FACTORS INFLUENCING AMINO ACID TRANSMITTER RELEASE *IN VITRO*

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ABSTRACT

The effects of a number of agents on synaptosomal amino acid release were examined.

Initially, the synaptosome preparations employed were shown to exhibit certain metabolic properties, such as respiration and glycolysis. Incubated synaptosomes were also shown to respond to potassium and veratrine stimulation, showing differential release of the putative amino acid transmitters, glutamate, aspartate, GABA and glycine, increased respiration and lactate production and in the case of veratrine stimulation a reduction in the synaptosomal K^+ content.

The uptake blockers p-chloromercuriphenyl sulphonic acid and 3-threo-hydroxy-aspartic acid were found to cause substantial increases in spontaneous release and in the case of the latter agent, stimulated amino acid release.

The glutamate analogues, Kainic acid, GDEE and HA966 were tested for their effects on synaptosomal amino acid release and uptake. Kainic acid increased the release of aspartate and glutamate in addition to causing an inhibition of ^{14}C-glutamate uptake in cerebro-cortical and striatal synaptosomes.

Evidence to suggest that the GABA antagonists, bicuculline and picrotoxin influence amino acid release in particular GABA efflux, was obtained. Picrotoxin (0.1mM) reduced the veratrine stimulated release of ^{14}C-GABA. At high concentrations picrotoxin inhibited the stimulated release of aspartate, glutamate and GABA. The results obtained with bicuculline were variable, reducing or increasing the spontaneous efflux of ^{14}C-GABA depending upon the concentration used.

β-bungarotoxin, an agent known to have a presynaptic mode of action, caused a massive increase in the spontaneous efflux of aspartate, glutamate and GABA and completely blocked the synaptosomal response to veratrine stimulation. Data suggesting that β-bungarotoxin functions as an uptake blocker for ^{14}C-glutamate (53% inhibition) and ^{14}C-GABA (35% inhibition), was obtained. Subsequent experiments gave results implying that this apparent inhibition of uptake may in fact result from a depolarizing action produced by the toxin.

A tremorgenic mycotoxin, Penitrem A (obtained from *Penicillium cyclopium*) caused a reduction in the veratrine stimulated release of glycine and GABA from rat spinal/ medullary synaptosomes, prepared from animals pretreated

with the toxin. In the cerebral cortex the spontaneous efflux of GABA, aspartate and glutamate was elevated. Preliminary experiments on the effects of the toxin on amino acid release from three neural regions (cerebral cortex, spinal cord and corpus striatum) obtained from sheep, were also conducted. The fungus Penicillium estinogenum, which contains the mycotoxin, verruculogen, was also shown to have an action on amino acid release in cerebrocortical synaptosome preparations, increasing the levels of aspartate and glutamate recovered in the medium.

Finally, the influence of varying K^+ concentrations and several biochemically active substances on spontaneous miniature end-plate potential activity in a locust neuromuscular junction preparation, was studied. In common with other workers, high K^+ concentrations and β-bungarotoxin were found to increase m.e.p.p. frequency. Kainate and glutamate on the other hand, substantially reduced spontaneous potential discharge.

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N.B. Some of the work presented in chapter 6. was done in collaboration with Mr. Peter Norris and will eventually appear in his Ph.D. thesis.

ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholine esterase
AOAA	amino-oxyacetic acid
BSA	bovine serum albumin
CNS	central nervous system
DABA	L-2,4 diamino-butyric acid
DA	dopamine
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethylene glycol bis-(2-aminoethyl) tetra-acetic acid
GABA	gamma-amino-butyric acid
GDEE	glutamate diethyl ester
HA966	1-hydroxy-3-amino-pyrrolid-2-one
5HT	5-hydroxytryptamine
LDH	lactate dehydrogenase
m.e.p.p.	miniature end plate potential
NA	nor-adrenaline
NMJ	neuromuscular junction
OPA	ortho-phthalaldehyde
PCA	perchloric acid
PCMPS	para-chloromercuriphenyl-sulphonic acid
p-HMB	p-hydroxymercuribenzoate
TCA	trichloractic acid
TTX	tetrodotoxin

CHAPTER ONE

GENERAL INTRODUCTION

1. INTRODUCTION

2. PROPERTIES OF SYNAPTOSOMES
   - a. Morphology and Synaptosomal Function
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4. NEUROTRANSMITTER RECEPTORS

Presynaptic receptors

5. THE PURPOSE OF THIS STUDY

1. INTRODUCTION

Interneuronal signalling in the nervous system occurs principally via chemical transmitter substances. Transmitter released by the nerve ending in response to an electrical impulse, traverses the synaptic cleft and interacts with a specific site (receptor) on the post-synaptic membrane producing a new wave of depolarization (excitatory transmitter) or hyperpolarization (inhibitory transmitter) in the post-synaptic cell. The mechanisms available for modulating neurotransmitter release and duration of action have not been fully elucidated, however, data obtained in recent years has led to several suggestions.

One mechanism by which the actions of released neurotransmitters on the post-synaptic membrane may be modified is by removal of the free transmitter from the synaptic cleft. Enzymic degradation is one way in which this may be achieved, e.g. acetylcholinesterase is responsible for the destruction of acetylcholine at cholinergic synapses. Non-destructive mechanisms appear to operate at most other chemically transmitting synapses, involving the physical removal of the released transmitter from its site of action by transport enzyme systems. These have been shown for nor-adrenaline (Bogdanski et al., 1968; Iversen, 1967), dopamine (Coyle and Snyder, 1969; Horn et al., 1971), 5-hydroxytryptamine (Marchbanks, 1966; Bogdanski et al., 1968), choline (Marchbanks, 1968; Diamond and Kennedy, 1969) and also the transmitter amino acids (Logan and Snyder, 1971; Iversen and Johnston, 1971; Balcar and Johnston, 1973; Davies and Johnston, 1976; Iversen, 1974), occurring in the pre-synaptic nerve terminal membrane and at other sites, e.g.

glia (Iversen, 1974; Iversen and Kelly, 1975). Apart from terminating transmitter action these uptake mechanisms may play a role in the economy of the system, transmitter recaptured by nerve-endings being later reused.

Longer term synaptic modulation may involve changes in the number of post-synaptic receptor sites available for transmitter interaction. Transynaptic control of enzyme synthesis could also be of importance and has been demonstrated for the dopaminergic system (Carlsson, 1975).

Calcium plays an important role in chemical synaptic transmission, neurotransmitter release being a calcium dependent process (reviewed by Koketsu, 1969; Abood, 1969; Baker, 1972). Using the luminescent protein aequorin it has been shown that following stimulation, the cytoplasmic concentrations of calcium increase in presynaptic isolated nerve-endings (Shimomura et al., 1963). Calcium is known to regulate membrane permeability, this being a function of the concentration of calcium bound to the membrane. In general, when the concentration of bound calcium is reduced the membrane permeability to Na^+, K^+ and Ca^{2+} ions is increased. Veratrine, potassium, scorpion venom, rubidium, caesium and lithium, all stimulate calcium uptake (Blaustein, 1975; Blaustein and Oborn, 1975) under suitable conditions. Recently, the calcium ionophore, A23187, has been shown to increase membrane permeability, causing a calcium influx, resulting in neurotransmitter (Redburn, Shelton and Cotman, 1975) release from synaptosomes and histamine secretion from mast cells (Foreman, Mongar and Gomperts, 1973). Therefore, it can be seen that agents affecting calcium binding and influx will exert some control over

neurotransmitter release. A further point of interest is that the results obtained with calcium ionophore, A23187, were thought to be evidence for neurotransmitter release not being dependent upon depolarization. However, Wonacott et al., (1978) found that calcium ionophore A23187 caused a decrease in the intrasynaptosomal levels of K^+ and an increase in the levels of Na^+ and Cl^-, suggesting that a depolarization process had occurred.

It has been proposed that neurotransmitter release might be controlled by a negative feedback mechanism (similar to negative feedback inhibition of enzyme synthesis) onto the presynaptic terminal (Langer, 1974; Bunney, 1975; Roth et al., 1975). It is believed that the neurotransmitter itself or other compounds (e.g. prostaglandins,cyclic AMP or other transmitters) released together with the transmitter, react with a pre -synaptic receptor causing a reduction in release. This hypothetical mechanism is discussed more fully in a later section.

Finally another phenomenon which may be of importance in the control of transmitter release is the existence of specialised interneuronal connections (e.g., axo-axonic and dendro-dentritic synapses) in certain regions of the nervous system. These specialised synapses are believed to be involved in inhibitory feedback loop mechanisms, e.g. dendro-dendritic synapses play a role in controlling mitral cell activity in the olfactory bulb (Shepherd et al., 1966, 1974, 1978).

The bulk of the work presented in this thesis was conducted using preparations enriched in nerve-terminals (synaptosomes) isolated from cerebral cortex, spinal cord and corpus striatum. Synaptosomes have been well

characterized in this and other laboratories and have been shown to retain the morphological and biochemical characteristics of the intact presynaptic terminal as seen *in situ*. They are also easily maintained under physiological conditions.

There now follows a description of the properties of synaptosomes.

2. PROPERTIES OF SYNAPTOSOMES

a. Morphology and Synaptosomal Function

Electron microscopy has shown that synaptosomes retain their normal morphological characteristics, having continuous membrane profiles enclosing synaptic vesicles, mitochondria and vacuoles (Jones and Bradford, 1971; Osborne et al., 1973). The continuity of the synaptosomal membrane has been shown by several biochemical parameters such as the retention of soluble cytoplasmic components, e.g. lactate dehydrogenase (Johnson and Whittaker, 1963), pyridine nucleotides (Lindall and Franz, 1967), diffusable ions such as potassium (Bradford, 1969), and putative transmitters including acetylcholine (Mangan and Whittaker, 1966; de Belleroche and Bradford, 1972a), glutamate, GABA and aspartate (de Belleroche and Bradford, 1972b). Bradford (1971) has estimated a theoretical synaptosomal membrane potential of -27mV, thus providing additional evidence for the integrity of the membrane. Furthermore, when synaptosomes are incubated in physiological salines they behave like intact neurons, showing high linear respiration, producing lactate and amino acids, generating phosphocreatine and ATP and have the ability to selectively accumulate K^+ against a concentration gradient (Bradford, 1969; Bradford

and Thomas, 1969). This demonstrates that synaptosomes retain the necessary cytoplasmic machinery for normal metabolism and this is contained within a functional membrane. The ability of synaptosomes to swell and shrink in media of different tonicities emphasises the existence of a continuous membrane (Marchbanks, 1967; Keen and White, 1970).

A portion of the post-synaptic membrane frequently remains attached to the nerve-ending showing that the integrity of the synapse is often maintained. Post-synaptic thickenings may sometimes be seen attached to the post-synaptic membrane, and it is possible to prepare fractions enriched in these structures using detergent extraction methods developed by De Robertis et al. (1967) and Cotman et al. (1971). However, Kornguth et al. (1969) have suggested that these postsynaptic thickenings are largely lost unless prepared using sucrose solutions containing 10mM $MgCl_2$.

Jones and Brearly (1972a, 1972b) have conducted a detailed morphological comparison of nerve endings in situ and in vitro (synaptosomes), finding only small differences. They showed that the hexagonal structure of the cytoplasmic filaments and pre-synaptic dense projections were more distinct in the in situ nerve-ending, also the post-synaptic thickenings of synaptosomes appeared to be less regular compared to that seen in situ.

Jones and Bradford (1971) have shown that synaptosomes incubated in physiological medium prior to fixation have a generally more spherical profile compared to those fixed directly from sucrose used during preparation. Also, no significant morphological differences, including simple

synaptic vesicle population size, have been detected between stimulated and unstimulated synaptosomes.

Finally, subfractionation of synaptosomes following their lysis has provided preparations enriched in synaptic vesicles. Several methods of rupture have been employed, including osmotic disruption with deionised water (Johnson and Whittaker, 1963; De Robertis et al., 1963) and successive freezing and thawing.

b. Respiration and Glucose Utilization

Synaptosomes when incubated in Na^+rich media containing 5 - 10mM glucose show a high linear rate of respiration (Bradford, 1969; Bradford and Thomas, 1969; de Belleroche and Bradford, 1972; Osborne et al., 1973), being approximately 60 - 70µmoles of O_2/100mg protein/hr for both cerebral cortex and medulla/spinal cord preparations. This respiration supports the accumulation of K^+ and the formation of ATP, phosphocreatine, lactate (16µmoles/100mg protein/hr) and numerous other metabolites (Bradford, 1969, Bradford and Thomas, 1969). On stimulation (electrical pulses, raised K^+, veratrine) the synaptosomal uptake of O_2 has been found to rapidly increase, lactate production also being increased (Bradford, 1970; de Belleroche and Bradford, 1972c; Osborne et al., 1973; Wedege et al., 1977).

Incubating synaptosomes for extended periods has shown that the concentrations of K^+, high energy phosphates and respiration are maintained for several hours (Bradford et al., 1975), respiration declining to about 25% of the original level after 15 hours.

The enzymes involved in glycolysis, Krebs cycle, oxidative phosphorylation, hexose monophosphate shunt, amino acid -

metabolism and putative transmitter synthesis, have all been shown to occur in synaptosomes, their subcellular localization being exactly as in the whole tissue. Thus, the glycolytic enzymes are found in the cytoplasm, while the Krebs cycle enzymes and those concerned with oxidative phosphorylation are found associated with the mitochondria.

c. The Na^+/K^+ ATPase system and ion transport

A Na^+/K^+ ATPase system has been detected in synaptosomes which allows the accumulation of K^+ (Escueta and Appel, 1969) and extrusion of Na^+ (Ling and Abdel-Latif, 1968; Bradford, 1969). The enzyme is tightly bound to membranes and in 1965, Albers et al. showed that in subcellular fractions prepared from rat brain, the maximal activity was associated with membranes prepared from nerve-endings.

Sodium transport is Mg^{2+} and energy dependent, temperature sensitive and closely linked with the K^+ transport system. This link is evidenced by the K^+ stimulated and ouabain inhibited efflux of Na^+ and the Na^+ stimulated and ouabain inhibited uptake of K^+ (Ling and Abdel-Latif, 1968; Diamond and Fishman, 1973). Apart from ouabain, active transport of Na^+ & K^+ is also blocked by oligomycin (Whittam et al., 1964) and digitoxin.

Potassium uptake occurs against a concentration gradient and requires the presence of an exogenous substrate such as glucose (Bradford et al., 1973); it is also inhibited by metabolic poisons such as KCN (Bradford, 1969). Verity (1972) has shown that the transmembrane gradients of Na^+ & K^+ play an important role in the control of synaptosomal respiration and oxidative phosphorylation.

The Na^+/K^+ ATPase system may be intimately involved in

the uptake of various transmitter substances. Sodium dependent uptake processes have been shown for choline (Schuberth et al., 1966; Marchbanks, 1968a; Potter, 1968; Diamond and Kennedy, 1969), noradrenaline (Bogdanski et al., 1968), 5HT (Marchbanks, 1968; Bogdanski et al., 1968), GABA (Weinstein et al., 1965; Kuriyama et al., 1969; Martin and Smith, 1972), glycine, glutamate and aspartate (Logan and Snyder, 1971).

Schuberth et al. (1966) concluded that choline uptake must be mediated by the Na^+/K^+ ATPase system as it was inhibited by ouabain and oligomycin, both ATPase inhibitors. Similarly, glutamate, aspartate and GABA uptake is also inhibited by ouabain (Tsukada et al., 1963).

Calcium uptake has also been shown to occur in synaptosomes. This process requires ATP and Mg^{2+} (Lust and Robinson, 1968) and is stimulated by K^+ (Blaustein and Wiesmann, 1970a) and a low external concentration of Na^+ (Blaustein and Wiesmann, 1970). Calcium uptake is not inhibited by ouabain and is therefore probably not mediated by Na^+/K^+-ATPase.

d. Putative transmitters - presence of and synthesis

Since the presynaptic terminal is the region believed to synthesize, store and release neurotransmitter substances, it has been an important task to show that synaptosomes are able to synthesize and concentrate putative transmitters. In fact acetylcholine, noradrenaline, serotonin and dopamine (Gray and Whittaker, 1962; de Robertis et al., 1962; Inouye et al., 1963; Levi and Maynert, 1964; Laverty et al., 1963) have all been found in high concentrations in synaptosomes. For example, several groups of workers (Whittaker, 1959;

de Robertis et al., 1962; Kurokawa et al., 1965) have shown that up to 90% of the total brain acetylcholine is recovered in the synaptosomal fractions. Fluroescence photomicrography has made it possible to visualize catecholamines and serotonin in neurons directly (Hillarp et al, 1966). The technique involves the condensation of these amines in tissue sections with formaldehyde in a humid environment to form fluroescent isoquinolenes. Serotonin can be distinguished from catecholamines by the wavelength of fluorescence; serotonin appears bright yellow and the catecholamines bright green. Using this method, cell bodies, axons and nerve terminals of the monoamine containing neurons have been mapped throughout the brain (Fuxe et al., 1968; Dahlstrom and Fuxe, 1964, 1965).

The putative amino acid transmitters include glutamate, aspartate, ~~taurine~~, GABA and glycine and perhaps taurine. These substances are found throughout the CNS, and occur at concentrations several orders of magnitude greater than those of ACh or the catecholamines, and because of their high potency these levels are far in excess of those required for neurotransmission. Despite their richness in the nervous system, amino acids are not particularly concentrated in synaptosomes, as evidenced by the failure to demonstrate any selective storage of either aspartate or glutamate within cerebral cortex nerve-endings (Ryall, 1964; Mangan and Whittaker, 1966; Whittaker, 1968; Bradford and Thomas, 1969).

The inhibitory neurotransmitter, glycine is found in the highest concentrations in the ventral gray matter of the spinal cord (Aprison and Werman, 1965), and has been shown to be selectively taken up into nerve-endings containing flat vesicles (Matus and Dennison, 1971, 1972). Another inhibitory

amino acid, taurine, occurs in high concentrations in the mammalian brain (Agrawal et al., 1966, de Belleroche and Bradford, 1973). It is particularly rich in the neurohypophysis and pineal body and also occurs in the retina. It is enriched in nerve-endings and may be implicated in epilepsy, low tissue concentrations being found in epileptic foci in animal and human subjects (Koyama, 1972; Van Gelder et al., 1972). Using ^{14}C-taurine, binding was found to be highest in synaptosomes prepared from brain stem and spinal gray regions (Ciria et al., 1975). Unlike the other putative amino acid transmitters, taurine is not a constituent of proteins.

Enzymes capable of synthesizing putative transmitters have been shown to occur in synaptosomal preparations. Using isotopically labelled precursors, such as ^{3}H- choline, ^{3}H -phenylalanine, ^{3}H -tyrosine, ^{14}C -tryptophan and ^{14}C -glucose, the formation of the radioactive products of enzyme action have been demonstrated. In this way the synaptosomal synthesis of ACh, noradrenaline, 5HT and the putative amino acid transmitters, has been shown (Schuberth et al., 1970; McGeer et al., 1967; Karobath and Baldessarini, 1972; Grahame-Smith, 1967; Bradford and Thomas, 1969).

e. Uptake Systems

Active transport systems play a vital role in maintaining normal function in the nervous system. Glucose uptake into nerve-endings is thought to be mediated by both high and low affinity mechanisms (Diamond and Fishman, 1971, 1972; Fletcher and Bachelard, 1978). The high-affinity system is carrier mediated, Na^{+} dependent and has a Km of 0.24mM. It appears to be unique to the nervous system and its function

may be to support the high rate of oxidative metabolism that occurs at the nerve-ending. On the other hand, low affinity uptake occurs throughout the tissues of the body and has a km of approximately 75mM.

Nerve-endings have also been shown to possess uptake systems for neurotransmitters. As previously mentioned, Na^+ dependent uptake processes for noradrenaline, choline, 5HT and the putative amino acid transmitters, have been demonstrated. Putative amino acid transmitter uptake appears to be mediated by both high (Km 1 - 50μM) and low (Km 100μM - 1mM) affinity uptake systems (Logan and Snyder, 1971; Balcar and Johnston, 1972, 1973; Iversen and Johnston, 1971; Wofsey et al., 1971; Curtis et al., 1968; Aprison and McBride, 1973). The high affinity systems appear to be associated with distinct populations of nerve-endings and are absolutely Na^+ dependent, on the other hand, the low affinity systems are found generally throughout the CNS and other tissues and are not Na^+ dependent. Of particular interest is glycine, which is an inhibitory transmitter in the mammalian spinal cord, but not the cerebral cortex. Low affinity uptake for glycine was shown for both tissues, however, a high affinity uptake system was detected only in the spinal cord (Johnston and I versen, 1971). It was therefore suggested that high affinity uptake may play a role in the termination of amino acid transmitter action (Aprison and McBride, 1968). However, this proposed function for high-affinity uptake is not accepted by all workers in this field. For example, results obtained recently by Cox et al. (1977) suggest that low affinity uptake may be of equivalent importance to high affinity uptake in the termination of excitatory amino acid neuro-

transmitter action. These workers found that the time for both onset and offset as well as the time for the rise to plateau of excitation by electrophoretically applied D-glutamate, L-glutamate, D-aspartate and L-aspartate are very similar, although D-glutamate differs from the other amino acids in not being taken up by a high-affinity carrier (Balcar and Johnston, 1972; Benjamin and Quastel, 1976; Davies and Johnston, 1976).

f. Stimulation of synaptosomes

Depolarization of the synaptosomal membrane potential may be achieved by (1) the application of electrical pulses (2) raising the medium concentration of potassium to 56mM, or (3) using veratrine. Veratrine is a mixture of plant alkaloids (cavadilline, sebadine, cevadine and the active component veratridine) and has gained considerable popularity in recent years as a depolarizing agent. ~~as it's mechanism of action has been fully elucidated.~~ It has been shown to produce its effect by causing a rapid influx of sodium ions into synaptosomes. This influx may be blocked using tetrodotoxin (Goldman and Blaustein, 1966) which is known to act on the "active" sodium channels associated with the action potential.

Stimulation elicits a metabolic response in synaptosomes increasing O_2 uptake and lactate production. Bradford (1970) showed that in cortical synaptosomes electrical pulses caused an immediate and rapid increase in respiration at an initial rate which was 50 - 60% higher than the level prior to stimulation. Lactate levels were also 50 - 60% higher than in unstimulated controls. Elevation of the medium potassium level to 56mM or using veratrine has similar effects on O_2

uptake and lactate production (de Belleroche and Bradford, 1972a; Wedege et al., 1977) and comparable results can be obtained using synaptosomes prepared from other tissues, e.g. hypothalamus (Bradford et al., 1973), spinal cord (Osborne et al., 1973) and corpus striatum (de Belleroche et al., 1976).

Depolarizing influences also cause a simultaneous calcium dependent release of neurotransmitter substances. De Belleroche and Bradford (1972a) have reported the release of acetylcholine from cortical synaptosomes, and electrical pulses and 56mM K^+ are known to cause the release of dopamine from nerve-endings prepared from sheep corpus striatum (de Belleroche et al., 1976). Noradrenaline is also released by veratrine and high K^+ concentrations from cortical synaptosomes in a Ca^{2+} dependent process (Blaustein et al., 1972). The release of the physiologically active amino acids glutamate, aspartate and GABA from cortical synaptosomes, has also been well documented (Bradford, 1970; de Belleroche and Bradford, 1972b; Raiteri et al., 1974; Wedege et al., 1977). Spinal cord synaptosomes release aspartate, glutamate, GABA and in addition glycine (Osborne et al., 1973; Osborne and Bradford, 1974).

3. NEUROTRANSMITTER ROLE FOR GLUTAMATE, ASPARTATE, GABA AND GLYCINE

a. Criteria for the identification of a neurotransmitter

Neurotransmitters are substances which mediate synaptic activity by altering the permeability of neuronal membranes to specific ions. They fall into two categories; excitatory neurotransmitters and inhibitory neurotransmitters.

Excitatory transmitters increase membrane permeability to sodium ions resulting in a depolarization. Alternatively, inhibitory transmitters increase the permeability to K^+ and/or Cl^- causing a hyperpolarization.

To be classed as a neurotransmitter a chemical substance must fulfill a certain set of criteria (Werman, 1966; Hebb, 1970), the basic criterion being its ability to mimic the electrophysiological action of the natural transmitters.

Other criteria are:

1. Nerves should possess the enzymes required to produce the chemical or be shown to accumulate it.
2. On stimulation, nerves should liberate the chemical.
3. The chemical should react with a specific receptor on the post-synaptic membrane and produce ~~a biological~~ the same physiological response as the neurally released transmitter.
4. Mechansims should be available to terminate the actions of the chemical rapidly.
5. The natural and proposed transmitters should be affected in the same way by pharmacological agents, e.g. agonists and antagonists.

Criteria 1, 2 and 4 are the province of neurochemistry to investigate, and synaptosome preparations have been useful for this purpose. These neurochemical criteria applied to putative amino acid transmitters are presented below.

b. <u>The excitatory amino acids, aspartate and glutamate.</u>

<u>Pharmacology/Physiology</u>

The acidic amino acids aspartate and glutamate are ubiquitous throughout the central nervous system occuring in very high concentrations (mM range). It was Hayashi

(1954, 1956) who first put forward glutamate as an excitatory transmitter candidate, basing his suggestion on its extensive and potent action when applied to the brain surface in vivo. However, the main body of pharmacological evidence for aspartate and glutamate's role as excitatory neurotransmitters has come from iontophoretic studies. Glutamate and aspartate will excite most neurons in the mammalian CNS when administered electrophoretically from micropipettes. Similarly, applied glutamate has been shown to have an excitatory effect on invertebrate neuromuscular junction preparations.

As the excitatory effects of these amino acids on both mammalian and invertebrate systems have been reviewed excellently elsewhere (Curtis and Johnston, 1974 Johnston, 1976 Gerschenfeld, 1973), it is not my intention to dwell on this subject here. However, some mention must be made of the neurophysiological work that has been conducted on glutamate analogues, as some of these compounds were tested for their effects on synaptosomal amino acid release.

Kainic acid and several related compounds (alpha-allo-kainic acid, domoic acid and quisqualic acid) have been found to be potent glutamate-like excitants of rat cerebral and cat spinal neurones. These compounds occur in certain marine algae and are as much as two orders of magnitude more potent than L-glutamate (Shinozaki and Konishi, 1970; Johnston, et al., 1974; Johnston, 1976; Biscoe et al., 1975). Several compounds have also been shown to reversibly block the excitatory effects produced by L-glutamate and L-aspartate. These antagonists include L-glutamate diethyl ester, DL-methyl-glutamate and HA966 (1-hydroxy-3-aminopyrrolidone-2)

and have been tested on spinal, ventrobasal thalamic, lateral geniculate, cerebral cortical and cuneate neurones (Curtis et al., 1972; Haldeman et al., 1972; McLennan and Haldeman, 1973; Davies and Watkins, 1973a, 1973b). On the crab neuromuscular junction, Wheal and Kerkut (1974, 1976) found that GDEE produced a similar effect to that seen in mammalian neurones, reversibly blocking the evoked excitatory junctional potentials.

Regional Distribution

Regional differences in the levels of these amino acids do occur, suggesting that they are of more importance in some parts of the nervous system than in others. However, their wide involvement in the metabolic and biosynthetic activities of neural tissue preclude a clear cut localization at nerve terminals.

Initial studies on the distribution of aspartate and glutamate were carried out on the spinal cord. The regional variation in the steady state levels of these amino acids in the cat spinal cord and nerves, together with changes following anoxic destruction of interneurones (induced by occlusion of the thoracic aorta) led to the suggestions that (1) glutamate is an excitatory transmitter released by primary afferents and (2) aspartate is a transmitter associated with excitatory interneurones (Graham et al., 1967; Davidoff et al., 1967a). It has been clearly shown that these amino acids are associated with defined regions of the spinal cord and roots.

In normal rat brain, L-glutamate levels are considerably higher in the cerebral hemispheres and cerebellum than in

the midbrain and pons-medulla, while aspartate is more evenly distributed (Shaw and Heine, 1965).

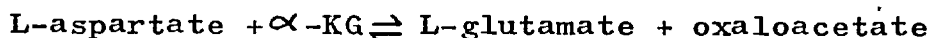
Subcellular fractionation studies on rat and guinea pig brain homogenates do not indicate a selective localization of L-glutamate or L-aspartate with nerve-ending particles (Mangan and Whittaker, 1966; Bradford and Thomas, 1969).

<u>Synthetic Enzymes</u>

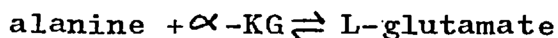
Glutamate may be synthesised in a transamination reaction involving the coupling of α-KG with an amino or oxo-acid (Albers et al., 1962; Graham and Aprison, 1969; Benuck et al., 1971).

The most important transaminases are aspartate, alanine and GABA:α-KG aminotransferases:

1. Aspartate aminotransferase


L-aspartate + α-KG $\rightleftharpoons$ L-glutamate + oxaloacetate

2. Alanine aminotransferase


alanine + α-KG $\rightleftharpoons$ L-glutamate

3. GABA aminotransferase

GABA + α-KG $\rightleftharpoons$ L-glutamate + succinic semialdehyde

These enzymes are all pyridoxal phosphate dependent and have been shown to occur in both mitochondrial and cytoplasmic fractions of homogenised brain and spinal cord tissue (Salvador and Albers, 1959; Baxter and Roberts, 1958; Pitts et al., 1965; Salganicoff and de Robertis, 1965; Graham and Aprison, 1969; Benuck et al., 1972). GABA-transaminase has also been detected in the CNS and peripheral nervous system of the lobster (Hall and Kravitz, 1967).

Glutamate may also be synthesised in a reaction involving the hydrolysis of glutamine. The enzyme

responsible for this reaction, glutaminase, has been shown to be enriched in brain mitochondrial fractions (Salganicoff and de Robertis, 1965; Bradford and Ward, 1976).

4. Glutaminase

L-glutamine + $H_2O \rightarrow$ L-glutamate + NH_3.

As can be seen from the reaction catalyzed by aspartate aminotransferase, it is reversible, therefore this provides one route for aspartate synthesis.

Studies on the regional distribution of these enzymes in spinal cord (Graham and Aprison, 1969) showed that their activities in the dorsal and ventral gray and white tissues were equal. However, GABA-aminotransferase activity exhibits regional variation in monkey brain (Salvador and Albers, 1959), the highest activities being detected in some of the subcortical structures, e.g. dentate nucleus, inferior colliculus, abducens nucleus, superior colliculus and inferior olivary nucleus.

<u>Release of aspartate and glutamate</u>

The release of amino acids from CNS tissue has been studied both under resting conditions ("spontaneous efflux") and under conditions whereby the preparation has been stimulated electrically, or by high K^+ concentration or veratrine ("evoked efflux").

In the case of aspartate and glutamate release studies have been primarily concerned with the latter, i.e. evoked efflux. This efflux has been examined both <u>in vivo</u> and <u>in vitro</u>.

<u>In vivo</u> studies have principally involved the collection of a perfusate from the surface of the exposed cerebral cortex

or from the ventricles, following direct or indirect stimulation of neuronal pathways. In this way Jasper and Koyama (1969) showed the release of endogenous glutamate and to a lesser extent aspartate, from the surface of the cerebral cortex following stimulation of the brain stem. Similarly Crawford and Connor (1973) detected endogenous glutamate efflux from the hippocampus in response to local or entorhinal stimulation. The release of radioactive glutamate from the surface of the cerebral cortex has also been investigated in relation to direct cortical stimulation (Roberts, 1973). A more sophisticated cannulation method for studying endogenous amino acid release has been developed by Dodd and Bradford (1974, 1976).

In vitro studies have involved a variety of different preparations. The stimulus-induced release of glutamate and aspartate has been shown for synaptosomes prepared from various CNS tissues including cerebral cortex, spinal cord, hypothalamus and corpus striatum (Bradford, 1970; de Belleroche and Bradford, 1972; Bradford et al., 1973; Osborne et al., 1973; Osborne and Bradford, 1975; de Belleroche et al., 1976). Slices have also been found to release glutamate (Mitchell et al., 1969; Hammerstad and Cutler, 1972; Roberts and Mitchell, 1972; Bradford and Richards, 1976), and aspartate (Roberts and Mitchell, 1972; Davies and Johnston, 1976), under similar conditions of incubation.

The stimulus induced release of glutamate has been demonstrated to occur from frog sciatic nerves where stimulation caused a 200% increase in the rate of release (Wheeler et al., 1966). Similarly, L-glutamate has been

detected in perfusates of the leg of the crab Carcinus maenus (Kerkut et al., 1965), cockroach Peripleneta americana (Kerkut et al., 1965), locust Schistocerca gregaria (Usherwood et al., 1968) and Helix (Kerkut et al., 1965).

Termination of glutamate and aspartate action

The termination of neurotransmitter action is vital for normal synaptic function. If a neurotransmitter is allowed to act for too long, serious and irreparable damage can result.

With the demonstration that ACh was inactivated by enzymic action (i.e. by acetylcholinesterase) the search was on for enzymes capable of breaking down the other transmitters. But, as far as the putative amino acid transmitters were concerned, no results were forthcoming. However, in 1967, Iversen demonstrated that noradrenaline inactivation involved a high-affinity uptake process, and so it was suggested that amino acid action might be terminated in a similar fashion. Indeed, as described in a previous section high-affinity uptake systems for the amino acid transmitters were discovered.

Logan and Snyder (1971, 1972) showed that unique high-affinity uptake systems existed for both glutamate and aspartate in synaptosomes prepared from both cerebral cortex and spinal cord. In the spinal cord the high-affinity uptake of glutamate was found to occur into the dorsal root at twice the rate as that for the ventral root (Hammerschlag, 1971; Roberts and Keen, 1973).

As previously mentioned some workers (Cox et al., 1977) have now suggested that low-affinity uptake may also be of

importance in terminating excitatory amino acid neurotransmitter action.

c. The inhibitory amino acids, GABA, glycine and taurine.

Pharmacology/Physiology

It has been known for some time that the neutral amino acids GABA and glycine have an inhibitory effect on neuronal firing, causing hyperpolarization and increased Cl^- permeability.

As with the excitatory amino acids (i.e. aspartate and glutamate) iontophoretic studies have played a major role in determining their mode of action. Thus, GABA and glycine have been shown to have a depressant effect on many mammalian neurones (reviewed by Werman and Aprison, 1968; Curtis and Watkins, 1965; Curtis and Johnston, 1974). GABA also increases the membrane conductance of lobster muscle fibres (Curtis and Watkins, 1965) and in this and other crustaceans, has a postsynaptic action similar to that of the naturally occurring inhibitory transmitter (Curtis and Watkins, 1965).

The acidic amino acid, taurine, has also been shown to inhibit neuronal firing, however, in general it has a weaker depressant action than GABA (Crawford and Curtis, 1964; Krnjević and Phyllis, 1963). Nevertheless, in some neural regions, e.g. cerebral cortex, taurine has a stronger depressant action than that of glycine. There is still considerable doubt as to taurine's proposed role as a neurotransmitter. I shall therefore concern myself mainly with GABA and glycine.

GABA

The depressant activity of GABA in both mammalian and

invertebrate systems, has been known for some years, however, attempts to investigate its pharmacology using various alkaloids, e.g. picrotoxin and bicuculline, has only met with variable success. Both toxins have been found to vary widely in their potencies (Straughan et al., 1971; Duggan, 1972) and some inconsistencies regarding their specificity of effects have also been reported (Krnjevic et al., 1966; Krnjevic, 1970; Curtis et al., 1968, 1969; de Groat, 1970; Hösli and Tebecis, 1970; Obata et al., 1967; Godfraind et al., 1970; Straughan et al., 1971; Hill et al., 1973). These partially contradictory results indicate that some caution is still necessary when interpreting the results of pharmacological experiments using bicuculline and picrotoxin. Further difficulties stem from the observation that picrotoxin seems to inhibit the release of GABA, whereas bicuculline enhances it (Johnston and Mitchell, 1971).

Bicuculline has been shown to be a relatively selective GABA antagonist in cuneate nucleus (Kelly and Renaud, 1971), spinal cord (Curtis et al., 1970a, 1971a), cerebral and cerebellar cortex (Curtis et al., 1970a; Curtis and Felix, 1971; Curtis et al., 1971b; Bistri et al., 1971), Deiter's nucleus (Curtis et al., 1970b), lateral vestibular nucleus and ventrobasal thalamic nucleus (Curtis et al., 1971b) neurones. Bicuculline also antagonizes GABA action on invertebrate systems, e.g. crayfish neuromuscular junction (Takeuchi and Onodera, 1972) and crayfish stretch receptor (McLennan, 1970). The evidence presented so far regarding bicuculline action has concentrated on its block of GABA induced postsynaptic inhibition, however, bicuculline is now known to have a presynaptic action. In the cuneate nucleus

bicuculline blocks GABA induced presynaptic inhibition (Davidson and Reisine, 1971). Several convulsant derivatives of bicuculline have also been shown to block presynaptic inhibition and the inhibitory effects of GABA: N-methylbicuculline (Pong and Graham, 1972; Davidoff et al., 1973) and bicuculline methochloride (Johnston et al., 1972).

Like bicuculline, picrotoxin antagonizes both the presynaptic and postsynaptic inhibitory actions of GABA. Its postsynaptic effect has been studied in the crayfish neuromuscular junction (Takeuchi and Takeuchi, 1969) and on neurons in the cuneate nucleus (Galindo, 1969), spinal cord (Engberg and Thaller, 1970), Deiter's nucleus (Ten Bruggencate and Engberg, 1971), cerebral cortex (Hill et al., 1972) and cerebellar cortex (Woodward et al., 1971). As with bicuculline, picrotoxin blocks presynaptic inhibition in the cuneate nucleus (Davidson and Reisine, 1971).

GLYCINE

Glycine is a major constituent of nervous tissue and is known to be the principle inhibitory transmitter in the spinal cord.

The inhibitory effect of glycine on spinal neurones was first demonstrated by Curtis and his coworkers (Curtis and Watkins, 1960a, 1960b; Curtis et al., 1961) using an iontophoretic technique. Initially, glycine was discounted as a neurotransmitter, however, in 1967, Werman et al., reported that glycine and the natural inhibitory transmitter had remarkably similar actions on spinal motoneurones. Since then glycine has been shown to have an equally effective

depressant action on Renshaw cells (Curtis et al., 1968a) and cuneate neurones (Galindo et al., 1967).

Pharmacological studies using the alkaloid, strychnine, have added greatly to our knowledge of glycine action (Curtis, 1963). Iontophoretically applied strychnine blocks both the hyperpolarization caused by glycine and the natural transmitter; it has no effect on GABA action. It is suggested that strychnine acts by blocking the action of glycine at the postsynaptic receptor (Curtis et al., 1967; 1968b; Curtis, 1969).

