AMINO ACID NEUROTRANSMISSION DURING CHEMICALLY-INDUCED EPILEPTOGENIC ACTIVITY IN THE RAT CEREBRAL CORTEX.

A thesis submitted to the University of London

for the degree of Doctor of Philosophy

by

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IN THE NAME OF GOD,

THE COMPASSIONATE, THE MERCIFUL

This thesis is dedicated to my dear parents, for their unwavering support and encouragement, and the rest of my family, Farzaneh, Farzad and Nader

ABSTRACT

This project was undertaken firstly to establish a relationship between chemically-induced epileptogenic activity and the release of amino acid neurotransmitters and secondly to try to compare the enhancement of γ-aminobutyric acid (GABA)-mediated inhibition with the blockade of excitatory amino acid receptors in the control of epileptogenic activity in rat cerebral cortex.

Using cortical cups incorporating platinum electrodes, it was possible to monitor epileptogenic activity in the electroencephalogram (EEG), quantified using a specially designed voltage integrator, at the same time as studying the release of endogenous amino acids. *In vitro* release studies were performed using cortical slices (0.4mm) in a continuous superfusion system. An automated high performance liquid chromatography (HPLC) procedure was employed to determine amino acid levels in the perfusates.

Bicuculline methiodide (BM) was chosen as the test convulsant to evaluate the relative importance of different amino acid receptors in the initiation and maintenance of epileptogenic activity in the rat cerebral cortex. EEG spiking was induced in urethane-anaesthetised rats by transient superfusion of BM across the exposed parietal cortex. Extracellular recording of a group of superficial cortical cells directly beneath the cup revealed that each interictal spike coincided with the sudden, synchronous burst firing of a group of cortical neurones. Compounds which enhance the action of GABA or block the excitatory amino acid receptors reduced such epileptogenic activity. $GABA_A$ receptor augmentation by muscimol and clonazepam reduced the size and, therefore, the severity of spiking whereas the $GABA_B$ agonist, baclofen, affected spiking by reducing the total number of spikes. NMDA

blockade lead to a $GABA_A$-like effect and only reduced the size of the spikes whilst non-NMDA receptor blockers reduced the number of spikes with little effect on their size.

Since intravenous infusion of pentylenetetrazole (PTZ) had been shown to lead to epileptogenic activity in urethane-anaesthetised rats its effect on the efflux of various amino acids from the cortex was monitored. Only glutamine release was in fact enhanced during seizure-like activity. Direct cortical superfusion of PTZ lead to an unspecific release of most amino acids. By contrast in cortical slices PTZ only increased the release of the neurotransmitter amino acids GABA, glutamate and aspartate, plus taurine. When BM was superfused across the cortex in concentrations producing spiking it had no effect on the release of amino acids whereas it induced the release of GABA, glutamate and aspartate from cortical slices but only in the presence of *p*-chloromercuriphenylsulphonic acid (PCMS). The bicuculline-induced release of amino acid neurotransmitters was Ca^{++}-dependent and it was reduced by the excitatory amino acid receptor antagonists.

The EEG studies together with release data show a close relationship between amino acid neurotransmitters and cortical epileptogenic activity. The results of the studies on amino acid manipulation also imply that a number of amino acid-related effects are involved in the initiation and severity of epileptogenic activity. Hence a clear understanding of their roles could facilitate a better use of drugs alone or together, in the control of epileptogenic activity.

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CONTENTS

PUBLICATIONS

The following publications have arisen from the work described in this thesis:

1) Zia-Gharib F. & Webster R.A. (1989) Manipulation of amino acid neurotransmission in the control of epileptogenic EEG activity in the rat. *Br. J. Pharmacol.* **96:** 84P.
2) Zia-Gharib F. & Webster R.A. (1991) Effect of compounds modulating amino acid neurotransmission on the development and control of bicuculline-induced epileptogenic spiking in the rat. *Neuropharmacology* **30:** 995-1009.

ABBREVIATIONS:

ACSF = Artificial cerebrospinal fluid

Ala = Alanine

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AOAA = Amino oxyacetic acid

AP5 = 2-Amino-5-phosphonovalproic acid

AP7 = 2-Amino-7-phosphonoheptanoic acid

Arg = Arginine

Asn = Asparagine

Asp = Aspartate

BM = Bicuculline methiodide

CNQX = 6-Cyano-7-nitroquinoxaline-2,3-dione

CNS = Central nervous system

CSF = Cerebrospinal Fluid

DIW = Deionised water

ECG = Electrocardiogram

EEG = Electroencephalogram

EGTA = Ethyleneglycol-bis-(β-amino-ethyl ether)n, n'-tetraacetic acid

GABA = γ-Aminobutyric acid

GABA-T = γ-Aminobutyric acid transferase

GAMS = γ-D-glutamylaminomethyl sulphonic acid

Gln = Glutamine

Glu = Glutamate

Gly = Glycine

His = Histidine

HPLC = High performance liquid chromatography

i.d. = Internal diameter

L.C.D. = Liquid crystal display

LTT = Leptazol threshold test

2-MCE = 2-Mercaptoethanol

MK-801 = (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten
-5,10-imine maleate

NMDA = N-methyl-D-aspartate

NMR-CT = Nuclear magnetic resonance computed tomography

n = Number of experiments

o.d. = Outer diameter

OPA = o-Phthalaldehyde

PCMS = ρ-Chloromercuriphenylsulphonic acid

PDS = Paroxysmal depolarizing shift

PET = Positron emission tomography

PTFE = Polytetrafluoroethylene

PTZ = Pentylenetetrazole

s.e. = Standard error

Ser = Serine

SPECT = Single photon emission computed tomography

Tau = Taurine

THF = Tetrahydrofuran

THIP = 4,5,6,7,-Tetrahydroisoxazolo-[5,4,-C]-pyridin-3-ol

Thr = Threonine

Tyr = Tyrosine

UV = Ultraviolet

V/F = Voltage to frequency

UNITS USED:

cm = centimetre

g = gram

G = Gauge

Hz = Hertz

Kg = Kilogram

M = molar

mg = milligram

min = minute

mm = millimetre

mm^2 = millimetre square

mM = millimolar

ms = millisecond

ηmol = nanomole

ρmol = picomole

μl = microlitre

μm = micrometre

μM = micromolar

μV = microvolt

V = volt

CHAPTER 1:

GENERAL INTRODUCTION

1:1: EPILEPSY: DEFINITION, PREVALENCE AND CLINICAL FEATURES:

Epilepsy is one of the most common neurological disorders in the world. Its prevalence is thought to be 3 to 6 people per 1000 with the annual incidence, which is a measure of new cases appearing, being 20 to 25 per 100000. It is thought that 0.5 to 2% of the world population is epileptic which could amount to as many as 80 million people in the world. The term epilepsy refers to a neuronal disorder in which the main manifestation is the abnormal, paroxysmal and synchronous firing of a large aggregate of central neurones leading to a transient disturbance of brain function. There are different types of epilepsy depending on the extent to which different areas of brain are affected. These could be as simple as a temporary loss of consciousness lasting only 10-15 seconds, as seen in petit mal epilepsy, or could lead to characteristic tonic-clonic convulsions, as in the case of grand mal epilepsy. Epilepsy is said to be *partial* if the abnormality only affects a focal part of brain without spreading to other parts. This could develop into a *partial epilepsy with secondary generalisation* if it spreads to other parts of the brain. Another class of epilepsy is *primary generalised* where the abnormal activity involves the whole of the brain at the onset (see Prince, 1978 and see Scambler, 1989).

1:2: ANIMAL MODELS OF EPILEPSY:

In common with most neurological disorders, epilepsy cannot be easily studied using human subjects and, therefore, the basic research is performed by using animal models of epilepsy. There are well over 50 different animal models of epilepsy which by far exceed the different types of epilepsy (see Fisher, 1989 and Snead III, 1983). It is, therefore, important to note that every animal model of epilepsy has its limitations and that none is perfect. However, each of these models

provide answers for some questions which are raised in the process of finding a cure for this illness, which still eludes us.

These models could be broadly divided into two groups. The first of these portraying partial epilepsy such as focal application of convulsants acutely or chronically (leading to secondary generalised epileptic activity) and the second group representing generalised tonic-clonic seizures such as those seen in genetically epileptic species (such as *papio papio* baboon, DBA/2 mice and genetically epilepsy-prone rats) or produced by systemic administration of convulsants or applied electrical stimulation whether acutely or chronically, as seen after kindling.

1:3: CHEMICAL CONVULSANTS:

It has long been established that certain substances produce epileptiform seizures when administered to animals. Most of these were plant extracts finding classical uses as poisons such as strychnine (from *Nux vomica*). However, it was soon evident that they could be used more productively as epileptogenic agents in animal models of epilepsy (Woodbury, 1980).

Pentylenetetrazole (PTZ) or leptazol is probably the most widely used convulsant in animal studies and the leptazol threshold test (LTT) in mice is a standard screening procedure for potential anticonvulsants affecting petit mal epilepsy. Systemic PTZ administration could also produce an animal model for generalised epilepsy such as grand mal (Kent & Webster, 1983). PTZ causes extensive stimulation of all levels of the central nervous system especially the higher centres (see Hahn, 1960; Piredda, Yonekawa, Whittingham & Kupferberg, 1985 and Bingmann & Speckmann, 1986) but its exact mode of action is still unclear. It is thought to reduce the inhibitory action of GABA on central neurones

either by blocking the chloride channels associated with the receptor, possessing an action similar to that of picrotoxin (Ramanjaneyulu & Ticku, 1984 and Ito, Chiu & Rosenberg, 1986), or by interacting with the benzodiazepine receptor of the GABA receptor complex (Rehavi, Skolnick & Paul, 1982). PTZ is also reported to alter the membrane properties of neuronal cells (Faugier & Willows, 1973 and Bingmann & Speckmann, 1986) whilst at a cellular level it has been postualated to facilitate a slow depolarizing Ca^{++} current through the cell membrane, uncovered by a depression of repolarizing K^{+} current (Louvel & Heinemann, 1981). PTZ has also been shown to cause bursting-type opening and closing of single potassium channels in cultured cortical neurons (Sugaya, Sugaya, Takagi, Tsuda, Kajiwara, Yasuda & Komatsubara, 1989) as well as increasing Ca^{++} release from endoplasmic reticulum in cells through cyclic AMP-dependent protein phosphorylation in rat cerebral cortex (Onozuka, Nakagaki & Sasaki, 1989).

Another commonly used chemical convulsant is bicuculline which was found to be a specific blocker of GABA two decades ago (Curtis, Duggan, Felix & Johnston, 1970 and Curtis, Duggan, Felix, Johnston & McLennan, 1971) and more recently a potent $GABA_A$ receptor antagonist (Simmonds, 1982). Bicuculline also has a direct effect on the cell membrane causing a persistent inward Ca^{++} movement, although this effect is mainly seen at higher concentrations (Heyer, Nowak & Macdonald, 1982). Another GABA blocker which is often used as a convulsant is picrotoxin which blocks the chloride ion channel associated with GABA stimulation in a non-competitive manner (Simmonds, 1982), although there is evidence suggesting that it produces a mixed (competitive and non-competitive) antagonism (Barolet, Li, Liske & Morris, 1985) of GABA fuction.

1:4: IMPORTANCE OF NEUROTRANSMITTERS IN EPILEPSY:

Spread of epileptic activity from a focal group of neurones is mostly achieved through neurotransmitters acting as chemical messengers and relaying the information from neurone to neurone. It is, therefore, not surprising that studying brain neurotransmitters has formed the bulk of research into epilepsy (Woodbury, 1984).

Amino acids provide the most universal and important inhibitory and excitatory neurotransmitters in the brain. The inhibitory amino acid neurotransmitters include GABA, glycine and possibly taurine and the excitatory neurotransmitters aspartate and glutamate. It is estimated that 30-40% of the neurones in the brain use GABA as the inhibitory neurotransmitter and 15-20% glutamate as the excitatory neurotransmitter (Walker, 1983). Amino acids appear to be the neurotransmitters which are most closely involved in epilepsy. Van Gelder & Courtois (1972) have shown a close correlation between the changing content of specific amino acids in the epileptogenic cortex of cats and the severity of epileptogenic activity. These workers reported a significant reduction in the levels of glutamate, aspartate, taurine, GABA and glycine in the close vicinity of the cobalt-induced epileptic focus with the decrease in the levels of GABA and aspartate persisting in the area immediately surrounding the focus.

Changes in the regional levels of amino acids have been reported in conjunction with seizures induced by a variety of convulsants (Nitsch, Schmude & Haug, 1983; Chapman, Westerberg, Premachandra & Meldrum, 1984 and Allen, Grieve & Griffiths, 1986). Such a relationship has also been reported in the cortex of electrically kindled rats (Leach, Miller, O'Donnell & Webster, 1983) and recently on altered levels of amino acids in the hippocampus of humans with temporal lobe epilepsy (Geddes, Cahan, Cooper, Kim, Choi & Cotman, 1990). Other investigators have also

studied biochemical and metabolic changes in epilepsy and have shown a relationship between amino acids and epileptic activity (Emson & Joseph, 1975; Emson, 1975 & 1978 and Dodd, Bradford, Abdul-Ghani, Cox & Continho-Netto, 1980).

Inspite of all the overwhelming information linking amino acid neurotransmitters to epilepsy, it is still difficult to reach a certain conclusion about their exact role. There are two major problems in studying amino acids. Firstly, there is the problem of not knowing whether the changes observed are causative in epilepsy or merely result from the abnormal, epileptogenic activity. Secondly, it is important to note that amino acids have an important metabolic role and the observed changes could simply be due to alteration in metabolism rather than epilepsy.

Another important consideration is that although there are fewer reports linking other neurotransmitters in the central nervous system to epilepsy their possible involvement in addition to that of amino acids in epilepsy cannot be overlooked (Craig, 1984 and Jobe, 1984).

1:5: STRATEGIES USED TO COMBAT EPILEPTOGENIC ACTIVITY IN ANIMAL MODELS OF EPILEPSY:

Epilepsy is thought to be caused by an imbalance between inhibition and excitation in the CNS which leads to seizures involving the uncontrolled, synchronous and repetitive discharge of a large number of neurones. If epileptic activity could be envisaged as a 'wave' of excessive, synchronous and excitatory activity 'surging' through the brain, it seems logical to tackle the problem of controlling epilepsy by either increasing the inhibitory mechanisms or decreasing the excitability in the brain. In fact these two different strategies form the basis of epilepsy research.

1:5:1: Increasing inhibition:

GABA is thought to be the inhibitory transmitter released by small interneurones in the cerebral cortex, neocortex, hippocampus and thalamus. The action of these interneurones is to either narrow or sharpen the area of activity or to shorten or terminate a period of activity. For this latter action, these GABA-releasing interneurones would appear to provide the major defence against the build-up or spread of epileptic activity (Meldrum, 1975). Failure of GABAergic inhibition has been considered as the key to local and global seizures (Balcar, Pumain, Mark, Borg & Mandel, 1978 and Roberts, 1986) and 'GABA replenishment therapy' has been suggested as the main way to combat epilepsy and to find new anticonvulsants (Fariello & Ticku, 1983). The importance of GABA-mediated inhibition is evident from the use of benzodiazepines and barbiturates and new GABA-related anticonvulsants which show promising results in animal models of epilepsy (Meldrum, 1981a and 1981b). Progabide which is a prodrug and is converted to GABA in the central nervous system has recently been introduced

clinically (Bergmann, 1985) and vigabatrin (γ-vinyl GABA) which inhibits the enzymatic breakdown of GABA is now in clinical use after successful clinical trials (Tartara, Manni, Galimberti, Hardenberg, Orwin & Perucca, 1986).

There are many ways of increasing the inhibitory effect of GABA and most of the measures taken appear to show a decrease in the epileptic activity in animal models of epilepsy. These measures include using GABA agonists such as muscimol and THIP (4,5,6,7,-tetrahydroisoxazolo-[5,4,-C]-pyridin-3-ol); compounds which inhibit the breakdown of GABA such as γ-vinyl GABA and amino oxyacetic acid (AOAA) and GABA uptake inhibitors, for instance nipecotic acid and its derivatives (Löscher, 1982). It is also important to note that some of these investigators have reported opposite effects and an increase in epileptic activity. This anomaly could be explained by the fact that the inhibitory action of GABA is often sharply focused to dampen a local excitatory abnormality and if the actions taken to increase its effect are too strong it could lead to extensive hyperpolarization which would prevent the delicate local inhibitory action of GABA. Additionally, potent GABA agonists may be acting on some of the GABA receptors acting as 'autoreceptors' and lowering the levels of endogenous GABA. They could also act on other GABA receptors present in the CNS that uncharacteristically lead to depolarization and an increase in excitation (Meldrum, 1984).

The other inhibitory amino acid neurotransmitters include glycine and probably taurine and although they have not been studied as extensively as GABA their involvement in controlling epileptogenic activity is also well documented. Intragastric glycine has been shown to be more effective in protecting young DBA/2 mice against all phases of sound-induced convulsions than GABA or taurine (Toth, Lajtha, Sarhan &

Seiler, 1983). The involvement of taurine in epilepsy has also been well documented (VanGelder 1972 and Huxtable, 1982) and Mutani, Durelli, Mazzarino, Valentini, Monaco, Fumero & Mondino (1977) have reported that the levels of taurine appeared to drop prior to epileptic activity in cats and this effect could be considered as the initiation point in the epileptogenic activity. In another words, the decrease in taurine could make certain neuronal populations more excitable since a controlling inhibitory factor has been diminished. Antagonism of cobalt-induced epilepsy by taurine has also been reported in cats and mice (VanGelder, 1972).

1:5:2: Decreasing excitation:

Coutinho-Netto, Abdul-Ghani, Collins & Bradford (1981) have shown that glutamate release could be considered as a 'trigger factor' in epilepsy. They reached this conclusion since glutamate was the main neurotransmitter released from a cobalt-induced epileptic focus in rats after the initial changes due to tissue damage. These workers have also modified the experiment and shown that application of L-glutamate, if anything, increased the epileptic activity and subsequent use of glutamate antagonists, such as 2-amino-5-phosphonovalproic acid (AP5) and γ-D-amino-adipic acid, decreased the levels of abnormal activity. Other workers have also shown that local or systemic application of excitatory amino acids could lead to epileptic activity in rats (Johnston, 1973 and Bradford & Dodd, 1977). The two main excitatory amino acids are glutamate and aspartate (Watkins & Evans, 1981 and Collingridge & Lester, 1989) and a great deal of pharmacological methodology in epilepsy has been geared to decreasing the activity of these neurotransmitters in the CNS. The study of excitatory amino acid neurotransmitters has led to classification of the receptors according to

the agonists which occupy them. These receptors have been divided broadly into two groups: N-methyl-D-aspartate (NMDA) and non-NMDA receptors [kainate and quisqualate or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), as it is now known] and antagonists of both groups have been shown to reduce epileptogenic activity. GAMS (γ-D-glutamylaminomethyl sulphonic acid) which is an antagonist preferentially blocking non-NMDA receptors (Davies & Watkins, 1985) has been shown to reduce audiogenic seizures in DBA/2 mice (Croucher, Meldrum, Jones & Watkins, 1984) as well as being effective in increasing the onset pressure for the initial tremor phase of the high pressure neurophysiological syndrome in rats (Wardley-Smith, Meldrum & Halsey, 1987). Further involvement of non-NMDA receptors in epileptogenic conditions is also provided by the demonstration of kainate-induced epileptic activity in mice (Turski, Meldrum, Turski & Watkins, 1987). However, the bulk of epilepsy research has concentrated on studying the effect of NMDA antagonists and these appear to provide the most promising results (see Croucher & Meldrum, 1984 and Meldrum, Chapman, Mello, Millan, Patel & Turski, 1986).

One of these antagonists is 2-amino-7-phosphonoheptanoic acid (AP7) and has been shown to reduce light-induced seizures in *papio papio* baboons (Meldrum, Croucher, Badman & Collins, 1983a) as well as reducing audiogenic epileptic activity in DBA/2 mice (Croucher, Collins & Meldrum, 1982). The anticonvulsant effect of AP7 in the latter study was equivalent to that of diazepam which is one of the most potent anticonvulsants known, being used in the emergency treatment of status epilepticus. Unfortunately AP7 appears to be very inefficient in crossing the blood-brain-barrier and, therefore, it cannot be administered peripherally unless in large doses. Further evidence

linking NMDA receptors with epilepsy has recently emerged from studies concerning the drug MK-801 {(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate} which is an orally active anticonvulsant and is found to be a NMDA channel blocker (Woodruff, Foster, Gill, Kemp, Wong & Iversen, 1987; Wong, Kemp, Priestley, Knight, Woodruff & Iversen, 1986 and Wong, Knight & Woodruff, 1988). MK-801 together with other non-competitive NMDA antagonists have also been shown to reduce sound-induced seizures in DBA/2 mice (Chapman & Meldrum, 1989) as well as retarding development of amygdala kindling in rats (Peterson, Collins & Bradford, 1983 and Sato, Morimoto & Okamoto, 1988) and being anticonvulsants in electroshock model of epilepsy (McNamara, Russell, Rigsbee & Bonhaus, 1988). What makes the study of NMDA antagonists interesting, is the fact that they appear to be active against a wide spectrum of neuronal disorders and present an exciting area in neuropharmacology (Fagg, Foster & Ganong, 1986).

1:6: CONCLUDING REMARKS:

Since there are two major approaches in controlling epilepsy by either augmenting the action of the inhibitory amino acid neurotransmitter GABA or decreasing that of the excitatory amino acid neurotransmitter glutamate, it was interesting to study the relative value of these two approaches in one experimental model that offers an accurate, quantitative measure of potency as well as providing some indication of specific receptors and synaptic mechanisms involved. The experimental work described in this thesis is an attempt to achieve this.

The work involved inducing EEG spiking by local application of convulsants pentylenetetrazole, bicuculline methiodide and picrotoxin across the parietal surface. The spiking was quantified to give measures of total spike voltage, total number of spikes as well as

average size of spikes. Drugs which augment $GABA_A$ and $GABA_B$ function or block NMDA and non-NMDA receptors were then applied in known concentrations to evaluate the relative importance of inhibitory and excitatory amino acid neurotransmission in the epileptogenic spiking and also to indicate how spiking develops by relating their effects in the test situations to their known electrophysiological effects on different cortical neurones. I also thought that the use of cortical cups would enable me to measure amino acid release during the development of convulsant-induced spiking and so obtain further details of their role in epileptogenesis. Some of this work was repeated in cortical slices because of problems encountered in measuring amino acid release *in vivo*. Whilst the use of an anaesthetic agent and concentrating mainly on one convulsant (bicuculline methiodide) may introduce some bias into the results and their interpretation I believe that this approach provides a useful and original method of studying amino acid neurotransmission in the development of epileptic spiking and an important link between the electrophysiological aspects of spike initiation and the chemical control of epilepsy.

CHAPTER 2

METHODS

2:1: INTRODUCTION:

The following section provides an outline of the techniques used to investigate neuronal activity during epileptogenesis and measure neurotransmitter release in various *in vivo* and *in vitro* preparations. Considerations which lead to the adoption of the methods used in the present work will also be explained.

There are numerous neuronal preparations used for electrophysiological studies ranging from the patch-clamp studies investigating the movement of ions in and out of the cell in *in vitro* preparations to the study of the electroencephalogram (EEG) *in vivo*. Many investigators consider the appearance of the paroxysmal depolarizing shift (PDS) as the cellular correlate of epilepsy. PDS is defined as a long-duration depolarization of the cell with burst firing during its most depolarized state. The effect of various drugs on the occurrence and the pattern of such a burst firing could then be studied to evaluate the importance of different prospective anticonvulsants. Although these *in vitro* experiments provide important results leading to various hypotheses they are always likely to be at a disadvantage as they investigate the problem in an unavoidably artificial situation where the neurones are somewhat isolated from their normal environment. Therefore, it is vital to investigate the effect of different drugs on epileptogenic activity *in vivo* to confirm or repudiate various hypotheses formulated in *in vitro* experiments. Since the appearance of interictal spikes in the EEG is the major characteristic of, and diagnostic tool in, epilepsy I studied the effects of various drugs on chemically-induced EEG spikes in the anaesthetised rat.

One of the major aims of the work undertaken was to establish whether there was a link between epileptogenic activity and the release of amino acids and, therefore, it was important to adopt a suitable *in*

vivo release technique. There are at least five different *in vivo* techniques used to investigate neurotransmitter release (see Marsden, 1984 and Bayðn & Drucker-Collin, 1985) these include: ventricular perfusion, cortical cups, push-pull cannulae, cerebral dialysis and voltammetry. Ventricular perfusion provides overall information about the efflux of neurotransmitters involving a number of neuronal compartments and hence the results are difficult to interpret. Voltammetry is a very sensitive technique used for measuring release of electrochemically active substances such as catecholamines but cannot be adopted for amino acids since they are not electrochemically active. Push-pull cannulae, cerebral dialysis and cortical cup techniques were, therefore, considered for these experiments. The first two techniques are very flexible and can be used in various cerebral sites making them quite attractive for studying the release of amino acids in various important sites during epileptogenic activity. However, drugs applied by these routes are less likely to have an overall effect on the EEG and their activity would have to be monitored by localized microelectrode recording plus the practical problem of not having enough sample volume to analyse for amino acid content since low flow rates had to be employed to minimize damage to the cerebral tissue. Although the cortical cup technique can only be used to investigate release from the cortex this was acceptable since the cerebral cortex has long been established as a major epileptogenic site. The cortical cup technique had the advantage of providing larger sample volumes for analysis and since EEG recording electrodes could be incorporated in the cup it provided a means of studying the release of amino acids from the cortical area from which epileptogenic activity was being recorded. The cups also provided a means of applying drugs in known concentrations. These considerations lead to the adoption of this technique for these

studies.

One of the major drawbacks in studying release in *in vivo* experiments is, however, the inhibitory effect of anaesthetic on neurotransmitter release and the limited number of experiments than can be performed. Therefore, it was decided to also study release in slices taken from the same area of cortex as used in cortical cup experiments so as to provide a direct comparison between *in vivo* and *in vitro* release.

2:2: GENERAL PROCEDURE:

2:2:1: Animals and anaesthesia:

Male Sprague-Dawley rats from the Joint Animal House, University College London were used throughout these experiments. The weight range used was 250 to 300g.

In the *in vivo* experiments the animals were anaesthetised with urethane. A 25% solution of urethane in saline was prepared by adding 7.8ml of saline to 2.5g of urethane. This solution was injected (0.6ml/100g) intraperitoneally to induce surgical anaesthesia (defined as the complete loss of blink and hind-limb-withdrawal reflexes) within 5 min. In practice urethane proved to be reliable and satisfactory in inducing surgical anaesthesia with the preparation surviving the whole of the experiment.

In *in vitro* experiments the animals were first placed in a perspex box (12×12×26cm) and anaesthetised with halothane to facilitate killing them by neck dislocation. 3% halothane in a 95%O_2:5%CO_2 mixture was delivered at a rate of 0.5l/min with the excess gas being sucked away via a tap-connected vacuum pump. The induction of anaesthesia took 3-4 min.

2:2:2: Electroencephalogram (EEG), Electrocardiogram (ECG) and temperature monitoring in *in vivo* experiments:

The EEG was monitored through platinum electrodes incorporated in the wall of the cortical cup. The signal was amplified 1000 times (Devices 3160 amplifier, 0.2s time constant, UK) and displayed on a paper oscillograph (400MD/2, George Washington, UK) providing a qualitative measure of the activity. The amplified signal was also fed into a

specially designed integrator (see section 2:2:6) to quantify the EEG spikes as well as a spike processor (Digitimer, D130, UK) to count the total number of spikes. The ECG was monitored in some experiments using two needle electrodes (23G) inserted beneath the skin of the animal in the chest area. The signal was amplified (Devices 3160, 0.2s time constant, UK) and the output was fed into an audio monitor (Devices 4010, UK) providing an auditory signal for the heart rate. This measure was used to detect any deterioration in the health of the animals in the early experiments although in later experiments there was no need to measure ECG as the animals stayed healthy throughout the experiment.

Body temperature was monitored and kept constant at 37 $\pm$ 0.2°C using an animal blanket unit with rectal thermometer (Harward Ltd., UK). Fig.2.1 shows a schematic diagram of the apparatus used.

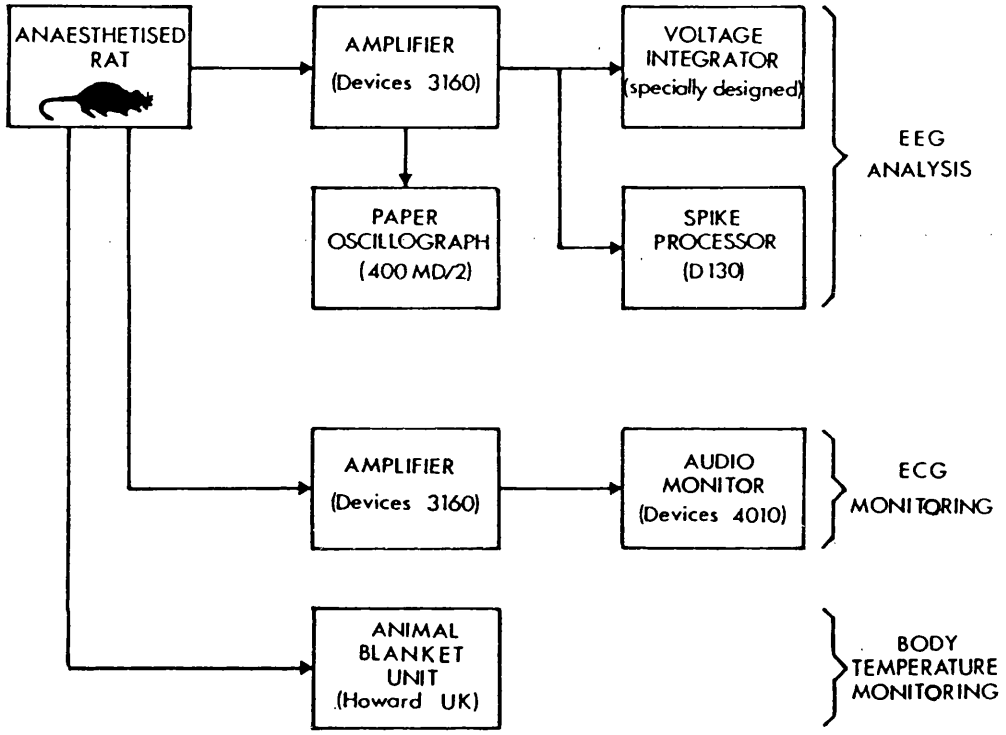

Fig.2.1: The schematic diagram of the apparatus used to monitor EEG, ECG and the body temperature during *in vivo* experiments.

2:2:3: Cortical cup technique:

The cortical cup technique was first described by Collier and Mitchell (1967). The cups used in these experiments were similar to those previously used in our laboratory (Croucher, 1985 and Kent & Webster, 1986).

After induction of urethane anaesthesia the animal was transferred onto the operating table. The head was secured using ear bars and a nose clip in such a way that the top of the skull was horizontal. A midline incision was made using a scapel, the flaps of the skin were deflected to either side and the connective tissue above the skull was carefully scraped away to reveal the skull sutures. A trephine drill (7mm o.d., Hager & Meisinger, West Germany) was used to remove a circular piece of skull from over the right hemisphere in the centre of the coronal plate (Fig.2.2.A). After carefully retracting the dura a cortical cup (Fig.2.2.B) which was firmly attached to a micromanipulator was slowly lowered into the hole ensuring that the base of the cup was exactly parallel to the surface of the exposed cortex. A slight pressure of the rim of the cup onto the exposed cortical surface was sufficient to produce a tight seal between the cup and the brain with no detrimental effect on the local blood vessels covering the cortex (this was visually inspected using a microscope). In the early pilot experiments it was necessary to use petroleum jelly to produce a tight seal between the cup and the brain surface. This was mainly due to the fact that the large outer circumference of the cups meant that they had to cover some curvature in the cortex. The smaller cups (5mm o.d., 4mm i.d.) eventually used in the present work could sit evenly on a flat area of cortex which made the use of sealant unnecessary. The cup covered an area of $\approx$12.57 mm^2 which included parts of the postcingulate cortex, frontoparietal cortex (motor area) and

striate cortex (area 18 and 17), situated directly above the hippocampus (from Paxinos & Watson, 1982).

Carboxygenated (95%O_2:5%CO_2) artificial cerebrospinal fluid (ACSF) with or without drugs, at approximately 37°C (checked routinely with a fine thermister probe), was introduced via the inlet tube into the centre and bottom of the cup using a Gilson minipuls 2 pump (Anachem, UK). The fluid accumulated in the cup until reaching the level of the outlet tube (7mm from the rim of the cup) when it was sucked away using a Watson Marlow pump (MC 10, UK). In these experiments special care was taken to keep the exposed cortex moist at all times, with carboxygenated ACSF, from the moment the dura was removed to when the cup was placed onto the cortex. The flow rate employed was 30μl/min which not only provided enough sample volume for the amino acid analysis but also was slow enough not to cause any visual damage to the cortical surface. The samples were collected on ice and stored at – 30°C for not longer than a few days before analysis of their amino acids content (as explained in section 2:2:5).

It was important to use PTFE tubing throughout as our pilot studies clearly showed that amino acids were somehow 'lost' in the system when other types of tubing were used (Fig.2.3). In addition, the whole system was cleaned at the end of each experiment by passing through a solution of detergent ('Haemosol', Alfred Cox Ltd., UK) in deionised water (DIW) at high flow rate with subsequent thorough rinsing with DIW.

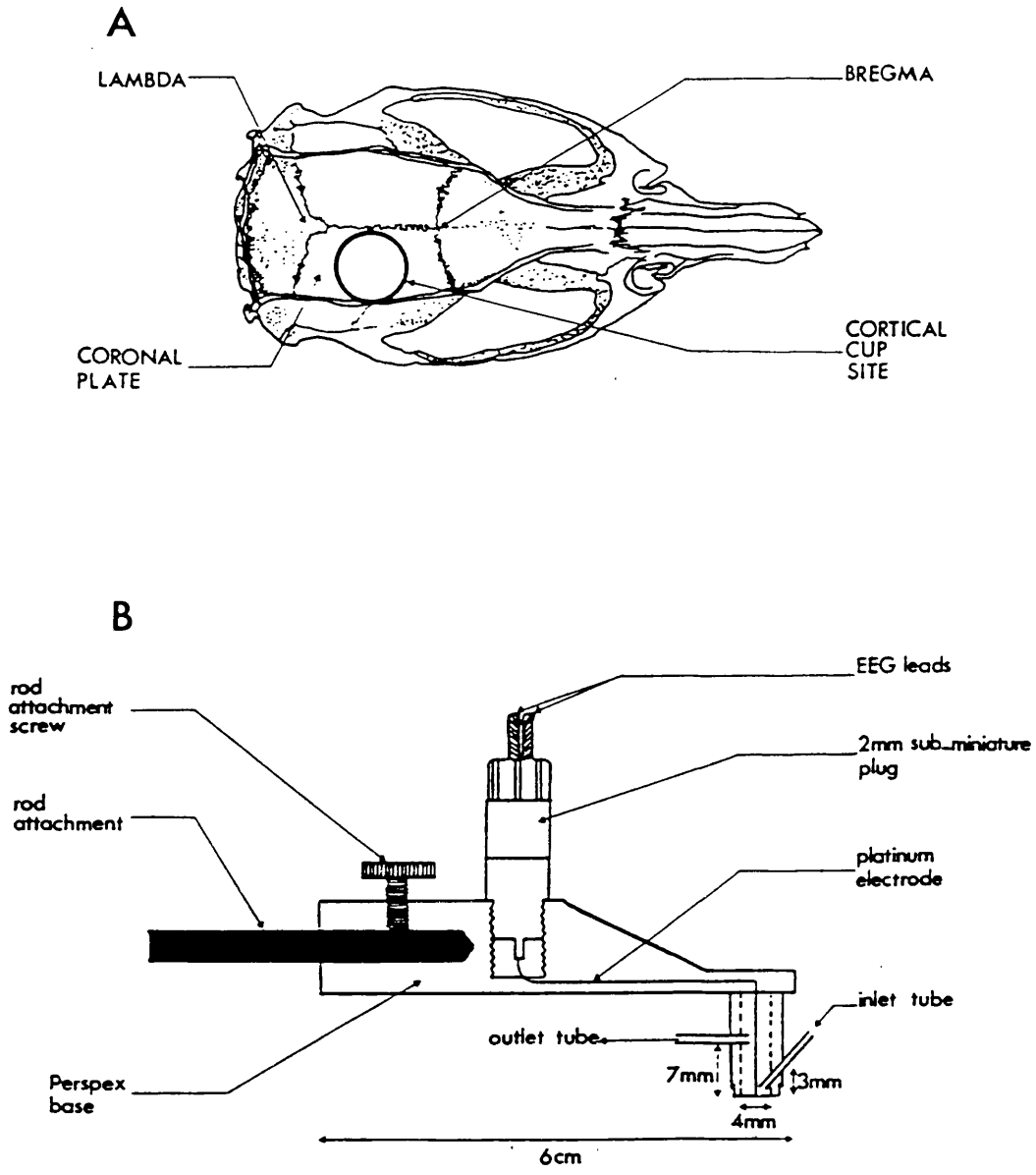

Fig.2.2: The cortical cup technique. A shows the dorsal view of the rat skull outlining the position of the trephine hole in the centre of the coronal plate. B is a schematic diagram of the cortical cup used showing the design and dimensions of the various components.

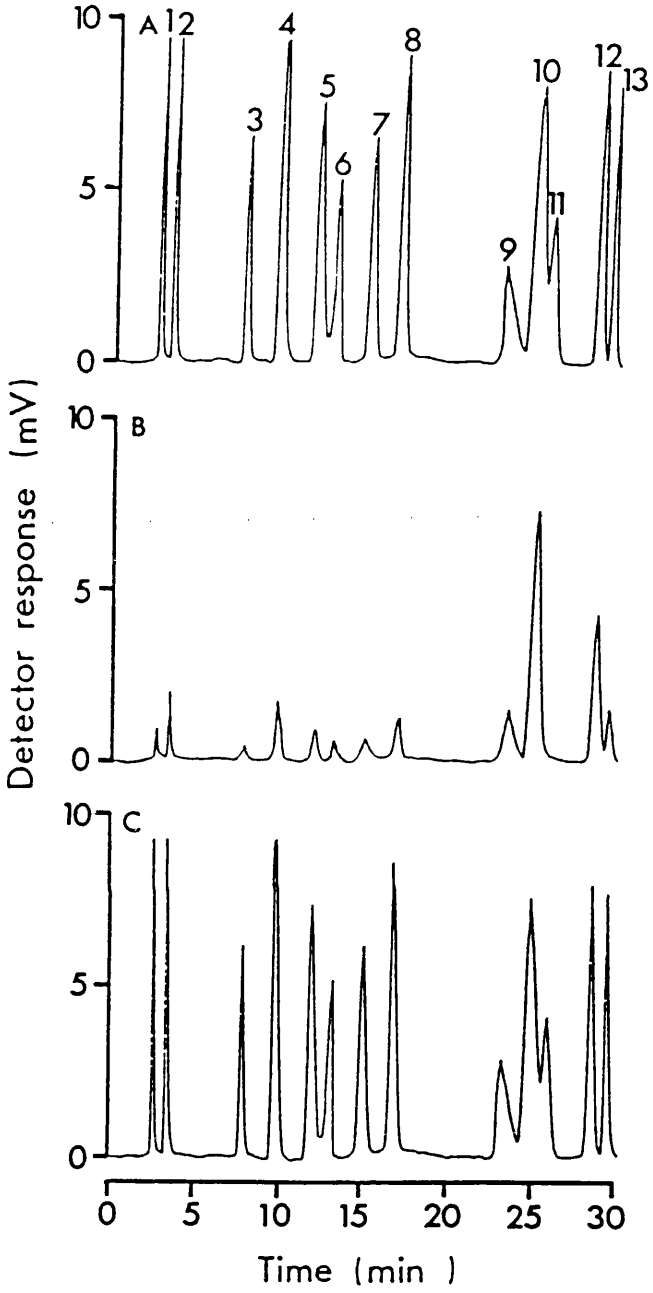

Fig.2.3: The importance of using PTFE tubings throughout the cortical cup system. Trace A shows a standard solution of amino acids (2μM in ACSF) before passage through the system. Trace B shows the same standard solution which has been passed once through the system, at the end of an experiment, when polyethylene tubings have been used. Trace C shows the recovery of amino acids in the standard solution when PTFE tubings are employed.
Aspartate(1), glutamate(2), asparagine(3), serine(4), glutamine(5), histidine(6), glycine(7), threonine(8), arginine(9), taurine(10), alanine(11), GABA(12) and tyrosine(13).

2:2:4: Cortical slice technique:

The technique employed was similar to that used by Vellucci & Webster (1985) when studying release of neurotransmitters from spinal cord slices. It involved a continuous superfusion of cortical slices in a submersion chamber.

The procedure is outlined in Fig.2.4.A. After the initial stunning of the animals with halothane, they were killed by dislocating the neck. The entire brain was dissected out and immediately submerged in cold (4° to 10°C) carboxygenated ACSF in a Petri dish. A rectangular piece of the cortex which corresponded to the area underneath the cortical cup in the *in vivo* experiments was dissected out. A scapel blade was used for the dissection. The brain was first cut into two hemispheres. Two coronal cuts were then made through the cortex in the right hemisphere (≙ 5mm apart) perpendicular to the first cut to isolate the central part of the parietal cortex. A further sagital cut was then made parallel to the midline and about 5mm away from it thus producing a square piece of approximately $5 \times 5 mm^2$. The cortex was then gently eased away from the cerebral structures underneath using fine curved scissors and was transferred into a fresh Petri dish and was again submerged in cold carboxygenated ACSF. The piece of the cortex was then placed with the striatal side uppermost onto a teflon platform and was cut coronally into 400μm slices using a McIlwain tissue chopper. The slicing was done slowly and with lowest force possible to minimise the disturbance of the cortical piece which could have lead to uneven slices. The edge of the cortical piece (away from the blade) was glued (grade C2 cyanoacrylate adhesive, Permabond Adhesive Ltd., UK) onto the teflon platform to keep the tissue steady. The slices were then transferred into an incubation chamber, using a fine paint brush, and incubated for 45 min at 37°C in carboxygenated ACSF. The incubation

chamber (Fig.2.4.B) consisted of a 250ml graduated flask into which a specially designed tissue holder was lowered. The slices rested on a coarse sintered-glass platform at the bottom of the tissue holder. The carboxygenation was done through the hose of a sintered glass tube positioned at the bottom of the flask. The use of cold medium during slicing has a protective value for the survival of the tissue but prolonged cooling reduces viability and quality of the slices. It is also important to note that in general speed of dissection seems not nearly so important as the care taken in removing and slicing the tissue (Dingledine, 1984). Therefore, it was important to strike a right balance between performing the slicing procedure as quickly as possible without being hasty. In practice, it took 4 min from killing the animal to transferring the cortical slices into the incubation chamber.

After the incubation period two slices were transferred, using the paint brush, into each of the tissue perfusion chambers (Fig.2.5.B). The tissue perfusion chambers consisted of glass tubes (5mm diameter) which were situated inside a heating glass jacket. The slices were placed on a platinum wire gauze 'sandwiched' between two fine nylon gauze discs (4.5mm diameter, 100μm aperture size, Gallenkamp, UK). The slices were completely submerged in carboxygenated ACSF in a continuous perfusion system in which the medium was introduced into the bottom of the chamber using a Gilson minipuls 2 pump (Anachem, UK). ACSF passed over the slices and gathered on top of them until it reached a height of approximately 7mm above the tissues from where it was rapidly sucked away and collected into sample vials using a Watson Marlow pump (MC10, UK), Fig.2.5.A. The slices were rested in the tissue perfusion chambers for a further 15 min, with ACSF being perfused over them during this period, before exposure to drugs. Solutions of these drugs in ACSF took 40-50 secs to reach the slices and they were

perfused over the tissue for 6 min followed by a further continuous superfusion of ACSF.

In a typical experiment ACSF, with or without drugs, was perfused at 300μl/min and 3 min samples were collected starting 6 min before and finishing 6 min after the exposure to drugs. The samples were stored at -30°C for subsequent analysis of their amino acid content. The slices were removed at the end of the experiments and their wet weights determined.

The optimal conditions for parameters such as the period of incubation, number of slices in each chamber and the flow rate were determined in the preliminary experiments.

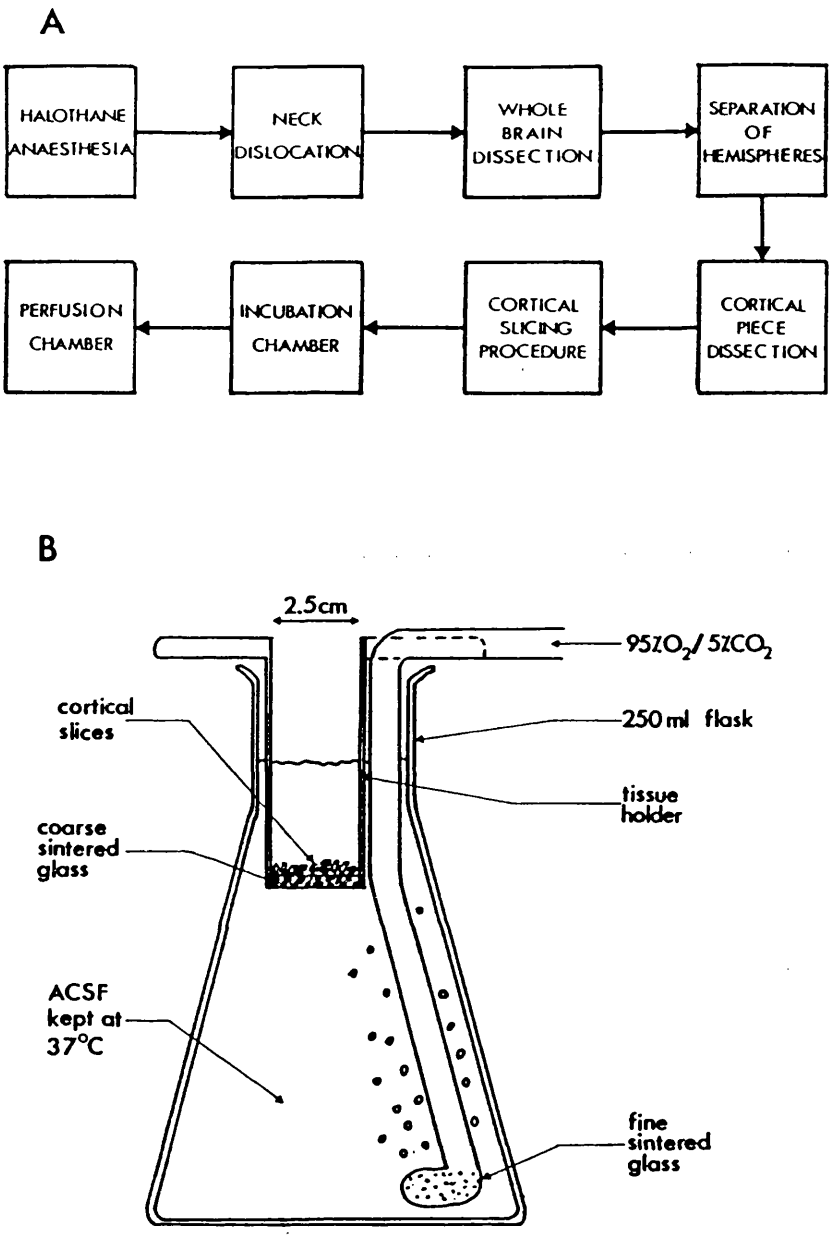

Fig.2.4: The *in vitro* cortical slices technique. A outlines the procedure involved from the moment the animal is anaesthetised to when the slices are placed into the tissue chambers. B shows the incubation chamber in more detail outlining its design and dimensions of the main components.

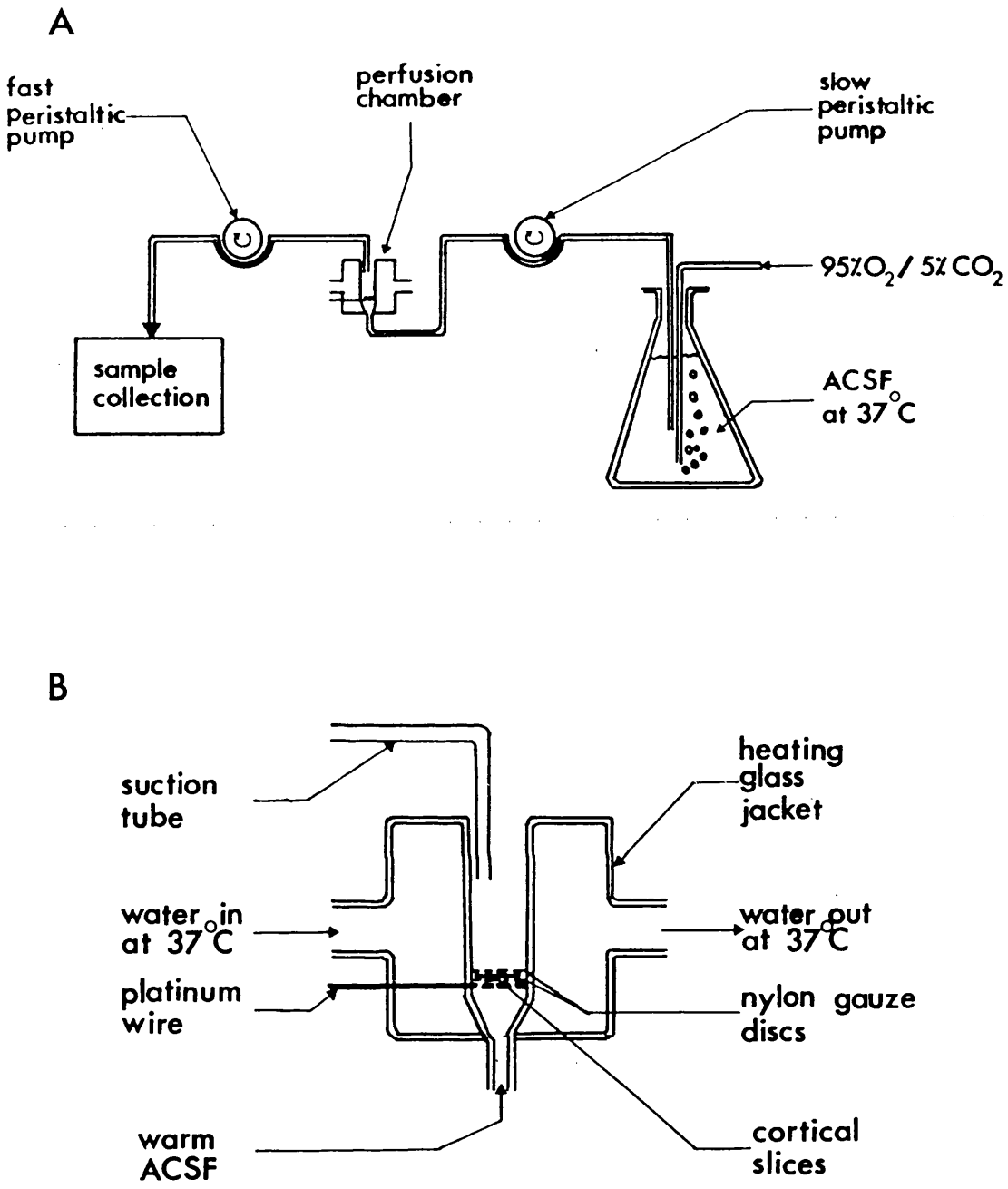

Fig.2.5: Schematic diagram of the *in vitro* cortical slices perfusion apparatus. A is a diagrammatic representation of the system used. B shows the perfusion chamber in more detail outling its design and dimensions.

2:2:5: Amino acid analysis by automated high performance liquid chromatography (HPLC):

An automated procedure in use in the laboratory was modified to measure more amino acids and improve their separation by using a new chromatographic column; a different solvent system and an improved gradient programme (Farrant, Zia-Gharib & Webster, 1987).

The procedure involved the separation of amino acids by passing the sample down a chromatographic column under high pressure before their detection by a UV-detector. Since amino acids are not naturally fluorescent, fluorescent derivatives had to be produced by pre-column derivatisation. This was achieved by mixing amino acids in the sample with o-phthalaldehyde/2-mercaptoethanol (OPA/2-MCE) reagent which reacts with the primary nitrogen group in the amino acid molecule forming a fluorescent complex (Fig.2.6).

A Varian LC 5000 liquid chromatograph (Varian Assoc., UK) was used. The column chosen was 15cm in length; it was densely packed (plate number in excess of 100000) with C18, reversed-phase, 3μ Hypersil beads (Jones chromatography, UK) and its temperature was kept at 32°C. The analytical column was protected by a low-volume guard coiumn (2cm × 2mm i.d.) incorporating 0.5μm stainless-steel frits and filled with Lichoprep RP-18 of 25-40 μm particle size (Upchurch Uptight, Anachem, UK). The solvents were withdrawn from their reservoirs through 10μm particulate filters and degassed on-line by an Erma ERC-3510 degasser (HPLC Technology, UK). Fluorescence of the column eluate was continuously monitored using a Varian Fluorichrom filter fluorescence detector (excitation 355nm; emission 450nm). Chromatographic data were recorded and processed by a Hewlett-Packard 3390A computing integrator(UK) which calculated the amino acid concentrations by relating peak areas in the sample to those obtained from a previously

run standard solution. Using a simple three-step gradient elution method in combination with some variation of the mobile phase flow-rate (Fig.2.7.A), it was possible to successfully separate the following amino acids: aspartate(Asp), glutamate(Glu), asparagine(Asn), serine(Ser), glutamine(Gln), histidine(His), glycine(Gly), threonine(Thr), arginine(Arg), taurine(Tau), alanine(Ala), γ-aminobutyric acid(GABA) and tyrosine(Tyr). A typical chromatogram showing the separation of these amino acids is shown in Fig.2.7.B.

