choline release from rat cortical slices in Krebs buffer containing 4.7 mM K⁺.
Open symbols represent untreated cortical slices. Filled symbols represent cortical slices treated with 65 μM phospholine.
∇ + ∇ = buffer without added CaCl₂.
All points are the means of two determinations. ACh was calculated as the difference between values represented by the open and closed symbols.
**Figure 7.2**

Time dependence of choline release from rat cortical slices containing 46 mM K⁺ buffer. Open symbols represent untreated cortical slices. Filled symbols represent cortical slices treated with 65 µM phospholine. All points are the mean of two determinations.

Range of values at 30 minutes for potassium-stimulated slices, ○ = 153 (134-199), ● = 73 (70-76).
Figure 7.3
The effect of calcium on the rate of choline release from unstimulated cortical slices. Open bars represent untreated cortical slices, filled bars represent cortical slices treated with 65 µM phospholine.
There were 6 determinations in each group.
The results shown are means ± SEM.
Figure 7.4

The effect of calcium on choline release from potassium stimulated cortical slices. Open bars represent untreated cortical slices, filled bars represent slices treated with 65 μm phospholine. There were 4 determinations in each group.

The results shown are means ± SEM.
Figure 7.5

The rate of choline release from cortical slices. Open bars represent untreated cortical slices, filled bars represent cortical slices treated with 65 μM phospholine.

There were 33 determinations in each group.

The results shown are means ± SEM.
Figure 7.6
The effect of halothane on potassium stimulated acetylcholine release from rat cortical slices. Control values were as quoted in the text. There were 5 determinations in each group except 0.4 % (n = 7) and 5.0 % (n = 6). Each point represents the mean ± SEM.
Figure 7.7

The effect of halothane on choline release from unstimulated phospholine treated rat cortical slices in Krebs buffer (4.7 mM K⁺). The results shown are means ± SEM.

There were 5 determinations in each group except 0.4 % (n = 7) and 5.0 % (n = 6). * p < 0.05.
Table 7.1

The Effect of Halothane on Acetylcholine Release From Unstimulated Cortical Slices

<table>
<thead>
<tr>
<th>Halothane (%)</th>
<th>Control</th>
<th>Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetylcholine Release (pmoles mg⁻¹ min⁻¹)</td>
<td>Mean (SEM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.30 (0.38)</td>
<td>0.42 (0.41)</td>
</tr>
<tr>
<td>0.2</td>
<td>0.16 (0.23)</td>
<td>0.29 (0.27)</td>
</tr>
<tr>
<td>0.4</td>
<td>0.13 (0.19)</td>
<td>0.49 (0.38)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.13 (0.14)</td>
<td>0.62 (0.44)</td>
</tr>
<tr>
<td>3.0</td>
<td>0.25 (0.16)</td>
<td>0.57 (0.13)</td>
</tr>
<tr>
<td>5.0</td>
<td>0.31 (0.16)</td>
<td>0.56 (0.38)</td>
</tr>
</tbody>
</table>

There were 5 determinations in each group except 0.4 % (n = 7) and 5.0 % (n = 6).
7.3.4 Radio-Receptor Assay

ACh standards displaced $[^3\text{H}]$ NMS from the rat cortical membranes (figure 7.8). The inter-assay variability is shown in table 7.2.

Twelve standards of 5,000 picomoles of ACh were measured in one assay. The mean (SEM) determined value was 6223 ± 268, this reflected the intra-assay variability of the radio-receptor assay.

Levels of ACh in samples measured with the radio-receptor assay were similar to those measured by the modified chemiluminescent assay (figure 7.9).

Samples from rat cortical slices that had not been pre-treated with phospholine did not displace $[^3\text{H}]$ NMS from the membranes, indicating that choline did not displace $[^3\text{H}]$ NMS.

The assay was also used to confirm the linearity of ACh release (figure 7.10), and the optimum K+ concentration for the release experiments (figure 7.11).
### Table 7.2

**Radio-Receptor Assay For Acetylcholine Inter-Assay Variability**

<table>
<thead>
<tr>
<th>ACh Standard (picomoles)</th>
<th>Amount Determined (picomoles)</th>
<th>Standard Error Mean (number of points)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50,000</td>
<td>44,666</td>
<td>3025 (n = 12)</td>
</tr>
<tr>
<td>500</td>
<td>656</td>
<td>142 (n = 12)</td>
</tr>
<tr>
<td>50</td>
<td>67</td>
<td>33 (n = 5)</td>
</tr>
</tbody>
</table>
Figure 7.8
Standard curve for estimation of ACh. Increasing concentration of ACh displaces radiolabelled $^3$H NMS. Non-specific binding in this experiment was $323 \pm 12$ DPM (mean of 5 determinations $\pm$ SD) and was subtracted from each determination shown above.
Figure 7.9
Comparison of two methods of determining ACh in experimental samples. Open bar represents radio-receptor assay, closed bar represents chemiluminescent assay. Results shown are the means of 9 samples ± SEM.
Figure 7.10
Time course of ACh release from rat cortical slices in buffer containing 46 mM K⁺. Acetylcholine determined using radio-receptor assay. Points are single determinations from a representative experiment. Correlation coefficient $r = 0.99$. 
Figure 7.11

Effect of increasing concentrations of potassium on rate of ACh release from cortical slices. ACh determined by radio-receptor assay. All points are single determinations.
7.4 Discussion

Acetylcholine was measured using a modification of the chemiluminescent assay of Israel and Lesbats (1981). The modified method enabled the signal choline signal derived from the hydrolysis of ACh to be distinguished from the choline released from the neural membranes. In addition the measured release of ACh was calcium dependent and was increased several fold upon potassium stimulation. The derived value for ACh release from the cortical slice preparation in this study was similar to previous investigations which had used different methods of measuring ACh release. In this study the rate potassium stimulated ACh release in the presence of carrier gas only was 1.98 pmoles mg wet weight\(^{-1}\) min\(^{-1}\) (SEM 0.15 for 33 observations). This value is consistent with that obtained by Richter (1976) who also recorded 2 pmoles mg wet weight\(^{-1}\) min\(^{-1}\) using a similar buffer, employing a radio enzymatic assay (Goldberg & McCaman, 1973). Levels of basal ACh release in 4.7 mM K\(^{+}\), approximately an eighth of the release stimulated by 46 mM K\(^{+}\), were similar to previous studies (Salehmoghaddam & Collier, 1976, Richter, 1976). Using the blood pressure response of the eviscerated cat to measure released ACh, the rate of release of potassium-stimulated (46 mM) ACh release has been estimated at 1.5 pmoles mg wet weight\(^{-1}\) min\(^{-1}\) from the rat cortical slice preparation and was eight fold higher than the basal release (Salehmoghaddam & Collier, 1976).

For this study omission of the first enzyme in the assay, exogenously added AChE, means that the activity of endogenous AChE can be used to separate the components of the total choline signal. Omission of the first
enzyme simplifies the assay but means that more cortical slices are required to perform the experiments as each group of phospholine treated slices is paired with an untreated group. However, the measurement of choline in supernatants in this study has avoided the use of radiolabelled precursors and has given levels of ACh release similar to previous methods. The advantage of the chemiluminescent assay is that it provides a direct estimate of released ACh and overcomes difficulties associated with dilution of radioactive "specific" activity and differential labelling of ACh pools when radioactive tracers are employed (Webster, 1989).

In the initial linearity experiments there was only a two fold increase in ACh release in the potassium-stimulated samples. The basal release of ACh was approximately 1 pmole mg⁻¹ min⁻¹, which was higher than in the study experiments. However, when the basal release is subtracted from the potassium-stimulated release, the evoked release obtained was similar for the linearity and study experiments. The most likely explanation for the decrease in basal ACh release in the latter experiments was improved experimental technique. Removal and dissection of the cortex post-mortem should be achieved within 5 minutes to prevent ACh and choline leak from the preparation (Dross & Kewitz, 1972).

A noticeable feature of the second series of experiments is the reduction in the choline signal in the phospholine slices exposed to potassium-stimulation. This reduction in choline is probably caused by a depolarisation induced stimulation of high affinity choline uptake (Murrin & Kuhar, 1976). The choline component that is reduced is the choline derived
from the neural tissue as the choline derived from the hydrolysis of ACh is unavailable owing to inhibition of AChE by phospholine.

The radio-receptor assay for ACh was employed to verify that the ACh signal released from the cortical slice preparation determined in chemiluminescent assays was indeed ACh. The radioligand was displaced by ACh standards and experimental samples that had been exposed to phospholine pre-incubation. The assay also confirmed that tissue that had not been exposed to phospholine did not subsequently release anything that displaced the radioligand. The assay did significantly overestimate the amount of ACh when the intra-assay variability was examined, but there was good agreement when the inter-assay variability was investigated. There was good agreement between the 2 methods when both were compared directly, and the linearity of the release of ACh from the cortical slice preparation was also confirmed using the NMS displacement assay.

Potassium evoked acetylcholine release from the rat cortical slice preparation was inhibited in the presence of halothane. The effect occurred over the dose range of clinical anaesthesia, with an IC50 of approximately 0.3 MAC. The potassium-evoked release of choline was not affected by most concentrations of halothane tested, however, there was a significant decrease in basal choline release at supra clinical concentrations of halothane above 3 %. There was an increase in non-stimulated ACh release in the slices exposed to halothane, but this effect did not show a dose-response relationship and was not statistically significant.

Potassium-stimulated ACh release from rat cortical slices was inhibited significantly by clinically relevant doses of halothane. Reduced ACh release
during anaesthesia has been implied from the 50% reduction in release seen in synaptosomes at 3% halothane concentration (Johnson & Hartzell, 1985). Observations in this study confirmed these findings and further demonstrated that the release process was sensitive to concentrations of halothane below MAC. The IC\(_{50}\) for the reduction in release occurred at approximately 0.3 MAC. Although these observations are from \textit{in vitro} experiments the reduction in evoked ACh release seen in these experiments suggests that ACh release may be involved in the process of anaesthesia.

In two previous studies on rat cortical slices, no effect on ACh release was observed with halothane 1.25%, methoxyflurane 0.2% or enfurane 3% (Bazil & Minneman, 1989a, Bazil & Minneman, 1989b). There are a number of differences between the study in this thesis the earlier work which may explain the contrasting results. First, the earlier studies did not measure ACh release, but measured total tritium release from the preparation that had been incubated with \(^{3}\text{H}\) choline. The endogenous AChE was not inhibited with an anticholinesterase and so the effect of volatile agents on total choline release from the cortical slice preparation was examined. No difference was observed in the presence of anaesthetic agents (Bazil & Minneman, 1989a, Bazil & Minneman 1989b). Our data agrees with these findings, as halothane had no effect on total choline release (from choline and ACh) at all concentrations tested.

Secondly, choline is released from the surface of neural preparations as soon as the cortex is removed from the animal (Dross & Kewitz, 1972), and the amount released varies depending on how quickly the particular neural preparation is made. Thus, the component of the choline signal from the
preparation that is not derived from the hydrolysis of ACh, can vary between preparations. If only the total choline signal is measured, as in the previous studies (Bazil & Minneman, 1989a, Bazil & Minneman, 1989b), any reduction in choline signal from the reduced release of ACh, may have been obscured by a choline signal derived from other sources. In this study we have measured both components of the choline signal and have demonstrated clearly a difference in the signal derived from ACh in the presence of halothane. Thirdly, differential labelling of intracellular pools of acetylcholine in the nerve terminal when labelled precursors are employed could account for the lack of effect seen in the presence of the volatile anaesthetic agents (Webster, 1989).