Regional Distribution

GABA and glycine have a wide distribution in the mammalian CNS.

A correlation between inhibitory action and GABA concentration has been observed in several regions of the CNS. For example, in the spinal cord, the concentration of GABA is much higher in the dorsal grey matter than in the white matter and roots, which is compatible with the role of this amino acid as a postsynaptic inhibitory transmitter (Aprison et al., 1965; Aprison and Werman, 1965; Graham et al., 1967). Studies on the GABA content of the cerebellum have shown that the highest concentrations are to be found associated with the inhibitory Purkinje cells (Hirsch and Robins, 1962; Kuriyama et al., 1966). High levels of GABA are also found in the outer layers of the cerebral cortex (Hirsch and Robins, 1962). Subcellular distribution studies indicate that GABA is apparently a cytoplasmic constituent of neurons without selective localization within nerve endings (Weinstein et al., 1963; Mangan and Whittaker, 1966).

GABA has also been shown to be associated with specific

neurones in invertebrate systems. Distribution studies on lobster excitatory and inhibitory neurones have shown that GABA was 100 times more concentrated in the inhibitory axon and that although glutamate decarboxylase (enzyme which synthesises GABA from glutamate) occurred in equal amounts in both types of axon, its activity was 11 times greater in the inhibitory neurone (Kravitz et al., 1965).

Like GABA, glycine levels are also highest in the gray matter of the spinal cord. However, whereas the concentration of GABA is higher in the dorsal than the ventral gray matter, the reverse situation obtains for glycine (Aprison and Werman, 1965; Aprison et al., 1969). Its distribution is therefore compatible with a possible role for glycine as a postsynaptic inhibitory transmitter. Aortic occlusion studies, have also provided evidence for a link between the spinal distribution of glycine and its role as an inhibitory transmitter (Davidoff et al., 1967a, 1967b).

High concentrations of glycine are also found in the medulla oblongata, and outside the spinal cord, in the dorsal root ganglia and some of the peripheral nerves, e.g. sural nerve and gastrocnemius nerve (Duggan and Johnston, 1970). The lowest concentrations are found in the cerebellum and cerebral cortex (Aprison et al., 1968).

Synthetic Enzymes

GABA is synthesised in a decarboxylation reaction from glutamate. The enzyme responsible for this reaction is glutamate decarboxylase and requires the cofactor pyridoxal phosphate for maximal efficiency. The decarboxylase has been shown to be at higher levels in grey matter than in white (Müller and Langeman, 1962; Albers and Brady, 1959) and has

been localized in nerve endings (Weinstein et al., 1963; Salganicoff and de Robertis, 1965; Balazs et al., 1966). The enzyme has also been shown to occur at high specific activities in the inhibitory neurones of the lobster (Kravitz et al., 1965).

The metabolism of glycine in the CNS is complex and poorly understood. In vivo labelling experiments with a variety of radioactive precursors indicate that glycine metabolism is connected to glucose and ribose metabolism, and that glycine is possibly in equilibrium with carbohydrate intermediates via more than one route (Shank et al., 1973). Serine and glyoxylate are the likely immediate precursors of glycine in the CNS. Serine and glycine are interconvertible by means of the enzyme serine hydroxymethyl transferase, which has been purified from brain (Broderick et al., 1972). Glyoxylate can be transaminated to glycine by extracts of CNS tissues in the presence of various amino group donors (Johnston et al., 1970; Benuck et al., 1971).

Release of GABA and glycine

Release of GABA from the brain to surrounding fluids has been demonstrated both in vitro and in vivo. It was released on local superfusion of the monkey brain (de Feudis et al., 1969) to fluids in contact with the neocortex of cats (Mitchell and Srinivasen, 1969) and also to those of the fourth ventricle (Obata and Takeda, 1969). GABA is also released from the surface of the cat visual cortex; stimulation of the cortex with bipolar electrodes or of the lateral geniculate nucleus raises the control release 3 - 6 fold (Iversen et al., 1971).

Stimulus induced release of GABA from brain slices and

synaptosomes prepared from various regions has also been demonstrated (Srinivasan et al., 1969; de Belleroche and Bradford, 1972; Osborne et al., 1973; Bradford et al., 1973; de Belleroche et al., 1975). This release was shown to be calcium dependent by de Belleroche and Bradford (1972).

A release of GABA in response to inhibitory nerve stimulation has been demonstrated at the neuromuscular junctions of the lobster (Otsuka et al., 1966).

Similarly, the stimulus induced release of glycine from the spinal cord has been shown both *in vivo* and *in vitro*. Jordan and Webster (1971) demonstrated ^{14}C-glycine release from the spinal cord of anaesthetized cats, when the glycine uptake blocker p-hydroxymercuribenzoate was included in the bathing saline.

Slices of rat spinal cord preloaded with ^{14}C-glycine have been shown to release ^{14}C-glycine in response to electrical stimulation (Hopkin and Neal, 1970). The stimulus induced release of endogenous glycine from spinal cord synaptosomes has also been found to occur (Osborne and Bradford, 1973).

Termination of GABA and glycine action

As for aspartate and glutamate, unique high affinity uptake systems for both GABA and glycine have been shown to occur in the mammalian nervous system (Logan and Snyder, 1971; Balcar and Johnston, 1972, 1973). These uptake systems are believed to be responsible for terminating GABA and glycine action.

The uptake of GABA into both invertebrate and vertebrate tissues has been demonstrated. Thus, a saturable Na^+ dependent uptake of GABA was found in the lobster nerve

muscle preparation (Iversen and Kravitz, 1968), rat cerebral cortex slices (Iversen and Neal, 1968) and synaptosomes (Logan and Snyder, 1971).

Mammalian spinal cord slices have been shown to accumulate glycine by a sodium dependent and temperature sensitive process (Neal and Pickles, 1969; Neal, 1971). In addition, it has been found that in cerebral cortex, glycine is accumulated by a low affinity system only, whereas in the spinal cord glycine is taken up by both low and high affinity systems (Johnston and Iversen, 1971; Logan and Snyder, 1971). This fact provides further evidence for glycine's role as an inhibitory neurotransmitter in the spinal cord.

4. NEUROTRANSMITTER RECEPTORS

As mentioned previously, one of the processes occurring during neurotransmission involves the interaction of the neurotransmitter molecule with a specific receptor site on the postsynaptic membrane. This elicits a metabolic response, which ultimately leads to an active physiological process such as an action potential.

Until recently, our knowledge of synaptic receptors came principally from physiological and pharmacological studies involving the iontophoretic injection of putative neurotransmitters and observing the effects of certain psychopharmacological agents which are now known to alter neurotransmitter receptor site function (Snyder and Bennett, 1976; Werman, 1972; Werman et al., 1968). Measurement of conductance changes at the cell membrane also gave a greater insight into the drug-receptor interaction (Werman, 1972). Pharmacologists also tried to interpret the possible structure of the receptor site from the stereochemical

conformation of specific agonists and antagonists.

These studies however, did not provide a direct analysis of the primary drug-receptor interaction. Some workers believed that for further progress to be made in this field it was necessary to isolate a pure preparation of receptor from its membrane. Organic solvents (e.g., chloroform/methanol) and detergents (e.g., Triton X-100) have been used for this purpose and synaptic receptors isolated so far, have been found to be hydrophobic proteins (i.e., proteolipids) intimately associated with the membrane lipids (de Robertis, 1975). However, the drastic conditions involved in receptor isolation, raises the question of whether the characteristics of the receptor are altered: there is some evidence for this occurring.

A number of workers claim that studying the receptor in its normal membrane environment provides the most biologically meaningful data concerning receptor function. Labelling of neurotransmitter receptors in brain membrane preparations is now possible involving the binding of radioactive forms of the neurotransmitter itself, specific agonists and antagonists (Hiley et al., 1972; Yamamura and Snyder, 1974; Yamamura et al., 1974; Zukin et al., 1974; Enna and Snyder, 1975). However, it has been found that most radioactive chemicals bind to biological membranes in a relatively non-specific fashion, there being an almost infinite number of non-specific binding sites. Therefore in order to distinguish the non-specific binding sites and the less numerous specific receptors, the use of ligands with high receptor affinity labelled to high specific radioactivity and employed at low concentrations, have been

used.

Neuronal receptors have been classified into two types depending upon their synaptic location, post-synaptic receptors and pre-synaptic receptors. Post-synaptic receptors have been excellently reviewed elsewhere (Snyder and Bennett, 1976; de Robertis, 1975; Hall, 1972; O'Brien et al., 1972; Usdin and Bunney, 1975). I shall therefore focus my attention on presynaptic receptors.

Presynaptic Receptors

As mentioned previously, neurotransmitter release may be controlled by a negative feedback mechanism onto the presynaptic terminal. Thus, the neurotransmitter itself or other compounds released together with the transmitter, react with a presynaptic receptor causing a reduction in release.

To date, the evidence for presynaptic receptors has come principally from experiments on dopaminergic and nor-adrenergic systems. Several groups (Kehr et al., 1972; Langer, 1974 Roth et al., 1973, 1974; Walters and Roth, 1974, 1975; Walters et al., 1974) have suggested that such a receptor may exist on central dopaminergic terminals and in addition to regulating transmitter release might also be involved in the modulation of dopamine synthesis. A presynaptic receptor may be responsible for modulating the activation of tyrosine hydroxylase, one of the enzymes involved in dopamine synthesis (Christiansen and Squires, 1974).

Noradrenaline release may be modulated by two mechansims. The first of these two mechanisms, proposed by Hedqvist (1970) and Stjärne (1973), suggests that the

release of noradrenaline is controlled by a negative feedback system which involves a local synthesis of prostaglandin E (PGE_1 and PGE_2). Alternatively, noradrenaline itself may inhibit its own release by an action on presynaptic α-adrenergic receptors (Enero et al., 1972; Starke, 1972). Findings reported by Szerb and Somogyi (1973) suggest that a similar "auto-inhibition" mechanism may exist in cholinergic neurones.

De Belleroche and Bradford (1978) have demonstrated that acetylcholine has a controlling effect on dopamine release from synaptosomes isolated from corpus striatum. Both muscarinic and nicotinic receptors are believed to be implicated in the cholinergic action on dopaminergic neurons of the striatum (Giorguieff et al., 1976; Westfall, 1974; Giorguieff et al., 1976). Presynaptic muscarinic cholinergic receptors may also be involved in controlling noradrenaline release. Sharma and Banerjee (1978) have reported that in the isolated rabbit heart, acetylcholine inhibits the release of noradrenaline evoked by nicotinic drugs, by KCl and by sympathetic nerve stimulation. Acetylcholine has also been reported to have a controlling effect on amino acid transmitter release from cerebral cortex slices (Benjamin and Quastel, 1977a, 1977b).

The putative amino acid transmitter, GABA, is known to evoke presynaptic inhibition in the cuneate nucleus (Davidson and Southwick, 1971; Davidson and Reisine, 1971), this inhibition being blocked by picrotoxin and bicuculline. This finding implies that a presynaptic receptor may be involved in this mechanism.

Finally, Giorguieff et al., (1977) have recently

obtained evidence showing that L-glutamate increases the spontaneous release of ^{3}H -dopamine in rat striatal slices. This effect was not produced by D-glutamate and was still observed in the presence of tetrodotoxin. On the basis of this data these workers concluded that the amino acid might be acting at a presynaptic site (receptor).

5. THE PURPOSE OF THIS STUDY

The purpose of the *in vitro* work presented in this thesis was to examine the effects of various analogues and uptake blockers on putative amino acid transmitter release, in the expectation that evidence for the existence of pre-synaptic receptors modulating transmitter release would be obtained. In addition, the effect of several tremorgenic mycotoxins on synaptosomal amino acid release were investigated in an effort to gain an insight into their basic mechanism of action in the CNS, since they too might act by influencing transmitter release rates.

Finally, as outlined in the last main chapter of this tnesis, the effects of a number of agents on synaptosomes were compared with their actions on the miniature end plate potential frequency in a locust leg muscle preparation. The latter provides a convenient method for measuring amino acid transmitter (glutamate) release by "physiological" mechanisms by employing a "built-in" bioassay, i.e. measurement of post-synaptic potentials in the muscle cells.

CHAPTER TWO

METHODS AND MATERIALS

1. Preparation of Synaptosomes

2. Synaptosome Suspensions
   a. Preparation of synaptosome suspensions
   b. Incubation of synaptosomes
   c. Stimulation of synaptosomes
   d. Incubation with uptake blockers, analogues, etc.
   e. Uptake studies
   f. Testing the action of tremorgenic mycotoxins on synaptosomes
   g. Preloading of synaptosomes with ^{14}C-GABA

3. Protein Assay

4. Amino Acid Analysis

5. Liquid Scintillation Counting

6. Potassium Determination

7. Lactate Assay

8. Superfusion Studies

1. Preparation of synaptosomes

Synaptosomes were prepared by the method of Gray & Whittaker (1962) as modified by Bradford et al. (1973). Whole cerebral cortex or medulla/spinal cord tissue was rapidly removed from adult female Sprague-Dawley rats, previously killed by stunning followed by exsanguination, and placed in ice-cold 0.32M sucrose. Using a glass-perspex homogenizer a 10% (w/v) homogenate was prepared

in ice-cold 0.32M sucrose. This homogenate was then centrifuged in a Beckman 30 rotor at 1,000g for 10 mins in a Beckman L265B ultracentrifuge. Two fractions were obtained, the pellet (P_1) was discarded, however, the supernatant (S_1) was retained and centrifuged in the 30 rotor for 20 mins at 20,000g to yield the P_2 and S_2 fractions. The crude mitochondrial pellet (P_2) was resuspended in 0.32M sucrose and 15mls of this suspension carefully layered onto a discontinuous sucrose density gradient composed of 20ml of 0.8M sucrose, above 20ml of 1.2M sucrose. The sucrose gradients were then centrifuged at 76,000g in a Beckman swing-out SW25 rotor for one hour, to yield three fractions; P_2A, P_2B and P_2C. The P_2B (synaptosomal) fraction was collected, ensuring that equal amounts of sucrose above and below it were taken, to give a final sucrose concentration of approx. 1.0M. From a burette cold deionized distilled water (0-4°C) was then added drop-wise to the P_2B fraction in a conical flask placed in ice. All the while the suspension was continuously shaken to prevent osmotic shock to the synaptosomes. Water was added until the sucrose concentration reached approx. 0.45M, at this point the external sucrose concentration is approx. iso-osmotic with the contents of the synaptosomes. The synaptosome suspension was then spun at 54,000g for 20min in a 30 rotor to yield a synaptosomal pellet. This synaptosomal pellet was then resuspended in a suitable volume of Krebs-phosphate medium, ready for experimentation.

2. Synaptosome Suspensions

a. Preparation of synaptosome suspensions

Following the final centrifugation step the synaptosomal pellets were gently resuspended in Krebs-phosphate medium (composition described below) using a loose fitting Teflon pestle and mortar with a radial clearance of 180 microns.

b. Incubation of synaptosomes

Synaptosomes were incubated in Warburg respirometer flasks (usually 1.5ml of suspension per flask), in Krebs-phosphate medium, which has the following composition: NaCl, 124mM; KCl, 5mM; Na_2HPO_4, 20mM; KH_2PO_4, 1.2mM; MgSO4, 1.3mM; $CaCl_2$, 0.75mM; glucose, 10mM; pH 7.4.

The centre well of each flask was fitted with a NaOH-soaked filter paper wick. The flasks were placed in a water bath at 37°C, gassed with O_2 followed by an equilibration period. Incubation was usually continued for a further 30 mins, pressure (manometer) readings being taken every 5 mins. A thermal barometer was set up for each experiment to enable variations in external temperature and pressure to be taken into account.

Stimulation of the nerve-endings was achieved using several methods (described below).

At the end of the incubation period the suspensions were centrifuged at 96,600g (40,000r.p.m.) in a Beckman 50 rotor for 5 mins, in order to separate the medium from the nerve-endings. The clear supernatant was then taken for amino acid and lactate analysis whilst the pellet was analysed for protein, amino acids and K^+ levels.

c. Stimulation of synaptosomes

(i) Potassium stimulation

Synaptosome suspensions were incubated for 35 mins, whereupon 125µl of 600mM KCl (made up in medium) was added from the side-arm of the Warburg-flask, giving a final K^+ concentration of 56mM. Incubation was continued for a further 10 mins before terminating the experiment.

(ii) Veratrine stimulation

Synaptosome suspensions were incubated as described, for 35 mins before the addition of veratrine (dissolved in medium). Final concentrations of 10µM and 75µM were usually used.

d. Incubation with uptake blockers, amino acid analogues etc.

Synaptosomes were incubated together with a variety of agents, at the concentrations specified in the results sections. Nerve-endings were suspended in Krebs-phosphate medium containing the compound to be tested and incubated as described previously. Controls consisted of synaptosomes made up in normal Krebs-phosphate medium.

e. Uptake studies

One ml. volumes of synaptosomes made up in Krebs-phosphate medium (previously saturated with O_2) were preincubated at 37°C, for 5 mins. Then ^{14}C-glutamate was added, to give a final concentration of usually 30µM and the incubation continued for a further 5 mins. The samples were then spun down in a bench-top microcentrifuge (Ole-Dich, Copenhagen), the synaptosomal pellets extracted with 10% TCA and the supernatants counted.

Using this method a variety of agents were tested for their effects on glutamate uptake. In addition, the effects of β-bungarotoxin and Penicillium cyclopium on ^{14}C-GABA uptake were investigated using a similar procedure. *R.T.O.

f. Testing the action of tremorgenic mycotoxins on synaptosomes

Penicillium cyclopium, Penicillium estinogenum and penitrem A were kindly supplied by Dr. Peter Mantle (Department of Biochemistry, Imperial College).

The effects of the mycelia and pure toxin (penitrem A) were investigated using synaptosomes prepared from animals previously injected with these agents. In addition, penitrem A was tested for its action in the purely in vitro situation.

Injection of animals

Rats were injected intraperitoneally with the mycelium (400mg/kg) made up in H_2O or the purified toxin (400μg/kg) dissolved in Et.OH/H_2O (1:1). After approximately 45 mins following the appearance of severe tremoring and paralysis of the hind limbs, the animals were sacrificed, spinal-medullary or cerebro-cortical synaptosomes prepared and incubated as described above.

Experiments were also performed on nerve-endings prepared from samples of spinal cord, cerebral cortex and corpus striatum obtained from sheep that had been orally dosed for 2-3 months with Penicillium cyclopium.

Incubation of synaptosomes with penitrem A

Rat spinal-medullary synaptosomes were incubated in Krebs-phosphate medium containing pure penitrem A at a

concentration of 100µm.

g. Preloading of synaptosomes with ^{14}C-GABA

A number of release experiments outlined in this thesis were conducted on synaptosomes which had been previously labelled with ^{14}C-GABA.

Following the final centrifugation step, the synaptosomal pellets were gently resuspended in Krebs-phosphate medium containing 10µM ^{14}C-GABA (specific activity 224mCi/mmol, Radiochemicals Amersham, U.K.) and incubated at 37°C for 10 mins. After this incubation period the synaptosomes were sedimented, washed and resuspended in fresh medium. The synaptosomal suspensions were then shared between Warburg flasks and the experiment continued in the usual fashion.

That the bulk of the radioactivity released during these experiments was associated with GABA was established using an amino acid autoanalyser linked to a scintillation counter. Figure 2.1 illustrates typical chromatrograms obtained using this system.

3. Protein Assay

10% TCA precipitates were assayed for protein according to the method of Lowry et al. (1951).

The TCA precipitates were digested for 1hr in 1 vol of water to 2 vols of 4.5N NaOH, with frequent mixing on a whirlimixer.

Bovine serum albumin (Sigma Grade V) was used as a standard and was made up prior to each assay. The alkaline copper reagent used was also made up freshly, prior to use. It consisted of 2% Na_2CO_3, 0.01% $CuSO_4$ and 0.02% Na-K-

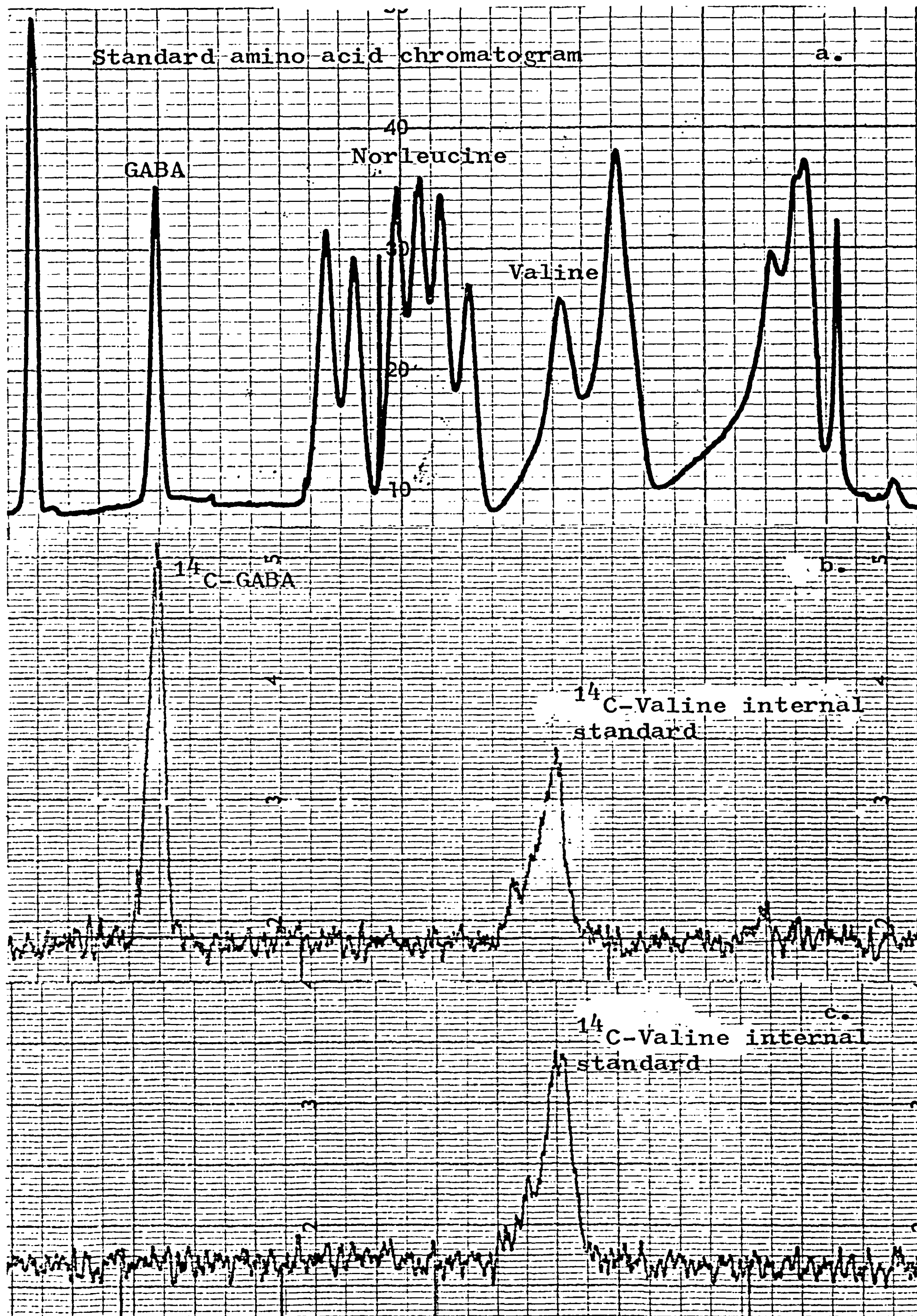
FIGURE 2.1

DETERMINATION OF PERCENTAGE OF TOTAL RELEASED RADIO-ACTIVITY ASSOCIATED WITH ^{14}C-GABA

Synaptosomes were prelabelled by incubating in Krebs-phosphate medium containing ^{14}C-GABA (final concentration, 10µM), as described in the text.

Following the subsequent period of experimentation, medium samples were treated with 10% TCA and the amino acids extracted and purified (as previously outlined). Samples were then analysed for both labelled and unlabelled amino acid, using the method of de Belleroche et al. (1976b). An internal standard of 250mCi of ^{14}C-Valine was included in each sample as well as nor-leucine.

As can be seen from chromatogram b, 100% of the label released upon stimulation was found to correspond to ^{14}C-GABA.


Standard amino acid chromatogram
a.
GABA
Norleucine
Valine
40
30
20
10
^{14}C-GABA
b.
^{14}C-Valine internal standard
c.
^{14}C-Valine internal standard

tartrate for the protein precipitate assays. For the BSA standard assays the Na_2CO_3 was made up in 0.1N NaOH. Folin ciocalteau reagent (BDH) was prepared just prior to use being diluted 1:1 with water.

A typical assay consisted of 100µl of sample, 1.1ml of H_2O and 3mls of alkaline copper reagent. This mixture was whirlimixed thoroughly and allowed to stand for 5 mins at room temperature. Then 0.3ml of diluted Folin-ciocalteau reagent was added and the solution thoroughly mixed. The mixture was then allowed to stand for 40 mins at room temperature and the O.D determined at 500mµ in a Unicam SP 600, fitted with a continuous flow cell. The BSA standards were assayed in the same fashion, a range of 0-200µg of BSA/tube being used.

4. Amino-acid analysis

TCA extracts obtained from both the incubation medium and synaptosome pellets were analysed for amino acids.

Prior to analysis the samples were purified to remove salts and other metabolites. This involved passing them through 10 x 0.5cm ion exchange columns containing Zeocarb 225 (52-100 mesh, 8% cross linked). The columns were first regenerated by washing with 1N HCl. Then the samples (each containing 1ml of 50nmol/ml nor-leucine, internal standard) were loaded onto the columns, followed by a water wash. Amino acids were eluted from the columns with 1M-NH_3 and the eluate evaporated to dryness using a Rotary Evapomix (Buchler Instruments) with the bath at 30-40°C. The dried extracts were taken up in 1ml of 0.025N HCl, ready for analysis. Recovery of amino acids from the purification columns was shown to be at least 95%.

The purified amino acid samples were analysed on an automated analyzer developed in this department (Bradford & Thomas, 1969). Each sample took approx. 3hrs for analysis and levels as low as 0.1nmol could be detected on this system.

Figure 2.2 shows a typical standard chromatogram obtained from this system. The quantities of each amino acid in a sample were determined on the basis of peak area with reference to the norleucine peak area which was equivalent to 50nmols/ml. These peak areas were either calculated by multiplying the peak height by the peak width at half the height or calculated automatically by a computer linked to the analyzer. As not all the amino acids reacted with OPA to the same extent (see Fig. 2.2) an amino acid colour factor had to be calculated. This colour factor was obtained by relating the peak areas of all the amino acids in a standard solution (50nmol/ml) with the peak area of norleucine, also at a concentration of 50nmol/ml. Therefore, if the colour factor for norleucine is regarded as 1, an amino acid giving a smaller degree of colour will have a colour factor greater than 1. And an amino acid giving a greater degree of colour than NL will have a colour factor less than 1. Thus the concentration of a particular amino acid in a sample was calculated using the following equation:

$$\frac{AA}{NL} \times C.F. \times 50 \times Vol$$

Where: AA = peak area of amino acid

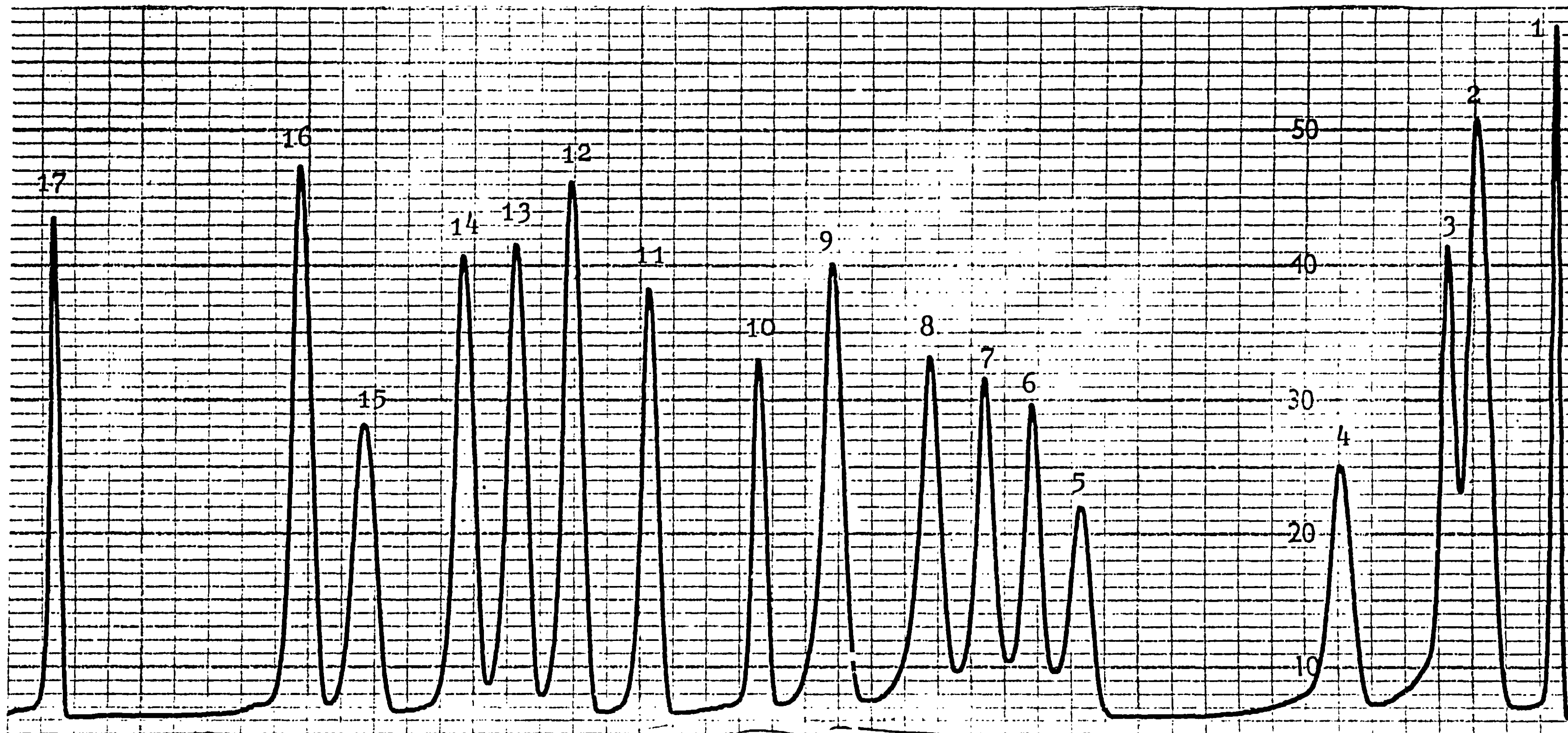
NL = peak area of norleucine

CF = colour factor of amino acid

Vol = volume of sample containing norleucine

FIGURE 2.2

STANDARD CHROMATOGRAPH OF AMINO ACIDS



1. Aspartate
2. Threonine
3. Serine
4. Glutamate
5. Proline
6. Glycine
7. Alanine
8. Citrulline
9. Valine
10. Cystine
11. Methionine
12. Isoleucine
13. Leucine
14. Norleucine
15. Tyrosine
16. Phenylalanine
17. GABA

at a concentration of 50nmol/ml

In most cases the amino acid concentration was expressed as nmols/100mg protein.

5. Liquid scintillation counting

A scintillant consisting of 5 parts of Toluene P.B.D. and 4 parts of 2-methoxyethanol was used. Toluene P.B.D. contained 250g naphthalene A.R. and 30g of butyl P.B.D. made up to 5 litres with toluene A.R.

Depending upon the volume of tissue extract to be counted volumes of 10 and 20ml of scintillant were used. A Packard-Tricarb scintillation counter was usually used for counting. A series of standards were used to achieve quench correction.

6. Potassium determination

Potassium determinations were done on the 10% (w/v) TCA extract obtained from the synaptosome pellets. Potassium was measured by flame photometry in an Eel flame photometer. A K^+ filter (730-850mμ) was used in the machine and calibration achieved using deionized water (zero deflection and 500μM K^+/1000μM Na^+ (100% deflection). Using a series of solutions of known potassium concentrations a standard curve of % deflection versus K^+ concentration was constructed. From this standard curve the K^+ concentrations in the synaptosomal extractions was determined.

7. Lactate assay

TCA extracts were assayed for lactate according to the method of Bergmeyer (1965). This is an enzymic method and

and depends upon the following reaction:

The assay relies on the fact that the amount of NADH produced is equivalent to the amount of lactate in the sample. Therefore, by measuring the absorption changes produced by standard solutions of lactate, the amount of this substance in tissue samples may be determined.

The assay buffer consisted of 14.7ml of hydrazine sulphate buffer (30g glycine, 20.8g hydrazine sulphate and 0.8g EDTA made up to 200ml with deionized water), 15.3ml of 2N NaOH, 4ml of 1% NAD and 22ml of deionized water.

0.2ml of tissue extract was added to 2.8ml of the assay buffer contained in a clean spectrophotometer cell. The solutions were thoroughly mixed and the absorbance (R_1) at 350nm measured in a Unicam SP500 spectrophotometer. Then 10μl of LDH (Boehringer, pig heart LDH) was added to the sample and the mixture thoroughly mixed. A reagent blank consisting of 2.8ml of assay buffer and 0.2ml of deionised water also had 10μl of LDH added to it, in order to measure any absorbance changes (Δ_1) occurring as a result of enzyme addition. ~~absorbance (R_3) determined.~~ After 45 mins, the absorbance (R_2) was measured whereupon 0.2ml of 0.5mM lactate was added, with mixing. The incubation was then continued for a further 45 mins and the final absorbance (R_3) determined.

The following equation was used to determine the

amount of lactate in the tissue extract:

1. Change in absorbance caused by unknown amount of lactate in extract = $(R_2 - R_1 - \Delta_1) = \Delta_2$

2. ditto. by 100µM standard lactate = $R_3 - R_2 = \Delta_3$

3. $\therefore$ the amount of lactate in 0.2ml of extract

$$= 100 \times \frac{\Delta 2}{\Delta 3} \text{ umoles}$$

8. Superfusion studies

The method used was a modification of the procedure described by Raiteri et al. (1974).

A crude mitochondrial extract (P_2) was prepared from normal rat medulla/spinal cord tissue. This extract was gently resuspended in Krebs-phosphate medium (gassed with O_2) to give a 10% (w/v) suspension. The P_2 suspension was preincubated for 15 mins at 37°C, whereupon ^{14}C-glycine was added (specific activity 10mCi/mmol Radiochemicals Amersham, U.K.) and incubation continued for a further 10 mins. Meanwhile the superfusion apparatus had been set up as in figure 2.3. The superfusion chambers consisted of a section of glass tubing (reservoir) connected to a Swinnex 13 millipore fitted with a 1.3cm filter of 0.8-1.2µM pore size. These chambers were connected to a multichannel Quickfit peristaltic pump with plastic tubing. The temperature was maintained throughout the experiment at 37°C by submerging the millipores in a water bath and the medium was gassed with O_2 by introducing a gas tube into the top of the medium reservoir.

Fifty microlitres of the P_2 suspension (approx. 2.0mg

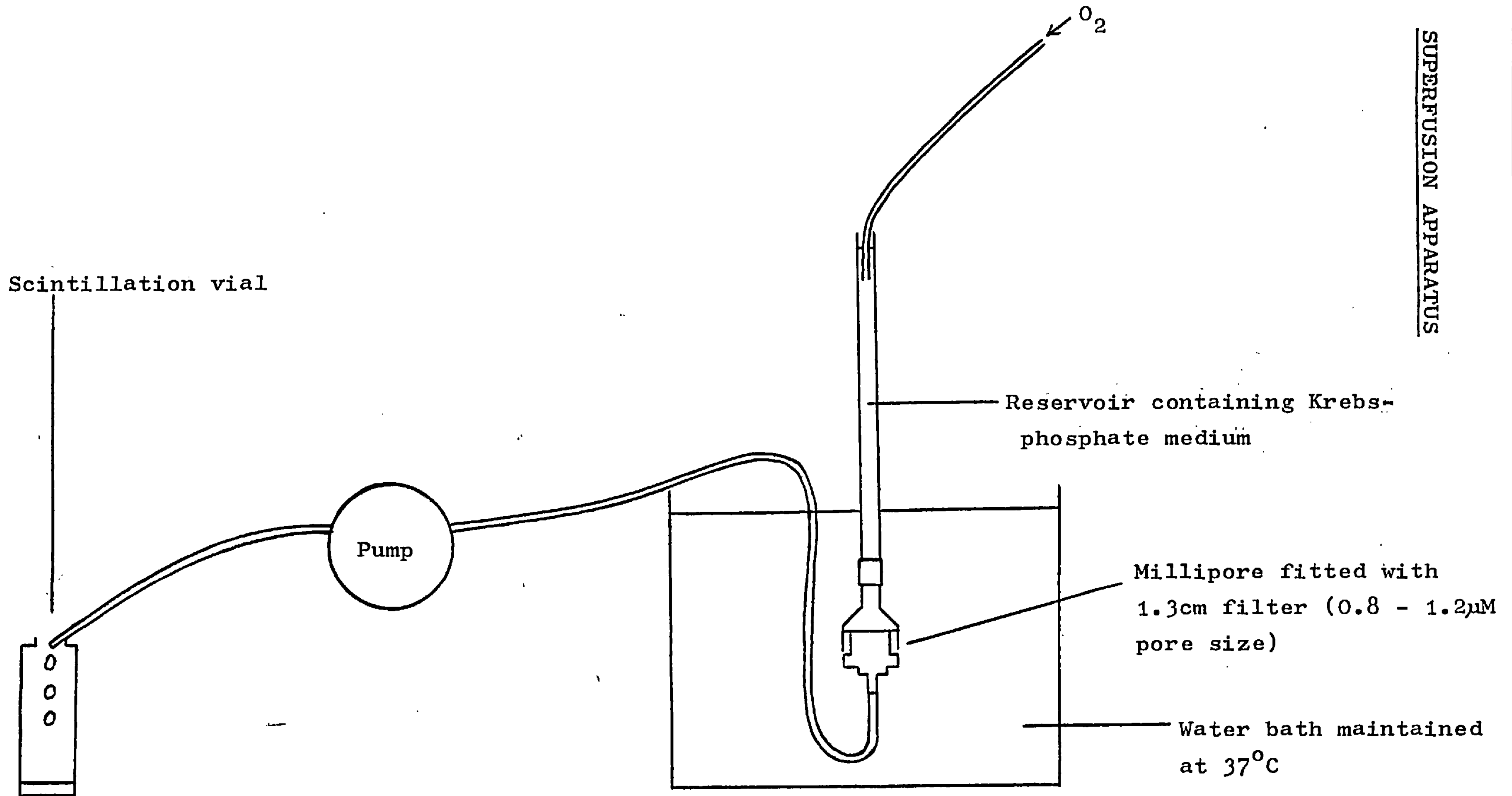


FIGURE 2.3

SUPERFUSION APPARATUS

protein) was loaded onto each millipore filter and the peristaltic pump switched on. The flow rate was set at 0.12ml/min and fractions were collected into vials every 4 mins. A number of fractions were collected before testing the effect of an agent on release, in order to remove excess isotope and to allow a steady spontaneous efflux level to be established. The radioactivity in the fractions was measured by liquid scintillation counting. In addition, the radioactivity remaining on the filters at the end of the experiment was determined and the percentage of the total radioactivity recovered calculated.