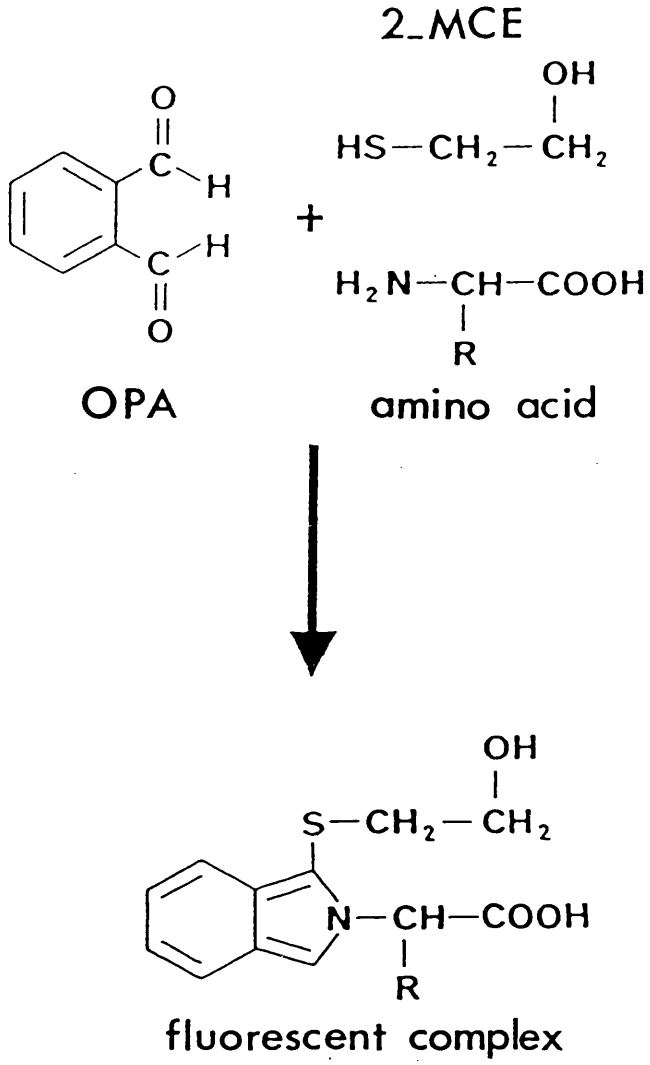

Fig.2.6: The schematic diagram of the reaction involved in the derivatisation of the amino acids with OPA/2-MCE reagent. OPA = o-phthalaldehyde, 2-MCE = 2-mercaptoethanol.

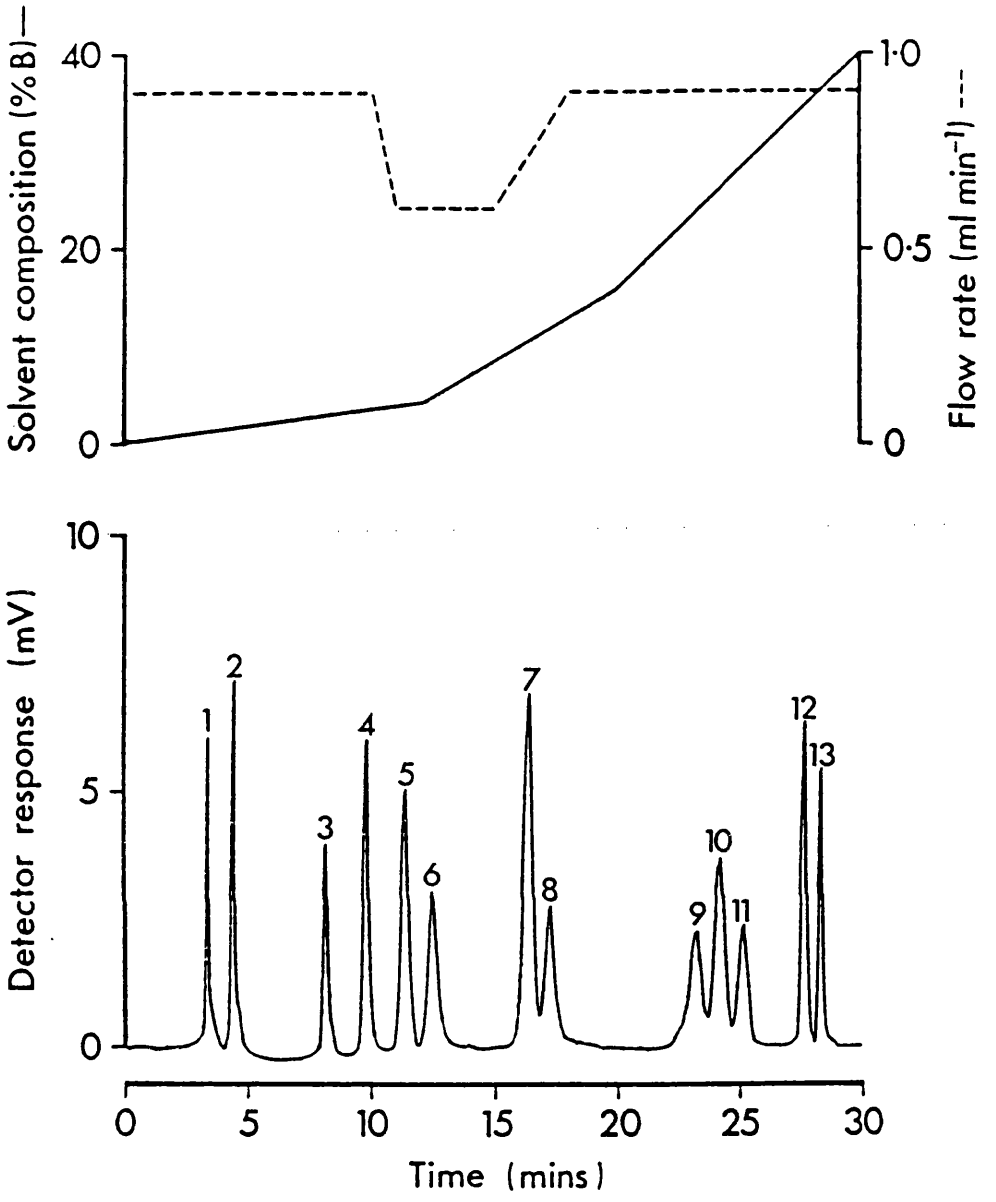

Fig.2.7: The analysis of a 13 component amino acid standard (1μM). A shows the variations in the mobile phase composition and its flow rate during the 'run'. B is a typical chromatogram showing the successful separation of aspartate(1), glutamate(2), asparagine(3), serine(4), glutamine(5), histidine(6), glycine(7), threonine(8), arginine(9), taurine(10), alanine(11), GABA(12) and tyrosine(13).

Automated sample derivatisation and injection was achieved using a modified Magnus M220 autosampler (Magnus Scientific, UK). Its sample probe was replaced with a short length of polytetrafluoroethylene (PTFE) tubing (0.3mm i.d.) connected, via a miniature solenoid valve (No. 1200218H, Lee products, UK) and a length of stainless-steel capillary tubing (0.18mm i.d.), to one arm of a low dead-volume T-piece (SSI, Anachem, UK). The opposite arm of the T-piece was connected to a 20-ml vial of OPA/2-MCE reagent. The third arm was attached to the inlet port of a pneumatically operated Rheodyne 7010 injection valve fitted with a 50μl sample loop. A peristaltic pump (Gilson Minipuls-2, Anachem, UK) was used to draw sample and reagent via the T-piece into the sample loop. The duration and speed of pumping was carefully adjusted so that it produced adequate mixing of the two liquids together with slight over-fill of the loop without using a large sample volume.

Solvents and reagents

A two-solvent system was employed. The more hydrophilic solvent, solvent 'A', consisted of 80% sodium acetate (20mM); 19% methanol and 1% tetrahydrofuran (THF). Solvent 'B' was a mixture of methanol (80%) and sodium acetate 20mM (20%). Both methanol and THF were of the purest type available (HPLC grade, May & Baker, UK). The accuracy in making these solvents was vital in producing consistent retention times for the amino acids and they were filtered through a 0.1μm Nylon 66 membrane (Pall, Ultipor N66T, Gallenkamp, UK) prior to use.

The OPA reagent was prepared by adding 1400μl of methanol to 60mg of OPA and shaking the mixture until all the OPA was dissolved. 600μl of 2-mercaptoethanol (2-MCE) was then added followed by addition of 18ml borate buffer. To make the borate buffer 24.732g boric acid was

dissolved in 800ml of deionised water; the pH of the solution was adjusted to 10.4 with 5M sodium hydroxide and the final volume was made to 1l with deionised water. Although some workers (Lindroth & Mopper, 1979 and Jones, Pååbo & Stein, 1981) suggest aging of the OPA/2-MCE reagent for 24 hours before analysis, in our experiments this reagent was used immediately after preparation and special care was taken not to use the reagent if it was more than 3-4 days old as an 'OPA' peak appeared on the chromatogram and increased in size on aging to affect the peaks for Arg, Tau and Ala.

Standard solution of amino acids

Individual amino acid stock solutions (10mM in 0.05M hydrochloric acid) were made directly from the crystalline solids. A working standard solution was then made by diluting each of the stock solutions 10000 times with DIW yielding a final concentration of 1μM for each of the amino acids studied. Normally 100ml of this working standard was prepared and frozen in 1ml aliquots at -70°C . When analysing a series of samples one of these 1ml samples was thawed and run to obtain the standard trace to which amino acid contents of the superfusates were compared and measured by the programmed computing integrator. Using this method it was possible to use the same standard batch for up to 3 months.

Reproducibility and linearity of the analysis procedure

In the preliminary experiments an internal-standardisation technique based on homoserine was used to measure the levels of amino acids. However, after developing the new automated HPLC technique it was evident that the variations in the levels of amino acids recorded in

the standard runs was less when using an external-standardisation technique and, therefore, the latter technique was adopted. The within-run precision of the automated procedure was evaluated following sixteen consecutive injections of the working standard solution. The coefficient of variation of the retention times of the majority of the amino acids was less than 1% (mean 0.74%), the largest deviation being for Arg (10.14s) and the smallest for Asp (1.44s). The mean variation of the measured peak areas was 2.2%. Gly showed the largest variation (3.6%) and Asn and Gln the lowest (1.2%), Table 2.1. The linearity of the analysis procedure was tested by running standard solutions containing amino acids in the concentration range of 0.1 to 5μM. Linear regression analysis of the peak area of each amino acid versus concentration gave a coefficient of determination of better than 0.999 in all cases (Table 2.2).

Amino acid	Mean retention time (min)	Coefficient of variation for retention time	Mean peak area (μV.s)	Coefficient of variation for peak area
Asp	3.44	0.79	264104.8	3.93
Glu	4.38	0.93	375828.1	2.16
Asn	8.13	0.97	265349.0	1.24
Ser	9.84	0.84	401714.1	5.06
Gln	11.56	1.16	618879.2	1.24
His	12.66	1.15	501102.6	1.30
Gly	16.25	0.66	764588.0	3.58
Thr	17.34	0.63	312531.5	1.89
Arg	23.46	0.72	290636.3	2.57
Tau	24.38	0.60	373023.1	2.19
Ala	25.47	0.44	353965.6	2.19
GABA	27.81	0.29	299076.4	2.01
Tyr	28.75	0.26	310716.9	1.54

Table 2.1: The with-in run precision of the automated procedure.
The above values were obtained by running sixteen consecutive injections of a thirteen-component amino acids standard (1μM).

Amino acid	Regression equation	Coefficient of determination (r^2)
Asp	y=229054x + 42732	0.9990
Glu	y=353407x + 27176	0.9994
Asn	y=239776x + 22818	0.9993
Ser	y=372229x + 49178	0.9991
Gln	y=568967x + 56462	0.9990
His	y=464858x + 41890	0.9992
Gly	y=686381x + 102361	0.9990
Thr	y=272830x + 34115	0.9991
Arg	y=284578x + 13332	0.9994
Tau	y=344091x + 27539	0.9993
Ala	y=326693x + 33969	0.9990
GABA	y=276594x + 17413	0.9994
Tyr	y=289009x + 25550	0.9991

Table 2.2: The linearity of response of the automated procedure. The relationship between peak area and amino acid concentration has been shown in the above table. The regression equation has the form y=mx + c, where y is the peak area (μV.s); m is the slope; x is the amino acid concentration (μM) and c is the intercept on the y axis.

2:2:6: Quantification of the EEG spikes:

An electronic circuit (Fig.2.8) was designed and constructed to provide a quantitative measure of epileptogenic spiking by converting integrated spike voltage to a digital read-out.

Amplified EEG signals were fed into a voltage-to-frequency (V/F) converter which produced output pulses at a frequency directly proportional to the amplitude of the incoming voltage (Fig.2.9). A comparator circuit was used to set a threshold voltage (equivalent to 0.1mV) above basal EEG so that only distinct spikes produced a positive response from the comparator. The output of both the V/F converter and the comparator were then fed into a logic gate which only allowed the pulses to go through if both signals were 'on' i.e. only spikes above the set threshold were counted. A digital counter then measured the frequency of pulses generated from the V/F converter by the spikes. A programmable timer reset the counter at minute intervals with the output of the counter being displayed on a digital L.C.D. Therefore, the integrated voltage recorded was representative of the total area under the voltage profile of all spikes above the set threshold in a min.

By applying rectangular pulses of different amplitude, frequency or width it was shown that the integrator gave an output that was linearly related to changes in all three parameters for pulses equivalent in size and frequency to the epileptogenic spikes (Fig.2.10). A detailed circuit diagram of the device is shown in appendix I.

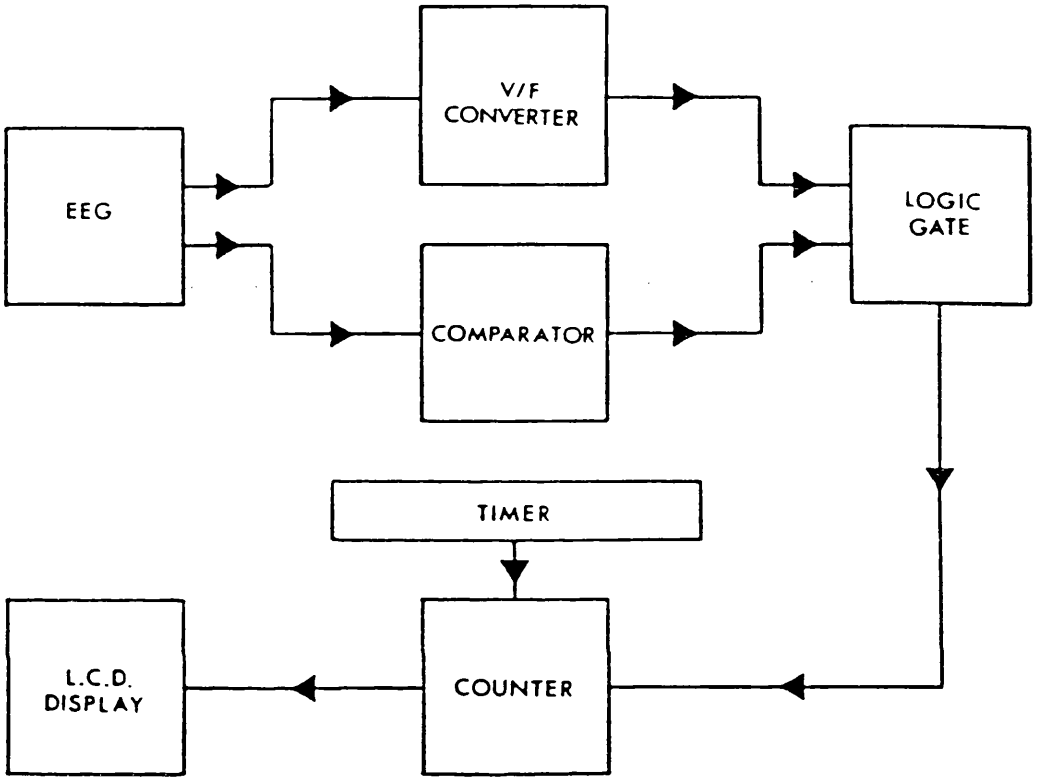

Fig.2.8. Block diagram of the spike voltage integrator showing the various components used in the device.
V/F = Voltage to frequency
L.C.D.= Liquid Crystal Display

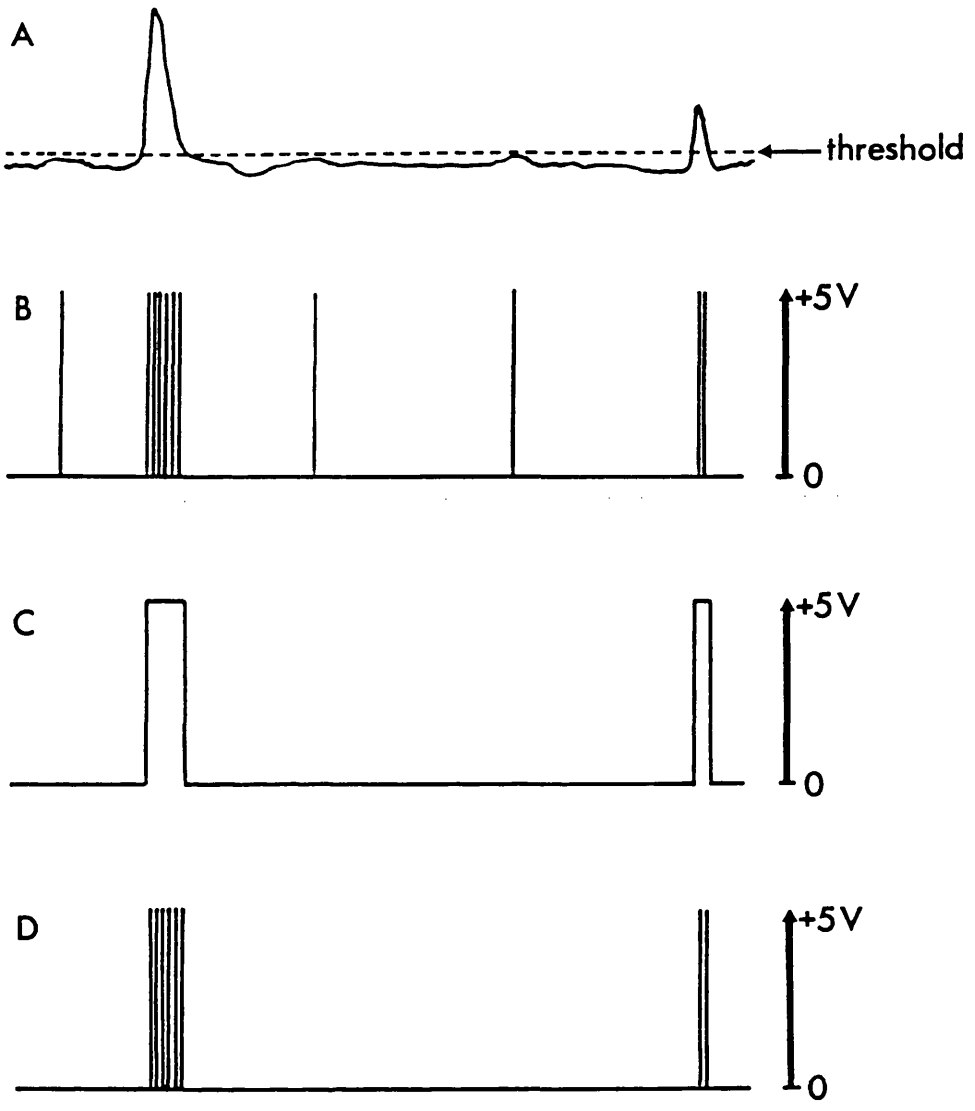

Fig.2.9. Diagramatic representation of the different stages in the integration of epileptogenic spikes. In A two imaginary spikes are shown superimposed on the basal EEG. B shows the output of the V/F converter which produces +5V pulses at a frequency which is directly proportional to the amplitude of the ingoing signal. In C the output of the comparator, which is only activated by spikes above the threshold setting, is shown. It produces +5V rectangular pulses of the same width as the spikes in A. Finally D shows the output which leaves the logic gate and enters the digital counter. Therefore, in this case the bigger spike registers a count of 6 compared to the smaller spike which is a third of the size and registers a count of 2.

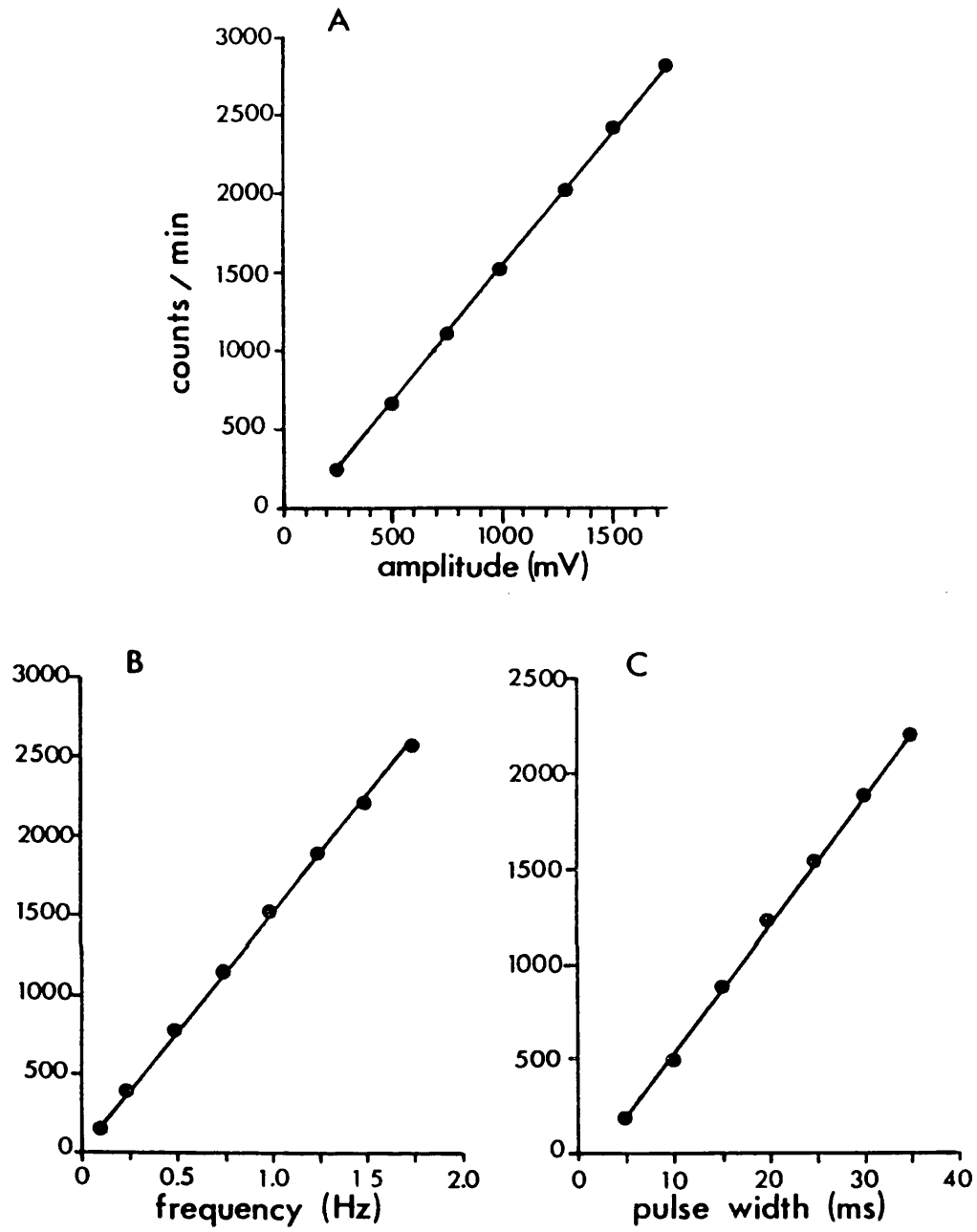

Fig.2.10: Calibration curves showing linearity of integrator output in relation to ingoing signal. A stimulator was used to produce rectangular pulses of differing amplitude, frequency or width. In A the width (25ms) and the frequency (1Hz) of the pulses were kept constant and their amplitudes were altered. In B the frequency plot demonstrates the linear response of the device to changes in frequency of the ingoing pulses if their amplitude (1V) and width (25ms) were unaltered. In C the amplitude (1V) and the frequency (1Hz) were kept the same and the pulse width was altered.

2:3: SPECIFIC PROCEDURES:

2:3:1: Intravenous infusion of pentylenetetrazole (PTZ):

The animals were anaesthetised with urethane. Prior to placement of cortical cups, as outlined above (2:2:3), a polyethylene cannula (Portex 3FG, UK) was inserted into the left femoral vein. A 500mM solution of PTZ in saline was infused at a rate of 30μl/min into the cannulated femoral vein. The EEG was monitored throughout the experiment through the cup electrodes.

2:3:2: Extracellular recording of neuronal activity:

In a few experiments extracellular neuronal recordings were performed with a glass-coated tungsten electrode lowered through the cup to a depth of approximately 300μm (layers I-III). After amplification (Neurolog NL104 & NL106, UK) the recorded potentials were displayed on a storage oscilloscope (Tetronix 5113, UK) and photographed.

2:3:3: Preparation of perfusion solutions:

The ACSF contained in mM: NaCl,137; KCl,2.68; $CaCl_2$,2.2; $MgCl_2$,1.04; $NaHCO_3$,11.9; Na_2HPO_4,0.42 and D-Glucose,10.

In some experiments a high K^+ solution was prepared by increasing the concentration of KCl to 60mM with the equivalent reduction in NaCl concentration to 79.68mM to keep the osmolarity of the solution constant.

Calcium-free ACSF was prepared by omitting $CaCl_2$ from the medium and adding 1mM ethyleneglycol-bis-(β-amino-ethyl ether)n, n'-tetraacetic acid (EGTA). Magnesium-free medium was prepared by removing $MgCl_2$ from ACSF. In the high Mg^{++} solution the ACSF concentration of $MgCl_2$ was 10mM.

All the drugs tested were soluble in ACSF except CNQX, which was first dissolved in DIW and pH adjusted to 7.2 with NaOH before a further 1:100 dilution of this solution in ACSF, and clonazepam, which was dissolved in ethanol with subsequent dilution (at least 1:100) in ACSF.

CHAPTER 3

EFFECTS OF VARIOUS CHEMICAL CONVULSANTS ON THE EEG OF URETHANE-ANAESTHETISED RATS AND THEIR MODIFICATION BY DIFFERENT COMPOUNDS.

3:1: INTRODUCTION:

Despite the relatively recent discovery of advanced new imaging techniques in the study of human epilepsy such as positron emission tomography (PET scan); single photon emission computed tomography (SPECT) and nuclear magnetic resonance computed tomography (NMR-CT) which are used to accumulate profiles of brain cross sections and reconstruct them into two-dimensional maps representing cerebral anatomy and function (Mazziotta & Engel Jr., 1985), the electroencephalograph (EEG) still forms an essential and basic tool in the examination of epileptic patients (Naquet, 1983 and Pedley, 1984). The EEG in some epileptic subjects exhibits distinctive periods of spikes or sharp waves superimposed on the background EEG. The cerebral cortex is established as an important site for the genesis and spread of epileptogenic activity (Bancaud, Telairach, Morel, Bresson, Bonis, Geier, Hemon & Buser, 1974; Piredda & Gale, 1985 and Fenwick, 1983). The intricate circuitry of the cerebral cortex makes this part of the brain quite unique in that epileptogenic activity can be initiated and controlled in isolated blocks of neocortex without the involvement of sub-cortical structures (Gutnick, Connors & Prince, 1982; Prince, 1965; Courtney & Prince, 1977; Connors, 1984 and Jones & Lambert, 1990). The experiments described in this chapter involve the study of the effects of various compounds on the EEG recorded from the exposed surface of parietal cortex in the urethane-anaesthetised rats.

The objective was two-fold; 1: to establish how various convulsants affect the basal EEG when applied to the cortex and then to use the most appropriate procedure to 2: ascertain the effects of various compounds that modulate amino acid transmission on any epileptogenic activity produced by the convulsants. The convulsants tested were pentylenetetrazole (PTZ), bicuculline methiodide (BM) and

picrotoxin.

The mode of action of PTZ is not fully understood. It is thought to antagonise the inhibitory action of GABA by blocking the chloride channel associated with GABA receptor stimulation or by interacting with the benzodiazepine portion of the GABA receptor, as well as by having a direct depolarizing effect on the cell membrane. Bicuculline and picrotoxin are thought to be GABA antagonists with differing modes of action. Bicuculline is a competitive $GABA_A$ receptor antagonist and picrotoxin appears to block the chloride ion channel associated with GABA stimulation (for a more detailed description of the mode of action of these compounds refer to chapter 1, section 1:3). The effect of 60mM K^+ solution which causes direct non-specific depolarization of neurones was studied on the basal EEG for comparison and to establish that the EEG could be modified by this procedure.

The convulsants were superfused across the cortex and having established a reproducible pattern of consistent spiking with a selected concentration of these convulsants its modification by compounds that alter amino acid neurotransmission was evaluated. These compounds included those which increase the inhibitory action of GABA as well as excitatory amino acid antagonists both of which have been shown to reduce epileptogenic activity in animal models of epilepsy (see chapter 1, section 1:4). Most of this work centred on the modification of spiking induced by BM since this convulsant was the most potent, also producing the most consistent spiking.

The importance of increasing the inhibitory action of GABA at its $GABA_A$ receptor site in reducing epileptogenic activity is well documented and benzodiazepines such as clonazepam, which augment the action of GABA at the $GABA_A$ receptor, are used clinically as anticonvulsants. The $GABA_B$ agonist, baclofen, is not anticonvulsant in

man (Terrence, Fromm, & Roussan, 1983) although it suppresses epileptogenic activity in hippocampal slices (Ogata, Matsuo & Inoue, 1986) as well as being active in other animal models of epilepsy (Benedito & Leite, 1981). The actions of the excitatory amino acids are mediated by two sets of receptors: the so-called non-NMDA (kainate and AMPA) receptors which are linked to the opening of Na^+ channels and fast synaptic excitation and the NMDA receptor which is linked to Ca^{++} as well as Na^+ influx and slower and less marked depolarization of the cell. It is thought that the release of L-glutamate initially leads to activation of non-NMDA receptors but when a certain level of membrane depolarization is reached NMDA-controlled channels, which are normally blocked by Mg^{++}, are opened (Macdermott & Dale, 1987). NMDA receptor antagonists, AP5 and AP7, have been used extensively to combat epileptogenic activity in different models of epilepsy with promising results (Croucher et al., 1982 and Meldrum et al., 1983a) and the potent anticonvulsant MK-801 is known to be a NMDA channel blocker (Wong et al., 1986). The non-NMDA receptors have not been studied as extensively, because of the lack of selective antagonists but, GAMS and CNQX [6-cyano-7-nitroquinoxaline-2,3-dione (Honoré, Davies, Drejer, Fletcher, Jacobsen, Lodge & Nielsen, 1988 and Blake, Yates, Brown & Collingridge, 1989)] are thought to preferentially block the non-NMDA receptors and the former has been shown to be effective in reducing sound-induced epileptic activity in DBA/2 mice (Croucher et al., 1984). For a more detailed account of the importance of these agents in combating epileptogenic activity see chapter 1, section 1:5.

In the present study I have monitored and quantified epileptogenic EEG spiking induced in urethane-anaesthetised rats by superfusing bicuculline methiodide (BM) across the cerebral cortex and then determined how it is modified by the various drugs discussed

above. It was hoped thereby not only to elucidate the relative importance of different amino acid receptors and the mechanisms to which they are linked, in the initiation and control of epileptogenic activity but also possibly to provide information that could facilitate the rational treatment of epilepsy.

The modulatory effect of AP7 on the epileptogenic spiking induced by PTZ and picrotoxin was also studied as a comparison to BM.

3:2: EFFECTS OF VARIOUS CONVULSANTS AND HIGH K^+ ON THE EEG OF URETHANE- ANAESTHETISED RATS:

Summary of the technique

After induction of urethane anaesthesia the head of the animal was secured and a cortical cup placed on the right parietal cortex. In some experiments two screw electrodes were also placed on the contralateral cortex to study the basal EEG from that site. In each experiment ACSF was superfused for 45 min before exposure to compounds under study. The changes in the basal EEG were monitored through the cup electrodes and the effects were compared to that recorded by screw electrodes when required.

3:2:1: High K^+:

After the initial recovery period, 60mM K^+ solution was superfused for 10 min followed by further continuous superfusion of ACSF. The addition of 60mM K^+ for 10 min lead to an immediate increase in the amplitude of basal EEG activity followed within 4 min by a marked reduction in the size of the basal EEG. A secondary decrease in the amplitude followed as the high K^+ solution was withdrawn. No changes in EEG were seen when K^+ was applied in Ca^{++} free medium although the absence of Ca^{++} did not appear to affect the basal EEG itself (Fig.3.1).

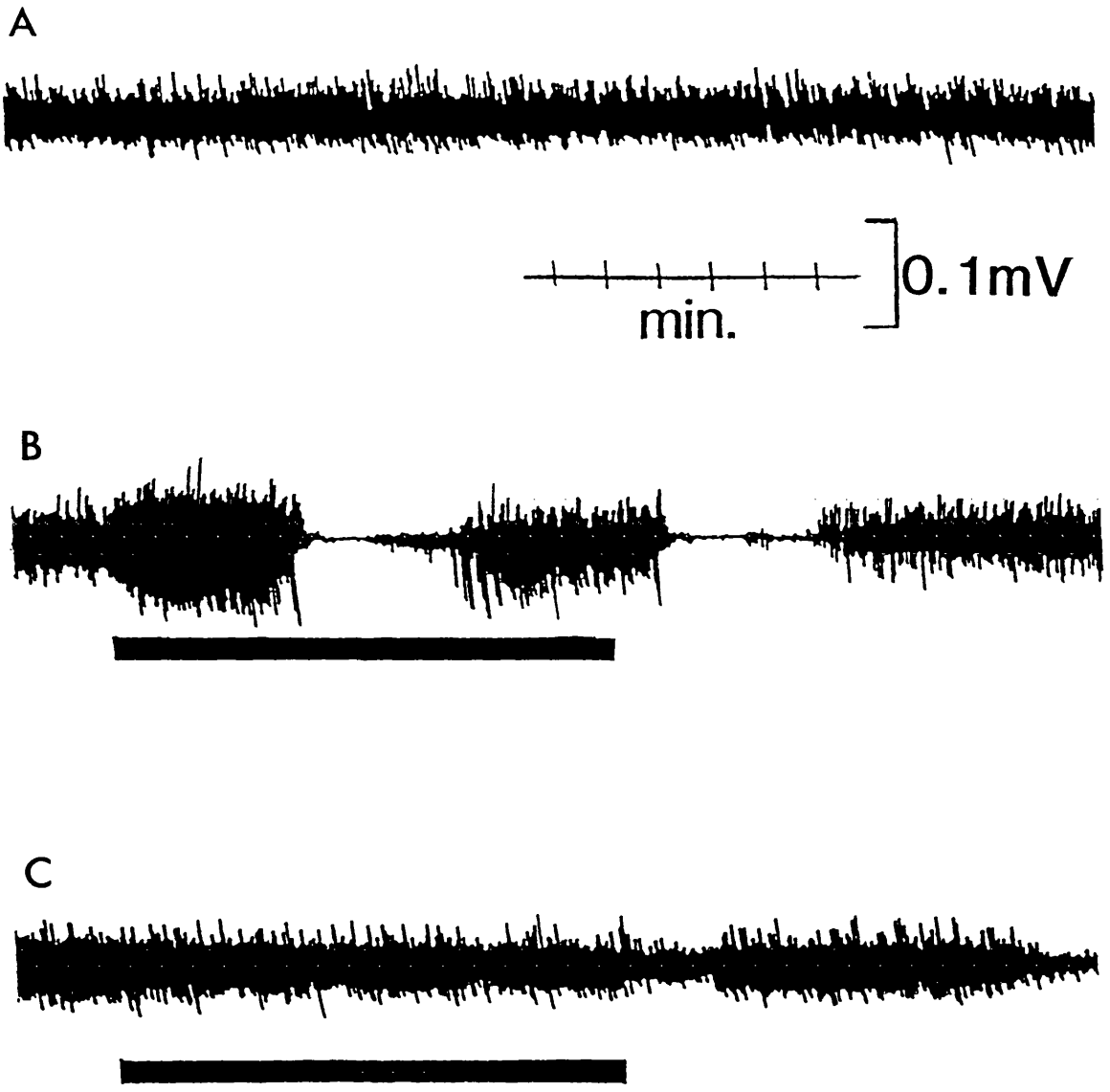

Fig.3.1. The effect of 60mM K^+ solution on the EEG of urethane-anaesthetised rat. Trace A shows the basal EEG; trace B shows the effect of 10 min application of 60mM K^+(denoted by black bar) on the basal EEG and trace C shows the effect of 60mM K^+ when Ca^{++}-free media were used.

3:2:2: Pentylenetetrazole (PTZ):

In these experiments two screw electrodes were placed on the contralateral cortex as well as placing a cortical cup on the right parietal cortex. After the initial 45 min rest period following surgery different concentrations of PTZ were superfused for 5 min followed by continuous superfusion of ACSF. Superfusion of PTZ lead to the appearance of distinctive, relatively low frequency spikes superimposed on the basal EEG (Fig.3.2) and although their size and number were dose-related they were few in number and were only seen with high concentrations (100 to 400mM) of PTZ.

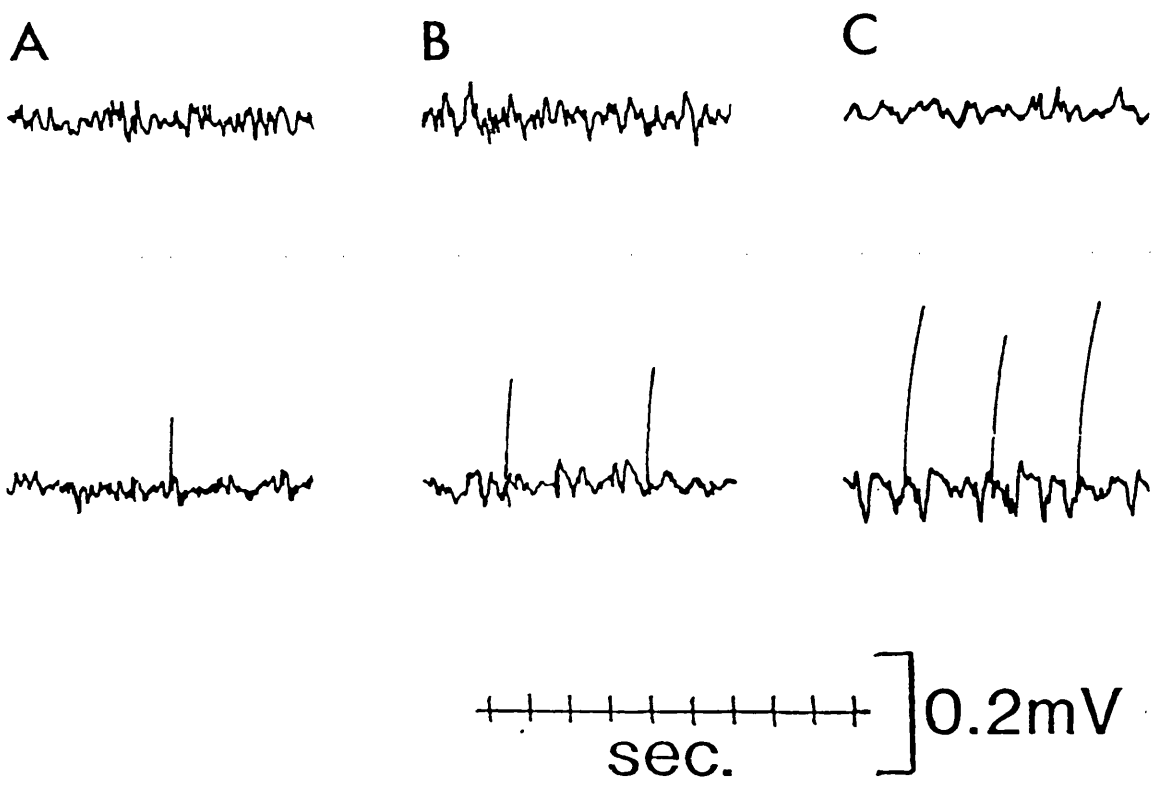

Fig.3.2. Spiking induced by cortical superfusion of PTZ. In each case the top trace shows the basal EEG recorded at the same time from the contralateral cortex using screw electrodes and the bottom trace represents the effect seen during the peak of activity when different concentrations of PTZ were superfused for 5min. over exposed cortex and the EEG recorded through cortical cup electrodes. A, B and C represent 100, 200 and 400mM PTZ, respectively. The peak of activity in each case was seen in the first min. after PTZ removal

3:2:3: Bicuculline methiodide (BM):

After the initial recovery period following placement of the cortical cup and screw electrodes different concentrations of BM were superfused for 5min followed by continuous superfusion of ACSF. BM lead to appearance of spikes superimposed on the basal EEG similar to those seen in the case of PTZ without any spread of spiking to the contralateral cortex or appearance of physical manifestation in the animal. The degree and duration of spiking was dose-related and at very high concentrations (≈750μM) high frequency (8Hz) spiking episodes could be seen intermingled with large spikes (Fig.3.3). It was evident from the preliminary pilot studies that the epileptogenic activity reached its maximum shortly (2 to 5 min) after removal of BM solution, however, the recovery from such epileptogenic activity was slow especially at higher doses of BM when it took anything up to 2 hours for the activity to subside. Therefore, to construct a dose-response curve it was decided to use a cumulative method of dosing. Ascending concentrations of BM were superfused with each concentration being in contact with the cortex for 5 min. The concentrations chosen were 100, 200, 400, 600, 800, 1000, 1250 and 1500μM BM and the levels of total integrated spiking they produced are plotted against concentration in Fig 3.4. An interesting observation was the absence of high frequency (8Hz) spiking seen during single-dose application of high doses of PTZ (see Fig.3.3D).

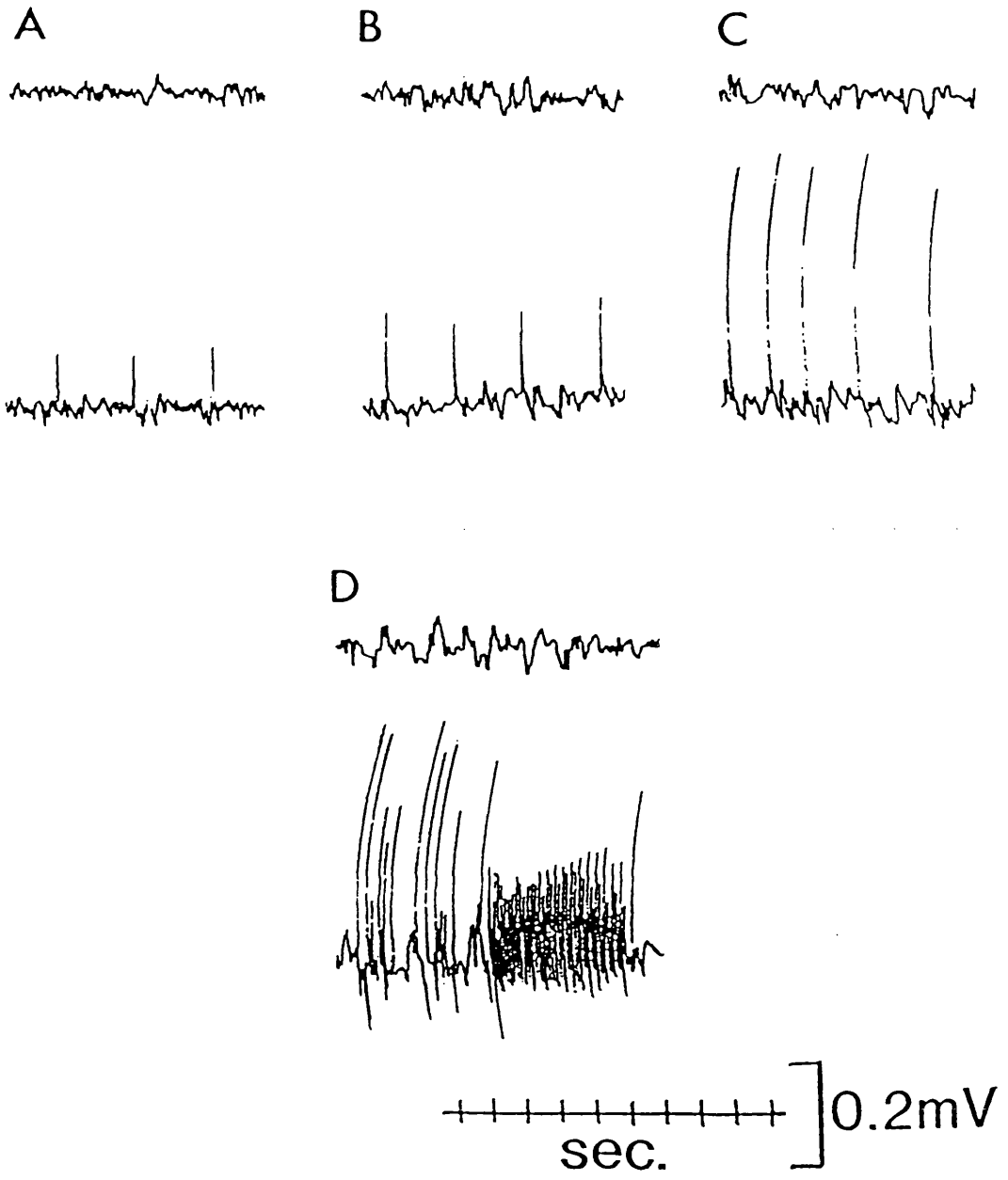

Fig.3.3. The effect of cortical superfusion of BM on the basal EEG. In each case the top trace represents the basal EEG recorded by screw electrodes from the contralateral cortex and the bottom trace shows the spiking activity seen during peak activity as a result of 5min superfusion of different concentrations of BM. A, B, C and D are 50, 100, 200 and 750μM BM respectively.

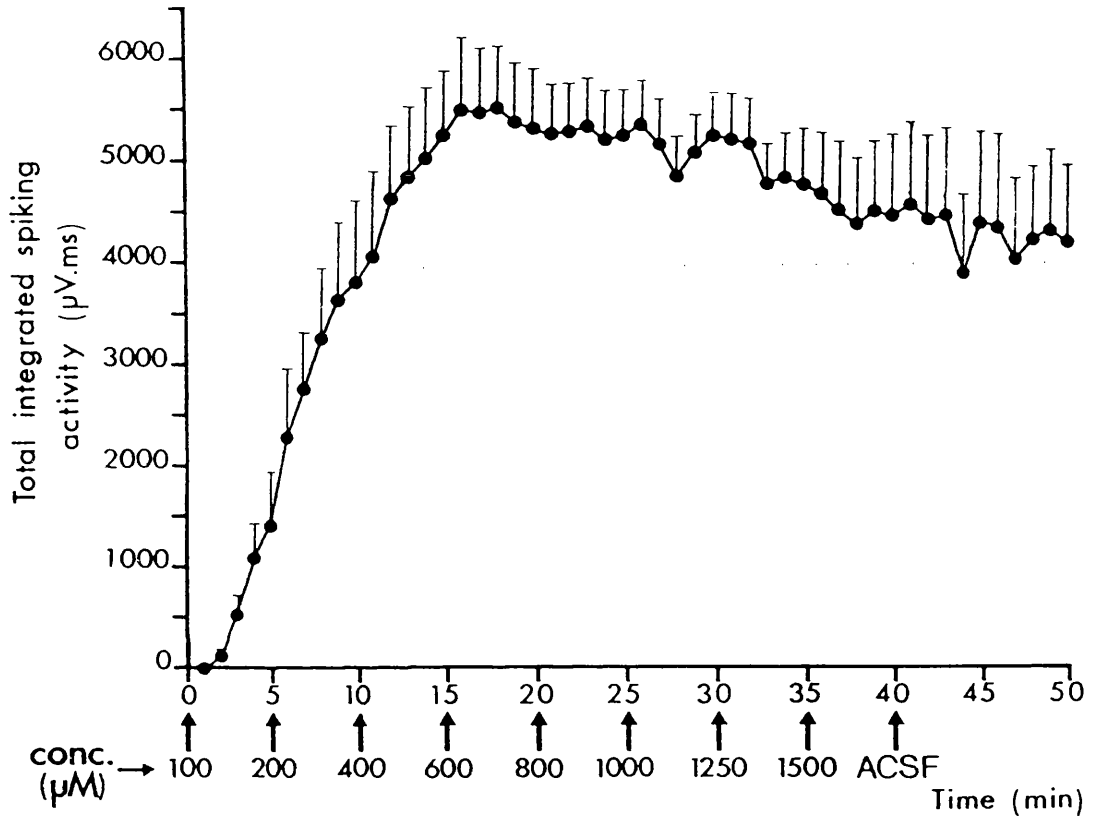

Fig.3.4. Cumulative dose-response curve for BM. Different concentrations of BM were superfused for 5 min each across the exposed cortex through cortical cups. A threshold, equivalent to 0.1mV above the basal activity was set and the total integrated spiking activity representing the total area under the voltage profile of all spikes above this level was integrated and displayed digitally as counts (μV.ms) per min. The values represented are mean $\pm$ s.e. (n=4).

From these experiments and the information gained from our early pilot studies concerning the pattern of spiking for each individual concentration it was decided to choose a sub-maximal dose of 200μM BM to study the epileptogenic effect of BM further.

At this concentration spiking started within 1-3 min of exposure and continued to increase in frequency and size even after the superfusion of BM had been stopped. It reached a peak at approximately 10 min but had subsided within 25-30 min (Fig.3.5). The time course of changes in the frequency and the size of the spikes during the course of epileptogenic activity produced by 200μM BM are shown in Fig.3.6.

The BM-induced spiking appeared to be Ca^{++}-dependent as the spiking became smaller and irregular with gaps appearing in the time-course of activity on removal of Ca^{++} (Fig.3.7).

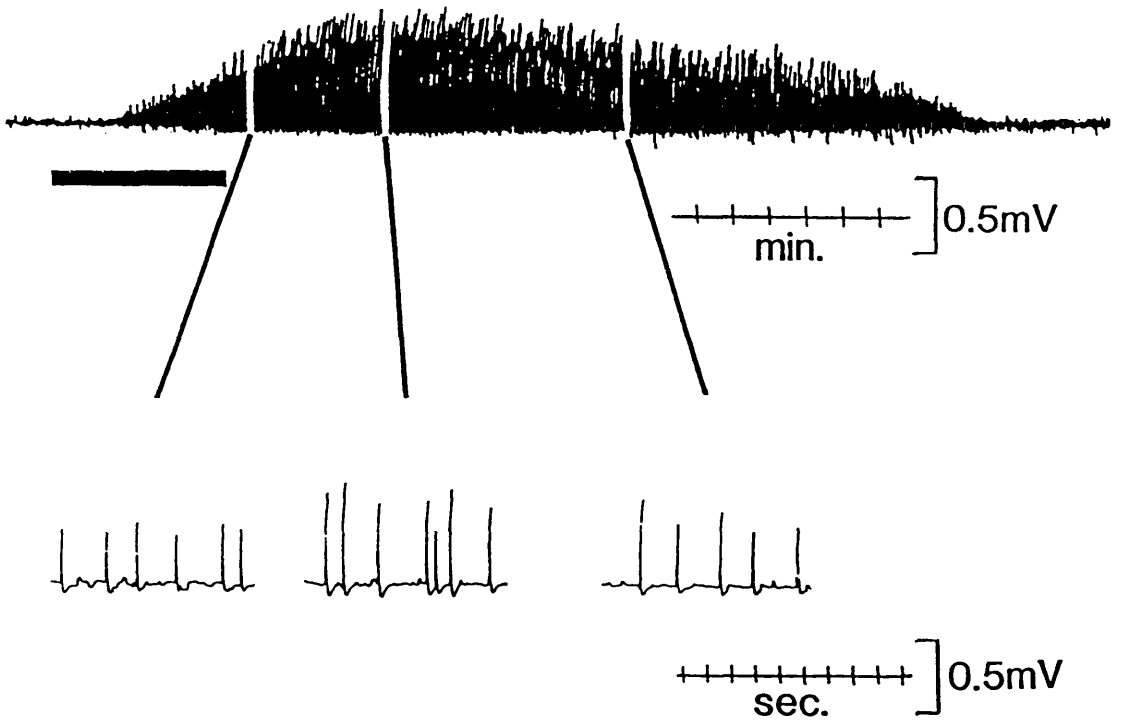

Fig.3.5. The qualitative time-course of epileptogenic spiking induced by 5 min superfusion of 200μM BM (black bar) across the exposed parietal cortex of the urethane-anaesthetised rat. The trace has been speeded up at three points (6, 10 and 17 min after BM exposure) to show individual interictal spikes.

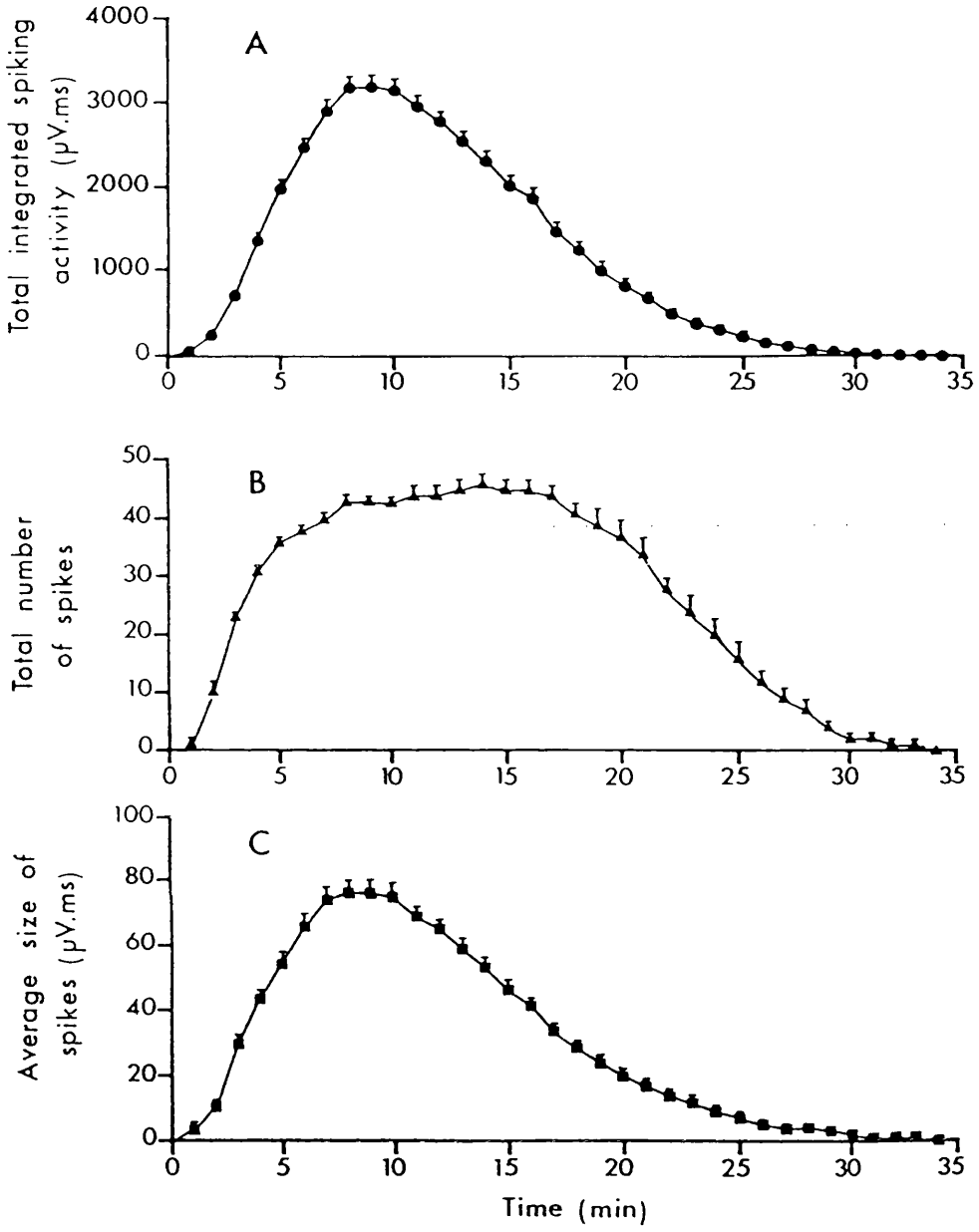

Fig.3.6. The quantitative time-course of spiking activity caused by 5 min cortical superfusion of 200μM BM across exposed parietal cortex through cortical cups in urethane-anaesthetised rats. A represents the changes in the total integrated spiking activity at minute intervals; B shows the variations seen in the number of spikes during BM induced spiking activity and C represents the changes in the size of the spikes during the time-course of such an activity. The values represented are mean ± s.e. (n=60).