It is noteworthy that apart from the release of ACh, the release of a number of other important neurotransmitters from neural preparations is sensitive only to supra clinical doses of various anaesthetic agents tested (Pocock & Richards, 1988, Hirose et al, 1992, El-Maghrabi & Eckenhoff, 1993). The high sensitivity of ACh release to halothane measured in this study together with the relatively high sensitivity of this process to ethanol (Carmichael & Israel, 1975) suggest that the inhibition of ACh release in vivo may play an important role in the depression of central activity. The rank potency for inhibition of release of a range of other transmitters by ethanol parallels that of the volatile anaesthetic agents (Carmichael & Israel, 1975). Moreover, the release of transmitters such as glutamate (Hirose et al, 1992) and dopamine (El-Maghrabi & Eckenhoff, 1993), which have been shown to be insensitive to clinically relevant concentrations of volatile anaesthetics also require lethal doses of ethanol to inhibit their release.
In vivo, the ACh release process in the mesencephalic reticular formation in rabbits has been shown to be more sensitive to ethanol than the cortex (Erickson & Graham, 1973). Release in the mesencephalic reticular formation was inhibited at sub-intoxicant doses whereas inhibition in the cortex was only seen at concentrations which produce intoxication in adults. Recently, a statistically significant reduction in ACh release from the pontine reticular formation of the cat has been reported (Keifer et al, 1994) in the presence of 1 MAC halothane. This in vivo observation compliments the earlier study by Kanai and Szerb (1965), in which increased dose of halothane from 1 to 1.5 % caused a reduction in ACh release from the cortex of rabbits which was also linked to EEG changes.  

Our study does not allow us to distinguish direct effects of the anaesthetics in the release mechanism from other activities that may affect ACh availability. We would suggest, however, that a direct action on ACh release is likely. Halothane induced inhibition of choline uptake, which is rat limiting for ACh synthesis, has been shown to occur at higher concentrations of halothane within the clinical range and also to reach a maximum inhibition of only 30 % of the original activity (chapter 5). However, the study on choline uptake was conducted under non depolarising conditions and we know that following depolarisation choline uptake is increased (Murrin & Kuhar, 1976). Therefore, the inhibition of choline uptake by inhalational agents could be much greater under depolarising conditions. Nevertheless the dose-response curve for the inhibition of choline uptake by halothane is to the right of that for ACh release, and it is likely that there is a direct effect of the
agent on the release mechanism which may act in concert with reduced choline uptake.

Anatomical and physiological studies have established a link between cholinergic transmission and cortical arousal (Steriade et al, 1990; Semba, 1991) and cholinergic neurones in the basal forebrain appear to have a crucial role in cortical arousal (Buzsaki et al, 1988). The present study demonstrates that the sensitivity of ACh release to halothane is within the clinically relevant anaesthetising range of this agent. This study on potassium evoked ACh release has reinforced the concept that neurotransmitters have different sensitivities to volatile anaesthetic agents. If anaesthesia was achieved by a general toxicological effect, then all transmitters studied would display the same inhibition or enhancement of release when exposed to the same dose of agent.

If the ACh release machinery is more sensitive to anaesthetics than other transmitters, how can this difference be investigated further? The first area to study would be the effect on early transmitter release. This study could only report on changes in ACh release that had occurred after an extended period. Earlier time points could not be investigated due to the small quantities of ACh present in nerve terminals. There are two phases to the ACh release process following potassium stimulation (Szuskiw & O'Leary, 1983), a rapidly terminating phase with a t½ of 18 seconds and a subsequent slow efflux that occurs during a prolonged depolarisation. It would not be possible to investigate the early release phase using either of the assay systems described in this study, as they would not be sufficiently sensitive. Labelled precursors enable the early release to be distinguished, but this
introduces inaccuracies owing to differential labelling of intra terminal depots (Suszkiw & O'Leary, 1982) that this study particularly wished to avoid.

This study may also be difficult to interpret because of the static system employed. The supernatants were in contact with the neural preparation for 30 minutes and many other neuroactive species would have been released from the tissue during this period. Accumulations of endogenous transmitters and modulators of release may have altered transmitter release and presynaptic receptor function. Acetylcholine release is inhibited through presynaptic GABA_B receptors (Taniyama et al, 1992), which complicates the interpretation of the data obtained. The static system can be replaced by superfusion systems (Pearce et al, 1991), but again labelled precursors are mandatory in order to assay such small quantities of released transmitter.

It is unknown as to which aspect of ACh release is initially affected by halothane in these investigations. It is known that there are two intraterminal pools of transmitter, a vesicular source and a cytoplasmic source (Arnaiz, 1993). It can be assumed that halothane affects the vesicular release of ACh as this release is calcium dependent (Arnaiz, 1993). In the cortical slice preparation, release was virtually undetectable in the absence of extracellular calcium. Further studies may be able to distinguish if vesicle fusion with the membrane or vesicle movement to the membrane are the prime targets for the action of the volatile agents. Preliminary experiments using the synthetic calcium ionophore A23187, have reversed the halothane induced inhibition of ACh release from rat cortical slices. This suggests that halothane is either preventing calcium entry into the neurone or is inhibiting calcium release from
intracellular stores. Further experiments may be able to distinguish these phenomena and may explain why ACh release is so sensitive.

The mechanics of ACh vesicle storage and fusion have been further understood by the discovery of the compound trans-2-(4-phenylpiperidino)cyclohexanol (vesamicol), which binds to and inhibits the ACh transporter. Active transport of ACh into synaptic vesicles and loss of ACh from the cytoplasm are both inhibited by vesamicol. However, when vesamicol is given \textit{in vivo} ACh release is also inhibited from the neuromuscular junction and the animals die from paralysis and respiratory arrest (Brittain \textit{et al.}, 1969). This makes the interpretation of \textit{in vivo} data difficult as there is no pure central effect. Vesamicol, however, may help in the understanding of the halothane induced inhibition of ACh release as if halothane was acting by a similar mechanism the two agents would be additive and a larger decrease in ACh release would be expected for a particular dose of halothane.

Already the identity of some of the proteins involved in vesicle fusion have been identified. A small G protein, rab3A and rab3B are involved in vesicle fusion to the active zone of release and a membrane protein synaptotagmin, is involved in the docking and fusion of synaptic vesicles (Südhof & Jahn, 1991). However, it appears that the vesicular release process is complex and has multiple steps and presents many possible sites of anaesthetic drug interaction.

A final cautionary note should be added regarding the physiological interpretation of any studies on ACh that necessitates the inhibition of the endogenous AChE present in the tissue. Phospholine was added in order to
separate the choline signal into two components, however, this manipulation may lead to prolonged presynaptic receptor activation by released ACh that is no longer subject to enzymatic hydrolysis by AChE. The presynaptic autoreceptors play a role in the regulation of evoked transmitter release, mobilisation of vesicles to the cell surface and also modulate the intraterminal metabolism of calcium (Langer et al, 1991), thus prolonged receptor activation by ACh may alter the homeostasis of the presynaptic region and lead to misleading results. There is no uptake mechanism for released ACh, as is the case for other transmitters, and the choline transporter is unable to carry ACh into the cell. Additionally, it is now believed that AChE may have other functions despite its role in the breakdown of ACh (Massoulié et al, 1993) such as during embryonic development and in the modulation of neuronal activity.

In conclusion this study demonstrates that potassium-evoked ACh release from rat cortical slices is sensitive to halothane. This inhibition may be responsible for the reductions in ACh release measured in vivo (Kanai & Szerb, 1965, Keifer et al, 1994) and it appears likely that central acetylcholine release is a target for volatile anaesthetic action.
8. Conclusions

8.1 Summary of The Findings of This Work

Three aspects of ACh metabolism that were investigated in this thesis were affected by the volatile anaesthetic agents tested. Total choline uptake into the synaptosome preparation was inhibited non-competitively with an IC$_{50}$ close to the MAC value for the rat. This in vitro investigation demonstrated that choline uptake was inhibited by clinically relevant doses of halothane.

The $K_m$ for choline of ChAT was reduced by enflurane. There was a dose response relationship for this effect, with greater reductions in $K_m$ at higher concentrations of enflurane. This in vitro effect may be a possible contributory factor to the excitatory effects seen in vivo with enflurane.

The potassium-stimulated release of ACh from the rat cortical slice preparation was inhibited with an IC$_{50}$ of approximately one third of MAC. The measurement of ACh release was achieved with a modification of a chemiluminescent assay and did not utilise labelled precursors. The inhibition of ACh release by halothane is the most potent effect of volatile anaesthetic agents on transmitter release reported using an in vitro preparation reported to date.

8.2 Implications of This Work

The results of this work suggest that volatile anaesthetic agents exhibit 'selectivity' for different aspects of ACh metabolism. If volatile agents achieved their effects by a generalised mechanism of action it would be expected that functions such as precursor uptake and transmitter
release would be influenced to a similar degree by equipotent doses of different agents. However, if anaesthetic agents have more specific effects on transmitter metabolism there would be differences in modulation of these functions by the drugs. The results herein favours the latter interpretation of a more specific mode of action, owing to the difference in sensitivity between choline uptake inhibition and reduction in ACh release.

A further finding of this work is the effect of enflurane on the intracellular enzyme, ChAT. If anaesthetic agents achieve their effect by interaction with an, as yet, unidentified protein or hydrophobic area of a protein, it seems probable that any side effects of the agents may also be explicable by interactions with proteins. The altered kinetics of ChAT by enflurane may contribute to the excitatory effects of this agent and indicate a possible protein target susceptible to modulation in vivo by enflurane.

An important point arising from this work is the apparent differential sensitivity of transmitter systems to volatile anaesthetic agents. Precursor uptake and transmitter release in the cholinergic system appear to be more sensitive to anaesthetic drugs than equivalent mechanisms for other transmitters. It is interesting to speculate that, as an integral part of the reticular activating system, the cholinergic system may be the switch that determines the conscious state from the anaesthetised state. However, although both precursor uptake and transmitter release, were affected with clinically relevant doses of agents, a smaller change in another transmitter system may reflect a greater functional change.
8.3 Further studies

The in vitro studies on the cholinergic system, which have demonstrated sensitivity of this system to anaesthetic agents should be further studied by both in vitro and in vivo investigations.

The in vitro studies should aim to examine in more detail the mechanisms of inhibition of ACh release, inhibition of precursor uptake and ChAT interaction.

The sensitivity of the release process to halothane is probably the most significant finding of this thesis. There are three main areas where the volatile anaesthetic agents could act to disrupt transmitter release. They may act by depletion of intracellular vesicles, the disruption of exocytosis or by activity on voltage-gated calcium channels. As the delay between the arrival of a nerve terminal impulse and synaptic transmission is less than 200 μs, depletion of intracellular vesicles is an unlikely mechanism. However, the rapidity of the response to a nerve impulse makes docking of synaptic vesicles to an active zone of the nerve terminal a candidate for the inhibition of release by volatile agents.

The two aspects that need to be investigated are the role of calcium in vesicle exocytosis and the family of proteins involved in vesicle docking and membrane fusion with the active zone.