CHAPTER THREE

SOME CRITERIA FOR DETERMINING THE VIABILITY OF SYNAPTOSOMES

INTRODUCTION

RESULTS

The effect of stimulation on respiratory rates, lactate production and K^+ levels in rat cerebrocortical synaptosomes.

The effect of veratrine and K^+ stimulation on synaptosomal amino acid release.

The effect of veratrine concentration on amino acid release from rat cerebrocortical synaptosomes.

The effect of veratrine concentration on synaptosomal respiration.

The effect of veratrine concentration on the K^+ content of rat cerebrocortical synaptosomes.

Effect of protein dilution on amino acid release from cerebrocortical synaptosomes.

The effect of pentobarbital on ^{14}C-GABA release from rat cerebrocortical synaptosomes.

The effect of pentobarbital on endogenous amino acid release from rat cerebrocortical synaptosomes.

The effect of pentobarbital on respiration in rat cerebrocortical synaptosomes.

DISCUSSION

The effect of stimulation on synaptosomal respiration, lactate production, K^+ levels and amino acid release.

The effect of veratrine concentration on amino acid release, respiration and K^+ retention in rat cerebrocortical synaptosomes.

The effect of protein dilution on amino acid release from cerebrocortical synaptosomes.

The effect of pentobarbital on synaptosomal amino acid release.

INTRODUCTION

Synaptosomes isolated from various neural regions and maintained under metabolic conditions have proved to be extremely useful for studying the processes concerned in the synthesis, storage and release of neurotransmitter compounds. They have been shown to respond to depolarizing stimuli with augmented respiration and glycolysis and differential release of the physiologically active amino acids. (Bradford, 1974, 1975 for reviews). Therefore, before testing the actions of a number of agents on synaptosomal amino acid release, the viability of our in vitro system was determined, using data previously published by this group as representative of the normal situation. Hence, the effects of veratrine and potassium stimulation on synapto-mal respiration, lactate production and amino acid release were examined. In addition the relationship between veratrine concentration, amino acid transmitter release and synaptosomal K^+ retention was studied.

The bulk of these initial studies were carried out using cerebrocortical synaptosomes, however, where applicable data obtained using spinal/medullary nerve-endings is referred to for comparative purposes.

Synaptosomes are known to possess rapid uptake systems for the transmitter amino acids (reviewed in Chapter 1) and until recently neurotransmitter release was thought to represent the net result of the inward and outward fluxes. Thus, the appearance of neurotransmitter in the incubation fluid after stimulation was interpreted as being due to a net enhancement of the outward over the inward flux and not

as a result of an inhibition of the specific uptake processes. That these processes of uptake and release are distinct is well established (reviewed by Cox and Bradford, 1978) however, they may be more intimately linked than previously supposed. It is interesting that a number of agents that are known to block radioactive transmitter uptake and are not depolarizing agents, appear to cause release of endogenous and labelled amino acids (de Belleroche and Bradford, 1972; Osborne and Bradford, 1975). It has therefore been proposed (de Belleroche and Bradford, 1976) that neurotransmitter release may be a continuous cyclical process of release and closely associated Na^+ dependent uptake, and that uptake blockers reduce or prevent the inwardly directed half of the process. This hypothetical mechanism has been referred to as a "transport shuttle" (de Belleroche and Bradford, 1976) and experiments designed to investigate this hypothesis were conducted. These experiments involved incubating similar quantities of synaptosomal protein in increasing volumes of medium and determining the extent of amino acid transmitter release. The rationale behind this procedure was that as the protein concentration was reduced so the amount of amino acid appearing in the medium would be elevated as a result of the decreased availability of the neurotransmitters for the uptake sites.

In the last section of this chapter, the effects of a barbiturate anaesthetic (pentobarbital) [handwritten: pentobarbitone] on synaptosomal respiration and amino acid release were examined.

General anaesthetics have been shown to depress post-synaptic excitatory transmission in the vertebrate central

and peripheral nervous systems (Weakly, 1969; Galindo, 1971; Westmoreland et al., 1971; Thomson and Turkanis, 1973; Richards, 1971; Richards, 1972) although preserving or prolonging both presynaptic and post synaptic inhibition (Eccles et al., 1963; Larson and Major, 1970; Eccles et al., 1971; Nicoll, 1972). The mechanisms underlying these cellular events and their precise relationship with the phenomenon of general anaesthesia in mammals have not been satisfactorily elucidated. Barker and his coworkers (Barker and Gainer, 1973; Barker, 1974) have used various invertebrate preparations to show that pentobarbital and other general anaesthetics operate at a postsynaptic level to depress Na^+ dependent postsynaptic excitation without affecting either Cl^- or K^+ dependent postsynaptic inhibition. These workers (Ransom and Barker, 1975) have also shown using intracellular recording from mouse spinal neurons grown in tissue culture, that pentobarbital depresses glutamate excitation and prolongs GABA inhibition in most cells, through a postsynaptic mechanism. Similar results for glutamate excitation have also been obtained by Richards and Smaje (1974), using guinea-pig prepiriform cortex slices.

On the basis of these observations several hypothetical mechanisms for pentobarbital and general anaesthetic action have been proposed. Firstly, as suggested by Weakly (1969) and Richards (1972) barbiturates could operate by reducing the output of transmitter from the presynaptic nerve terminal. Secondly, pentobarbital action may result from a reduction in the sensitivity of the post-synaptic membrane to the released transmitter substance (Richards, 1972).

Alternatively as suggested by Cutler and Dudzinski (1974) pentobarbital may stimulate the release of the inhibitory transmitter, GABA, increasing postsynaptic inhibition. Indeed, in their hands, pentobarbital was found to increase the electrically induced release of ^{3}H-GABA from cerebral cortex slices. These workers have also shown pentobarbital to inhibit ^{3}H-GABA uptake and spontaneous release. In this last respect the work of Cutler and Dudzinski (1974) conflicts with that of Crowshaw et al. (1967) who reported an increase in the spontaneous release of endogenous amino acids, including GABA, from cat cerebral cortex *in vivo*.

Pentobarbital is known to influence respiration. Himwich (1951) has shown that it inhibits O_2 uptake into cerebral tissues, this inhibition being more pronounced in adult rats than in infants.

Finally, pentobarbital is believed to influence other neural functions. For example, pentobarbital has been demonstrated to elevate brain glucose (Taberner, 1973) and histamine (Taylor and Snyder, 1972) levels and inhibit acetylcholine synthesis (Aquilonius et al., 1973). Pentobarbital may also have a depressant effect on acetylcholine excited neurons in the brain stem (Bradley and Dray, 1973).

RESULTS

The effect of stimulation on respiratory rates, lactate production and K^+ levels in rat cerebro-cortical synaptosomes

When cerebrocortical synaptosomes were incubated in Krebs-phosphate medium containing 10mM glucose, they showed linear rates of respiration over the 30 mins of experimentation (Fig. 3.1)

In the presence of potassium (56mM) and veratrine (75µM), administered as described in the materials and methods, there was a significant increase in O_2 uptake and lactate production (Figs. 3.1, 3.2; Table 3.a), as shown previously by this group (reviewed by Bradford, 1974). The effect of elevated K^+ on oxygen uptake was to cause an increase from 58.5 ± 3.1 (8) to 94 ± 18 (4) µmol O_2/100mg protein/h. Similarly, administration of veratrine raised the O_2 consumption from 45.6 ± 1.7 (10) to 134.2 ± 4.4 (8) µmol O_2/100mg protein/h.

Veratrine (75µM) stimulation was found to reduce the intrasynaptosomal levels of K^+ by 50% (Table 3.b). In spinal/medullary nerve-endings this reduction was not so marked, however, decreases of approximately 28% were recorded (Table 3.b)

The effect of veratrine and K^+ stimulation on synaptosomal amino acid release

As shown previously (reviewed by Bradford, 1976) cerebrocortical synaptosomes responded to veratrine and K^+ stimulation with the differential release of the three phsyiologically active amino acids aspartate, glutamate

FIGURE 3.1

RESPIRATION OF CEREBROCORTICAL SYNAPTOSOMES

Cerebrocortical synaptosomes were incubated in Krebs-phosphate medium, at 37°C, containing 10mM glucose. After 35 mins of incubation (this includes a gassing and equilibration period of 15 mins), veratrine (final concentration, 75μM) was added and incubation continued for a further 10 mins. Values are mean ± S.E.M. for 6-12 determinations.

FIGURE 3.1

RESPIRATION OF CEREBROCORTICAL SYNAPTOSOMES

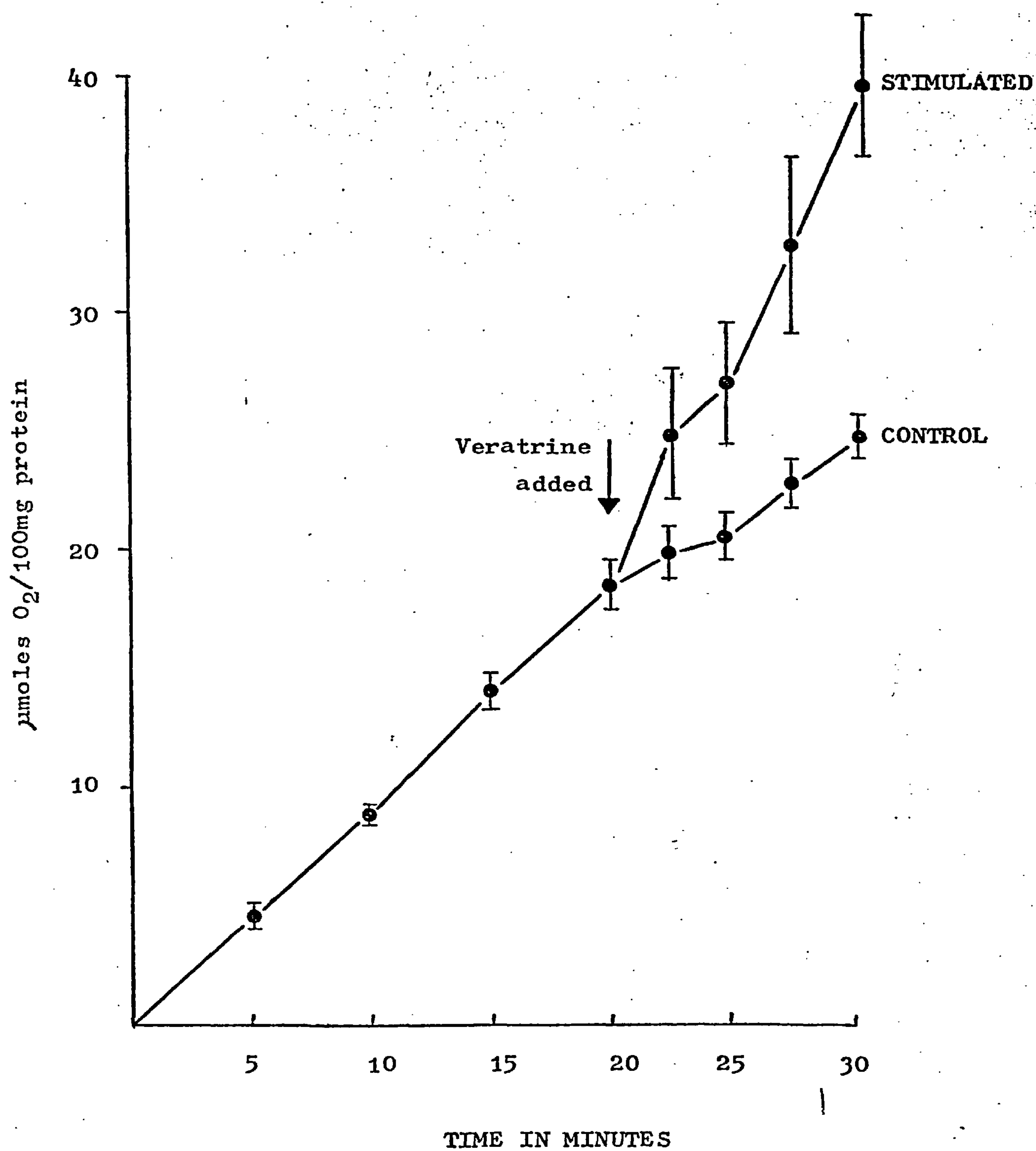
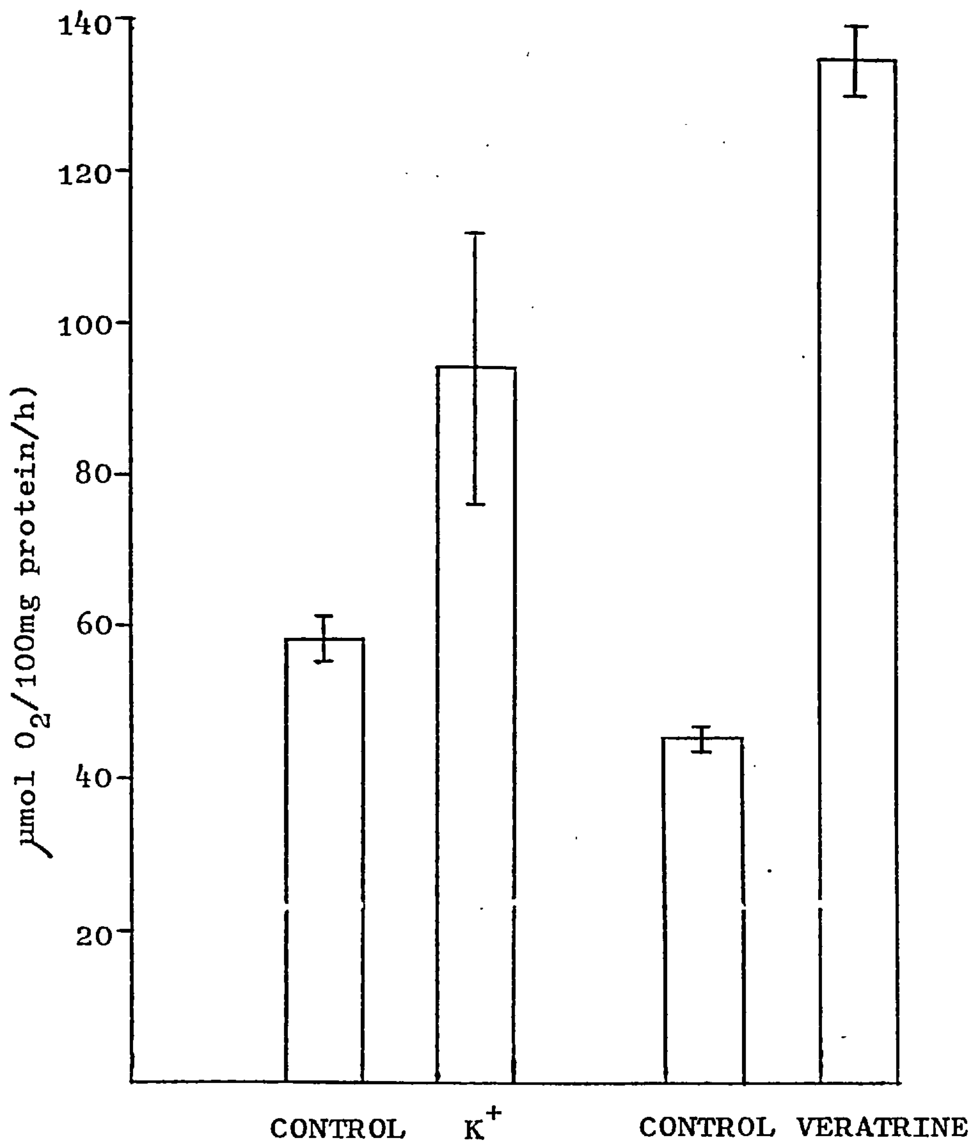


FIGURE 3.2

EFFECT OF VERATRINE AND K^+ STIMULATION ON SYNAPTOSOMAL RESPIRATION



Cerebrocortical nerve-endings were incubated in Krebs-phosphate medium, at 37°C, for 45 mins. Veratrine (final concentration, 75μM) and K^+ (final concentration, 56mM) stimulation was applied for 10 mins.

Values are mean ± S.E.M. for 4-10 determinations.

TABLE 3.a

LACTATE PRODUCTION BY CEREBROCORTICAL SYNAPTOSOME SUSPENSIONS

	Lactate produced (μmol/100mg protein/h)
Control	15.5±2.5 (4)
Stimulated	24.3±2.8 (4)

Synaptosomes were incubated in Krebs-phosphate medium for 45 mins. Potassium stimulation was applied during the last 10 minutes of incubation. Values are means ± S.E.M. for the number of samples in brackets.

TABLE 3.b

POTASSIUM CONTENT OF CEREBROCORTICAL AND MEDULLA/SPINAL CORD SYNAPTOSOMES

Potassium content (μequivs/100mg protein)

	Control	Stimulated
Cerebrocortical synaptosomes	20±1 (14)	10±0.3 (8)
Medulla/spinal cord synaptosomes	22±1 (8)	16±1 (8)

Synaptosomes were incubated in Krebs-phosphate medium at 37°C. After 35 mins incubation veratrine was added to give a final concentration of 75μM. Values are mean ± S.E.M. for the number of samples in brackets.

and GABA (Table 3.c).

With spinal/medullary synaptosomes veratrine only caused the selective release of glycine and GABA (Table 3.d). This result is not in agreement with that of Osborne and Bradford (1973) who also demonstrated the release of aspartate and glutamate. However, unlike these workers who used synaptosomal beds, our experiments were performed using suspensions which have a much higher fluid to tissue ratio. Perhaps under these conditions the nerve-endings had greater difficulty in establishing an efficient amino acid reuptake system. This would also appear to be confirmed by the high control levels obtained.

Figures 3.3a and 3.4 show typical amino acid chromatograms for control and veratrine stimulated medium samples obtained from rat cerebrocortical and spinal/medullary synaptosomal incubations. Similarly, Figure 3.3b shows typical chromatograms obtained with rat cerebrocortical synaptosomes on K^+ stimulation.

The effect of veratrine concentration on amino acid release from rat cerebrocortical synaptosomes

Introduction

Osborne and Bradford (1975) demonstrated that the release of physiologically active amino acids from spinal/medullary synaptosomes was directly proportional to the K^+ concentration of the incubation medium, a sharp increase being observed at 15mM K^+. It is interesting that this concentration corresponds to the threshold level shown by Blaustein and Wiesman (1970) to cause Ca^{2+} uptake occurring at a time when the synaptosomal membrane would theoretically be depolarized by 30mV. As Katz and Miledi (1967) have observed that the presynaptic terminal

TABLE 3.c

THE EFFECT OF VERATRINE AND POTASSIUM STIMULATION ON AMINO ACID RELEASE FROM CEREBROCORTICAL SYNAPTOSOMES

Amino acid in incubation medium (nmol/100mg protein)

	Control (6)	K^+ stimulated (6)	Veratrine stimulated (6)
Aspartate	44±5	*236±22	*886±50
Threonine	138±16	†212±25	160±8
Serine	289±12	*478±34	**413±27
Glutamate	75±20	*423±60	*1812±68
Glycine	318±34	**450±23	*534±27
Alanine	536±22	583±26	*784±27
GABA	39±7	**102±17	*645±25

Rat cerebrocortical synaptosomes were incubated for 35 mins in Krebs-phosphate medium, whereupon KCl (final concentration, 56mM) or veratrine (final concentration, 75µM) was added. Incubation was then continued for a further 10 mins. Values are means ± S.E.M. for the number of samples in brackets. Change from control significant $P < 0.001$*, $p < 0.01$** or $p < 0.05$†.

Reference to the above table reveals that in some cases the release of threonine, serine, glycine and alanine was significantly increased in the presence of 56mM KCl or 75µM veratrine. However, these increases were not of the same order of magnitude as those recorded for the physiologically active amino acids, aspartate, glutamate and GABA.

TABLE 3.d

VERATRINE STIMULATED AMINO ACID RELEASE FROM SPINAL/ MEDULLARY SYNAPTOSOMES

Amino acid in incubation medium (nmol/100mg protein)

	Control (10)	Veratrine stimulated (10)
Aspartate	1618±179	1860±195
Threonine	431±81	574±102
Serine	816±153	1188±175
Glutamate	2899±340	3197±301
Glycine	1565±228	†2545±314
Alanine	993±139	1260±166
GABA	91±7	*761±65

Synaptosomes were incubated in Krebs-phosphate medium at 37°C for 45 mins. Values are means ± S.E.M. for the number of determinations in brackets. Change from control significant, $P < 0.05^{\dagger}$ or $P < 0.001^{*}$.

FIGURE 3.3a

RELEASE OF AMINO ACIDS TO THE MEDIUM CAUSED BY VERATRINE (75μM) STIMULATION OF CEREBROCORTICAL SYNAPTOSOMES

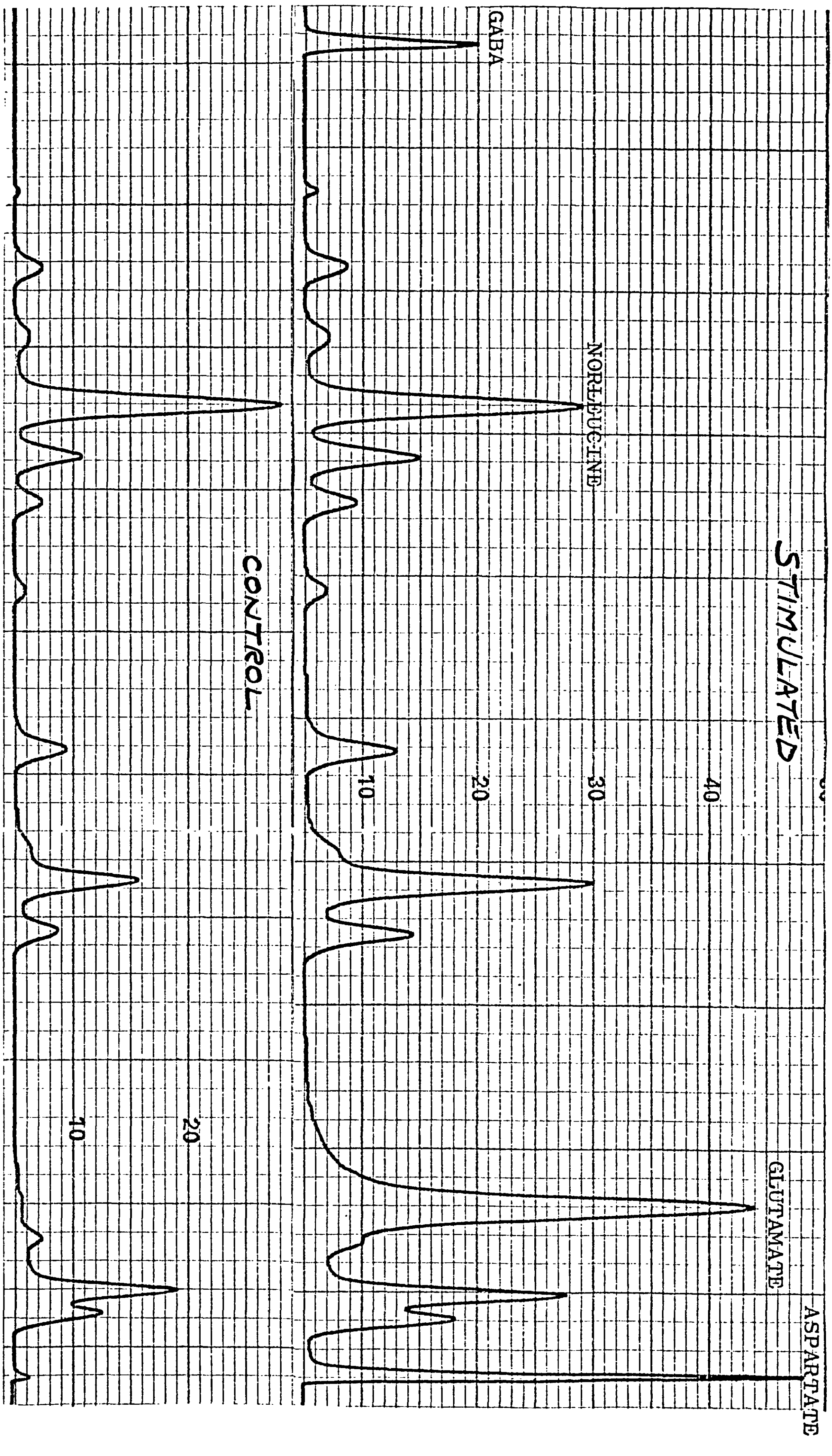


FIGURE 3.3b

RELEASE OF AMINO ACIDS TO THE MEDIUM CAUSED BY POTASSIUM (56mM) STIMULATION OF CEREBROCORTICAL SYNAPTOSOMES

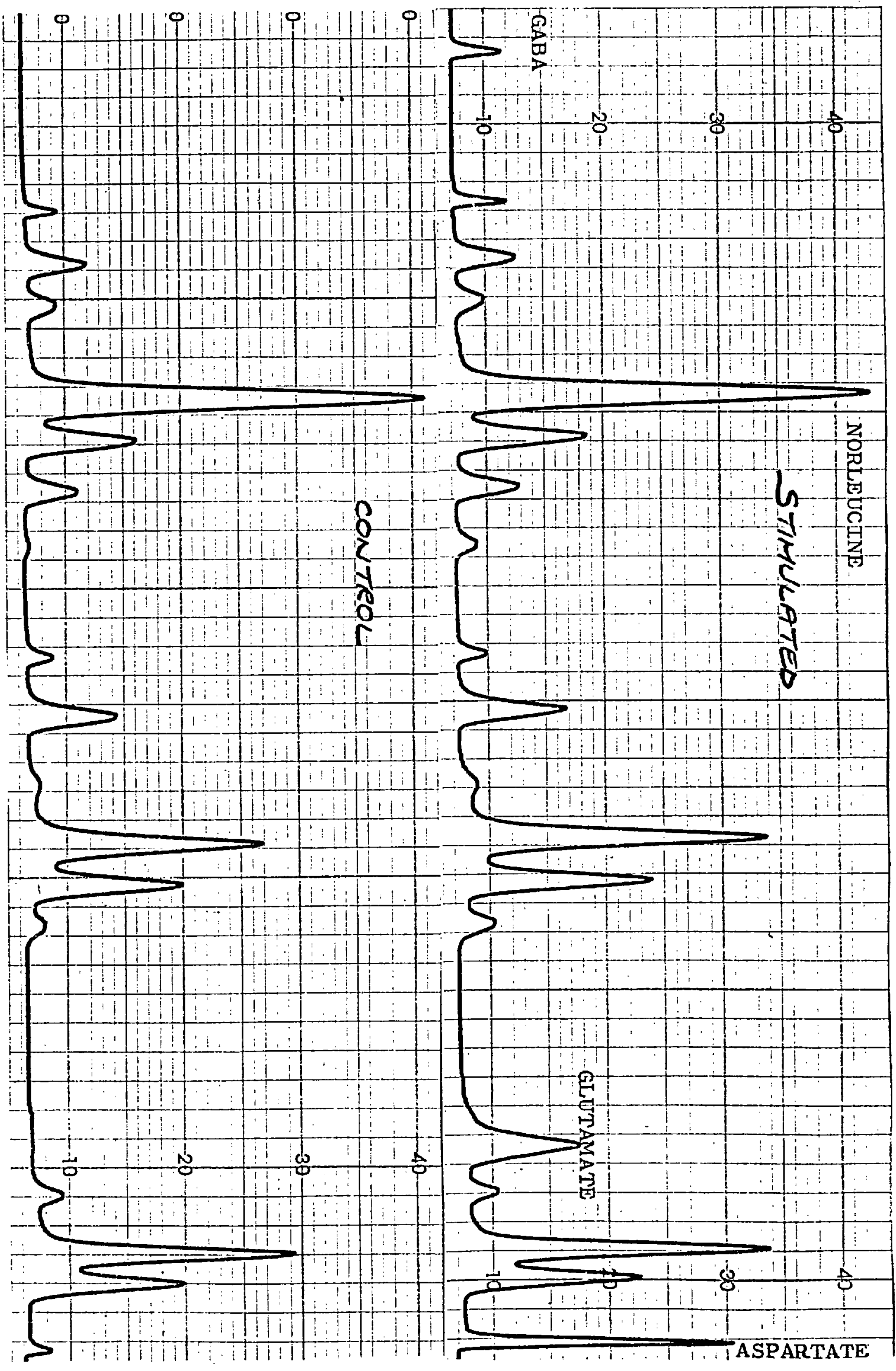
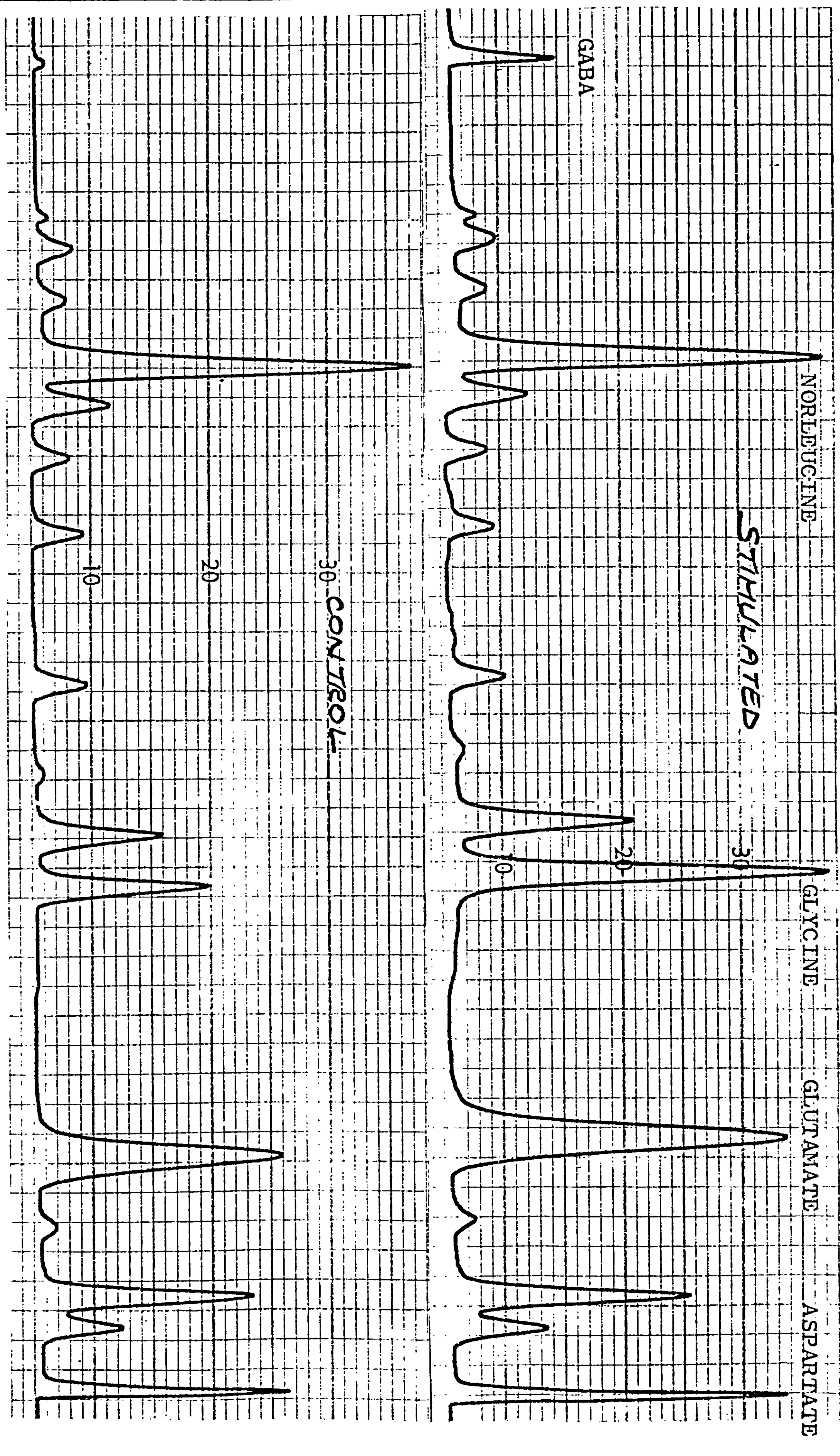


FIGURE 3.4

RELEASE OF AMINO ACIDS TO THE MEDIUM CAUSED BY VERATRINE (75μM) STIMULATION OF SPINAL/MEDULLARY SYNAPTOSOMES



had to be depolarized by about 30mV to elicit a post-synaptic response in the squid giant axon, Osborne and Bradford (1975) concluded that some link must exist between depolarization and amino acid release.

Therefore, as veratrine is an extremely efficient depolarizing agent, the relationship between veratrine concentration and amino acid release in cerebro-cortical synaptosomes was investigated.

Results

Cerebrocortical synaptosomes were incubated in Krebs-phosphate medium for 35 mins. At the end of this period of incubation, veratrine was added to give the following final concentrations: 1, 10, 25, 50, 75, 150 and 300µM.

Maximal release of the putative amino acid transmitters aspartate, glutamate and GABA occurred at veratrine concentrations between 25 and 50µM (Fig. 3.5)

However, as can be seen from figure 3.5 substantial amino acid release was also obtained with concentrations of 1 and 10µM. This effect on release was not so marked for the non-physiologically active amino acids threonine, serine, glycine and alanine, which showed irregular levels of release in response to the increasing veratrine concentrations.

Examination of the intrasynaptosomal amino acid levels revealed a picture which mirrored the release data, i.e. a decrease in the synaptosomal content of aspartate, glutamate and GABA with increasing veratrine concentration (Fig. 3.6).

FIGURE 3.5

THE EFFECT OF VERATRINE CONCENTRATION ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

Nerve-endings were incubated in Krebs-phosphate medium at 37°C. After 35 mins incubation veratrine was added to give the following final concentrations: 1, 10, 25, 50, 75, 150 and 300μM. Incubation was then continued for a further 10 mins.

Values are mean ± S.E.M. for 3-15 determinations.