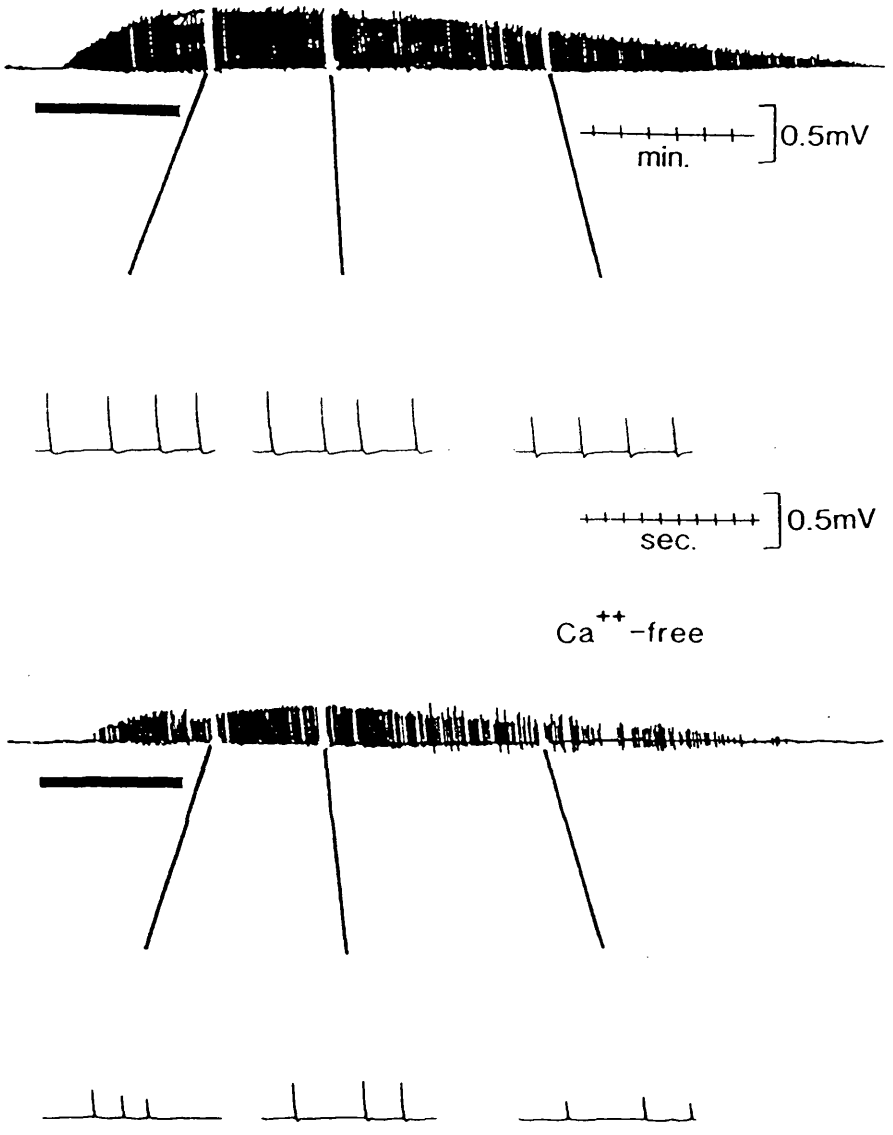

Fig.3.7. The effect of Ca^{++} removal on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification in Ca^{++}-free medium. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

3:2:4: Picrotoxin:

Following the initial 45 min rest period after placement of cortical cup and screw electrodes, different doses of picrotoxin were superfused for 5 min followed by continuous superfusion of ACSF. Picrotoxin lead to the appearance of spikes superimposed on the basal EEG as in the case of BM (Fig.3.8). However, the pattern of activity appeared to be different. The spiking started after a relatively long delay and the recovery from the activity was slower than BM. As in the case of BM a cumulative dose-response curve was constructed as shown in Fig.3.9. According to information gained from this cumulative dose-response curve and the knowledge gained concerning the pattern of picrotoxin-induced spiking during our single-dose studies a sub-maximal concentration of 300μM picrotoxin was chosen to study the effect further.

The differences in the time-course of spiking activity between BM and picrotoxin can be readily seen by comparing Fig.s 3.6 and 3.10. The BM-induced activity starts almost immediately; it reaches a maximum $\simeq$ 5 min after the end of BM superfusion with the activity subsiding after $\simeq$ 30 min whereas in the case of picrotoxin the onset of activity is 4 to 5 min; the peak of epileptogenic spiking is not reached until at least 10 min after the end of picrotoxin superfusion and the spiking activity lasts for 50 min.

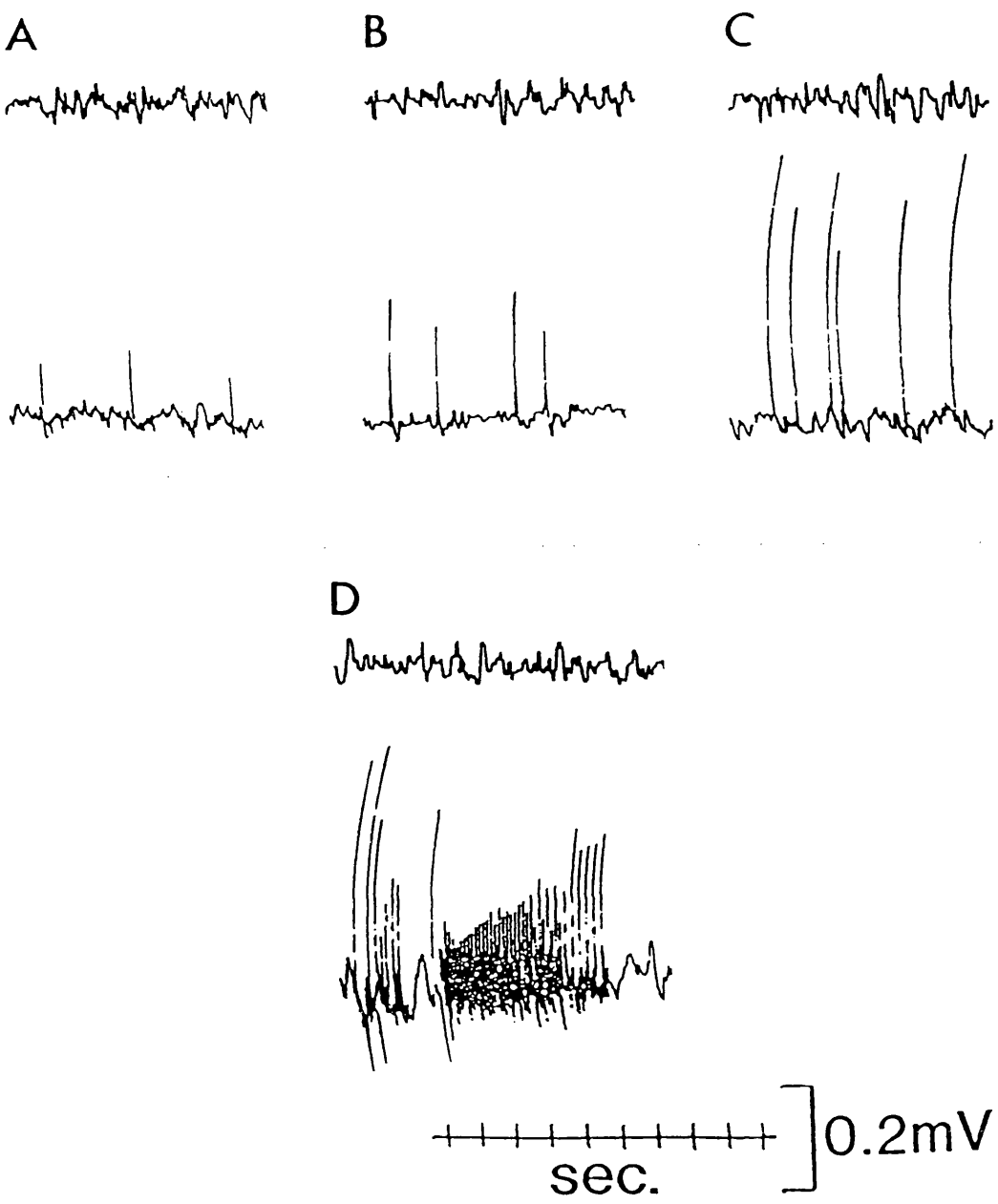

Fig.3.8. The effect of cortical superfusion of picrotoxin on the basal EEG. In each case the top trace represents the basal EEG recorded by screw electrodes from the contralateral cortex and the bottom trace shows the spiking activity seen during peak activity as a result of 5 min superfusion of different concentrations of picrotoxin. A, B, C and D are 75, 150, 300 and 1500μM picrotoxin, respectively.

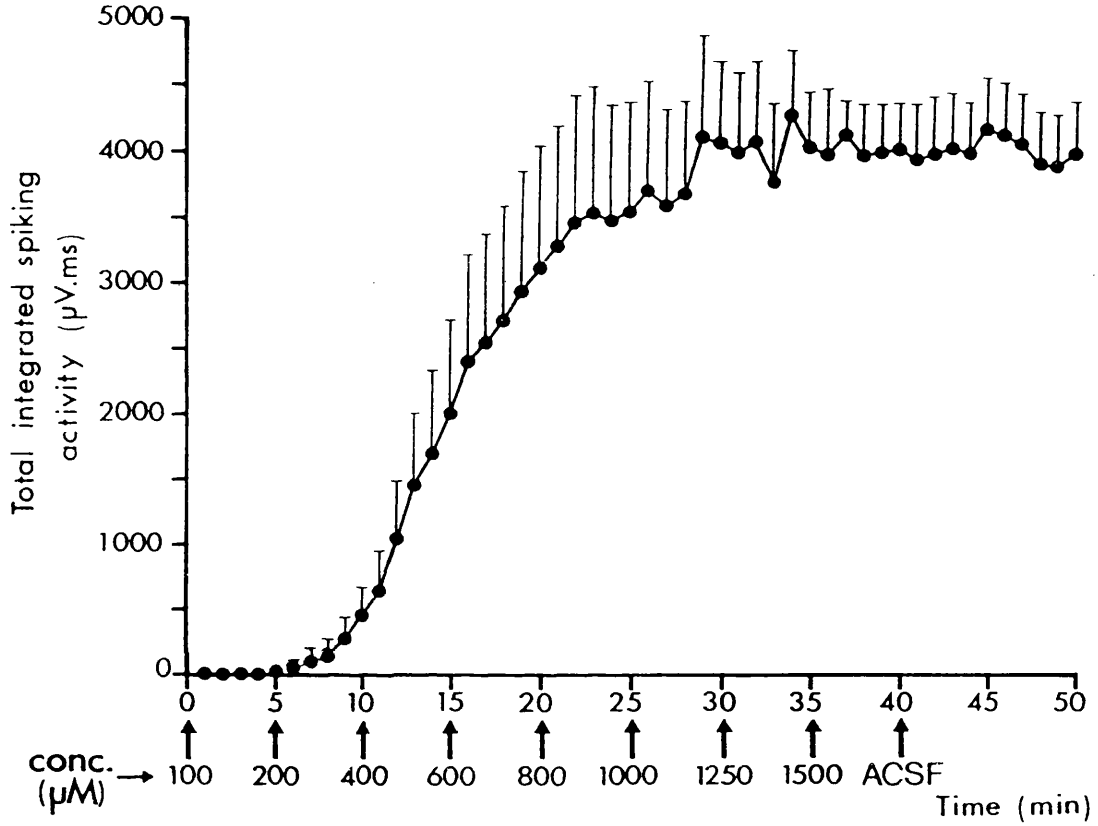

Fig.3.9. Cumulative dose-response curve for picrotoxin. Different concentrations of picrotoxin (as denoted on the graph) were superfused for 5 min each across the exposed cortex through cortical cups. A threshold, equivalent to 0.1mV above the basal activity was set and the total integrated spiking activity representing the total area under the voltage profile of all spikes above this level was integrated and displayed digitally as counts per min. The values represented are mean ± s.e. (n=5).

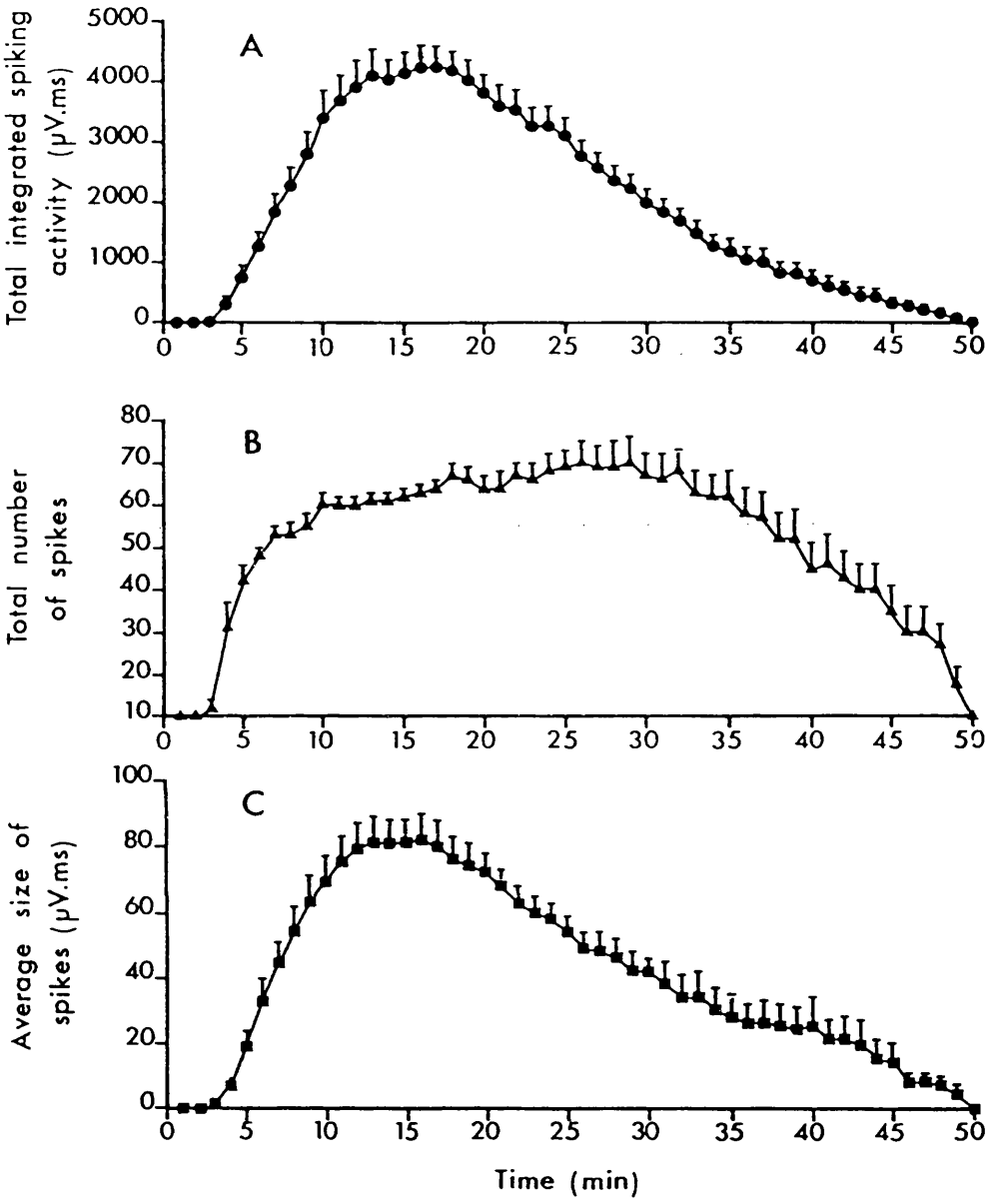

Fig.3.10. The quantitative time-course of spiking activity caused by 5 min cortical superfusion of 300μM picrotoxin across exposed parietal cortex through cortical cups in urethane-anaesthetised rats. A represents the changes in the total integrated spiking activity at minute intervals; B shows the variations seen in the number of spikes during BM induced spiking activity and C represents the changes in the size of the spikes during the time-course of such an activity. The values represented are mean ± s.e. (n=12).

3:2:5: Cellular origin of EEG spiking:

Extracellular neuronal recording was performed as outlined in the methods chapter (chapter 2, section 2:3:2). This showed that each EEG spike, induced by 200μM BM superfused across the cortex, coincided with a sudden, synchronous discharge of a group of cortical cells approximately 300μm below the surface which corresponds to layers II-III in the rat (Paxinos & Watson, 1982). The frequency of discharge and the number of neurones taking part in the synchronous firing were directly related to the size of the EEG spikes (Fig.3.11).

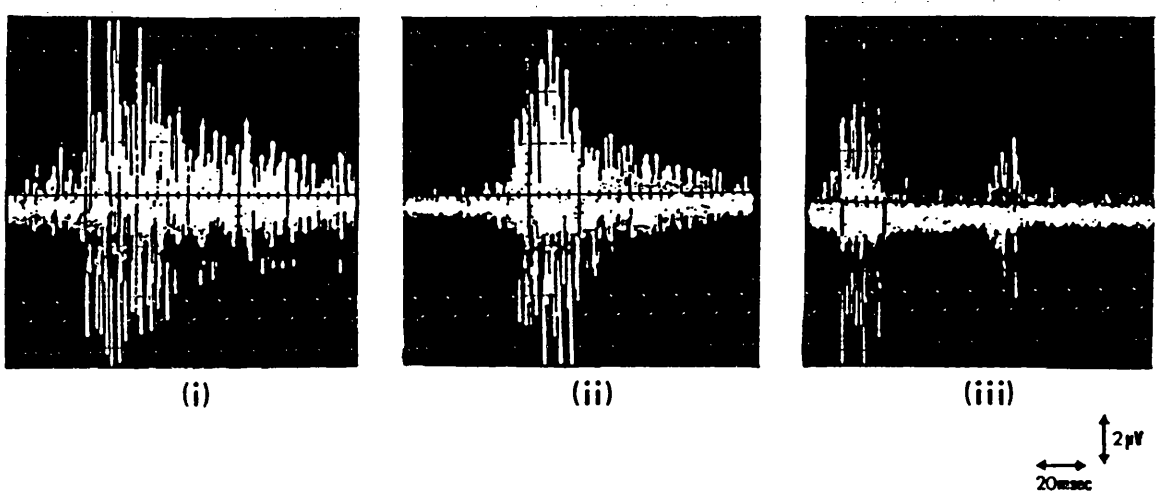

Fig.3.11. The cellular origin of EEG spikes. The photographs illustrate the discharge of a group of superficial cortical neurones (layers I-III) during EEG spiking, recorded with a glass-coated tungsten microelectrode. The burst firing in (i) correspond with a large EEG spike and that in (ii) and (iii) with medium and small spikes, respectively

3:3: DISCUSSION:

3:3:1: High K^+:

Application of the 60mM K^+ solution is thought to lead to depolarization of both cortical neurones and glia. However, since the alterations in the EEG induced by high K^+ in these studies were Ca^{++}-dependent the effect could result from a reduced release of neurotransmitters from a neuronal rather than glial source, since release from the latter is not Ca^{++}-dependent. The immediate increase in the amplitude of the basal EEG signifies a degree of synchronization in the firing of the neurones which has also been shown by Moroni, Corradetti, Casamenti, Moneti & Pepeu, 1981. This is then followed by a deep depression of activity. This spreading depression has also been reported by other investigators (Clark & Collins, 1976). No spiking like that obtained with the convulsants (see later) was seen.

3:3:2: Pentylenetetrazole (PTZ):

The spiking seen after cortical superfusion of PTZ provided a quantifiable means of measuring the epileptogenic activity since the spikes were mono-phasic and could be easily counted.

The main disadvantage with PTZ, however, was the high concentration which had to be used. One of the reasons for needing such a high concentration could be the dampening effect of the anaesthetic used since even an intravenous infusion of PTZ required much larger dose of PTZ than expected. To reach full body convulsion a total of at least 300mg/Kg PTZ had to be injected in our experiments which is much higher than the threshold convulsive dose of PTZ in rats (Marcucci, Airoldi, Mussini & Garattini, 1971).

3:3:3: Bicuculline methiodide (BM):

Superfusion of BM across the parietal cortex of the urethane-anaesthetised rat led to the appearance of distinctive spikes in the EEG the number and size of which were related to dose (Fig.3.3). Although the corpus callosum is intact in this *in vivo* preparation the spiking activity did not spread to the contralateral cortex. Thus it appears that this local spiking activity is confined to the cortical cells beneath the cup and does not spread to other parts of the cortex. This focal epileptogenic spiking which resides only in the cortex produces a direct experimental model for studying cortically initiated partial or focal seizures as well as being important in studying seizures which are generalized at onset, some of which have been thought to also originate from cortex (Connors & Gutnick, 1984). The cumulative dose-response studies enabled us to choose a sub-maximal dose of BM to study the BM-induced spiking further. Another interesting observation during these cumulative studies was the absence of any high frequency (8Hz) spiking as seen after single application of high doses of BM (Fig.3.3D). One possible explanation for such an effect could be the secretion of a 'natural' anticonvulsant which is progressively built up as the cortex comes into contact with lower concentrations of BM as in the cumulative dose-response studies. When the cortex is, however, suddenly subjected to an acute high dose of BM as in our single application studies there is no time for sufficient amount of the 'natural' anticonvulsant to be secreted and high frequency (8Hz) spiking is seen. Two possible candidates for such 'natural' anticonvulsant could be adenosine (Franklin, Tripp, Zhang, Gale & Murray, 1988; Maitre, Ciesielski, Lehmann, Kempf & Mandel, 1974 and Dragunow, 1986) or taurine (Joseph & Emson, 1976; Durelli, Mutani, Delsedime, Quattrocolo, Buffa, Mazzarino & Fumero, 1976 and Huxtable, 1982).

Although the concentration of BM (200μM) used in these experiments to induce spiking is in excess of that employed as a $GABA_A$ antagonist in *in vitro* systems (pA_2=5.93, Simmonds, 1980) it is important to realize that in our preparation not only is there some initial dilution of the BM solution in the cup, because it is only applied for 5 min of a continuing superfusion of normal ACSF, but also BM has to diffuse down to the deeper layers of the cortex to reach $GABA_A$ receptors and so initiate its effect. Therefore, although a relatively high concentration of BM is applied it is likely that very little of it reaches the site of action where the concentration could be nearer to the pA2 (i.e. μM concentrations). The spiking activity in our system started within 1-3 min of bicuculline exposure and it reached a peak of 46 $\pm$ 2 (n=60) spikes per min which agree favourably with other similar *in vivo* studies (Matsumoto & Ajmone Marsan, 1964; Prince, 1965; Elger & Speckmann, 1980 and Elger, Speckmann, Prohaska & Caspers, 1981). However, in *in vitro* experiments spontaneous burst firing can only be observed after 10-20 minutes and it only occurs at a rate of less than 3 per min (Horne, Harrison, Turner & Simmonds, 1986 and Jones, 1988). Such differences could stem from the fact that in an *in vitro* situation the synaptic connections are damaged and so it is not as easy to recruit cells to fire in unison and produce interictal spikes. Whilst BM was not used in our experiments under equilibrium conditions that permit the precise concentration at its site of action to be determined its effects were dose-related and the response to the sub-maximal test concentration chosen (200μM) was reversible and reproducible for at least 4 applications after 1 hour rest periods and was modified by drugs (as seen later). It thus provides a good test model on which to investigate the effects of drugs that modify amino acid neurotransmission.

3:3:4: Picrotoxin:

The spiking seen after cortical superfusion of picrotoxin was very similar to that seen with BM. The main difference in the pattern of activity was that there was a greater delay in time to spiking after picrotoxin than BM. Following cortical superfusion of 300μM picrotoxin there was a gap of 4 min before any spiking activity could be seen whereas the spiking in the case of BM was almost immediate (Fig.s 3.6 & 3.10). This difference in the onset of activity could result from the different modes of action of these two GABA blockers. It would be more difficult for a channel blocker such as picrotoxin to bring about its disinhibitory effect as it relies on the GABA receptor to be activated first and the channel opened before it can block the activity whereas a receptor antagonist such as BM can 'sit' on the receptor and block the activity regardless of whether the receptor is already activated or not. It is also possible that picrotoxin takes a longer time to diffuse down and reach the site of spike initiation.

3:3:5: Cellular origin of spikes:

Extracellular recording showed that the spikes induced by cortical superfusion of 200μM BM coincided with a burst of firing in cortical neurones monitored in layers II-III. Such spikes could be considered as 'interictal spikes', which are believed to result from the abortive, restricted paroxysmal discharge of a number of neurones (Matsumoto & Ajmore Marsan, 1964 ; Prince, 1978 and Fenwick, 1983). This type of chemically-induced epileptogenic activity originating in the cortex has been extensively studied in an attempt to elucidate their cellular mechanisms. These spikes are most likely to originate from neurones in layer IV and the upper parts of layer V that generate intrinsic, all-or-none burst potentials when depolarized with current

pulses or by synaptic activation (Connors & Gutnick, 1984 and Ebersole & Chatt, 1986). They thus act as 'pace makers' projecting paroxysmal synaptic excitation radially onto the neurones of deeper and superficial layers, such as those mentioned above (Connors, 1984 and Connors, Gutnick & Prince, 1982) eventually leading to spikes in the EEG. Binding studies show a high density of $GABA_A$ sites in layer IV and upper layer of layer V (Olsen, Snowhill & Wamsley, 1984) and the distribution of GABA is also shown to be highest at these layers (Emson & Lindvall, 1979). It is, therefore, not surprising that bicuculline, which is a $GABA_A$ receptor antagonist, is thought to initiate its epileptogenic activity here (Connors, 1984 and Campbell & Holmes,1984), where axosomatic GABA-mediated inhibition occurs on deep pyramidal cells.

3:4: CONCLUSIONS:

In view of the above findings it was decided to concentrate on the manipulation of spiking induced by BM rather than picrotoxin or PTZ in order to study the effects of modifying amino acid transmission on the development of epileptogenic spiking. The effect of AP7 was, however, also studied in the case of the other two convulsants for comparison.

3:5: EFFECTS OF VARIOUS DRUGS MODIFYING AMINO ACID TRANSMISSION ON THE CONVULSANT-INDUCED EEG SPIKING:

3:5:1: Bicuculline methiodide-induced spiking:

The EEG spikes induced by BM in the preceeding studies are similar to interictal spikes which are brief epileptiform events providing the simplest experimental system for investigating some of the basic mechanisms of epilepsy (Fenwick, 1983; Prince & Connors, 1986). Bicuculline methiodide was also the most potent and consistent of the convulsants used. The response to 200μM BM showed no tachyphylaxis if it was applied to the cortex a further three times at hourly intervals (Fig.3.12) and formed a sound basis for studying the effects of various drugs on spiking activity. Thus drug modification of spiking could be determined by comparing the level of spiking during the application of BM in the presence of drug with that obtained with its first (control) application. For these reasons it was chosen as the test convulsant for a comprehensive study of the role of a series of compounds modulating amino acid transmission on epileptogenic spiking. All the compounds tested were applied for 30 min before and during the second application and wash out of 200μM BM and none of them appeared to have any effect on the basal EEG (Fig.3.13).

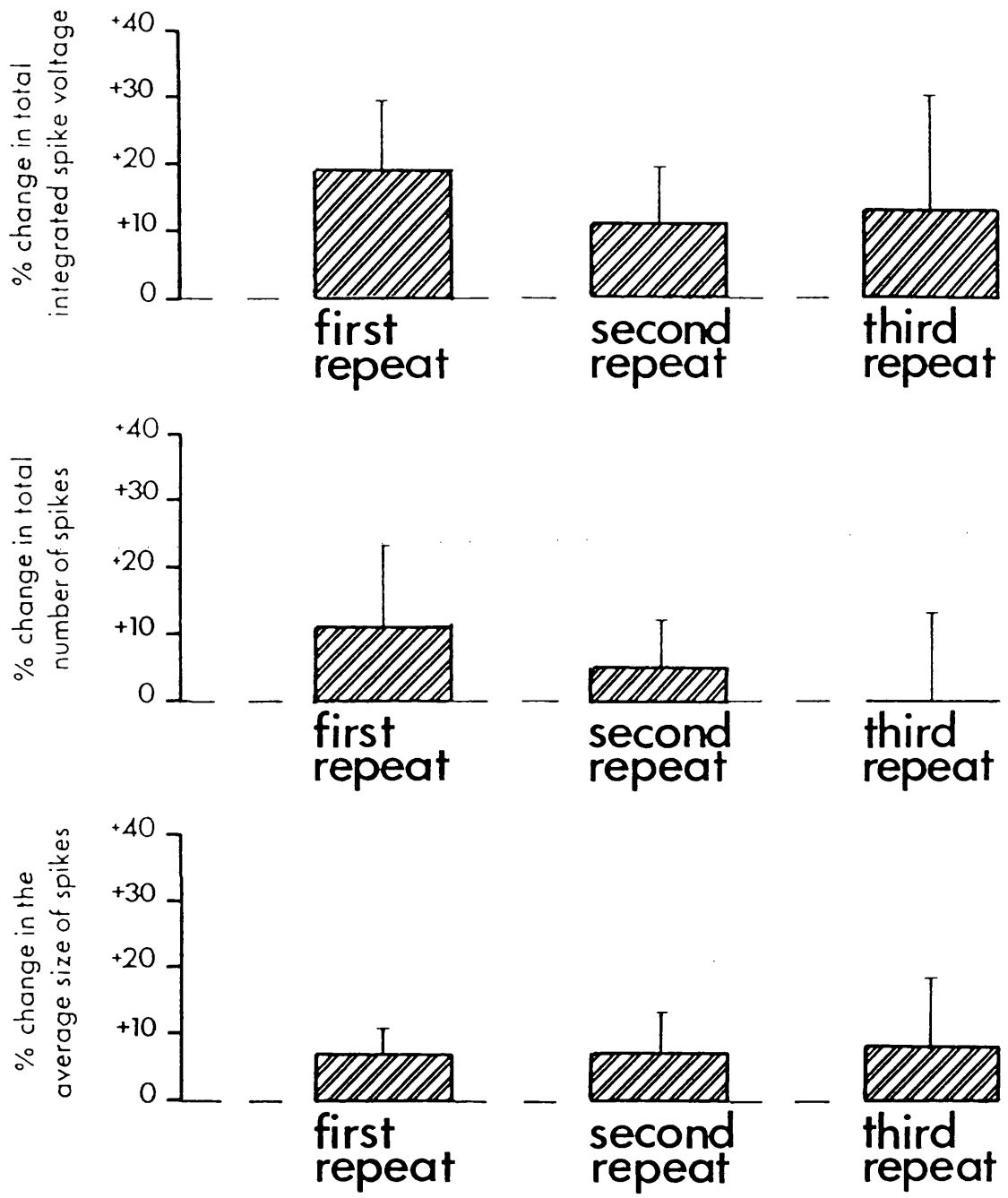

Fig.3.12. The variations in the epileptogenic activity induced by repetitive 5 min cortical superfusion of 200μM BM at hourly intervals, compared to the first application. The top part of the figure shows the mean ($\pm$ s.e., n=5) percentage changes in total integrated spike voltage; the middle part shows the effect on the total number of the spikes and the bottom part the effect on the average size of the spikes.

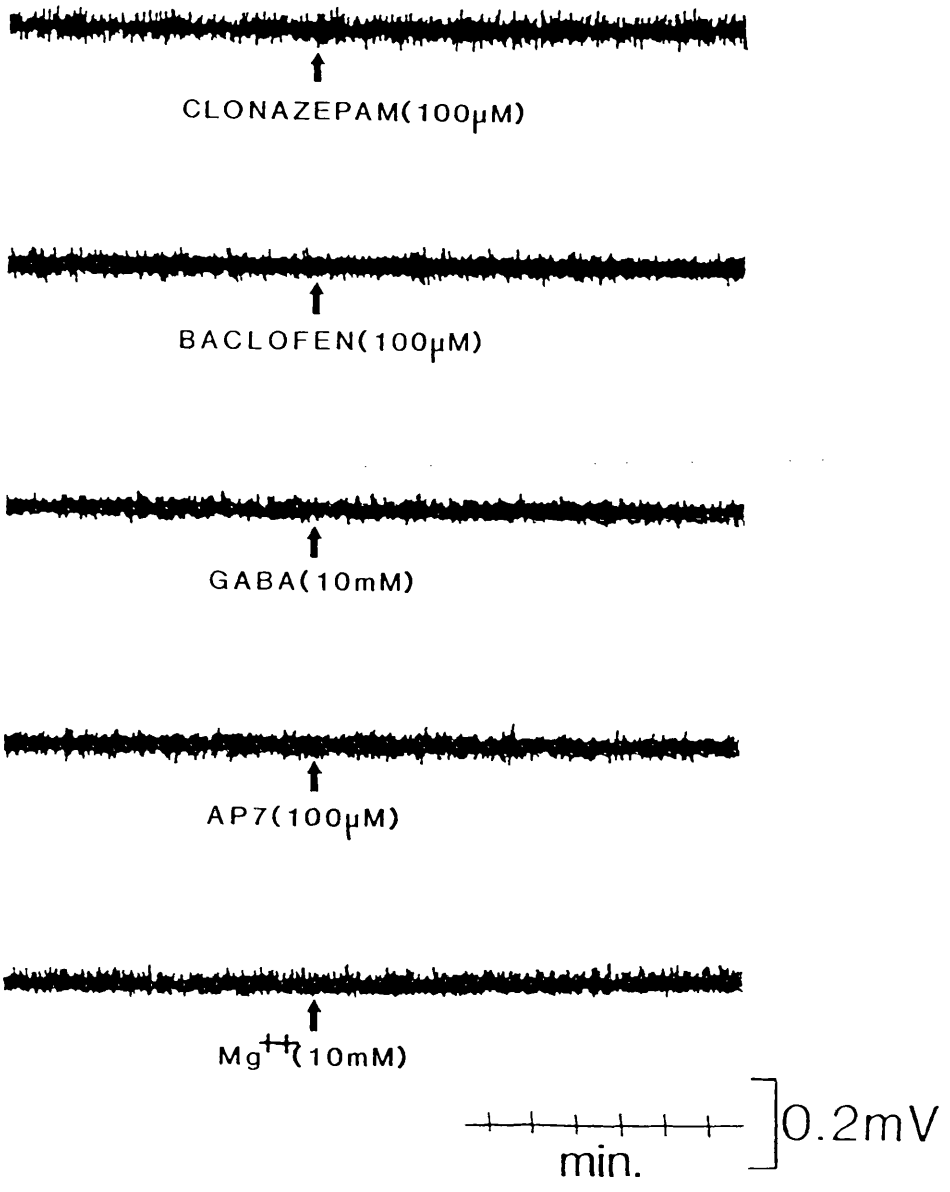

Fig.3.13. The effect of some of the compounds used to modulate amino acid transmission on the basal EEG recorded from the exposed surface of rat parietal cortex using cortical cups. The traces show the basal EEG before and after exposure to these compounds. These were introduced into the system 30 min before any subsequent exposure to 200μM BM and were present throught the spiking activity (see the text for more details).

Compounds increasing inhibitory action of GABA:

The compounds used were muscimol, clonazepam and (±)baclofen as well as GABA itself. The $GABA_A$ receptor agonist muscimol and the benzodiazepine clonazepam were used to increase the $GABA_A$-mediated inhibition and (±)baclofen which is a $GABA_B$ receptor agonist was used as a tool to investigate the involvement of $GABA_B$ receptors in such an activity.

Muscimol (30μM) reduced spiking by mainly affecting the size of the spikes as shown qualitatively in Fig 3.14. However, dose-response studies revealed that at the highest concentration tested (100μM) it also reduced the total number of spikes (Fig.3.15). The benzodiazepine clonazepam (1-100μM) also diminished activity mainly by size reduction and there was no effect on the frequency of spikes even at the highest concentration tested (Fig.s 3.16 & 3.17).

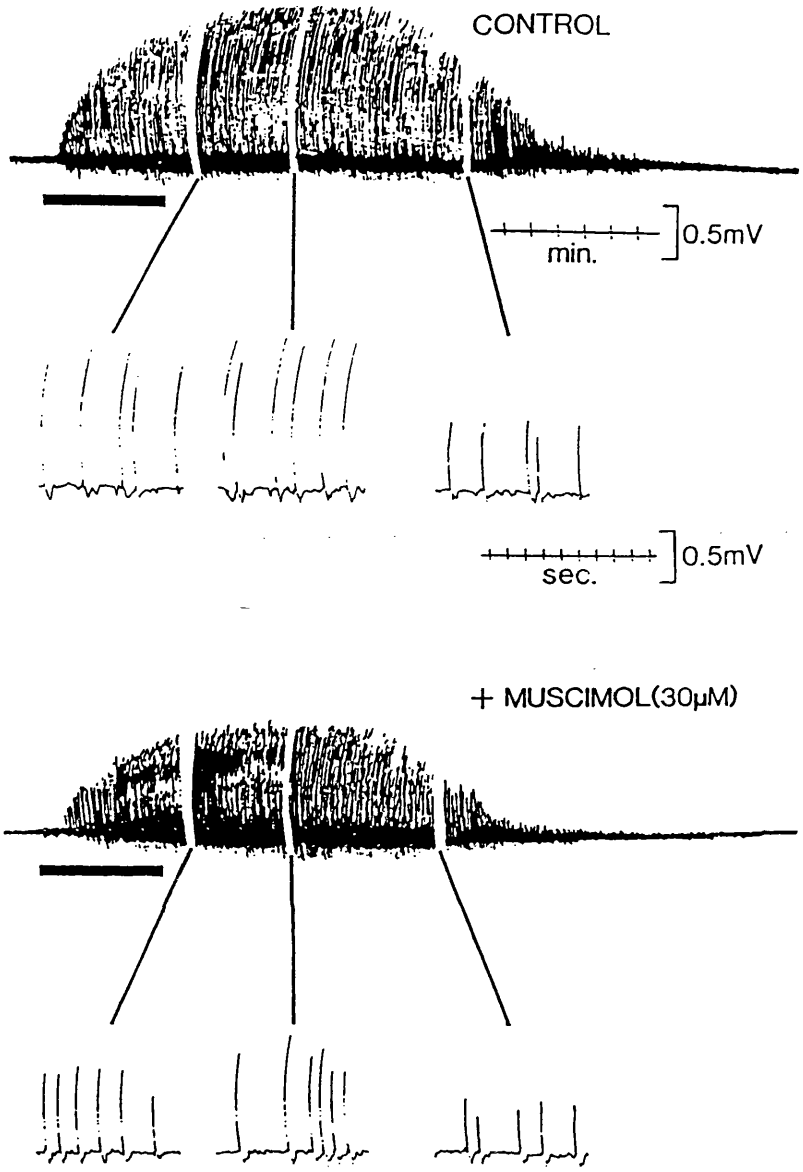

Fig.3.14. The effect of muscimol on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 30μM muscimol superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

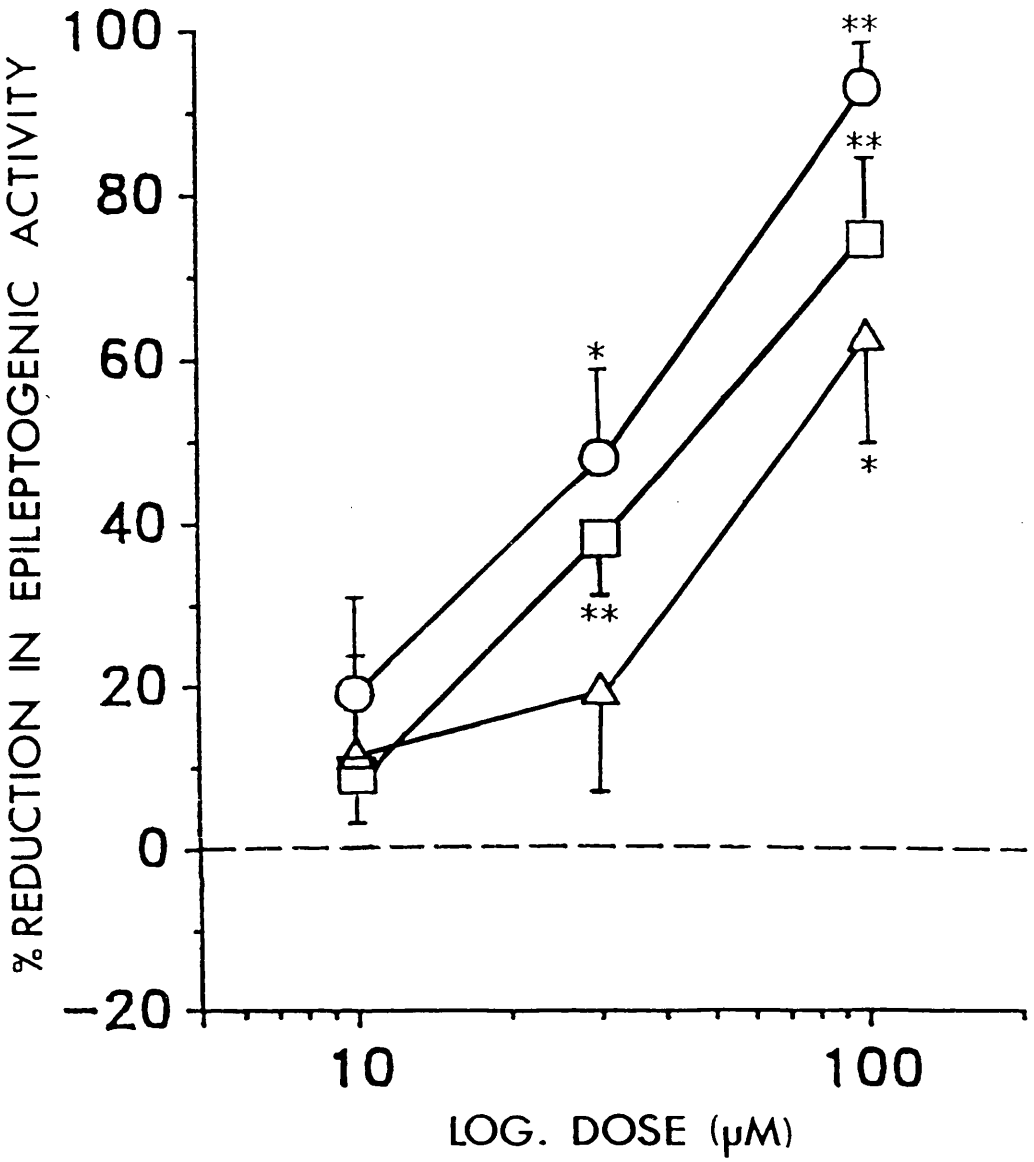

Fig.3.15. The effect of muscimol on BM-induced spiking. The graphs represent percentage reductions in total integrated spike voltage (O—O) as well as reductions in total number of spikes (Δ—Δ) and the average size of the spikes (□—□) for different doses of muscimol. n=5 in all cases except 30μM muscimol where the n-value was 4. *P<0.05 and **P<0.005 show significant difference (paired t-test) compared to the control appilcation of BM (200μM).

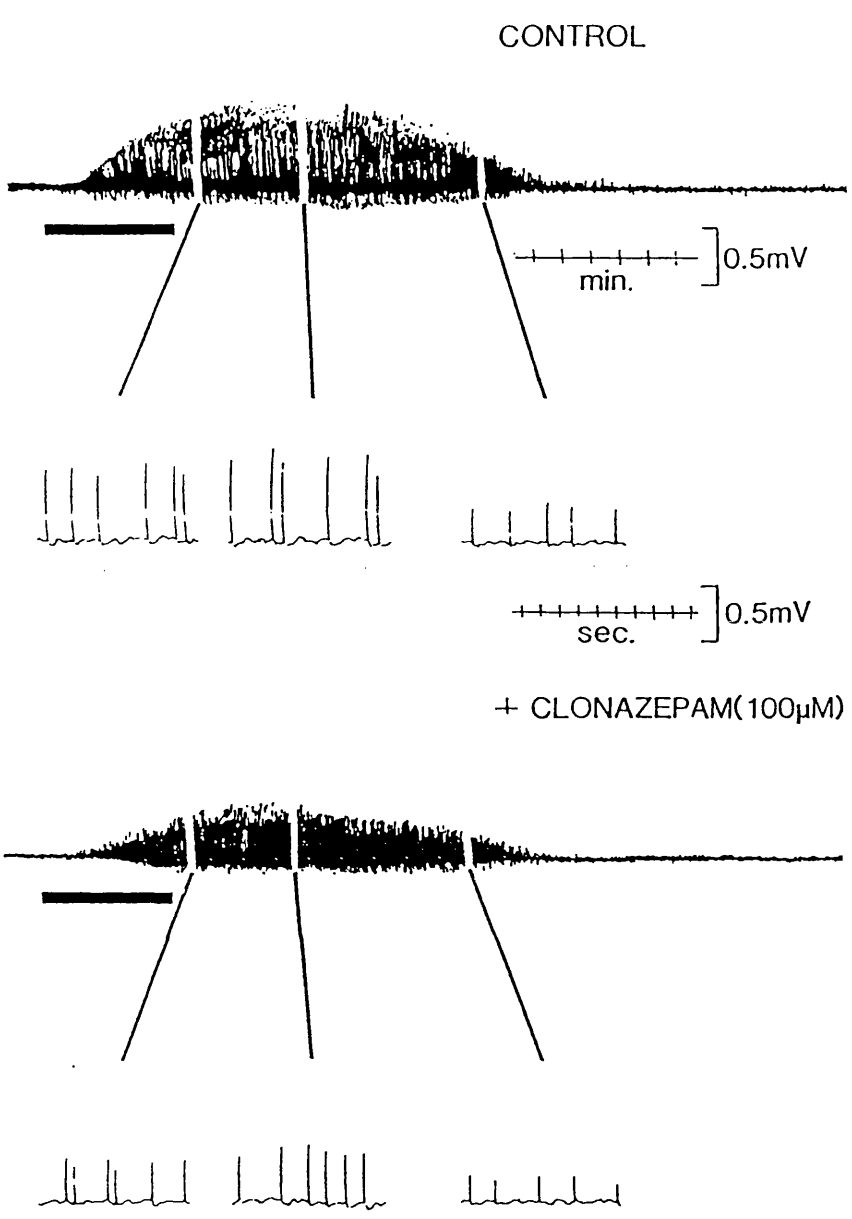

Fig.3.16. The effect of clonazepam on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 100μM clonazepam superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

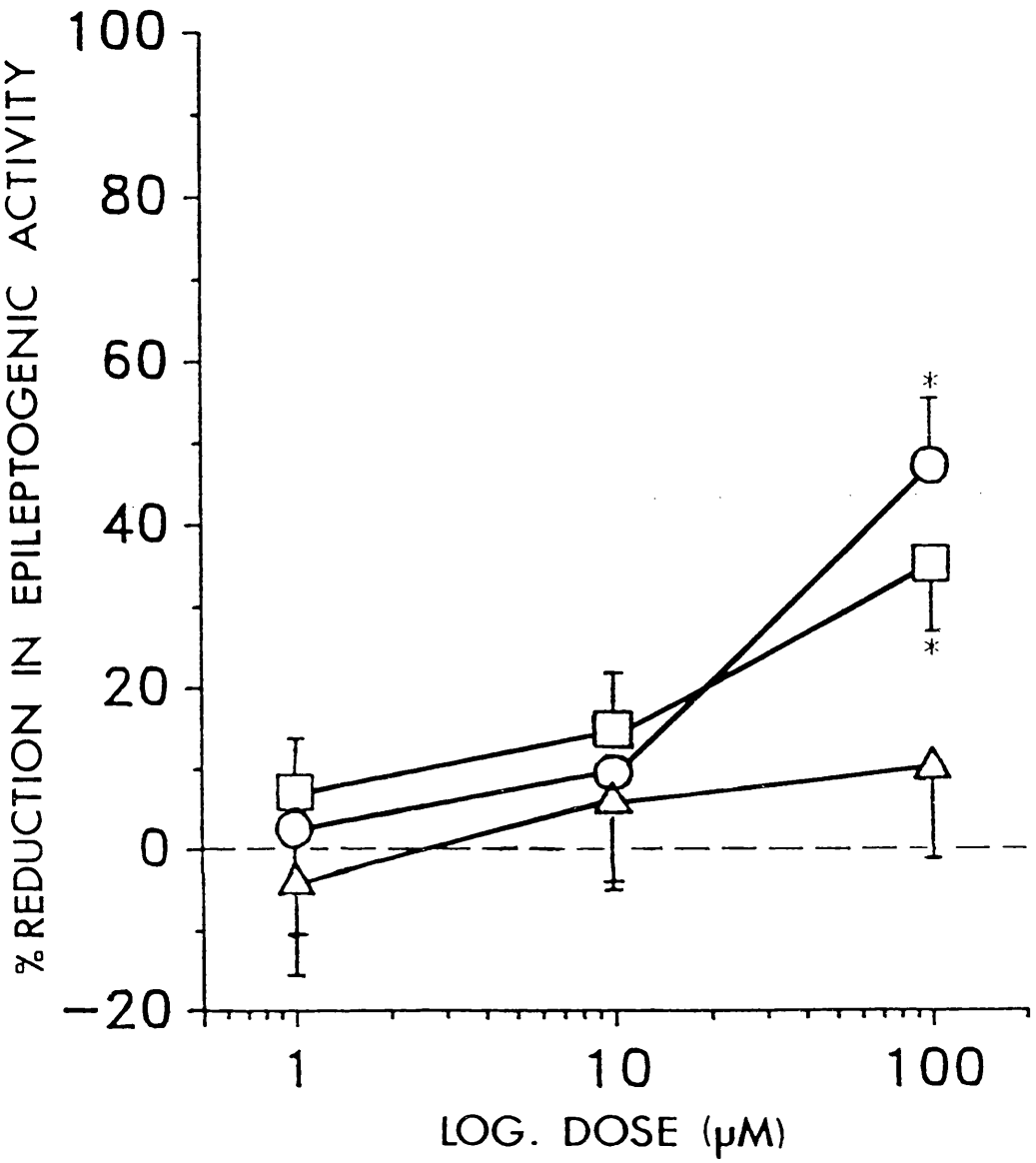

Fig.3.17. The effect of clonazepam on BM-induced spiking. The graphs represent percentage reductions in total integrated spike voltage (O—O) as well as reductions in total number of spikes (Δ—Δ) and the average size of the spikes (□—□) for different doses of clonazepam (n=5). *P<0.05 and **P<0.005 show significant difference (paired t-test) compared to the control application of BM (200μM).

(±)Baclofen decreased spiking by producing distinctive gaps in the spiking pattern as shown in Fig.3.18. It significantly reduced BM-induced spiking at 30 and 100μM but at neither concentration did it reduce the size of the spikes (Fig.3.19). The involvement of $GABA_B$ receptors was further studied by using phaclofen which is a $GABA_B$ receptor antagonist (Kerr, Ong, Prager, Gynther & Curtis, 1987 and Karlsson, Pozza & Olpe, 1988). Phaclofen had no effect on either the basal EEG or BM-induced spiking up to a concentration of 1mM.

GABA itself reduced spiking in a dose-related manner but only at concentrations in excess of 1mM. At 3mM (Fig.3.20) it significantly reduced only the number of the spikes, like baclofen, whereas increasing the concentration to 10mM produced a reduction in the size as well as the number of the spikes (Fig.3.21).