Further studies on transmitter release have been commenced to investigate the influence of calcium on the halothane-sensitive component of ACh release. Initial data suggests the synthetic calcium ionophore, A23187, can overcome halothane inhibition of release which suggests it may be working by preventing calcium entry or preventing
calcium release from intracellular stores. It is known that the increase in cellular calcium following transmitter mobilisation and release probably only occurs transiently and is also restricted to micro-domains under the plasma membrane, therefore the interference with calcium is probably caused by prevention of external calcium gaining access to the cell. If halothane affects the calcium influx following the stimulation of ACh release, it seems likely that the release process for ACh is more sensitive to anaesthetic modulation as other calcium dependent transmitter systems are not so susceptible to inhibition by volatile agents.

Synaptotagmin is one of the vesicle proteins that is involved in the binding of calcium and the docking of the vesicles to the active zone. The neurexins and epimorphins are two further protein families that are probably located in the active zone and may interact with synaptotagmin. The receptor for a component of the black widow spider venom, α-latrotoxin, is a member of the neurexin family. α-Latrotoxin causes massive neurotransmitter release from presynaptic nerve endings which may be reduced by halothane if the volatile agent was interacting with the function of the docking proteins. Interestingly, the transmitter release caused by α-latrotoxin is calcium independent and occurs one step further down the sequence from the involvement of calcium with the active zone. Thus, it may be possible to separate different anaesthetic actions on transmitter release.

A further membrane protein, mediatophore, participates in the calcium-dependent release of ACh in synaptosomes produced from the *Torpedo* electric organ (Israel *et al*, 1984). Drugs derived from cetiedil may potentiate or inhibit the release of ACh from synaptosomes or from artificial membranes in which the ACh release machinery has been
incorporated. By the use of these drugs together with halothane it may be possible to enhance or overcome the inhibition of release promoted by the volatile agents. Although, not directly related to the study of ACh from mammalian species, data obtained from the *Torpedo* electric organ may help to understand the anaesthetic interaction with the ACh release process.

Further studies will also need to investigate the influence of the volatile agents on pre-synaptic receptors. Transmitter release is influenced by endogenous and exogenous ligands acting on presynaptic autoreceptors. Nicotinic and muscarinic effects may be distinguished by the use of specific agonists and antagonists. In the *Torpedo* electric organ synaptosomes muscarinic activation can inhibit transmitter release (Dolezal *et al*, 1988). Again, it may prove useful to study the anaesthetic interaction with this preparation before attempting similar experiments in mammalian species.

Similarly, the differential sensitivity of the precursor transport systems, suggests that the choline uptake mechanism is more sensitive to volatile agents than other precursor transporters. The amino acid sequences of the transporters share sequence similarity and structure but are not equally affected by volatile agents. In order to understand the anaesthetic interaction, more detailed information on the structure-activity relationship of the transporters is required. It is likely that this information will come from the field of molecular biology, and may reveal an anaesthetic-sensitive component of the choline uptake transporter that is not shared by the other uptake systems.

Modulation of ChAT activity by enflurane may also be shared by other anaesthetic/convulsant ethers, such as the convulsant
hexafluorodiethylether ether and its anaesthetic isomer isohexafluorodiethylether. From this study it would be expected that the convulsant ether would affect the enzyme and the anaesthetic ether would have no effect similar to halothane and isoflurane. These agents should also be screened for their effect on ChAT activity. A difficulty will be the safe delivery of a convulsant ether in a laboratory environment. These studies may be able to add substance to the hypothesis that the excitatory phenomena observed with enflurane are partially mediated through the cholinergic system, although the precise nature of the interaction may be difficult to examine as the possible rate limiting role of ChAT in cholinergic function is still not fully understood.

The studies of cholinergic function in vitro may be extended to functional aspects of the cholinergic system in vivo. This study has shown that volatile anaesthetic agents do modulate the activity of the cholinergic system in vitro. There has been a tremendous interest in cholinergic pharmacology and physiology related to the deterioration in the central cholinergic system that occurs due to ageing. The in vitro effects of volatile agents, if present in vivo, may contribute to post operative impairment of cholinergic function which may be manifested as post operative cognitive impairment. New cholinergic drugs, aimed at improving cholinergic function, such as anticholinesterases and selective muscarinic agonists, may improve the cognitive impairment and attenuate the effects of anaesthesia. To date there is no specific ACh releasing drug available although, ondansetron, a selective 5-HT3 antagonist increases ACh release indirectly. Studies have already commenced to examine the benefit that ondansetron may have in improving perioperative cognitive function following general anaesthesia.
8.4 Conclusions

The *in vitro* sensitivity of choline uptake and ACh release to modulation by clinically relevant doses of anaesthetic agents suggest that the cholinergic system may be a target for the action of these agents. Suggestions from the findings of this study of these findings are already being investigated in clinical studies into the effects of volatile anaesthetic agents in the post-operative period. It seems likely that the volatile group of anaesthetic drugs do exhibit specific effects and do not affect all transmitter systems similarly.
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EFFECTS OF ANAESTHETICS ON UPTAKE, SYNTHESIS AND RELEASE OF TRANSMITTERS

R. GRIFFITHS AND R. I. NORMAN

While the molecular basis of the action of most anaesthetic agents is unknown, it is a commonly held view that general anaesthetic drugs have a more pronounced effect on synaptic mechanisms in the central nervous system (CNS) than on the propagation of electrical signals along axons [39]. A more contentious issue relates to whether or not anaesthetic agents act generally, producing a range of metabolic alterations which together result in anaesthesia or at, as yet, unidentified specific sites in neuronal mechanisms [25].

Sensory information is passed from cell to cell in the CNS by neurotransmitters. These transmitter substances are released in the synaptic cleft between adjacent cells and, depending on the particular synapse, can interact with presynaptic and/or postsynaptic receptors. The postsynaptic actions of transmitter substances are mediated classically via excitatory postsynaptic potentials (EPSP) or inhibitory postsynaptic potentials (IPSP) and the size of these can be modulated by the regulation of transmitter release from the nerve terminal by the interaction of neurotransmitter substances at receptors in the presynaptic membrane.

The diversity of different transmitter substances and the complex interplay between transmitter systems results in a large number of potential sites of action of anaesthetic agents (fig. 1).

EXPERIMENTAL SYSTEMS

Although there have been a number of in vivo studies [4, 9, 55–57, 65], by far the majority of studies on the action of anaesthetic agents on transmitter systems have been carried out in in vitro preparations. Three major experimental paradigms, namely synaptosomes, brain slices and cells in culture, have been used in in vitro studies (fig. 2). Each of these systems has limitations and caution must be exercised in extrapolating conclusions from these studies to the clinical situation.

Synaptosomes are pinched off nerve endings that are formed when brain tissue is homogenized. The synaptosome fraction is separated from particulate material containing mitochondria and other cell debris by centrifugation in density gradients of either sucrose [27], Ficoll [11] or Percoll [22]. The resulting synaptosomes are sealed plasma membrane vesicles which contain the secretory machinery and mitochondria necessary for transmitter metabolism and are a good model for the study of the uptake and release of neurotransmitters (fig. 3). The synaptosome preparation consists of a mixed population of nerve endings containing many different transmitter types which permits the study of the metabolism of a number of neurotransmitters in a single system. The major limitations of the synaptosome preparation are that there is the potential for non-physiological cross-talk between transmitter systems and that the influence of supporting cells and other

KEY WORDS
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neurones has been removed by the process of shearing off the nerve endings. For a fuller consideration of the synaptosome preparation see McMahon and Nicholls [40].

Brain slice (pyramid) preparations have also been used extensively to study neurotransmitter metabolism. This is a more complex preparation, as some functional connections are maintained. Possibly the simplest in vitro preparations are neuronal and neuronal-like cells in culture, which permit the study of intact, isolated cell types. Again, the absence of functional tissue organization cannot be ignored and the relevance of observations made on non-neuronal cells, such as chromaffin cells, and cells which may be only partially differentiated must be considered.

NEUROTRANSMITTER CLASSIFICATION

Neurotransmitter systems have been subdivided according to their classification into (1) amino acids, (2) other classical transmitters and (3) neuropeptide systems [40]. In this review we concentrate on the group 1 transmitters, including aspartate, glutamate, gamma-aminobutyrate (GABA) and glycine and the group 2 transmitters, including the catecholamines,
dopamine, noradrenaline and adrenaline, 5-hydroxytryptamine (5-HT) and acetylcholine, as little work has been directed to the effects of anaesthetic agents on group 3 transmitter systems.

GROUP 1—AMINO ACID TRANSMITTERS

Excitatory transmitters: glutamate and aspartate

Glutamate and aspartate are both believed to act as excitatory neurotransmitters in the mammalian brain [24]. In particular, neurotransmission mediated by glutamate has been implicated in learning and memory, neurodegenerative disorders, epilepsy and the effects of hypoxia, ischaemia and possibly other neurological and psychiatric states [16, 20].

Biosynthesis of glutamate can occur via two pathways, either from the deamination of glutamine or from the tricarboxylic acid cycle [33] (fig. 4). It would appear that most of the glutamate released following depolarizing stimuli is derived originally from glutamine [33]. After release into the synapse, signal transmission by the excitatory amino acids is mediated at the postsynaptic membrane by receptors for these neurotransmitters. These are classified into three classes, N-methyl-D-aspartate (NMDA), quisqualate and kainate, depending on their pharmacology [69]. The NMDA receptor subclass is of particular importance to the anaesthetist as ketamine is a non-competitive antagonist at this receptor [72]. Postsynaptic events are terminated largely by the uptake of glutamate and aspartate into neighbouring glial cells. In view of their wide distribution and suggested roles in the CNS, the excitatory amino acid transmitter systems constitute an important potential target for anaesthetic action.

Release. In early studies of brain slices, barbiturates were shown to decrease the release of aspartate from electrically stimulated rat lateral olfactory tract slices in a dose-dependent manner [19]. Similarly, depression of potassium-stimulated aspartate release has been observed also in rat brain slices in the presence of pentobarbitone, methohexitone and thiopentone [5, 52, 68]. However, a biphasic pattern of release was observed in a rat thalamic slice preparation in which small concentrations of thiopentone \(10^{-4} \text{ mol litre}^{-1}\) and methohexitone \(10^{-3} \text{ mol litre}^{-1}\) enhanced release and greater doses decreased release of D-aspartate and GABA [37].

![Diagram of a presynaptic nerve terminal showing the relationship between the metabolism of excitatory amino acid neurotransmitters and GABA.](image-url)
The greater concentration of thiopentone was within the clinical range but that of methohexitone was greater than clinical concentrations. The mechanism of the biphasic response in excitatory amino acid release in response to barbiturates remains unresolved but may be indirect effects on GABA transmission [37]. Depolarization-induced calcium uptake in the nerve terminal, which triggers transmitter release, has been shown to be inhibited by thiopentone and pentobarbitone [10]. Hence, alteration in calcium handling by the nerve terminal may provide an explanation for the action of barbiturates on excitatory amino acid transmission.

The steroid anaesthetic hydroxydeone is the only other agent which has been shown to inhibit potassium-stimulated aspartate efflux [52]. Other anaesthetic agents, including Althesin (a combination of alphaxalone and alphadalone), ketamine, urethane and halothane have been reported to have no effect or to enhance the release of aspartate from rat brain slice preparations [5, 37, 52]. At clinical doses, 1-2% halothane had no significant effect on the efflux of [3H]aspartate from rat brain slices [5], although increased aspartate efflux has been observed at larger concentrations of halothane (4-8%) which are considerably greater than the clinical dose range.