FIGURE 3.5a

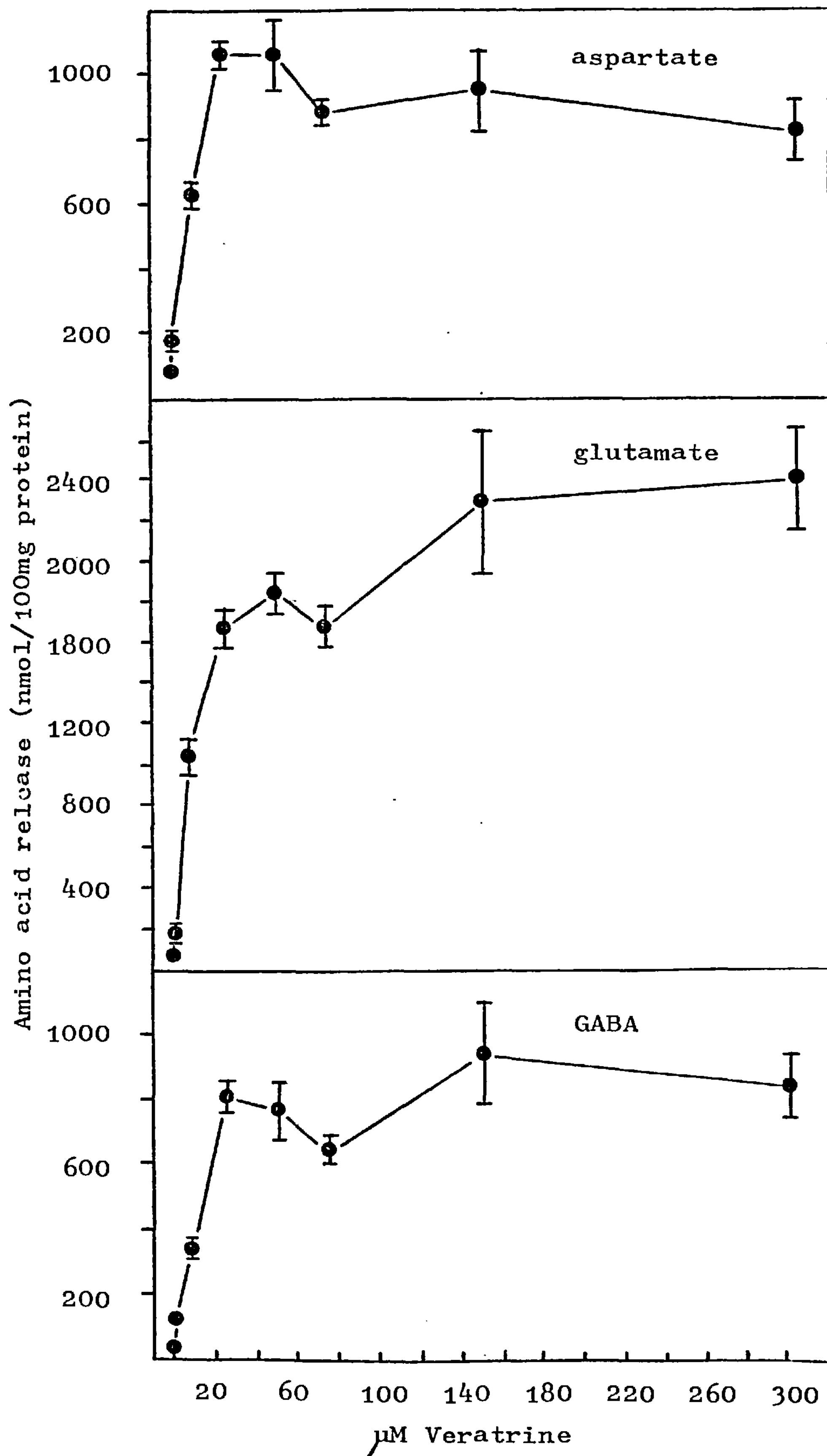


FIGURE 3.5b

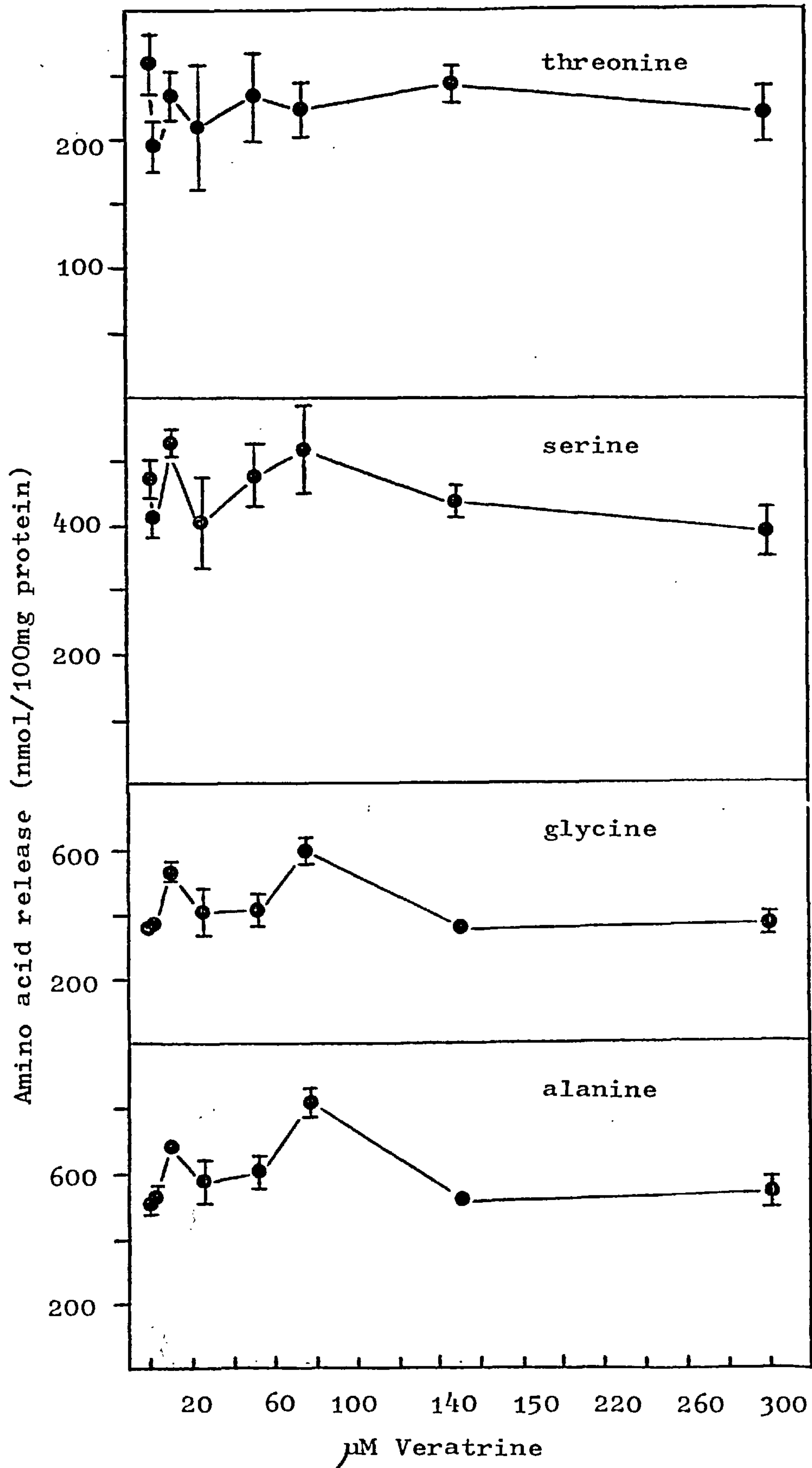


FIGURE 3.6

THE EFFECT OF VERATRINE CONCENTRATION ON THE AMINO ACID CONTENT OF RAT CEREBROCORTICAL SYNAPTOSOMES

Synaptosomes were incubated in Krebs-phosphate medium at 37°C, for 35 mins. Veratrine (final concentrations, 1, 10, 25, 50, 75, 150 and 300μM) stimulation was then applied for 10 mins.

Values are mean ± S.E.M. for 3-13 determinations.

FIGURE 3.6a

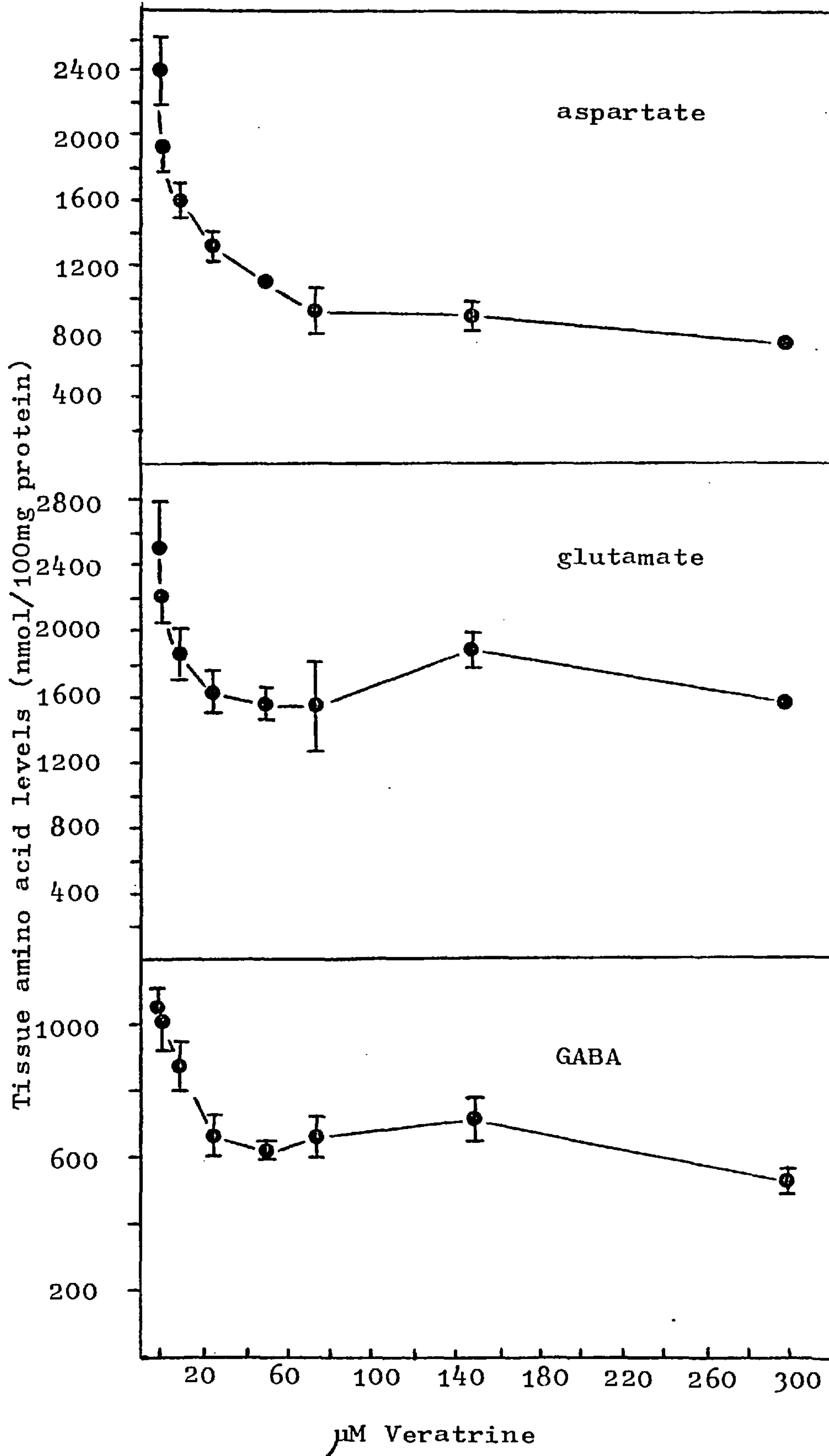
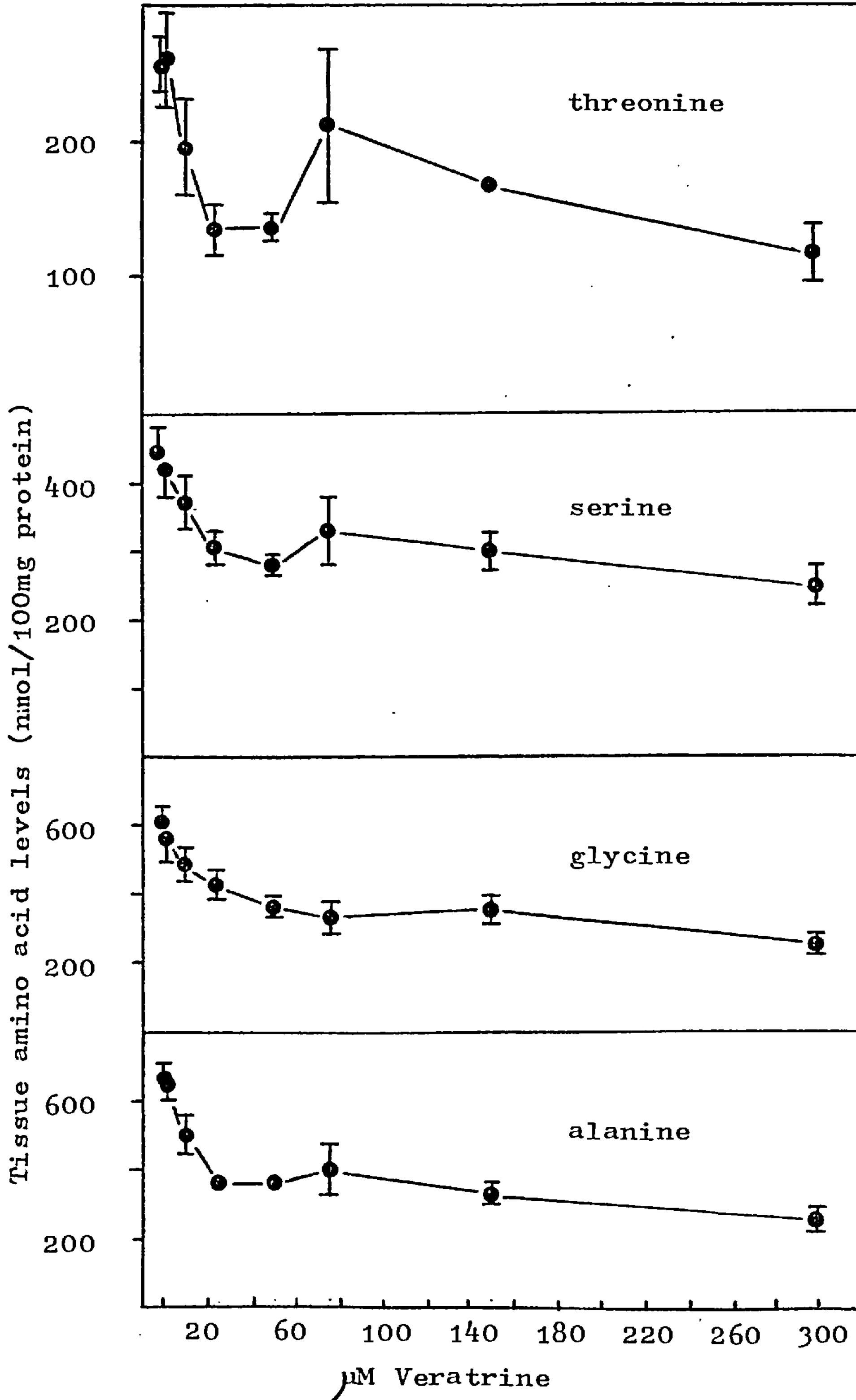


FIGURE 3.6b



The effect of veratrine concentration on synaptosomal respiration

Figure 3.7 illustrates the effect increasing veratrine concentration has on synaptosomal respiration. Maximal respiratory rates were obtained with a veratrine concentration of 75µM.

The effect of veratrine concentration on the K^+ content of rat cerebrocortical synaptosomes

25µM veratrine was found to reduce the synaptosomal K^+ content by 50%, this being the maximum response elicited by this agent. (Table 3.e).

This result is interesting when one considers that maximal release of the putative amino acid transmitters was obtained with approximately this concentration.

Effect of protein dilution on amino acid release from cerebrocortical synaptosomes

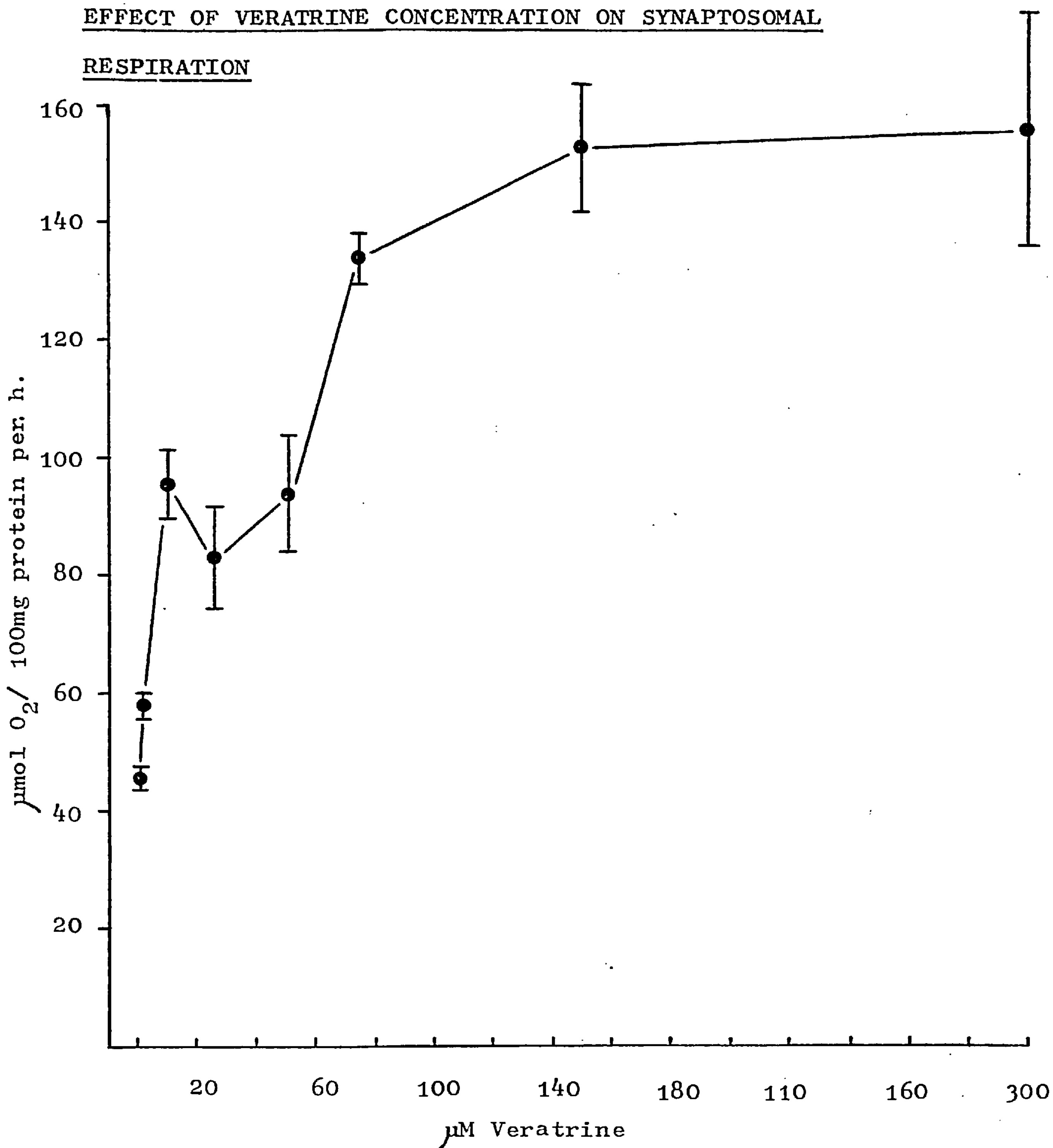
Similar quantities (5-6mg synaptosomal protein) of cerebrocortical synaptosomes were incubated in the following volumes of Krebs-phosphate medium 0.75, 1.5, 2.25, 3.0 and 3.75ml, for 45 mins.

Under these conditions the levels (expressed as nmols/ 100mg protein) of the amino acids recovered in the medium increased as the volume increased, maximum increases being recorded for the physiologically active amino acids aspartate, glutamate and GABA (Table 3.f).

When the same data was expressed as concentrations, i.e. nmols of amino acid/ml of medium, the overall concentrations of aspartate, glutamate and GABA were found to remain

FIGURE 3.7

EFFECT OF VERATRINE CONCENTRATION ON SYNAPTOSOMAL RESPIRATION



Synaptosomal suspensions were incubated at 37°C in Krebs-phosphate medium for 35 mins, whereupon veratrine (final concentrations 1, 10, 25, 50, 75, 150 and 300μM) was added and incubation continued for a further 10 mins.

Values are the mean ± S.E.M. for 3-10 determinations.

TABLE 3.e

THE EFFECT OF VERATRINE CONCENTRATION ON THE K^+ CONTENT OF RAT CEREBROCORTICAL SYNAPTOSOMES

Condition	K^+ content (µequivs/100mg protein)	n
Control	20±1.1	14
1µM veratrine	18±0.8	6
10µM veratrine	12±0.8	6
25µM veratrine	9±0.5	6
50µM veratrine	9.3±0.4	9
75µM veratrine	10±0.3	8
150µM veratrine	10.6±0.9	3
300µM veratrine	8.4±0.4	3

Synaptosomes incubated in Krebs-phosphate medium for 35 mins, whereupon veratrine added and incubation continued for a further 10 mins. Values are mean ± S.E.M. for the number of samples shown.

TABLE 3.f

EFFECT OF PROTEIN DILUTION ON AMINO ACID RELEASE FROM CEREBROCORTICAL SYNAPTOSOMES

Amino acid in incubation medium (nmol/100mg protein)

	Incubation Volume (ml)					
	0.75 (a)	1.5 (b)	2.25 (c)	3.0 (d)	3.75 (e)	(e) expressed as % over (a)
Aspartate	103±14	93±8	116±17	215±11	248±32	140
Threonine	263±21	279±33	273±40	349±41	356±32	35
Serine	445±40	483±53	518±56	633±47	649±35	46
Glutamate	91±13	96±23	114±19	192±11	201±21	121
Glycine	327±41	402±1	464±29	581±14	656±49	101
Alanine	422±44	459±76	545±76	647±74	671±55	59
GABA	29±9	62±21	55±9	68±9	83±26	186

Cerebrocortical synaptosomes were incubated in Krebs-phosphate medium for 45 mins. Values are mean ± S.E.M. for three experiments.

relatively constant, whereas the non-physiologically active amino acids threonine, serine, glycine and alanine rapidly decreased as the protein concentration was reduced (Fig. 3.8). Similarly, examination of the synaptosomal amino acid content showed that the levels of aspartate, glutamate, alanine and GABA remained unchanged or even increased as the protein concentration was reduced, whereas the quantities of threonine, serine and glycine decreased (Table 3.g). In the case of aspartate the total (medium and tissue) levels would appear to rise with dilution, suggesting that increased synthesis had occurred.

The effect of pentobarbital on ^{14}C-GABA release from rat cerebrocortical synaptosomes

Pentobarbital was tested for its effect on synaptosomal ^{14}C-GABA release, at the same concentrations as those used by Cutler and Dudzinski (1974), i.e. 0.1mM and 5mM.

When synaptosomes were incubated in Krebs-phosphate medium containing 0.1mM pentobarbital no effect was observed on the spontaneous or K^+ stimulated release (Fig. 3.9). However, at a concentration of 5mM the release was increased by approx. 200% (Fig. 3.10).

The effect of pentobarbital on endogenous amino acid release from rat cerebrocortical synaptosomes

As with ^{14}C-GABA release, 0.1mM pentobarbital did not appear to influence the efflux of any of the endogenous amino acids assayed (Table 3.h).

On the other hand, 0.5mM pentobarbital had a marked

FIGURE 3.8

THE EFFECT OF PROTEIN DILUTION ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

Similar amounts of synaptosomal protein (5-6mg) were incubated in varying volumes (0.75, 1.5, 2.25, 3.0 and 3.75ml) of Krebs-phosphate medium, for 45 mins.

Values are mean ± S.E.M. for 3 experiments.

NB Take special note of scales used in 3.8a and 3.8b when considering data.

FIGURE 3.8a

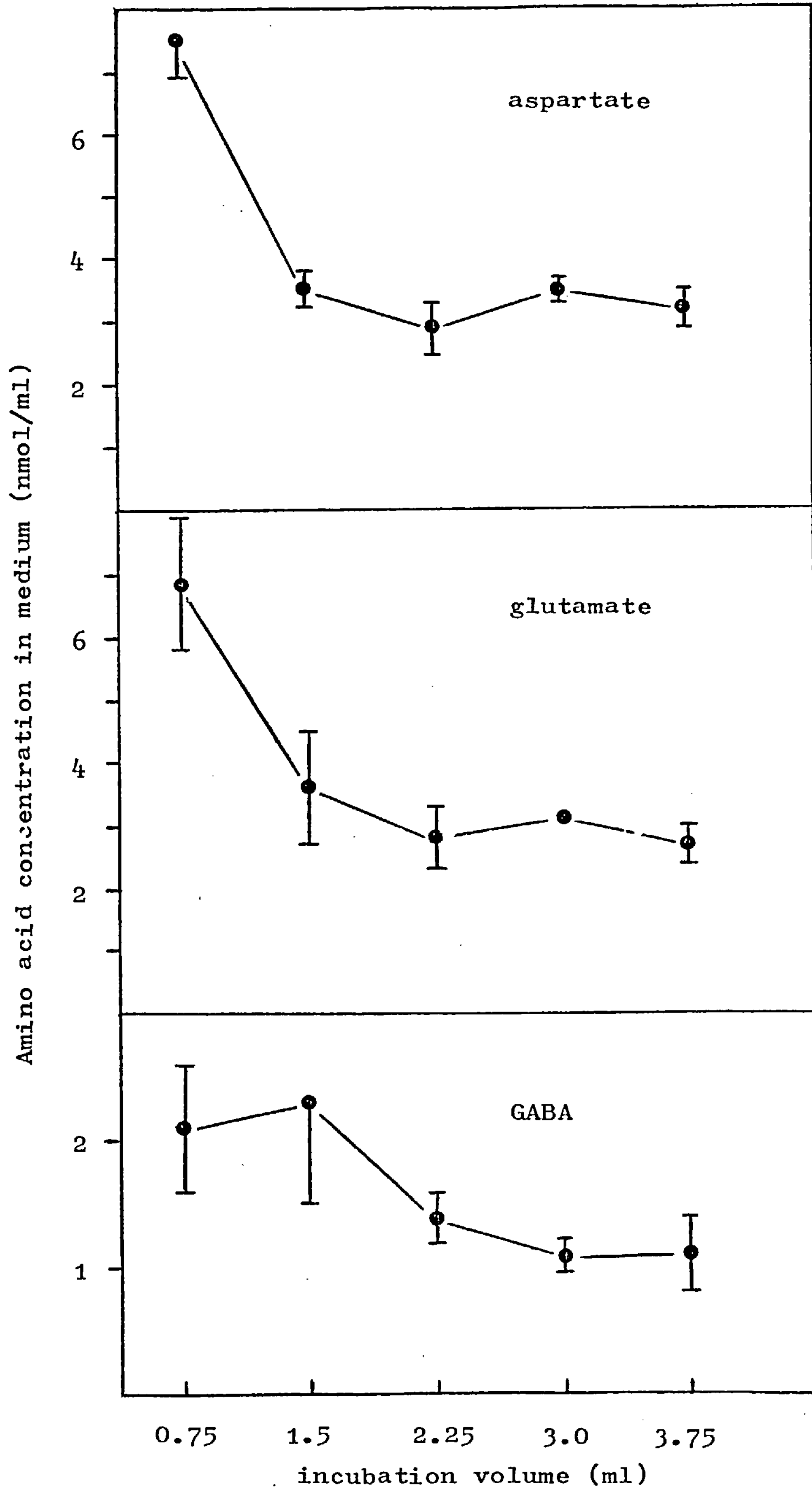


FIGURE 3.8b

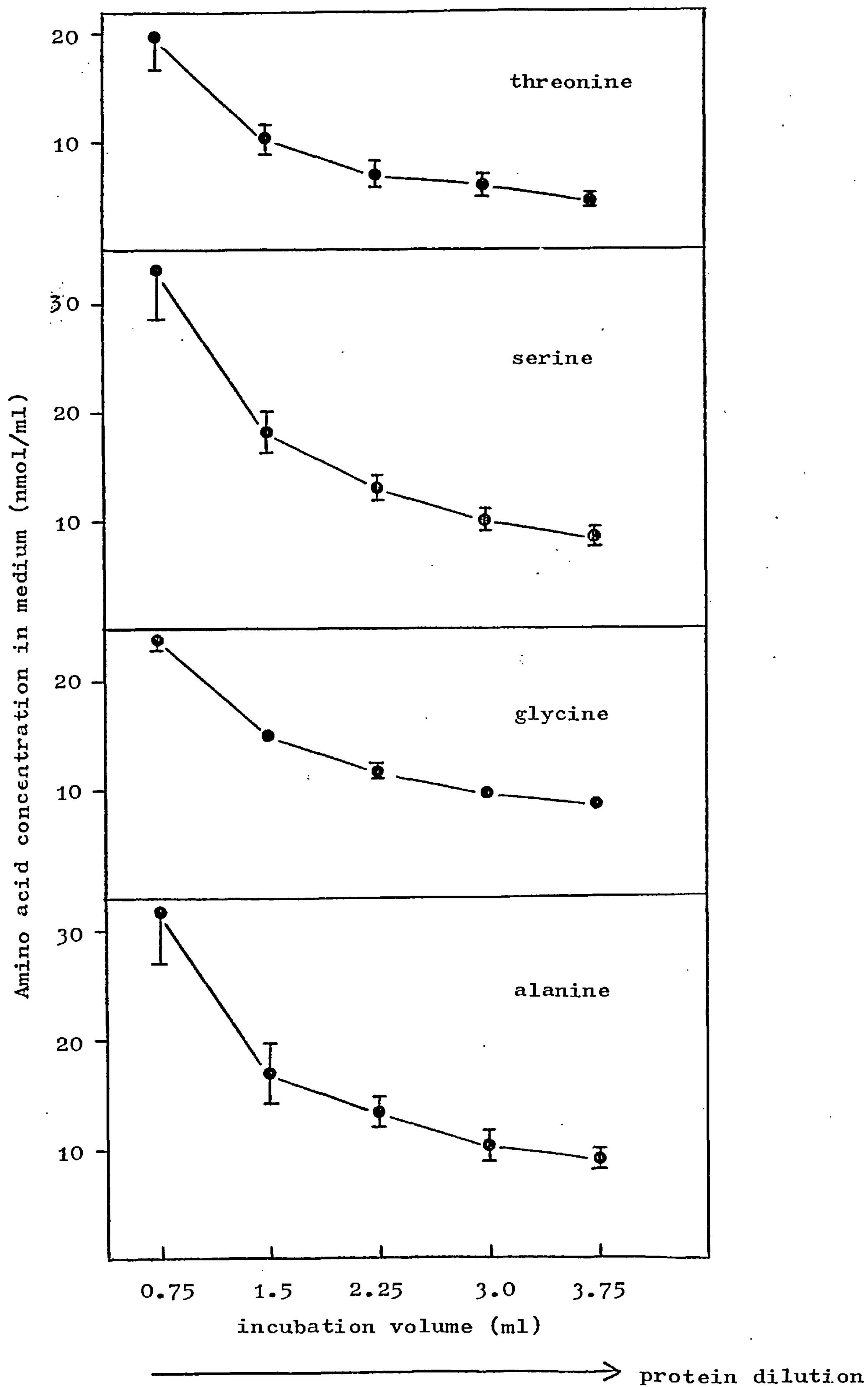


TABLE 3.g

EFFECT OF PROTEIN DILUTION ON THE AMINO ACID CONTENT OF RAT CEREBROCORTICAL SYNAPTOSOMES

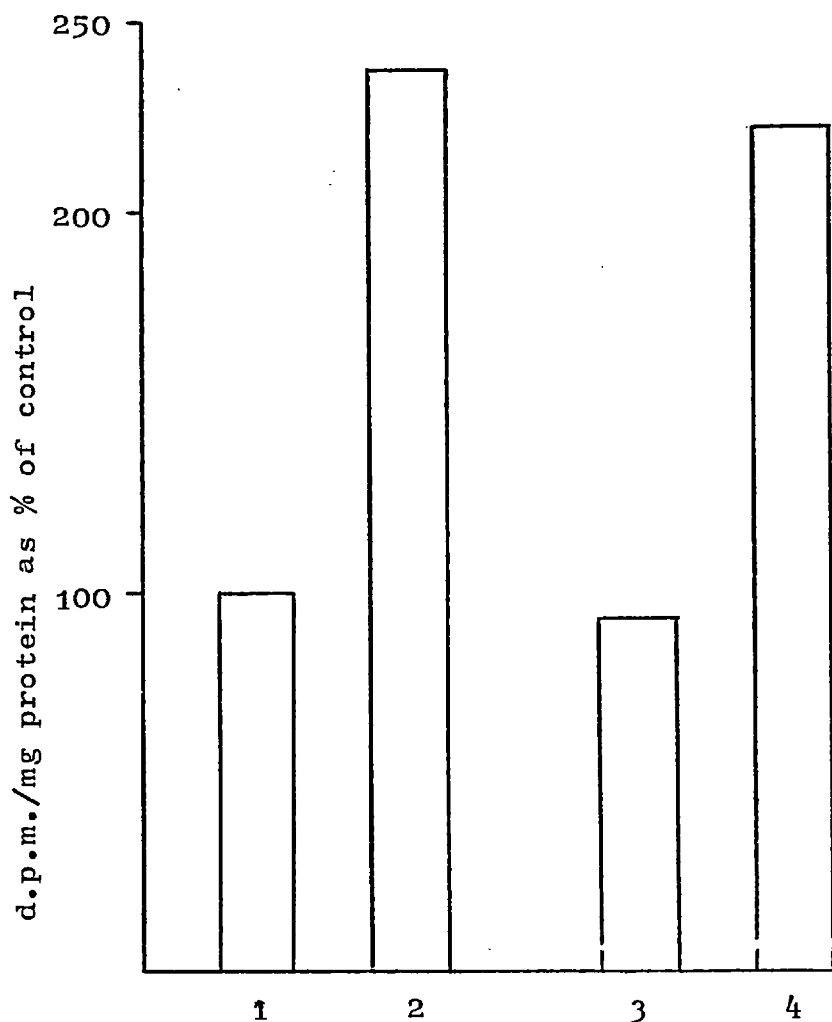
Amino acid content (nmol/100mg protein)

	Incubation Volume (ml)				
	0.75	1.5	2.25	3.0	2.75
Aspartate	2386±182	2454±275	2747±49	3084±340	3160±170
Threonine	210±13	115±5	129±9	140±18	123±7
Serine	418±12	291±18	300±4	377±106	260±53
Glutamate	3200±180	2631±392	2759±115	3020±395	2922±130
Glycine	588±12	543±47	505±13	462±104	348±75
Alanine	769±2	629±55	995±56	1006±64	941±45
GABA	1066±126	933±200	1292±60	1304±235	1288±94

Cerebrocortical synaptosomes were incubated in Krebs-phosphate medium for 45 mins. Values are mean ± S.E.M. for three experiments.

FIGURE 3.9

THE EFFECT OF 0.1mM PENTOBARTICAL ON ^{14}C-GABA RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

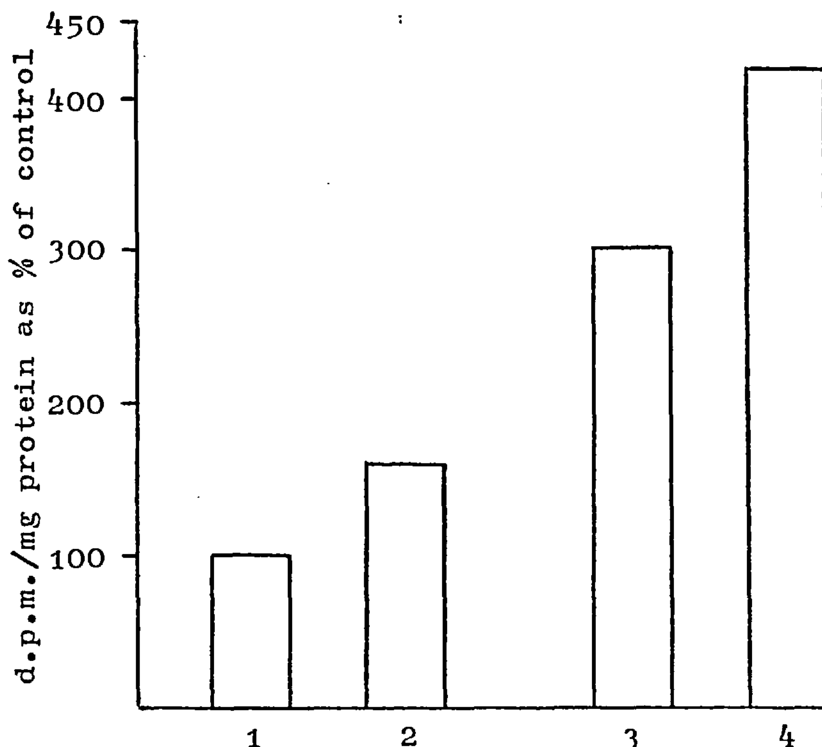


Cerebrocortical nerve-endings were preloaded with ^{14}C-GABA as outlined in Chapter 2. Synaptosomal suspensions were then incubated in Krebs-phosphate medium for 45 mins (controls) or for 35 mins followed by 10 mins K^+ (final concentration, 56mM) stimulation. Values are the mean for 2 determinations.

1. - control
2. - K^+ stimulated
3. - control } 0.1mM pentobarbital in medium
4. - K^+ stimulated } 0.1mM pentobarbital in medium

FIGURE 3.10

THE EFFECT OF 5mM PENTOBARBITAL ON ^{14}C-GABA RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES



Synaptosomes were prelabelled with ^{14}C-GABA as described in the experimental section (Chapter 2).

Synaptosomal suspensions were then incubated at 37°C in Krebs-phosphate medium for 45 mins (controls) or for 35 mins followed by 10 mins K^+ (final concentration,56mM) stimulation. Values are the mean for 2 determinatons.

1. - control
2. - K^+ stimulated
3. - control } 5mM pentobarbital in medium
4. - K^+ stimulated } 5mM pentobarbital in medium

TABLE 3.h

AMINO ACID RELEASE FROM CEREBROCORTICAL SYNAPTOSOMES IN THE PRESENCE OF 0.1mM PENTOBARBITAL

Amino acid in incubation medium (nmol/100mg protein)

	No addition		0.1mM pentobarbital in medium	
	Control (2)	Stimulated (2)	Control (2)	Stimulated (2)
Aspartate	25	647	28	656
Threonine	202	202	184	206
Serine	265	282	307	317
Glutamate	26	1498	24	1610
Glycine	225	259	203	226
Alanine	346	416	335	413
GABA	12	573	16	644

Synaptosomes were incubated in Krebs-phosphate medium with or without pentobarbital. Veratrine (final concentration, 75µM) was used for stimulation. Values are means for two determinations.

effect on the veratrine stimulated release of aspartate and GABA, reducing the former by 29% and raising the latter by 35%. The other amino acids measured in the medium showed little or no response to the presence of the anaesthetic (Fig. 3.11).

The inclusion of 0.5mM pentobarbital in the medium also appeared to significantly alter synaptosomal amino acid synthesis, as evidenced by the 12 - 114% increases in the total synaptosomal levels (i.e. medium and tissue levels combined) of aspartate, glutamate and GABA.

The amino acid levels detected in the tissue are shown in (Table 3.i)

The effect of pentobarbital on respiration in rat cerebrocortical synaptosomes

Pentobarbital showed evidence of inhibiting synaptosomal respiration to a greater or lesser extent, at all the concentrations used, 5mM pentobarbital blocking this process completely. (Table 3.j).

In view of the amino acid data obtained using 0.5mM pentobarbital, the respiratory results are perhaps a little surprising as a decrease in the respiratory rate might have been expected to result in a reduction in synaptosomal metabolism and hence a decrease in amino acid synthesis. Alternatively, it may be argued that the increase in the total amino acid levels is due to a decrease in the utilisation of these substances as respiratory fuels.

FIGURE 3.11

THE EFFECT OF 0.5mM PENTOBARBITAL ON AMINO ACID RELEASE FROM CEREBROCORTICAL SYNAPTOSOMES

Cerebrocortical nerve-endings were incubated in Krebs-phosphate medium at 37°C, for 45 mins. Veratrine (final concentration, 75μM) was used to achieve stimulation. Values are the mean ± S.E.M. for 6 determinations. Change in stimulated release due to presence of pentobarbital significant with $p < 0.05$* or $p < 0.001$**.