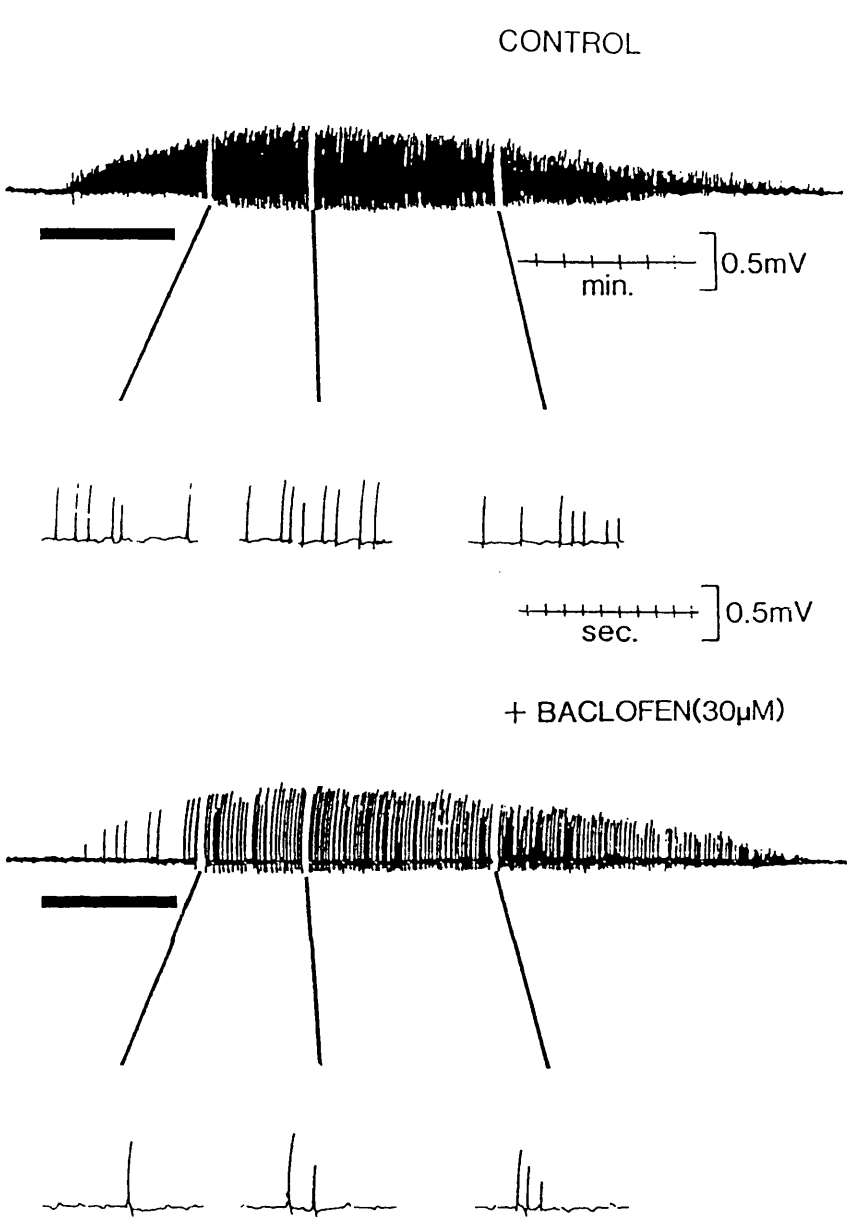

Fig.3.18. The effect of (±)baclofen on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 30μM (±)baclofen superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

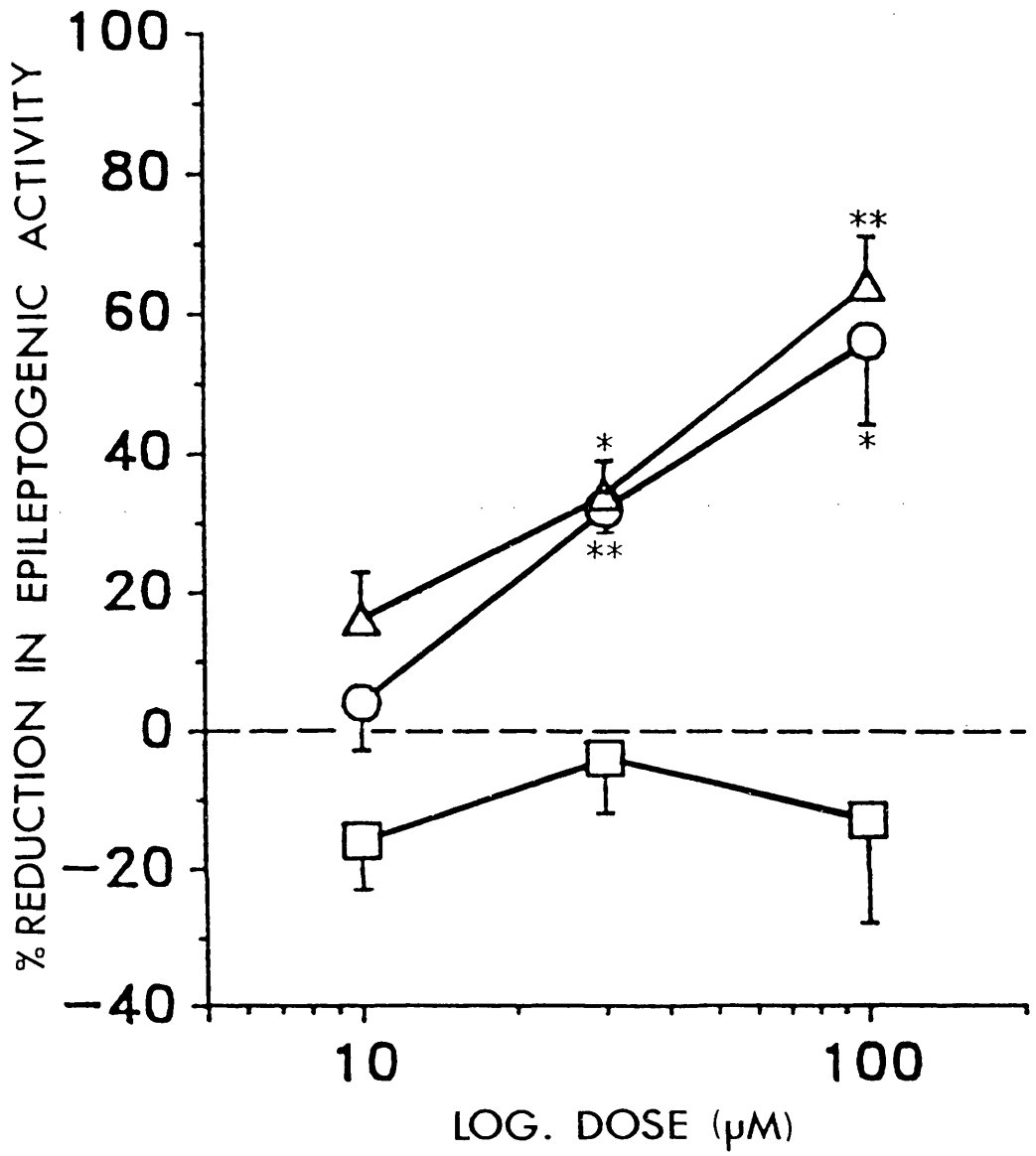

Fig.3.19. The effect of (±)baclofen on BM-induced spiking. The graphs represent percentage reductions in total integrated spike voltage (O——O) as well as reductions in total number of spikes (Δ——Δ) and the average size of the spikes (□——□) for different doses of (±)baclofen (n=4). *P<0.05 and **P<0.005 show significant difference (paired t-test) compared to the control application of BM (200μM).

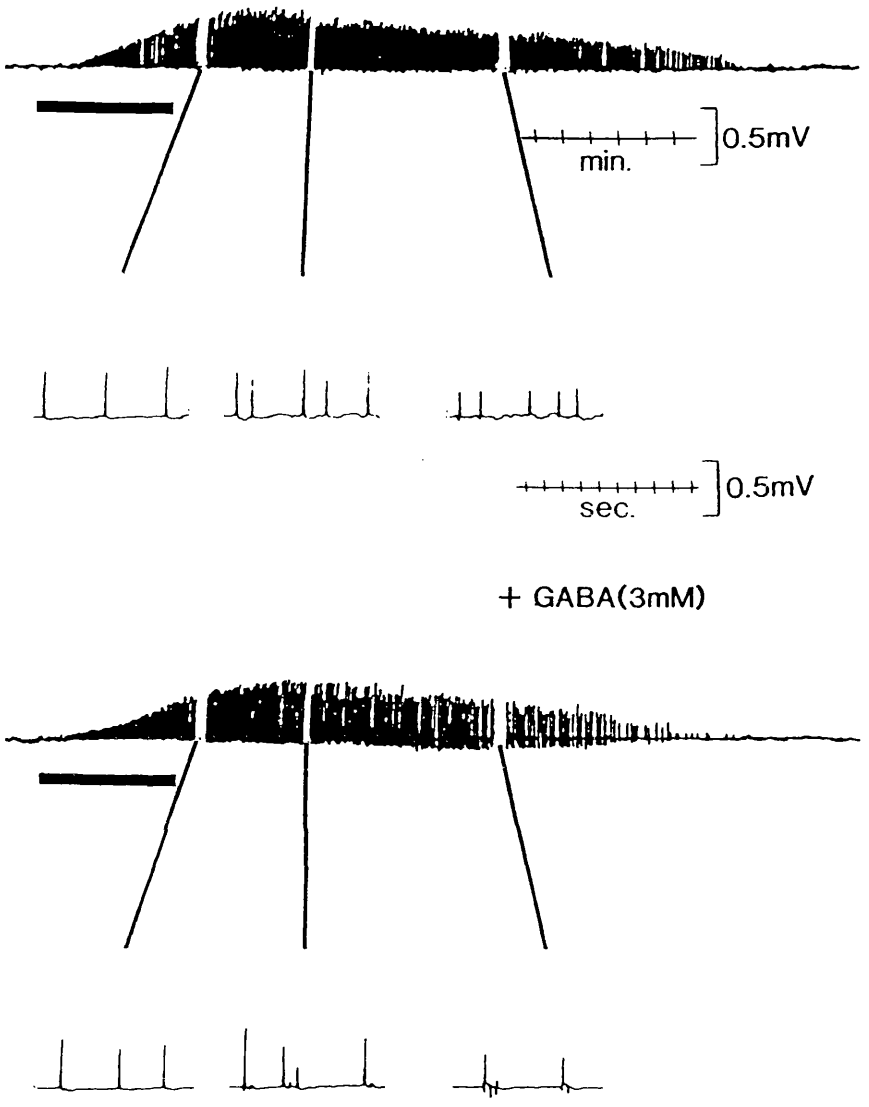

Fig.3.20. The effect of GABA on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 3mM GABA superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

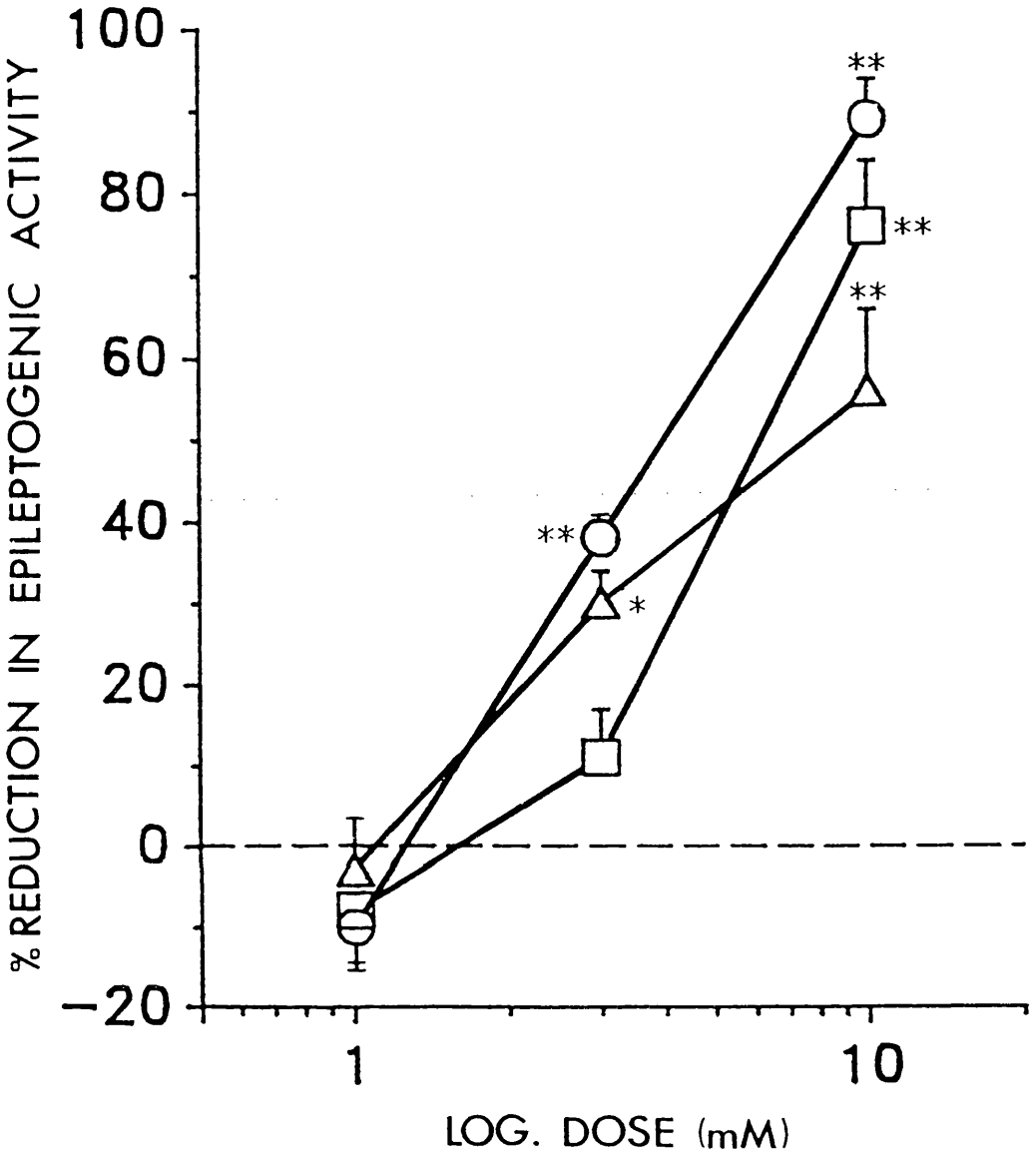

Fig.3.21. The effect of GABA on BM-induced spiking. The graphs represent percentage reductions in total integrated spike voltage (O—O) as well as reductions in total number of spikes (Δ—Δ) and the average size of the spikes (□—□) for different doses of GABA. n=3 for 1mM, 4 for 3mM and 7 for 10mM. *P<0.05 and **P<0.005 show significant difference (paired t-test) compared to the control application of BM (200μM).

Compounds inhibiting excitatory amino acids:

The compounds used were AP7, AP5, MK-801, high Mg^{++}, GAMS, CNQX and kynurenic acid. The NMDA receptor antagonists AP7 and AP5 (1-100μM) both achieved a significant reduction in spiking by decreasing the average size of the spikes with no effect on their frequency (Fig.s 3.22 & 3.24). The dose-response relationship for these two NMDA receptor antagonists against all three parameters studied are shown in Fig.s 3.23 & 3.25. By considering the effect of these two drugs on the total integrated spike voltage it was evident that AP5 had approximately 1/5 of the potency of AP7.

The NMDA channel blocker MK-801 (1-100μM) had a dual effect. Although it reduced the size of the spikes (see the example shown in Fig.3.26) its overall depressant effect on epileptogenic activity was counteracted by a significant increase in the frequency of spikes at high concentrations (Fig.3.27). Increasing the concentration of Mg^{++} from 1 to 10mM in the ACSF had similar effects (Fig.3.28).

NMDA itself at 100μM only increased the size of the spikes (Fig.3.29) whilst application of Mg^{++}-free medium appeared to increase the number of spikes with burst firing being seen during BM-induced epileptogenic spiking activity (Fig.3.30) although it had no effect on the basal EEG, on its own.

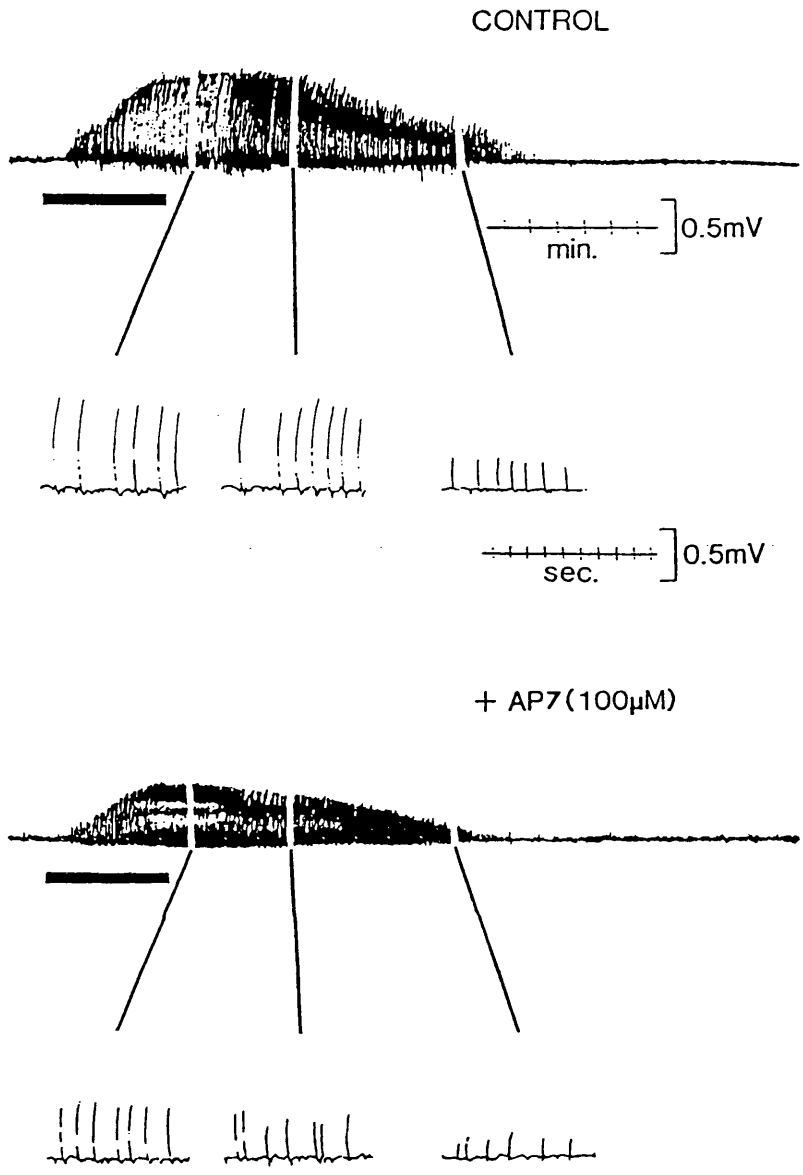

Fig.3.22. The effect of AP7 on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 100μM AP7 superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

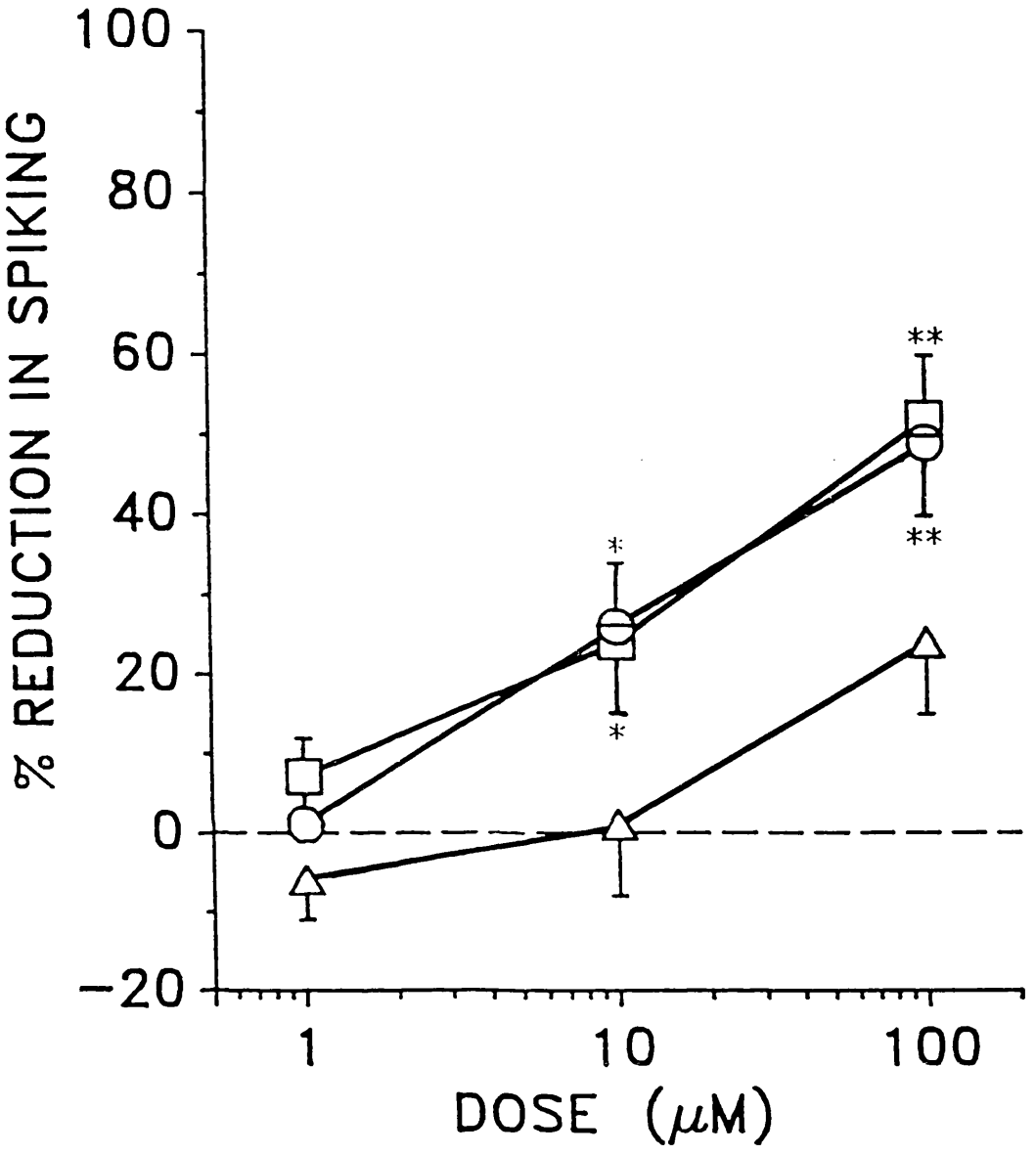

Fig.3.23. The effect of AP7 on the BM-induced spiking. The graphs represent percentage reductions in total integrated spike voltage (O—O) as well as reductions in total number of spikes (Δ—Δ) and the average size of the spikes (□—□) for different doses of AP7 (n=6). *P<0.05 and **P<0.005 show significant difference (paired t-test) compared to the control application of BM (200μM).

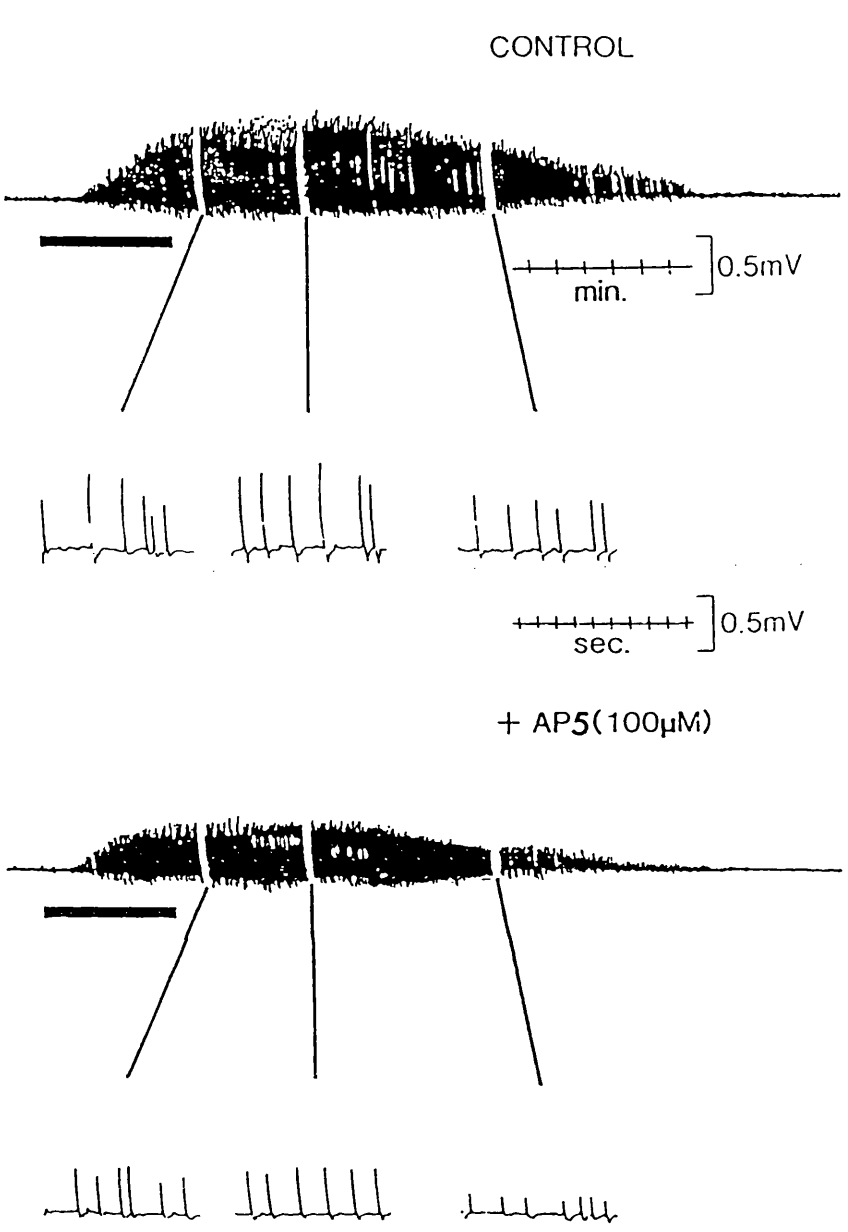

Fig.3.24. The effect of AP5 on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 100μM AP5 superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

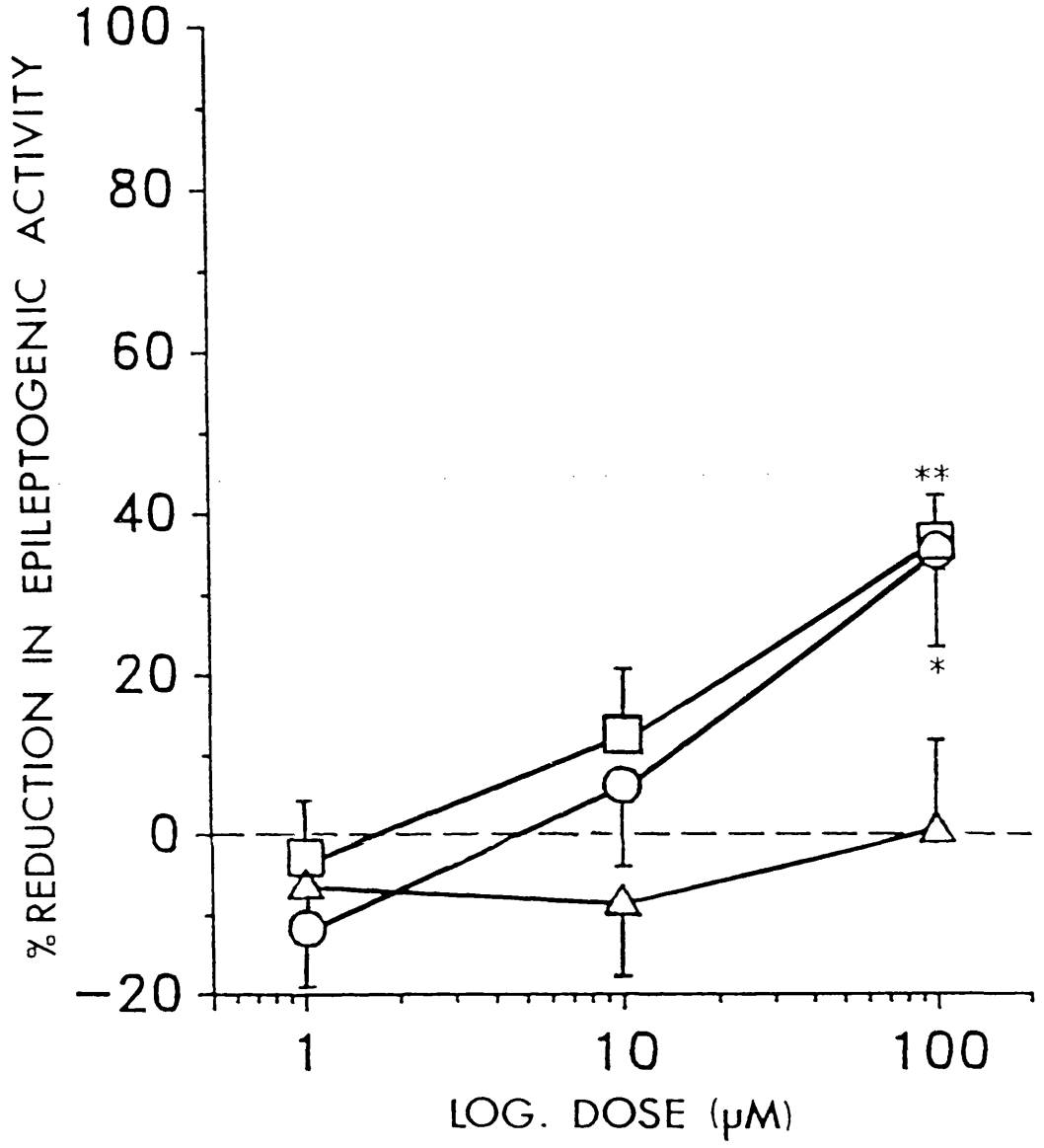

Fig.3.25. The effect of AP5 on the BM-induced spiking. The graphs represent percentage reductions in total integrated spike voltage (O—O) as well as reductions in total number of spikes (Δ—Δ) and the average size of the spikes (□—□) for different doses of AP5 (n=4). *P<0.05 and **P<0.005 show significant difference (paired t-test) compared to the control application of BM (200μM).

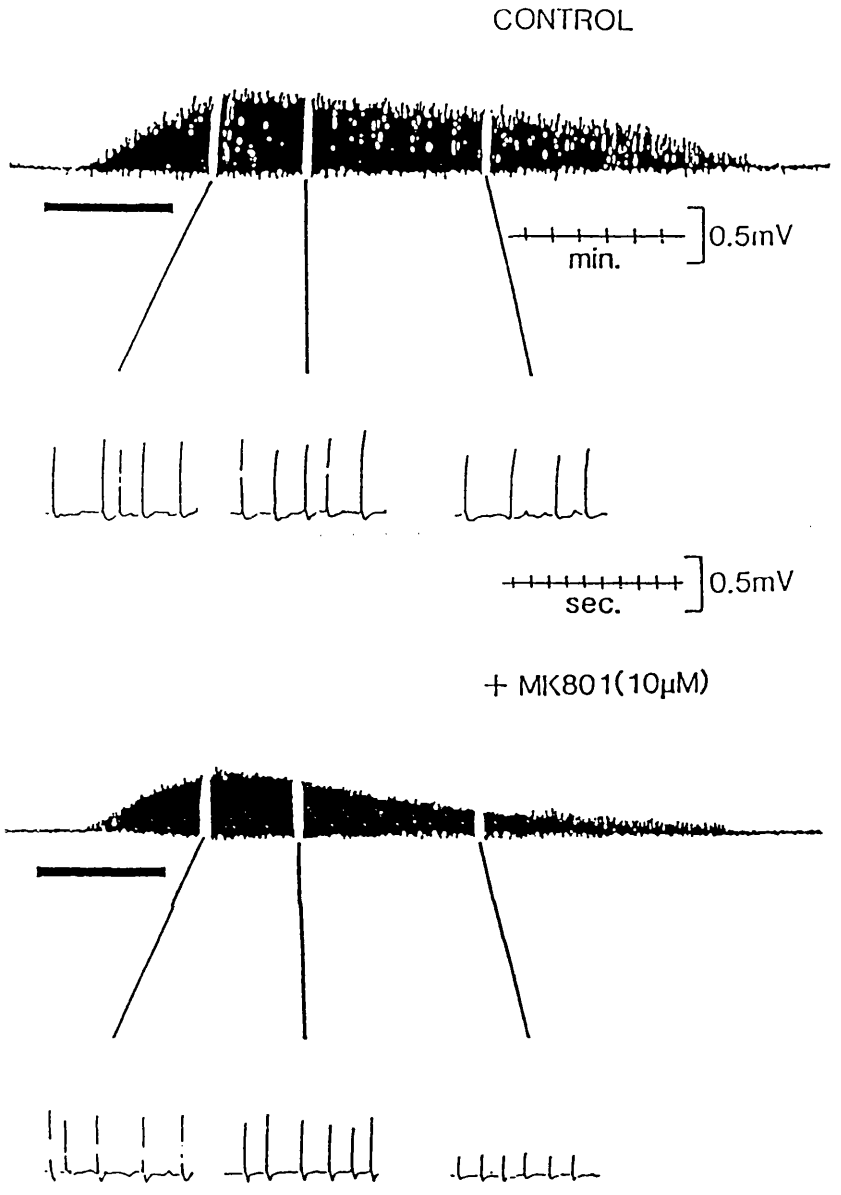

Fig.3.26. The effect of MK-801on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 10μM MK801 superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

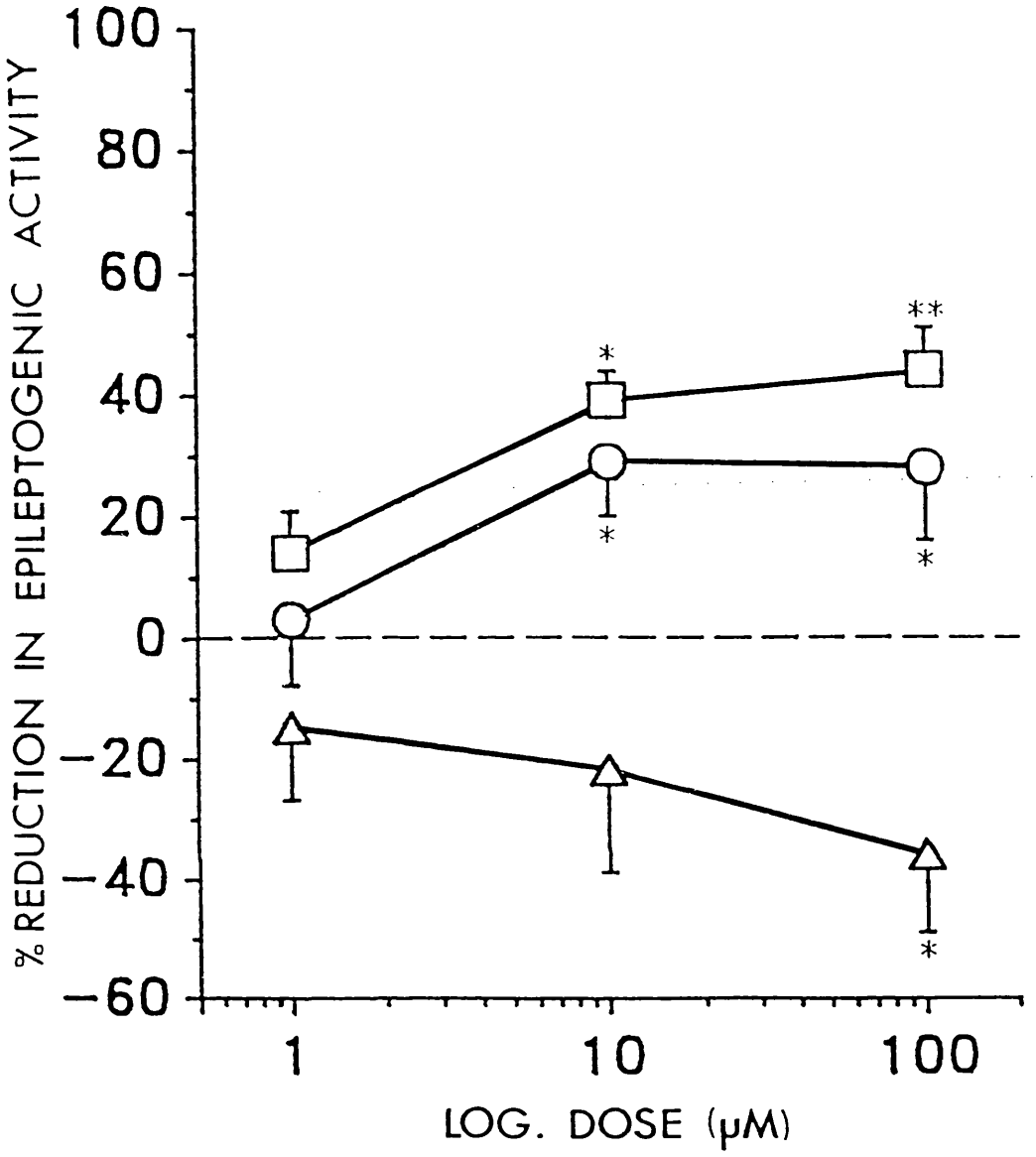

Fig.3.27. The effect of MK-801 on the BM-induced spiking. The graphs represent percentage reductions in total integrated spike voltage (O—O) as well as reductions in total number of spikes (Δ—Δ) and the average size of the spikes (□—□) for different doses of MK801 (n=6). *P<0.05 and **P<0.005 show significant difference (paired t-test) compared to the control application of BM (200μM).

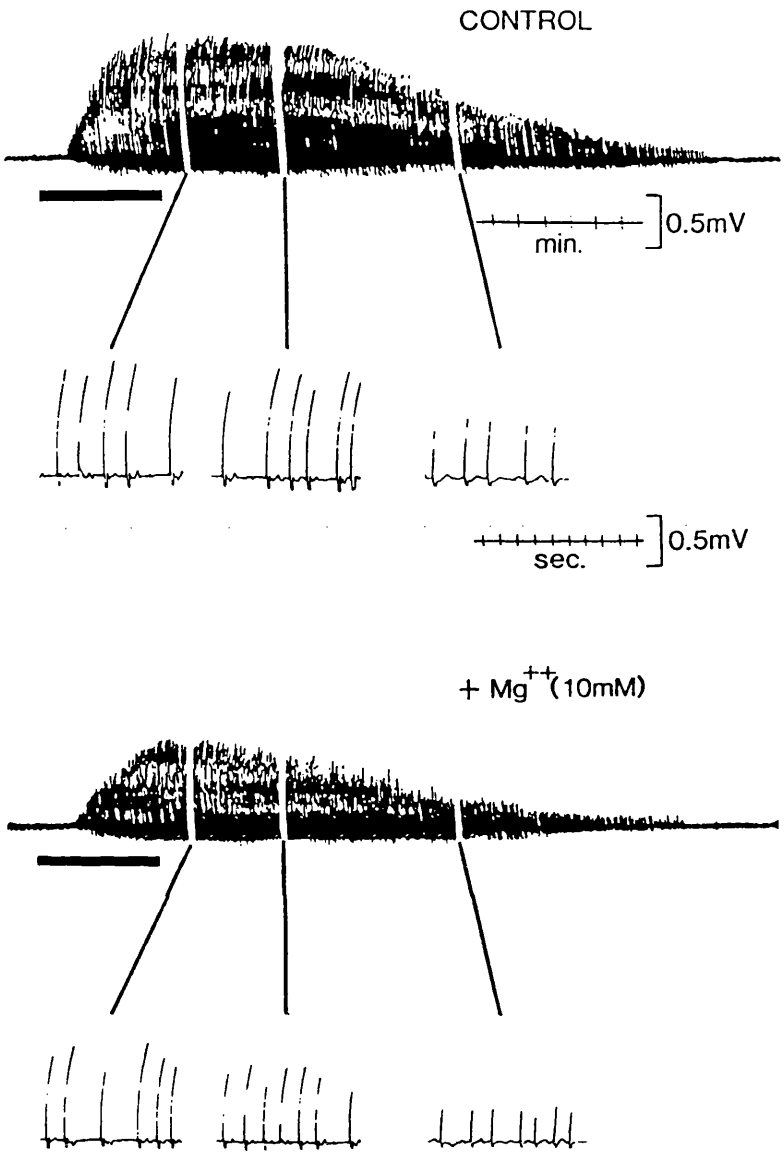

Fig.3.28. The effect of high Mg^{++} on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 10mM Mg^{++} superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

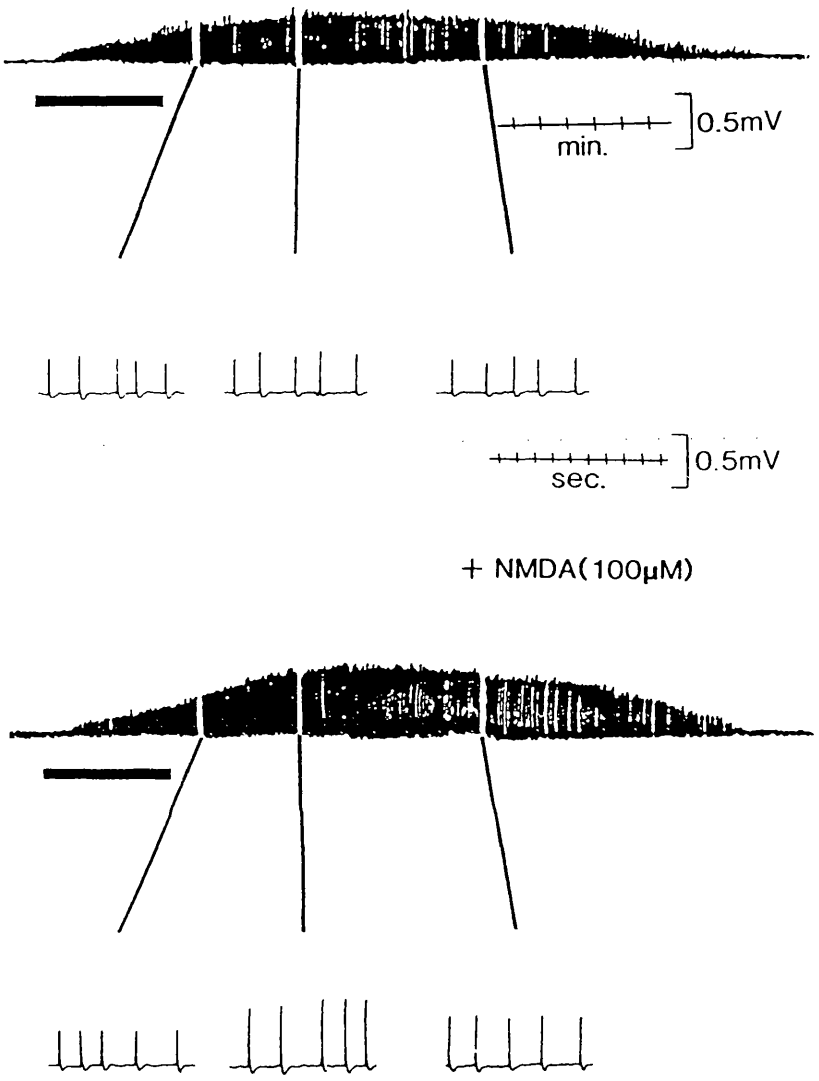

Fig.3.29. The effect of NMDA on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 100μM NMDA superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

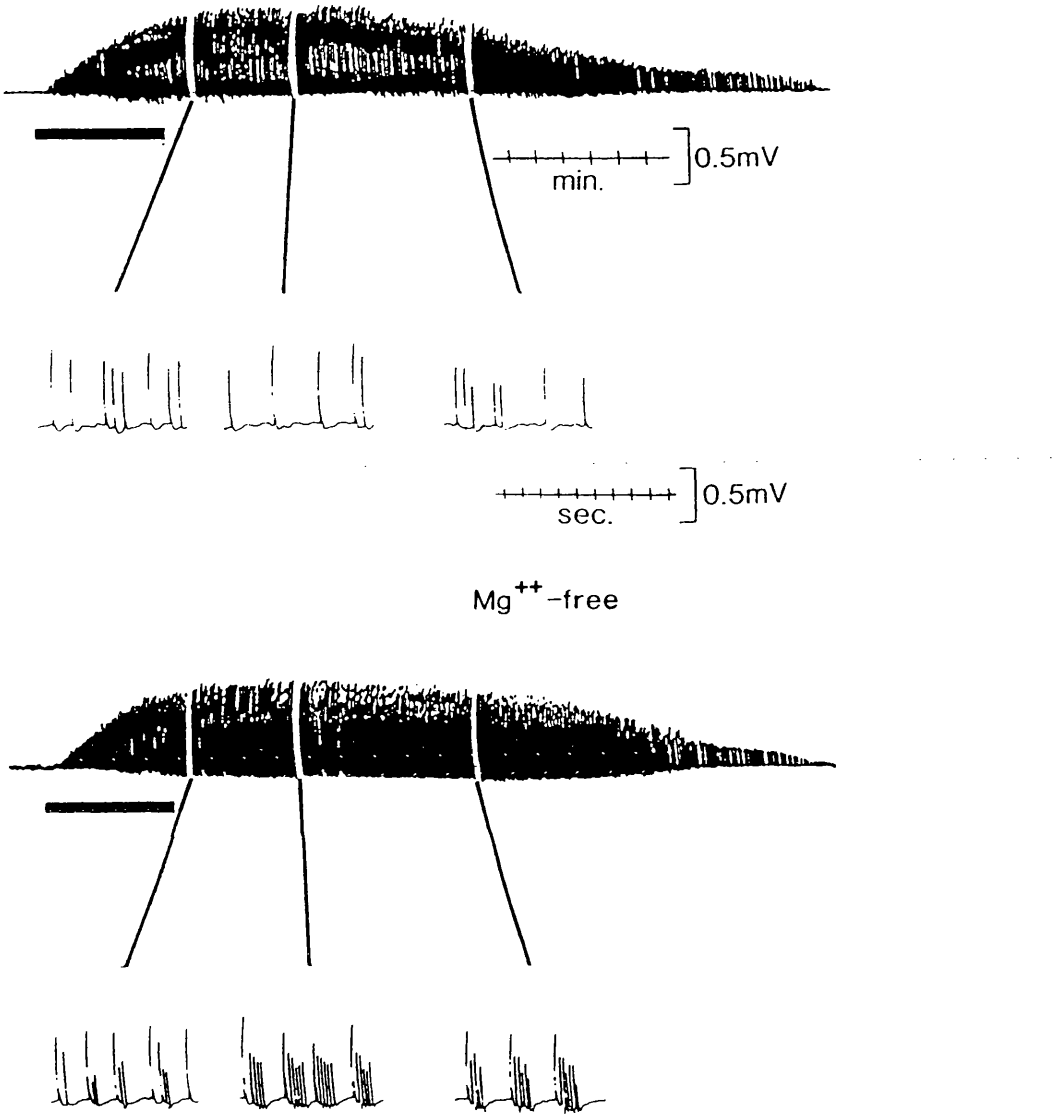

Fig.3.30. The effect of Mg^{++}-free solution on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification when Mg^{++}-free solution is superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

In order to investigate the role of non-NMDA receptors in BM-induced spiking activity CNQX and GAMS which are thought to preferentially block the non-NMDA receptors were used together with the non-specific excitatory amino acid blocker kynurenic acid.

The non-NMDA antagonist CNQX (20μM) significantly decreased epileptogenic activity by mainly reducing the frequency of spiking (Fig.3.31). A similar effect was seen with GAMS (Fig.3.32) but at higher concentration (10mM) it also reduced the average size of the spikes (Fig.3.33). Kynurenic acid (30-1000μM) had a significant effect in diminishing the epileptogenic activity by reducing both the size and the frequency of the spikes (Fig.s 3.34 & 3.35).

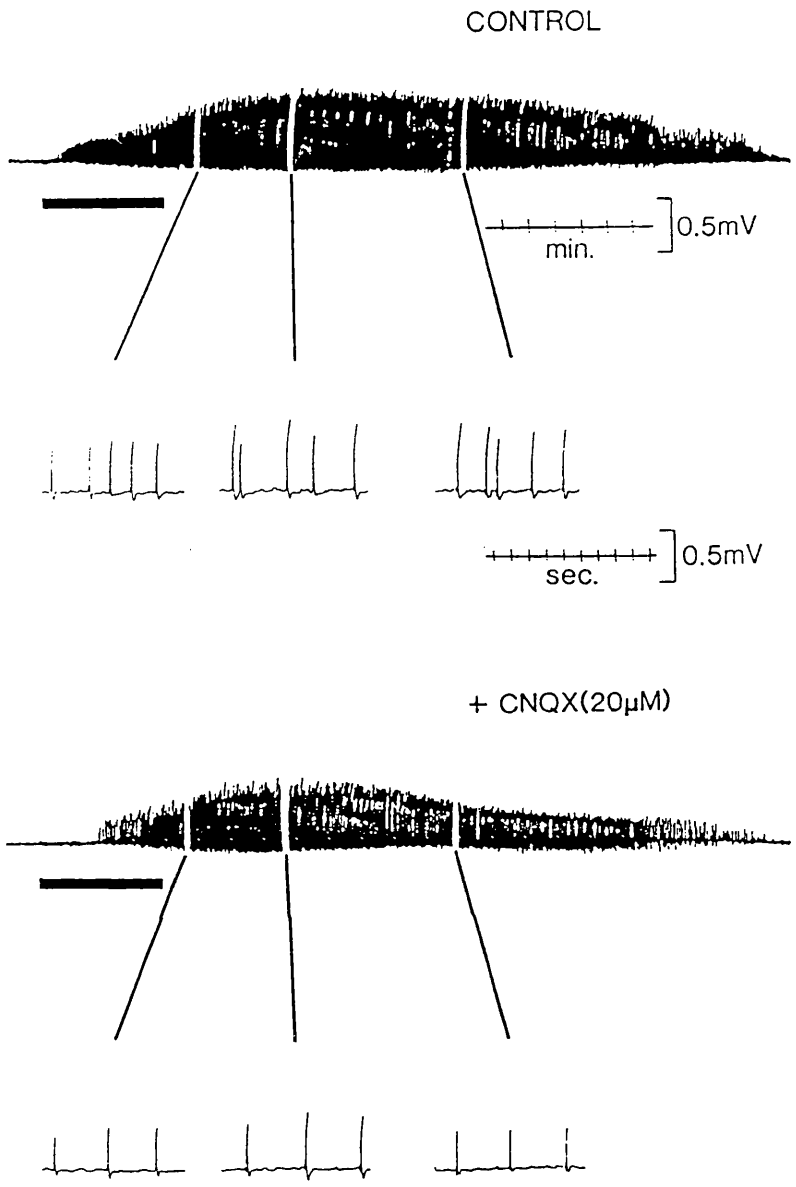

Fig.3.31. The effect of CNQX on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 20μM CNQX superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

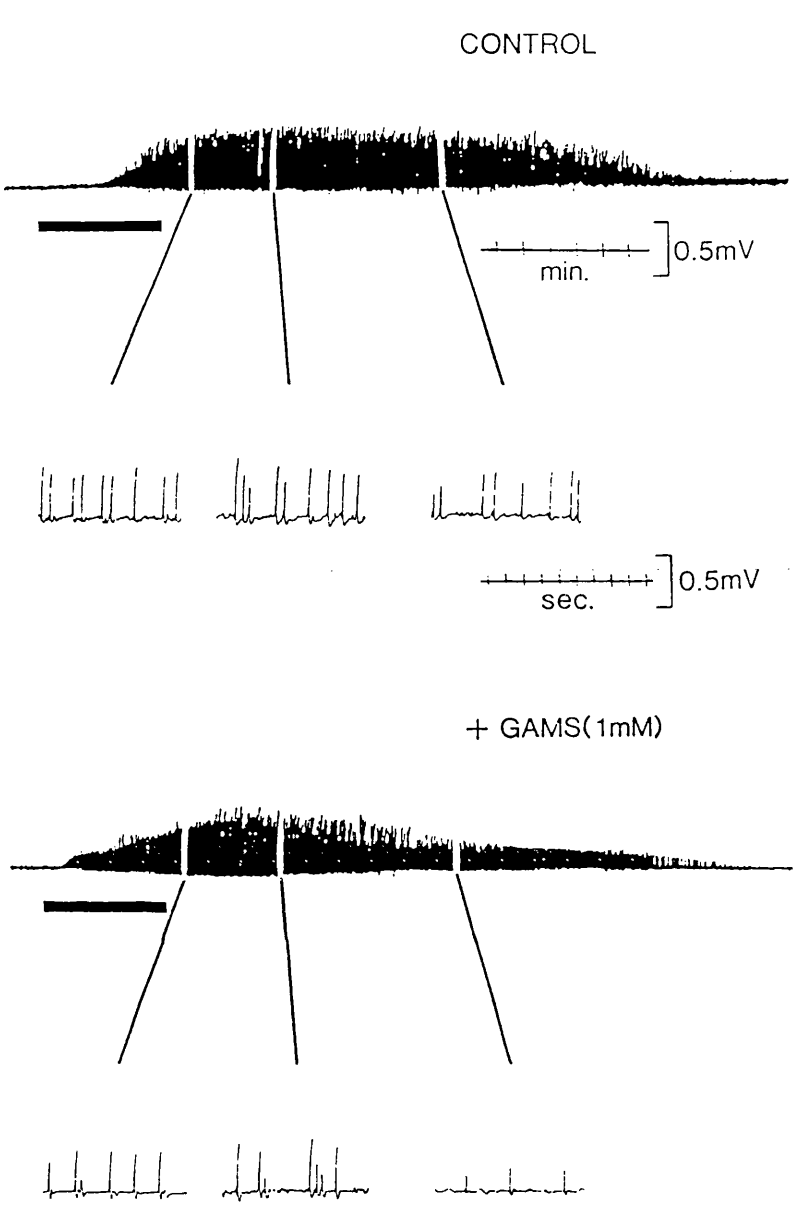

Fig.3.32. The effect of GAMS on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 1mM GAMS superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

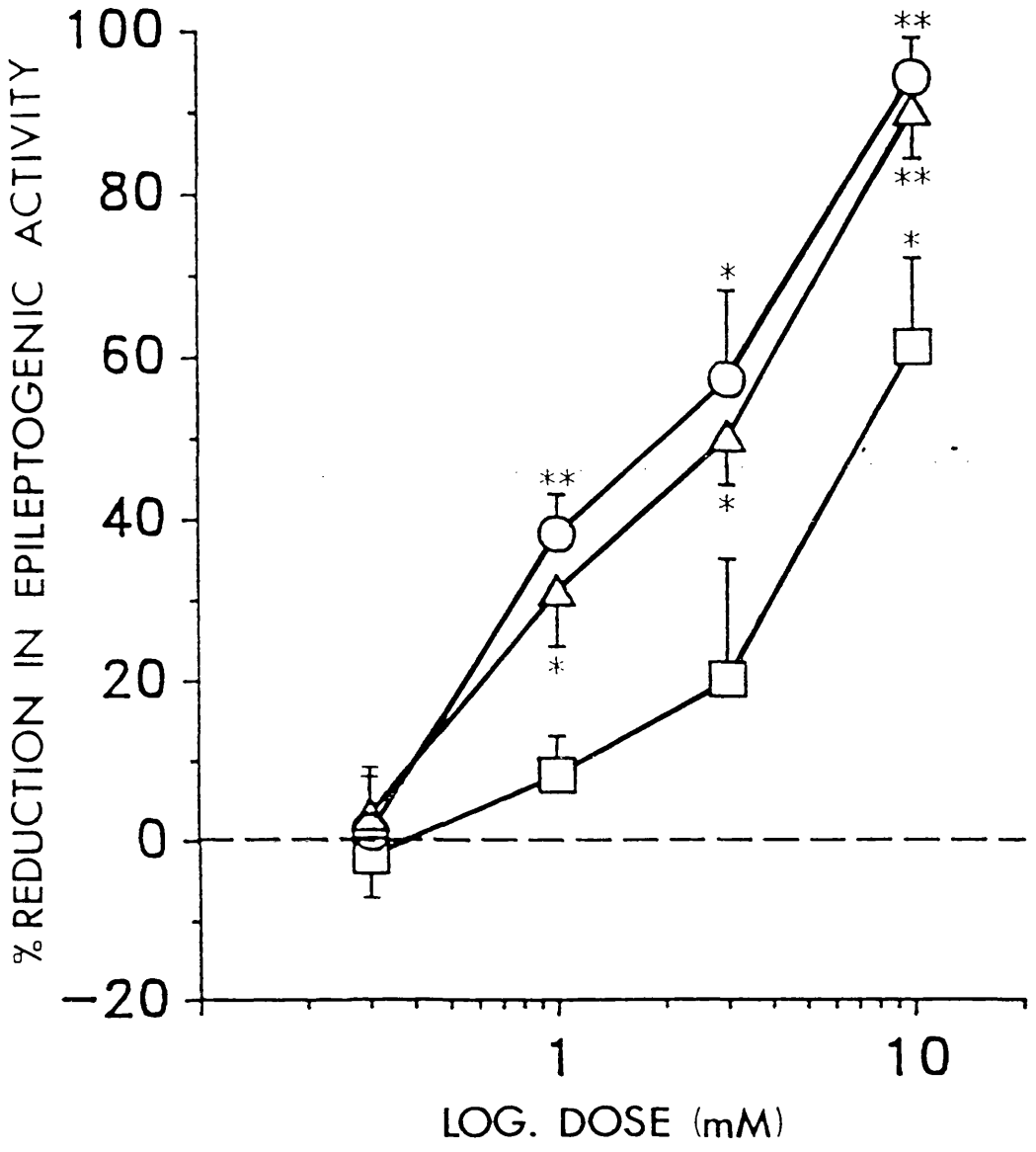

Fig.3.33. The effect of GAMS on the BM-induced spiking. The graphs represent percentage reductions in total integrated spike voltage (O—O) as well as reductions in total number of spikes (Δ—Δ) and the average size of the spikes (□—□) for different doses of GAMS. n=4 for 0.3mM, 6 for 1mM, 5 for 3mM and 3 for 10mM. *P<0.05 and **P<0.005 show significant difference (paired t-test) compared to the control application of BM (200μM).