In contrast, a recent study investigating glutamate release from mice synaptosomes [32] demonstrated an increase in release when the preparation was exposed to clinical concentrations of halothane and enflurane; the increase in release being greater for enflurane than for halothane at equivalent concentrations. Interestingly, the excitatory amino acids are implicated in the pathogenesis of some forms of epilepsy [20]: it is possible that the enhanced release of glutamate in the presence of enflurane could contribute to the electroencephalographic abnormalities seen with this agent [67].

In vivo measurements of excitatory amino acid release, by monitoring brain concentrations of ascorbate voltametrically, have shown that anaesthesia with chloral hydrate reduces the release of excitatory neurotransmitters and that transmitter release recoveries as the animal recovers [56]. These observations provide in vivo evidence for the interference of excitation amino acid neurotransmitters to anaesthetic agents [37, 52, 56, 60]. Perhaps as important, other agents would appear not to be implicated [5, 52]. Together, these results suggest a possible selectivity of action of some anaesthetic agents for the excitatory amino acid release mechanism.

From the limited evidence available, it is possible that some anaesthetics may act on synaptic transmission mediated by the excitatory amino acids [19, 32, 42, 52, 56, 60]. Perhaps as important, other agents would appear not to be implicated [5, 52]. Together, these results suggest a possible selectivity of action of some anaesthetic agents for the excitatory amino acid release mechanism.

Recently, a new compound riluzole (54274 RP), has been found to have possible anaesthetic actions and pharmacologically this compound appears to exert its effects by interrupting glutamate neurotransmission [15]. Riluzole has been shown to reduce the presynaptic release of glutamate [15] and also to affect some postsynaptic properties [62]. In one study, riluzole induced loss of righting reflex in rats and in smaller doses it also reduced the MAC of halothane [42]. The inability of riluzole to bind at glutamate receptors [70] suggests that the hypnotic state induced by this agent is different from that produced by ketamine, which is known to be an NMDA receptor antagonist [72]. Interestingly, riluzole protects against glutamate-induced neural injury following ischaemia [41] and this may also provide a further possible use for this novel compound in neurosurgical intensive care. Further investigation of the anaesthetic mechanisms, possibly mediated by altered excitatory amino acid transmission, is certain to follow the initial observations on riluzole action.

Uptake. When glutamate is released from nerve endings, its actions are terminated by uptake into the surrounding glial cells and neurones. In addition, glutamate uptake occurs in the nerve terminal where it is recycled to glutamine [33]. This carrier also transports aspartate into the nerve terminal.

Uptake of aspartate was not affected by thiopentone, methohexitone, ketamine or urethane in a rat brain slice preparation [52]. Similar findings were reported in rat thalamic slices using anaesthetic doses of thiopentone, methohexitone, urethane and ketamine [57]. Pentobarbitone, in the same study, did reduce slightly the uptake of aspartate but the authors concluded that this effect was unlikely to contribute to the mechanism of action of the barbiturates.

At present, there is little evidence to link the excitatory amino acid neurotransmitters to anaesthetic mechanisms, despite their possible relevance to consciousness. More work in this area is required to clarify the significance of the anaesthetic effects reported to date [5, 32, 42].

**Inhibitory transmitters: GABA and glycine**

**GABA.** GABA is formed from the irreversible decarboxylation of glutamate, catalysed by the enzyme glutamic acid decarboxylase (GAD) (fig. 4). After release into the synapse, GABA transmission is terminated by uptake of the transmitter by surrounding glial cells and nerve terminals and this process, similar to that for glutamate, limits the activity of GABA in the synapse.

GABA is the major inhibitory neurotransmitter in the central nervous system [33]. By acting on both pre- and postsynaptic receptors, it causes an increase in chloride ion conductance which results in hyperpolarization of the nerve membrane. The barbiturate and steroid anaesthetic agents increase this GABA-stimulated chloride conductance, resulting in enhanced hyperpolarization [64], whereas the volatile anaesthetics have a weaker effect on the GABA receptor-Cl channel complex [54].

**Catabolism of GABA.** In a study using rat brain slices, it was found that 5% halothane had no effect on either the uptake or release of GABA [14]. However, the catabolism of GABA in the preparation...
was reduced. This could lead to increased concentrations of GABA in the synapse. Similar effects of halothane on GABA catalysis have also been observed in synaptosomes prepared from rat brain [13]. In addition, clinical concentrations (1 MAC) of chloroform, enflurane and diethyl ether significantly affect GABA catalysis [13]. Of the barbiturates studied, only thiopentone inhibited GABA catalysis at clinical concentrations: pentobarbitone and phenobarbitone had effects only at very large doses [13]. The different actions that have been described, namely reduction in GABA catalysis by the barbiturates, illustrate the possibility that different classes of anaesthetic agents may achieve the same physiological effect via different pharmacological mechanisms.

**GABA uptake**. No effect was found on the uptake of GABA into rat brain slices in the presence of thiopentone, methohexitone, Althesin, ketamine, halothane or urethane [52]. Similarly, pentobarbitone had no effect in rabbit hippocampal slices [34], or thiopentone, methohexitone, urethane and ketamine in rat thalamic slices [37]. It would appear that the uptake process does not play a significant role in the anaesthetic action of these compounds.

**GABA release**. Potassium-stimulated GABA release from rat brain slices has been shown to be reduced by the i.v. barbiturates, hydroxydione and pentobarbitone [52]. However, in the rat olfactory cortex preparation, there appeared to be an increase in the release of GABA in the presence of pentobarbitone [19]. In a third study, a biphasic response was observed in the rat thalamus slice preparation, in which small concentrations of thiopentone and methohexitone produced enhanced release, and larger concentrations reduced release [37]. The contrasting effects of barbiturates on GABA release from different brain regions probably reflect the complexity of action of these agents such that it is the balance of the effect on excitatory and inhibitory transmission that results in net excitatory or inhibitory synaptic events. Results obtained from slice preparations may be complicated by secondary cellular cross-talk, and the synaptosome preparation may be more useful for distinguishing primary effects at the level of the GABA releasing terminal.

The conflicting results concerning the effects of anaesthetic agents on the release of GABA from different areas of the brain in vivo [19, 37, 52, 68], necessitate caution in the extrapolation of observations to the whole animal. In one study, the effect of halothane on the concentration of GABA release in an in vivo preparation was investigated [57]. A microdialysis technique was used in which catheters were placed into the extracellular fluid (ECF) surrounding the brain and the concentrations of neurotransmitters measured. A reduction in GABA content of the ECF was observed in the animals anaesthetized with halothane. Although it was not possible to tell if this reduction was a result of a decrease in release or an increase in uptake, this technique did indicate alteration in transmitter homeostasis in the intact animal.

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In summary, the evidence points to a number of potential sites of action of anaesthetic agents on the GABA transmitter system, but as in other transmitter systems, further work is required.

**Glycine.** Glycine is the major inhibitory neurotransmitter in the spinal cord and medulla and is also implicated widely in other brain regions. This transmitter acts on a receptor-operated chloride channel complex, similar to the GABA receptor–Cl channel complex [36]. The effects of anaesthetics on glycine transmission have not been studied extensively, but one study on rat brain slices has indicated that pentobarbitone has no effect on the potassium-stimulated release of glycine [68]. Further study is necessary to investigate the possible role of glycine transmission in anaesthetic mechanisms.

**GROUP 2—CATECHOLAMINES, ACETYLCHOLINE AND 5-HT**

**Noradrenaline and adrenaline**

The characteristics of release of noradrenaline from peripheral nervous tissue have been documented widely, owing to the availability of the chromaffin cell preparation derived from bovine adrenal medullary glands. This cultured cell preparation has been used as a model to investigate the action of anaesthetics on the process of neurotransmission [1, 53, 58, 59]. While contributing to the understanding of anaesthetic mechanisms and, in particular, to their effect on stimulus-secretion coupling, it should be remembered that this is only a model of the mechanisms in the CNS.

The barbiturates, at clinical concentrations, have been shown to reduce both the potassium- and carbachol-stimulated release of noradrenaline from chromaffin cell preparations [58], and from potassium-stimulated synaptosomes [31]. When the volatile anaesthetic agents halothane (fig. 5), enflurane, methoxyflurane and isoflurane were studied, a similar reduction in adrenaline and noradrenaline...

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**Fig. 5.** Dose-response relationship for the effect of halothane on noradrenaline/CA secretion from bovine chromaffin cells. := Basal secretion; – – after stimulation with carbachol 500 μmol litre⁻¹; – after stimulation with potassium 77 mmol litre⁻¹ (reproduced with permission from [59]).
release was seen when carbachol stimulation was used. However, in contrast to the barbiturates, much larger doses of the volatile anaesthetic agents were needed to inhibit the release after potassium-stimulation. Again, evidence from this experimental system indicates possible selectivity for different classes of anaesthetic agents. However, this apparent selectivity could simply reflect the ability of different anaesthetic agents to reduce calcium concentrations in cells [58, 59] (see below). In contrast to observations on chromaffin cells, reduced potassium-evoked release of noradrenaline has been observed in rat brain slice preparations in the presence of volatile anaesthetic agents at clinical doses (fig. 6) [7, 8]. When applied at equipotent concentrations for producing loss of righting reflex in rats, halothane, enflurane and methoxyflurane were found to reduce potassium-stimulated noradrenaline release by 30% [7].

It has been suggested that anaesthetic agents may increase the ionic permeability of synaptic vesicles and thereby lead to a decrease in the proton gradients that are essential for synaptic vesicles to accumulate transmitters [6]. This "pump leak" hypothesis has been investigated for noradrenaline in the chromaffin cell system. Halothane, diethyl ether, butanol and ethanol were found to cause only a small insignificant loss of noradrenaline from chromaffin granules [1], inconsistent with the pump leak hypothesis. By inference, it appears unlikely that depletion of noradrenaline or other neurotransmitters from secretory vesicles could lead to loss of efficiency of synaptic transmission and subsequent anaesthetic state. However, taken together, in vitro observations for noradrenaline and adrenaline release suggest susceptibility of stimulated release mechanisms to modification by anaesthetic agents.

Complimentary studies have been performed in vivo. By manipulation of central noradrenaline concentrations, it has been shown that the MAC of halothane increases with increasing concentrations and decreases with decreasing concentrations, suggesting that reduced noradrenaline transmission may be an important contributory factor to the induction of the anaesthetic state [50]. Thus observations from both in vitro and in vivo investigations indicate that central noradrenaline metabolism is worthy of further study in unravelling the actions of anaesthetic drugs.

**Dopamine**

Dopamine has a better understood and more localized central action than noradrenaline. As a result, Parkinson's disease and schizophrenia may both be treated using drugs that manipulate central dopamine metabolism. It is noteworthy that amphetamine, which leads to a state of arousal, stimulates dopamine release centrally. By inference, therefore, the dopaminergic system is a potential target for anaesthetic action.

Concentrations of dopamine in the CNS can be manipulated by either increasing concentrations of the dopamine precursor, L-dihydroxyphenylalanine (L-Dopa), to increase concentrations, or by depleting dopamine using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is an analogue of pethidine that selectively kills central dopaminergic neurones by inhibiting respiratory chain enzymes via a metabolic product. When mice were subjected to these two treatments, L-Dopa decreased the MAC of halothane and MPTP treatment increased the MAC of halothane [65]. Furthermore, when the concentrations of dopamine of MPTP-treated mice were repleted with L-Dopa, the MAC of halothane was restored to control levels.