1. - control
2. - veratrine stimulated
3. - control } 0.5mM pentobarbital in medium
4. - veratrine stimulated } 0.5mM pentobarbital in medium

FIGURE 3.11

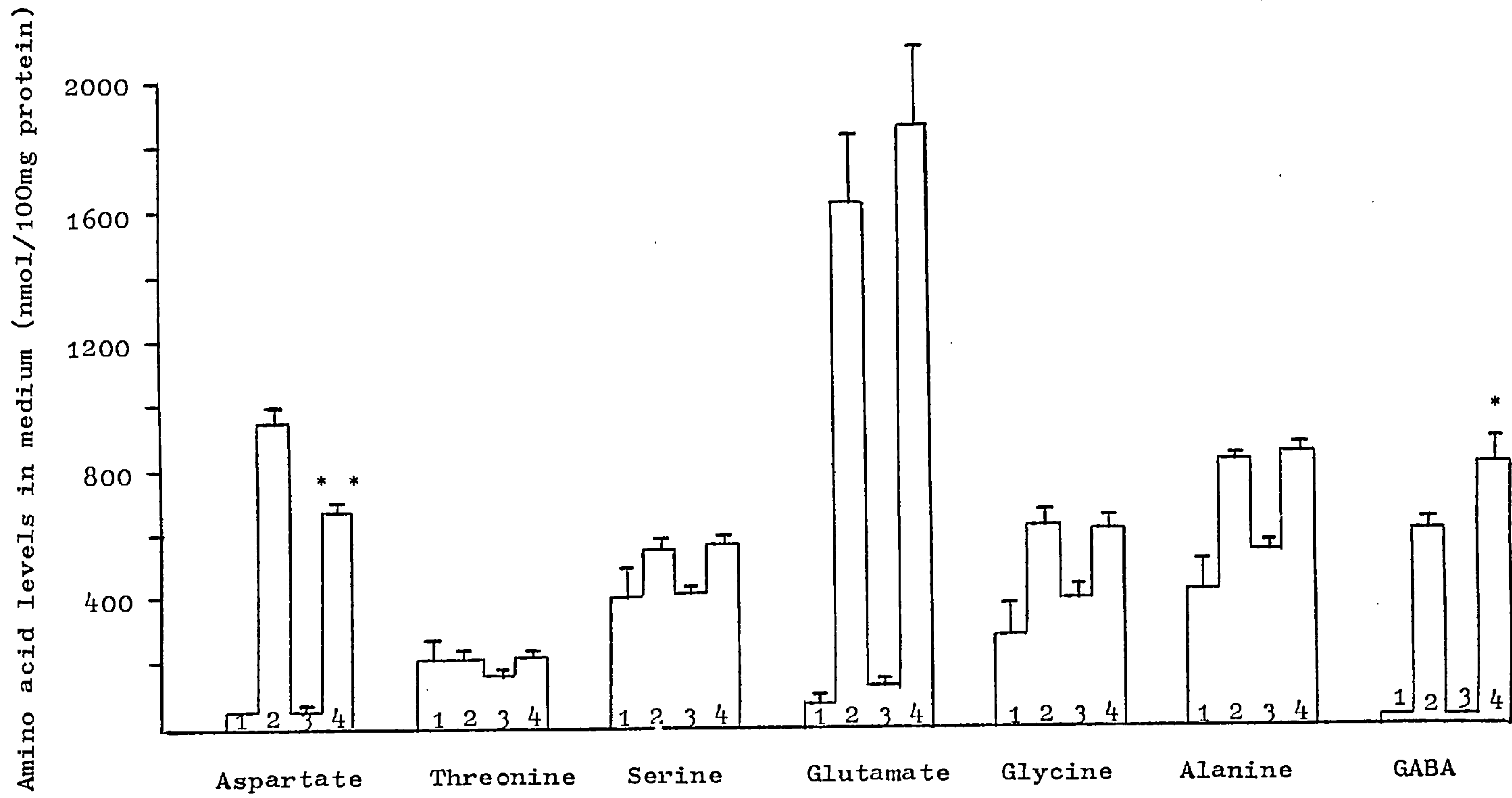


TABLE 3.i

THE EFFECT OF 0.5mM PENTOBARBITAL ON THE AMINO ACID CONTENT OF RAT CEREBROCORTICAL SYNAPTOSOMES

Amino acid content nmol/100mg protein

	Control (4)	Stimulated (4)	Control (4)	Stimulated (4)
Aspartate	1906±246	946±146	4142±406	1467±214
Threonine	274±46	272±39	169±21	186±26
Serine	427±19	331±52	385±47	409±21
Glutamate	2392±115	1568±268	4280±215	3187±99
Glycine	615±97	394±127	575±70	384±33
Alanine	629±99	409±80	629±83	473±23
GABA	963±111	674±61	2032±199	915±77

Synaptosomes were incubated in Krebs-phosphate medium with or without pentobarbital (0.5mM). Veratrine (final concentration, 75µM) was used for stimulation. Values are mean ± S.E.M. for the number of determinations in brackets.

TABLE 3.j

THE EFFECT OF PENTOBARBITAL ON RESPIRATION IN CEREBRO-CORTICAL SYNAPTOSOMES

Conditions	Respiratory rate (µmol O_2/100mg protein/h)	n
Control incubation	58.5±3.1	8
Control incubation with 0.1mM pentobarbital	54.1±5.5	4
Control incubation with 0.5mM pentobarbital	42.8±3.8	6
Control incubation with 5mM pentobarbital	No respiration	2
Potassium stimulated	94.0±18	4
Potassium stimulated with 0.1mM pentobarbital	75.5	2
Potassium stimulated with 5mM pentobarbital	No respiration	2
Veratrine stimulated	113.5±12.8	6
Veratrine stimulated with 0.1mM pentobarbital	88.7	2
Veratrine stimulated with 0.5mM pentobarbital	102.3±10.2	6

Cerebrocrotical synaptosomes were incubated in Krebs-phosphate medium for 45 mins. Values are mean ± S.E.M. for the number of samples shown, except where indicated.

DISCUSSION

The effect of stimulation on synaptosomal respiration, lactate production, K^+ levels and amino acid release

The data presented in this chapter regarding synaptosomal respiration, lactate production, K^+ retention and amino acid release, tends to confirm the work previously published by this group, the one exception being the results obtained using spinal/medullary nerve-endings. As stated in the results section, Osborne and Bradford (1973) have demonstrated the stimulus-induced release of aspartate, glutamate, glycine and GABA, using synaptosomal beds, whereas with suspensions we could only show the selective release of the inhibitory amino acids. A proposed explanation for this apparent anomaly is given in the aforementioned section.

The effect of veratrine concentration on amino acid release, respiration and K^+ retention in rat cerebro-cortical synaptosomes

Osborne and Bradford (1975) showed that maximal release of the putative amino acid transmitter candidates occurred at an external K^+ concentration of 15mM. As this concentration is also known to cause rapid increases in both Ca^{2+} uptake and theoretical membrane depolarization they concluded that depolarization must be a prerequisite for stimulated amino acid release.

The results obtained using increasing concentrations of veratrine would suggest that maximum depolarization occurs at a concentration somewhere between 25 and 50µM. This is born out by the release data and also the amino

acid and K^+ levels measured in the intrasynaptosomal fractions. It would be interesting to determine whether the membrane potential change elicited by this concentration corresponds to the 30mV depolarization quoted by Katz and Miledi (1967).

The fact that maximal release of the putative amino acid transmitters was produced by a veratrine concentration of 25-50μM would seem to support the idea of a well defined releasable transmitter pool. Hence, the data would suggest that it is possible to completely deplete the releasable pool of a particular neurotransmitter merely by applying a sufficiently strong stimulus.

As a postscript it must be mentioned that with respiration, maximal rates were not obtained until the veratrine concentration had reached 75 - 150μM. This is perhaps surprising in view of the fact that maximal responses as far as amino acid release and intrasynaptosomal K^+ depletion are concerned, were achieved with much lower veratrine concentrations, i.e. 25 - 50μM. However, it may be the case that the link between respiration and depolarization is not so rigid.

<u>The effect of protein dilution on amino acid release from cerebrocortical synaptosomes</u>

As described in the introduction to this chapter, de Belleroche and Bradford (1976) have proposed a hypothetical mechanism for neurotransmitter release, which they refer to as a "transport shuttle". They have suggested that release is part of a continuous cyclical process in which efflux and Na^+ dependent uptake are closely linked. This system would be organised such that no net uptake or release

occurred. However, where exogenous neurotransmitter was introduced there would be net uptake if the capacity of the inward process exceeded the capacity of the outward flux. Similarly, any factor which intefered with the inwardly directed half of this cycle would result in raised neurotransmitter levels in the medium.

The experiment used in this study to test the above hypothesis was found to yield the predicted result, i.e. as the protein dilution was increased so the total levels of all the amino acids recovered in the medium increased. Comparison of the relative quantities released over the whole range of dilution revealed that the largest increases were recorded for the putative transmitters.

When the release data was expressed as concentrations (i.e. nmols. amino acid per ml of medium) a more interesting picture was obtained. The levels of threonine, serine, glycine and alanine were found to decrease as the protein concentration decreased, whilst the concentrations of aspartate, glutamate and GABA remained relatively constant. The significance of this result becomes clearer when one considers the fact that the levels of the non-physiologically active amino acids appearing in the medium would be expected to be directly proportional to the protein concentration. This is because, unlike the putative transmitters, resynthesis of these amino acids does not occur, i.e. their tissue levels would not be maintained.

In contrast with the non-physiologically active amino acids, the putative transmitters are known to be subject to high-affinity uptake (outlined in Chapter 1). These uptake processes have been shown to be dependent upon the

concentration, rather than the absolute amount of neurotransmitter present in the medium. It is believed that the primary purpose of these uptake systems is to maintain the extracellular neurotransmitter concentration at a very low level, whilst the nerve-ending is at rest. The data presented in this chapter would therefore appear to lend support to this idea.

Reference to Figure 3.8 shows that over the 0.75 - 1.5ml incubation volume range, the concentrations of aspartate and glutamate drop quite sharply (N.B. Similar trend observed for threonine, serine, glycine and alanine), before levelling off. This observation may provide further evidence for the suggestion made by Bradford et al. (1975) that at high protein concentrations, during the initial stages of incubation the amino acid levels present in the medium are extremely high. It may be the case that for the 0.75ml suspension a longer period of incubation was necessary in order to allow the tissue to equilibrate and set up fully operational uptake systems. The high concentrations observed for aspartate and glutamate in the high-protein incubations may also provide indirect evidence for a common uptake system.

The fact that the graph for GABA concentration (Fig. 3.8) is constant over the entire range of dilution suggests that the rate of uptake for this amino acid is more rapid than for aspartate and glutamate.

As mentioned in the results section, the total level of asparate was found to rise as the protein concentration decreased, suggesting that protein dilution may play a role in influencing amino acid synthesis. This increase in

synthesis is probably required to maintain the intracellular level of this amino acid as the amount released to the medium increases. It would have been interesting to have seen whether this apparent increase in synthesis occurred to a greater extent in incubations containing even smaller quantities of protein.

The data presented in this section provides some interesting information regarding amino acid release and uptake. However, it cannot be claimed that conclusive evidence for the existence of a "transport shuttle" was obtained. Indeed, the concept of such a shuttle may in fact be totally incorrect.

As described earlier, de Belleroche and Bradford (1976) visualise their hypothetical mechanism as a cyclical process in which release and uptake are intimately linked. However, data reported recently by Redburn (1978) would suggest that these two processes operate completely independently of each other. In this study, the effects of a number of agents on ^{14}C-GABA release and uptake in synaptosomes was examined. None of the compounds tested caused simultaneous changes in rates of uptake and release of GABA from synaptic pools. Thus, when uptake processes were inhibited, at the time release was assayed, there was no apparent effect on release. Similarly, when release processes were inhibited, at the time uptake was assayed, there was no apparent effect on uptake.

As stated by Redburn (1978) the common regulating factor for both uptake and release is the size of the synaptosomal neurotransmitter pool. Simon and Kuhar (1975) have shown that depletion of the intrasynaptosomal pool

following prolonged, stimulated release will eventually result in an increase in the rate of choline uptake. Similarly, the activity of the uptake process can lead to changes in the size of the neurotransmitter pool, thus affecting the amount of neurotransmitter available for release. It must be stressed that this apparent coupling of these two processes represents only an indirect link, due to the fact that they share a common pool, the respective rates of uptake and release being governed by the size of this pool. A more direct coupling could be envisaged as involving some common regulatory cofactors which would have simultaneous effects on both uptake and release.

Despite the information outlined above refuting the idea of a close link between uptake and release, there still remains the fact that Bradford and his coworkers (de Belleroche and Bradford, 1972; Osborne and Bradford, 1975; Cox and Bradford, 1978) have obtained results with various uptake blockers and amino acid analogues, explainable by their hypothetical mechanism. Suggestions that these effects may result from an inhibition of the inwardly directed component of classical homoexchange are unfounded, as by definition, homoexchange would require the initial presence of external amino acid to activate the process. Data consistent with a membrane transport shuttle has also been demonstrated for dopamine by labelling its endogenous and exogenous pools with different isotopes (de Belleroche and Bradford, 1976).

As regards the data presented in this chapter only evidence for an active uptake process can be claimed. The

same cannot be said for the release component of the proposed cycle. Obviously, a great deal more work remains to be completed, in order to test the hypothesis further.

The effect of pentobarbital on synaptosomal amino acid release

The results obtained using pentobarbital would appear to fit in with several proposed mechanisms for general anaesthetic action.

As mentioned previously, pentobarbital has been shown to depress the postsynaptic response to applied glutamate and prolong the inhibition produced by GABA (Ransom and Barker, 1975; Richards and Smaje, 1974). It has been suggested that pentobarbital produces this effect by reducing the sensitivity of the postsynaptic membrane to the released excitatory transmitter. Alternatively, pentobarbital may reduce the output of excitant from the presynaptic terminal.

In our *in vitro* system pentobarbital was found to reduce the release of the excitatory amino acid, aspartate, this observation tending to confirm the latter theory.

The observation that pentobarbital also increased the synaptosomal release of GABA agrees with the hypothesis put forward by Cutler and Dudzinski (1974). They have suggested that general anaesthetics may act by stimulating the release of inhibitory transmitter (GABA), resulting in increased postsynaptic inhibition. Indeed, using cerebrocortical slices these workers found this to be the case, pentobarbital increasing the electrically induced release of ^{3}H-GABA. The results presented in this chapter differed in one respect

from those of Cutler and Dudzinski (1974) in that the spontaneous release of GABA was not inhibited.

It is interesting that in our hands pentobarbital was also found to markedly increase the total synaptosomal levels of aspartate, glutamate and GABA, suggesting that this agent apart from affecting the release of these amino acids also influenced their synthesis. Several proposed explanations for this observation are given in the results section. However, it is still perhaps surprising that despite the common effect observed on synthesis, the release of GABA should be increased, whilst the release of aspartate was depressed and glutamate unaffected.

Measurement of synaptosomal respiration in the presence of this anaesthetic yielded data similar to that of Himwich (1951), i.e. a depression in the respiratory rate.

CHAPTER FOUR

SUPERFUSION STUDIES ON THE RELEASE OF GLYCINE FROM CRUDE NERVE-ENDING PREPARATIONS

INTRODUCTION

RESULTS

The potassium stimulated release of ^{14}C-glycine from superfused crude spinal cord (P_2) fractions.

The effect of Na^+ on ^{14}C-glycine release from crude spinal cord (P_2) fractions.

The effect of unlabelled glycine (100μM) on the release of ^{14}C-glycine from superfused crude spinal cord (P_2) extracts.

DISCUSSION

INTRODUCTION

Synaptosomes provide a useful model for studying the uptake, storage and release of putative neurotransmitters *in vitro* and for analyzing the effects of drugs and other agents on these phenomena (reviewed by Bradford, 1974). Often, for release studies, synaptosomes are first pre-loaded by incubating them in a medium containing a particular radio-active neurotransmitter (or precursor), then sedimented, washed and resuspended in fresh unlabelled medium; aliquots of this suspension are then incubated in tubes/flasks containing the agent under study and the release of radioactivity into the medium measured. This method has a number of disadvantages, including the length of the procedure and also the fact that some of the radio-activity is released during the washing and resuspension steps. However, the major disadvantage lies in the fact that synaptosomal reuptake of neurotransmitter released into the medium is not prevented. The prevention of this process is particularly important since nerve-endings have high affinity transport systems capable of taking up neuro-transmitters present in the surrounding medium at very low concentrations (Snyder et al., 1970; Iversen & Johnston, 1971). Even in the method developed by de Belleroche and Bradford (1972a, b, c) in which synaptosomes are sand-wiched between two pieces of nylon gauze to form a 'synaptosome bed', the reuptake of released neurotransmitter from the medium trapped in the 'bed', cannot be prevented.

Thus the technique of superfusion has been put forward as an answer to some of these problems, most importantly that of synaptosomal reuptake. The idea of superfusion is

not a recent innovation and has been applied to neural tissue previously (Baldessarini and Kopin, 1967; Srinivasan et al., 1969; Hopkin and Neal, 1970; McIlwain and Snyder, 1970). Hopkin*and Neal (1970) have studied the electrically induced release of ^{14}C-glycine from rat spinal cord slices, using a superfusion method. The effect of electrical stimulation and high K^+ concentrations on the efflux of ^{3}H-GABA from brain slices has also been examined (Srinivasan, Neal and Mitchell, 1969). Similarly, McIlwain and Snyder (1970) used a superfusion system for studying the release of various putative neurotransmitters from cerebral tissue. In their method, guinea pig piriform or neo-cortical slices were mounted in quick transfer holders and maintained at 38°C in a continuous flow system; stimulation of the slices was achieved electrically via the electrodes of the quick transfer holder. In this way McIlwain and Snyder studied labelled glycine, 5HT and NA release. In addition the effect of superfusion on cerebral tissue metabolism was determined.

It was not until 1974 (Raiteri et al.) however, that a method of superfusing synaptosomes was developed. Prelabelled synaptosomes were deposited on a millipore filter (0.65µm pore) at the bottom of a superfusion chamber and medium passed through. Raiteri and his coworkers have studied the K^+ stimulated release of ^{3}H-GABA from cerebral cortex synaptosomes and homoexchange in cerebral cortex and spinal cord synaptosomes using this technique (Levi and Raiteri, 1974; Raiteri et al., 1975). In the latter study they demonstrated that unlabelled GABA will stimulate the release of ^{3}H-GABA from cerebrocortical nerve-endings,

* PTO

similarly unlabelled glycine caused ^{3}H-glycine efflux from spinal cord synaptosomes. These workers have also shown that the GABA (unlabelled) stimulated release of ^{3}H-GABA does not occur in Na^{+}-free medium, suggesting that under these conditions the high-affinity uptake system for this amino acid is completely inhibited.

Recently, Brennan and Cantrill (1978) have examined the effects of DABA, alanine and β-alanine on the efflux of ^{3}H-GABA from rat brain synaptosomes, using the method of Raiteri et al. (1974). They found that at high concentrations (0.75mM-5mM) all three of these agents enhanced ^{3}H-GABA release.

Redburn et al. (1975) have also developed an advanced system for studying neurotransmitter release from isolated nerve-endings. Using their "rapid perfusion apparatus" they have successfully monitored the stimulus induced release of ^{14}C-GABA from cerebrocortical synaptosomes. Furthermore modification of their superfusion system (described by McIlwain and Snyder, 1970) has enabled McIlwain and his coworkers (Kuroda and McIlwain, 1974; Pull and McIlwain, 1975) to investigate factors influencing the release and uptake of adenine and its derivatives, in synaptosomal beds.

In this chapter the release of ^{14}C-glycine from crude spinal cord subcellular fractions was examined, using a modified version of the Raiteri et al. (1974) superfusion method. It was hoped that this technique would provide a rapid and simple method for determining the actions of a number of drugs on synaptosomal amino acid release.

RESULTS

The potassium stimulated release of ^{14}C-glycine from superfused crude spinal cord (P_2) fractions

Figure 4.1 shows the effect of potassium stimulation on ^{14}C-glycine release from a crude (P_2) extract.

The peak of efflux was found to range between 186 and 280% (results expressed as percent over basal efflux).

Measurements of the amount of radioactivity remaining associated with the millipore filter following superfusion yielded values of 5 - 9% of the total recovered.

Subsequent experiments run with AOAA included in the superfusion medium did not show a significant alteration in the pattern of release.

The effect of Na^+ on ^{14}C-glycine release from crude spinal cord (P_2) fractions

The effect of sodium ions on ^{14}C-glycine release was also evaluated. It was felt that the result obtained using 56mM potassium might have been due to a tonicity effect. Hence, the experiments were repeated substituting 194mM NaCl for 56mM. KCl.

Indeed, superfusion with medium containing 194mM Na^+ did elicit a response, peak efflux values of 175 - 190% being obtained (Fig. 4.2). This result therefore cast some doubt on the efficiency of the system.

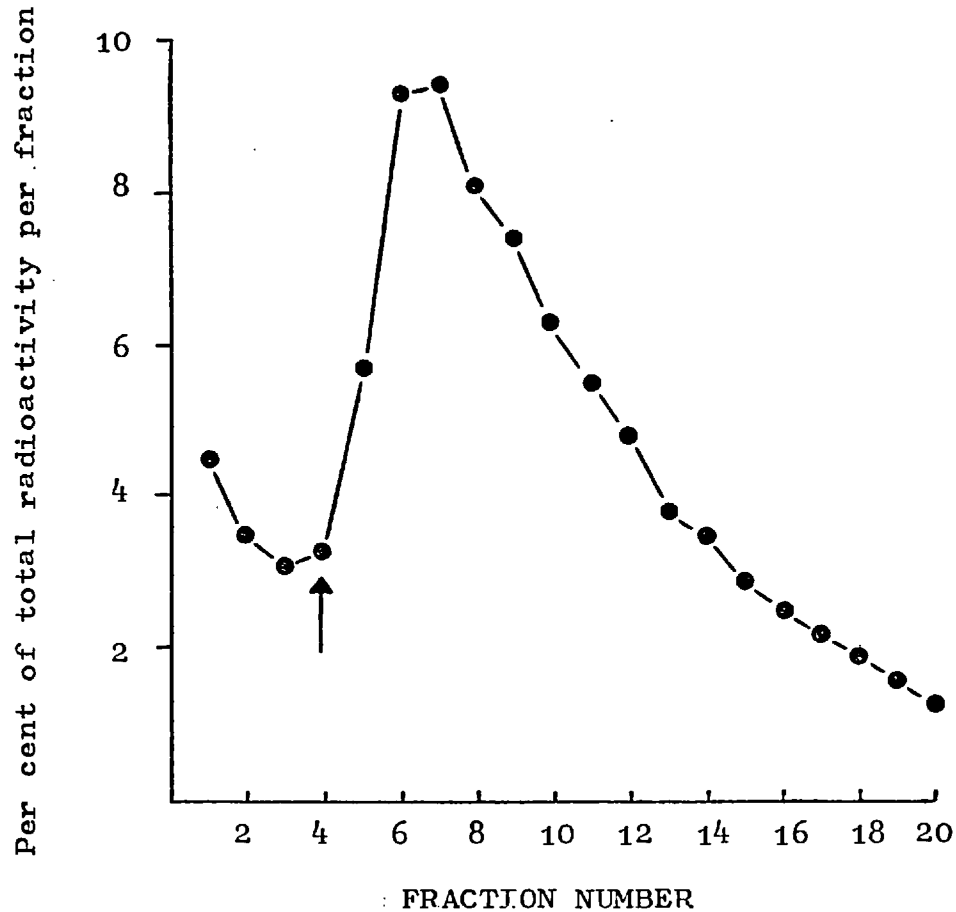
12 -14% of the total radioactivity recovered was found to remain associated with the P_2 extract on the millipore filter.

The effect of unlabelled glycine (100µM) on the release of ^{14}C-glycine from superfused crude spinal cord (P_2) extracts

Levi and Raiteri (1974) tested the effects of a range

FIGURE 4.1

THE K^+ STIMULATED RELEASE OF ^{14}C-GLYCINE FROM SUPERFUSED CRUDE SPINAL CORD (P2) FRACTIONS

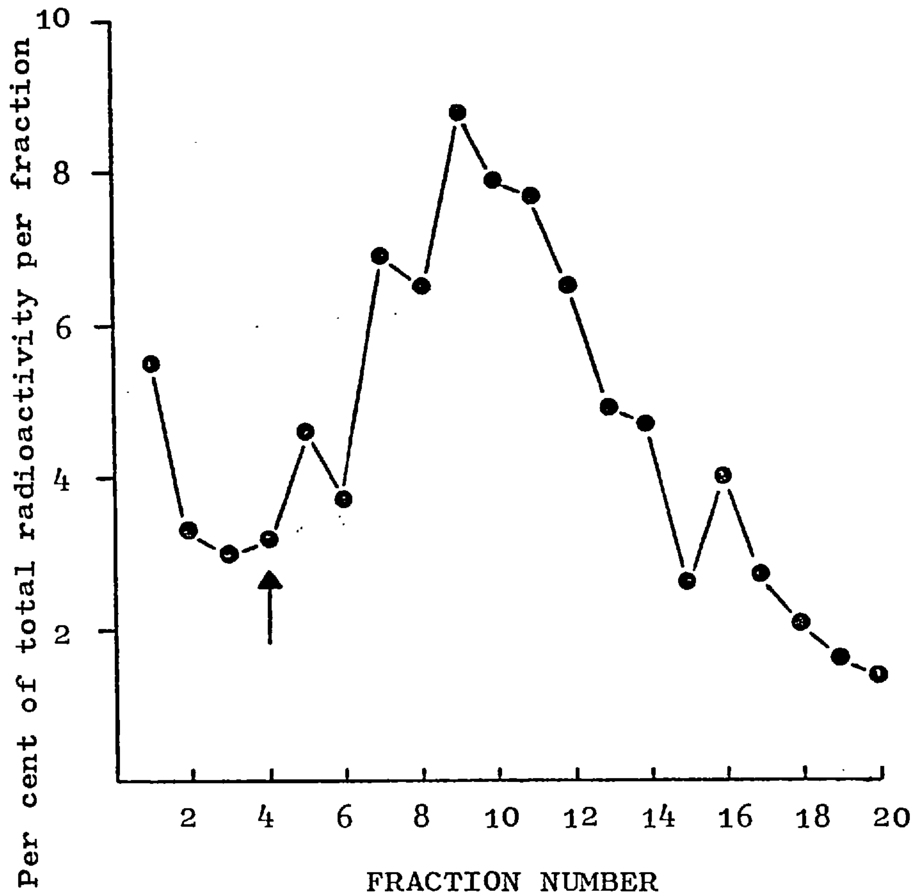


Nerve endings were prelabelled in a medium containing 20 µM ^{14}C- glycine, as described in Chapter 2. Fifty microlitre portions of the suspension were superfused with standard Krebs-phosphate medium until a steady spontaneous efflux level was established. At the point indicated by the arrow the medium was replaced by a medium containing 56mM K^+.

The above curve represents a typical result from 3 experiments.

FIGURE 4.2

THE EFFECT OF Na^+ ON ^{14}C-GLYCINE RELEASE FROM CRUDE SPINAL CORD (P2) FRACTIONS



Prelabelled nerve-endings were superfused with 194mM Na^+. Experimental conditions as described in the legend for Figure 4.1.

Curve represents a typical result from 3 experiments.

of concentrations (10 - 100μM), of unlabelled glycine on the release of ^{3}H-glycine from spinal synaptosomes.

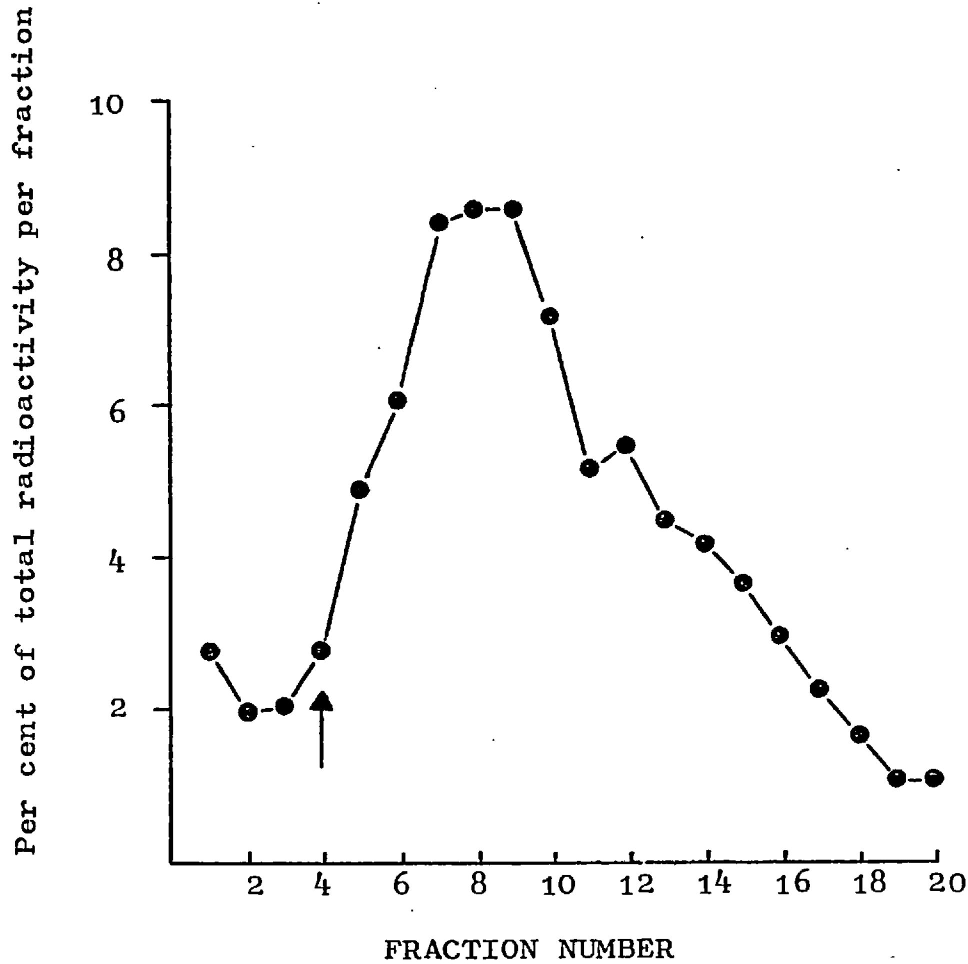
Under their conditions a maximal release was obtained at a glycine concentration of 100μM.

The results obtained using our system were extremely variable, however, unlabelled glycine appeared to elicit a response, peak effluxes of 125 - 336% being produced. Figure 4.3 shows a typical result from three experiments.

The amount of radioactivity remaining on the millipore filter at the conclusion of the experiment was approximately 7% of the total recovered.

FIGURE 4.3

THE EFFECT OF UNLABELLED GLYCINE (100µM) ON THE RELEASE OF ^{14}C-GLYCINE FROMSUPERFUSED CRUDE SPINAL CORD (P2) EXTRACTS



Prelabelled nerve-endings were superfused with medium containing 100µM glycine. Experimental conditions as described in the legend for Figure 4.1.

The curve represents a typical result from 3 experiments

DISCUSSION

Superfusion would obviously provide an ideal method for examining the processes going on in nerve-endings. Raiteri et al. (1974) have listed the advantages of their method over the other procedures described in the literature for studying synaptosomal amino acid release. These advantages include: (1) continuous superfusion should largely prevent the reuptake of released substrates; (2) the method is extremely simple and allows the utilization of synaptosomes immediately after their preincubation with the radioactive substrates; (3) a complete pattern of the release process can be obtained which gives improved reliability and allows more accurate kinetic studies, as compared with methods in which sampling is discontinuous; (4) the method allows the introduction of changes in the composition of the medium during the superfusion or even the replacement of the fluid with a medium of different composition; (5) the quantity of synaptosomes required for a typical experiment with radioactive substrates is very low: 0.05 - 0.1mg of synaptosomal protein per filter may be sufficient for most experiments; (6) superfusions run simultaneously are reproducible within 2 - 3% and results obtained on different days also show very little variation.

Despite the advantages outlined above, the results obtained using our system were most unsatisfactory. Although the effects described by Raiteri et al. (1974, 1975) were observed, the results obtained between experiments were inconsistent. This phenomenon may have been due to a number of factors.

Primarily, the design of the system may have been at fault. Raiteri et al. (1974) used a superfusion chamber specifically designed for the purpose and we have been advised (personal communication from Levi) that the dimensions of this chamber are critical. Their apparatus consisted of a superfusion chamber provided with a thermostatic jacket maintained at 37°C. The chambers were fitted with 2.5cm (0.65µm pore size) Millipore filters. Our apparatus comprised a Millipore swinnex 13 (corresponding to the superfusion chamber) fitted with a 1.3cm (0.8 or 1.2µm) filter. The superfusion medium capacity of this system was much smaller than that of Raiteri et al. (1974) and this may not have been sufficient to prevent the reuptake of the released amino acid. The amount of protein loaded onto each filter may also have been critical when taking into consideration this phenomenon of synaptosomal neurotransmitter reuptake. In our system approximately 2mg of protein was loaded onto each filter, whereas, Raiteri et al. (1974) used 0.05mg - 0.1mg; this amount may have been excessive. It is also interesting to note that Raiteri and his coworkers (1974) made up their synaptosome suspensions in a 0.32M glucose/ Krebs-Ringer mixture, whereas, we used Krebs-phosphate medium alone. The glucose may well have aided the sedimentation of the synaptosomes onto the filter.

Another factor which may have contributed to the failure of our system was the speed of superfusion used, Raiteri et al. (1974, 1975) collected 0.5ml/min fractions, in contrast to our superfusion rate of 0.12ml/min, fractions, being collected every 4 mins. This rate may have been too

slow and again not sufficient to prevent synaptosomal amino acid reuptake.

It is interesting that we should have obtained results similar to those described by Raiteri et al. (1974, 1975), i.e. K^+ stimulated ^{14}C-glycine release and unlabelled glycine stimulated ^{14}C-glycine efflux, however, it is worrying that 194mMNaCl should have produced a similar response, suggesting that a tonicity effect may have played a considerable role in this process.

Another cause for concern with our system was the fact that only 5 -14% of the total radioactivity was retained by the millipore filter following superfusion. Raiteri et al. (1974, 1975) claim a 75% retention, however, it must be pointed out that they used pure synaptosomal preparations, whereas, we used a crude P_2 extract. Even so one would have expected a greater percentage of the counts to have remained associated with the millipore filter following experimentation. This is unfortunate as the prime reason for developing our system was to enable us to study the actions of a number of drugs on amino acid release and uptake, using each nerve-ending laden filter to test several agents in succession, between washes.

The apparatus described by Raiteri et al. (1974) has now been constructed in our workshops and the future will tell whether their results are repeatable in our laboratory. As previously mentioned, Brennan and Cantrill, (1978) have used an apparatus of the type and dimensions described by Raiteri et al. (1974) and have obtained

comparable results.

CHAPTER FIVE

THE EFFECT OF VARIOUS AMINO ACID ANTAGONISTS/ANALOGUES, AND UPTAKE BLOCKERS ON PUTATIVE AMINO ACID TRANSMITTER RELEASE FROM SYNAPTOSOMES

INTRODUCTION

RESULTS

The effect of p-chloromercuriphenyl sulphonic acid and 3-threo-hydroxy aspartic acid on amino acid release from rat cerebrocortical synaptosomes.

The effect of PCMPS and 3-threo-hydroxy aspartic acid on synaptosomal respiration.

The effect of bicuculline on ^{14}C-GABA and endogenous amino acid release from rat cerebrocortical synaptosomes.

The effect of picrotoxin on ^{14}C-GABA and endogenous amino acid release from rat cerebrocortical synaptosomes.

The effect of kainic acid on amino acid release from rat cerebrocortical and sheep striatal synaptosomes.

The effect of kainic acid on the amino acid content of rat cerebrocortical and sheep striatal synaptosomes.

The effect of kainic acid on respiration and K^+ levels in rat cerebrocortical and sheep striatal synaptosomes.

The effect of kainic acid on ^{14}C-glutamate uptake into rat cerebrocortical and sheep striatal synaptosomes.

The effect of HA966 and GDEE on amino acid release and uptake in rat cerebrocortical synaptosomes.

The effect of β-bungarotoxin on the medium and tissue amino acid levels of rat cerebrocortical synaptosomes.

The effect of β-bungarotoxin on the K^+ content of rat cerebrocortical synaptosomes.

The effect of β-bungarotoxin on synaptosomal respiration.

The effect of β-bungarotoxin on amino acid uptake into rat cerebrocortical synaptosomes.

The effect of acetylcholine and carbachol on amino acid release from rat cerebrocortical synaptosomes.

DISCUSSION

The effect of p-chloromercuriphenyl sulphonic acid (PCMPS) and 3-threo-hydroxy aspartic acid on cerebrocortical synaptosomes.

The effect of picrotoxin and bicuculline on ^{14}C-GABA and endogenous amino acid release from rat cerebrocortical synaptosomes.

The effect of kainic acid, a glutamate agonist, on rat cerebrocortical and sheep striatal synaptosomes.

The effect of β-bungarotoxin on cerebrocortical synaptosomes.

The effect of ACh and carbachol on amino acid transmitter release.

Evidence for presynaptic receptors.

INTRODUCTION

Iontophoretic studies have yielded a considerable amount of information about the neurophysiological and neuropharmacological effects of the glutamate and GABA analogues on neuronal excitation and inhibition. However, as regards their biochemical mode of action, in particular their effects on synaptosomes and tissue slices, our knowledge is extremely limited.

The primary purpose of the work presented in this chapter, was to investigate the effects of some of these agents on the synaptosomal release of the putative amino acid transmitters, in order to demonstrate a correlation between the considerable body of neurophysiological data in the literature and their biochemical effects. In addition, their action on synaptosomal metabolism was determined, using synaptosomal respiration as a measure of metabolic activity. It was hoped that the data obtained would also provide further evidence for the existence of presynaptic receptors.

As outlined in Chapter 1, it has been suggested that neurotransmitters may control their own release through a feedback mechanism onto the presynaptic membrane. It was therefore proposed that an amino acid agonist would mimic the natural transmitter, combining with the presynaptic receptor to inhibit further release. Similarly, it may be suggested that an antagonist would have the opposite effect, interaction with the presynaptic receptor resulting in an increased release, the transmitter having been prevented from gaining access to the receptor. The agonists and antagonists used were in some cases tested at very high

concentrations (1 - 5mM) as evidence has been obtained in recent years to suggest that during depolarization the levels of neurotransmitter occurring at the synapse are extremely high (Kuffler and Yoshikami, 1975).

The glutamate analogues Kainic acid (agonist), glutamate diethyl ester and 1-hydroxy-3-aminopyrrolid-2-one (antagonists) have been used extensively in iontophoretic studies on both vertebrate and invertebrate systems (Shinozaki and Konishi, 1970; Johnston et al., 1974; Biscoe et al., 1975; Curtis et al., 1972; Haldeman et al., 1972; Haldeman and McLennan, 1972; McLennan and Haldeman, 1973; Davies and Watkins, 1973; Wheal and Kerkut, 1974; 1975, 1976; Bonta et al., 1971; Johnston et al., 1974). In these studies Kainic acid has been shown to behave as an excitant, whereas, glutamate diethyl ester (GDEE) and 1-hydroxy-3-amino-pyrrolid-2-one (HA966) have a depressant action.

The chemical structures of these agents have been elucidated, Kainate and GDEE being structurally related to glutamic acid, whereas, HA966 closely resembles GABA (Figures 5.1 to 5.3). It is interesting to note that HA966's parent pyrrolidone bears structural similarities, particularly as regards charge distribution to both the excitatory and inhibitory amino acids. It has therefore been proposed that the mechanism of the depressant actions of HA966 in the CNS may involve a direct interaction with receptors for either the putative excitatory transmitters, glutamate and aspartate or the putative inhibitory transmitters, GABA and glycine (Davies and Watkins, 1972, 1973).