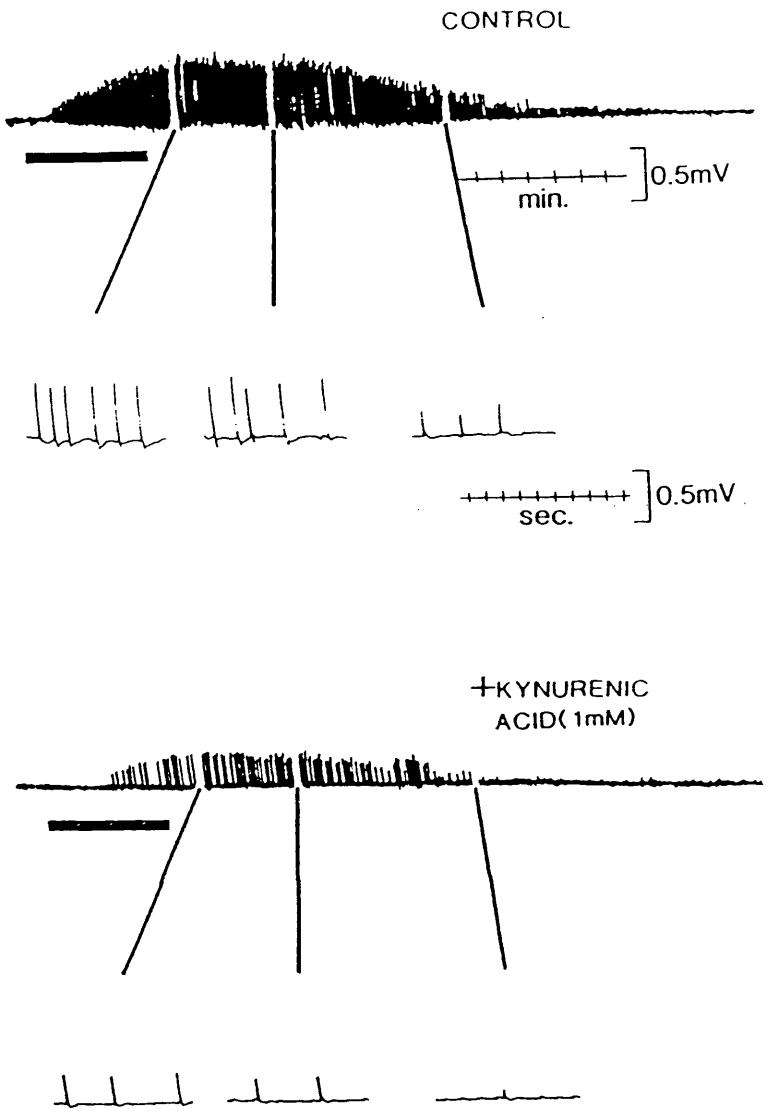

Fig.3.34. The effect of kynurenic acid on BM-induced EEG spiking. The upper trace illustrates the control response to bicuculline and the lower trace its modification by 1mM kynurenic acid superfused across the cortex 30 min before and during BM-induced epileptogenic activity. The traces have been speeded up at 6, 10 and 17 min after BM exposure to show changes in the interictal spikes.

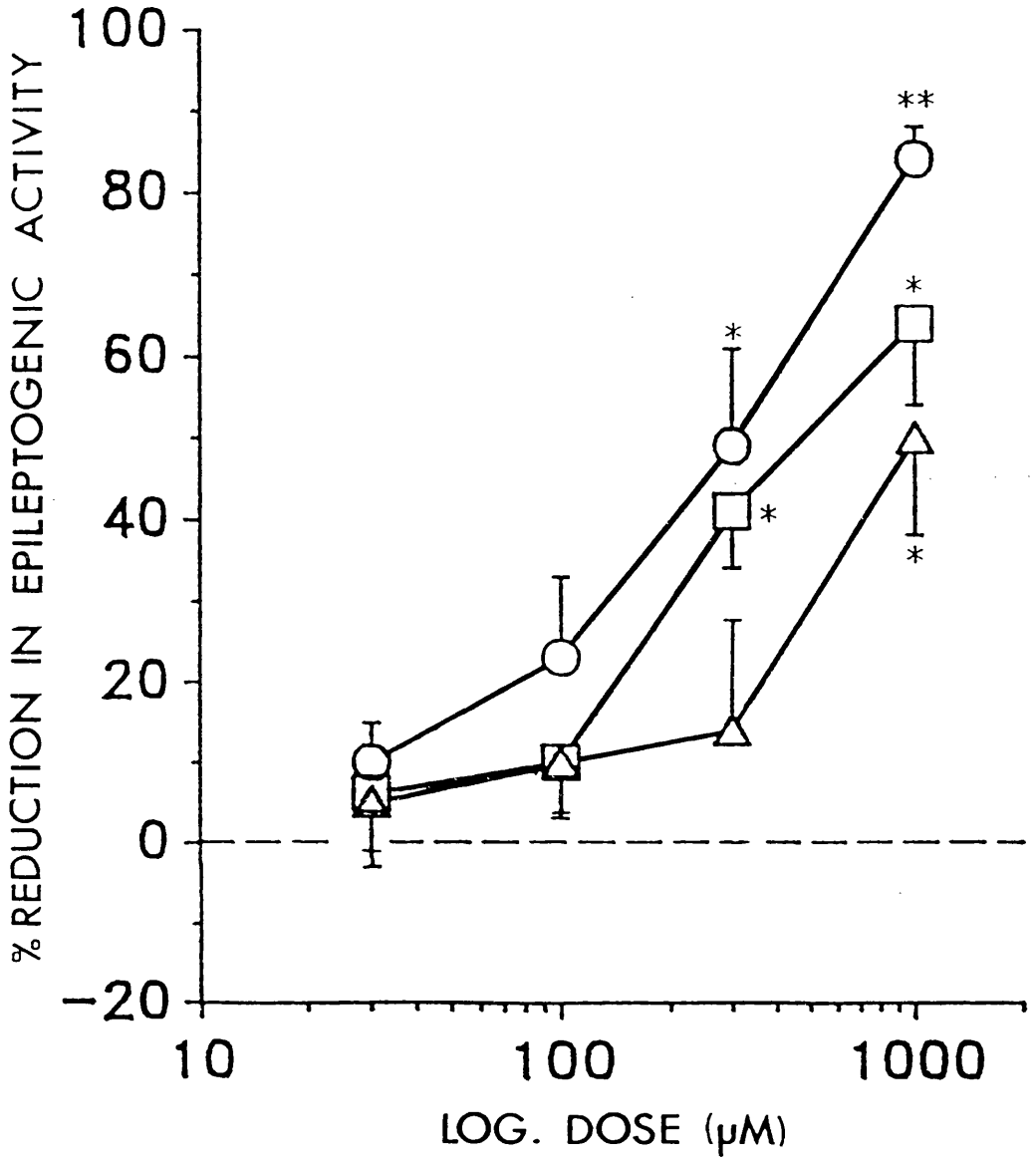

Fig.3.35. The effect of kynurenic acid on the BM-induced spiking. The graphs represent percentage reductions in total integrated spike voltage (O—O) as well as reductions in total number of spikes (Δ—Δ) and the average size of the spikes (□—□) for different doses of kynurenic acid. n=4 for 30μM, 6 for 100μM, 5 for 300μM and 3 for 1000μM. *P<0.05 and **P<0.005 show significant difference (paired t-test) compared to the control application of BM (200μM).

The effect of compounds modulating amino acid neurotransmission were not studied in detail on the spiking activity caused by PTZ or picrotoxin and only the effect of AP7 was evaluated for comparison.

3:5:2: Pentylenetetrazole-induced spiking:

Two different protocols were used to study the effect of AP7 on PTZ-induced spiking. In the first, three concentrations of PTZ (100, 200 and 400mM) were superfused across the exposed cortex cumulatively with each dose being in contact with the cortex for 5 min. This cumulative application of PTZ was repeated, after a one hour rest period, in the presence of 100μM AP7 which was introduced 30 min before the exposure to PTZ and was present until cessation of spiking. The representative traces in Fig. 3.36 show the degree of spiking in the last minute of application of each concentration of PTZ both in the absence and presence of AP7. In the second set of experiments a single dose of 400mM PTZ was applied for 5 min after an initial 45 min rest period. The same single dose application of PTZ was repeated after one hour rest in the presence of 100μM AP7, which was again introduced 30 min before the second exposure of PTZ and monitored until cessation of spiking activity. The peak of activity in both cases was observed during the first minute after PTZ removal and the traces shown in Fig.3.37 show the spiking observed during such peak activity in the presence and absence of AP7. It was evident from these studies that AP7 had reduced both the size and the frequency of spikes induced by PTZ.

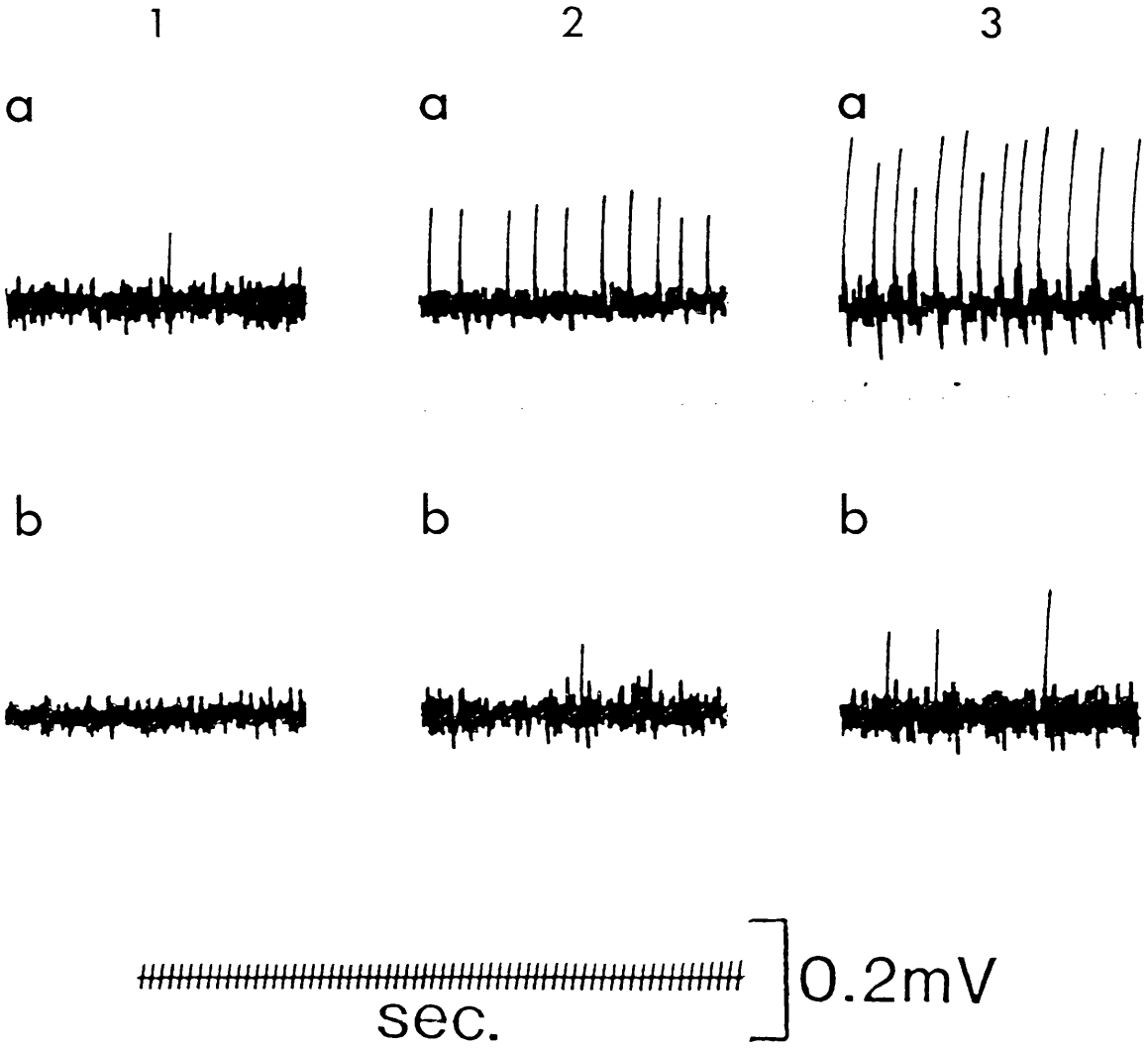

Fig.3.36. The effect of 100μM AP7 on the EEG spiking induced by cumulative application of PTZ. In each case the top traces (1a, 2a & 3a) show spiking activity seen as a result of cumulative application of different doses of PTZ and the bottom traces (1b, 2b & 3b) represent the spiking seen at the same stage in the presence of 100μM AP7. 1, 2 and 3 represent 100, 200 and 400mM PTZ which were superfused cumulatively for 5 min each and the spiking shown were seen during the last min of each application.

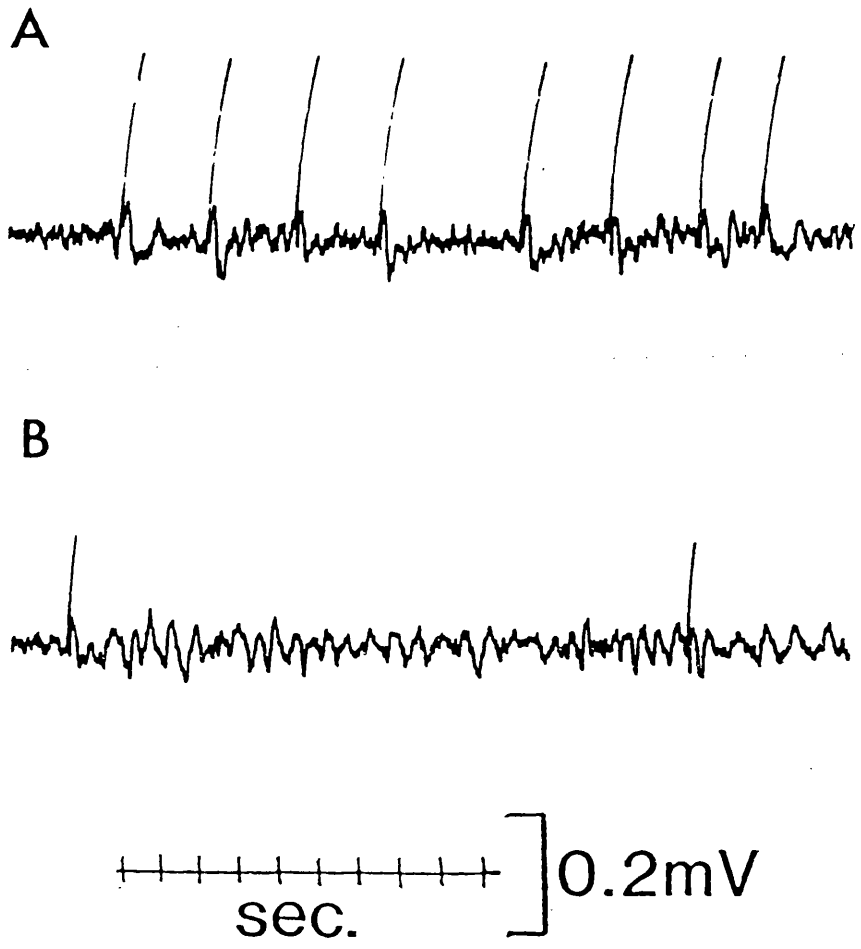

Fig.3.37. The effect of 100μM AP7 on the EEG spiking induced by a single 5 min application of 400mM PTZ. A represents peak spiking seen as a result of 400mM PTZ application and B shows the spiking seen at the same time after 400mM PTZ but in the presence of 100μM AP7. The peak activity shown in each case were seen during the first min after PTZ removal.

3:5:3: Picrotoxin-induced spiking:

As in the case of BM the spiking showed no tachyphylaxis if the application of picrotoxin was repeated at hourly intervals (Fig.3.38). AP7 significantly reduced the spiking activity by both reducing the frequency and the size of the spikes (Fig.s 4.39 & 4.40).

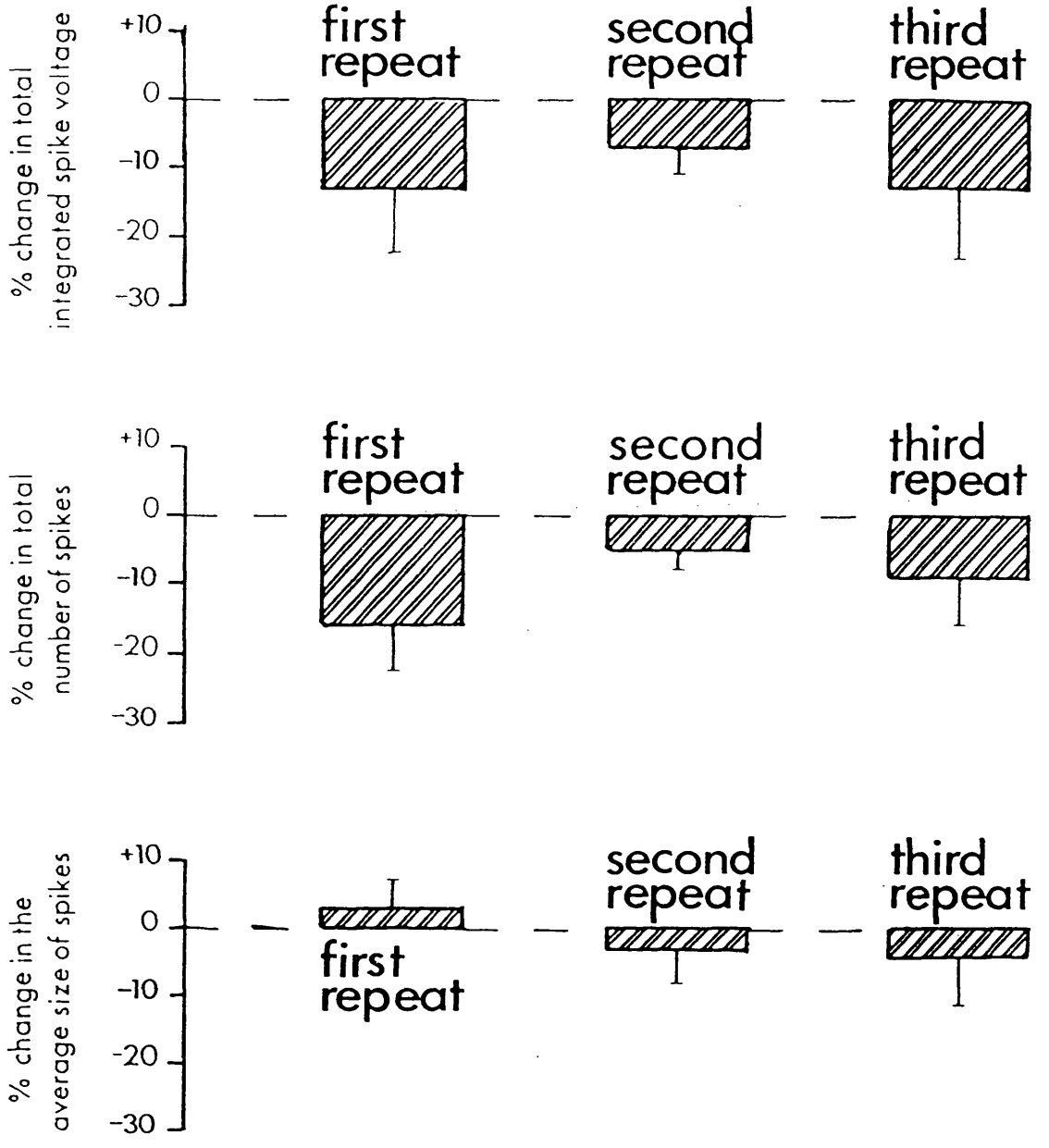

Fig.3.38. The variations in the epileptogenic activity induced by repetitive 5 min cortical superfusion of 300μM picrotoxin at hourly intervals, compared to the first application. The top part of the figure shows the mean ($\pm$ s.e., n=4) percentage changes in total integrated spike voltage; the middle part shows the effect on the total number of the spikes and the bottom part the effect on the average size of the spikes.

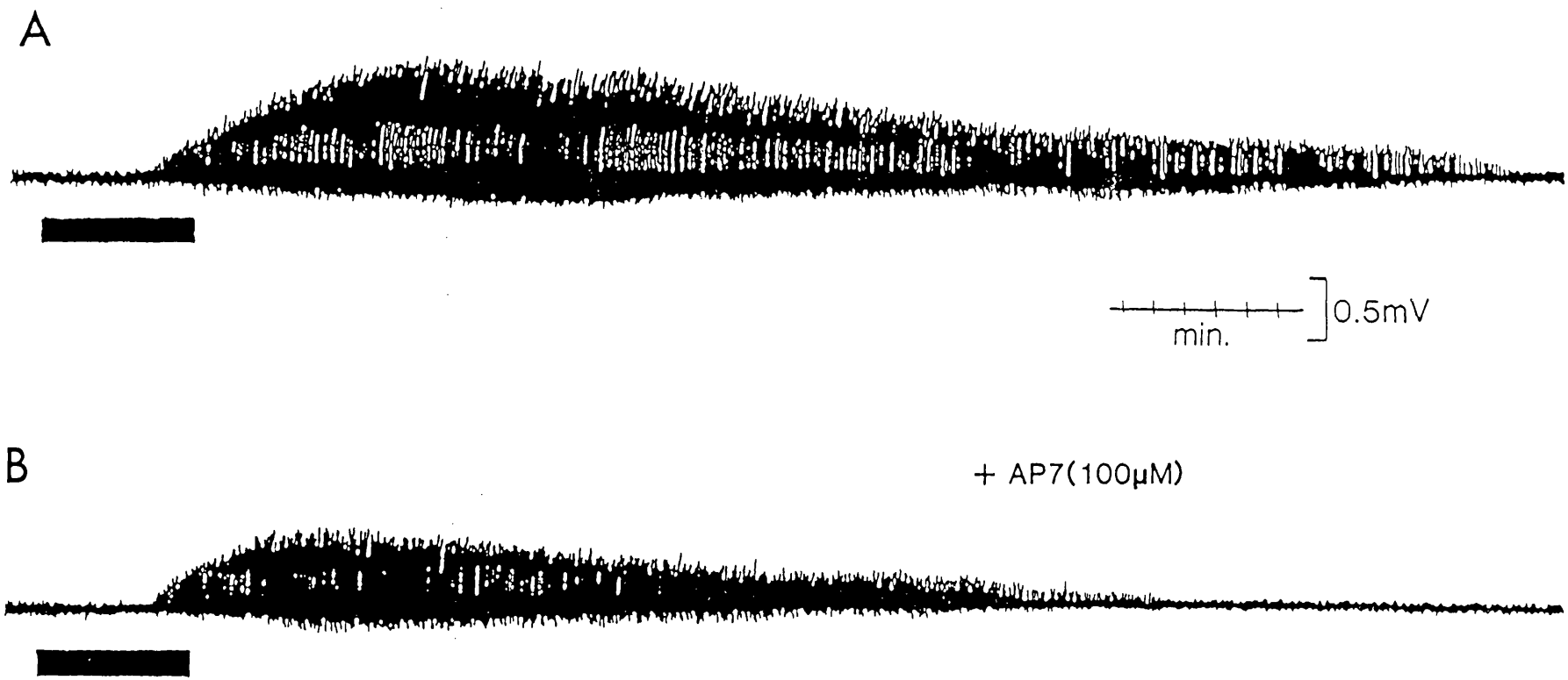

Fig.3.39. The effect of AP7 on picrotoxin-induced EEG spiking. A illustrates the control response to 5 min cortical superfusion of picrotoxin (300μM, black bar) and B its modification by 100μM AP7 superfused across the cortex 30 min before and during picrotoxin-induced epileptogenic activity.

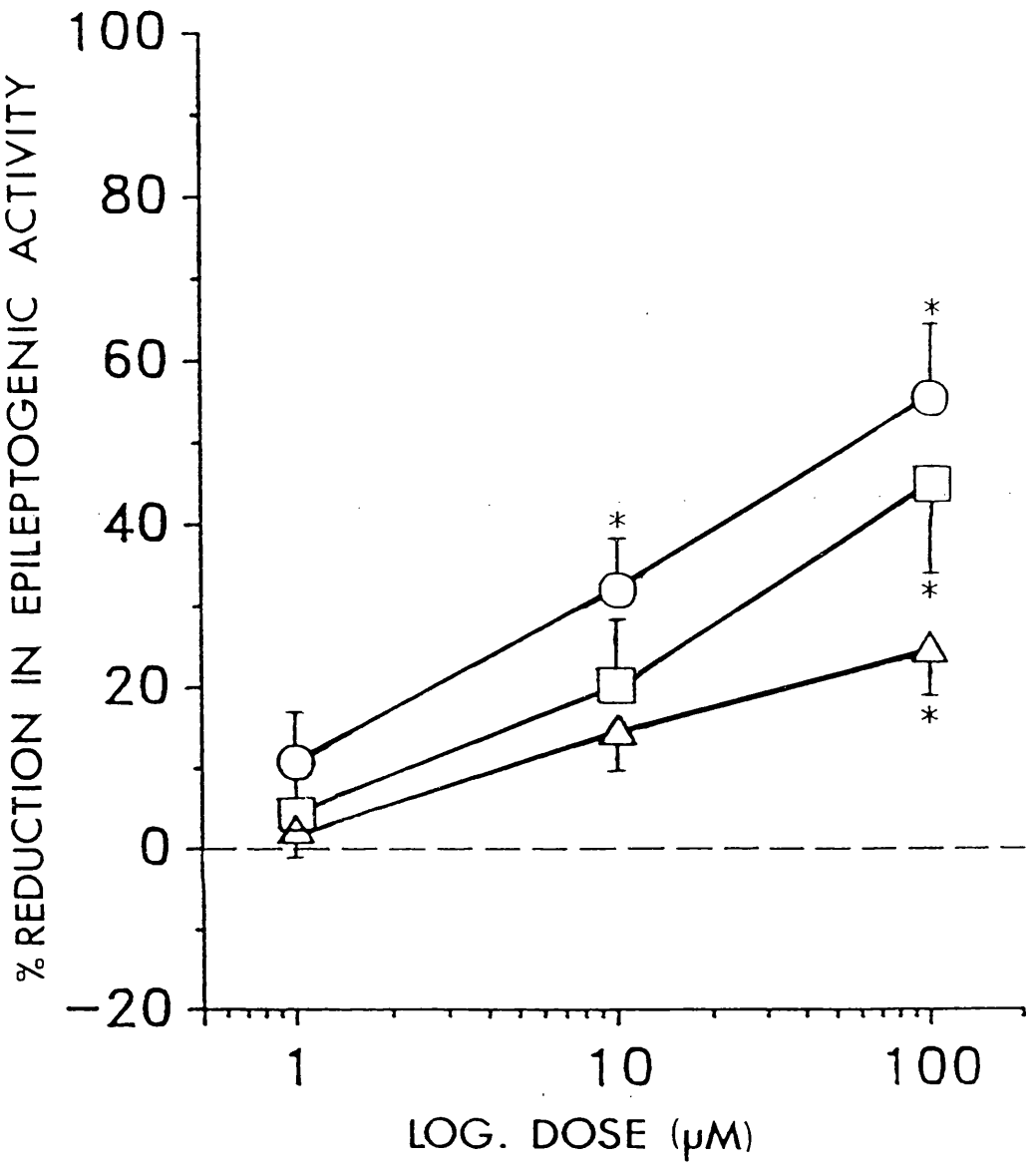

Fig.3.40. The effect of AP7 on the picrotoxin-induced spiking. The graphs represent percentage reductions in total integrated spike voltage (O—O) as well as reductions in total number of spikes (Δ—Δ) and the average size of the spikes (□—□) for different doses of AP7 (n=4). *P<0.05 and **P<0.005 show significant difference (paired t-test) compared to the control application of picrotoxin (300μM).

3:6: DISCUSSION:

3:6:1: Bicuculline methiodide-induced spiking:

As the response to the chosen sub-maximal test concentration of BM (200μM) showed no tachyphylaxis if it was applied a further three times at hourly intervals, drug modification of spiking could be determined by comparing the level of spiking as a result of the application of BM in the presence of drug, with that obtained with its first (control) application. In all such studies test drugs were superfused for 30 min before, as well as during BM-induced epileptogenic activity, in an attempt to achieve equilibrium conditions. None of these test drugs had any detectable effect on the basal EEG although those which cross the blood-brain-barrier, for example clonazepam, depress it if given intravenously. Possibly the localisation of the drugs to the cortical area beneath the cup means that they cannot influence a basal EEG, which has derivation beyond the confines of the cup, but can modify the effects on it of a convulsant also applied locally.

The effects of a wide range of drugs, which manipulate amino acid neurotransmission on bicuculline-induced spiking are summarized in Fig.3.41. From this it can be seen that at concentrations which produced similar overall depressions of spiking some compounds (baclofen, GAMS and CNQX) primarily affected the number of spikes generated, whilst others (AP7, clonazepam and muscimol) mostly affected the size rather than the number of spikes. GABA and kynurenic acid affected both parameters.

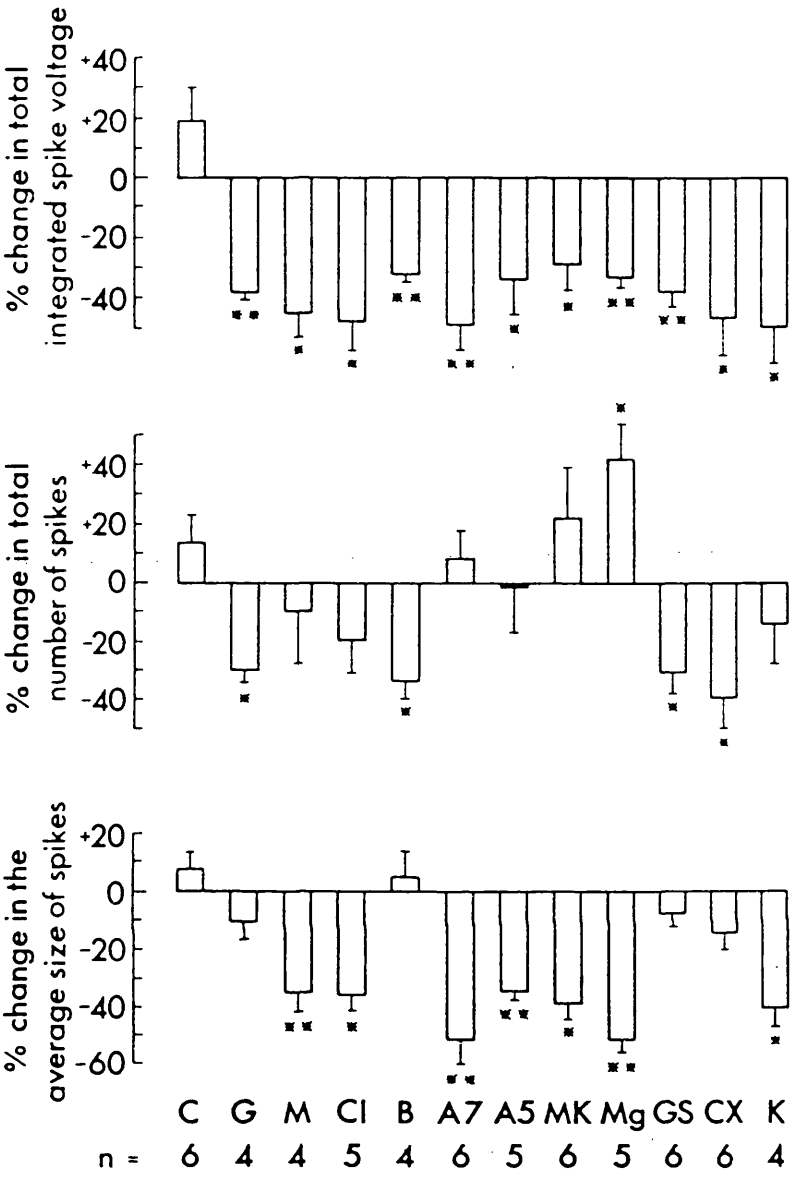

Fig.3.41. The effect of selected doses of various drugs tested on the epileptogenic activity induced by cortical superfusion of 200μM BM. The top part of the figure shows the mean ($\pm$ s.e.) percentage changes in total integrated spike voltage; the middle part shows the effect on the total number of the spikes and the bottom part the effect on the average size of the spikes. C=control, G=GABA(3mM), M=muscimol(30μM), Cl=clonazepam(100μM), B=($\pm$)baclofen(30μM), A7=AP7(100μM), A5=AP5(100μM), MK=MK801(10μM), Mg=Mg^{++}(10mM), GS=GAMS(1mM) , CX=CNQX(20μM) and K=kynurenic acid(300μM). *P<0.05 and **P<0.005 show significant difference (paired t-test) compared to the control application of BM in each case.

Czuczwar, Frey & Löscher (1985) have shown that both enhancement of GABA-mediated inhibition and reduction of NMDA-produced excitation antagonizes NMDA-induced convulsions in mice and Turski, Urbanska, Dziki, Parada-Turska & Ikonomidou (1990) have also shown that compounds which increase GABA-mediated inhibition or are excitatory amino acid antagonists can both protect mice against bicuculline-induced seizures. Unfortunately, these measures unlike EEG recording do not permit further analysis to distinguish between drug effects. Thus whilst EEG spiking is a compound response and may be somewhat removed from both specific neurotransmitter receptor function and clinical epilepsy it provides a useful functional link between them and I have, therefore, attempted to explain the observed changes in EEG activity produced by drugs in this study in terms of neurotransmitter activity in the hope that it will provide some explanation of their mode of action in controlling seizures.

By testing GABA itself and compounds relatively specific for its A and B receptors I hoped to evaluate their relative importance in the overall effects of GABA. Muscimol, a $GABA_A$ agonist, only reduced the size of the spikes at concentrations below 30μM and clonazepam, which augments $GABA_A$ receptor function, showed a similar specificity up to 100μM. Although these findings link $GABA_A$ receptor activation to the control of spike size, higher concentrations of muscimol(100μM) also reduced spike number. Whether this is an effect of muscimol not mediated through the $GABA_A$ receptor is unclear. Although clonazepam had no effect on spike frequency the highest concentration tested (100μM) did not depress activity as much as muscimol (Fig.s 3.15 & 3.17) and it is possible that higher doses could have also affected spike number in a similar way to muscimol. In fact Gartside (1978) has reported a reduction in the size as well as frequency of

penicillin-induced epileptic spiking in rats by another benzodiazepine, diazepam. These results are comparable with those seen in cortical slices in which muscimol and GABA lead to a reduction in the size of the spikes in the bursting episodes (Horne et al., 1986).

The $GABA_B$ agonist, baclofen (Hill & Bowery, 1981), had a striking effect on epileptogenic spiking by reducing the number of spikes without any effect on their size (Fig.3.18). A similar finding has also been reported in hippocampal (Ault, Gruenthal, Armstrong, Nadler & Wang, 1986) and cortical (Horne et al., 1986) slices where baclofen reduces the frequency of burst firing episodes without altering the profile of individual bursts. This suggests that it is able to reduce the likelihood of a spike occuring and, therefore, presumably of a number of neurones firing together but is not able to affect the frequency of their firing once activated, or the recruitment of other neurones. Baclofen is known to reduce the chemically-induced firing rate of cat cortical neurones (Davies & Watkins, 1974) and it is thought to have both a pre and postsynaptic action (Peet & McLennan, 1986). Presynaptically it reduces the release of excitatory (Johnston, Hailstone and Freeman, 1980 and Olpe, Baudry, Fagni & Lynch, 1982) as well as inhibitory (Harrison, 1990) neurotransmitters presumably in this instance on to pyramidal neurone dendrites. Postsynaptically baclofen depresses firing by membrane hyperpolarization, primarily of dendrites, through increased K^+ efflux (Newberry and Nicoll, 1984 and Ong, Kerr & Johnston, 1990) as well as reducing the voltage-dependent inward Ca^{++} current as shown in cultured rat dorsal root ganglion cells (Dolphin & Scott, 1986). These effects of baclofen would be most likely to occur in the superficial layers (I-III) of the neocortex where excitatory synapses on pyramidal cell dendrites predominate and where binding studies show a high density of $GABA_B$ sites, although some are found in layer IV with the

$GABA_A$ receptors (Gehlert, Yamamura & Wamsley, 1985; Olsen et al., 1984). Thus $GABA_B$ agonists may reduce the synaptic initiation of a spike by their action on the superficial dendrites of more deeply located neurones. If this fails then the subsequent spread and recruitment of activity from the deeper layers (IV-V) could go unimpeded with no attenuation of spike size especially in these experiments with reduced $GABA_A$-mediated inhibition in the presence of bicuculline. Since, however, some $GABA_B$ binding sites are found in layer IV, where spikes may be initiated, they could be of particular importance in producing local inhibition in that area. The effect of the $GABA_B$ antagonist, phaclofen was also studied on the EEG. This agent did not affect the basal EEG and had no effect on the BM-induced spiking up to a concentration of 1mM. Therefore, it appears that although $GABA_B$ receptor activation is important in attenuating BM-induced spiking activity its antagonism does not lead to any epileptogenic activity on its own. Also since phaclofen did not augment BM-induced spiking it could be that there is no attenuation of spiking by endogenous GABA through the $GABA_B$ receptor.

GABA itself reduced both the size and the number of spikes although, as in other studies (Horne et al., 1986; Löscher, 1982), high concentrations (mM) were required, probably because of its active uptake. At the lower concentrations tested (3mM) it preferentially affected spike number (Fig.3.20), which is a $GABA_B$-like effect. Since $GABA_B$ receptors are situated more superficially than $GABA_A$ sites the concentration of GABA could be higher in their locality, despite being applied for 30 min, and as bicuculline is blocking $GABA_A$ receptors it would be more difficult for GABA to activate these receptors even under equilibrium conditions.

Differential effects on spiking were also observed by manipulating activity mediated through the different excitatory amino acids receptors. Studies with the NMDA antagonists AP5 and AP7 showed that NMDA receptor blockade leads to a reduction in the average size with little effect on the total number of spikes (Figs. 3.22 to 3.25). The results obtained show that the initiation of spikes does not involve the stimulation of NMDA receptors but NMDA receptors are certainly involved in the recruitment of neurones and hence the severity of spiking. Similar results have also been seen in *in vitro* cortical and hippocampal slices where blockade of NMDA receptors leads to a reduction in the frequency and the height of the after potentials in the paroxysmal depolarizing shift (PDS), which is the cellular correlate of interictal spikes (Ayala, Dichter, Gumnit, Matsumoto & Spencer, 1973; Fenwick, 1983), without affecting the initial action potential in the burst firing episode (Hwa & Avoli, 1989; Jones, 1988; Avoli & Olivier, 1987; McBain, Boden & Hill, 1988; Baldino, Wolfson, Heinemann & Gutnick, 1986; Ashwood & Wheal, 1987 and Gean & Chang, 1991). It is, therefore, reasonable to assume that the initial action potential in the PDS is responsible for the initiation of the interictal spikes with the after potentials determining its size as more cells become recruited.

The inability of NMDA blockers to stop the initiation of spikes produced by bicuculline could be the reason for AP7 not being active against bicuculline-induced convulsions in mice (Czuczwar & Meldrum, 1982). It is also interesting to note that although NMDA blockade considerably reduces the severity of epileptogenic activity in various models of epilepsy it often fails to lead to complete abolition of activity (Hwa & Avoli, 1989; Patel, Millan, Mello & Meldrum, 1986; Horne et al., 1986; Dingledine, Hynes & King, 1986 and Herron, Williamson & Collingridge, 1985). In agreement with other studies (Meldrum et al.,

1983a; Meldrum, Croucher, Czuczwar, Collins, Curry, Joseph and Stone, 1983b) our results indicate that AP7 is slightly more potent in reducing epileptogenic activity than AP5 and this difference could be due to the greater potency of AP7 in blocking excitatory responses to NMDA in rat cortical neurones (Perkins, Stone, Collins & Curry, 1981).

Blockade of NMDA activated channels by MK-801 and high Mg^{++} (10mM) reduced the size of the spikes but this was offset by an increase in their frequency. The latter effect could result from a more rapid repolarization of neurones, because of their shorter depolarization under reduced NMDA activity, to a toxic effect of MK-801 at higher concentrations and in the case of Mg^{++} to a reduction in inhibitory neurotransmitter release (Del Castillo & Katz, 1954). The effect of Mg^{++}-free solution was very interesting. Although it had no effect on the basal EEG its reported epileptogenic activity (Gean & Shinnick-Gallagher, 1988) was only evident during BM-induced spiking when it lead to a significant increase in the number of spikes (Fig.3.30). This suggests that in these experiments any epileptogenic spiking produced by the lack of Mg^{++} can only be observed if the GABAergic inhibition is reduced.

The compounds which preferentially block non-NMDA receptors, CNQX (Fig.3.31) and GAMS (at low concentrations, see Fig.s 3.32 & 3.33) mainly reduced the frequency of spikes with little effect on the average size of the spikes. This implies that non-NMDA receptors, like $GABA_B$ receptors, are more involved in the initiation of spikes rather than their subsequent development although the pattern of reduction was different from that seen with baclofen. GAMS loses it specificity for non-NMDA receptors at high concentrations (Davies & Watkins, 1985) and is, therefore, seen to affect the size of the spikes as well as their total number at higher concentrations (Fig.3.33). After non-NMDA receptor

blockade spike frequency is lowered but remains steady (Fig.s 3.31 & 3.32) whereas with baclofen distinct gaps occur in spiking and this effect is more pronounced at the begining of spiking (Fig.3.18).

It is possible that baclofen mainly affects the rate of firing of cells which participate in the production of a spike reducing the chance of their synchronized discharge. Thus when the cells fire synchronously the spike will be the same size as normally but when the firing is not synchronous no spikes are seen. Binding experiments have shown that non-NMDA receptors are located mainly in the superficial dendritic layers I-III but not in layer IV although some are found in layers V and VI (Unnerstall & Wamsley, 1983; Monaghan, Yao & Cotman, 1984 and Cotman, Monaghan, Ottersen & Storm-Mathisen, 1987). Their occurrence in layers I-III, like $GABA_B$ receptors, could explain the similar depressant effects of baclofen and CNQX on the spike number. Differences between them on the pattern of spiking may depend on the additional location of $GABA_B$ receptors in layer IV (Gehlert et al., 1985) where baclofen would be better placed to produce local inhibition and stop spiking at its onset hence producing distinct gaps in the spiking activity.

The effect of Kynurenic acid in reducing both the total number of spikes and their size (Fig.s 3.34 & 3.35) could be explained by the fact that it is a non-specific excitatory amino acid antagonist blocking both the NMDA and non-NMDA receptors.

3:6:2: Pentylenetetrazole-induced spiking:

Although at the time of performing the experiments with PTZ I had no means of accurately measuring the integrated voltage due to spikes and subsequently calculating the average size of the spikes it was evident from the EEG traces that AP7 not only reduced the number of spikes but it also attenuated their height. These results indicate a role for NMDA receptors both in the initiation and the development of PTZ-induced spiking.

3:6:3: Picrotoxin-induced spiking:

As in the case of PTZ, AP7 lead to a reduction in both the total number and size of picrotoxin-induced spikes.

The effect of AP7 against picrotoxin and PTZ is in contrast to that seen in the case of BM. Since like BM both PTZ and picrotoxin are GABA blockers this difference is surprising although picrotoxin (and possibly PTZ) are thought to act more directly on the Cl^- channel rather than GABA receptor. However, it is possible that a part of the spiking induced by picrotoxin and PTZ is due to some other pharmacological effects which again involved NMDA receptors but this time in an initiatory capacity.

3:7: CONCLUSIONS:

The epileptogenic activity of the three convulsants used again underlines the importance on GABAergic inhibition in epilepsy as they are all GABA antagonists. It is evident from the results that bicuculline-induced epileptogenic spiking activity can be affected differentially by manipulating various aspects of amino acid neurotransmission.

CHAPTER 4

EFFECTS OF CONVULSANTS ON THE EFFLUX OF ENDOGENOUS AMINO ACIDS FROM RAT CEREBRAL CORTEX.

4:1: INTRODUCTION:

Amino acids, as discussed earlier (chapter 1, section 1:4) have an established role in the initiation and development of epileptogenic activity. Numerous investigators have studied their efflux from different sites in the CNS linking the changes in their release to the mode of action of various convulsants and anticonvulsants (Skerritt & Johnston 1984; De Boar, Stoof & Duijn, 1982; De Belleroche, Dick & Wyrley-Birch, 1982; Collins, 1980; Crowder & Bradford, 1987 and Abdul-Ghani, Coutinho-Netto, Druce & Bradford, 1981).

In the present study we have monitored changes in the efflux of endogenous amino acids from the cortex both *in vivo* and *in vitro* during exposure to three convulsants, pentylenetetrazole (PTZ), bicuculline methiodide (BM) and picrotoxin administered either intravenously, by superfusion across the cortex or applied directly to cortical slices.

PTZ is a weak convulsant but it has been used extensively to induce epileptogenic activity both *in vivo* and *in vitro*. Its intravenous infusion leads to the appearance of distinctive phases in the EEG of the urethane-anaesthetised rat (Kent & Webster, 1983) and since PTZ may also initiate its epileptogenic action in the cortex (Starzl, Niemer, Dell & Forgrave, 1953 and Zouhar & Mareš, 1972), it seemed appropriate to ascertain whether the distinctive phases corresponded to specific changes in amino acid efflux. The *in vivo* effect of PTZ was further investigated by direct cortical superfusion of different concentrations across the exposed cortex through cortical cups. The *in vitro* effects of PTZ on amino acid efflux from the cortex were studied using the cortical slices technique and a continuous perfusion system.

The spiking activity caused by cortical superfusion of BM was extensively studied in chapter 3 (section 3:2:3). It was, therefore,

appropriate to study its effect on amino acid efflux during cortical superfusion, as well as in cortical slices. The effect of picrotoxin was also studied but only for its effect on the efflux of amino acids *in vivo* by direct cortical superfusion. High K^+ was used as a standard procedure to test the viability of amino acids release in both the *in vivo* and *in vitro* systems.

In an attempt to link the release of amino acids with electrophysiological changes I investigated the effects of selected compounds modulating amino acid transmission and shown to reduce BM-induced spiking (chapter 3, section 3:5:1), on BM-induced changes in the efflux of amino acids from cortical slices. These included compounds which increase the action of GABA through $GABA_A$ or $GABA_B$ receptor subtypes and antagonists of the excitatory amino acids at both NMDA and non-NMDA receptors.

4:2: *IN VIVO* STUDIES:

Summary of technique:

Urethane-anaesthetised rats with a cortical cup positioned on the right hemisphere were used. Oxygenated, warm ACSF was superfused for 45 min to allow sufficient time for the preparation to recover from the surgery before exposure of the cortex to convulsants. Although the release of amino acids from the exposed cortex was high immediately after placement of the cortical cup it quickly fell to a plateau within the first two 10 min samples collected. A flow rate of 30μl min^{-1} was employed, the samples were collected on ice and stored at -30°C for later analysis of their amino acid content (for full details see chapter 2, section 2:2:3).

4:2:1: Pentylenetetrazole (PTZ):

Intravenous infusion:

In these experiments the left femoral vein was cannulated before placement of the cortical cup. Following the 45 min recovery period 500mM PTZ solution in saline was infused intravenously at a rate of 30μl min^{-1}. 10 min samples were collected starting before the start of infusion until appearance of full body convulsion. The intravenous infusion of PTZ lead to the appearance of distinctive phases in the EEG of the animal, eventually leading to full body convulsion as reported by Kent & Webster, 1983 (Fig 4.1). No significant changes in the efflux of any amino acid apart from that of glutamine could be seen. The increased release of glutamine was statistically significant in the fourth sample collected (Fig.4.2), during which the epileptogenic activity was maximal. The overall time course of the release of all the amino acids studied is shown in Fig.4.3.

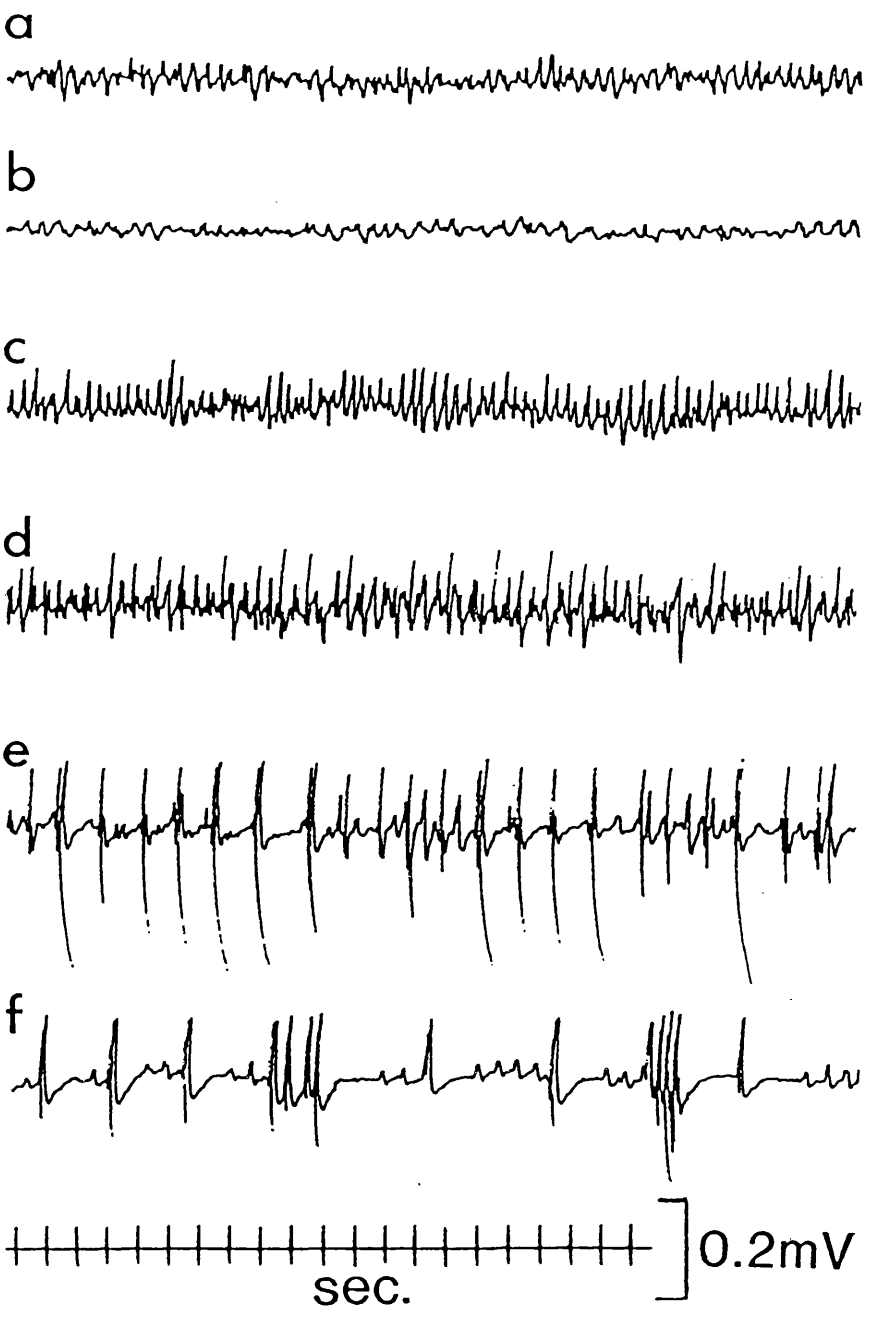

Fig.4.1. Different phases of activity seen during slow intravenous infusion of PTZ. The distinctive phases seen are as follows:

a) basal EEG

b) arousal phase

c) appearance of large slow waves

d) appearance of spiking

e) established spiking

f) grouped spiking.

The phases have been lettered according to the criteria adopted by Kent & Webster (1983).