In a second complex study, using an in vivo microdialysis technique, the effect of halothane anaesthesia on dopamine secretion was investigated [57]. Halothane was found to increase basal concentrations of dopamine when rats were anaesthetized. Results from both these studies conflict with expectations arising from the effects of amphetamine on dopamine metabolism. In vivo and in vitro studies are urgently required to characterize the effects of anaesthetic agents on dopamine metabolism in more detail. In view of the number of patients with alteration in central dopamine pharmacology, an enhanced understanding of anaesthetic mechanisms in the dopaminergic system is likely to have important clinical implications.

**5-HT**

A precise functional role for 5-HT has yet to be fully established. However, 5-HT has been implicated as an important transmitter in the feeding, sleep, mood, behaviour and cardiovascular control pathways [3].

5-HT is synthesized from the amino acid L-tryptophan in a two-step pathway. The first, a rate limiting step, involves conversion of L-tryptophan to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase. This is followed by decarboxylation of 5-hydroxytryptophan to 5-HT, catalysed by L-aromatic acid decarboxylase. L-tryptophan is transported actively into the nerve terminal and this may be a further limiting factor in the synthesis of 5-HT. After stimulated release into the synapse, the
postsynaptic response is terminated by removal of 5-HT from the cleft by a high-affinity active transport mechanism.

To date, only two aspects of 5-HT metabolism have been investigated in the presence of anaesthetic agents, 5-HT uptake and release. There have been no studies on the effects of anaesthetic agents on the uptake of L-tryptophan, or on the rate limiting enzyme tryptophan hydroxylase, which may represent important control points in this transmitter system.

**5-HT uptake.** In a series of experiments using the rat synaptosome preparation, ketamine, enflurane, halothane and isoflurane [43-45, 47] inhibited the uptake of 5-HT into the synaptosomal fraction. It is possible that inhibition of uptake may increase the effect of the transmitter at its postsynaptic receptor site as the synaptic activity of 5-HT is terminated by an active re-uptake mechanism. 5-HT is believed to exert its influence on analgesic mechanisms by activating inhibitory mechanisms in the dorsal horn of the spinal cord, hence a decrease in re-uptake may contribute to the anaesthetic effects of these drugs. In contrast, during the same series of experiments, pentobarbitone and etomidate were found to have little effect on the activity of the 5-HT uptake process, again suggesting selectivity for anaesthetic agents on different neuronal activities.

To further investigate the selectivity of action of anaesthetic agents on the 5-HT uptake mechanism, competition binding studies have been carried out using [3H]paroxetine, which binds competitively to the substrate recognition site of the 5-HT transporter [46]. Ketamine caused a concentration-dependent inhibition of specific [3H]paroxetine binding, whereas halothane had no effect. Interestingly, ketamine and halothane were found to induce a similar level of inhibition of 5-HT uptake at clinically relevant concentrations of anaesthetic agents. Thus while ketamine may cause inhibition of 5-HT uptake by direct interaction with the active site of the transport protein, inhibition of uptake by halothane must be mediated, at the very least, by a distinct site on the transporter protein or possibly by mechanisms located elsewhere in the membrane. 

Ethanol in sufficient quantities causes general anaesthesia in humans [2]. When an equivalent dose of ethanol was applied in vivo to a synaptosome preparation, the uptake of 5-HT was stimulated [2], in contrast to other anaesthetic agents studied above [43-47]. These contrasting effects on the 5-HT transmitter uptake mechanism do not offer a simple explanation for the possible role of this process in anaesthesia.

**5-HT release.** To date, only the effect of pentobarbitone on 5-HT release from rat midbrain slices has been studied. A small decrease (29%) in 5-HT release was reported, although this was not significantly different to untreated control samples [68].

The complex interaction between neurotransmitter systems was well documented in an in vivo study [9] in which a push–pull cannula technique was used to study the influence of acetylcholine on 5-HT release in the cat caudate nucleus. In the control group there was an acetylcholine-dependent reduction in the release of 5-HT, which was not present in the halothane anaesthetized group. Further experiments suggested that the reduction in 5-HT release observed after application of acetylcholine may be a result of stimulation of GABA interneurons. This work suggests that 5-HT concentrations could vary in response to anaesthetic agents, because of indirect effects on other transmitter systems, and demonstrates the complex interconnections between neurotransmitter systems that are seen in whole animal preparations which cannot be studied easily in in vitro systems.

**Acetylcholine**

Acetylcholine is well established as an excitatory neurotransmitter. A number of in vivo observations have suggested that cholinergic transmission may be a target for anaesthetic action. In a classical study on acetylcholine transmission, the release of acetylcholine from the surface of the rabbit brain was shown to decrease during barbiturate anaesthesia and increase after a period of feeding and walking, indicating a role for acetylcholine in the maintenance of consciousness [18]. Clinical anaesthetists will be aware also of the prolonged sedative effects that anticholinergic drugs produce, particularly in the elderly population [66]. In a recent study, central cholinergic activity was shown to oppose ketamine anaesthesia [51] and this effect on ketamine anaesthesia was antagonized when the anticholinergic drug 1-hyoscymamine, was given as premedication. These lines of evidence emphasize the important role of acetylcholine in the control of conscious level.

Acetylcholine is synthesized in the nerve terminal from choline and acetyl coenzyme A in a reaction catalysed by choline acetyltransferase (ChAT) (fig. 7). After release, the acetylcholine signal in the synapse is terminated by hydrolysis by acetylcholinesterase (AChE) and choline is taken up by the presynaptic cell for resynthesis of acetylcholine. The rate limiting step in the synthesis of acetylcholine is
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... (SEM) of 3-4 determinations (reproduced with permission from choline (ACh: release from rat midbrain slices. Values are mean

2.4 MAC. A similar reduction was seen also when enflurane and isoflurane were studied. Whether the action of the volatile anaesthetics on precursor

2.4 MAC, enflurane has been shown to alter ChAT activity in rat brain synaptosomes [29] by reducing the Michaelis constant ($K_m$) of the enzyme for choline. In vivo, such an effect could potentiate the synthetic pathway for acetylcholine and contribute to the central effects on the electroencephalogram [53] and to the seizure activity that may develop with large inspired concentrations of enflurane. This may also explain the maintenance of acetylcholine concentrations in subcortical structures under enflurane anaesthesia in the in vivo study described above [55].

**Acetylcholine release.** Few studies of the effects of anaesthetic agents on release of acetylcholine have been performed. In rat slice preparations, potassium-stimulated acetylcholine release has been shown to be decreased in the presence of pentobarbitone (fig. 8) [60, 61, 68] and methohexitone [61]. Similarly, halothane, at a concentration of 2.5 MAC, has been shown to reduce potassium-stimulated release of acetylcholine by approximately 50%, from a rat brain synaptosome preparation [35]. A reduction in acetylcholine release has also been demonstrated with 1 and 2% halothane in the cat superior cervical ganglion preparation [12]. However, conflicting results have been obtained in other studies. Halothane, methoxyflurane and enflurane, at concentrations of 1.25%, 0.2%, and 3%, respectively, were reported to have no effect on acetylcholine release from a rat brain slice preparation (fig. 9) [7, 8] and in an isolated cat superior cervical ganglion preparation, chloral hydrate was found to either decrease potassium-stimulated release [61] or to have no effect [48].

In the above studies, total acetylcholine release was measured in synaptosomes [35], while release of [$^3$H]acetylcholine was monitored in the brain slice studies [7, 8]. Differential labelling of intracellular pools of acetylcholine in the nerve terminal [71] could underlie some of the differences in the observations [7, 8, 35]. In addition, rat brain slices could be known to be more sensitive to potassium depolarization than synaptosomes [63] and this could also account for differences in actions of anaesthetics between the preparations. Comparisons between these studies may be further complicated by the different doses of anaesthetic agents applied in the various studies [8, 35].

**OTHER TRANSMITTERS**

A number of other transmitter systems, in particular those classified as group 3, including neuropeptides such as substance P, the opioids and thyrotrophin...
releasing hormone (TRH), have received little or no attention with respect to the effects of anaesthetics. At present, 40 neuroactive peptides have been identified, but not all satisfy the criteria for neurotransmitters [38]. Therefore there remains a large amount of work before questions regarding selectivity of anaesthetic action can be answered fully.

THE ROLE OF CALCIUM IN NEUROTRANSMITTER RELEASE: EFFECT OF ANAESTHETIC DRUGS

Influx of extracellular calcium is essential for the coupling of electrical excitation to neurotransmitter release [17]. Two phases of depolarization-induced calcium entry into the nerve terminal have been characterized, a rapid phase and a slower plateau phase [40]. The release of different neurotransmitters may be regulated on different phases of the calcium current [40]. As calcium is crucial to neurotransmitter release, this aspect of stimulus-secretion coupling may be a potential site of action of anaesthetic agents and may present a mechanism by which some selectivity of anaesthetic action may occur.

Depolarization-induced calcium uptake into synaptosomes has been shown to be reduced in the presence of a wide range of anaesthetic agents [10, 21, 30]. Furthermore, in bovine chromaffin cells, anaesthetic-induced reduction in calcium influx has been linked to a decrease in evoked noradrenaline release in the presence of pentobarbitone, halothane (fig. 10a), isoflurane, enfurane and methoxyflurane [58, 59, 73]. When noradrenaline release from chromaffin cells was stimulated with carbachol or potassium, carbachol-evoked release appeared to be more susceptible to the action of clinical doses of volatile anaesthetic agents than potassium-evoked release. Inhibition of potassium-stimulated release was affected only at suprachronic doses of anaesthetic agents. These results are most likely to be explained by the concentration of calcium in the cell achieved after the two stimulation procedures. When stimulated with potassium, the influx of calcium was much greater than when stimulation was with carbachol, thus halothane-induced reduction in intracellular calcium concentrations to threshold concentrations for transmitter release was more easily achieved under carbachol stimulation. These results are consistent with an important role for calcium homeostasis in anaesthetic-induced modulation of neurotransmitter release.

The possibility that reductions in calcium influx in the presence of anaesthetic agents may be linked to the membrane disordering properties of these drugs has been investigated further [21, 30]. No simple relationship between the ability of anaesthetic agents to reduce calcium influx and disorder membrane structure was shown. A stronger correlation was found between the effect on sodium influx and membrane perturbation [30].

DIRECT VERSUS INDIRECT ACTIONS OF ANAESTHETIC AGENTS ON PRESYNAPTIC TRANSMITTER MECHANISMS

So far in this discussion, consideration of anaesthetic effects at the level of the presynapse has focused on specific aspects of synaptic transmission mediated by a variety of transmitter agents. A variety of effects have been reported, some of which may be compatible with the onset of anaesthesia. An important question that remains is how the anaesthetic agents exert their effects on such a range of biochemical systems.

From a pharmacological perspective, most general anaesthetic agents would appear to be essentially non-specific in their actions at the level of transmitter metabolism and release. However, as can be seen from the above discussion, not all anaesthetics produce the same response in the assay systems exploited, indicating some degree of selectivity of action (see tables I and II). Indeed, it is possible that certain anaesthetic agents are found to exert their primary effects in a specific manner at the level of discrete proteinaceous acceptors, for example ketamine acts at the NMDA receptor [51] and at the active site of the 5-HT transporter [46], the barbiturate acts at the GABA receptor–Cl complex [65] and isoflurane produces stereoselective activation of a potassium channel subtype [26]. However, when agents such as the volatile anaesthetic agents are considered, a wide spectrum of effects in different transmitter systems is observed, involving not only presynaptic but also postsynaptic events. In these cases, anaesthetic effects may reflect the susceptibility of discrete cellular functions to non-specific, anaesthetic-mediated changes in the local environment rather than specific interactions with proteinaceous acceptors. Hence, anaesthesia may be the net result of a combination of a large number of non-specific toxicological effects on CNS function that
any particular agent exerts. If so, the "physiological mechanism" of anaesthesia may well be different for each agent.