Biochemical studies using the aforementioned agents have been restricted mainly to determining their effect on

FIGURE 5.1

STRUCTURE OF KAINIC ACID

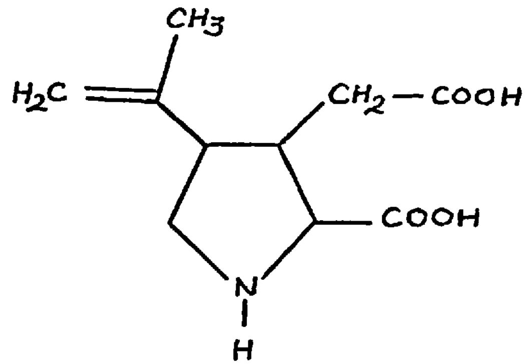


FIGURE 5.2

STRUCTURE OF GLUTAMATE DIETHYL ESTER

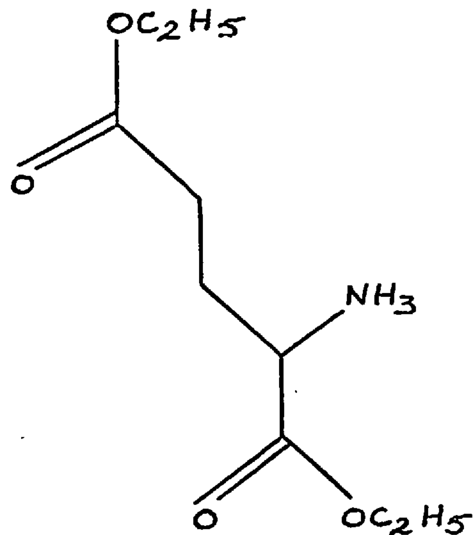
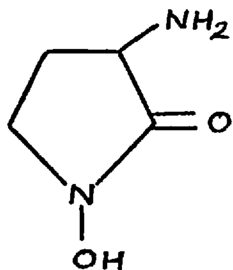
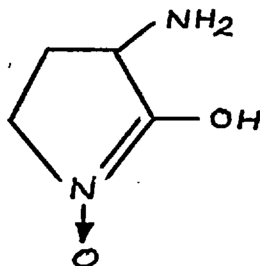


FIGURE 5.3

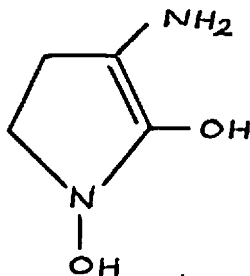
CHEMICAL STRUCTURE OF HA966



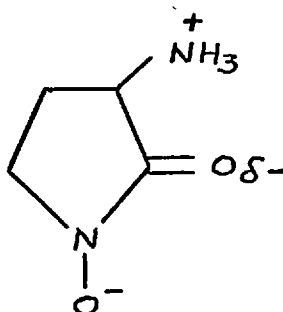
a



b



c



d

The structure of HA966 is usually written as a., however, the compound may exist in several different tautomeric forms, including b and c. It could also occur in the zwitterionic form shown in d.

L-glutamate uptake. However, even these theoretically simple experiments have been beset with problems, as the results obtained by various workers have tended to vary. Balcar and Johnston (1972) testing the effects of GDEE on high and low affinity uptake into cerebrocortical slices, found the agent to be inactive. Similar results were also obtained by Roberts and Watkins (1975), using synaptosomes and glia. However, McLennan (1975) claims that GDEE inhibits L-glutamate uptake. Similarly, Roberts and Watkins (1975) claim to have shown that kainic acid (and HA966) is inactive as an inhibitor of L-glutamate uptake, whereas, both Lakshmanan and Padmanaban (1974) and McGeer et al., (1978) have reported that kainate at concentrations of 0.3 - 1.0mM reduced the uptake of glutamate (1 -10μM) into crude striatal (P_2) or whole brain (P_2B) synaptosome fractions by 60 - 75%. These uptake studies underline the fact that one must exercise extreme caution when interpreting uptake data. Inhibition is a relative phenomenon and depends upon the relative concentrations of the inhibitor and substrate used. Also, one must take into account the position of the concentrations of inhibitor on the dose response curve of inhibition of uptake of a given concentration of substrate versus concentration of inhibitor. As can be seen from all the published observations, relatively high concentrations of kainate (0.3 - 1mM) are necessary to significantly inhibit glutamate uptake. In addition under normal circumstances kainate would not be expected to cause strong inhibiton of glutamate uptake as it is a potent excitant.

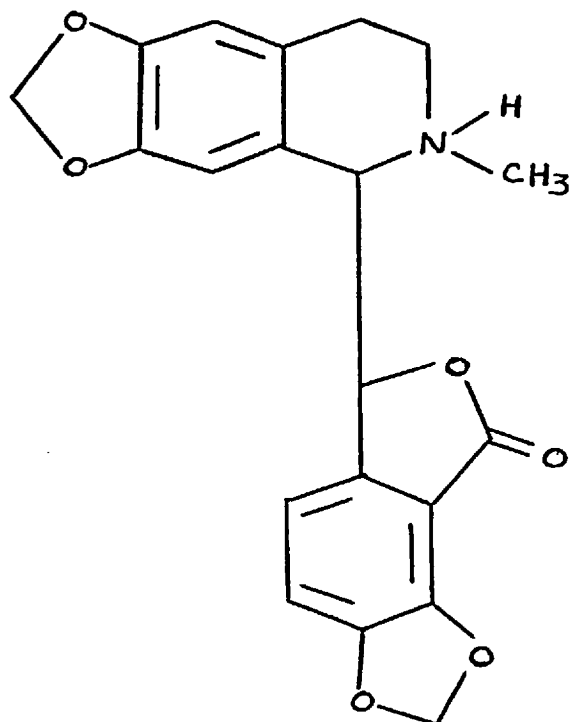
Cox et al. (1977) have shown that kainic acid increases oxygen uptake by cerebrocortical slices but has no effect on lactate production or NADH levels.

Kainic acid is also known to have a neurotoxic action. Subcutaneous or oral administration of kainic acid at sub-convulsive doses, like glutamate, causes neuronal degeneration in the circumventricular organs of the rodent brain, the arcuate nucleus of the hypothalamus, and in the inner retina (Olney et al., 1974, 1977; Friedle et al., 1978). It is believed that these neuropathological changes involve an action on postsynaptic sites rather than on the presynaptic axons (Olney et al., 1974).

The alkaloids, bicuculline and picrotoxin (structures shown in Figures 5.4 and 5.5) have been shown to behave to a greater or lesser degree as GABA antagonists and are believed to have a presynaptic as well as postsynaptic mode of action (Davidson and Reisine, 1971; Davidson and Southwick, 1971; Johnston and Mitchell, 1971; Curtis et al., 1974). As with the glutamate analogues, very little information has been forthcoming, regarding their biochemical effects. Johnston and Mitchell (1971) have shown that bicuculline potentiates the electrically evoked release of ^{3}H-GABA but not the spontaneous release, whereas picrotoxin inhibits the electrically evoked release. Similar results with picrotoxin have also been obtained by Collins (1973). Uptake studies have failed to establish bicuculline or picrotoxin as inhibitors of GABA uptake <u>in vitro</u> (Iversen and Johnston, 1971; Johnston and Mitchell, 1971). Bicuculline has been shown to influence GABA binding in rat cerebral

FIGURE 5.4

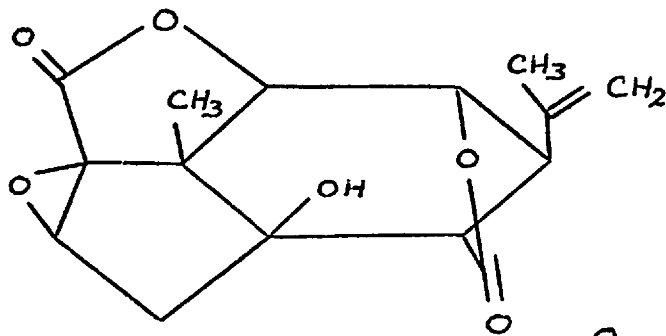
BASIC CHEMICAL STRUCTURE OF BICUCULLINE



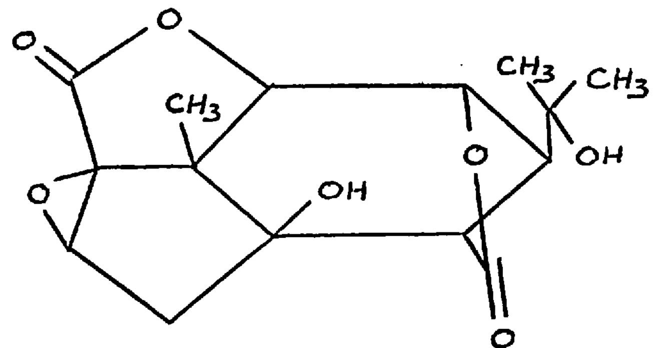
The hydrochloride form of this agent was used in this study.

FIGURE 5.5

CHEMICAL STRUCTURES OF THE TWO COMPONENTS OF PICROTOXIN



PICROTOXININ



PICROTIN

Picrotoxin is a mixture of the two above closely related compounds, picrotin and picrotoxinin. The latter is probably responsible for the GABA antagonism of picrotoxin, being approximately 50 times more potent as a convulsant than picrotin (Ramwell and Shaw, 1963; Jarboe et al,, 1968).

cortex (De Robertis et al., 1975).

Initial studies involved testing the effects of two agents, p-chloromercuriphenyl sulphonic acid (PCMPS) and 3-threo-hydroxy aspartic acid, known to inhibit the active transport of the putative amino acid transmitters, on synaptosomal amino acid release. The results obtained from these experiments were used as a reference when considering the data obtained subsequently, using the amino acid analogues. Para-chloromercuriphenylsulphonate (0.1mM) has been shown to inhibit GABA uptake by ~~as much as~~ 90% (Iversen and Johnston, 1971); it also has an inhibitory effect on glutamate uptake (Curtis et al., 1970; Roberts and Watkins, 1975). 3-threo-hydroxy aspartate is known to block both aspartate and glutamate uptake (Balcar and Johnston, 1972).

A compound closely related to p-chloromercuriphenyl sulphonic acid, p-hydroxymercuribenzoate, has been reported to increase the spontaneous release of aspartate, glutamate, GABA and glycine from spinal/medullary synaptosomes, however, it also reduces the stimulus-induced release (Osborne and Bradford, 1975).

The effect of an agent, β-bungarotoxin, known to have a presynaptic mode of action (Chang and Lee, 1963; Chang et al., 1973; Kelly and Brown, 1974; Oberg and Kelly, 1976a, b; Howard and Wu, 1976; Wernicke et al., 1975), was tested on synaptosomal amino acid release. Certain aspects of its mechanism of action on synaptosomal preparations have been studied previously (Sen et al., 1976) and it has been shown to stimulate neurotransmitter release (Kelly et al., 1975; Dolly et al., 1978). β -bungarotoxin inhibits noradrenaline,

GABA, 5HT and glutamate uptake into nerve-endings (Wernicke et al., 1974; Dolly et al., 1978), this effect being Ca^{2+} dependent. It has also been shown in electrophysiological studies, using neuromuscular junction preparations to initially cause an increase in the frequency of miniature endplate potentials and subsequently block neuromuscular transmission by inhibiting nerve impulse induced release of ACh (Chang et al., 1973; Kelly and Brown, 1974).

It has been suggested (Wernicke et al., 1975; Howard 1975; Kelly et al., 1975; Strong et al., 1976) that the activity of β-bungarotoxin depends on its phospho-lipase A activity. However, these results have been largely discredited by Sen et al. (1976) who have shown that β-bungarotoxin retains its neurotoxic properties in the presence of an inhibitor of phospholipase A activity.

Recently, it has been proposed that β-bungarotoxin may function as a depolarizing agent (Sen and Cooper, 1978).

A number of the agents tested on synaptosomal amino acid release were also tested for their effects on uptake. Control uptake experiments involved incubating synaptosomes at 0°C and in ouabain, both of which are known to block amino acid transmitter uptake (Roberts and Watkins, 1975; Gonda and Quastel, 1962). Ouabain has also been shown to increase the spontaneous and stimulus-induced release of the putative amino acid transmitters, from both spinal/ medullary (Osborne and Bradford, 1975) and cerebrocortical synaptosomes (de Belleroche and Bradford, 1972).

RESULTS

The effect of p-chloromercuriphenyl sulphonic acid and 3-threo-hydroxy aspartic acid on amino acid release from rat cerebrocortical synaptosomes

Introduction

Both p-chloromercuriphenyl sulphonic acid and 3-threo-hydroxy aspartic acid have been shown to inhibit putative amino acid transmitter uptake in a variety of preparations. Para-chloromercuriphenyl sulphonate inhibits the uptake of glutamate and GABA (Curtis et al., 1970; Iversen and Johnston, 1971; Balcar and Johnston, 1972; Roberts and Watkins, 1975) and 3-threo-hydroxy aspartate the uptake of glutamate (Balcar and Johnston, 1972).

The effects of these two agents on synaptosomal amino acid release, was examined.

Results

Effect of p-chloromercuriphenyl sulphonic acid (PCMPS) on amino acid release

Synaptosomes were incubated in Krebs-phosphate medium or medium containing PCMPS (final concentrations 100µM and 200µM) for 35 mins. At the end of this period, in those suspensions to be stimulated the K^+ concentration was raised to 56mM and incubation continued for a further 10 mins.

At a concentration of 100µM, PCMPS did not appear to alter the spontaneous or stimulated release of aspartate, glutamate or GABA. However, it reduced the spontaneous and stimulus-induced efflux of glycine (Figure 5.6).

On the other hand, 200µM PCMPS raised the control levels of aspartate (424%), glutamate (181%) and GABA (78%).

FIGURE 5.6

EFFECT OF PARA-CHLOROMERCURIPHENYL SULPHONIC ACID ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

Synaptosomes were incubated in Krebs-phosphate medium for 45 mins (controls) or for 35 mins followed by 10 mins K^+ (final concentration, 56mM) stimulation. Values are means $\pm$ S.E.M. for 4 - 6 determinations. Change in release due to presence of p-chloromercuriphenyl sulphonate, $p<0.05^*$, $p<0.001^{**}$, $p<0.02^{\dagger}$ or $p<0.01^{\dagger\dagger}$.

1. - control
2. - K^+ stimulated
3. - control } 0.1mM PCMPS in medium
4. - K^+ stimulated }
5. - control } 0.2mM PCMPS in medium
6. - K^+ stimulated }

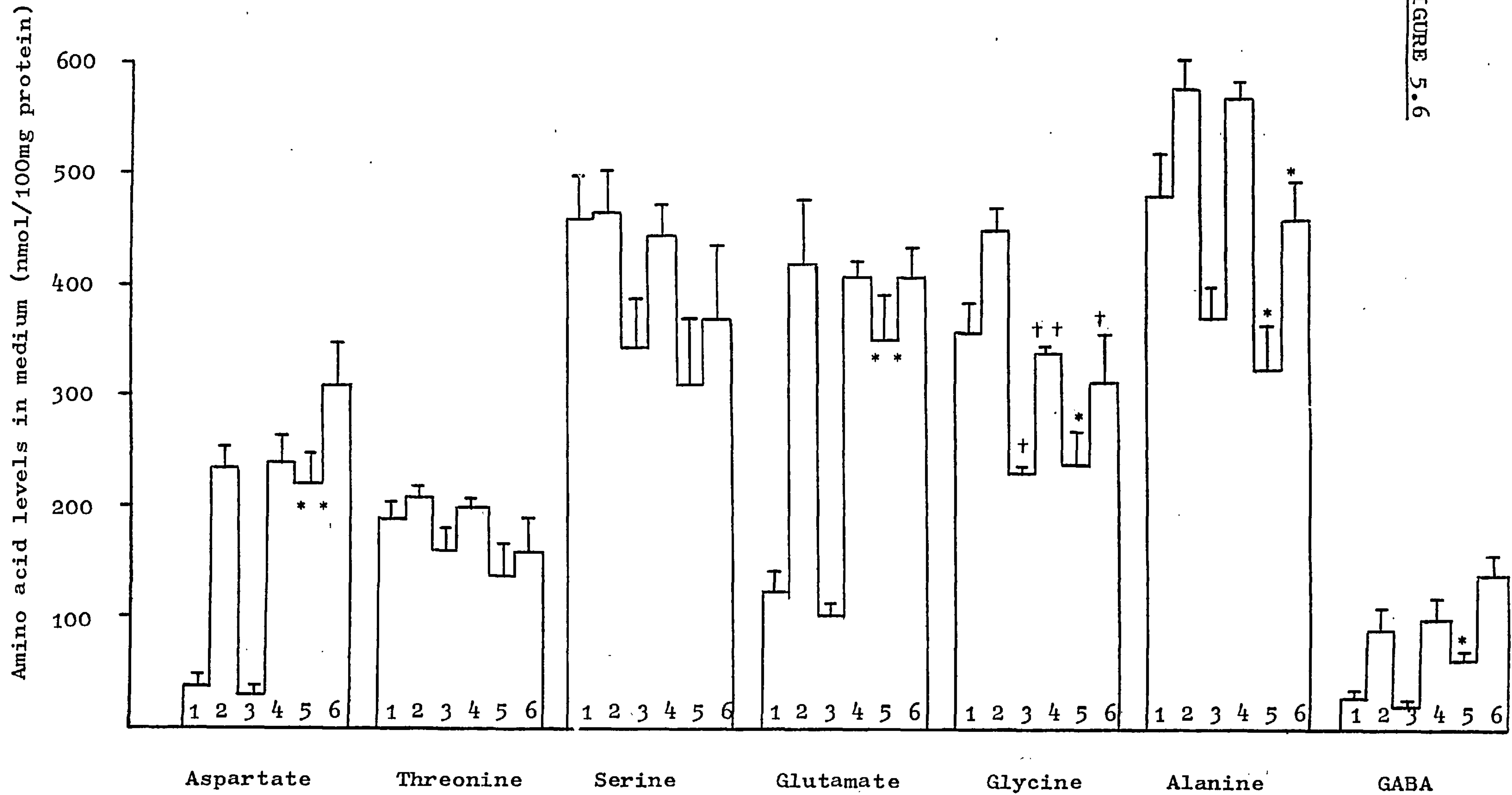


FIGURE 5.6

Again, the control and stimulated release of glycine was reduced, this also being the case for alanine (Figure 5.6).

Glycine and alanine were the only amino acids whose intrasynaptosomal levels were significantly altered by incubation with PCMPS. Glycine being reduced in both control and stimulated samples, alanine only in the latter (Table 5.a). The total levels of glycine (i.e., tissue and medium levels combined) were also reduced, suggesting that PCMPS had in some way interfered with its synthesis.

Effect of 3-threo-hydroxy aspartic acid on amino acid release

3-threo-hydroxy aspartic acid differed in its action from PCMPS.

When cerebrocortical synaptosomes were incubated in Krebs-phosphate medium containing 3-threo-hydroxy aspartic acid (final conc. 100μM), the spontaneous efflux of aspartate and glutamate was increased by 680% and 504% respectively. In addition, unlike PCMPS, 3-threo-hydroxy aspartate increased the stimulus induced release of aspartate (111%) and glutamate (79%) (Figure 5.7).

Examination of the total amino acid levels (i.e. tissue and medium levels combined; Figure 5.7 and Table 5.b) showed no appreciable changes having occurred as a result of 3-threo-hydroxy aspartate action.

The effect of PCMPS and 3-threo-hydroxy aspartic acid on synaptosomal respiration

3-threo-hydroxy aspartate had no effect on synaptosomal

TABLE 5.a

THE EFFECT OF PARA-CHLOROMERCURIPHENYL SULPHONIC ACID ON THE AMINO ACID CONTENT OF RAT CEREBROCORTICAL SYNAPTOSOMES

	Amino acid content (nmol/100mg protein)					
	No addition		0.1mM PCMPS in medium		0.2mM PCMPS in medium	
	Control (6)	Stimulated (6)	Control (4)	Stimulated (4)	Control (4)	Stimulated (4)
Aspartate	2433±236	2593±213	2003±101	2183±181	1724±119	2326±136
Threonine	146±20	120±13	107±9	95±14	85±15	116±21
Serine	364±35	315±20	329±19	289±6	293±45	276±26
Glutamate	2323±162	2247±154	2082±69	2011±126	2309±106	2569±117
Glycine	573±43	412±12	*407±27	**339±18	**315±43	*332±30
Alanine	620±54	436±11	471±31	**366±14	469±56	437±46
GABA	844±62	863±85	853±127	788±54	835±50	836±31

Synaptosomes were incubated in Krebs-phosphate medium for 45 mins (controls) or 35 mins followed by 10 mins K^+ (final concentration, 56mM) stimulation. Values are mean ± S.E.M. for the number of samples in brackets. Change in content due to presence of p-chloromercuriphenyl sulphonic acid significant with $p < 0.05$ (*) or $p < 0.01$ (**) when comparing control values and stimulated values.

FIGURE 5.7

EFFECT OF 3-THREO-HYDROXY ASPARTIC ACID ON AMINO ACID RELEASE

Cerebrocortical synaptosomes were incubated in Krebs-phosphate medium with or without 3-threo-hydroxy aspartic acid (final concentration 100μM) at 37°C. Potassium stimulation was applied after 35 mins incubation, by elevating the external potassium concentration to 56mM, and lasted 10 mins. Values are mean ± S.E.M. for 4 - 6 determinations. Change in release due to presence of 3-threo-hydroxy aspartate, $p < 0.05$* or $p < 0.01$**

1. - control
2. - K^+ stimulated
3. - control } 3-threo-hydroxy aspartate in medium
4. - K^+ stimulated } 3-threo-hydroxy aspartate in medium

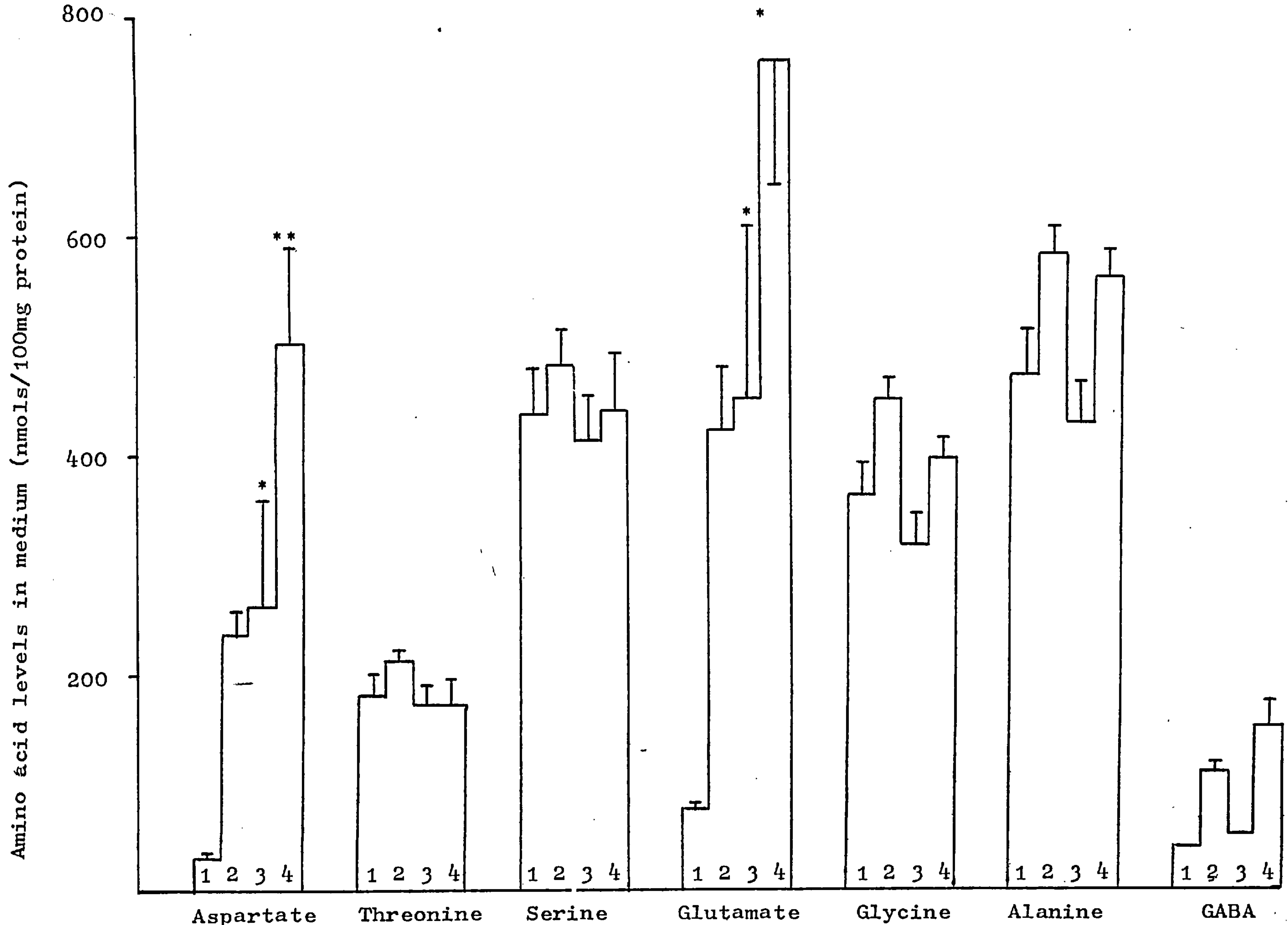


FIGURE 5.7

TABLE 5.b

THE EFFECT OF 3-THREO-HYDROXY ASPARTIC ACID ON THE AMINO ACID CONTENT OF RAT CEREBROCORTICAL SYNAPTOSOMES

Amino acid in tissue (nmols/100mg protein)

			3-threo-hydroxy aspartate in medium	
	Control (6)	Stimulated (6)	Control (6)	Stimulated (6)
Aspartate	2433±236	2593±213	2472±298	1972±107
Threonine	146±20	120±13	136±15	110±12
Serine	364±35	315±20	367±36	321±13
Glutamate	2323±162	2247±154	2383±131	2310±144
Glycine	573±43	410±12	476±30	362±24
Alanine	620±54	436±11	543±49	382±24
GABA	844±62	863±85	972±103	954±69

Synaptosomes incubated in Krebs-phosphate medium with or without 3-threo-hydroxy aspartic acid (final concentration 0.1mM). K^+ (final concentration, 56mM) was used for stimulation. Values are means ± S.E.M. for the number of samples in brackets.

respiration, however, PCMPS reduced the respiratory rate in control incubations by 24% (Table 5.c).

Curiously, in this series of experiments K^+ stimulation did not elicit the usual response, i.e. an increase in O_2 consumption, as outlined in Chapter 3.

The effect of bicuculline on ^{14}C-GABA and endogenous amino acid release from rat cerebrocortical synaptosomes

Johnston and Mitchell (1971) have reported that 10µM bicuculline increases the electrically stimulated efflux of ^{3}H-GABA from cerebral cortex slices, concentrations of 1µM and 100µM eliciting no response.

Figure 5.8 shows the effect of bicuculline (final concentrations 100µM and 200µM) on ^{14}C-GABA release from rat cerebrocortical synaptosomes. Like Johnston and Mitchell (1971), at these relatively high concentrations, little or no effect on the stimulus-induced release was observed. However, in contrast with these workers, bicuculline at these concentrations was found to influence the spontaneous efflux of ^{14}C-GABA.

At a concentration of 100µM bicuculline significantly reduced the spontaneous release. However, when the concentration was raised to 200µM the level of counts recovered in the medium was increased by 22% over control levels.

Neither, 100µM or 200µM had any effect on endogenous amino acid release (Table 5.d).

The effect of picrotoxin on ^{14}C-GABA and endogenous amino acid release from rat cerebrocortical synaptosomes

Under our conditions, picrotoxin (final concentration, 100µM) depressed the veratrine (final concentration,

TABLE 5.c

THE EFFECT OF P-CHLOROMERCURIPHENYL SULPHONIC ACID AND 3-THREO-HYDROXY-ASPARTIC ACID ON RESPIRATION IN RAT CEREBRO-CORTICAL SYNAPTOSOMES

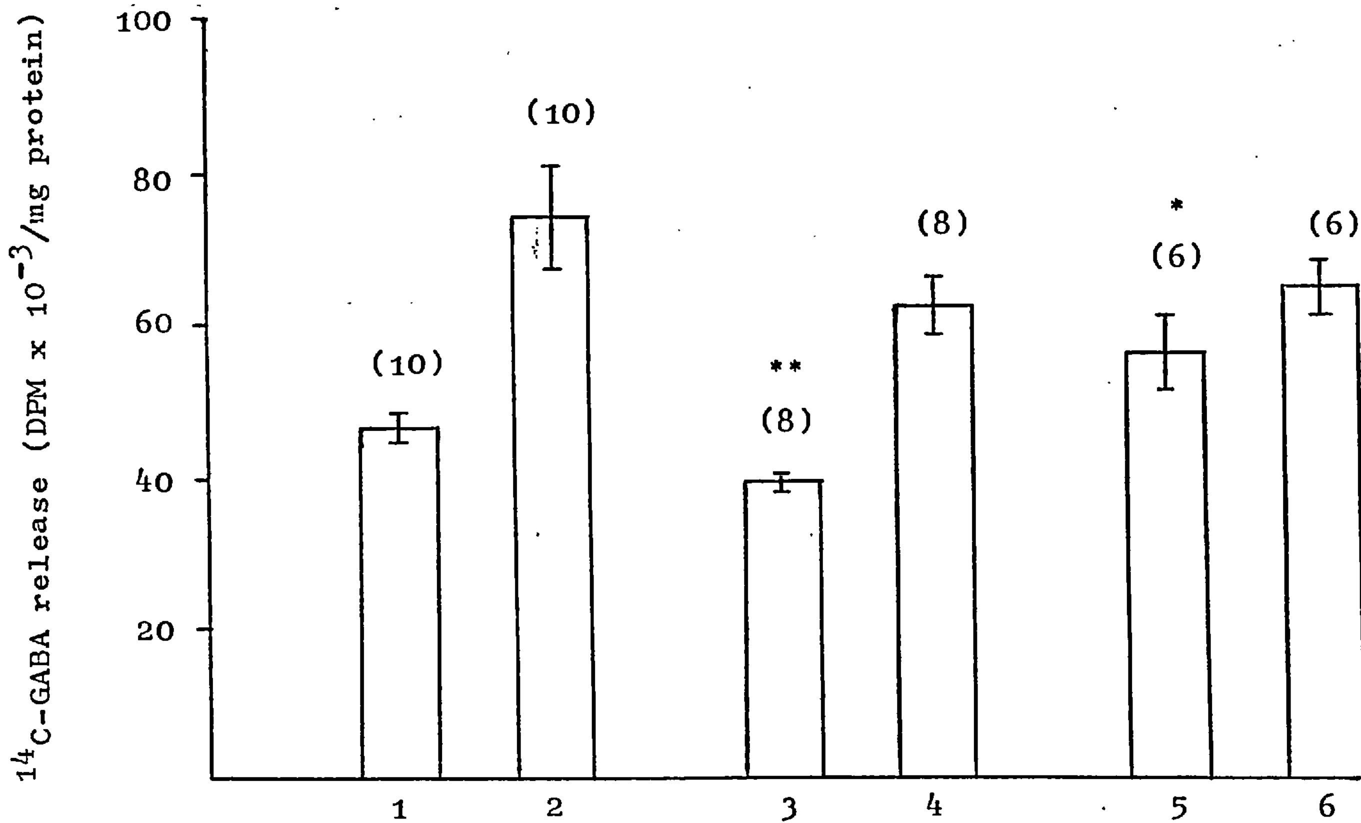
Respiratory rate (μmol O_2/100mg protein/h)

	No addition	PCMPS (final concentration, 200μM) in medium	3-threo-hydroxy aspartate (final concentration, 100μM) in medium
CONTROL	48.7±1.5 (5)	*37.0±0.8 (4)	52.2±2.7 (6)
STIMULATED	55.5±5.1 (6)	43.7±3.2 (4)	47.6±2.8 (6)

Synaptosomes were incubated at 37°C for 45 mins in Krebs-phosphate medium. K^+ (final concentration, 56mM) was used for stimulation. Values are mean ± S.E.M. for the number of samples in brackets. Change from control significant, $p < 0.001$*.

FIGURE 5.8

EFFECT OF BICUCULLINE ON ^{14}C-GABA RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES



Rat cerebrocortical nerve endings were prelabelled in Krebs phosphate medium containing 10μM ^{14}C-GABA, sedimented, washed and resuspended in fresh medium. Samples were then incubated for 45 mins (controls) or 35 mins followed by 10 mins K^+ (final concentration, 56mM) stimulation. The suspensions were then sedimented and the amount of radioactivity occurring in the medium determined by liquid scintillation counting.

Histobars represent the mean ± S.E.M. for the number of determinations shown in brackets. Change in spontaneous release due to presence of bicuculline significant with $p<0.05$* and $p<0.002$**

1. - control
2. - K^+ stimulated
3. - control } 0.1mM bicuculline in medium
4. - K^+ stimulated
5. - control } 0.2mM bicuculline in medium
6. - K^+ stimulated

TABLE 5.d

THE EFFECT OF BICUCULLINE ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

	Amino acid in incubation medium (nmol/100mg protein)					
	No addition		0.1mM bicuculline in medium		0.2mM bicuculline in medium	
	Control (8)	Stimulated (8)	Control (6)	Stimulated (6)	Control (6)	Stimulated (6)
Aspartate	35±8	313±55	61±16	318±39	44±5	*216±15
Threonine	155±8	164±16	143±8	167±10	171±4	168±11
Serine	277±18	293±33	266±27	291±20	259±16	251±24
Glutamate	54±13	476±90	86±23	475±56	60±5	373±18
Glycine	185±8	249±30	182±16	211±12	210±7	250±51
Alanine	386±24	473±58	368±43	482±45	328±9	376±20
GABA	49±6	123±15	49±7	120±9	71±9	139±12

Synaptosomes were incubated in Krebs-phosphate medium with or without bicuculline at 37°C. After 35 mins KCl (final concentration, 56mM) was added and incubation continued for a further 10 mins. Values are means ± S.E.M. for the number of determinations in brackets. * Not significant.

75µM) stimulated release of labelled GABA by 21% (Figure 5.9). This result is comparable with the 50% decrease in electrically induced efflux, observed by Johnston and Mitchell (1971).

At this concentration, picrotoxin appeared to have no influence on endogenous amino acid release (Table 5.e). However, at a concentration of 1mM, picrotoxin produced a 40 - 50% decrease in the veratrine (final concentration 10µM) stimulated release of aspartate, glutamate and GABA (Figure 5.10). The veratrine induced release of the non-physiologically active amino acids serine, glycine and alanine, exhibited a similar trend.

Nerve-endings treated with picrotoxin (final concentration, 1mM) showed no decrease in their total amino acid levels (combine data from Figure 5.10 and Table 5.f) when compared with control synaptosomes. Consequently, the toxin was not producing its effect by diminishing the endogenous levels of the compounds whose release it reduced.

Table 5.g shows that picrotoxin had no effect on synaptosomal respiration.

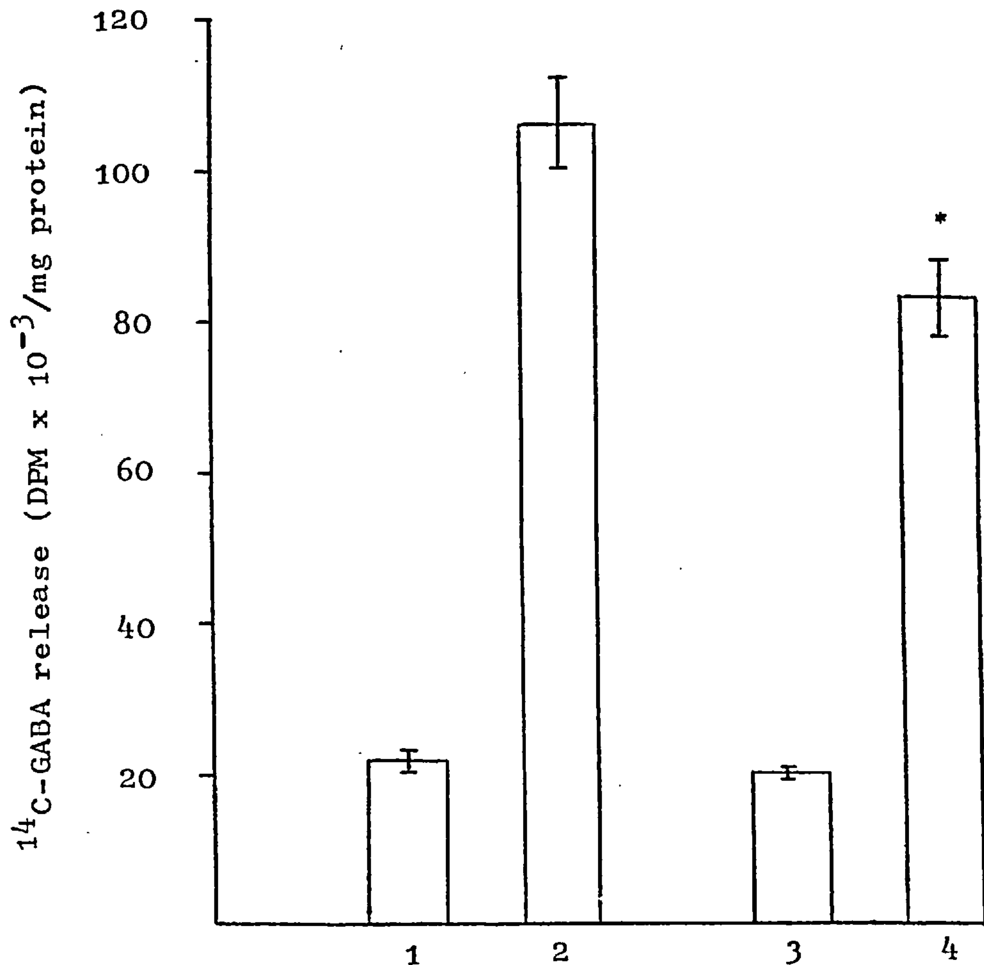
<u>The effect of kainic acid on amino acid release from (1a, 1b) rat cerebrocortical and (2) sheep striatal synaptosomes</u>

1a) Rat cerebrocortical synaptosomes were incubated in Krebs-phosphate medium with or without kainic acid (final concentrations, 0.1mM, 0.5mM and 1mM).

Initially, stimulation was achieved using 75µM veratrine, however, this was subsequently thought to have masked any effects occurring. Therefore, in view of the results obtained in Chapter 3 concerning the relationship

FIGURE 5.9

EFFECT OF PICROTOXIN ON ^{14}C-GABA RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES



Prelabelled nerve-endings were treated under similar conditions to those described in the legend for Figure 5.8. However, veratrine (final concentration, 75µM) was used for stimulation as opposed to K^+.