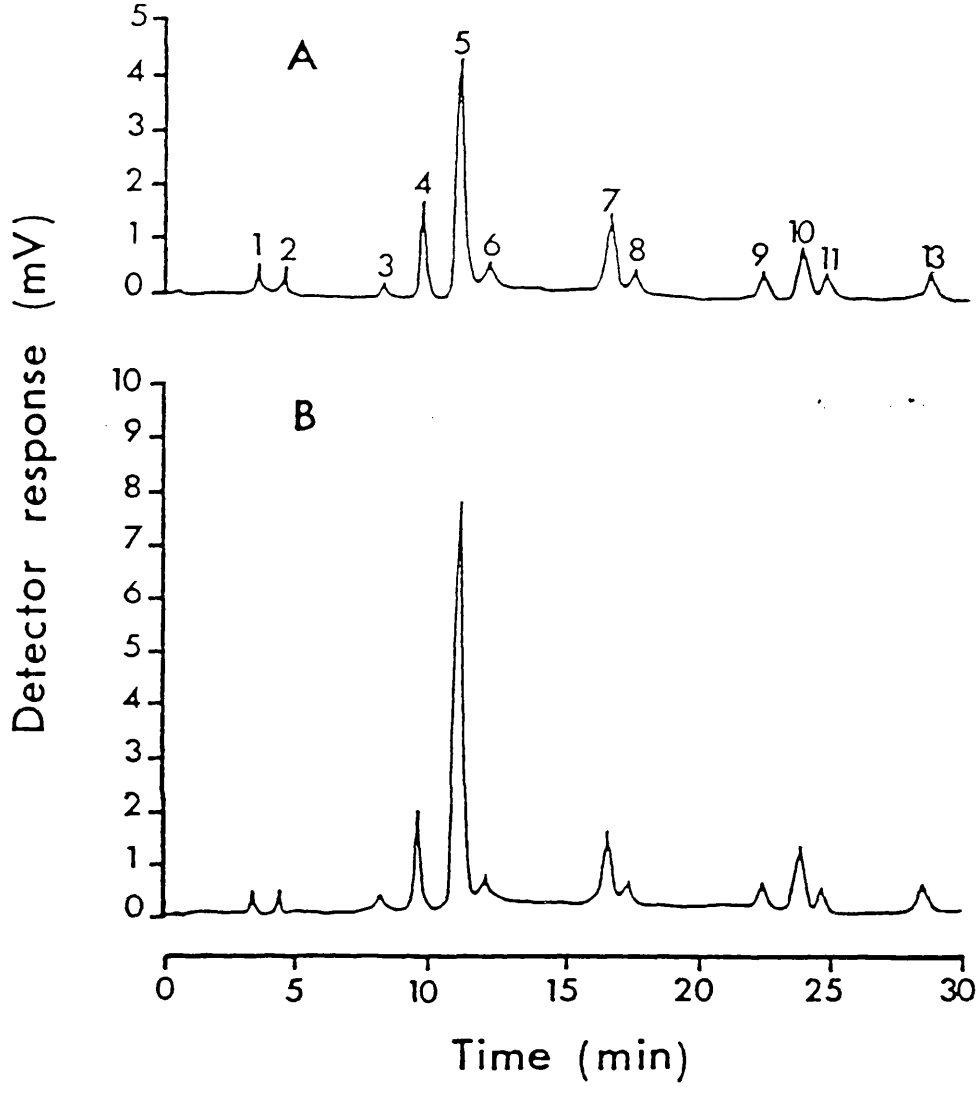

Fig.4.2. Representative chromatograms showing the effect of intravenous infusion of PTZ on the release of amino acids from cortex. Trace A shows the basal release of amino acids before the start of PTZ infusion and trace B the changes in the release of various amino acids in the same animal between 30 to 40 min into PTZ infusion when the epileptogenic activity was at its maximum. Aspartate(1), glutamate(2), asparagine(3), serine(4), glutamine(5), histidine(6), glycine(7), threonine(8), arginine(9), taurine(10), alanine(11) and tyrosine(13).

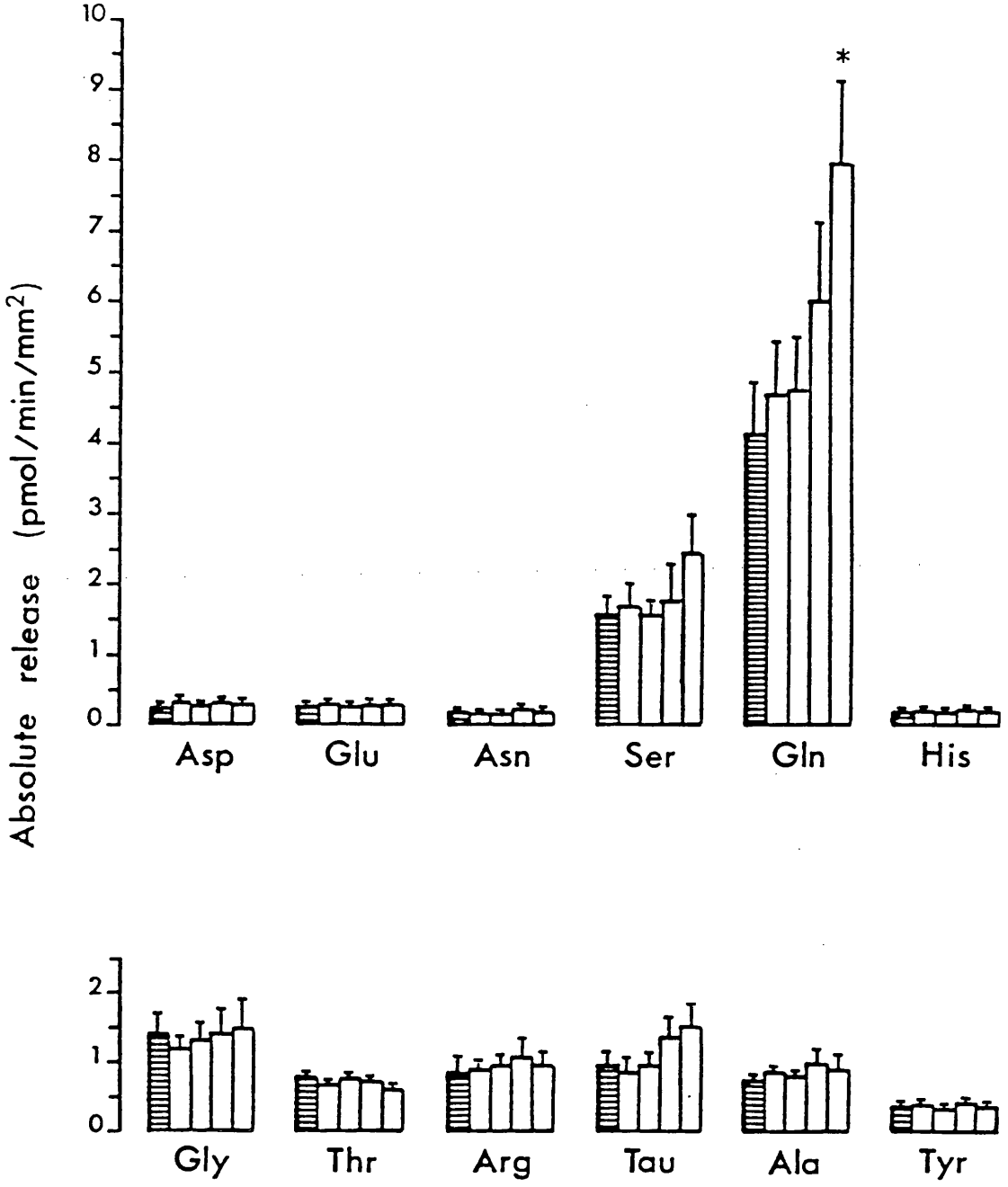

Fig.4.3: The overall time course of amino acid release during intravenous infusion of PTZ. (▤) represents the basal sample collected 10 min before the start of PTZ infusion and (▢) are successive 10 min samples collected during PTZ infusion. The histograms represent mean absolute release, ρmol/min/mm² of exposed cortical surface, $\pm$ s.e. (n=7). *P<0.05 is the significance of results compared to the basal release (paired t-test).

Since no changes in the efflux of excitatory amino acids, glutamate and aspartate, could be seen despite such an intensive spiking and as GABA was not detected in any samples, it was decided to include *p*-chloromercuriphenylsulphonic acid (PCMS) in the ACSF in an attempt to block the uptake of amino acids and so to ascertain whether their efficient uptake in the cortex precluded the detection of any change in their efflux. Concentrations of up to 1mM PCMS had no effect on the release of amino acids but by increasing the concentrations of PCMS to 10mM significant increases could be seen in the basal release of amino acids and GABA could also be detected. In the presence of this high concentration of PCMS, infusion of PTZ lead to significant increases in the release of most of the amino acids studied (Fig.4.4).

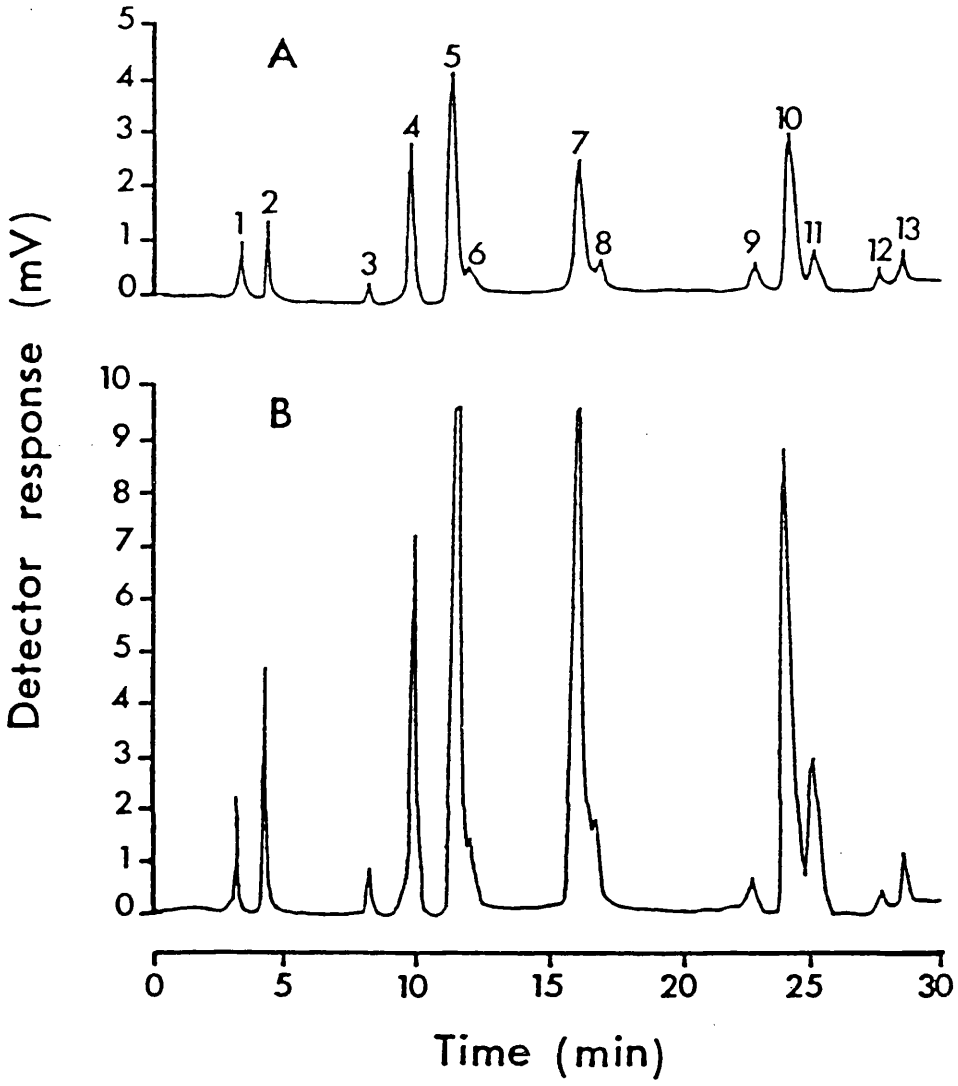

Fig.4.4: Representative chromatograms showing changes in the release of various amino acids during intravenous infusion of PTZ in the presence of 10mM PCMS. Trace A represents the basal release and trace B the increase in the release of various amino acids in the same animal 30 to 40 min into PTZ infusion when the epileptogenic activity was at its maximum. Aspartate(1), glutamate(2), asparagine(3), serine(4), glutamine(5), histidine(6), glycine(7), threonine(8), arginine(9), taurine(10), alanine(11), GABA(12) and tyrosine(13).

Cortical superfusion:

In these experiments the effects of three increasing concentrations of PTZ (100, 200 and 400mM in ACSF) were investigated on the efflux of amino acids. Normal ACSF was superfused for 45 min before successive 5 min periods of superfusion of three ascending concentrations of PTZ followed by continuous superfusion of normal ACSF. 5 min samples were collected, starting before PTZ superfusion until the end of spiking. Cortical superfusion of PTZ lead to a dose-related increase in the release of all amino acids studied with the exception of aspartate (Fig.4.5). This showed a significant reduction on exposure to 100mM PTZ only (Fig.4.6). PCMS was not included in the ACSF and GABA was not detected.

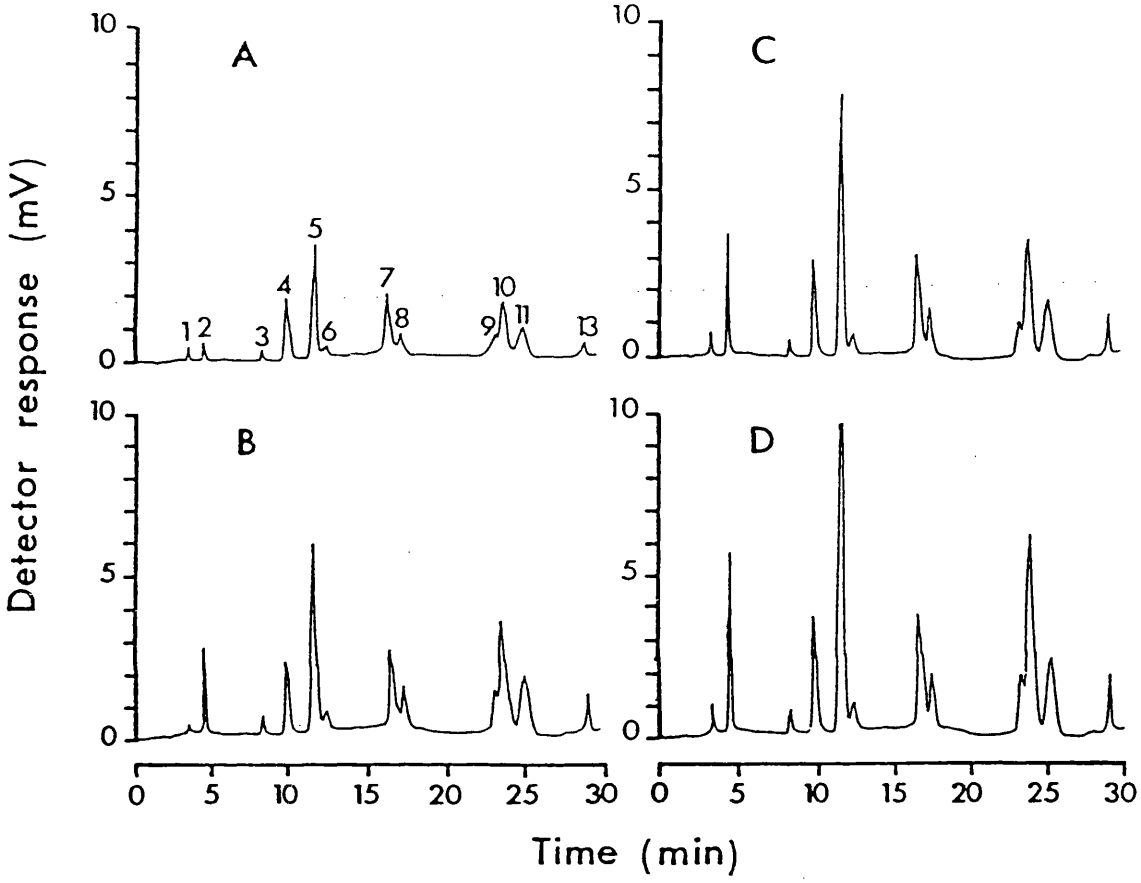

Fig.4.5: Representative chromatograms showing the effect of cortical superfusion of PTZ on the release of amino acids *in vivo*. Trace A represents the basal release of amino acids and traces B,C and D show the changes in release of amino acids in the same animal during the superfusion of 100, 200 and 400mM PTZ, respectively. Aspartate(1), glutamate(2), asparagine(3), serine(4), glutamine(5), histidine(6), glycine(7), threonine(8), arginine(9), taurine(10), alanine(11) and tyrosine(13).

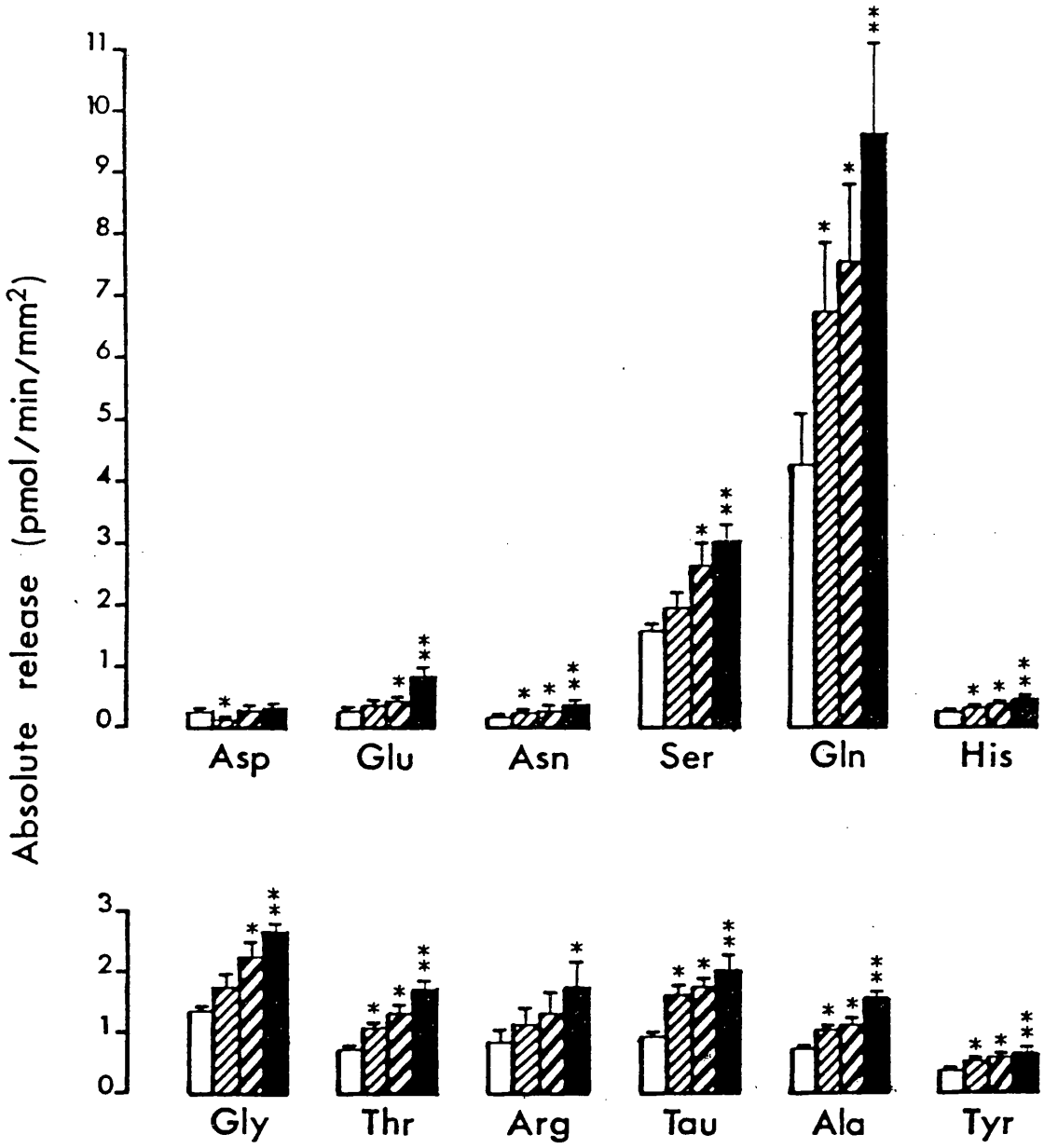

Fig.4.6: Effect of cortical superfusion of PTZ on the release of amino acids from cortex *in vivo*. The histograms represent successive 5 min samples. (☐) shows the basal release, (▨) the release during 100mM PTZ superfusion, (▨) the release during 200mM PTZ superfusion and (■) the release of amino acids during 400mM PTZ superfusion. Values are mean absolute release, ρmol/min/mm² of exposed cortical surface, ± s.e. (n=7). *P<0.05 and **P<0.005 show significance of results compared to the basal release (paired t-test).

4:2:2: Bicuculline methiodide (BM):

Since cortically superfused PTZ yielded more pronounced changes in the efflux of amino acids than its intravenous infusion it was decided only to study cortical superfusion of BM for any possible changes in the efflux of amino acids. This also provided direct comparison with our electrophysiological studies where epileptogenic changes in the EEG were investigated after transient cortical superfusion of 200μM BM (see chapter 3). After the initial 45 min rest period, 200μM BM in ACSF was superfused for 5 min followed by continuous superfusion of ACSF. Samples were collected, every 10 min, starting before BM introduction until the cessation of spiking activity. The results showed no significant changes in the release of the various amino acids studied (Fig.4.7). Introduction of PCMS (up to 1mM) had no effect on amino acid efflux.

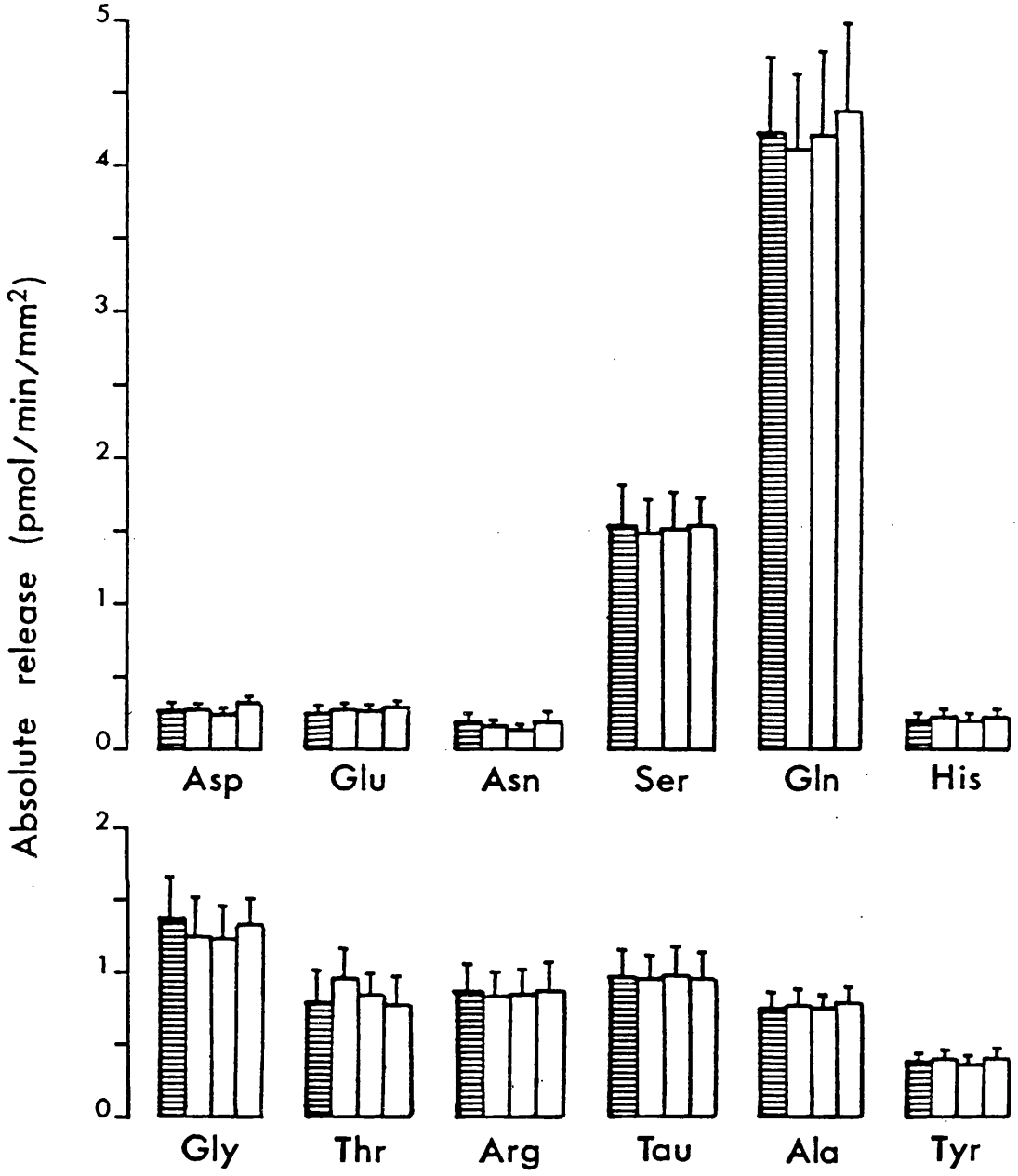

Fig.4.7: Overall time-course of amino acid release during cortical superfusion of 200μM BM in ACSF *in vivo*. (▤) shows the basal release before the 5 min superfusion of 200μM BM through the cortical cup and successive 10 min samples collected as soon as BM was in contact with the cortex until cessation of spiking is shown by (□). The histograms represent mean absolute release , $p$mol/min/mm^2 of exposed cortical surface, $\pm$ s.e. (n=5).

4:2:3: Picrotoxin:

The effect of cortical superfusion of picrotoxin on the *in vivo* efflux of amino acids from cerebral cortex was studied as in the case of BM. ACSF was superfused for 45 min before 5 min superfusion of 300μM picrotoxin, which was shown to induce extensive EEG spiking (chapter 3, section 3:2:4), followed by continuous superfusion of ACSF. Samples were collected, every 10 min, before picrotoxin exposure until cessation of spiking activity. Analysis of the amino acid content of the samples collected revealed no significant changes in their efflux (Fig.4.8).

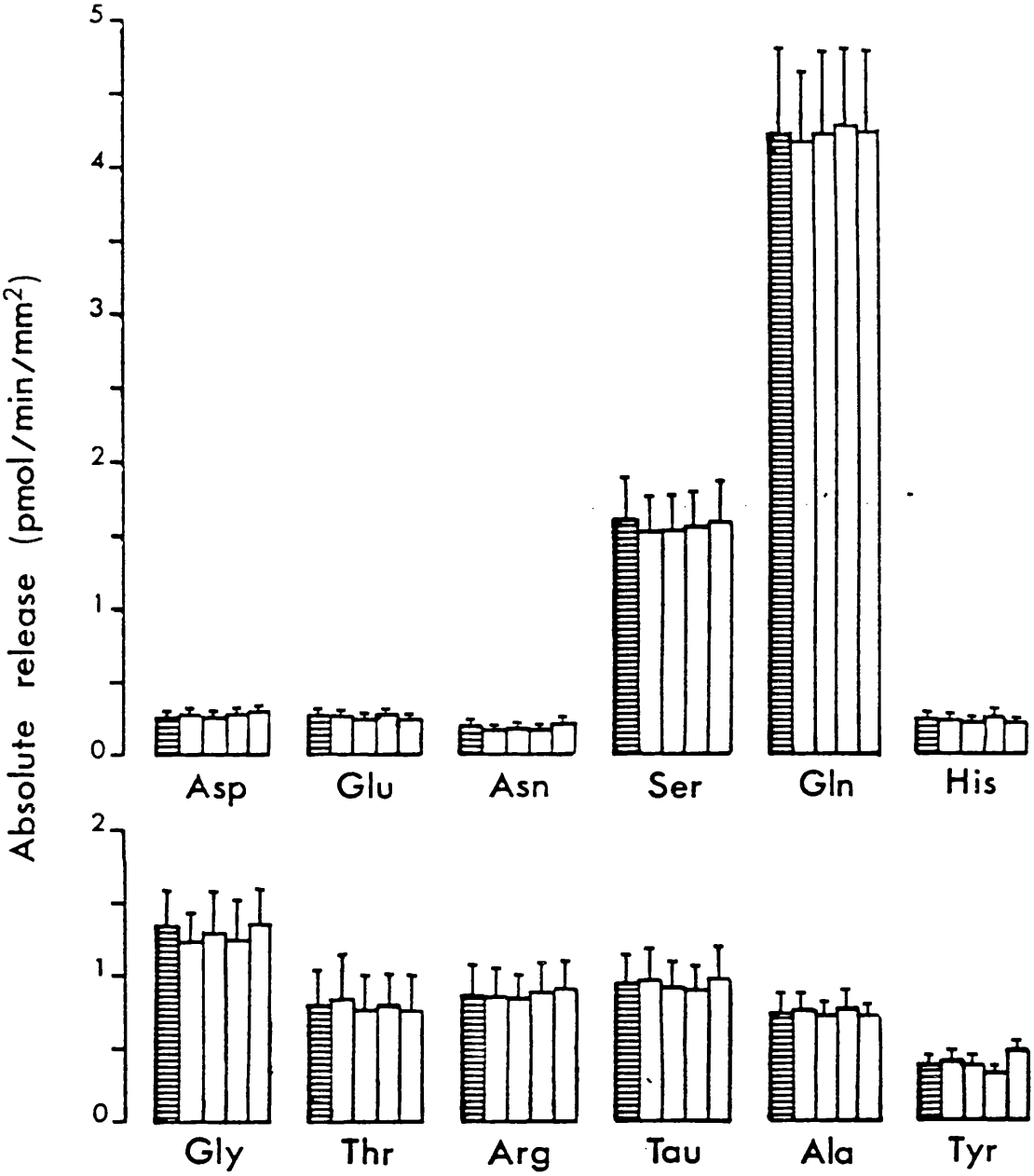

Fig.4.8: Overall time-course of amino acid release during 300μM picrotoxin superfusion *in vivo*. (▤) shows the basal release before the 5 min superfusion of 300μM picrotoxin through the cortical cup, (□) represent successive 10 min samples collected as soon as picrotoxin was in contact with the cortex until cessation of spiking. The histograms represent mean absolute release , ρmol/min/mm^2 of exposed cortical surface, $\pm$ s.e. (n=5).

4:2:4: High K^+:

The failure to detect any changes in the efflux of amino acids during BM and picrotoxin superfusion, despite the fact that extensive EEG spiking could be seen (chapter 3), lead me to test the viability of the cortical cup system in detecting changes in the release of amino acids. The effect of a high concentration of K^+, which is used as a standard depolarizing agent, on the release of amino acids was, therefore, studied. Again after the initial 45 min recovery period 60mM K^+ ACSF solution was superfused for 10 min followed by further continuous superfusion of ACSF. Three continuous 10 min samples were collected starting before the introduction of the high K^+ solution. 60mM K^+ lead to a significant increase in the efflux of GABA and taurine from the cerebral cortex as well as reducing the release of glutamine. The increase in GABA release subsided after removal of the high K^+ solution whereas the changes in glutamine and taurine release persisted even after its removal. Specimen traces showing the variations in the release are given in Fig.4.9 and the overall results summarized in Fig.4.10. The changes in glutamine and GABA release were not seen when the 60mM K^+ ACSF was substituted by one containing no Ca^{++} whereas the induced increase in taurine also occured when Ca^{++} free medium was used (Fig.4.11)

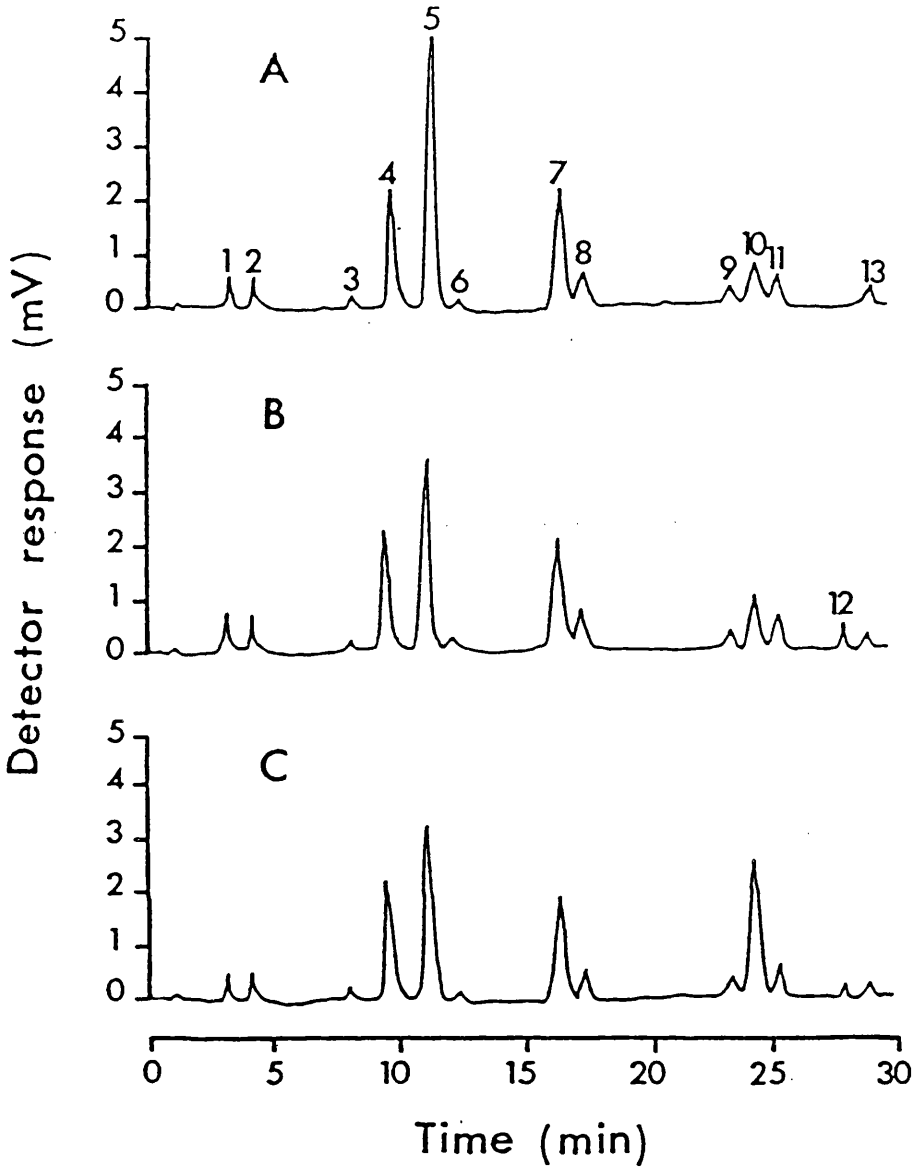

Fig.4.9. Representative chromatograms of the effect of cortical superfusion of 60mM K^+ solution on amino acids release. The basal release is shown in trace A, trace B illustrates the efflux of amino acids during 10 min exposure to 60mM K^+ and trace C shows the changes in the release of various transmitters in the sample collected in the 10 min immediately after 60mM K^+ superfusion. Aspartate(1), glutamate(2), asparagine(3), serine(4), glutamine(5), histidine(6), glycine(7), threonine(8), arginine(9), taurine(10), alanine(11), GABA(12) and tyrosine(13).

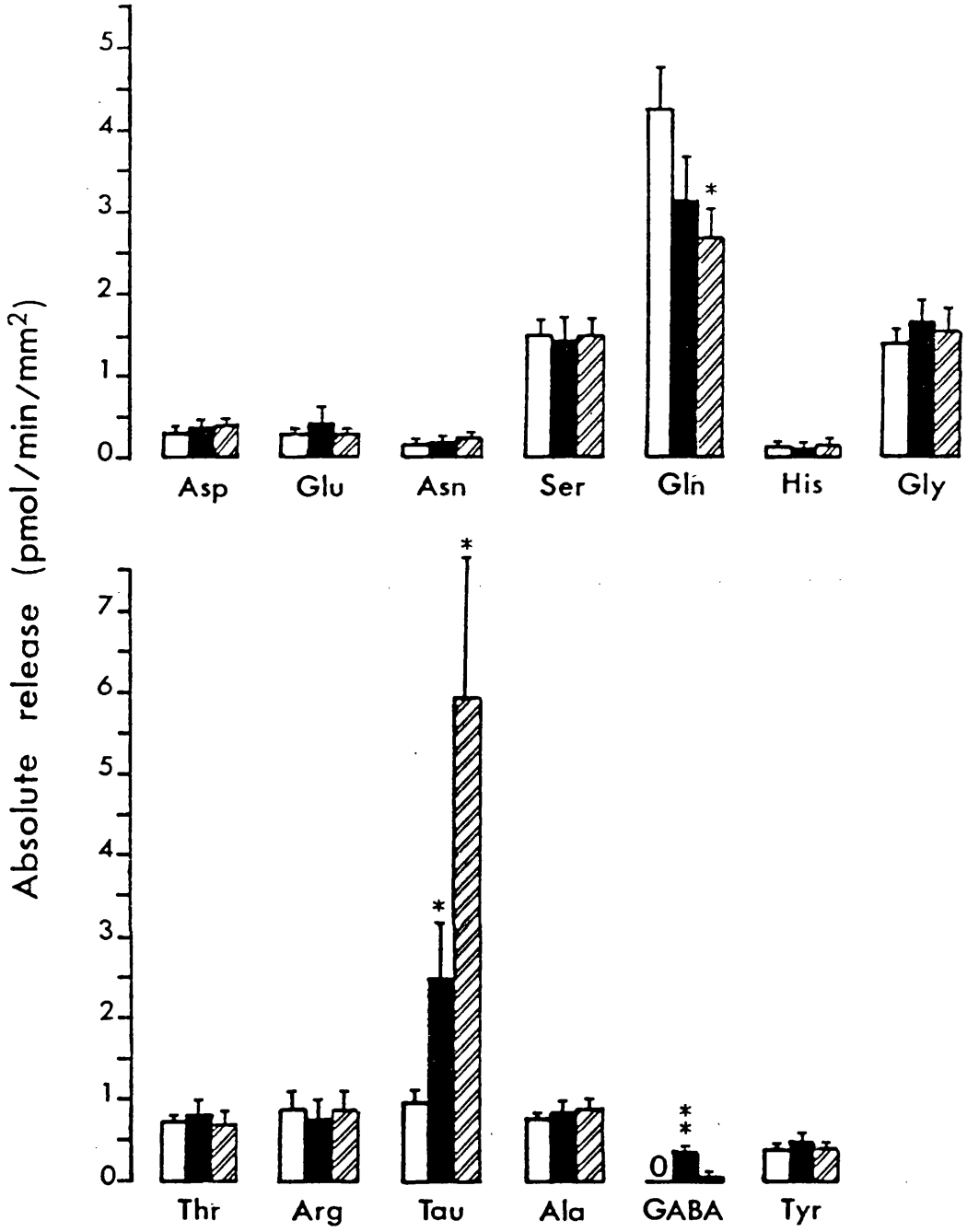

Fig.4.10. Changes in the release of all the amino acids studied prior, during and after *in vivo* cortical superfusion of 60mM K^+. (□) shows the basal release, (■) is the release during 60mM K^+ exposure and (▨) illustrates the release of amino acids immediately after such an exposure. The histograms are successive 10 min samples and represent mean absolute release, pmol/min/mm² of exposed cortical surface, ± s.e. (n=5). *P<0.05 and **P<0.01 show significance of results (paired t-test) compared to the basal release of different amino acids.

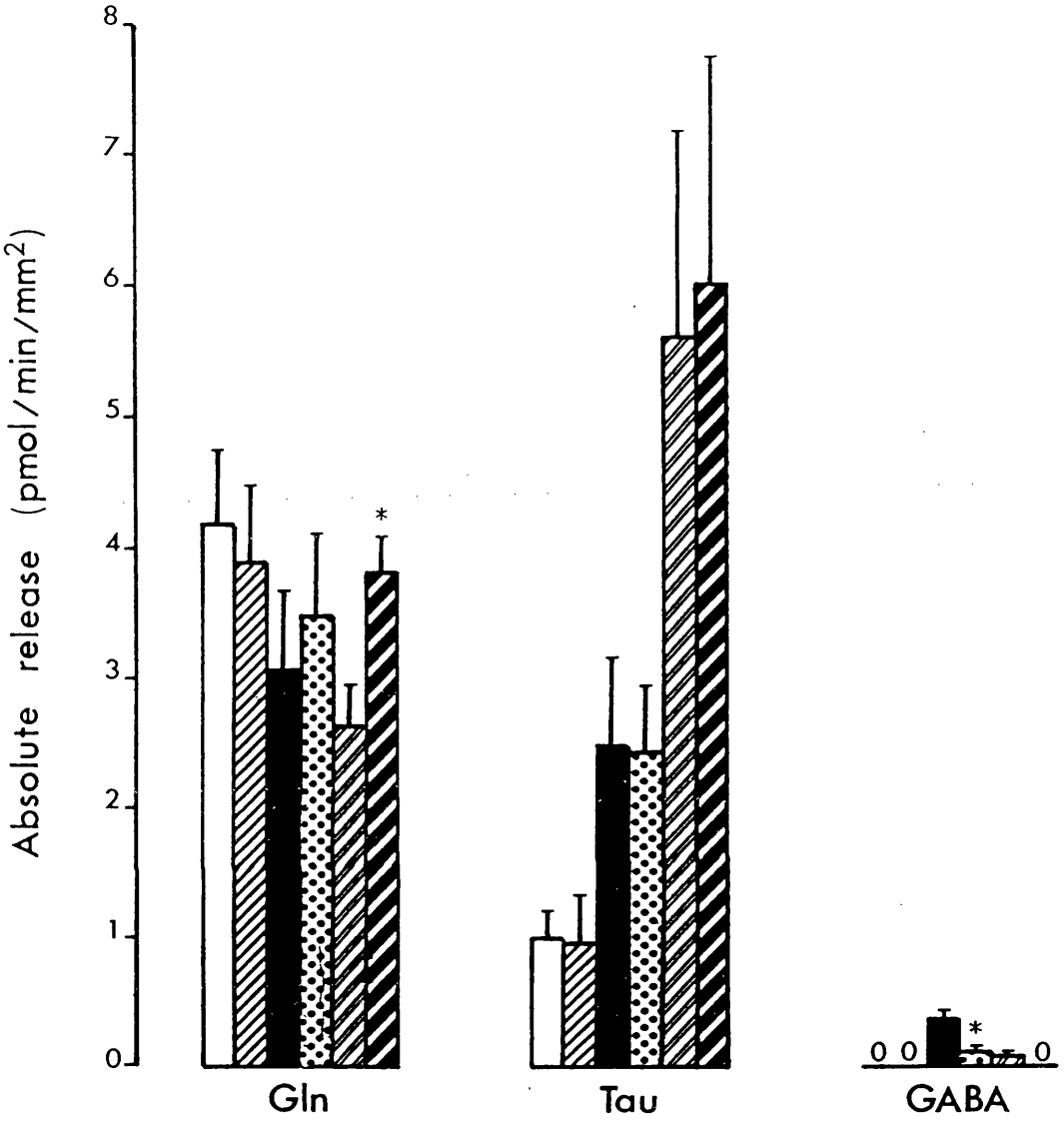

Fig.4.11. Ca^{++}-dependency of 60mM K^+-induced changes in the release of amino acids *in vivo*. The histograms represent mean absolute release, ρmol/min/mm² from exposed cortical surface, ± s.e. (n=5 for control experiments and 4 for Ca^{++}-free). *P<0.05 shows significant difference between normal and Ca^{++}-free release before, during and after 60mM K^+ (unpaired t-test).

(□)= basal release (control)

(▨)= basal release (Ca^{++}-free)

(■)= release during 60mM K^+ exposure (control)

(⊡)= release during 60mM K^+ exposure (Ca^{++}-free)

(▨)= release after 60mM K^+ exposure (control)

(◪)= release after 60mM K^+ exposure (Ca^{++}-free)

4:2:5: Discussion:

Basal amino acid efflux:

The basal release of endogenous amino acids in these experiments were similar to those reported by other investigators. Table 4.1 shows a comparison between my data and those reported by other investigators in three other similar studies.

Although the cortical cups used by these investigators had an internal diameter of 4mm, which was exactly the same size as cups used in these experiments, other experimental differences make simple comparisons difficult. In the experiments performed by Connick & Stone (1988b) and Clark & Collins (1976) the rats were anaesthetised using urethane which makes their findings directly comparable to mine. Abdul-Ghani et al. (1981), however, used reversible anaesthetic during the surgical procedure involved in placing the cortical cup and studied the release of endogenous amino acids in free moving, unrestrained rats. The cortical area used by Connick & Stone (1988b) was very similar to my experiments being in a central position between the coronal and lambdoid sutures. Abdul-Ghani et al. (1981) studied the release from the sensorimotor cortex and Clark & Collins (1976) the visual cortex. Other differences between these experiments and those of Connick & Stone (1988b) and Clark & Collins (1976) was that I have used a continuous superfusion system whereas these workers have placed a set volume of ACSF (100μl in the case of Connick & Stone, 1988b and 50μl in the case of Clark & Collins, 1976) into the cup replacing it every 10 min and they have also drained the CSF, by puncturing the cisterna, to prevent cerebral oedema in their experiments. Abdul-Ghani et al. (1981), on the other hand, have used a continuous superfusion system but the flow rate used (0.1ml/min) was more than three times faster than the flow

rate used in my experiments (30μl/min).

Although at first sight the results of Abdul-Ghani et al. (1981) and Clark & Collins (1976) appear to be much higher than the levels detected in these experiments it is important to note that Abdul-Ghani et al. (1981) have expressed the results in ρmol per cm^2 of exposed cortical surface whereas my data are only presented as ρmol per mm^2 and will, therefore, be 100 times less. Clark & Collins (1976) showed the total level of amino acids released in 50μl in a period of 10 min. The results presented by Connick & Stone (1988b) are, however, considerably higher than my results and also other results mentioned in table 4.1. In order to convert the results by Connick & Stone (1988b) to ρmol/min/mm^2 the values have to be multiplied by a factor of 10^3 which would make them appreciably higher than the other results. Apart from such an apparent discrepancy and although there are differences in the design of the experiments some unifying aspects can be seen in the data presented.

Amino acid	Basal release according to: My experiments (ρmol/min/mm²) n=28	Connick & Stone (1988b) (μmol/10min/cm²) n=10	Abdul-Ghani et al., (1981) (ρmol/min/cm²)	Clark & Collins (1976) (ρmol/10min) n=24
Asp	0.26 ± 0.009	1.32 ± 0.17	48 ± 9(27)	229 ± 30
Glu	0.27 ± 0.008	2.95 ± 0.33	37 ± 6(18)	509 ± 59
Asn	0.18 ± 0.004	NM	NM	NM
Ser	1.53 ± 0.023	NM	*304 ± 27(14)	NM
Gln	4.15 ± 0.026	5.34 ± 0.06	*304 ± 27(14)	904 ± 112
His	0.15 ± 0.019	NM	NM	NM
Gly	1.36 ± 0.012	0.72 ± 0.01	186 ± 20(12)	117 ± 19
Thr	0.76 ± 0.015	NM	NM	NM
Arg	0.85 ± 0.004	NM	NM	NM
Tau	0.94 ± 0.012	0.80 ± 0.16	NM	223 ± 27
Ala	0.73 ± 0.007	0.82 ± 0.08	119 ± 14(25)	129 ± 14
GABA	ND	0.34 ± 0.05	ND	32 ± 4
Tyr	0.37 ± 0.008	NM	NM	NM

Table 4.1. Comparison of basal release of endogenous amino acids in my experiments with that reported by other investigators in similar *in vivo* studies.

The values represented are all mean ± s.e. The n-values for Abdul-Ghani et al. (1981) are shown in the brackets and *shows the combined value for glutamine and serine.

NM = not mentioned

ND = not detected

In all experiments glutamine exhibited the highest basal release followed by glutamate in the systems where static perfusion was used (Connick & Stone, 1988b and Clark & Collins, 1976) and by glycine and serine in my experiments and that of Abdul-Ghani et al. (1981) where a continuous superfusion system was undertaken. It is possible that in a static system more glutamine and possibly glycine and/or serine are converted to glutamate than in a continuous system when it is quickly removed and there is less chance of this conversion. Direct comparison between a static and continuous superfusion system is provided by comparing my data with that reported by Connick & Stone (1988b). The levels of glutamine released in these experiments are 15 times more than glutamate whereas such a ratio is only approximately 2 in the case of experiments by Connick & Stone (1988b). Although the superfusion rate used in these experiments was 3 times slower than those employed by Abdul-Ghani et al. (1981) the ratio of glutamine over glutamate in their superfusates was much lower than mine. This discrepancy could be due to the inhibitory effect of urethane on the release of glutamate (Moroni et al., 1981) in my experiments. It is also important to note that Abdul-Ghani et al. (1981) not only removed the dura but also pierced the pia membrane 10-15 times at avascular points which could also have lead to an increased basal level for glutamate.

I was not able to detect any basal release of GABA in the superfusates which is in agreement with Abdul-Ghani et al. (1981) and could be due to the efficient uptake of GABA into cortical neurones and glia and/or its avid breakdown. Infact Abdul-Ghani et al. (1981) succeeded in detecting small amounts of GABA only after systemic or local administration of γ-vinyl GABA, which is a GABA-transaminase (GABA-T) inhibitor, emphasizing the latter point. Connick & Stone (1988b) and Clark & Collins (1976) have used static superfusion systems

and have been able to detect GABA although the levels detected were the lowest of all the amino acids studied. Other workers who have reported a small basal release of GABA *in vivo* using cortical cups have superfused a larger part of the cortex (Moroni et al., 1981) and have also added a GABA-T inhibitor such as AOAA (Iversen, Mitchell & Srinivasan, 1971). The basal release of amino acids was not Ca^{++}-dependent in these experiments.

Stimulated amino acid efflux:

The intravenous infusion of PTZ produced developing epileptogenic activity but even when this was maximal the only change seen in amino acid efflux was an increase in glutamine. This increase could be due to the classical 'glutamine cycle' (see Shank & Aprison, 1981) representing an increase in the activity of the glutamate containing neurones in the cortex. In short, the increase in the activity of glutamate containing neurones increases the release of glutamate into the extracellular space. This is rapidly taken up by glial cells and converted into glutamine which is in turn released into the extracellular fluid. Glutamine is then taken up into the neurones to act as the precursor for glutamate and the cycle continues. This could result in a build up of glutamine which is detected in these experiments. PTZ infusion also leads to a significant increase in blood pressure leading to possible contamination of the samples with blood even if this was not visible. This, however, could not account for the increase in glutamine since although such contamination is shown to alarmingly increase the levels of aspartate, glutamate, asparagine, glycine and alanine it has no effect on glutamine levels (Kornhuber, Kornhuber, Kornhuber & Hartmann, 1986).

Although amino acids have been shown to be important in

epilepsy, epileptogenic-induced changes in their release has not been consistently shown by other investigators (Lehmann, Hagberg, Jacobson & Hamberger, 1985 & Lehmann, 1987). The inability to detect any changes in the levels of the putative neurotransmitters aspartate, glutamate or GABA could be indicative of: 1) a very efficient uptake system for these amino acids and their enzymatic breakdown before detection, and 2) PTZ-induced epileptogenic activity being initiated in the deeper layers of the cortex so that any changes in the efflux of amino acids would not be detected on the surface of the cortex. Thus either the uptake and breakdown of the amino acids precludes their detection in the cup perfusates and/or these amino acids are released from deeper layers of cortex.

The efficient uptake of amino acid neurotransmitters could play a vital part in the failure to detect changes although this has recently been disputed at least in the case of glutamate by Millan, Obrenovitch, Sarna, Lok, Meldrum & Symon (1990) who used glutamate uptake blockers, which induced an increase in the basal release of glutamate, and were still unable to detect changes in glutamate release during epileptogenic activity. In some of my experiments the non-specific uptake blocker, PCMS, was used in an attempt to study the effect of uptake on the release of endogenous amino acids. This agent had previously been used successfully in our laboratories to increase amino acid efflux in the cat spinal cord *in vivo* (Fagg, Jordan & Webster, 1976) and had the added advantage of not interfering with our HPLC detection system. Higher concentrations of PCMS (10mM) than used previously (100μM), however, had to be used to observe any increase in the basal release of amino acids. In the presence of 10mM PCMS the basal release of most amino acids showed an increase and the intravenous infusion of PTZ lead to increases in the efflux of almost all of the amino acids

studied with the maximum change seen in the last sample collected (Fig.4.4). The results obtained could signify the importance of amino acid uptake systems in detecting any possible changes in their efflux. However, it is important to note that high concentrations of PCMS had to be used and these could result in effects related to the toxicity of mercurial compounds rather than their effect in blocking the uptake of amino acids (see later in the case of cortical superfusion of BM and picrotoxin).

Any PTZ-induced amino acid release should be more evident if the convulsant is applied directly to the cortex and, therefore, PTZ was applied by cortical superfusion. This was associated with distinct epileptogenic spiking in the EEG (chapter 3, Fig.3.2) and lead to increases in the release of most of the amino acids studied (Fig.s 4.5 & 4.6). This increased release of amino acid neurotransmitters could be related to the increased firing rate of cortical neurones. However, the high concentrations of PTZ required and the rather non-specific nature of the release could be due to an increase in the metabolism of the neuronal tissue, as amino acids also play an important role in metabolic reactions. Also it is important to note that although the epileptogenic EEG activity seen during the intravenous infusion of PTZ was similar to that recorded after its local application, intravenous infusion did not have the same extensive effect on amino acid release. It is likely that the local application of PTZ could have increased the release by causing some damage to the neuronal tissue and the leakage of amino acids. It would have been useful to investigate the Ca^{++}-dependency of this effect, together with that of the interesting reduction in aspartate release on exposure to 100mM PTZ to establish whether the release was neuronal or glial.

No changes in the efflux of any of the amino acids studied could

be seen after exposure to either 200μM BM (Fig.4.7) or 300μM picrotoxin (Fig.4.8). Inclusion of PCMS (up to 1mM) did not affect the basal release or the release during epileptogenic spiking activity, induced by these convulsants. It was decided not to use 10mM PCMS as it had a detrimental effect on the spiking activity.

Although spiking activity induced by cortical superfusion of BM or picrotoxin was very similar to, and infact more extensive than, that produced by PTZ cortical superfusion, striking differences were seen in the levels of amino acids released during these separate spiking activities. This suggests that either PTZ induces spiking by a mechanism different from that of BM or picrotoxin or more probably the increases seen in the case of PTZ are not related to spiking but are due to some other non-physiological effect.