At the present time, there have been insufficient studies to allow any definitive statements to be made concerning the relevance of the action of anaesthetic agents on presynaptic transmitter release mechanisms to the overall mechanisms of general anaesthesia (see tables I and II). In many cases, our knowledge of the effects of anaesthetic agents on discrete presynaptic activities depends on one or two studies. Often these have been carried out on more than one experimental system and under different experimental conditions, such that a true comparison of results is not possible. More systematic studies of the actions of general anaesthetic agents on individual presynaptic activities in defined experimental systems and under comparable conditions are urgently required. Furthermore, these should include both in vivo and in vitro studies to allow primary and secondary effects of anaesthetic agents to be dissected. While in vitro studies may allow effects on specific functions to be studied more easily, the importance of in vivo studies must be stressed to permit the discrimination of statistically significant changes measured in vitro which have no physiological relevance in vivo. Since a universal action of general anaesthetic agents cannot be ruled out, one aim of these studies should be to identify central activities that are universally altered by general anaesthetic agents in a manner consistent with the induction of the anaesthetic state. The likelihood is, however, that physiological mechanisms of anaesthesia will prove to be much more complex.

The feature that unifies all general anaesthetic agents is their relatively high hydrophobicity. Indeed, lipid solubility is the only property of all anaesthetic agents that appears to correlate with anaesthetic potency [49]. Hence, anaesthesia may depend solely on the ability of any agent to disrupt local hydrophobic environments such that central mechanisms in the control of consciousness become disrupted. Selectivity of agents may, therefore, be related to their ability to penetrate hydrophobic domains of different central functional structures [23]. It is likely that many of the effects of anaesthetic drugs will be mediated via their disordering effects in the lipid bilayer of the cell membrane [49]. Where anaesthetic effects on membrane functions are shown, it will be important to investigate the possible relationship of these changes with alterations in the membrane environment.

When individual central activities are considered, the anaesthetic agents appear not to act in concert but to exert some selectivity. For example, the barbiturates exert different effects on excitatory amino acid transmitter release than the volatile anaesthetics [5, 19, 32, 37, 52]. Further study of presynaptic transmitter systems may allow agents to be grouped functionally which, in turn, may simplify the investigation of their mechanisms.

REFERENCES
ANAESTHETICS AND TRANSMITTER UPTAKE, SYNTHESIS AND RELEASE


Choline acetyltransferase activity of rat synaptosomes is sensitive to enflurane, but not halothane or isoflurane†

R. Griffiths, E. Boyle, J. M. C. Greiff, D. J. Rowbotham and R. I. Norman

SUMMARY

We have examined the activity of choline acetyltransferase (ChAT) in rat cortical synaptosomes in the presence of three volatile anaesthetic agents: enflurane, halothane and isoflurane. The Michaelis constant $K_m$ for choline was reduced significantly ($P = 0.012$) in the presence of 6.5% enflurane (3 rat MAC) compared with control samples exposed to carrier air only, while maximum reaction velocity ($V_{\text{max}}$) remained unaltered. The reduction in $K_m$ was also significant at enflurane concentrations of 4.4% (2 rat MAC) ($P = 0.043$) and 2.2% (1 rat MAC) ($P = 0.043$). Halothane 3% (2.5 rat MAC) and 4.5% isoflurane (3 rat MAC) had no effect on either kinetic property. If present in vivo, an enflurane-induced alteration in acetylcholine metabolism, through modified ChAT, may contribute to the convulsive properties of this anaesthetic. (Br. J. Anaesth. 1994; 72: 577-580)

KEY WORDS

Acetylcholine, Anaesthesia, volatile, Complications, epilepsy.

A role for the excitatory neurotransmitter acetylcholine (ACh) in the maintenance of consciousness has been suggested from observations of spontaneously released ACh from the surface of the cortex of conscious and anaesthetized animals [1-3]. The release was greater during periods of increased activity and attention and decreased during periods of rest [1]. On induction of barbiturate anaesthesia, release of ACh decreased to much lower levels than resting conscious values [1] and the level of release was related to the depth of anaesthesia [2]. The increase in output of ACh as animals regained consciousness, further supports a role for this transmitter in contributing to the level of consciousness [1].

Anaesthetic agents may modify presynaptic ACh metabolism either by reducing the synthesis or release of this transmitter or by reducing reuptake of choline into the nerve terminal [4]. At partial pressures at the top of the range administered clinically, halothane has been shown to reduce the potassium-evoked release of ACh from rat cortical synaptosomes by approximately 50% [5]. Halothane-induced reduction in high affinity choline uptake has also been observed, although it is not clear if this occurs by a competitive [5] or non-competitive mechanism [6]. While high affinity choline uptake is believed to be the rate limiting step in ACh biosynthesis [7] and thus a possible central point for anesthetic action [5], the further possibility of additional anaesthetic modification of ACh metabolism at the level of synthesis cannot be excluded.

The committing reaction in ACh biosynthesis is catalysed by choline acetyltransferase (ChAT) and involves the transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to choline [8]. This enzyme has been reported to be insensitive to halothane at concentrations of 3 and 4% [5]. We have investigated further the effects of the halogenated anaesthetic agents enflurane, halothane and isoflurane on ChAT activity in rat cortical synaptosomes.

MATERIALS AND METHODS

Preparation of synaptosomes

Synaptosomes were prepared from the cerebral cortex of female Wistar rats (200-250 g) using a method modified from that described by Gray and Whittaker [9]. Rats were killed by decapitation and the cortex removed. All subsequent procedures were performed at 4°C. The cortex was washed with ice cold sucrose 0.32 mol litre$^{-1}$ (10 ml/g tissue) and then homogenized in sucrose 0.32 mol litre$^{-1}$ (10 ml/g tissue) by 12 strokes in a motor driven Potter homogenizer (Teflon glass). The homogenate was centrifuged at 5000 x g for 10 min. The supernatant was retained and the pellet re-homogenized in sucrose 0.32 mol litre$^{-1}$ (10 ml/g original tissue) and centrifuged at 5000 x g for 10 min. The second pellet was discarded and the two supernatants were combined and centrifuged at 20000 x g for 60 min. The supernatant was discarded and the pellet re-suspended in sucrose 0.32 mol litre$^{-1}$, potassium phosphate 5 mmol litre$^{-1}$ (pH 7.4) at an approximate
protein concentration of 1 mg ml\(^{-1}\). The suspension was layered onto discontinuous sucrose gradients consisting of sucrose 0.6, 0.8, 1.0 and 1.2 mol litre\(^{-1}\), in potassium phosphate 5 mmol litre\(^{-1}\) (pH 7.4). Centrifugation of gradients was for 105 min at 100,000 x g at 4 °C.

Synaptosomes were recovered from the 1.0/1.2 mol litre\(^{-1}\) interface, resuspended in sucrose 0.32 mol litre\(^{-1}\), potassium phosphate 5 mmol litre\(^{-1}\) at a protein concentration of 0.4 mg ml\(^{-1}\) and centrifuged at 150,000 x g for 30 min. The supernatant was discarded and the pellet taken up in 0.32 mol litre\(^{-1}\), potassium phosphate 5 mmol litre\(^{-1}\) to a final protein concentration of 5-12 mg ml\(^{-1}\). Protein concentration was measured by the method of Lowry and colleagues [10] using bovine serum albumin as standard.

Anaesthetic apparatus

Anaesthetic agents were delivered to synaptosome samples via a manifold of 10 outlets, each delivering 60 ml min\(^{-1}\) of humidified air. The flow of air was controlled by a flowmeter block and each outlet was calibrated using a bubble flowmeter. Anaesthetic agents were delivered at a partial pressure equivalent to four times the human MAC for each agent (6.5% enflurane, 3% halothane and 4.5% isoflurane) from calibrated vaporizers (Cyprane Tec 3).

Air only was delivered to control samples.

Choline acetyltransferase assay

ChAT activity was determined using the method of Fonnum [11]. Synaptosomes were suspended in an incubation medium containing NaCl 300 mmol litre\(^{-1}\), ethylenediaminetetra-acetic acid (EDTA) 20 mmol litre\(^{-1}\), neostigmine 1 mmol litre\(^{-1}\), 0.5% Triton X-100 and sodium phosphate 50 mmol litre\(^{-1}\) (pH 7.4), to a protein concentration of 0.5 mg ml\(^{-1}\). A substrate mixture (0.5 ml) containing choline bromide at concentrations of 0.2-4.0 mmol litre\(^{-1}\), acetyl CoA 2 mmol litre\(^{-1}\) and [\(^{3}H\)]acetyl-CoA 0.5 μCi in the above medium, without Triton X-100, was placed in a 10-ml plastic test tube and exposed to air or air with anaesthetic agent to equilibrate the solutions at 37 °C. After 10 min, 0.5 ml of synaptosome suspension was added to the substrate mixture and incubated at 37 °C. Immediately after addition of the synaptosomal suspension, a 100-μl aliquot was withdrawn and ACh extracted using kalignost [11]. These samples were used as time zero controls for background subtraction from the test samples. After incubation for 60 min, 300-μl samples were obtained in duplicate and the ACh content measured by scintillation counting after kalignost extraction [9].

Kinetic parameters

Michaelis-Menten kinetic parameters, maximum reaction velocity (V\(_{\text{max}}\)) and Michaelis constant (K\(_{\text{m}}\)) were calculated for ChAT in each experiment using Pharm/Pcs computer software [12]. Results were compared using analysis of variance. Student's paired t test and Wilcoxon's signed rank test. A value of P < 0.05 was considered statistically significant.

RESULTS

In the absence of anaesthetic agents, ACh synthesis was directly proportional to time during the period of the assay used in this study (r = 0.99). V\(_{\text{max}}\) and K\(_{\text{m}}\) for ChAT activity were determined for synaptosomes exposed to air only or air containing either 3% halothane, 6.5% enflurane or 4.4% isoflurane (table I).

Enflurane 6.5%, reduced significantly (P = 0.012) the K\(_{\text{m}}\) for choline determined for ChAT by 37%, while V\(_{\text{max}}\) was not altered significantly (fig. 1, table I). In contrast, at equivalent human anaesthetic doses, 3% halothane and 4.5% isoflurane did not produce any significant effect on V\(_{\text{max}}\) or K\(_{\text{m}}\) of the synaptosomal ChAT activity (table I). V\(_{\text{max}}\), determined in the presence of all three anaesthetic agents, remained the same compared with air treated controls (table I).

V\(_{\text{max}}\) and K\(_{\text{m}}\) values were determined also for synaptosomes exposed to air or air containing 1.1%, enflurane, 2.2% enflurane and 4.4% enflurane. Enflurane at 2.2% and 4.4%, reduced significantly the K\(_{\text{m}}\) determined for ChAT (P = 0.043) by 13% and 26%, respectively. The K\(_{\text{m}}\) for ChAT was reduced also by 1.1% enflurane (ns). The relation-

<table>
<thead>
<tr>
<th>Choline acetyl bromide (mmol litre(^{-1}))</th>
<th>Air Agent</th>
<th>Enflurane</th>
<th>Isoflurane</th>
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</table>

TABLE I. Mean (SEM) V\(_{\text{max}}\) and K\(_{\text{m}}\) values for ChAT activity determined for synaptosomes exposed to air or containing halothane, enflurane and isoflurane. n = 12 for each agent.