Histobars represent the mean ± S.E.M. for 4 determinations. Change in stimulated release due to presence of picrotoxin (final concentration, 0.1mM) significant with $p < 0.05$*.

1. - control
2. - veratrine stimulated
3. - control
4. - veratrine stimulated

(3 and 4: picrotoxin (final concentration, 0.1mM) in medium)

TABLE 5.e

THE EFFECT OF 0.1mM PICROTOXIN ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

Amino acid in incubation medium
(nmol/100mg protein)

	No addition		0.1mM picrotoxin in medium	
	Control (4)	Stimulated (4)	Control (4)	Stimulated (4)
Aspartate	34±6	1063±96	62±26	869±26
Threonine	116±7	147±11	136±5	114±6
Serine	290±23	392±37	356±8	261±46
Glutamate	142±12	1908±152	158±24	1607±56
Glycine	285±17	428±65	306±46	394±13
Alanine	467±19	585±82	456±75	538±26
GABA	38±3	649±43	42±4	543±14

Synaptosomes were incubated in Krebs-phosphate medium for 45 mins. Veratrine (final concentration, 75μM) was used for stimulation. Values are mean ± S.E.M. for the number of samples in brackets.

FIGURE 5.10

THE EFFECT OF PICROTOXIN (1mM) ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

Cerebrocortical nerve-endings were incubated at 37°C in Krebs-phosphate medium with or without picrotoxin (final concentration, 1mM), for 45 mins. Veratrine (final concentration, 10μM) was used to achieve stimulation. Values are the mean ± S.E.M for 4-10 determinations. Change in stimulated release due to presence of picrotoxin significant with $p < 0.02$* or $p < 0.001$**.

1. - control
2. - veratrine stimulated
3. - control } picrotoxin in medium
4. - veratrine stimulated } picrotoxin in medium

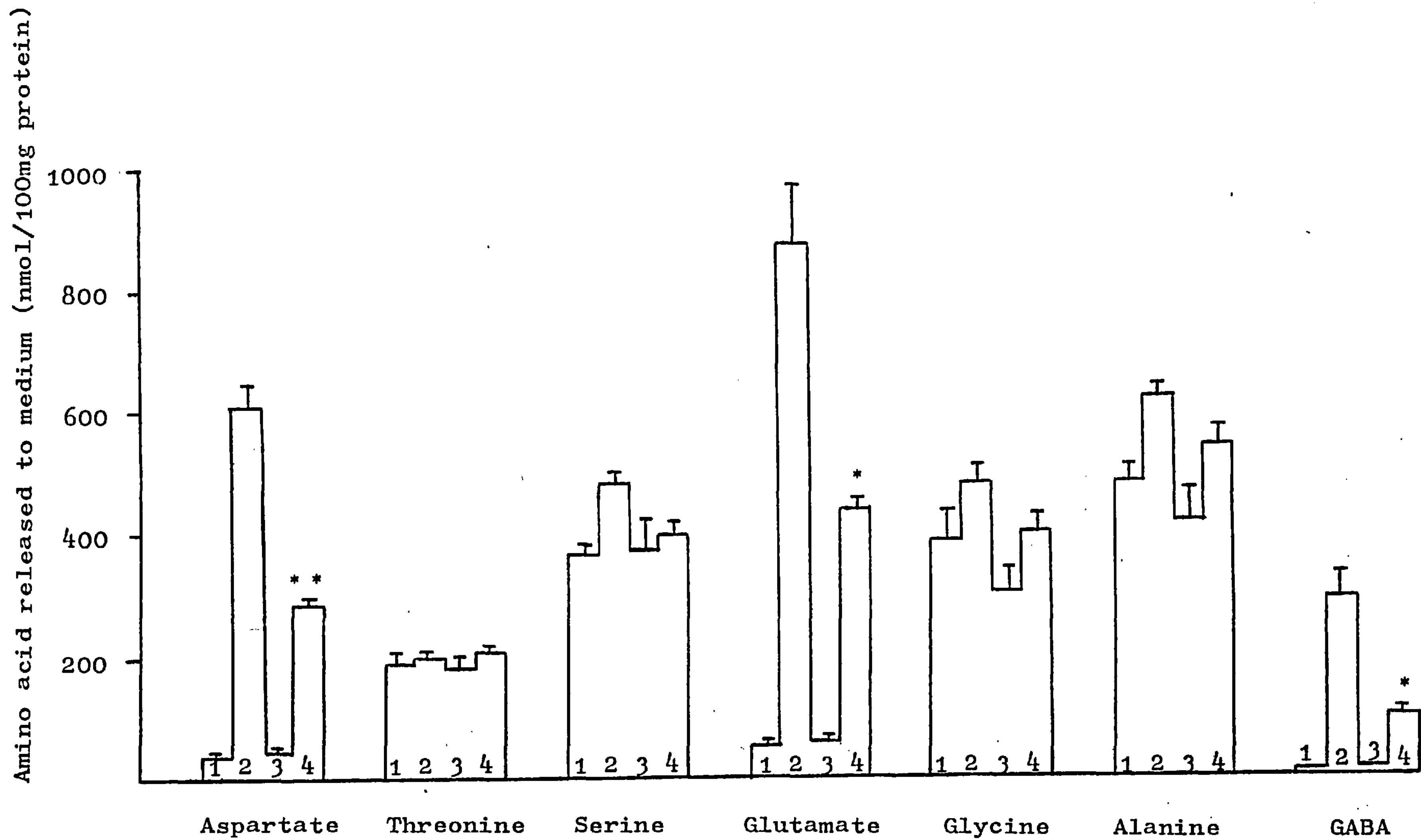


FIGURE 5.10

TABLE 5.f

THE EFFECT OF PICROTOXIN (1mM) ON THE AMINO ACID CONTENT OF RAT CEREBROCORTICAL SYNAPTOSOMES

Amino acid content (nmol/100mg Protein)

	No addition		1mM picrotoxin in medium	
	Control (6)	Stimulated (6)	Control (4)	Stimulated (4)
Aspartate	3203±195	1490±127	2801±426	1591±193
Threonine	265±28	154±26	136±35	101±22
Serine	632±73	468±11	625±83	400±49
Glutamate	3530±296	3040±130	3333±378	2872±259
Glycine	655±65	427±26	574±88	425±65
Alanine	732±66	473±35	590±84	395±40
GABA	1070±74	1067±57	1079±144	1017±91

Synaptosomes were incubated in Krebs-phosphate medium with or without picrotoxin. Stimulation was achieved using veratrine (final concentration, 10μM). Values are means ± S.E.M. for the number of samples in brackets.

TABLE 5.g

THE EFFECT OF PICROTOXIN (1mM) ON RESPIRATION IN RAT CEREBROCORTICAL SYNAPTOSOMES

Respiratory rate (μmol O_2/100mg protein/h)

	No addition	1mM picrotoxin in medium
CONTROL	46.3±1.7 (6)	55.3±7.3 (4)
STIMULATED	117.9±4.9 (6)	96.9±5.4 (4)

Synaptosomes were incubated in Krebs-phosphate medium. Veratrine (final concentration, 10μM) was used for stimulation. Values are mean ± S.E.M. for the number of determinations shown in brackets.

between amino acid release and veratrine concentration (see Figure 3.5), a final veratrine concentration of 10μM was chosen for these studies, in order to provide a small but easily detectable release signal whose size was readily modulated by the agents under examination. This concentration was used in all subsequent experiments involving the glutamate analogues.

At a concentration of 0.1mM, kainic acid had no effect on the release of any of the amino acids assayed (Table 5.h). However, 0.5mM and 1mM kainate was found to raise the control levels of aspartate and glutamate in the medium by 66 - 149% and 50 - 114%, respectively. A marginal increase in the stimulated release of aspartate was also observed. Kainate did not appear to affect the release of any of the other amino acids determined (Figure 5.11)

1b) The question of how kainic acid functions as a modulator of amino acid release was also examined using another method.

As previously mentioned, there is evidence to suggest that kainate acts as a depolarizing agent, depolarization being a process that is usually accompanied by an increase in O_2 consumption. Therefore, several experiments were conducted in which cerebrocortical synaptosomes were incubated in ordinary Krebs-phosphate medium for 35 mins whereupon a quantity of kainic acid was added (to give final concentrations of 0.1mM and 1mM) instead of veratrine and incubation continued for a further 10 mins.

Under these conditions 0.1mM kainate was found to have no effect on synaptosomal amino acid release.

However, at a concentration of 1mM the release of aspartate

TABLE 5.h

AMINO ACID RELEASE FROM CEREBROCORTICAL SYNAPTOSOMES INCUBATED IN MEDIUM CONTAINING KAINIC ACID (100µM)

Amino acid in incubation medium (nmol/100mg protein)

	Control (4)	Stimulated (4)	Control (4)	Stimulated (4)
Aspartate	34±4	571±92	47±8	550±31
Threonine	157±31	206±48	208±	261±49
Serine	351±18	430±43	370±20	561±60
Glutamate	97±13	1167±100	126±21	1141±27
Glycine	310±14	543±46	351±29	583±28
Alanine	528±43	724±44	547±25	754±15
GABA	28±14	277±33	24±12	346±41

Synaptosomes were incubated for 45 mins (controls) in Krebs-phosphate medium or for 35 mins followed by 10 mins veratrine stimulation (final concentration, 10µM). Values are means ± S.E.M. for the number of determinations in brackets.

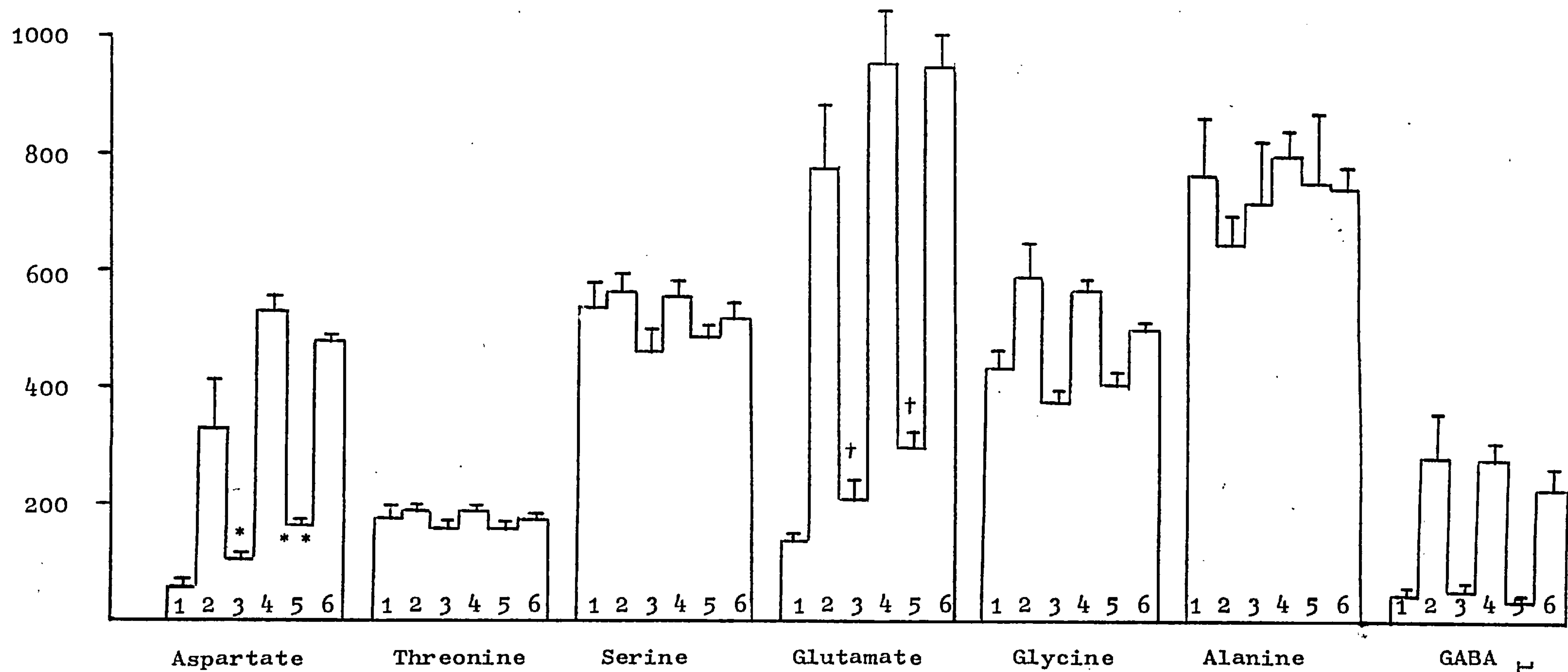
FIGURE 5.11

THE EFFECT OF KAINIC ACID ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES (1a - LONG INCUBATION)

Cerebrocortical nerve-endings were incubated at 37°C in Krebs-phosphate medium for 45 mins. Veratrine (final concentration, 10µM) was used to produce stimulation. Values are the mean ± S.E.M. for 6 determinations. Changes in release due to presence of kainate, $p < 0.05$*, $p < 0.01$**, $p < 0.001$†.

1.	-	control	
2.	-	veratrine stimulated	
3.	-	control	0.5mM kainate in medium
4.	-	veratrine stimulated	
5.	-	control	1mM kainate in medium
6.	-	veratrine stimulated	

FIGURE 5.11



and glutamate was increased by 236% and 191%, respectively (Figure 5.12).

2) In sheep striatal synaptosomes kainic acid (final concentrations 0.5mM and 1mM) completely blocked the response to veratrine (final concentration 10μM) stimulation with respect to aspartate and glutamate. The spontaneous efflux of glycine alanine and GABA in the presence of 1mM kainate and glycine in the presence of 0.5mM kainate, was also increased (Figure 5.13)

The effect of kainic acid on the amino acid content of (1) rat cerbrocortical and (2) sheep striatal synaptosomes

Kainic acid (final concentrations, 0.5mM and 1mM) was found to have no significant effect on the amino acid content of rat cerebrocortical or sheep striatal synaptosomes (Tables 5.i and 5.j). When this data was combined with the release data no drastic changes were observed in the total synaptosomal amino acid levels.

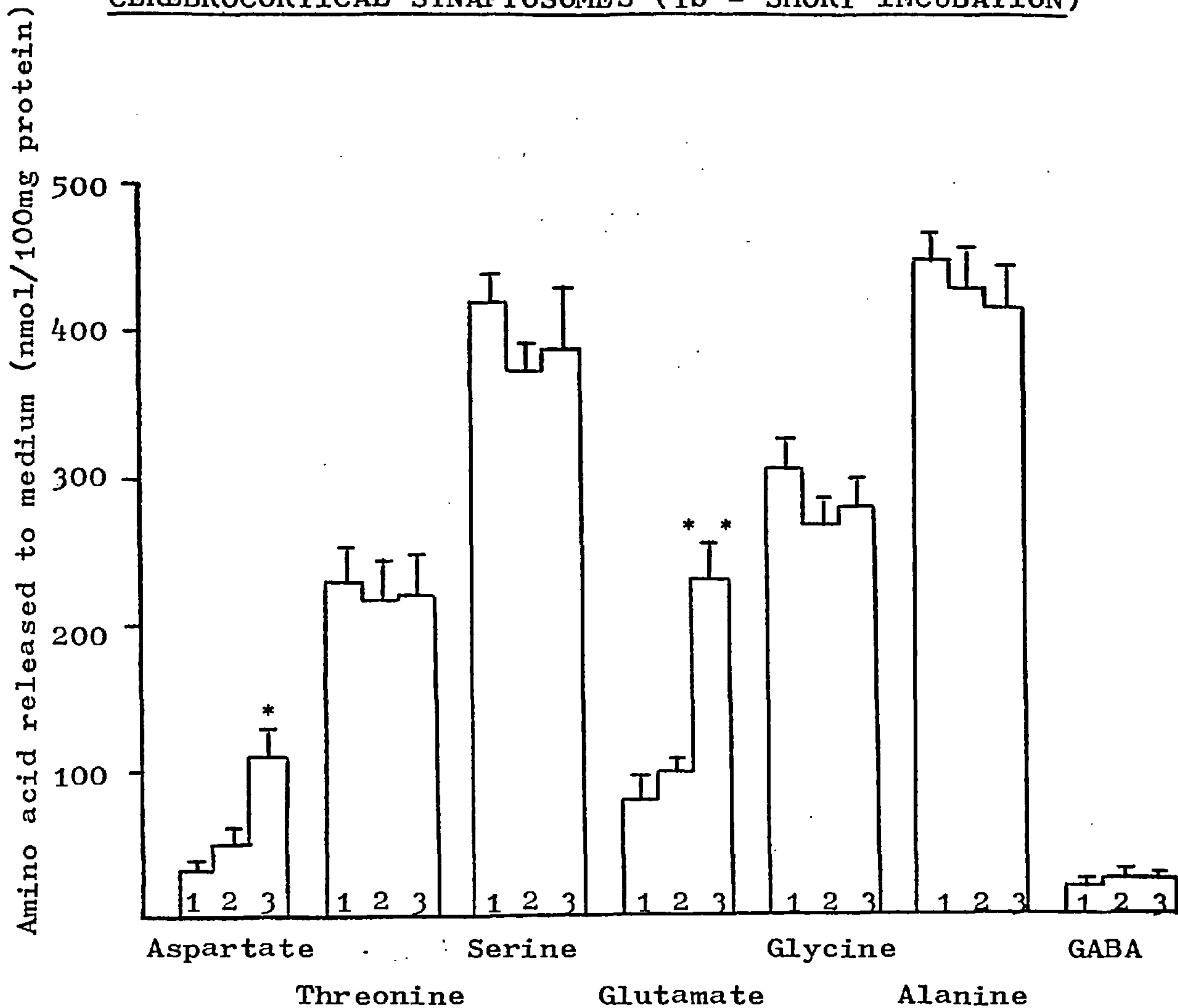
The effect of kainic acid on respiration and K^+ levels in rat cerebrocortical and sheep striatal synaptosomes

Kainic acid (final concentrations, 0.5mM and 1mM) was not found to significantly influence respiratory rates or K^+ levels in sheep striatal synaptosomes or rat cerebrocortical nerve-endings incubated as described in section 1a (Tables 5.k, 5.l).

However, when cerebrocortical synaptosomes were treated as described in section 1b, 1mM kainate produced a 30 - 40% increase in O_2 consumption (Table 5.m). This result would tend to agree with the respiratory data obtained by Cox et

FIGURE 5.12

THE EFFECT OF KAINIC ACID ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES (1b - SHORT INCUBATION)



Synaptosomes were incubated at 37°C in Krebs-phosphate medium for 35 mins, whereupon a quantity of kainic acid was added (to give final concentrations of 0.1mM and 1mM) and incubation continued for a further 10 mins. Values are the mean ± S.E.M. for 6-7 determinations. Change in release due to presence of kainate significant with $p < 0.01$* and $p < 0.001$**.

1. - control

2. - kainate added to give a final concentration of 0.1mM

3. - kainate added to give a final concentration of 1mM

FIGURE 5.13

THE EFFECT OF KAINIC ACID ON AMINO ACID RELEASE FROM SHEEP STRIATAL SYNAPTOSOMES

Striatal synaptosomes were incubated at 37°C in Krebs-phosphate medium. Stimulation was achieved using veratrine (final concentration, 10μM). Values represent the mean ± S.E.M. for 4 determinations. Change in release due to kainate action, $p<0.05$*, $p<0.02$** and $p<0.01$†.

1. - control
2. - veratrine stimulated
3. - control } 0.5mM kainate in medium
4. - veratrine stimulated } 0.5mM kainate in medium
5. - control } 1mM kainate in medium
6. - veratrine stimulated } 1mM kainate in medium

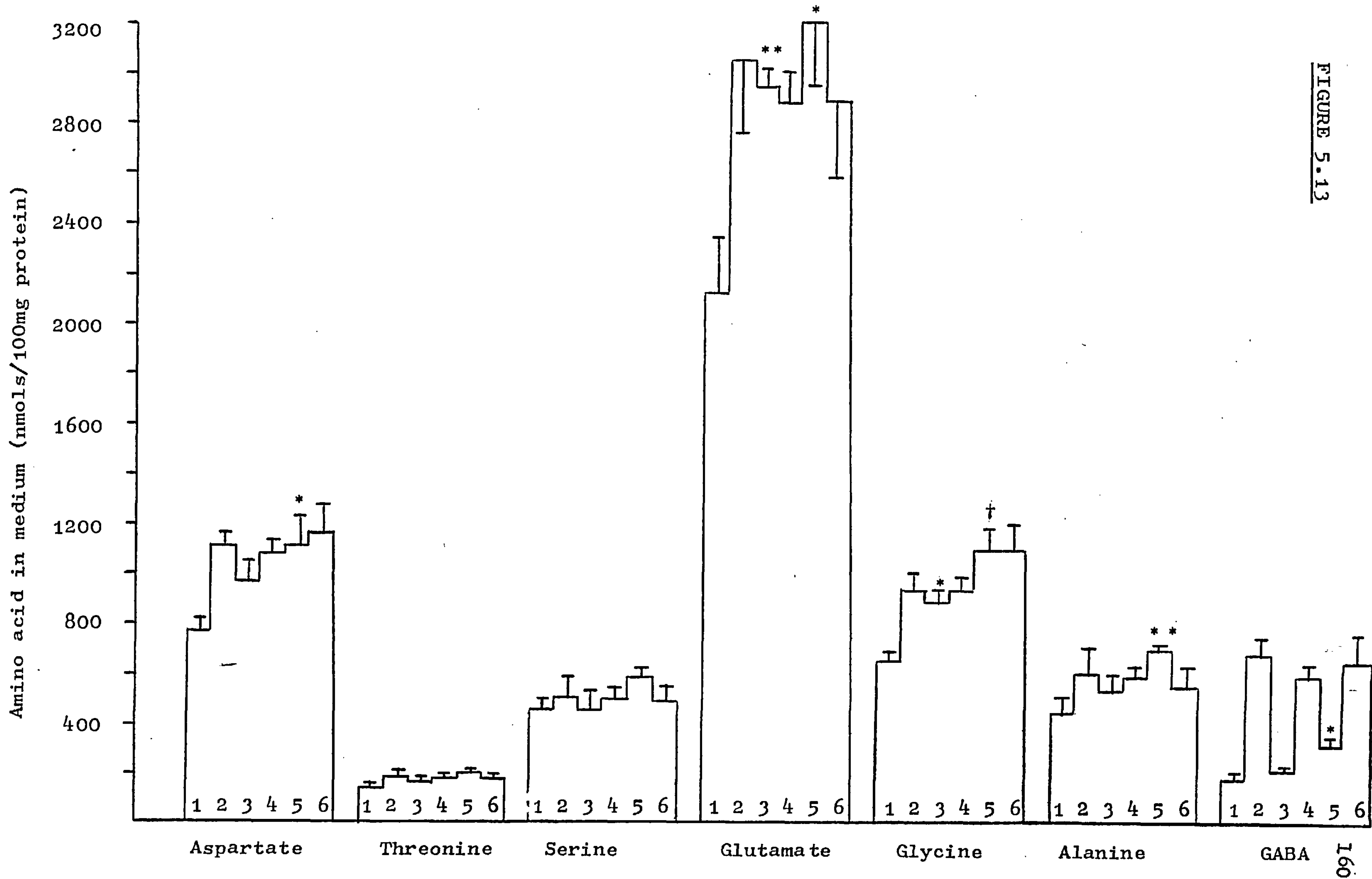


FIGURE 5.13

TABLE 5.i

THE EFFECT OF KAINIC ACID ON THE AMINO ACID CONTENT OF RAT CEREBROCORTICAL SYNAPTOSOMES

Amino acid content (nmols/100mg protein)

	No addition		0.5mM kainic acid in medium		1mM kainic acid in medium	
	Control (6)	Stimulated (6)	Control (6)	Stimulated (6)	Control (6)	Stimulated (6)
Aspartate	2899±237	1532±125	2492±191	1779±223	2479±177	1609±64
Threonine	239±30	172±19	171±12	143±17	166±9	148±9
Serine	578±80	416±26	430±30	415±31	435±28	431±43
Glutamate	3195±357	2945±128	3038±231	3146±170	2944±193	2771±139
Gycine	665±62	428±25	528±39	442±44	555±28	405±22
Alanine	724±68	483±33	622±47	437±11	629±32	451±18
GABA	1044±78	1068±56	903±87	932±74	995±67	941±53

Cerebrocortical synaptosomes were incubated in Krebs-phosphate medium with or without kainic acid (final concentrations, 0.5mM and 1mM). Stimulation was achieved using veratrine (final concentration, 10µM). Values are mean ± S.E.M. for the number of determinations in brackets

TABLE 5.j

THE EFFECT OF KAINIC ACID ON THE AMINO ACID CONTENT OF SHEEP STRIATAL SYNAPTOSOMES

	Amino acid content (nmols/100mg protein)					
	No addition		0.5mM kainic acid in medium		1mM kainic acid in medium	
	Control (4)	Stimulated (4)	Control (4)	Stimulated (4)	Control (4)	Stimulated (4)
Aspartate	1620±317	1849±433	2046±496	1474±153	1824±567	1564±238
Threonine	268±47	299±45	401±11	200±39	273±31	281±38
Serine	864±145	1012±280	1241±59	645±40	1012±196	869±97
Glutamate	5729±776	4519±379	5440±279	3998±128	5390±342	4620±231
Glycine	2193±610	1599±119	2149±275	1396±183	2236±538	1830±316
Alanine	794±141	670±51	916±68	576±42	942±107	738±52
GABA	972±113	942±68	1473±236	1013±53	1052±35	999±50

Sheep striatal synaptosomes incubated in Krebs-phosphate medium. Veratrine (final concentration, 10μM) was used for stimulation. Values are mean ± S.E.M.

TABLE 5.k

THE EFFECT OF KAINIC ACID ON RESPIRATION IN RAT CEREBROCORTICAL (1a) AND SHEEP STRIATAL SYNAPTOSOMES

Respiratory rate (μmol O_2/100mg protein/h)

Rat cerebrocortical synaptosomes

	No addition	0.1mM kainic acid in medium	0.5mM kainic acid in medium	1mM kainic acid in medium
CONTROL	59.8±5.4 (6)	70.6±14 (3)	58.7±4.1 (6)	59.3±3.8 (6)
STIMULATED	105±6.3 (9)	98.6±11 (4)	117.6±8.7 (4)	98.3±9.2 (6)

Sheep striatal synaptosomes

	No addition	0.5mM kainic acid in medium	1mM kainic acid in medium
CONTROL	33.0±1.7 (6)	41.8±4.9 (6)	32.5±3 (5)
STIMULATED	60.9±3.7 (4)	62.9±6.9 (4)	59.7±6.8 (3)

Synaptosomes were incubated at 37°C for 45 mins in Krebs-phosphate medium. Veratrine (final concentration, 10μM) was used for stimulation. Values are mean ± S.E.M. for the number of samples in brackets.

TABLE 5.1

THE EFFECT OF KAINIC ACID ON THE K^+ CONTENT OF RAT CEREBROCORTICAL (1a) AND SHEEP STRIATAL SYNAPTOSOMES

Condition	Potassium content (µequiv/100mg protein)	
	Rat cerebrocortical synaptosomes	Sheep striatal synaptosomes
Control	20.4±1.1 (6)	20.3±1.5 (4)
Stimulated	12.1±1.0 (6)	15.4±1.1 (4)
Control with 0.5mM kainic acid	17.5±0.8 (6)	24.2±0.9 (4)
Stimulated with 0.5mM kainic acid	12.2±0.7 (6)	14.4±0.8 (4)
Control with 1mM kainic acid	17.4±1.0 (6)	22.3±0.9 (4)
Stimulated with 1mM kainic acid	12.3±0.7 (6)	15.9±0.9 (4)

Synaptosome suspensions were incubated in the presence or absence of kainic acid in Krebs-phosphate medium at 37°C for 45 mins. Veratrine (final concentration 10µM) stimulation was applied for 10mins. Values are means ± S.E.M. for the number of determinations shown in brackets.

TABLE 5.m

EFFECT OF KAINIC ACID ON RESPIRATION IN RAT CEREBROCORTICAL SYNAPTOSOMES (1.b)

Condition	Respiratory rate (μmols O_2/100mg protein/h)	n
Control	34.1±1.3	(4)
0.1mM kainic acid	*48.1±6.5	(4)
1mM kainic acid	**44±1.2	(4)

Cerebrocortical synaptosomes were incubated for 35 mins in Krebs-phosphate medium whereupon kainic acid was added to give the final concentrations indicated above. The incubation was then continued for a further 10 mins. Values are means ± S.E.M. for the number of samples shown.

When compared with control * not significant and ** significant with $p < 0.01$

al. (1977), using cerebrocortical slices.

The effect of kainic acid on ^{14}C-glutamate uptake into rat cerebrocortical and sheep striatal synaptosomes

Introduction

Prior to studying the effects of kainic acid and the other glutamate analogues on ^{14}C-glutamate uptake into rat cerebrocortical synaptosomes, several control experiments were conducted. These experiments involved testing the effects of ouabain (Gonda and Quastel, 1962) and incubation at $0^{o}C$ (Roberts and Watkins, 1975) on the synaptosomal uptake processes occurring in our *in vitro* system. Ouabain (final concentration, 100µM) was found to produce a 56% decrease in the uptake of ^{14}C-glutamate (final concentration, 30µM), whilst incubating synaptosomes at $0^{o}C$ resulted in an 83% decrease (Table 5.n.).

Results

Kainate (final concentration, 0.5mM) was found to inhibit ^{14}C-glutamate (final concentration, 30µM) uptake into rat cerebrocortical synaptosomes by approx. 20% (for details of experimental procedure see Table 5.n). A similar result was obtained when synaptosomes were incubated in medium containing 1.0mM kainic acid and ^{14}C-glutamate at a concentration of 1µM (Table 5.n).

This result contrasts with that of Lakshmanan and Padmanaban (1974) who have reported that kainic acid (final concentration, 1.0mM) reduced the uptake of ^{14}C-glutamate (final concentration, 1µM) into whole young rat brain synpatosomal preparations, by 60 - 70% (Table 5.n).

When crude (P_2) fractions prepared from sheep corpus

TABLE 5.n

EFFECT OF SOME AGENTS ON AMINO ACID UPTAKE IN NERVE-ENDING PREPARATIONS

Preparation	Experimental conditions	Uptake as % of control
Cerebrocortical synaptosomes	37°C, 30µM glutamate, 0.1mM ouabain	44±4 (4)
Cerebrocortical synaptosomes	0°C, 30µM glutamate	17±1 (4)
Cerebrocortical synaptosomes	37°C, 30µM glutamate, 0.5mM kainic acid	80±5 (4)
Cerebrocortical synaptosomes	37°C, 1µM glutamate, 1.0mM kainic acid	80±4 (4)
Cerebrocortical P_2 fraction	37°C, 1µM glutamate, 1.0mM kainic acid	94±10 (6)
Striatal P_2 fraction	37°C, 1µM glutamate, 1.0mM kainic acid	74±4 (6)
Cerebrocortical synaptosomes	37°C, 30µM glutamate, 1mM GDEE	95±9 (4)
Cerebrocortical synaptosomes	37°C, 30µM glutamate, 1mM HA966	87±6 (4)
Cerebrocortical synaptosomes	37°C, 30µM glutamate, 0.22µM β-bungarotoxin	47±4 (7)
Cerebrocortical synaptosomes	37°C, 20µM GABA 0.22µM β-bungarotoxin	65±8 (4)

Nerve-ending preparations were incubated under the conditions shown above for 5 mins, whereupon, labelled amino acid was added and the incubation continued for a further 5 mins. The suspensions were then sedimented and the amount of radioactivity occurring in the TCA extracts determined.

Values are mean ± S.E.M. for the number of determinations in brackets.

striatum were incubated in the presence of kainic acid (final concentration, 1mM), the uptake of ^{14}C-glutamate (final concentration, 1µM) was reduced by 26% (Table 5.n). This result does not agree with the observations of McGeer et al. (1978) who showed that kainic acid inhibited the accumulation of radioactive glutamate by the P_2 fraction of rat striatal homogenates, by approximately 75%.

Kainic acid was not found to influence ^{14}C-glutamate uptake into crude (P_2) extracts prepared from cerebrocortical homogenates (Table 5.n).

The effect of HA966 and GDEE on amino acid release and uptake in rat cerebrocortical synaptosomes

When synaptosomes were incubated in Krebs-phosphate medium containing the glutamate antagonist HA966 (final concentrations, 0.1mM, 0.5mM,1mM and 5mM) no change occurred in the pattern of amino acids released to the medium (Table 5.0). Similarly HA966 (final concentrations, 1mM) had no effect on ^{14}C-glutamate uptake (Table 5.n).

Preliminary experiments with GDEE gave the impression that it increased the spontaneous and stimulated release of aspartate and glutamate from synaptosomes. However, the data obtained was later shown to have been due to the chemical breakdown of GDEE to aspartate and glutamate, as a result of the TCA extraction method employed when preparing amino acid samples. As for HA966, GDEE was found to exert no influence over ^{14}C-glutamate uptake (Table 5.n). This result contrasts with that reported by McLennan (1975) who claims to have shown that GDEE functions as an uptake blocker.

TABLE 5.o

THE EFFECT OF HA966 ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

Amino acid in incubation medium (nmol/100mg protein)

	No addition		0.1mM HA966 in medium		0.5mM HA966 in medium		1mM HA966 in medium		5mM HA966 in medium	
	Control (10)	Stimulated (10)	Control (4)	Stimulated (4)	Control (4)	Stimulated (4)	Control (4)	Stimulated (4)	Control (4)	Stimulated (4)
Aspartate	43±8	400±16	28±3	501±53	50±6	507±66	80±22	394±13	56±9	412±46
Threonine	241±43	297±43	172±26	237±23	361±49	388±48	386±50	401±52	237±11	227±13
Serine	466±47	541±40	447±40	545±31	616±88	660±63	584±46	591±111	396±37	468±38
Glutamate	107±18	825±76	160±27	932±38	121±60	1038±166	85±29	857±162	72±15	690±97
Glycine	313±42	440±39	259±86	358±73	373±39	498±40	321±32	489±74	314±30	391±38
Alanine	600±46	798±49	532±32	817±85	754±70	947±91	680±30	876±74	473±22	687±55
GABA	27±8	329±38	21±2	316±31	26±6	499±83	18±2	395±34	17±2	325±32

Synaptosomes were incubated at 37°C in Krebs-phosphate medium Veratrine (final concentration, 10μM) was used for stimulation. Values are mean ± S.E.M. for the number of samples in brackets.

Neither agent was found to affect synaptosomal respiration (Tables 5p and 5q).

The effect of β-bungarotoxin on the medium and tissue amino acid levels of rat cerebrocortical synaptosomes

Kelly et al. (1975) claim that β-bungarotoxin stimulates neurotransmitter release. The results obtained (Figure 5.14) using our in vitro system would tend to confirm this. Incubations were for 45 mins and stimulation was achieved using veratrine (final concentration 10μM).

In the presence of β-bungarotoxin (final concentrations, 0.22μM and 0.43μM), there was a marked increase in the levels of aspartate, glutamate and GABA released to the medium, whilst glycine and alanine showed only slight increases. β -bungarotoxin also appeared to greatly reduce, if not completely block the response to veratrine stimulation, as regards the release of aspartate, glutamate, glycine, alanine and GABA.

The amino acid levels detected in nerve-endings treated with the toxin showed a substantial reduction when compared with control synaptosomes (Table 5.r). As far as threonine, serine, glutamate, glycine, alanine and GABA were concerned, this reduction merely mirrored the situation encountered in the medium, thus, the total amino acid levels (i.e. tissue and medium levels combined) remained unaffected by the toxin. However, when the total levels of aspartate were considered, they were found to be reduced by approximately 47%.

TABLE 5.p

THE EFFECT OF HA966 ON RESPIRATION IN RAT CEREBROCORTICAL SYNAPTOSOMES

Respiratory rate (μmol O_2/100mg protein/h)

	No addition	0.1mM HA966 in medium	0.5mM HA966 in medium	1mM HA966 in medium	5mM HA966 in medium
CONTROL	52.9±3.4 (11)	49.8±2.9 (4)	50.3±4.6 (4)	45.2±5.7 (3)	53.7±4.0 (4)
STIMULATED	94.2±5.9 (10)	83.6±2.5 (4)	108.7±7.7 (4)	85.0±7.9 (4)	69.1±9.7 (3)

Synaptosomes were incubated at 37°C for 45 mins in Krebs-phosphate medium. Veratrine (final concentration, 10μM) was used for stimulation. Values are mean ± S.E.M. for the number of determinations in brackets.

TABLE 5.q

THE EFFECT OF GDEE ON RESPIRATION IN RAT CEREBROCORTICAL SYNAPTOSOMES

	Respiratory rate (μmol O_2/100mg protein/h)			
	No addition	0.1mM GDEE in medium	0.5mM GDEE in medium	1mM GDEE in medium
CONTROL	58.2±6.7 (6)	52.1±5.2 (3)	67.3±3.3 (6)	70.6±1.6 (5)
STIMULATED	105.3±8.0 (6)	101.3±14 (3)	103.6±2.7 (5)	107.1±3.0 (6)

Cerebrocortical synaptosomes were incubated in Krebs-phosphate medium. Veratrine (final concentration 10μM) was used for stimulation. Values are mean ± S.E.M. for the number of determinations in brackets.

FIGURE 5.14

THE EFFECT OF β-BUNGAROTOXIN ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

Cerebrocrotical synaptosomes were incubated at 37°C in Krebs-phosphate medium. Veratrine (final concentration, 10μM) was used for stimulation. Values represent the mean ± S.E.M. for 4 determinations. Change in release due to presence of toxin $p < 0.05$*, $p < 0.01$** or $p < 0.001$†.

1. - control
2. - stimulated
3. - control } 0.22μM toxin in medium
4. - stimulated }
5. - control } 0.43μM toxin in medium
6. - stimulated }

NB β-bungarotoxin (free of phospholipase activity (in the absence of detergent, e.g. deoxycholate) was kindly supplied by Dr. E. Thompson (Institute of Neurology, Queen Square, London).