The epileptogenic site of action of BM is thought to be in layer IV or upper parts of layer V of the cortex (Connors, 1984; Campbell & Homes, 1984 and Jones & Lambert, 1990). Any changes in the efflux of amino acids in these deeper layers could only be indirectly measured if they diffuse onto the pial surface, by which time the amounts will be too low to be detected because of the breakdown and/or uptake of these amino acids.

In an attempt to investigate the ability of the system to detect changes in amino acid release if neurones or glia are depolarized in the superficial layers of cortex, It was decided to superfuse a high K^+ solution across the cortex. This only showed increases in the efflux of three amino acids glutamine, GABA and taurine with the efflux of others remaining unchanged (Fig.s 4.9 & 4.10). In a similar set of experiments Clark & Collins (1976) reported that 50mM K^+ induced an increase in the levels of GABA and taurine together with a decrease in the level of glutamine which agree with the results presented here. Again in

agreement with these investigators, It was found that the K^+-induced increase in GABA together with the reduction in glutamine were Ca^{++}-dependent whereas the increase in taurine release was not affected when Ca^{++}-free media were used. The changes in the levels of GABA and glutamine could be due to glutamine acting as the GABA precursor (Ward, Thanki & Bradford, 1983) and the main amino acid 'pool' for production of GABA during high K^+-evoked depolarization (Tapia & Gonzalez, 1978) leading to a reduction in glutamine and a resultant increase in GABA release. My results fail to show any increase in the levels of aspartate or glutamate. A similar finding was reported by Clark & Collins (1976), who had to increase the extracellular concentration of K^+ to 100mM before detecting any increase in the release of these two excitatory amino acid neurotransmitters which again could be due to their efficient uptake in the cortex. Another possible reason for failing to detect a K^+-induced increase in the release of glutamate could be the removal of the dura, which has been shown to affect the K^+-induced release of this excitatory amino acid from cortex (Moroni et al., 1981). Although taurine release is increased on exposure to 60mM K^+ the change was not Ca^{++}-dependent suggesting a non-neuronal source of its release. It is also important to note that in the *in vivo* experiments the animal is anaesthetised and the dampening effect of urethane on amino acid release has been shown by other investigators (Skerritt & Johnston, 1984; Evans & Smith, 1982 and Moroni et al., 1981).

4:2:6: Conclusions:

The changes seen in the efflux of glutamine during intravenous infusion of PTZ as well as other amino acids during its cortical superfusion provide important evidence linking the epileptogenic activity of this convulsant to amino acids neurotransmission although in each instance high concentrations had to be used. Such a link could not be established for other convulsants studied. However, given the apparant insensitivity of the cortical cup system to detect changes in the release of amino acid neurotransmitters it was considered important to study the effect of these agents in an *in vitro* system using cortical slices, when any changes in amino acid release may be more readily detected.

4:3: *IN VITRO* STUDIES:

Summary of technique:

Rats were anaesthetised with halothane and then killed by neck dislocation. The piece of cortex on which the cortical cup would have been placed was dissected out and 0.4mm slices were made. These were incubated for 45 min before transferring two slices into each of the perfusion chambers. Oxygenated ACSF, at 37°C, was perfused over the slices for a period of 15 min before a 6 min exposure to drugs under investigation. The flow rate employed was 300μl min^{-1} and continuous 3 min samples were collected starting 6 min before drug exposure until 6 min after drug removal. Samples were immediately stored at -30°C for later analysis of their amino acid content (for full details refer to chapter 2, section 2:2:5).

4:3:1: High K^+:

It was decided to first study the effect of the standard depolarizing agent, high K^+, to ascertain the viability of the *in vitro* technique before investigating the influence of convulsants on the amino acid efflux from cortical slices. Perfusion of 60mM K^+ solution lead to significant increases in the release of aspartate, glutamate, glycine and GABA from cortical slices. The changes seen were greatest in the second sample collected during high K^+ exposure but the induced increase quickly subsided on removal of high K^+ solution. Fig.4.12 shows representative traces of the release of amino acids in the sample collected before high K^+ exposure and that in the second sample collected during 60mM K^+ exposure, when the induced release was at its maximum. An overall summary of the results is shown in Fig.4.13. The 'basal' release was calculated by averaging the amount of amino acids in

the two samples collected prior to 60mM K^+ exposure and the 'induced' release was determined by averaging the amount of amino acids in the two samples collected during 60mM K^+ exposure. High K^+ induced significant increases in the release of glutamate, aspartate, GABA and glycine which, except for the latter, were Ca^{++}-dependent (Fig.4.14).

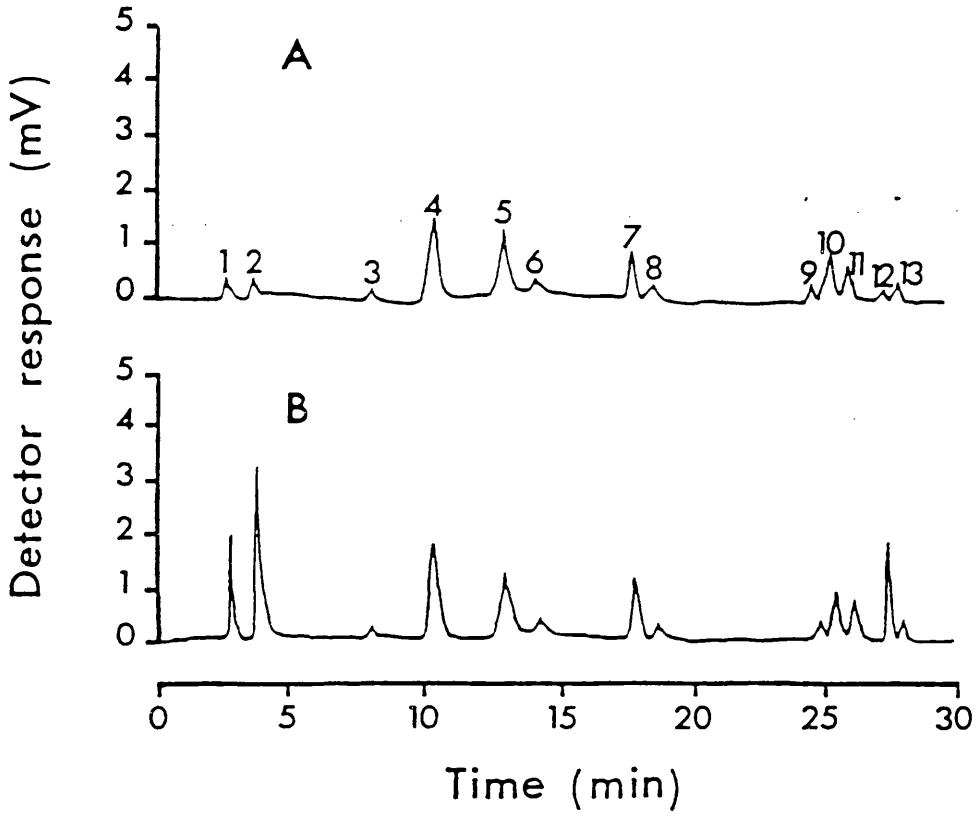

Fig.4.12: Representative chromatograms of the effect of 60mM K^+ solution on the release of amino acids from cortical slices. Trace A shows the basal release of various amino acids in the sample collected just before high K^+ exposure and trace B represents the release in the second sample collected during 60mM K^+ exposure when the changes seen were maximal. Aspartate(1), glutamate(2), asparagine(3), serine(4), glutamine(5), histidine(6), glycine(7), threonine(8), arginine(9), taurine(10), alanine(11), GABA(12) and tyrosine(13).

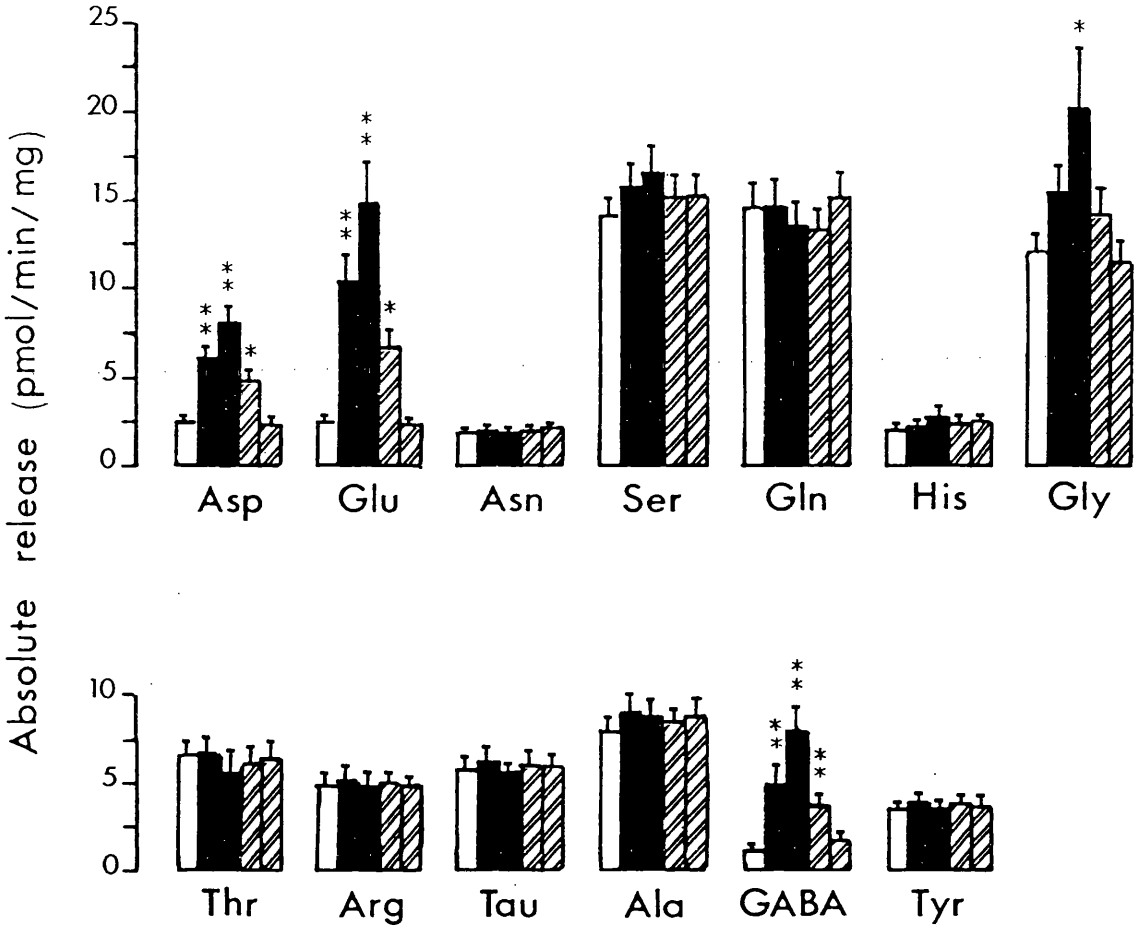

Fig.4.13: The overall time-course of amino acid release before, during and after 60mM K^+ exposure *in vitro*. Each histogram represents mean absolute release (pmol/min/mg) wet weight of slices ± s.e. in 3 min samples. (□) is the 'basal' release, (■) represent the samples collected during 60mM K^+ exposure and (▨) show the samples collected immediately after such an exposure. *P<0.05 and **P<0.0005 show the significance of the results compared to the 'basal' release (paired t-test, n=8).

'basal' release= mean of two samples collected before high K^+ exposure

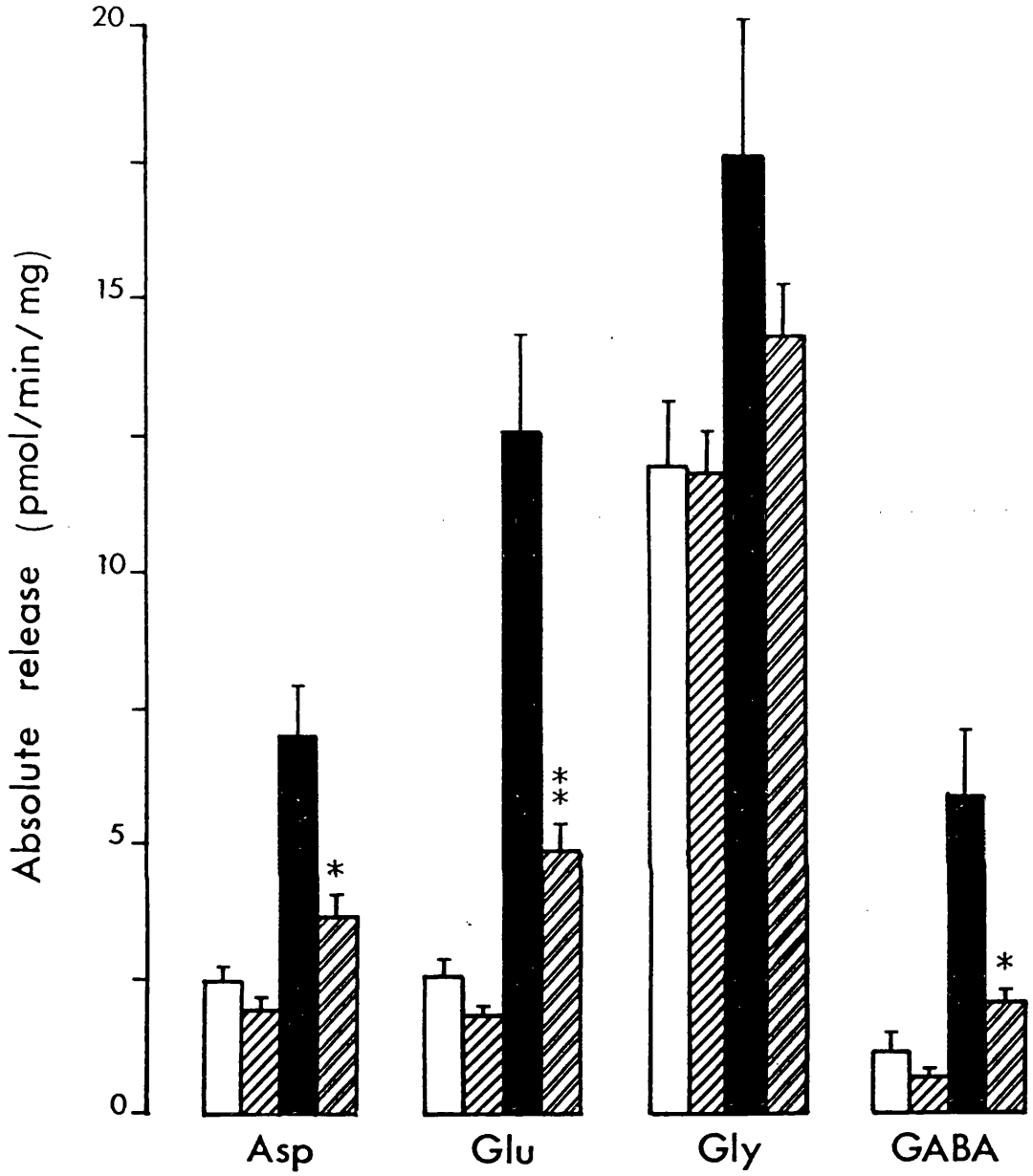

Fig.4.14: Ca^{++}-dependency of K^+-induced increase in release of amino acids from cortical slices. (▭) is the 'basal' release in the presence of Ca^{++} with (▨) representing the 'basal' release in the Ca^{++}-free medium, (■) shows the 'induced' release in the presence of Ca^{++} whereas (▨) is the 'induced' release in the absence of Ca^{++}. The histograms represent mean absolute release, pmol/min/mg wet weight of slices, ± s.e. (n=8). *P<0.05 and **P<0.005 show the significance of results obtained in the Ca^{++}-free medium compared to the corresponding values in the control studies (unpaired t-test).

'basal' release = mean of two samples collected before high K^+ exposure

'induced' release = mean of samples collected during high K^+ exposure

As high K^+-induced release established the viability of the *in vitro* technique, it was adopted to study the effect of PTZ and BM on the efflux of amino acids from cortical slices.

4:3:2: Pentylenetetrazole (PTZ):

Three concentrations of PTZ (200, 300 and 400mM in ACSF) were chosen for preliminary studies. Perfusion of PTZ lead to a dose-related increase in the release of aspartate, glutamate, taurine and GABA only. The 'induced' release (calculated as in the case of 60mM K^+) of all these four amino acids appeared to be lower in the absence of Ca^{++} although not significantly (Fig.4.15). As the osmolarity of the PTZ solutions in ACSF were higher than in the case of control ACSF a solution of sodium citrate in ACSF which had the same osmolarity as the 400mM PTZ solution was prepared; this had no effect on the efflux of amino acids (Fig.4.16).

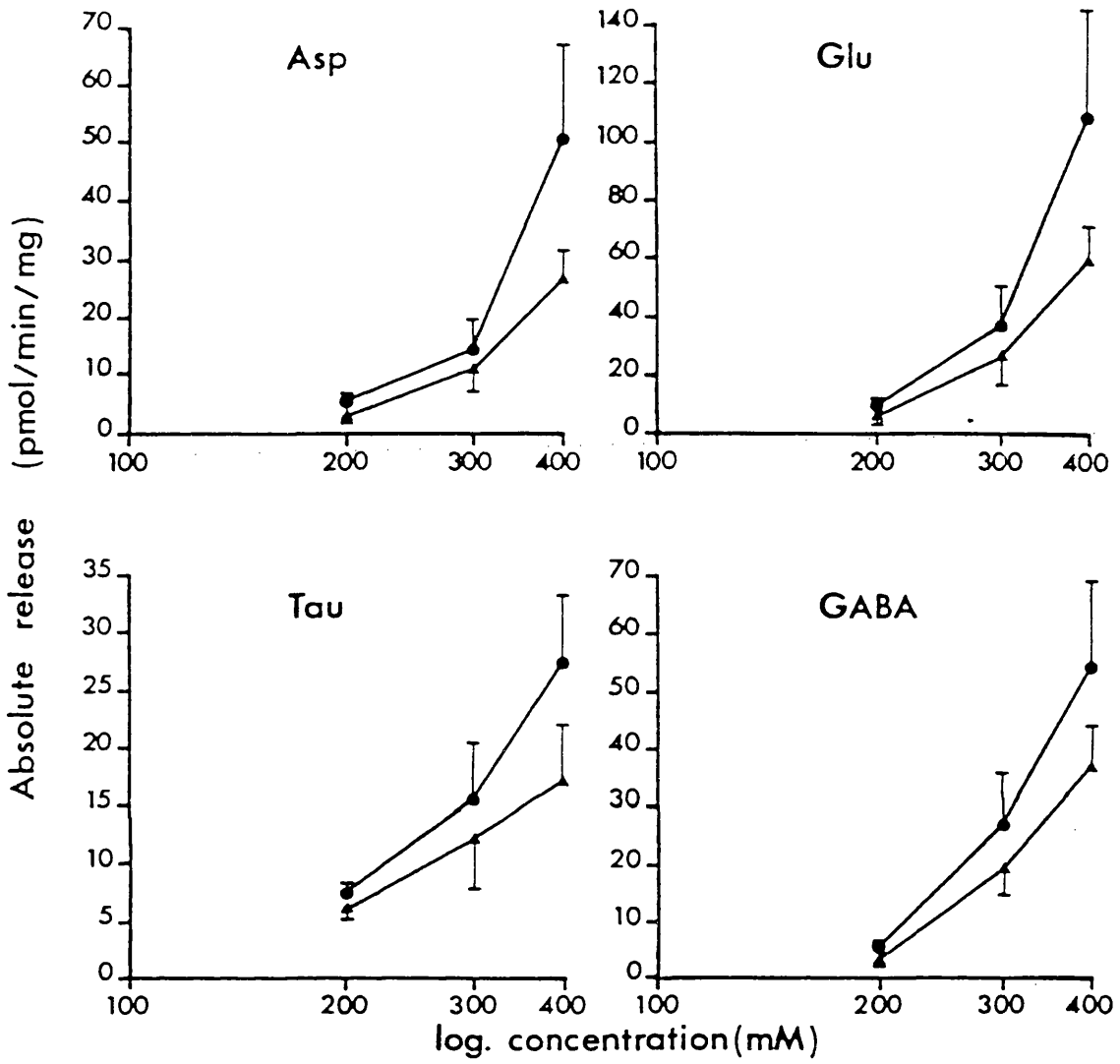

Fig.4.15: Dose-related effect of PTZ on the release of aspartate, glutamate, taurine and GABA from cortical slices and its Ca^{++}-dependency. (●——●) shows the increase in release in the control experiments with (▲——▲) representing the results obtained when Ca^{++}-free media were used (n=5). The values presented are mean ± s.e. absolute release, ρmol/min/mg wet weight of slices.

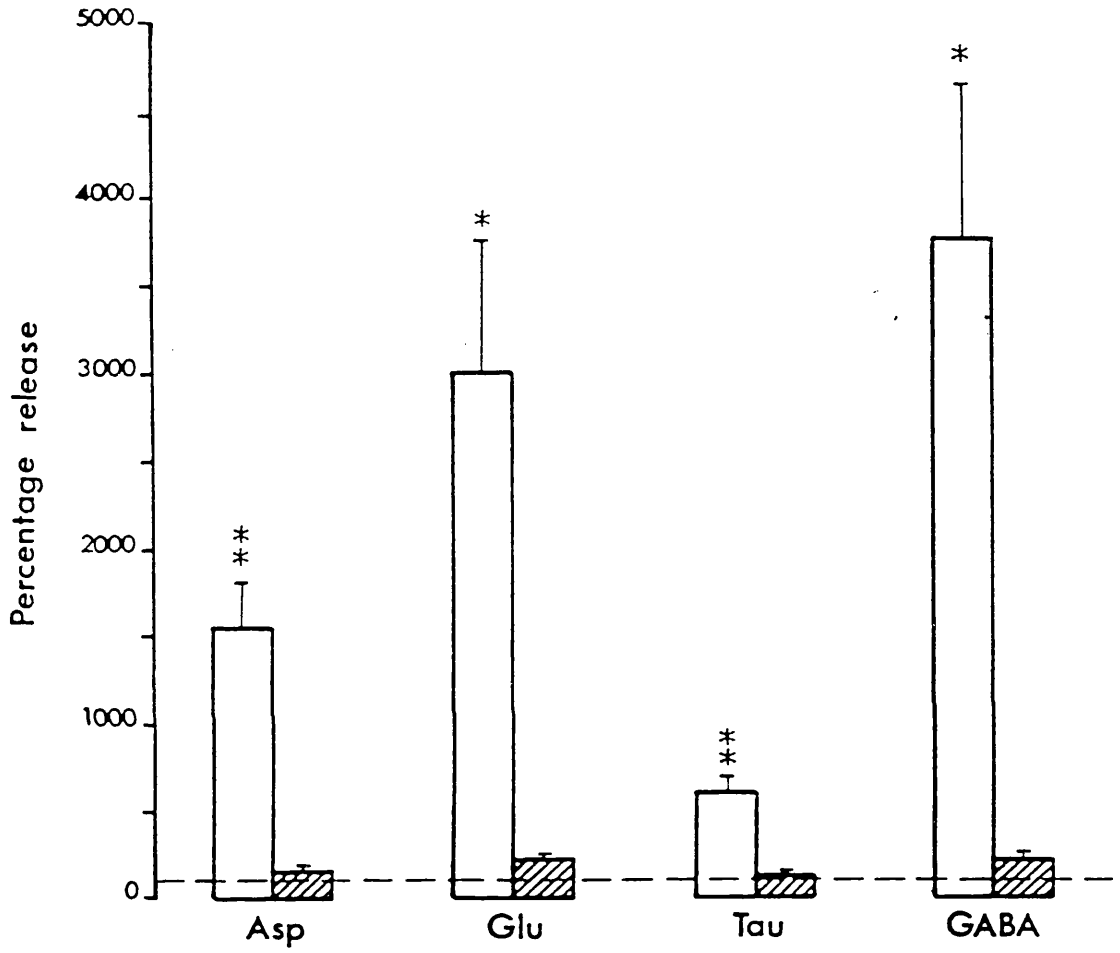

Fig.4.16: Effect of osmolarity on the release of aspartate, glutamate, taurine and GABA from cortical slices. (▭) represents the percentage release of amino acids studied as a result of 400mM PTZ in ACSF perfusion and (▨) shows the percentage release when 130mM sodium citrate in ACSF is perfused. 400mM PTZ and 130mM sodium citrate solutions in ACSF had equivalent osmolarity. The dotted line represents the basal release. *P<0.05 and **P<0.01 show the significance of the increase in release compared to the basal value, paired t-test (n=5 for the control experiments and 4 when sodium citrate was used).

A submaximal dose of 250mM PTZ was chosen to further study the induced release. Analysis of the time course of release showed that release of the amino acids affected continued to increase even after cessation of PTZ superfusion with the release being maximal in the sample collected immediately after PTZ superfusion (Fig.4.17). Representative traces comparing this sample to that collected immediately before PTZ exposure are shown in Fig.4.18.

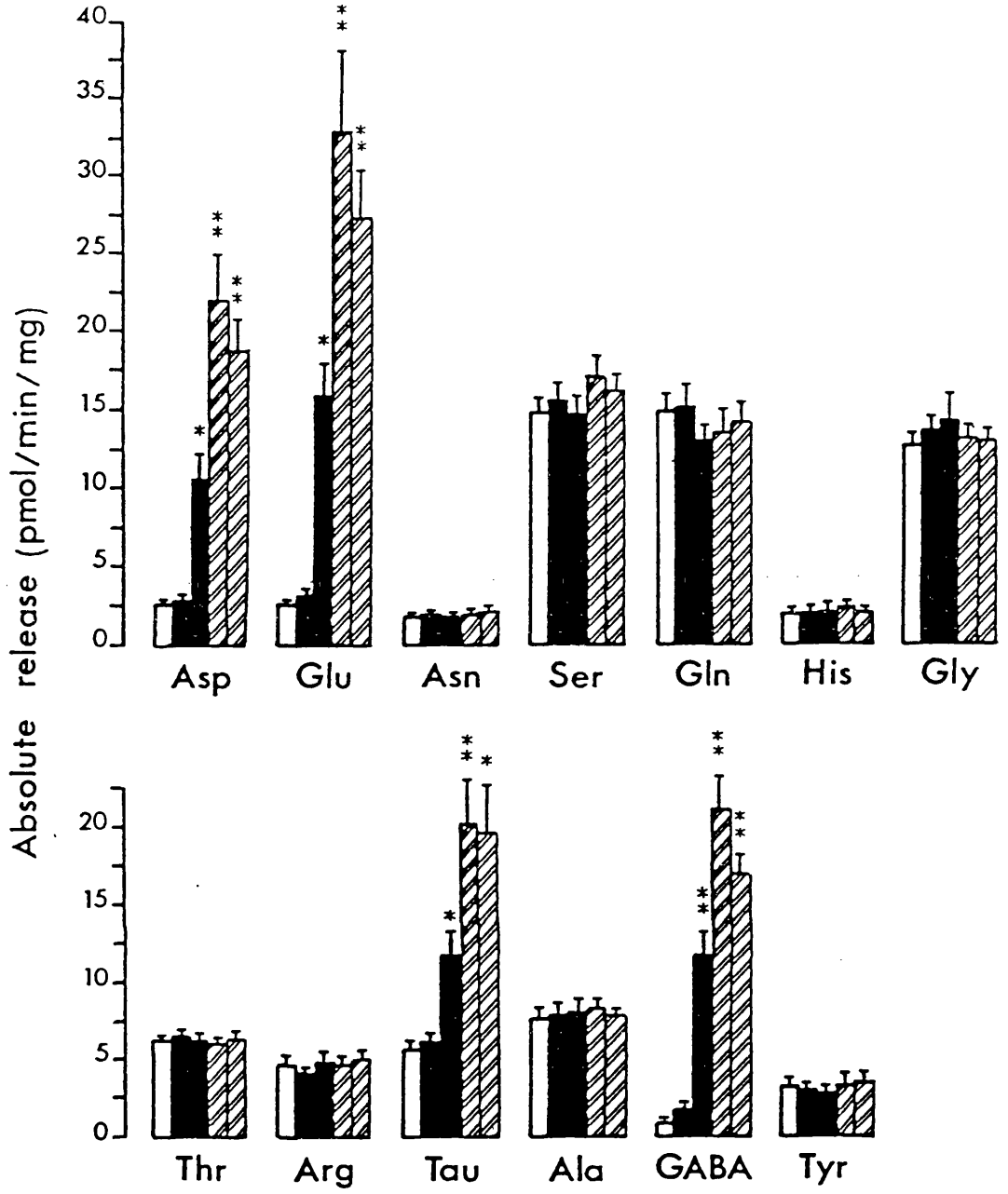

Fig.4.17: The overall time-course of amino acid release from cortical slices as a result of 250mM PTZ perfusion. (□) is the 'basal' release, the two 3 min samples collected during 250mM PTZ perfusion are shown by (■) and the two 3 min samples collected immediately after PTZ perfusion by (▨). The histograms represent mean absolute release, pmol/min/mg wet weight of slices, ± s.e. (n=11). *P<0.005 and **P<0.0005 are the significance of results compared to the 'basal' release in each case (paired t-test).

'basal' release = mean of two samples collected before PTZ exposure.

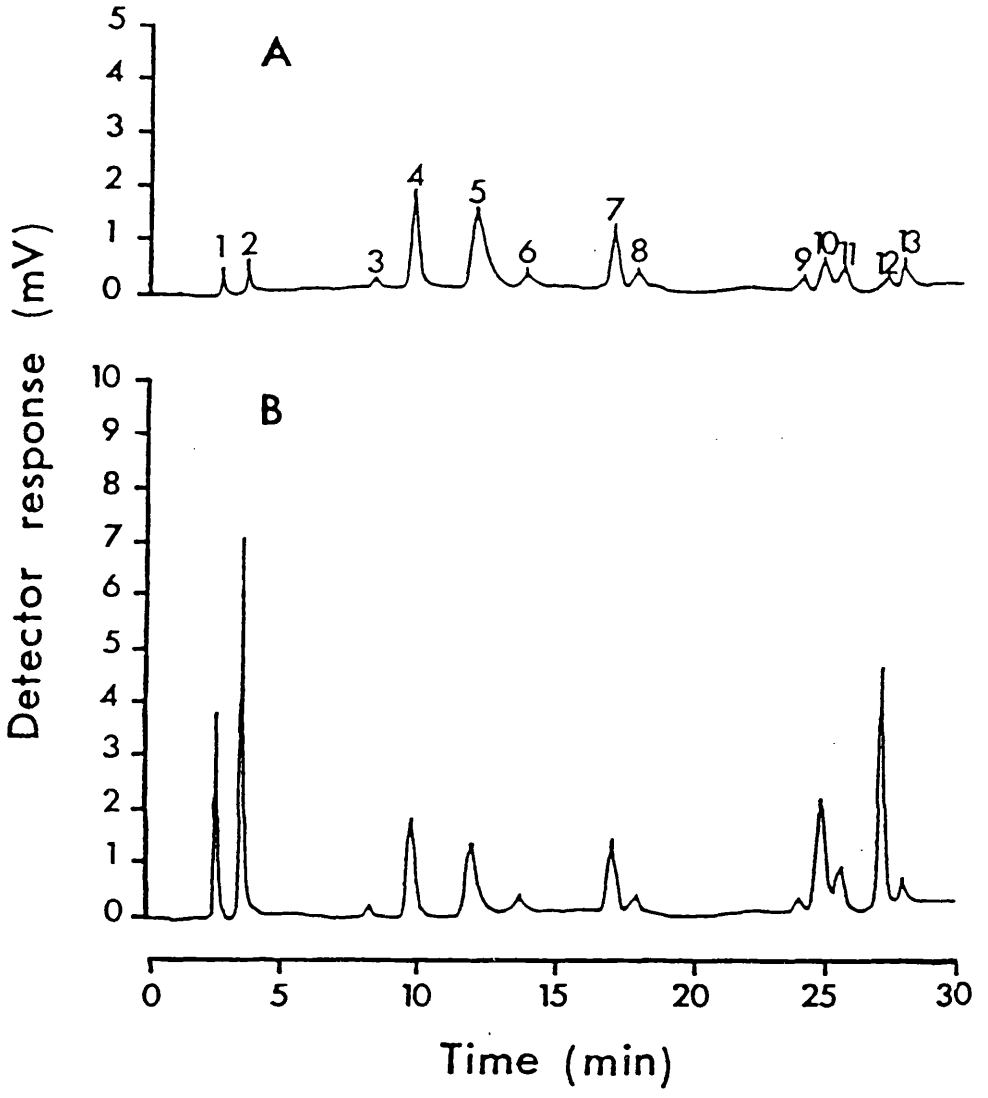

Fig.4.18. Representative chromatograms of the effect of 250mM PTZ perfusion on the release of amino acids from cortical slices. Trace A shows the release of amino acids in the sample collected just before 250mM PTZ perfusion and trace B represents the sample collected immediately after PTZ exposure in which the increase in release was greatest. Aspartate(1), glutamate(2), asparagine(3), serine(4), glutamine(5), histidine(6), glycine(7), threonine(8), arginine(9), taurine(10), alanine(11), GABA(12) and tyrosine(13).

4:3:3: Bicuculline methiodide (BM):

The effect of BM on the efflux of amino acids was determined using 200μM BM which was the same concentration as used in the EEG (chapter 3, section 3:2:3) and *in vivo* release studies (earlier, section 4:2:2). Bicuculline methiodide on its own did not produce a significant change in the efflux of amino acids, however, in the presence of 100μM PCMS, changes in the efflux could be seen on addition of BM. *p*-Chloromercuriphenylsulphonic acid (PCMS) was introduced as soon as the slices were placed in the tissue perfusion chamber. Six 3 min samples were collected as explained in the case of 60mM K^+. The basal release of various amino acids increased when PCMS was added (Fig.4.19) and subsequent exposure to BM, in the presence of 100μM PCMS, induced a further increase in the levels of aspartate, glutamate and GABA only, with other amino acids unaffected (Fig.4.20). The induced release was maximal in the last sample collected and representative HPLC traces of this sample and that collected just before BM exposure are shown in Fig.4.21. The 'basal' release was calculated as in the case of 60mM K^+ but to take into account the increase in release in the samples after BM exposure the 'induced' release was considered to be the average of the four samples collected during and after BM exposure. Both 'basal' and the 'induced' release were Ca^{++}-dependent (Fig.4.22)

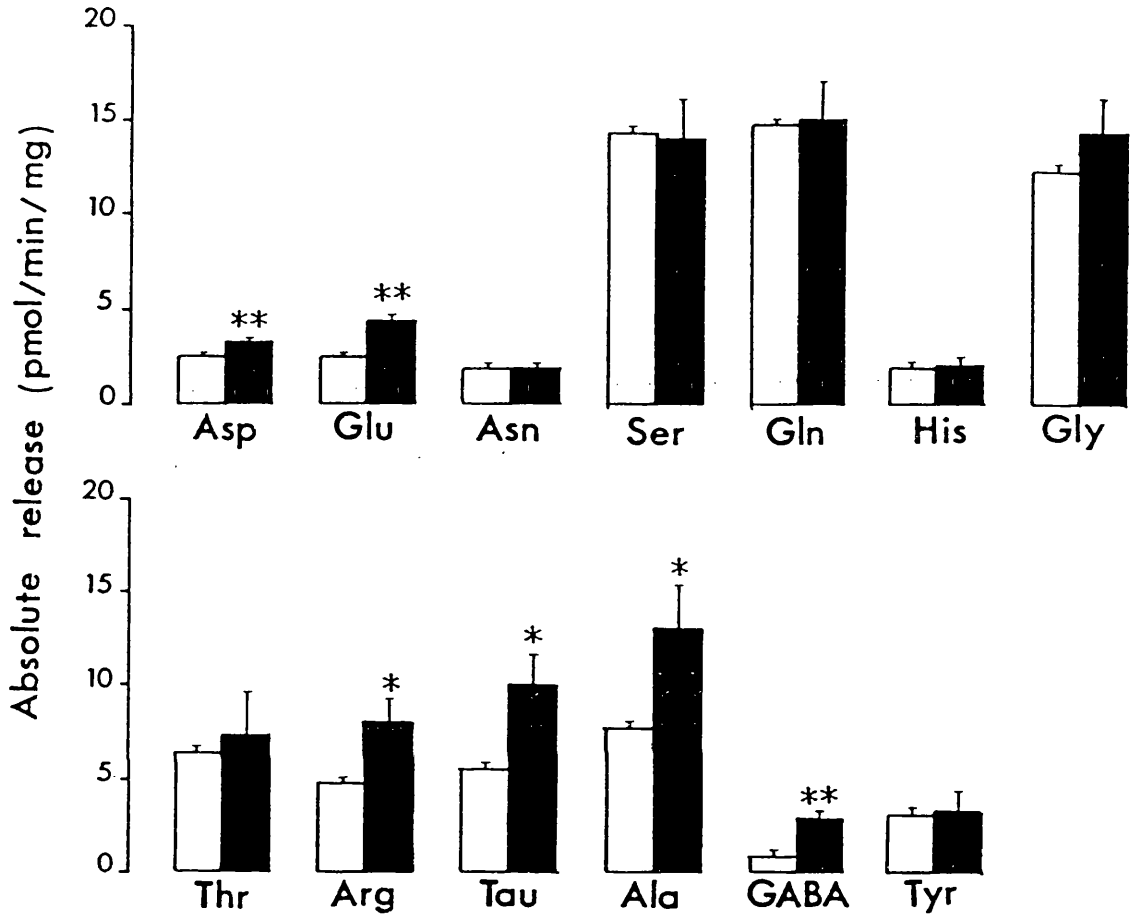

Fig.4.19: The effect of 100μM PCMS on the basal release of various amino acids from rat cortical slices. (☐) is the basal release in the absence of 100μM PCMS and (■) represents the basal release in the presence of 100μM PCMS. The histograms represent mean absolute release, ρmol/min/mg wet weight of slices, $\pm$ s.e. (n=10). *P<0.05 and **P<0.0005 are the significance of results compared to the basal release in the absence of PCMS in each case (unpaired t-test).

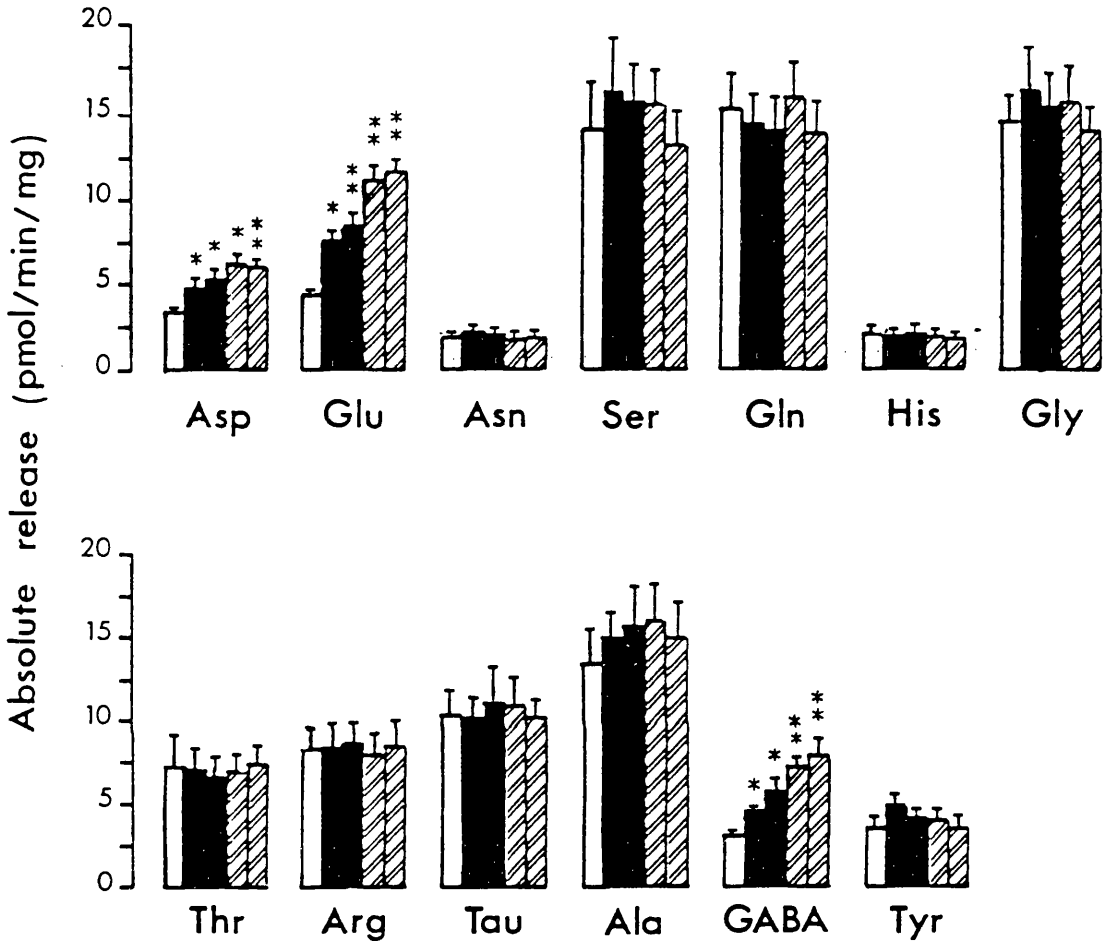

Fig.4.20: The overall time-course of amino acids release before, during and after 200μM BM in ACSF perfusion, from cortical slices in the presence of 100μM PCMS. (□) is the 'basal' release before BM perfusion, (■) represent the two 3 min samples collected during BM exposure and (▨) show the last two samples collected immediately after BM removal. The histograms represent mean absolute release, ρmol/min/mg wet weight of slices, $\pm$ s.e. (n=10). *P<0.05 and **P<0.0005 show the significance of results compared to 'basal' release (paired t-test).

'basal' release = mean of two samples collected before BM exposure

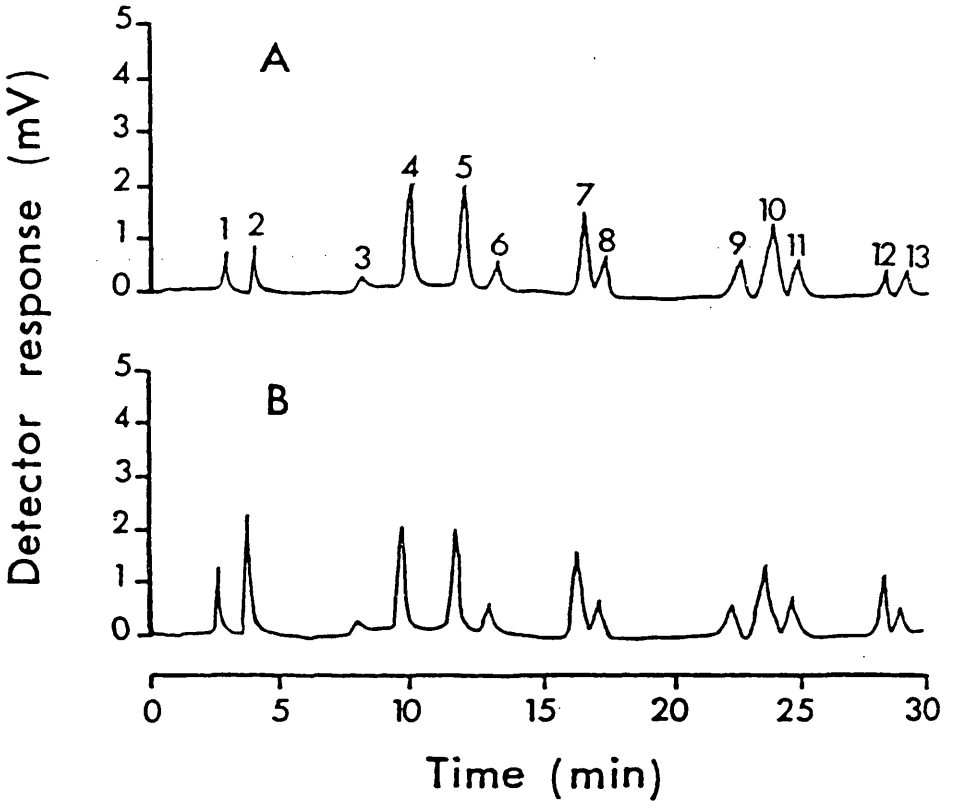

Fig.4.21: Representative chromatograms showing the effect of 200μM BM perfusion on the release of amino acids from cortical slices in the presence of 100μM PCMS. Trace A shows the basal release in the presence of 100μM PCMS and trace B is a representative of the second sample collected after BM removal when the increase in the release was maximal. Aspartate(1), glutamate(2), asparagine(3), serine(4), glutamine(5), histidine(6), glycine(7), threonine(8), arginine(9), taurine(10), alanine(11), GABA(12) and tyrosine(13).

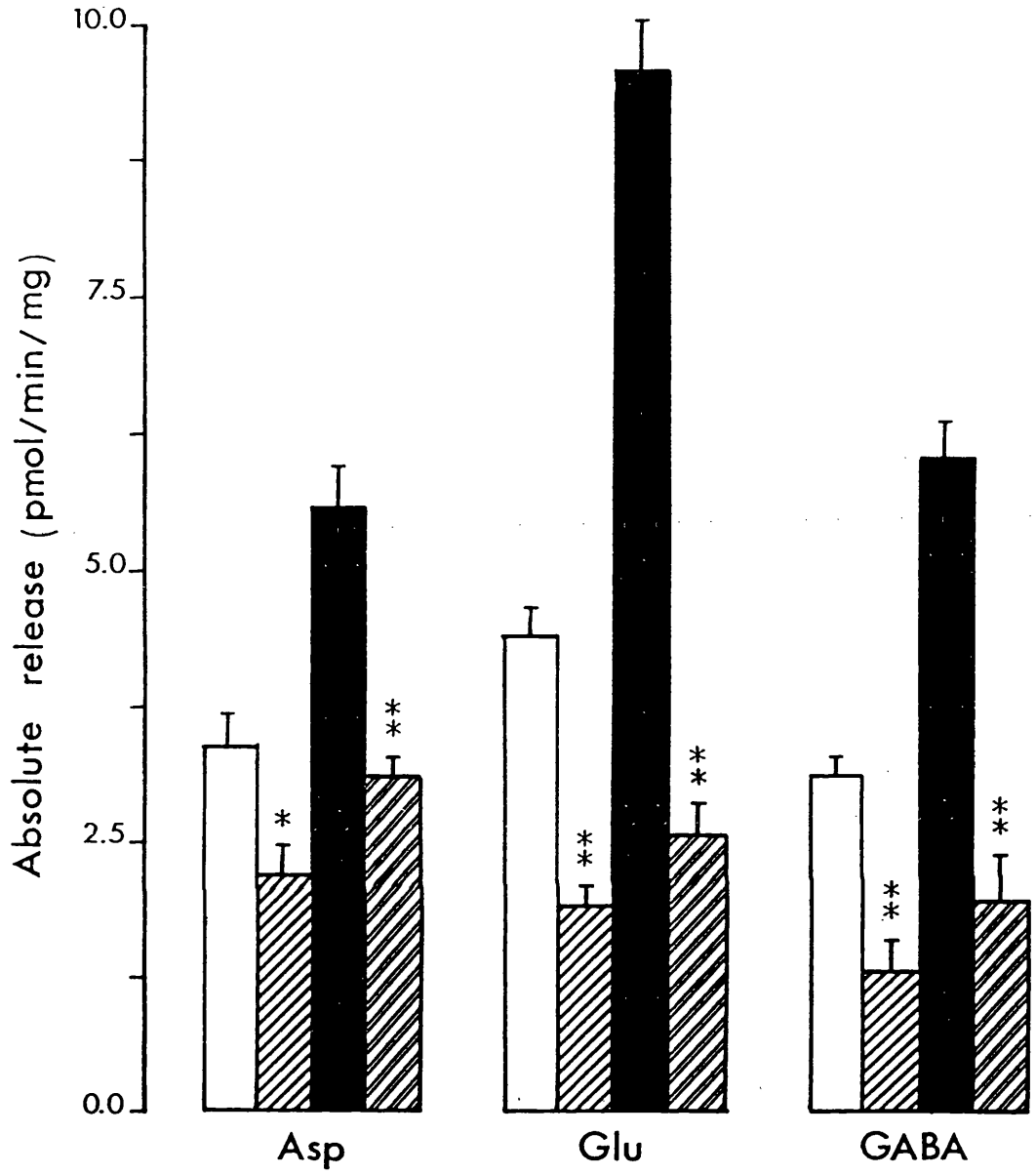

Fig.4.22: Ca^{++}-dependency of the basal and BM-induced increase in amino acid efflux in the presence of 100μM PCMS. The histograms represent mean absolute release, ρmol/min/mg wet weight of slices, $\pm$ s.e. with n=10 for control and 5 for Ca^{++}-free. *P<0.01 and *P<0.001 show the significant effects (unpaired t-test) of Ca^{++} removal.

(□)= 'basal' release (control), (▨)= 'basal' release (Ca^{++}-free)

(■)= 'induced' release (control), (▨)= 'induced' release (Ca^{++}-free)

'basal' release= mean of two samples collected before BM exposure

'induced' release= mean of 4 samples collected during and after BM exposure

4:3:4: Discussion:

Basal amino acid release:

My findings were similar to those reported by other investigators. It is rare to find experiments studying the endogenous amino acid release *in vitro* since most investigators study the release of exogenous amino acid neurotransmitters. However, Table 4.2 shows a comparison between these data and those reported by other investigators in three similar experiments.

Bradford & Richards (1976) studied the release of endogenous amino acids from guinea pig piriform cortex. The slices prepared were 0.3 to 0.35mm thick; they were preincubated at 37°C for 30 min before successive 10 min incubation in 5ml of ACSF at 37°C. Waller & Richter (1980) studied endogenous amino acid release from rat midbrain slices (tissue prisms) made by cutting the tissue at 0.4mm intervals in two planes at 45° angle; the tissue suspension was then transferred to the perfusion chamber and ACSF was continuously perfused over it at 0.5ml/min. In the third set of experiments, Smith, Bowen & Davison (1983) studied the release in prisms prepared from the entire rat cortex. Immediately after dissecting the tissue it was placed in oxygenated, ice-cold medium, was chopped at 0.3mm intervals at two different planes at 45° angle and then was preincubated for 45 min. A static perfusion system was used as in the case of Bradford & Richards (1976) by placing a set volume of tissue suspension in a vial and incubating it for 10min.

Amino acid	Basal release according to: My experiments (ρmol/min/mg) n=24	Bradford & Richards, (1976) (ηmol/g/10min) n=10	Waller & Richter (1980) (ηmol/g/ml) (n)	Smith et al. (1983) (ηmol/100mg) n=22
Asp	2.56 ± 0.051	65 ± 4	5.08 ± 0.55(36)	82 ± 40
Glu	2.50 ± 0.098	88 ± 5	13.5 ± 1.08(38)	128 ± 42
Asn	1.92 ± 0.016	NM	NM	NM
Ser	14.36 ± 0.285	178 ± 8	NM	NM
Gln	14.81 ± 0.326	335 ± 12	NM	242 ± 102
His	2.01 ± 0.038	NM	NM	NM
Gly	12.27 ± 0.339	198 ± 10	16.3 ± 1.34(37)	NM
Thr	6.43 ± 0.130	NM	NM	NM
Arg	4.80 ± 0.100	NM	NM	NM
Tau	5.70 ± 0.047	NM	NM	NM
Ala	7.77 ± 0.075	55 ± 3	16.1 ± 0.89(38)	NM
GABA	1.06 ± 0.070	ND	1.19 ± 0.27(37)	44 ± 24
Tyr	3.36 ± 0.105	NM	NM	NM

Table 4.2. Comparison of basal release of endogenous amino acids in my experiments to that reported by other investigators in similar *in vitro* studies.

The values represented are all mean ± s.e. The n-values for Waller et al. (1980) are shown in the brackets.

NM = not mentioned

ND = not detected

As in the case of *in vivo* results the design of different sets of experiments have to be considered when comparing the data presented in Table 4.2. Bradford & Richards (1976) and Waller & Richter (1980) have presented the release as ηmol/g wet weight of the tissue, which was the same criterion used in these experiments (ρmol/mg wet weight). Although Waller & Richter (1980) have also used a continuous perfusion technique the flow rate used was faster than that used in my experiments (0.5ml/min as compared to 0.3ml/min). Bradford & Richards (1976), on the other hand, have used a static system by placing the tissue in a beaker containing 5ml of saline and incubating it for 10 min before analysis of the supernatant. The results reported by Smith et al. (1983) are higher than others but it is important to note that the release has been presented as ηmol/100mg protein weight which represents a considerably higher wet weight of the tissue. They have also used a static technique by placing their tissue suspension (0.45ml equivalent to 0.5 to 1mg of prism protein) into a vial, adding a further 50μl of ACSF and incubating the suspension for a further 10 min.

Apart from these differences in the design of the experiments similarities are seen between the data presented in Table 4.2. As in the case of the *in vivo* experiments glutamine exhibited the highest level of release with GABA showing the lowest basal release. The basal levels of amino acids are generally lower in my experiments than in the others quoted and this could be due primarily to the fact that other investigators have used more finely chopped tissue resulting in a greater surface area and subsequently larger release values. The ratio of glutamine to glutamate is more than 10 in my experiments which is much higher than the ratio reported by Smith et al. (1983) who have used a similar part of the cortex but a static perfusion technique which could have facilitated conversion of glutamine to glutamate. Higher

levels of glutamate reported by Waller & Richter (1980) could be due to the fact that midbrain slices, consisting of hippocampus, striatum and septum as well as cerebral cortex, were used and there could be a larger population of glutamate-releasing neurones in this preparation than cerebral cortex itself.

The levels of amino acids released in these experiments were higher than corresponding values in the *in vivo* experiments. This may be due to the greater surface area of release in *in vitro* experiments with the amino acids being released not only from the pial surface but also from the striatal surface together with the cut surfaces.

Interestingly, the Ca^{++}-dependency of the basal release could only be seen in the presence of PCMS and not under normal conditions (compare Fig.s 4.14 & 4.22). This is perhaps because in the absence of the uptake blocker, PCMS, the net amount of neurotransmitter released is too low to be significantly affected by Ca^{++} removal as it can proceed using the residual Ca^{++}.