*P < 0.05

FIG. 1. Representative Lineweaver-Burk plot of choline acetyltransferase activity in the absence [□] and presence of 6.5% enflurane. Eleven similar experiments were performed in air controls and in the presence of enflurane.
ship between the partial pressure of enflurane and the reduction in $K_m$ for ChAT is shown in figure 2A. $V_{\text{max}}$ did not alter significantly at any of the concentrations (fig. 2B).

**DISCUSSION**

This study has demonstrated that enflurane, at concentrations of 6.5%, lowered the $K_m$ of ChAT without altering $V_{\text{max}}$, while 3% halothane and 4.5% isoflurane had no effect on either variable. These doses of anaesthetic agents correspond to approximately four human MAC. However, these doses of agents correspond to 3 rat MAC for enflurane and isoflurane and 2.5 rat MAC for halothane [13]. A subsequent dose-response analysis of the enflurane-induced effect on $K_m$ showed a progressive reduction from a concentration of 1.1% (0.5 rat MAC) up to 6.5% (3 rat MAC), although the reduction at 0.5 MAC was not statistically significant.

For this study, we used a synaptosome preparation from rat cortex. Synaptosomes are produced by homogenization of brain tissue so that isolated nerve terminals are produced. The synaptosomes contain the necessary machinery for neurotransmitter uptake, synthesis and release [14]. However, there are some limitations with the preparation, the most notable being the heterogeneous population of transmitter types obtained. This may lead to non-physiological communication between types of synapses when one particular transmitter is being investigated. During this investigation, however, the synaptosomes were lysed with detergent in order to gain access to the intracellular enzyme, ChAT, which removed the possibility of cellular cross-talk in this experiment. As intra- and extracellular components were still present in the preparation, an indirect secondary effect on ChAT cannot be excluded.

There are few studies on the effects of anaesthetic agents on presynaptic ACh metabolism. However, a previous study [5] indicated that halothane had no effect on ChAT activity in concentrations up to 4%, which corresponds to 3.2 rat MAC. Our observations on halothane agree with this study.

Our results strongly suggest a selective and direct effect of enflurane on ChAT. The molecular basis of this apparent selectivity of enflurane for ChAT remains to be elucidated. It is possible that there is molecular selectivity at the level of ChAT. Stereo-specificity of enantiomers of isoflurane has been reported for anaesthetic-induced increase in $K^+$ efflux from Lymnea stagnalis neurones via a potassium channel [15]. It seems unlikely that the reduction in $K_m$ could be a simple chemical concentration effect of the anaesthetic agents in solution, as 4.5% isoflurane (1.08 mmol litre$^{-1}$) and 3% halothane (0.84 mmol litre$^{-1}$) showed no effect, whereas statistically significant effects of enflurane were observed at a concentration of 2.2% (0.68 mmol litre$^{-1}$).

Enflurane has been implicated in the development of EEG abnormalities and in the genesis of seizure [16]. The appearance of these abnormalities in the presence of enflurane is related to the dose of agent received by the patient and is particularly evident at high inspired enflurane concentrations [17]. Involvement of ACh in seizure activity is suggested by the reduction in enflurane-induced spike activity by hyoscine [19]. Measurement of ACh turnover under enflurane anaesthesia has been shown to be reduced in cortical but not subcortical structures in the brain, whereas halothane reduces ACh turnover in both areas [20]. If present in vivo, an enflurane-induced reduction in $K_m$ of ChAT
could lead to facilitation of ACh synthesis in susceptible brain regions and may thereby contribute to the convulsant properties of enflurane. The lack of effect on $K_m$ or $V_{max}$ of ChAT at equipotent clinical concentrations of the structural isomer of enflurane, isoflurane or of halothane, which are both non-epileptogenic, is consistent with the above suggestion.

In summary, we have shown that ChAT activity is susceptible to kinetic modification in the presence of partial pressures of enflurane towards the top of the clinical relevant range. The relationship of these in vitro observations to possible in vivo mechanisms of seizure genesis remains to be investigated.

ACKNOWLEDGEMENTS

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REFERENCES


Volatile Anesthetic Agents Inhibit Choline Uptake into Rat Synaptosomes


Abstract

Acetylcholine is an excitatory neurotransmitter associated with the maintenance of consciousness. Choline uptake is the rate-limiting step in acetylcholine synthesis and may be a target for the action of volatile anesthetic agents.

Methods: Methyl-$^3$Hcholine uptake was investigated using rat cortical synaptosomes. The preparation was exposed to air, as control, or equipotent partial pressures (2.4 rat MAC) of enflurane, halothane or isoflurane. In addition, the dose-response relation for halothane on [methyl-$^3$H]choline uptake was studied.

Results: The maximum rate of uptake was reduced significantly by 24% in the presence of enflurane (5.5%, 2.4 rat MAC) and isoflurane (3.5%, 2.4 rat MAC) and by 38% in the presence of halothane (5%, 2.4 rat MAC) with no change in Michaelis constant in the presence of each agent. A linear relation between the inhibition of [methyl-$^3$H]choline uptake and the concentration of halothane was observed up to 3% halothane above which there was no further inhibition. The concentration of halothane resulting in half-maximum inhibition of total choline uptake was 1.5%.

Conclusions: Noncompetitive inhibition of [methyl-$^3$H]choline uptake by volatile anesthetic agents has been demonstrated in the in vitro synaptosome preparation. If present in vitro reduction in anesthetic-sensitive choline uptake may reduce the presynaptic availability of acetylcholine and hence contribute to the process of anesthesia. (Key words: Anesthetics. volatile: enflurane; halothane; isoflurane. Brain: synaptosomes. Neurotransmitters. acetycholine. choline uptake.)

A role for cholinergic neurons in the electroencephalographic arousal response was suggested from the levels of released acetylcholine (ACh) measured from exposed rabbit cortex. The level of ACh measured at the surface of the cortex was reduced when animals were anesthetized with halothane and recovered to resting levels when the animals regained consciousness. More recently, anatomic and physiologic studies have established a strong link between central cholinergic neurotransmission and cortical arousal. A reduction in release of ACh by volatile anesthetic agents may contribute to anesthesia and the mechanisms responsible for reduced release in response to volatile agents remains to be resolved.

Reduced release of ACh by volatile anesthetics may be mediated by reduction in synthesis, evoked release of ACh or reuptake of choline into the synapse. A role for action of volatile anesthetics on the committing enzyme in ACh synthesis, choline acetyltransferase is unlikely as it has been shown that this enzyme is insensitive to clinically relevant partial pressures of halothane and isoflurane. In addition, enflurane has been shown to reduce the Michaelis constant (Km) of choline acetyltransferase with no change in the maximum rate of uptake (Vmax), again inconsistent with a role for the enzyme in reduced ACh release. Roles for reduced depolarization-evoked ACh release and choline uptake in decreasing ACh release in anesthesia have been implied from observations of the effects of halothane on these processes in rat cortex synaptosomes.

Sodium-dependent high-affinity choline uptake is the rate-limiting step in the synthesis of ACh and is, therefore, a potential sensitive mechanism by which anesthetics may bring about reductions in ACh release. In this study the possibility that sodium dependent choline uptake is sensitive to clinically relevant doses of volatile anesthetic agents was investigated in rat cortical synaptosomes.

Materials and Methods

Preparation of Synaptosomes

Sucrose Gradients. Synaptosomes were prepared from the cerebral cortex of female Wistar rats (200-
250 g) using a method modified from that described by Gray and Whittaker. Rats were killed humanely by a Schedule 1 method under the Animals (Scientific Procedures) Act 1986, the head removed, and the cortex dissected. All subsequent procedures were performed at 4°C. The cortex was washed with ice-cold 0.32 m sucrose (10 m g⁻¹ tissue) and then homogenized in 0.32 m sucrose (10 m g⁻¹) by 12 strokes in a motor-driven homogenizer (Teflon glass, Potter). The homogenate was centrifuged at 5,000 × g for 10 min. The supernatant was retained and the pellet rehomogenized in 0.32 m sucrose (10 m g⁻¹ of original tissue) and centrifuged at 5,000 × g for 10 min. The second pellet was discarded, and the two supernatants were combined and centrifuged at 20,000 × g for 60 min. The supernatant was discarded and the pellet resuspended in 0.32 m sucrose 5 mM K phosphate (pH 7.4) at an approximate protein concentration of 1 mg ml⁻¹. Two milliliters of the suspension was layered onto 35-ml discontinuous sucrose gradients consisting of 0.65, 0.8, 1.0, and 1.2 m sucrose, in 5 mM K phosphate (pH 7.4). Centrifugation of gradients was for 105 min at 100,000 × g at 4°C.

Synaptosomes were recovered from the 1.0/1.2 m interface, resuspended in 0.32 m sucrose 5 mM K phosphate (pH 7.4) at 0.4 mg ml⁻¹ protein and centrifuged at 150,000 × g for 30 min. The supernatant was discarded and the pellet taken up in 0.32 m sucrose 5 mM K phosphate to a final protein concentration of 5–12 mg ml⁻¹. Protein concentration was measured by the method of Lowry et al. using bovine serum albumin as standard.

Percoll Gradients. Alternatively, synaptosomes were prepared from the cortex of female Wistar rats (200–250 g) using the method described by Dunkley et al. One gram of rat cerebral cortex was homogenized in 10 ml of 0.32 m sucrose by 12 strokes in a motor-driven homogenizer (Teflon glass, Potter). The homogenate was sedimented at 5,000 × g for 10 min. The supernatant was retained and made up to a total volume of 14 ml with ice-cold 0.32 m sucrose. Two milliliters of the suspension were layered over freshly prepared 8 ml Percoll gradients, comprising 25%, 15%, 10%, and 5% Percoll (v/v) in 0.32 m sucrose. in a 10-ml polycarbonate tube. Gradients were centrifuged in a fixed-angle rotor for 10 min at 20,000 × g. The fractions at the 25%/15% and 15%/10% interfaces were collected and washed by centrifugation with ice-cold Krebs buffer three times at 20,000 × g for 10 min before use in the experiments. Protein concentration was measured by the method of Lowry et al. using bovine serum albumin as standard.

Anesthetic Apparatus

Anesthetics were delivered to synaptosome samples via a manifold of 10 outlets, each delivering 60 ml min⁻¹ of humidified air. The flow of air was controlled by a rotameter block and each outlet was calibrated using a bubble flow meter. Anesthetics were delivered at concentrations equivalent to 2.4 times the rat minimum alveolar concentration for each agent (enflurane 5.5%, halothane 3% and isoflurane 5.5%) from calibrated vaporizers (Cyprane TEC 3) which had recently been calibrated using a laser refractometer (Index Instruments) by General Anaesthetic Services (Keighley, UK). The aqueous concentration of halothane in buffer and the time to reach equilibrium of 10 min was confirmed by n-heptane extraction and measurement in a gas chromatograph (8410, Perkin-Elmer). After a 10-min preincubation with 1.3, and 5% halothane the aqueous concentrations were 0.22, 0.62, and 1.19 ms, respectively. Air only was delivered to control samples.