Stimulated amino acid efflux:

The *in vitro* system appears to be more sensitive in detecting changes in the efflux of amino acids compared to the cortical cup technique. On exposure to 60mM K^+ the release of aspartate, glutamate, glycine and GABA increased significantly (Fig.s 4.12 & 4.13). The main increases seen were in GABA ($\simeq$700%), glutamate ($\simeq$500%) and aspartate ($\simeq$300%) with glycine also showing a small ($\simeq$150%) but nevertheless statistically significant increase in release. The K^+-evoked release was Ca^{++}-dependent in all cases except glycine (Fig.4.14). The K^+-evoked release of aspartate, glutamate and GABA and the Ca^{++}-dependency of such a release provides further evidence for their neurotransmitter role in the rat cerebral cortex. The induced release was not, however,

entirely Ca^{++}-dependent in agreement with reports by other investigators (Mulder & Snyder, 1974). This could be due to either insufficient removal of all the Ca^{++} ions from the media or Ca^{++}-independent release from glia and neurones (Arias & Tapia, 1986). The increase in the release of glycine is rather unexpected as it is thought to act as a neurotransmitter in the spinal cord but not cerebral cortex (Davidoff & Adair, 1976 and McBride, Flint, Ciancone and Murphy, 1983). Waller & Richter (1980) reported a significant increase in the efflux of glycine in the rat mid brain slices but as in the case these experiments Ca^{++}-dependency could not be established. Vargas, Del Carmen Doria De Lorenzo & Orrego (1977), in contrast, showed a K^{+}-evoked, Ca^{++}-dependent increase in glycine from rat cortical slices. However, in my experiments and those mentioned above the endogenous release of amino acids were studied whereas Vargas et al. (1977) used labelled glycine and discrepancies in the release of endogenous and exogenous neurotransmitters may be seen (Ferkany & Coyle, 1983 and Herdon, Strupish & Nahorski, 1985). However, despite this fact other investigators have also failed to show Ca^{++}-dependency for electrically stimulated (Orrego, Miranda & Saldate, 1976) and K^{+}-evoked (Lopez-Colomé, Tapia, Salceda & Pasantes-Morales, 1978) release of exogenous glycine from rat cerebral cortex. In the latter set of experiments, Lopez-Colomé et al. (1978) studied the K^{+}-evoked exogenous release of amino acids from slices of several regions of rat CNS including cerebral cortex and reported an increase ($\simeq$150%) in glycine which was not Ca^{++}-dependent suggesting that the release was either glial or due to some damage to the tissue by the high K^{+} solution.

The *in vitro* perfusion of PTZ lead to a more specific change in the efflux of amino acids than cortical superfusion *in vivo*, only increasing the release of aspartate, glutamate, GABA and taurine (Fig.s

4.17 & 4.18). *In vitro* application of PTZ has been shown to lead to repetitive epileptogenic firing of neurones (Piredda et al., 1985) and the increases in release detected could be an indirect measure of such a repetitive firing of cells. PTZ has also been shown to increase the release of calcium from the endoplasmic reticulum of rat cerebral cortex neurones (Onozuka et al., 1989) which could lead to an increase in the release of amino acid neurotransmitters. Despite this fact, the Ca^{++}-dependency of the release could not be shown statistically for PTZ (Fig. 4.15), suggesting a non-neuronal source of release for these amino acids. The release of taurine is interesting as although its status as a neurotransmitter is still controversial, its importance in epilepsy is well documented (Huxtable, 1982). The release of taurine has been shown to be increased by electrical stimulation and K^+-induced depolarization of rat cortical slices (Orrego et al., 1976 and Collins & Topiwala, 1974) as well as being released extracellularly in the rabbit hippocampus during kainate-induced status epilepticus (Lehmann et al., 1985). Another important factor to consider is the large amounts of amino acids released by PTZ. For example, they were some 5 to 6 times more than high K^+ exposure in the case of 400mM PTZ which suggests other factors are involved apart from the simple increase in the firing rate of the neurones. The PTZ solutions in ACSF had high osmolarities; the osmolarity of the 400mM PTZ, as an example, being approximately 2.5 times that of control ACSF. It was decided to test the effect of such a hyperosmotic solution on the release of these amino acids by using solutions of inert compounds in ACSF which had equivalent osmolarity to my PTZ solutions. Unfortunately, there appeared to be a problem associated with using sugars such as sucrose or mannose in that they interfered with the detection of amino acids in the HPLC system which lead me to use sodium citrate instead. The extra addition of Na^+ is

unlikely to have any effect as the amount of Na^+ is already maximal in the ACSF. The effect of a solution of sodium citrate (130mM in ACSF) which had an equivalent osmolarity to that of 400mM PTZ was studied on the efflux of amino acids and was shown to have no effect on their release (Fig.4.16). An increase in the levels of amino acids is more likely to result from exposure to a hypoosmotic solution as this could cause bursting of the cells and the leakage of their content into the extracellular space. In fact, Solis, Herranz, Herreras, Lerma & Martin del Rio (1988) studied the efflux of exogenous taurine in rat dentate gyrus and have posed the question whether taurine acts as an osmoregulatory substance in the rat brain as its level increases in response to hypoosmotic solutions. Similar findings have also been reported by Lehmann, Carlström, Nagelhus & Ottersen (1991) who have shown elevated levels of taurine in the hippocampal and cerebrospinal fluids of acutely hypoosmotic rats.

The PTZ-induced release of amino acids *in vitro* was further studied by choosing the sub-maximal dose of 250mM which lead to ≃600% increase in aspartate; ≃900% increase in glutamate; ≃300% increase in taurine and ≃1400% increase in GABA release compared to the basal release.

200μM BM perfusion across cortical slices had no effect on the release of amino acids studied. However, this appeared to be partly due to the efficient uptake of amino acids from cortical slices since addition of 100μM PCMS not only increased the basal release but also 200μM BM, in its presence, evoked a significant increase in the release of the putative amino acid neurotransmitters aspartate, glutamate and GABA (Fig.s 4.20 & 4.21). The basal and the induced release of these amino acids, in the presence of 100μM PCMS, were Ca^{++}-dependent (Fig.4.22) implicating a neuronal source of release. An important observation both

in the case of PTZ- and BM-induced release of amino acids from cortical slices was the pattern of the release. Both convulsants induced a long-standing release which persisted even after their removal. This was consistent with our electrophysiological studies (chapter 3) where the local application of these agents lead to spiking activity which reached a peak after removal of the convulsants.

4:4: EFFECTS OF VARIOUS COMPOUNDS MODULATING AMINO ACID NEUROTRANSMISSION ON THE STIMULATED RELEASE FROM CORTICAL SLICES:

4:4:1: High K^+:

The effect of NMDA blockade on 60mM K^+-induced release was studied by using the NMDA receptor antagonist, AP7, and high Mg^{++}, which is thought to block the ion channel associated with the NMDA receptors. The percentage release represented in the results (Fig.4.23) was calculated using the following formula:

$$\text{percentage release} = \frac{\text{'induced' release}}{\text{'basal' release}} \times 100$$

with the 'induced' release being the mean of the two samples collected during 60mM exposure and the 'basal' release the average of the two control samples collected before high K^+ exposure.

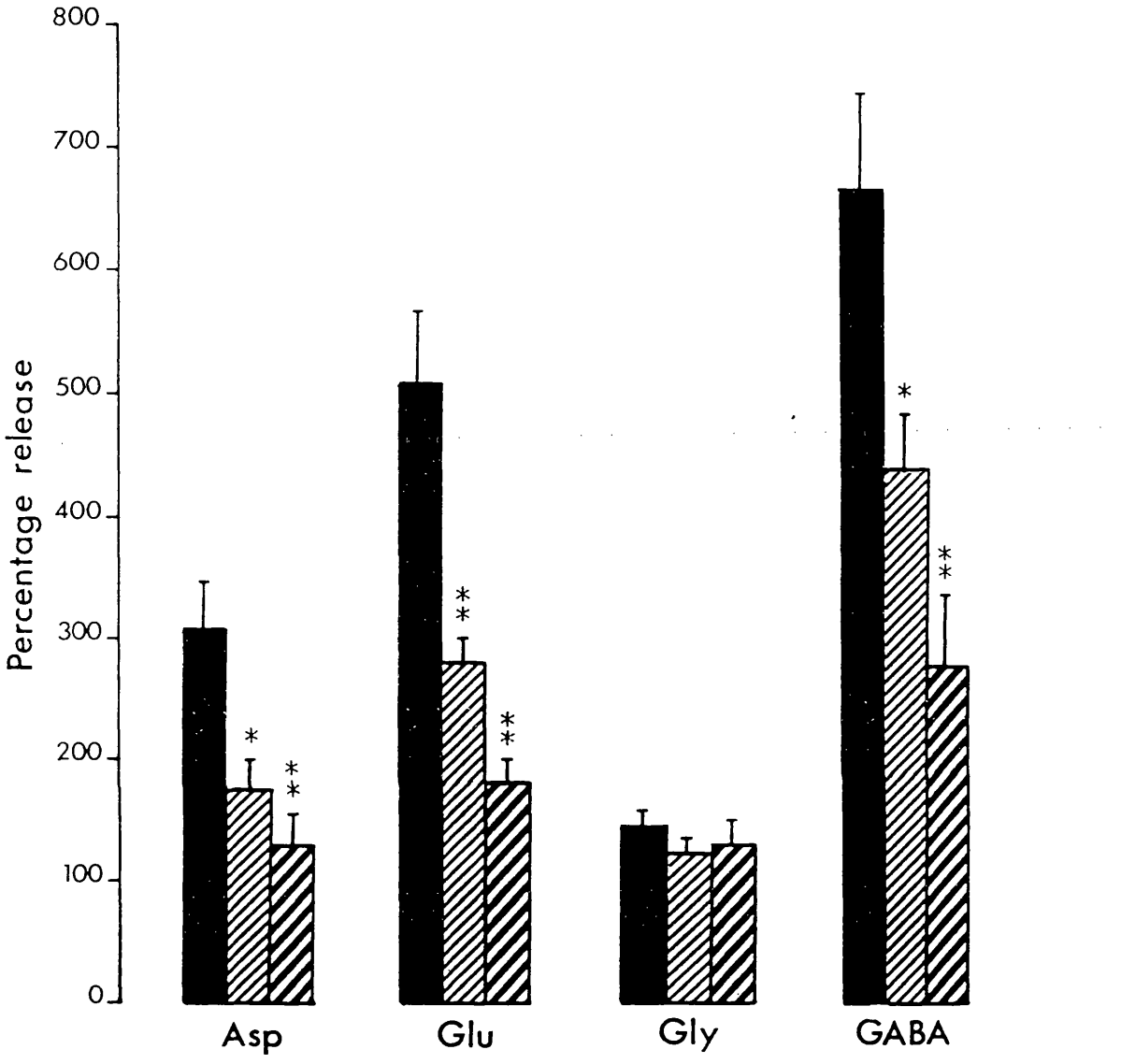

Fig.4.23: The effect of AP7 and high Mg^{++} on the K^{+}-induced increase in release of selected amino acids from cortical slices. (■) represents the percentage release in the control experiments (n=8), (▨) is the percentage release in the presence of 100μM AP7 (n=5) and (▨) shows the percentage release when ACSF containing 10mM Mg^{++} is used (n=4). The histograms represent mean $\pm$ s.e. *P<0.05 and **P<0.005 show the significance of results compared to the control release (unpaired t-test).

4:4:2: Pentylenetetrazole (PTZ):

The effect of NMDA blockade was studied on PTZ (250mM)-induced release by using the NMDA channel blocker MK-801 and high Mg^{++} solution. 10μM MK-801 produced a non significant effect on the PTZ-induced increase in release whereas 10mM Mg^{++} significantly reduced it for all four amino acids studied (Fig.4.24). Percentage release was calculated as in the case of high K^{+} exposure with the 'induced' release being the average of the four samples collected during and immediately after PTZ perfusion.

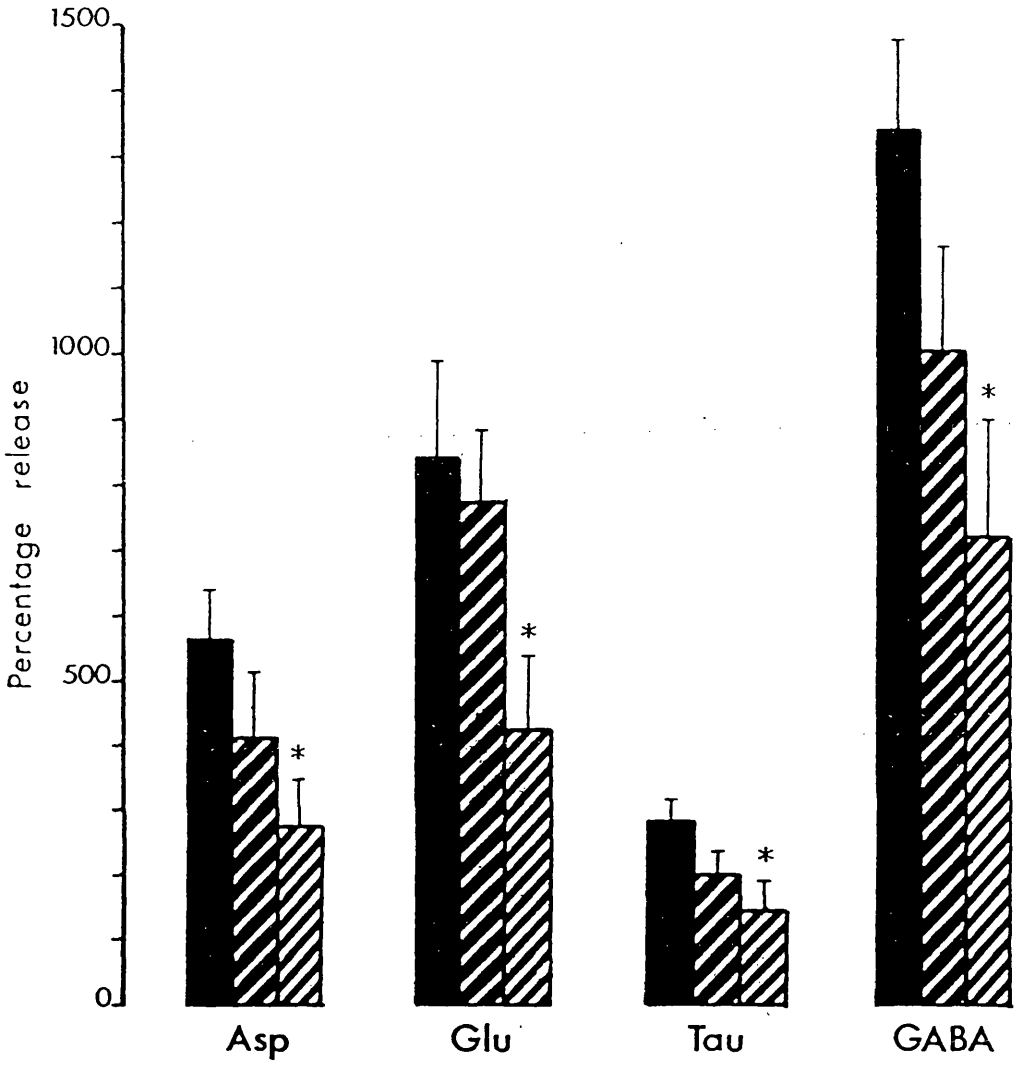

Fig.4.24: Effect of 10μM MK-801 and 10mM Mg^{++} on the PTZ -induced increase in the release of selected amino acids. (■) represents the percentage release as a result of 250mM PTZ in ACSF perfusion compared to that in the presence of 10μM MK-801 (▨) and 10mM Mg^{++} (▨). The histograms represent mean values $\pm$ s.e. (n=11 in the case of control results, 4 in the case of MK801 and 5 for Mg^{++}). *P<0.05 shows the significance of results compared to control values (unpaired t-test).

4:4:3: Bicuculline methiodide (BM):

As seen earlier, BM, in the presence of ١٥٥μM PCMS, induced an increase in the release of aspartate, glutamate and GABA only (Fig.s 4.20 & 4.21). The percentage release of these three putative amino acid neurotransmitters was calculated as in the case of PTZ and the effects of two sets of compounds modifying amino acid neurotransmission on such an induced release were studied. These included drugs which augment the inhibitory action of GABA (clonazepam, muscimol and baclofen) or excitatory amino acid blockers (AP7, MK-801, CNQX and high Mg^{++}) and the results are shown in Fig.s 4.25 to 4.27.

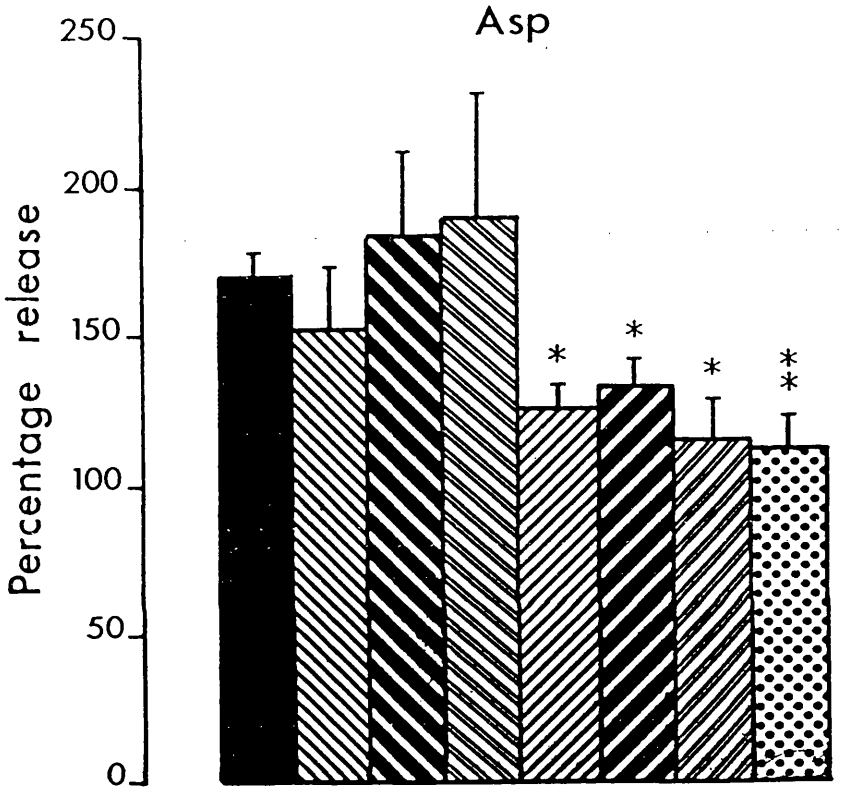

Fig.4.25: Effect of various agents on the BM-induced increase in the release of aspartate in the presence of 100μM PCMS. The results are presented as the percentage increase in the release $\pm$ s.e. *P<0.05 and **P<0.005 are the significance of the results compared to control (unpaired t-test).

(▬)= control, 200μM BM, n=10; (▨)= muscimol, 100μM, n=4;

(▨)= clonazepam, 100μM, n=4; (▨)= (+)baclofen, 100μM, n=4

(▨)= AP7, 100μM, n=6; (▨)= MK-801,10μM, n=6;

(▨)= CNQX, 20μM, n=5 & (▨) = Mg^{++}, 10mM, n=5.

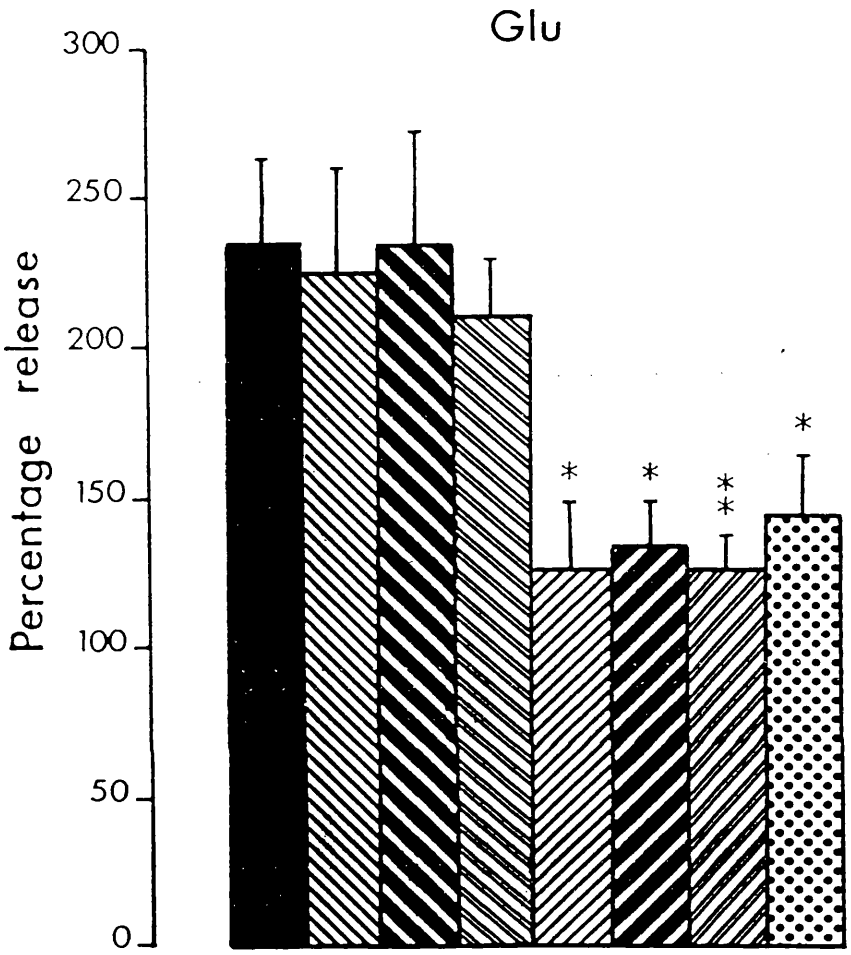

Fig.4.26: Effect of various agents on the BM-induced increase in the release of glutamate in the presence of 100μM PCMS. The results are presented as the percentage increase in the release $\pm$ s.e. *P<0.05 and **P<0.005 are the significance of the results compared to control (unpaired t-test).

(▬)= control, 200μM BM, n=10; (▨)= muscimol,100μM, n=4;

(▨)= clonazepam, 100μM, n=4; (▨)= ($\pm$)baclofen, 100μM, n=4

(▨)= AP7, 100μM, n=6; (▨)= MK-801,10μM, n=6;

(▨)= CNQX, 20μM, n=5 & (▨) = Mg^{++}, 10mM, n=5.

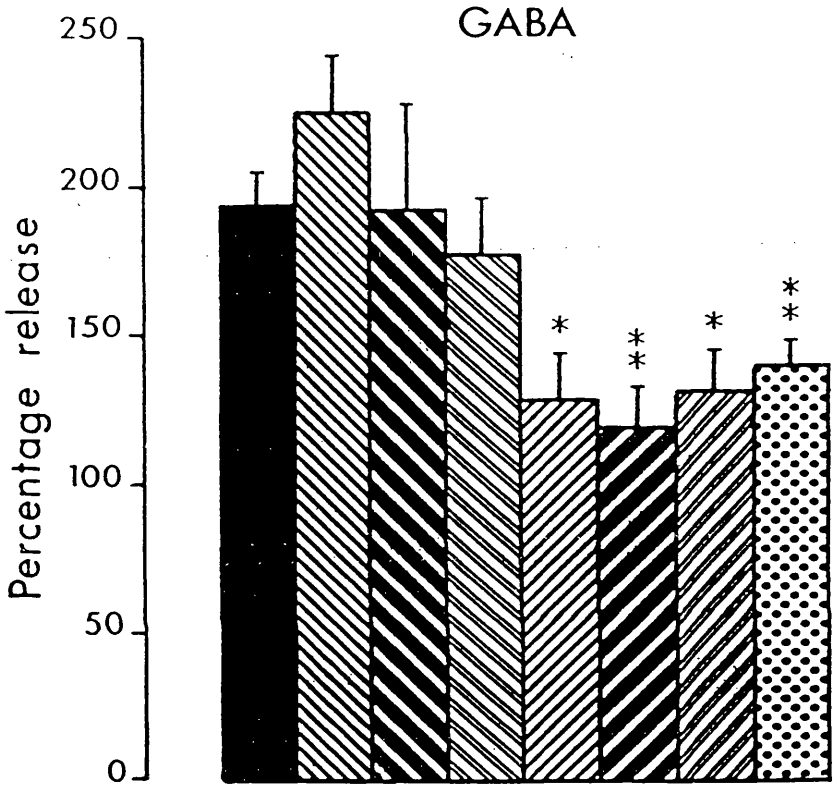

Fig.4.27: Effect of various agents on the BM-induced increase in the release of GABA in the presence of 100μM PCMS. The results are presented as the percentage increase in the release $\pm$ s.e. *P<0.05 and **P<0.005 are the significance of the results compared to control (unpaired t-test).

(▬)= control, 200μM BM, n=10; (▨)= muscimol, 100μM, n=4;

(▨)= clonazepam, 100μM, n=4; (▨)= ($\pm$)baclofen, 100μM, n=4

(▨)= AP7, 100μM, n=6; (▨)= MK-801,10μM, n=6;

(▨)= CNQX, 20μM, n=5 & (▨) = Mg^{++}, 10mM, n=5.

4:4:4: Discussion:

The results in the case of high K^+ exposure showed that the evoked release of aspartate, glutamate and GABA but not that of glycine were significantly reduced by AP7 and 10mM Mg^{++} (Fig.4.23). AP7 is a specific NMDA receptor antagonist and its effect, therefore, suggests a regulatory effect of NMDA receptors on the K^+-evoked release of these putative amino acid neurotransmitters. Although the idea of receptor-mediated control of neurotransmitter release from brain slices or synaptosomes is well established (Middlemiss, 1988) it has mainly been shown for the classic neurotransmitters noradrenaline and 5-hydroxytryptamine and there are relatively few studies showing such a receptor-regulated control for the release of amino acid neurotransmitters (see Chesselet, 1984). Contrasting results of investigating NMDA receptor-mediated modifications of neurotransmitter release can be found in the literature. Collins, Anson & Surtees (1983) reported a decrease in the levels of K^+-evoked release of aspartate and glutamate but not GABA from rat olfactory cortex in the presence of the excitatory amino acids NMDA and kainate. This effect was shown to be tetrodotoxin resistant and reversible by the corresponding excitatory amino acid antagonists which lead to the conclusion that such a regulatory effect was presynaptic. However, such an effect was not seen in rat hippocampal slices (McBean & Roberts, 1981) and in fact autoradiographic studies have recently provided evidence that NMDA-coupled channels are located postsynaptically and not presynaptically in the hippocampus (Bekenstein, Bennett Jr., Wooten & Lothman, 1990). There are also numerous studies suggesting a regulatory mode of action for excitatory amino acids leading to an increase in the levels of different neurotransmitters including amino acids, themselves (McBean & Roberts, 1981; Lehmann & Scatton, 1982;

Harris & Miller, 1989; Moghaddam, Gruen, Roth, Bunney & Adams, 1990 and Gannon & Terrian, 1991). The excitatory amino acid receptors can be subdivided into NMDA and non-NMDA receptors (Watkins & Evans, 1981 and MacDermott & Dale, 1987). The reported NMDA-mediated effects on the release of neurotransmitters are mentioned here reserving the discussion of the non-NMDA effects for the section concerned with the BM-evoked release of amino acids (see later).

NMDA-mediated stimulation of rat cortical (Fink, Bönisch & Göthert, 1990) and hippocampal (Pittaluga & Raiteri, 1990) noradrenaline, striatal acetylcholine (Scatton & Lehmann, 1982) as well as striatal dopamine (Moghaddam et al., 1990 and Krebs, Desce, Kemel, Gauchy, Godeheu, Cheramy & Glowinski, 1991) release are well documented. However, the latter effect has been suggested to result from a toxic and pathological dose of NMDA and not a pharmacological effect of this drug (Moghaddam & Gruen, 1991). NMDA-evoked [^{3}H] GABA release from cultured mouse striatal neurones has also been shown (Weiss, 1990). Connick & Stone (1988a) have studied the NMDA-mediated release of endogenous aspartate and glutamate from hippocampal slices and have reported a reduction in the K^+-induced release of both of these excitatory amino acid neurotransmitters by NMDA receptor antagonists AP5 and AP7. The same investigators have also studied the effect of quinolinic acid, which is a NMDA-preferring receptor agonist, on the efflux of aspartate and glutamate from rat cortex and have shown an increase in the endogenous release of these neurotransmitters *in vivo* although not *in vitro* (Connick & Stone, 1988b). Quinolinic acid has also been shown to increase acetylcholine release in striatal slices (Lehmann, Schaefer, Ferkany & Coyle, 1983). There are also a number of reports showing a NMDA-mediated increase in intracellular Ca++ (see Mayer & Miller, 1990; Burgoyne, Pearce & Cambray-Deakin, 1988 and Daniell, 1991),

which is involved in the process of exocytosis and could subsequently lead to an increase in the release of amino acid neurotransmitters whose release is now strongly thought to involve exocytosis like other more classic neurotransmitters (see Maycox, Hell & Jahn, 1990). Our results indicate a regulatory role for NMDA receptors in the K^+-evoked release of aspartate, glutamate and GABA. I have not used tetrodotoxin in these studies which would have blocked Na^+ channels and those events which were dependent on spread of action potentials. According to Collins et al. (1983) NMDA receptor activation in the olfactory cortex leads to a reduction in the K^+-induced release of some endogenous amino acids. The results presented here, however, indicate an opposite regulatory effect for NMDA receptors in that their blockade leads to a decrease in release of amino acids. It is possible that there are two different regulatory effects present in the rat cortex depending on whether the stimulation of NMDA receptors is pre or postsynaptic. The effect reported by Collins et al. (1983) was tetrodotoxin resistant and, therefore, presynaptic, whereas the effect seen in these experiments may be similar to those reported by Lehmann & Scatton (1982) and mainly due to NMDA receptors being stimulated postsynaptically exciting the axons of the amino acid containing neurones leading to generation of action potentials and subsequent further release of the neurotransmitter into the extracellular space. Another reason for such a discrepancy could be the possibility of different parts of rat cortex having different properties as far as this NMDA-regulatory effect is concerned as Fink, Göthert, Molderings & Schlicker (1989) have failed to show any NMDA-receptor mediated stimulation of amino acid neurotransmitter release in the occipitoparietal rat cortex.

The reduction in the K^+-evoked release seen by Mg^{++} could be due to its long established effect in reducing any Ca^{++}-dependent

release of neurotransmitters by replacing this cation (Del Castillo & Katz, 1954). However, Mg^{++} has also been shown to block the ionic channel associated with the NMDA receptor (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984) and the reduction in the K^{+}-evoked release seen here could also be due to this action of Mg^{++} since high Mg^{++} has been shown to have a more pronounced effect on the reduction of K^{+}-evoked release than simple removal of Ca^{++} ions (Flint, Rea & McBride, 1981). However, the reduction in release seen by 10mM Mg^{++} in these results is comparable to that seen after removal of Ca^{++} (comparing Fig.s 4.14 & 4.23) suggesting that the reduction in release by Mg^{++} is mainly due to its replacement of Ca^{++} and not its blocking action on the NMDA channels. This could be due to the level of Mg^{++} (1.04 mM) in the control ACSF already being sufficient to block the NMDA activated channels. None of the compounds tested had any effect on the K^{+}-evoked release of glycine.

The PTZ-evoked release of amino acids could not be blocked by 10μM MK-801, which is a NMDA channel blocker (Wong et al., 1986). It was decided to use MK-801 instead of AP7, as in the high K^{+} studies, as a large PTZ-evoked release of glutamate would have coincided with the AP7 peak on the chromatograph. It is possible that the amount of MK-801 used was not sufficient to block the PTZ-evoked release. Additionally 10mM Mg^{++}, which also blocks the NMDA-associated channel, significantly blocked the evoked release (Fig.4.24) which could not have resulted simply from its replacement of Ca^{++} since Ca^{++}-dependency of release could not be shown (Fig.4.15).

BM-induced release was attenuated by compounds reducing excitatory amino acid neurotransmission but not those increasing GABA action (Fig.s 4.25 to 4.27). This is perhaps surprising since presynaptic GABA receptors modulating neurotransmitter output has been reported

by Pickles (1979). Brennan, Cantrill, Oldfield & Krogsgaard-Larsen (1981) have shown that GABA agonists acting at $GABA_A$ subtype reduce the release of GABA and similar results were reported by Lockerbie & Gordon-Weeks (1985) when studying the K^+-evoked release of exogenous GABA from isolated neuronal growth cones generated from neonatal rat forebrain. Vellucci & Webster (1985) have also shown an inhibitory effect of GABA and benzodiazepines on the K^+-evoked release of exogenous glutamate from spinal cord slices using the perfusion system used in these experiments. This again involves $GABA_A$ receptors and I was unable to detect any modulating effect for muscimol or clonazepam (at doses sufficient to significantly reduce the epileptogenic activity of 200μM in the electrophysiological studies, see chapter 3, section 3:5:1) on the BM-evoked release of amino acids. It is possible that in my experiments the presence of BM would block these $GABA_A$ autoreceptors and, therefore, such modulating effect on the amino acid release could not be seen.

The release-modulating GABA receptors may, however, be of the $GABA_B$-type as shown in the human cortex (Bonanno, Cavazzani, Andrioli, Asaro, Pellegrini & Raiteri, 1989). (-)Baclofen has been shown to inhibit K^+-evoked excitant amino acid release from rat cortical slices (Johnston et al., 1980) as well as reducing kainate-enhanced release of labelled aspartate from guinea pig cerebral cortex (Potashner & Gerard, 1983). Paradoxically (-)baclofen has also been shown to act presynaptically reducing the release of GABA from rat hippocampal neurones (Harrison, 1990) and human cortical synaptosomes (Bonanno et al., 1989). (+)Baclofen, on the other hand, is reported to increase the release of exogenous GABA from rat globus pallidus, *in vitro* (Kerwin & Pycock, 1978). The latter finding has, however, not been shown in rat cortical slices where baclofen is known to inhibit electrically-evoked exogenous

GABA release (Baumann, Wicki, Stierlin & Waldmeier, 1990). These results do not show any such modulating effect of baclofen on the BM-evoked release of aspartate, glutamate or GABA.

The modulating effect of excitatory amino acids on the efflux of putative amino acid neurotransmitters has been widely reported. The NMDA-modulated effects on the release of neurotransmitters have already been discussed earlier. According to my results, NMDA blockade (whether by AP7 or MK-801) reduces the BM-evoked release of aspartate, glutamate and GABA. 10mM Mg^{++} also significantly reduced the evoked release which could be partially due to its blockade of the NMDA-associated channel as already explained. Such regulating effects of the NMDA receptor is similar to that seen in the case of high K^{+} exposure and similar mechanisms in reducing the release could be involved.

The non-NMDA-modulated release of neurotransmitters has also been documented. Kainate-induced increase in the release of aspartate has been reported in the cerebral cortex and striatum of guinea pig (Potashner & Gerard, 1983) as well as mouse cerebellum where it also increases glutamate levels (Ferkany, Zaczek & Coyle, 1982). The release of GABA has also been found to be regulated by kainate as well as glutamate, itself (Perouanski & Grantyn, 1990). Holopainen & Kontro (1990) have shown that non-NMDA excitatory amino acids increase the K^{+}-evoked release of aspartate from cerebellar astrocytes whereas inhibitory amino acids reduce such an evoked release. It has also been shown recently that the release of endogenous and newly synthesized glutamate and of other amino acids can be induced by non-NMDA receptor activation in cerebellar granule cell cultures (Levi, Patrizio & Gallo, 1991).

6-Cyano-7-nitroquinoxaline-2,3-dione, CNQX (Honoré et al., 1988)

was used to investigate the effect of non-NMDA blockade on the BM-evoked release of amino acid neurotransmitters. 20μM CNQX was employed which is shown to be insufficient to block NMDA-mediated [^{3}H]GABA release from cultured rat cortical neurones (Harris & Miller, 1989) as well as being the dose used in the electrophysiological studies which only showed a non-NMDA blocking effect (see chapter 3, Fig 3.31). The ability of 20μM CNQX to reduce the BM-evoked release of aspartate, glutamate and GABA, therefore, suggest a non-NMDA as well as NMDA-modulating effect in the BM-evoked release.

The overall effect of 60mM K^+, 250mM PTZ and 200μM BM on the release of aspartate, glutamate and GABA from cortical slices together with their modification by various compounds blocking NMDA receptors is summarized in table 4.3.

Although at the concentrations used BM produced more extensive spiking than PTZ it had much less effect on slices in increasing the release of amino acids. This is perhaps because BM does not produce burst firing as extensively as PTZ *in vitro*. It also, at concentrations used, only blocks GABAergic inhibition and does not have the additional non-specific effect on the cell membrane as seen with PTZ. Since high K^+ also induces direct depolarization of neurones and glia hence it could yield a larger increase in release than BM.

	Percentage release from cortical slices								
	Aspartate			Glutamate			GABA		
	K^+	PTZ	BM	K^+	PTZ	BM	K^+	PTZ	BM
Control	308	562	169	509	841	235	663	1339	194
(n)	(8)	(11)	(10)	(8)	(11)	(10)	(8)	(11)	(10)
100μM AP7	*175		*125	‡279		*127	*436		*129
[% reduction]	[43]	ND	[26]	[45]	ND	[46]	[34]	ND	[34]
(n)	(5)		(6)	(5)		(6)	(5)		(6)
10μM MK-801		410	*134		774	*134		1003	‡119
[% reduction]	ND	[29]	[21]	ND	[8]	[43]	ND	[25]	[39]
(n)		(4)	(6)		(4)	(6)		(4)	(6)
10mM Mg^{++}	‡134	*272	‡112	‡181	*427	*147	‡275	*720	‡141
[% reduction]	[56]	[52]	[34]	[64]	[50]	[37]	[59]	[46]	[27]
(n)	(4)	(5)	(5)	(4)	(5)	(5)	(4)	(5)	(5)

Table 4.3. The overall effect of 60mM K^+, 250mM PTZ and 200μM BM on the release of aspartate, glutamate and GABA from cortical slices and their modification by various NMDA blockers. The average values have been presented in each case. *$P<0.05$ and ‡$P<0.005$ are the significance of reductions in percentage release compared to the control (unpaired t-test).

ND = Not Determined

A large proportion of PTZ-induced release is likely to be due to its direct effect on the cell membrane rather than just its GABAergic inhibition as NMDA blockade had no modulating effect on such a release whereas AP7 and MK-801 both reduced BM-induced release. Also 10mM Mg^{++} which has a similar effect to MK-801 on BM-induced amino acid release had more effect than MK-801 on the PTZ-induced release. This probably, therefore, reflects the Ca^{++} antagonist property of Mg^{++} to reduce neurotransmitter release rather than its blockade of NMDA channels. The reverse is seen when comparing the NMDA effect in modulating BM-induced release to that of high K^{+}. AP7 is more potent in reducing high K^{+} than BM-induced release implying that the NMDA modulation of the release is more evident when the release is induced by a direct action on the neurones and glial cells and not indirectly as a result of GABA blockade.

4:5: GENERAL CONCLUSIONS:

The *in vitro* technique used provided a more sensitive method for detecting changes in the efflux of amino acids from cerebral cortex as a result of exposure to convulsants. Both PTZ and BM lead to specific increases in the release of putative amino acid neurotransmitters linking them to the mode of action of these convulsants.

CHAPTER 5

GENERAL DISCUSSION

The object of this final chapter is to combine the more important results of chapters 3 and 4 and to discuss whether the original aims of the project have been met and to suggest further experiments which would help to fulfil them.

5:1: DISCUSSION OF THE OVERALL RESULTS:

The aims of this work were essentially two-fold: firstly to study the effect of various selected convulsants on the EEG of urethane-anaesthetised rats and evaluate the role of compounds modulating amino acid neurotransmission in the initiation and control of such an epileptogenic activity and secondly to establish a direct relationship between epileptogenic spiking activity and release of amino acids.

The effect of convulsants on the EEG of urethane-anaesthetised rats and the modification of such an effect by potential anticonvulsants was successfully studied using cortical cups. Using this technique convulsants could be superfused across the exposed cortex leading to easily quantifiable EEG spiking.

Bicuculline Methiodide (BM) proved a more potent convulsant in inducing epileptogenic spiking when superfused across the cortex and was adopted as the main agent to study the role of various compounds, modulating amino acids neurotransmission, on such spiking. Perhaps the most important finding of this project emerged from these studies which provided data distinguishing between the different modes of action of these potential anticonvulsants. It is evident that the initiation of these spikes involves activation of $GABA_B$ and non-NMDA receptors whereas $GABA_A$ and NMDA receptors are only involved in the control of subsequent spread of epileptogenic activity. The anatomical distribution of various amino acid receptors formed an essential consideration in discussing the results in chapter 3 and it is useful to combine the

information in a simplified diagram showing the laminar distribution of these receptors (Fig.5.1).

The spiking activity is initiated by a 'pace-maker' neurone (neurone X in Fig.5.1) which is excitatory and normally under the controlling inhibitory influence of GABAergic interneurones (neurones denoted by A in Fig.5.1). These pace-maker neurones which are located in layer IV and upper region of layer V start to fire abnormally and excessively as soon as the inhibitory action of GABA is diminished by the addition of BM which blocks the $GABA_A$ receptors. The activity spreads in all directions also reaching the upper part of the cortex where it recruits other excitatory cells ('follower' cells) leading to their synchronous burst firing as these cells are extensively interconnected. These episodes of synchronous burst firing lead to the appearance of 'interictal spikes' in the surface EEG.

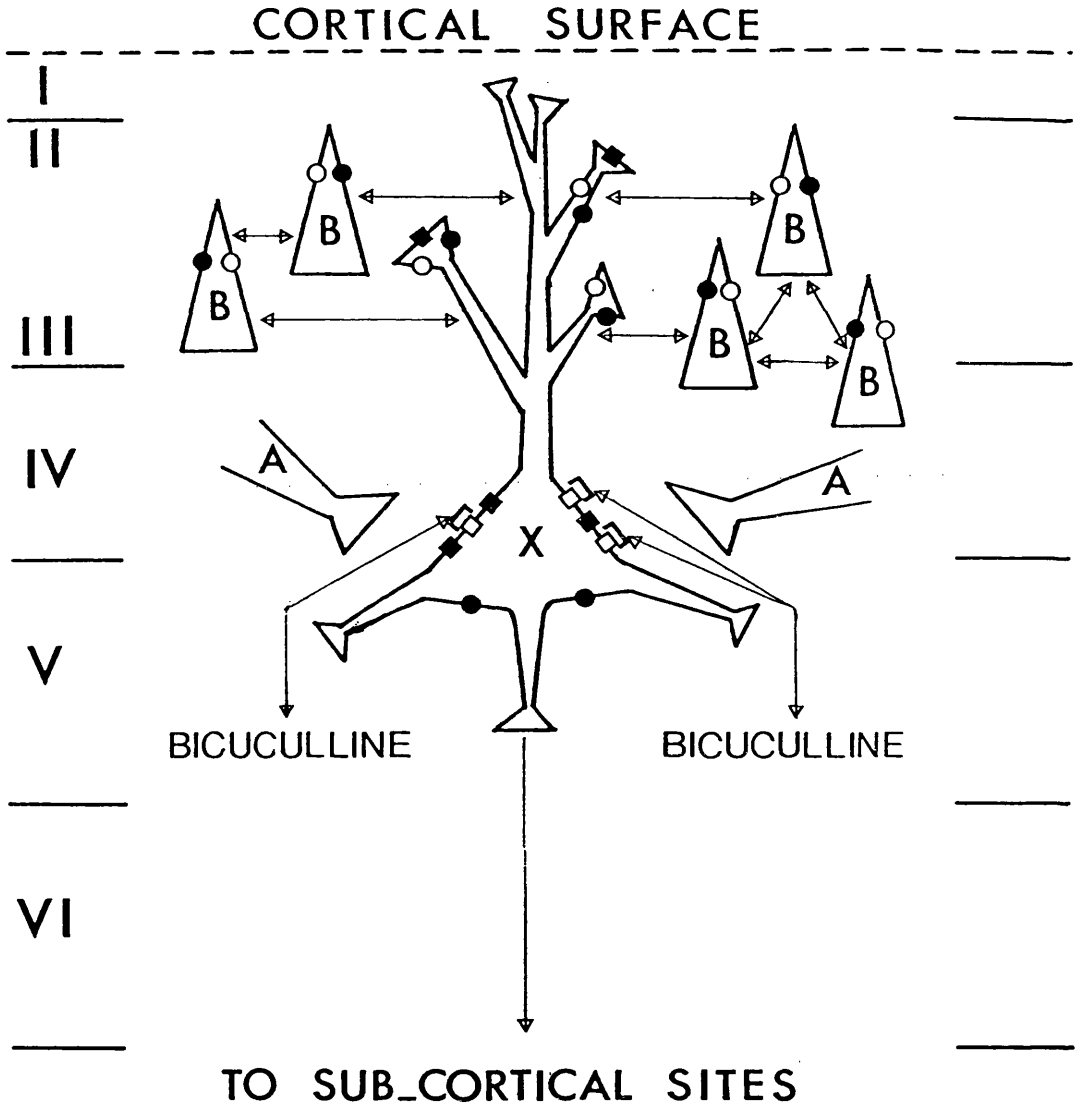

Fig.5.1. A schematic diagram showing the laminar distribution of various amino acid receptors on the 'pace-maker' and 'follower' cells (see the text for more details).

X= 'pace-maker' cell

B= 'follower' cells

A= GABAergic interneurones

(□)= $GABA_A$ receptors

(■)= $GABA_B$ receptors

(O)= NMDA receptors

(●)= non-NMDA receptors

The potential involvement of various amino acid receptors in the initiation and control of epileptogenic spiking is also indicated in Fig.5.1. $GABA_B$ receptors have both a postsynaptic action in layer IV and presynaptic effect in layers I-III. Their activation can either lead to postsynaptic inhibition of the 'pace-maker' cells terminating the activity at its onset in layer IV or stop the synchronization of the 'follower' cells in layers I-III by a presynaptic action stopping the release of excitatory amino acid neurotransmitters. NMDA receptors are mainly situated in the superficial layers (I-III) and their blockade stops the recruitment of the 'follower' cells leading to a reduction in the size of the spikes. Blockade of non-NMDA receptors, on the other hand, affects the initiation of spikes leading to a reduction in their total number. The reduction in the total number of spikes seen in the case of non-NMDA blockade is not as pronounced as that seen with baclofen. This may be due to lack of non-NMDA receptors in the layer where the activity mainly starts (i.e. layer IV). The role of $GABA_A$ receptors appears to be rather complex as although they are optimally situated to stop the initiation of the spikes their activation merely leads to a reduction in the recruitment of 'follower' cells. This apparent discrepancy may be due to the presence of BM which interferes with their activation and also the nature of the inhibitory effect of $GABA_A$ receptor activation which is appreciably shorter in duration than the inhibitory effect of $GABA_B$ activation.

Epileptogenic spiking induced by cortical superfusion of PTZ and picrotoxin was also monitored and the effect of the NMDA receptor antagonist, AP7, on such spiking was studied. NMDA receptor activation appeared to be important in both the initiation and control of spiking induced by these convulsants as AP7 reduced the number of spikes as well as their size in both cases. As explained in chapter 3 (section

3:5:3) this may be due to other actions of these convulsants.

I hoped that the second aim of the project would be achieved mainly by using cortical cups which would allow a direct comparison of the efflux of amino acids from the exposed part of the cortex within the cup to the epileptogenic activity produced in the same area and recorded using electrodes incorporated in the wall of the cortical cup. This technique, however, proved rather inadequate in detecting changes in the release of various amino acids studied although careful steps were taken to minimize the significant loss of amino acids in the tubing (see chapter 2, Fig.2.3) as well as the rapid uptake of these amino acids. It was, therefore, important to adopt another technique in studying the release of amino acids if the objectives of the project were to be met and the use of cortical slices technique proved more fruitful as significant changes in the release of amino acids could be seen as a result of exposure to convulsants.

PTZ was the only convulsant which induced a detectable increase in the release, be it unspecific, of most of the amino acids tested when cortically superfused through cortical cups. A relationship between PTZ-induced epileptogenic activity and amino acid release was further emphasized when intravenous infusion of this convulsant lead to a significant increase in the release of glutamine from the surface of the cortex. The PTZ-induced release of amino acids showed a degree of specificity when it was applied to the cortical slices suggesting a more pharmacological action of PTZ especially as only putative amino acid neurotransmitters were released together with taurine which is thought to be important in epilepsy. It can, therefore, be claimed that the first aim in relating epileptogenic activity of PTZ and the release of amino acids was successfully met.

The relationship between the epileptogenic spiking activity

produced by BM and release of amino acids could not be established directly using cortical cups. However, exposure of cortical slices to this convulsant in the presence of a non-specific uptake blocker, PCMS, lead to a significant, Ca^{++}-dependent increase in the release of putative amino acid neurotransmitters only suggesting a genuine pharmacological effect. The effect of excitatory amino acid antagonists in inhibiting the induced release could be another reason for their anticonvulsant properties although all compounds had a similar effect including high Mg^{++}.

The last convulsant used, picrotoxin, was not studied as extensively and no relationship between its epileptogenic activity and amino acid release could be established.

5:2: FUTURE CONSIDERATIONS:

The relationship between the release of cortical amino acids in response to PTZ exposure could be further studied by using other more specific uptake blockers as well as using agents which stop the metabolic breakdown of amino acid neurotransmitters. A suggestion for further experiments would also be to study the effect of AP7, which reduced both the number as well as the size of the PTZ-induced spikes, on the seemingly non-specific release of amino acids into the cortical cup during the cortical superfusion of this agent.

As far as the EEG studies involving BM are concerned it will be important to investigate the combined effect of compounds with different modes of action on the spiking (i.e. AP7 or MK-801 with baclofen).

5:3: CONCLUSIONS:

It is important to realize that BM-induced epileptogenic activity could be attenuated differentially by various compounds modulating amino acid neurotransmission. Drugs which appear to reduce excitatory synaptic activity in dendritic layers I-III of the cortex either by blocking non-NMDA receptors or activating $GABA_B$ receptors reduce the likelihood of spike generation in pyramidal cells lacking their normal inhibitory somatic inputs through $GABA_A$ receptors that are blocked by bicuculline. To what extent my results are biased by the use of one anaesthetic and a particular convulsant is uncertain but the latter might contribute to the effectiveness of baclofen seen in these experiments but not found clinically. Certainly these results show that not only may a number of compounds reduce BM-induced epileptogenic activity but they do so by differing mechanisms. This latter finding may provide a scientific basis for combining drugs with differing modes of action to achieve a better control of epileptogenic activity with fewer side effects.

Another important finding of these results was that both in the case of high K^+ and BM-evoked release, from cortical slices, NMDA blockade played an important regulating part leading to a reduction in the evoked release. This could provide another explanation for the anticonvulsant action of NMDA blockers.

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APPENDIX I: CIRCUIT DIAGRAM OF THE SPIKE VOLTAGE INTEGRATOR

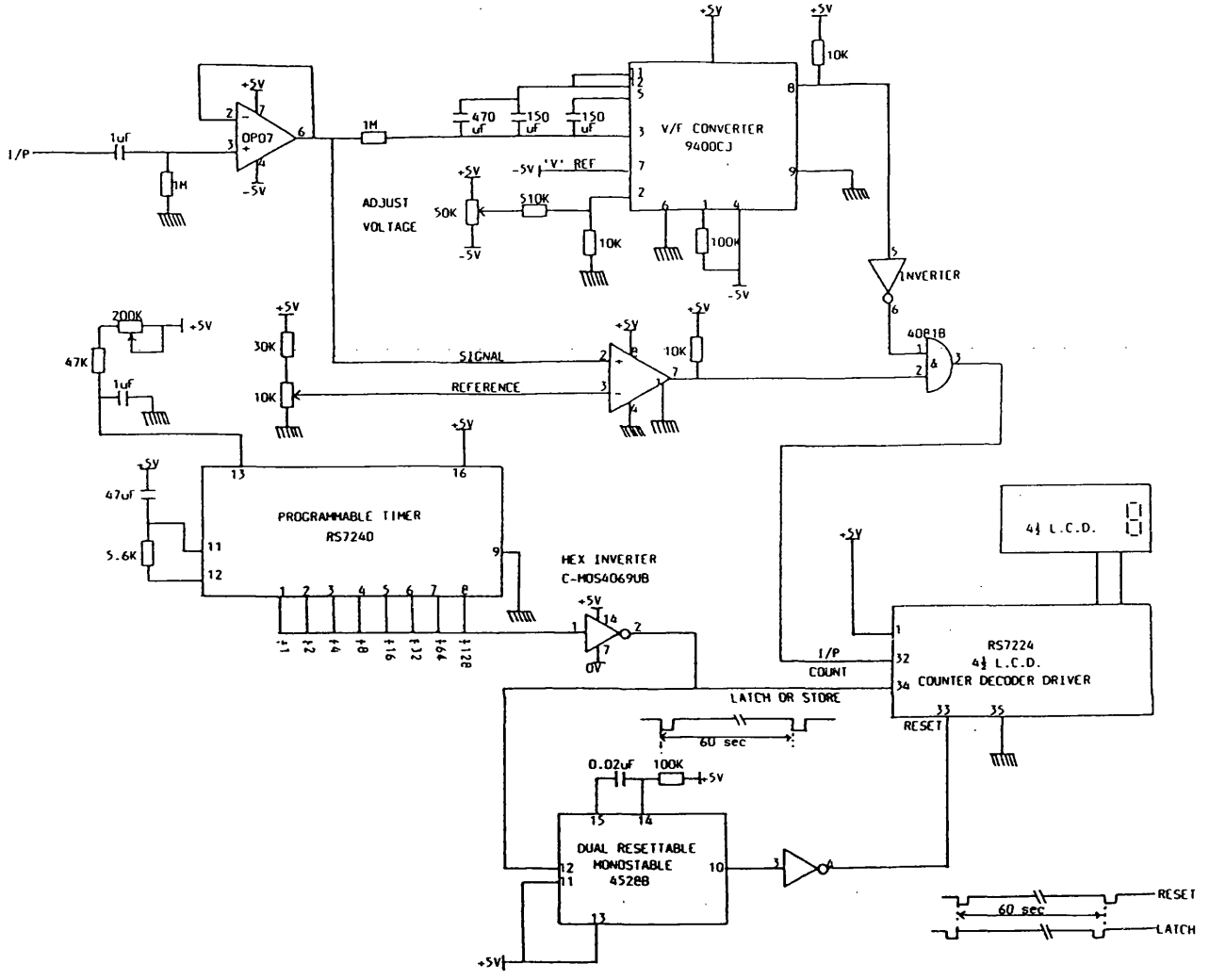

Fig.A.1. Detailed circuit diagram of the spike voltage integrator. The device was used to quantify the EEG spikes as fully explained in chapter 2 (section 2:2:6). All the components were obtained from Radio Spares.

APPENDIX II: DATA ANALYSIS

All the statistical analysis of the data were computed by using the Penn State University statistical package, MINITAB, running on EUCLID at University College London.