Methyl-³H]Choline Uptake Assay

All assays were performed within 10 min of preparation of the synaptosome preparations. The synaptosomal suspension was diluted with salt medium containing 140 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 20 mM hydroxyethylpiperazineethanesulfonic acid and 100 µM sodium ethylenediaminetetraacetate to a final protein concentration of 0.62 mg ml⁻¹. One milliliter of the diluted synaptosomal preparation was placed in a series of polycarbonate test tubes and exposed to air or air containing anesthetic agent for 10 min in a water bath at 37°C. Following equilibration with gases. 0.25 ml of a reaction mixture containing choline chloride (final assay concentration ranging from 2 to 20 µM), neostigmine 100 µM, sodium pyruvate 5 mM and 50 µCl of [methyl-³H]choline chloride (1.0 mCi ml⁻¹) (Amersham International plc, UK) was added.

A 500 µl sample was withdrawn and added to 1.0 ml of ice-cold salt medium containing 200 µM choline chloride and centrifuged for 5 min in a refrigerated microfuge. The pellet was washed, resuspended and recentrifuged twice before being resuspended in 0.25 ml of distilled water, and allowed to lyse for 4 h before addition of 4 ml of Oluiphase X (LKB Pharmacia) and scintillation counting. Radioactivity in these samples was used to establish baseline at the start of the assay.
VOLATILE AGENTS INHIBIT CHOLINE UPTAKE

Assay mixtures were exposed to air or air containing anesthetic agents for 60 min for the sucrose gradient prepared synaptosomal suspension and 15 min for the Percoll gradient prepared synaptosomal suspension. At the end of the incubation period two 300 μL samples were withdrawn from each assay, centrifuged, resuspended and washed twice before being lysed in 0.25 ml distilled water and scintillation counting.

The effect of halothane on low-affinity choline uptake was determined by replacing the sodium chloride in the salt medium with an equimolar concentration of lithium chloride.

**Kinetic Parameters**

Michaelis-Menten kinetic parameters, $V_{\text{max}}$ (as maximum reaction velocity), and $K_m$ were calculated for choline uptake in each experiment using Pharm/Pc's computer software. Results were compared using paired analysis of variance and Student's paired $t$ test. A value of $P < 0.05$ was considered statistically significant. The dose–response curve was analyzed statistically by analysis of variance, and computer-assisted curve-fitting (nonlinear regression model) with Instat GraphPad version 2.0 software was used to determine the concentration of anesthetic resulting in half-maximum inhibition of choline uptake.

**Results**

Choline uptake was shown to be linear over the time course of the experiments, 60 and 15 min for synaptosomes prepared by the sucrose method and and the Percoll method, respectively (not shown). Uptake rates were significantly higher in the Percoll-prepared synaptosomes (2.31 ± 0.20 pmol·mg⁻¹·min⁻¹, n = 16, vs. 1.02 ± 0.08 pmol·mg⁻¹·min⁻¹, n = 24, in the sucrose-prepared synaptosomes). Values are means ± SE.

The kinetic parameters $V_{\text{max}}$ and $K_m$ for choline uptake were determined in synaptosomes prepared by the sucrose method exposed to air only or air containing equipotent clinical concentrations (2.4 rat MAC) of enflurane (5.5%), halothane (3.0%), and isoflurane (3.5%) [table 1]. Kinetic variables determined in this study were comparable to those obtained previously.

A significant reduction in $V_{\text{max}}$ was observed for choline uptake in the presence of halothane (fig. 1). Similar reductions were observed in the presence of enflurane and isoflurane. There was no significant change in $K_m$ in the presence of any agent tested (compare fig. 1).

<table>
<thead>
<tr>
<th>$V_{\text{max}}$ (pmol·mg⁻¹·min⁻¹)</th>
<th>$K_m$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Air</strong></td>
<td><strong>Agent</strong></td>
</tr>
<tr>
<td>Enflurane</td>
<td>0.97 ± 0.10</td>
</tr>
<tr>
<td>Halothane</td>
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<tr>
<td>Isoflurane</td>
<td>0.92 ± 0.09</td>
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</table>

Values are mean ± SE.

* n = 11 for enflurane ($P < 0.05$).
† n = 12 for halothane ($P < 0.05$).
‡ n = 11 for isoflurane ($P < 0.05$).

Low-affinity choline uptake was approximately 25% of the total at a substrate concentration of 5 μM and was not altered significantly in the presence of 3% halothane (fig. 2). Total choline uptake was investigated in all other experiments.

To investigate the dose–response relation of the halothane-induced inhibition of choline uptake $V_{\text{max}}$ and $K_m$ were determined on synaptosomes prepared by the Percoll method at halothane concentrations ranging from 1% to 10%. At each partial pressure tested halothane reduced significantly the uptake of choline. Maximum inhibition was observed at 3% halothane above which no further inhibition of uptake was observed. The concentration of halothane resulting in half-maximum inhibition of total choline uptake was 1.38% ± 0.32% (fig. 3).

**Discussion**

ACh release from cortex has been shown to be reduced during anesthesia. Further evidence implicating cholinergic processes in anesthetic mechanisms comes from the clinical 'anticholinergic syndrome', in which excessive doses of anticholinergic agents result in excessive sedation and even coma, especially in the elderly population.

Choline uptake is the rate-limiting step for ACh synthesis and therefore represents a possible sensitive point in ACh metabolism at which volatile anesthetic agents may exert their action. To investigate the sensitivity of choline uptake to volatile anesthetics the alteration in [methyl-³H]choline uptake in rat cortical synaptosomes was investigated in the absence and presence of either enflurane, halothane or isoflurane. three volatile anesthetics in common
Fig. 1. Lineweaver-Burk analysis of choline uptake into rat cortical synaptosomes. This representative experiment shows the effect of 3% halothane on synaptosomes prepared by the sucrose method. There is a significant reduction in the $V_{\text{max}}$ (reciprocal of intercept on the y axis) and no significant change in the $K_m$ (reciprocal of intercept on the x axis). Circles = control; triangles = halothane.

Clinical use. Initially, these agents were studied at a supra-anesthetic concentration equivalent to 2.4 times the rat minimum alveolar concentration (2.4 rat MAC) using synaptosomes prepared on sucrose gradients. In the presence of each agent $V_{\text{max}}$ for choline uptake was reduced significantly with no significant change in $K_m$, indicative of noncompetitive inhibition of uptake.

Investigation of the dose-response relation for halothane was performed on synaptosomes prepared from Percoll gradients. Both preparations showed similar reductions in $V_{\text{max}}$ for choline uptake when exposed to 3% halothane. 38% for synaptosomes prepared on sucrose gradients and 32% for synaptosomes prepared on Percoll gradients. The Percoll method was employed for the dose-response part of the study for three main reasons. First, the preparation time is considerably shortened. 2 h compared with 6 h for the sucrose method. Second, the Percoll method allows the separation of viable from nonviable synaptosomes. Third, the method allows the preparation to remain isotonic throughout preparation. These factors are responsible for the improved uptake rates seen with the Percoll synaptosomes.

The dose-response for halothane revealed an apparently linear inhibition of choline uptake with increasing partial pressures of halothane up to a maximum inhibition of 32% at 3% concentration, above which there was no further inhibition by halothane. The concentration of halothane producing a half-maximum inhibition of the anesthetic-sensitive component of choline uptake of 1.5% was close to the rat MAC value for halothane of 1.24%, which would be consistent with a role for choline uptake in the process of anesthesia. Low-affinity, sodium independent uptake contributed 25% of choline uptake to the total choline uptake measured. This component of choline uptake was insensitive to concentration of 3% halothane.

Little work has been directed to the study of the alteration of presynaptic ACh metabolism by volatile anesthetic agents. In contrast to the above results, a previous study reported a "competitive-like" inhibition of choline uptake by 3% halothane. In these experiments on a small number of samples, $K_m$ was reported to increase in the presence of halothane with no change in $V_{\text{max}}$. We have reanalyzed the data presented (Graphpad version 2.02) and found that the differences were not statistically significant. From the high statistical significance of our results we conclude that, as choline and the volatile anesthetic agents are structurally unrelated, the noncompetitive inhibition indicated by our study is more likely.

Fig. 2. The contribution of low-affinity uptake to total choline uptake for synaptosomes prepared by the Percoll method. Halothane 3% had no effect on low-affinity uptake compared with control ($P > 0.80$). Values are means ± SE (n = 4).
VOLATILE AGENTS INHIBIT CHOLINE UPTAKE

Fig. 3. Effect of halothane on synapticosomal uptake of choline for synaptosomes prepared by the Percoll method. There were 10 determinations for 1%, 2%, and 3% halothane. Numbers in parentheses indicate numbers of determinations for 5% and 10% halothane. Data points are means ± SE.

A significant decrease in ACh release in the presence of 1 MAC halothane has been shown recently, in vivo, from the pontine reticular formation of the cat. In addition, inhibition of depolarization-evoked release of ACh from rat cortical synaptosomes by halothane has been reported. The contribution of the inhibition of choline uptake to these repeated decreases in presynaptic ACh release is not known. This inhibition may result from reduced availability of ACh due to inhibition of the choline uptake mechanism. However, a direct inhibition of the ACh release process cannot be ruled out at this stage.

Choline uptake in rat brain synaptosomes is also sensitive to ethanol, being inhibited in a noncompetitive manner. At ethanol concentrations of 50 mM, a concentration that in the human would bring on advanced inebriation, choline uptake was reduced by 15%. Interestingly, in the same study uptake of the inhibitory neurotransmitter γ-aminobutyric acid was unaffected by ethanol. Similar insensitivity to volatile anesthetic agents has been reported for γ-aminobutyric acid uptake. By contrast, 5-hydroxytryptamine uptake is reduced in the presence of halothane although this has been shown to be due to an indirect interaction with the transporter. The amino acid sequences of transporters for a number of neurotransmitters and transmitter metabolites have been deduced recently including those for choline, γ-aminobutyric acid, and 5-hydroxytryptamine. This family of transporters share structural similarities, with 12 proposed transmembrane domains, and are likely to share functional mechanisms. Although similar in structure, and possibly function, the response of these transporters to the presence of volatile anesthetic agents and ethanol differs. Such differential sensitivity could underlie apparent selectivity of agents for individual transmitter systems.

Regarding the mechanism by which choline transport activity is modified in the presence of exogenous agents, we favor the hypothesis that volatile anesthetics and ethanol mediate their effects via general, nonspecific membrane perturbation and that the choline transporter is more susceptible to these changes than other transporters. However, we cannot rule out the possibilities that other regulatory influences such as allosterism due to the direct interaction of anesthetic with the transporter or responses to cellular signaling mechanisms may be involved. For example, volatile anesthetics have been shown to stimulate the activity of protein kinase C. In insect species, choline uptake activity has been found to be increased by activation of protein kinases A and C. In addition, increased choline transport activity has been shown to be related to phospholipase A, activation, suggesting a possible role for fatty acids in modulating choline uptake activity. Whether the inhibition of choline uptake in the presence of volatile anesthetics is the result of membrane perturbation or a response to altered signalling pathways remains to be resolved.

In conclusion, the results of this study show that the activity of the choline transporter in cortical synaptosomes is inhibited in the presence of volatile anesthetic agents. Such inhibition may underlie the decreases in release of ACh observed in vivo from the surface of brains of anesthetized animals. Whether inhibition of choline uptake contributes to reduced ACh release and thence to the process of anesthesia remains to be determined.

References


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