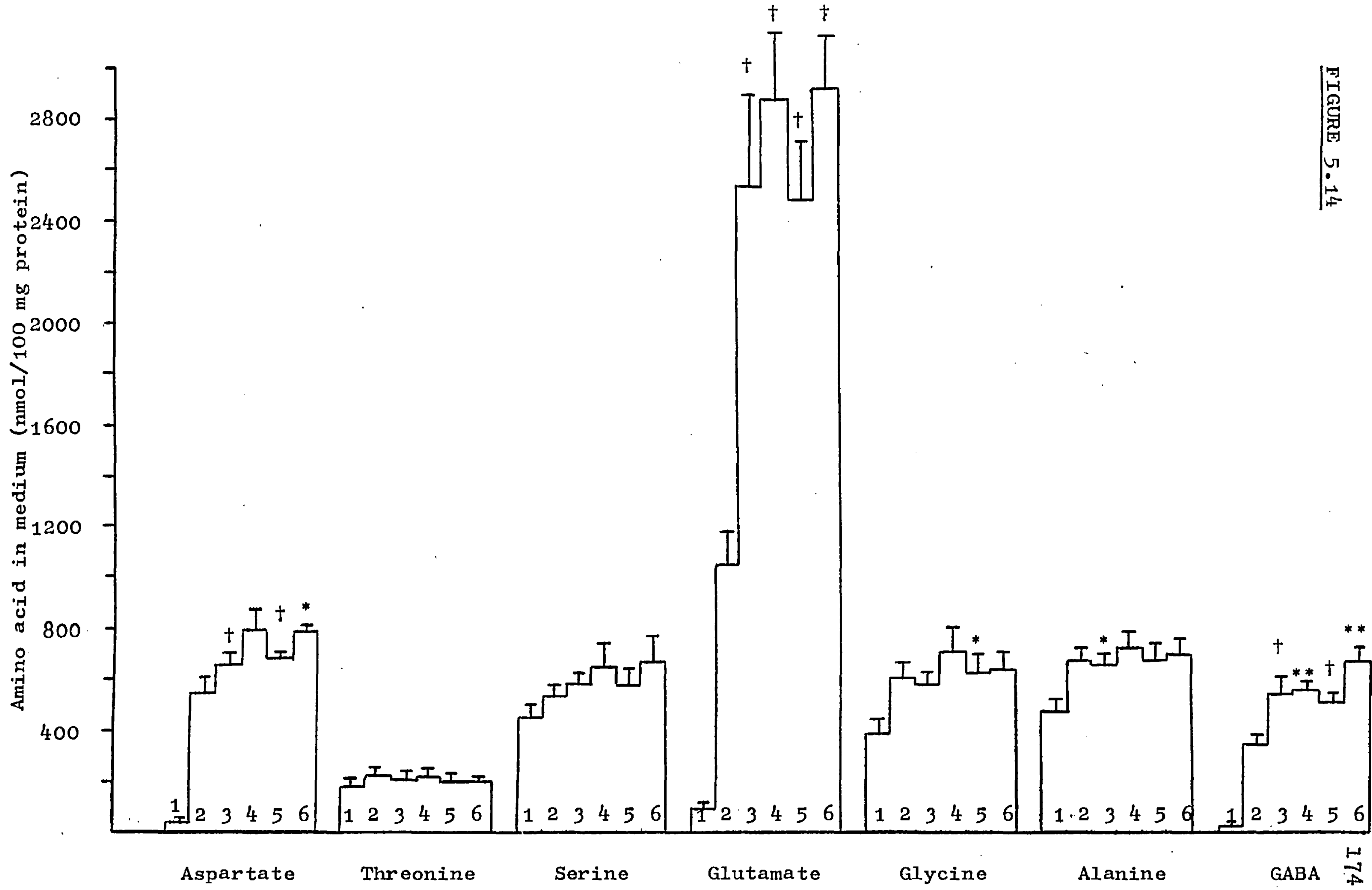


FIGURE 5.14

TABLE 5.r

THE EFFECT OF β-BUNGAROTOXIN ON THE AMINO ACID CONTENT OF RAT CEREBROCORTICAL SYNAPTOSOMES

	Amino acid content (nmol/100mg protein)					
	No addition		0.22µM β-bungarotoxin in medium		0.43µM β-bungarotoxin in medium	
	Control (10)	Stimulation (10)	Control (4)	Stimulation (4)	Control (4)	Stimulation (4)
Aspartate	2668±233	1518±83	†756±56	†768±74	†799±57	†821±26
Threonine	220±23	149±16	**96±26	*79±6	**96±24	*83±15
Serine	520±60	378±24	383±95	318±46	400±82	370±42
Glutamate	3206±399	2958±244	1956±414	*1893±184	1988±354	2446±530
Glycine	663±58	433±17	*385±91	*346±16	429±82	370±46
Alanine	706±52	487±20	†296±22	†290±14	†328±18	†301±11
GABA	1098±75	1038±53	991±123	1000±68	978±77	1175±164

Synaptosomes were incubated at 37°C in Krebs-phosphate medium with or without β-bungarotoxin. Stimulation was achieved using veratrine (final concentration, 10µM). Values are mean ± S.E.M. for the number of determinations in brackets. Change in amino acid content due to presence of toxin significant with $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (†) when comparing control values and stimulated values.

The effect of β-bungarotoxin on the K^+ content of rat cerebrocortical synaptosomes.

Table 5.s shows the effect incubating in medium containing β-bungarotoxin (final concentrations, 0.22µM and 0.43µM) has on the K^+ content of rat cerebrocortical synaptosomes.

The K^+ levels in nerve-endings treated with the toxin showed a substantial decrease when compared with those levels in control synaptosomes. The response to stimulation also appeared to be blocked, as the change in the synaptosomal K^+ content usually associated with veratrine action, was not observed.

From the above data we may conclude that β-bungarotoxin may function as a depolarizing agent. The negative response to veratrine stimulation suggests that the tissue may have been stimulated to exhaustion by the toxin, any further stimulation being made impossible.

The effect of β-bungarotoxin on synaptsosomal respiration

β -bungarotoxin had no effect on the normal basal synaptosomal respiratory rates, however, it was found to completely block the increase in synaptosomal O_2 consumption usually associated with veratrine stimulation (Table 5.t).

It was suggested in the previous section that β-bungarotoxin may function as a depolarizing agent, however, the respiratory data would appear to disagree with this hypothesis. If indeed the toxin is a depolarizing agent one might have expected much higher respiratory rates than those observed for control incubations.

TABLE 5.s

THE EFFECT OF β-BUNGAROTOXIN ON THE K^+ CONTENT OF RAT CEREBROCORTICAL SYNAPTOSOMES

Condition	K^+ content (μequivs/100mg protein)	n
Control	18.8±1.2	10
Stimulated	12.2±0.9	10
Control with 0.22μM β-bungarotoxin	*8.6±1.1	4
Stimulated with 0.22μM β-bungarotoxin	**8.8±1.5	4
Control with 0.43μM β-bungarotoxin	*8.9±1.3	4
Stimulated with 0.43μM β-bungarotoxin	**8.8±1.1	4

Synaptosomes were incubated in Krebs-phosphate medium with or without β-bungarotoxin. Incubations were for 45 mins, stimulation being achieved using veratrine (final concentration, 10μM). Values are mean ± S.E.M. for the number of determinations shown above. Change in K^+ content due to presence of toxin significant with $p < 0.001$ (*).

NB ** - not significant

TABLE 5.t

THE EFFECT OF β-BUNGAROTOXIN ON RESPIRATION IN RAT CEREBROCORTICAL SYNAPTOSOMES

Respiratory rate(μmol O_2/100mg protein/h)

	No addition	0.22μM β-bungarotoxin in medium	0.43μM β-bungarotoxin in medium
CONTROL	49.7±3.2 (8)	59.0±10.2 (4)	53.3±5.7 (4)
STIMULATED	110.8±7.8 (8)	*42.5±3.3 (4)	*51.8±6.7 (4)

Synaptosomes were incubated in Krebs-phosphate medium with or without the toxin. Veratrine (final concentration, 10μM) was used for stimulation. Values are mean ± S.E.M. for the number of samples in brackets. Change in respiratory rate due to presence of toxin significant with $p < 0.001$ (*) when comparing stimulated samples.

The effect of β-bungarotoxin on amino acid uptake into rat cerebrocortical synaptosomes

Introduction

It has been reported that β-bungarotoxin inhibits the uptake of noradrenaline, GABA and 5HT into nerve terminals (Wernicke et al., 1974). Therefore, the effect of the toxin on (1) ^{14}C-GABA and (2) ^{14}C-glutamate uptake, in our in vitro system, was tested.

Results

1) Effect on ^{14}C-GABA uptake

β -bungarotoxin (0.22 μM) gave an apparent inhibition for ^{14}C-GABA uptake of 35% (Table 5.n) as compared with 67% reported by Dolly et al (1978) using 1 μM β-bungarotoxin.

2) Effect on ^{14}C-glutamate uptake

β -bungarotoxin appeared to reduce the synaptosomal uptake of ^{14}C-glutamate (Table 5.n). However, as mentioned previously one must exercise extreme caution when interpreting amino acid uptake data.

There is some controversy as to whether β -bungarotoxin functions as an uptake blocker or as a releasing agent. The data obtained when β-bungarotoxin's action on synaptosomal amino acid release was examined would tend to favour the latter. Therefore, the apparent inhibition of uptake observed for both GABA and glutamate may represent the net result of uptake and stimulated release. The properties of amino acid transmitter release differ from those of uptake, e.g. (a) release is Ca^{2+} dependent whereas uptake is not (de Belleroche and Bradford, 1972; Bradford, H.F. and Richards, C.D., 1976; Takagaki et al., 1959; Tsukada et al., 1963), (b) tetrodotoxin blocks veratrine-induced and

electrically induced release but does not affect uptake (Wedege et al., 1977; Balcar and Johnston, 1972; Benjamin and Quastel, 1976). Therefore, an experiment was designed whose purpose was to distinguish between these two processes.

Synaptosomes were incubated at 37°C in either (1) Ca^{2+}-free medium containing EGTA (final concentration 0.5mM) or (2) medium containing tetrodotoxin (final concentration, 1µM), and the effects on ^{14}C-glutamate uptake assessed.

The result obtained (Figure 5.15) would suggest that β-bungarotoxin's action is to some extent Ca^{2+} dependent and partially affected by tetrodotoxin. As previously mentioned under normal circumstances, β-bungarotoxin appeared to reduce the synaptosomal uptake of ^{14}C-glutamate by 53%. However, in the presence of Ca^{2+}-free medium or TTX, the uptake was only reduced by 20% and 36%, respectively.

Finally, it is interesting to note that Wernicke et al., (1974) showed that in their system, β-bungarotoxin's action on NA, 5HT and GABA uptake was Ca^{2+} dependent.

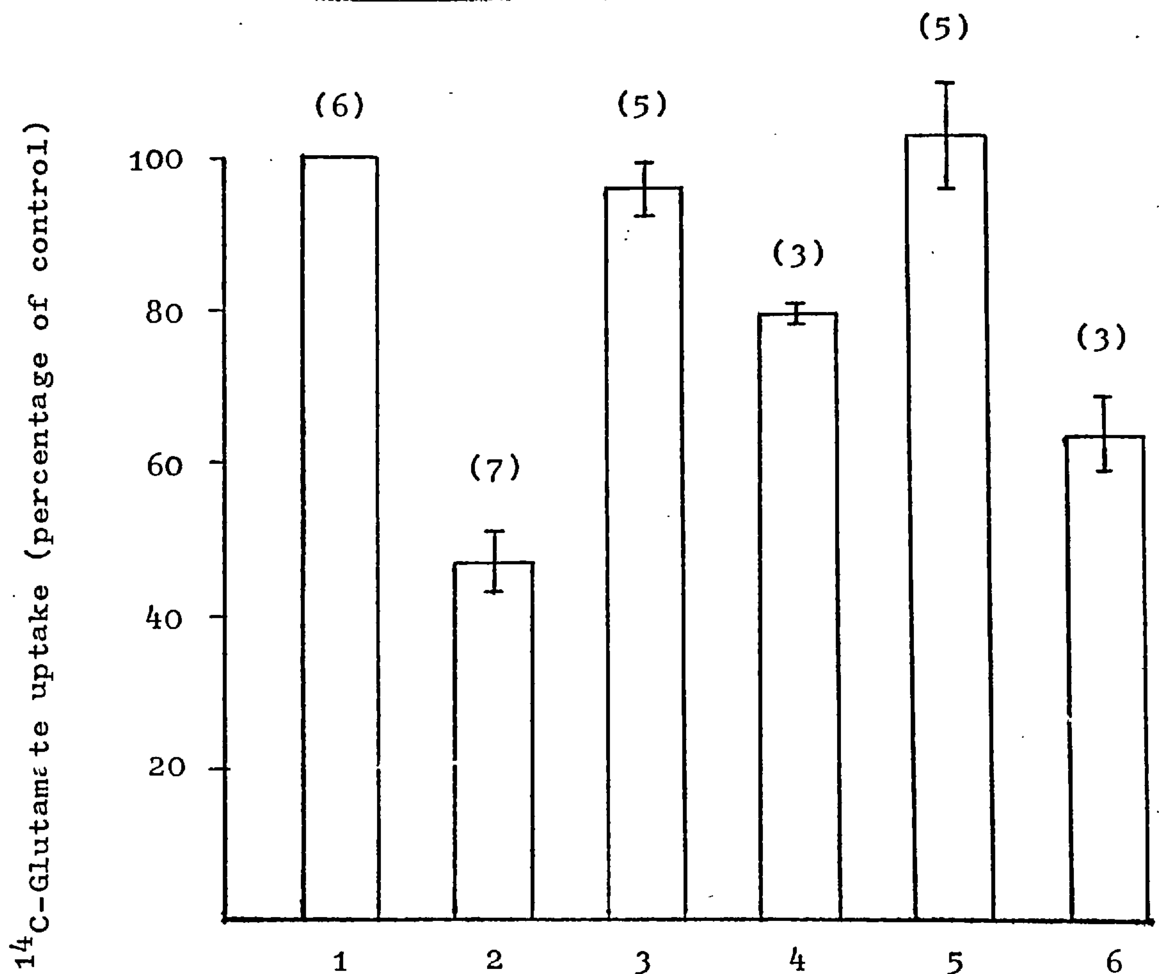
The effect of acetylcholine and carbachol on amino acid release from rat cerebrocortical synaptosomes

Introduction

Benjamin and Quastel (1977a, 1976b) have shown acetylcholine to influence putative amino acid transmitter release from cerebral cortex slices. Several groups (Giorguieff et al., 1976a, 1976b; de Belleroche and Bradford, 1978; Luqmani and Bradford, 1978) have suggested that such a mechanism may involve a presynaptic receptor. Therefore, the effects of acetylcholine and carbachol

FIGURE 5.15

EFFECT OF β-BUNGAROTOXIN ON ^{14}C-GLUTAMATE UPTAKE INTO CEREBROCORTICAL SYNAPTOSOMES



Rat cerebrocortical nerve-endings were incubated under the conditions described in the text and in Table 5.n.

Values are mean ± S.E.M. for the number of samples in brackets.

1. - control
2. - 0.22μM β-bungarotoxin in medium
3. - Ca^{2+} free medium containing 0.5mM EGTA
4. - Ca^{2+} free medium containing 0.5mM EGTA & 0.22μM β-bungarotoxin
5. - 1μM tetrodotoxin in medium
6. - 1μM tetrodotoxin & 0.22μM β-bungarotoxin in medium

(acetylcholine agonist) on amino acid release from cerebrocortical synaptosomes was studied.

Results

Synaptosomes were incubated in Krebs-phosphate medium for 35 mins. After this period of preincubation an aliquot of acetylcholine or carbachol was added to give the final concentrations shown in Table 5.s.

Under these conditions neither acetylcholine or carbachol were found to influence synaptosomal amino acid release (Table 5.u) or respiration (Table 5.v).

TABLE 5.u

THE EFFECT OF ACh AND CARBACHOL ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

	Amino acid in incubation medium (nmol/100mg protein/h)						
	Control (9)	Control with neostigmine (4)	0.1mM ACh (4)	0.25mM ACh (4)	0.5mM ACh (4)	0.1mM carbachol (3)	0.5mM carbachol (3)
Aspartate	33±4	36±4	28±3	42±2	36±2	42±1	37±2
Threonine	219±16	216±12	169±17	240±26	239±12	237±10	215±28
Serine	491±48	565±46	473±65	533±21	554±70	542±28	390±48
Glutamate	130±9	175±12	126±10	164±23	145±20	127±9	87±15
Glycine	299±43	414±52	275±56	405±4	402±35	397±20	298±25
Alanine	555±30	650±42	547±23	569±4	560±41	558±23	481±43
GABA	12±3	22±5	21±7	20±11	22±12	14±1	0

Synaptosomes were incubated in Krebs-phosphate medium for 35 mins, whereupon ACh or carbachol was added to give the final concentrations shown above. Incubation was then continued for a further 10 mins. Values are mean ± S.E.M. for the number of samples in brackets.

NB. Incubations to which ACh was added had neostigmine included in the medium to prevent enzyme breakdown of ACh by AChE.

TABLE 5.v

THE EFFECT OF ACETYLCHOLINE AND CARBACHOL ON SYNAPTOSOMAL RESPIRATION

Condition	Respiratory rate (μmols O_2/100mg protein/h)	n
Control	52.9±3.4	9
Control incubation with neo-stigmine	47.4±2.6	4
0.1mM ACh	50.6±6.1	4
0.25mM ACh	49.1±2.2	4
0.5mM ACh	49.7±6.5	4
0.1mM Carbachol	58.1±6.3	5
0.5mM Carbachol	-	

Cerebrocortical synaptosomes were incubated at 37°C, for 35 mins, whereupon an aliquot of ACh or carbachol (to give the final concentrations shown above) was added and the incubation continued for a further 10 mins. Values are mean ± S.E.M. for the number of determinations indicated above.

DISCUSSION

The effect of p-chloromercuriphenyl sulphonic acid (PCMPS) and 3-threo-hydroxy aspartic acid on cerebrocortical synaptosomes

The results obtained using PCMPS and 3-threo-hydroxy aspartic acid bore a striking resemblance to those reported by Osborne and Bradford, (1975), who studied the effects of the uptake blockers p-hydroxymercuribenzoate (p-HMB) and ouabain on amino acid release from spinal/medullary synaptosomes. Therefore, like p-HMB, PCMPS increased the levels of GABA, glutamate and aspartate in the medium, also greatly reducing the response to stimulation. Similarly, as with ouabain (Osborne and Bradford, 1975; de Belleroche and Bradford, 1972b), 3-threo-hydroxy aspartate produced a marked increase in the spontaneous and stimulus induced release of aspartate and glutamate.

Thus, 3-threo-hydroxy aspartate appears to act by reducing the uptake of amino acids released into the medium and PCMPS, while also inhibiting uptake, significantly reduces the stimulus-induced release of the putative amino acid transmitters. Therefore, in the normal situation, the amount of each amino acid present in the medium represents the difference between that which has been released and the fraction that has been reabsorbed into the synaptosomes.

Examination of the synaptosomal amino acid content did not reveal any drastic changes, apart from significantly lowered glycine and alanine levels in those samples obtained from synaptosomes treated with PCMPS. The reduction in intrasynaptosomal glycine levels was also reflected in a

lowering of the amount of this amino acid released. Therefore, in this respect PCMPS differed in its action from that of p-HMB. Thus, in the case of glycine, PCMPS may produce its effect on release by inhibiting its synthesis and as a result limit the amount available for release.

Finally, it is interesting that PCMPS reduced synaptosomal respiration in control incubations by approximately 25%. Under normal circumstances this reduction might be expected to be accompanied by a lowering in amino acid synthesis, however, on the whole, the data outlined above does not comply with this theory.

<u>The effect of Picrotoxin and Bicuculline on ^{14}C-GABA and endogenous amino acid release from rat cerebrocortical synaptosomes</u>

As mentioned previously, the results obtained using the GABA antagonist, picrotoxin (final concentration, 100μM), were found to closely resemble those of Johnston and Mitchell (1971) who demonstrated that the stimulated release of ^{3}H-GABA from rat brain slices was inhibited in the presence of this agent. However, the magnitude of the effect was found to be considerably smaller than that observed by these workers. Whereas, they obtained inhibitions of 50 - 60%, using cerebrocortical synaptosomes an inhibition of only 20% was produced. The discrepancy between these results may be due to a number of factors, e.g. the difference as far as the two types of <u>in vitro</u> system used may be of importance. It must also be pointed out that whereas Johnston and Mitchell (1971) used electrical

pulses for stimulation we used veratrine (final concentrations, 75μM). Perhaps electrical stimulation is more susceptible to picrotoxin action.

Picrotoxin at a concentration of 1mM was also found to influence endogenous amino acid release, reducing the veratrine stimulated release of aspartate, glutamate and GABA. This coupled with the fact that the efflux of serine, glycine and alanine followed the same trend, leads one to conclude that perhaps picrotoxin is more general in its action than previously thought and that the assumption that it behaves only as a GABA antagonist is not strictly correct. Indeed, the question of this agents potency and selectivity as a GABA antagonist has been put in doubt by several workers (Duggan, 1972; Kelly and Beart, 1975) and there is evidence to suggest that it influences brain acetylcholine levels.

Picrotoxin at lower concentrations, i.e. 100μM, was found to have no effect on endogenous amino acid release. Similarly, picrotoxin did not appear to influence synaptosomal respiration or amino acid synthesis as evidenced by the total amino acid levels detected in nerve-endings treated with the toxin. We may, therefore, conclude that picrotoxin does not influence amino acid release by decreasing the quantities of those compounds released upon stimulation.

The results obtained using another GABA antagonist, bicuculline, unlike those described for picrotoxin, differed considerably from the observations made by Johnston and Mitchell (1971). They claim to have shown that bicuculline increases the electrically induced efflux of labelled GABA,

however, under our conditions the agent was only found to affect the spontaneous release. At a concentration of 100µM, bicuculline was shown to reduce the spontaneous release by 15%, whereas, at 200µM the release was increased by 25%. This is interesting when one considers that in order to obtain their result Johnston and Mitchell (1971) used a concentration of 10µM, no action being observed at concentrations higher than this. However, it must be remembered that whereas these workers used cerebral cortex slices for their in vitro system, our experiments were conducted on relatively pure preparations of cerebrocortical nerve-endings.

The above concentrations of bicuculline were found to have no significant effect on the synaptosomal release of endogenous amino acids. This apparent lack of effect raises the question of whether the observations made using radiolabelled GABA are in fact valid and truly representative of the physiological condition.

As with picrotoxin, the matter of bicuculline's specificity and potency has been questioned (Godfraind et al., 1970; Straughan et al., 1971; Kelly and Beart, 1975) and the seemingly conflicting results obtained using 100µM and 200µM bicuculline underlines this phenomenon. It must also be remembered that bicuculline has been shown to affect non-GABA-ergic systems for example, Miller and McLennan (1974) have shown that bicuculline causes a marked enhancement of the excitation of neurons induced by acetylcholine. This effect is believed to be consistent with the report that bicuculline is active as an anti-cholinesterase (Svenneby and Roberts, 1973).

In view of the variability of the results obtained by various groups using these GABA antagonists and the question of their specificities (especially for bicuculline), considerable doubt has been cast on their usefulness as probes for studying inhibitory pathways in the mammalians CNS, mediated by GABA. However, the results obtained using these agents would suggest that they have a presynaptic site of action as proposed by Davidson and Southwick (1971) and Davidson and Reisine (1971). The latter authors have also suggested that these two alkaloids may act by preventing the release of GABA and the results obtained using picrotoxin would tend to agree with this theory. The potentiation of electrically evoked ^{3}H-GABA efflux by bicuculline, over a limited concentration range, as reported by Johnston and Mitchell (1971) has been interpreted as resulting from the antagonism by bicuculline of GABA-induced postsynaptic inhibition leading to increased activity of GABA-releasing neurons, which are normally inhibited by other GABA-releasing neurons. An alternative explanation suggesting that bicuculline might 'disinhibit' a presynaptic inhibitory action of GABA on its own release as has been proposed for the potentiation of ACh release by atropine (Polak, 1971) was also put forward.

Our data obtained using cerebrocortical synaptosomes does not appear to fit either theory, however, though evidence exists to the contrary (Iversen and Johnston, 1971; Johnston and Mitchell, 1971), bicuculline at a concentration of 200μM could possibly have been behaving as an uptake blocker whilst also reducing the stimulus-induced release of GABA, as has been proposed for p-HMB (Osborne and Bradford,

1975). At a concentration of 100 μM, bicuculline appeared to function as an inhibitor of ^{14}C-GABA release.

The effect of kainic acid, a glutamate agonist, on rat cerebrocortical and sheep striatal synaptosomes

In attempting to determine the mechanism of action of the excitant, kainic acid, extremely confusing and apparently conflicting results were forthcoming.

It has been proposed that kainate may operate as a depolarizing agent (Biscoe et al., 1975) and indeed some of the data obtained using rat cerebrocortical and sheep striatal synaptosomes goes some way to supporting this hypothesis. For example, cerebrocortical synaptosomes exposed to a ten minute incubation with kainate exhibited a 30 - 40 per cent increase in O_2 consumption as compared with a 110% increase elicited by 10 μM veratrine. This coupled with the fact that kainate apparently caused the release of aspartate and glutamate would at first sight appear to be consistent with it having functioned as a depolarizing agent. However, when one considers that it was only these two amino acids that were released, GABA efflux being totally unaffected, some doubt must be cast on this hypothesis.

The picture obtained with sheep striatal synaptosomes differed considerably. Striatal neurons are believed to be particularly rich in glutamatergic nerve-endings (McGeer and McGeer, 1976) and as kainate is a glutamate analogue it may be argued that the results obtained with sheep striatal synaptosomes are more valid. In this situation kainate was found to release GABA in addition to aspartate and glutamate,

again raising the question of whether the agent was acting as a depolarizing agent or not. However, closer examination of the data revealed a significantly enhanced release of glycine and alanine having occurred suggesting that kainic acid is not as specific in its action, as previously supposed. Another factor must also be taken into consideration, this being that kainate may produce its effects as a result of a toxicological action. Its neurotoxic effects in various neural regions, including the corpus striatum, have been well documented (Olney et al., 1974; McGeer and McGeer, 1976; McGeer et al., 1978) and it may be the case that it causes the selective breakdown of neuronal membrane components, especially those mechanisms responsible for amino acid reuptake. The observation made by McGeer and McGeer (1976) that kainate produces its most dramatic neuropathological changes in those brain regions (including the corpus striatum) thought to contain high concentrations of glutamate receptors mediating excitation, underlines the importance of the results obtained with striatal synaptosomes.

No effect was observed on respiration in sheep striatal synaptosomes, this also being the case for rat cerebrocortical synaptosomes given a 45 min. as opposed to a 10 min exposure (as described earlier) to kainate. This data coupled with the fact that another parameter usually associated with depolarization, i.e. a decrease in the intracellular K^+ levels, was not satisfied, would tend to discount kainate as being a depolarizing agent. Kainate was also found to exert no influence on amino acid synthesis as evidenced by the total synaptosomal amino acid content.

The suggestion that kainate may function as an uptake blocker was not unequivocally born out by our observations. Unlike Lakshmanan and Padmanaban (1974) and McGeer et al., (1978) who have reported that kainate reduces the uptake of glutamate into crude striatal (P_2) or whole brain (P_2B) extracts by 60 - 75%, we in common with other workers (Roberts and Watkins, 1975) could not obtain effects of similar magnitude. Under our conditions kainate only showed inhibitions of 20 - 25%, and as mentioned earlier the concentrations of kainate required to produce this effect were extremely high calling into question the validity of such results. However, when one considers the enhanced release of glutamate and aspartate caused by kainate (Figures 5.11 - 5.13) it may be possible that a slow effect on uptake may be responsible.

Finally, as outlined above two possible mechanisms for kainate action have been proposed (1) an effect on release and (2) an inhibition of uptake. In order to distinguish between these two suggested mechanisms, release and uptake experiments will have to be repeated in which synaptosomes are incubated in (1) Ca^{2+} free, EGTA containing medium and (2) tetrodotoxin containing medium. Knowing that amino acid transmitter release is TTX sensitive and Ca^{2+} dependent, whereas uptake is not, it will be possible to determine whether kainate relies upon Na^+ or Ca^{2+} ions for its action.

The effect of β-bungarotoxin on cerebrocortical synaptosomes

Electrophysiological and pharmacological studies suggest that β-bungarotoxin may block neurotransmission by acting

primarily on the presynaptic membrane and affecting transmitter release (Chang et al., 1973; Kelly, R.B. and Brown, F.R., 1974; Chang and Lee, 1964; Wernicke et al., 1975; Oberg and Kelly, 1976b). The data presented in this thesis would appear to confirm this idea.

Several groups of workers (Kelly et al., 1975; Oberg and Kelly, 1976a; Dolly et al., 1978) have shown β-bungarotoxin to stimulate neurotransmitter release. We found the toxin to enhance aspartate, glutamate and GABA efflux and in common with Dolly et al., (1978) this process and the release produced as a result of stimulation (veratrine) were shown to be non-additive.

Recently, it has been proposed that β-bungarotoxin may function as a depolarizing agent (Sen and Cooper, 1978) and the release data outlined above would certainly appear to be consistent with this hypothesis. Measurement of the intrasynaptosomal levels of K^+ showed that they were significantly reduced in synaptosomes treated with the toxin. Certainly, this information adds further support to the proposal that β-bungarotoxin functions as a depolarizing agent, however, the increase in respiration usually associated with depolarization was not observed and in fact the toxin blocked the respiratory response to stimulation, normally produced by veratrine. This conflicts with the observations of Dolly et al. (1978) who showed β-bungarotoxin to increase synaptosomal respiration by about 2.5 fold. However, whereas these workers used a β-bungarotoxin concentration of 1μM, our concentrations were considerably lower, being 0.22 and 0.43uM.

Various workers (Wernicke et al., 1974; Dolly et al.,

1978) have suggested that β-bungarotoxin may act as an uptake blocker and at first sight our data would appear to agree with this idea, the uptake of ^{14}C-glutamate and ^{14}C-GABA being reduced by 53% and 35% respectively. However, as this apparent effect on uptake was found to be Ca^{2+} dependent and to some extent TTX sensitive, we may infer that β-bungarotoxin is acting on release rather than uptake (reasons given previously). The toxin is known to require Ca^{2+} for its action in vivo (Strong et al., 1976) as well as in vitro (Sen et al., 1976), on cholinergic systems.

Finally, β-bungarotoxin has been shown to reduce neurotransmitter storage in brain synapses (Wernicke et al., 1974) and when we consider the amino acid levels detected in nerve-endings treated with the toxin (see Table 5r, this certainly would appear to be the case. The total synaptosomal levels of glutamate and GABA, remained unaltered. However, the total levels of aspartate were found to be significantly reduced. Therefore, it can be concluded that apart from reducing neurotransmitter storage, β-bungarotoxin may also influence synthesis.

The effect of ACh and Carbachol on amino acid transmitter release

Several groups of workers have shown acetylcholine to influence neurotransmitter release. This has been put forward as evidence for the existence of pre-synaptic receptors, and it has been suggested that ACh would combine with such a receptor in order to control the release of another transmitter.

Benjamin and Quastel (1977a, 1977b) have demonstrated that ACh reduces the K^+ stimulated release of aspartate, glutamate, glycine and GABA from rat cerebrocortical slices, this effect being inhibited by the muscarinic antagonist, atropine. On the other hand, de Belleroche and Bradford, (1978) found that ACh stimulated dopamine release from striatal synaptosomes, this action being modified by both muscarinic and nicotinic antagonists. Similarly, a former worker in this laboratory (Luqmani and Bradford, 1978) obtained evidence to suggest that ACh and the ACh agonist, carbachol, stimulate putative amino acid transmitter release.

Therefore, it was decided to test the effects of a range of concentrations of ACh and carbachol on amino acid release from cerebrocortical synaptosomes. The experiments were conducted as described in the results section, however, neither ACh nor carbachol were found to modify the release of any of the amino acids assayed.

Evidence for Presynaptic Receptors

On the basis of the hypothesis outlined in the introduction, none of the data presented in this chapter could be taken to have shown direct evidence for the existence of presynaptic receptors. In fact, kainic acid, picrotoxin and bicuculline produced effects completely opposite to those predicted by this theory. In addition, the glutamate antagonists, GDEE and HA966, were found to be totally inactive.

Despite this seemingly disappointing result, some of the agents yielded data explainable by the theory proposed

by de Belleroche and Bradford (1976), for a "transport shuttle". As described previously (see Chapter 3), Bradford and his coworkers (de Belleroche and Bradford, 1972 ; Osborne and Bradford, 1975) have shown that a number of agents including N-acetyl-L-glutamate, ouabain, L-2-4-diaminobutyrate and p-hydroxymercuri-benzoate, which block uptake of radioactive amino acid transmitter into tissues over short periods (e.g. 5min) often cause "release" of endogenous or preloaded radioactive transmitter. This was also found to be the case for PCMPS, 3-threo-hydroxy-aspartate, kainic acid and β-bungarotoxin.

CHAPTER SIX

THE EFFECTS OF SEVERAL TREMORGENIC MYCOTOXINS ON PUTATIVE AMINO ACID TRANSMITTER RELEASE FROM SYNAPTOSOMES

INTRODUCTION

RESULTS

Behavioural and neurological changes induced in rats by penitrem A and Penicillium cyclopium.

The effect of penitrem A on the medium and tissue amino acid levels of rat spinal/medullary synaptosomes.

The effect of penitrem A and Penicillium cyclopium on amino acid release from cerebrocortical synaptosomes prepared from rats given an i.p. injection of the pure toxin or mycelium.

The effect of Penicillium cyclopium on synaptosomal respiration.

Effect of penitrem A on the K^+ content of rat cerebrocortical and spinal/medullary synaptosomes.

The effect of Penicillium cyclopium on putative amino acid neurotransmitter uptake into rat cerebrocortical synaptosomes.

The effect of orally administered Penicillium cyclopium on amino acid release from synaptosomes prepared from sheep CNS.

The effect of Penicillium cyclopium on striatal neurotransmitter release.

The effect of Penicillium cyclopium on amino acid release from sheep cerebrocortical synaptosomes.

The effect of Penicillium cyclopium on amino acid release from sheep spinal/medullary synaptosomes.

The effect of Penicillium estinogenum on rat cerebrocortical synaptosomes - amino acid release and respiration.

DISCUSSION

The effect of penitrem A and Penicillium cyclopium on rat synaptosomes.

The effect of Penicillium cyclopium on sheep synaptosomes.

The effect of Penicillium estinogenum on rat cerebrocortical synaptosomes.

How an alteration in transmitter release may cause tremor.

Further remarks.

INTRODUCTION

Penitrem A and verruculogen represent a group of toxic secondary metabolites of certain fungal mycelia referred to as "tremorgenic mycotoxins". These tremorgenic substances have been isolated primarily from the Penicillium and Aspergillus genera of fungi and produce a unique neurotoxic syndrome in vertebrate animals characterized by sustained tremors, limb weakness, ataxia and convulsions.

It was Wilson et al, (1968) who first described the isolation of a toxin from Penicillium cyclopium, which they subsequently termed penitrem A. These authors demonstrated that a dose as small as 250μg/kg injected intraperitoneally was sufficient to cause perceptible tremors in rats and mice, which persisted for several hours. Increasing the dosage caused several behavioural and neurological signs including increased general irritability, weakness of limbs, grasping loss, pinna loss in addition to marked tremors. With doses of 2.5mg/kg and higher, the initial tremors soon progressed to clonic or tetanic convulsions often terminating in the death of the animal.

Attempts to determine the chemical structure of penitrem A have so far been unsuccessful, however, it is known to have the chemical formula, $C_{37}H_{44}NO_{6}Cl$ (Wilson et al., 1968; Wilson, 1971).

Verruculogen, has the chemical formula $C_{30}H_{37}N_{3}O_{7}$. Its chemical structure has been elucidated and has been shown to contain an indole nucleus.

Verruculogen may be extracted from the mycelia of Penicillium verruculosum and Penicillium estinogenum (Cole

et al., 1972; Cole and Kirksey, 1973). As regards its toxicological actions, they are very similar to those of penitrem A, being characterized by general immobility and whole body tremor, best described as an "intentional" tremor since it generally occurs when the animal moves (Sobotka et al., 1978).

The mechanism of tremor induction by penitrem A and verruculogen has been studied by Stern (1971) and Hotujac et al. (1973) using antitremorgenic substances with known modes of action. Stern (1971) found that glycine, GABA, mephenesine, meprobamate, and diazepam, substances known to inhibit interneurones in the spinal cord, prevented penitrem A induced tremors in mice, while administration of glutamic acid, an excitant of spinal interneurones, enhanced penitrem action. He has suggested that penitrem A may produce its effect by acting as an inhibitor of glycine and GABA synthesis.

In similar studies on verruculogen induced tremors in mice and 3 - 7 day old chicks. Hotujac et al. (1973) reported that verruculogen acted as an antagonist of GABA function in the CNS. They were able to abolish verruculogen induced tremors in chicks with injections of GABA, which in these animals easily traverses the blood brain barrier. Similarly Stern (1971) has shown that AOAA, a drug that raises brain GABA levels by inhibiting GABA-transaminase, prevented penitrem A induced tremor in rats and mice. In follow-up studies, Hotujac et al. (1976) found that brain GABA levels were substantially reduced in mice treated with the toxin. This, therefore, substantiated their earlier suggestion that verruculogen induced tremor

was mediated by a loss of inhibitory function. Thus, both penitrem A and verruculogen were found to antagonize the effect of naturally occurring neurotransmitters, causing a loss of central control of tone and movement.

Wilson et al. (1972) have studied the effects of penitrem A on miniature end-plate potential frequency in a rat phrenic nerve-diaphragm preparation in an effort to gain an insight into the basic mechanism of action of the toxin in the CNS. They reported an increase in the frequency of m.e.p.p.'s suggesting the tremorgenic effect of penitrem A may arise from an action on the nerve terminal. This increased m.e.p.p. activity was interpreted to indicate that the toxin increased the excitability of the nerve ending.

The effect of verruculogen on neuromuscular transmission in the rat phrenic nerve-diaphragm preparation has also been investigated (Cole, 1977) and preliminary results suggest that verruculogen like penitrem A may have a pre-junctional site of action. Post-tetanic potentiation has been shown to be enhanced by verruculogen, providing further support for a pre-junctional mode of action. However, evidence that verruculogen produces post-junctional and musculotropic effects has also been forthcoming.

The possible involvement of the cholinergic and catecholaminergic systems in penitrem A and verruculogen induced tremors has been discounted. Sobotka et al. (1978) have shown that even at high neurotoxic levels neither verruculogen nor penitrem A produce changes in NA or DA levels. Verruculogen was also found to have no effect on brain AChE activity. Similarly, Wilson (1971) and Wilson

et al. (1972) reported no penitrem A related effects on cholinesterase activity.

It has been proposed that tremorgenic mycotoxins may be responsible for several naturally occurring neurological disorders generally described as a "staggers" syndrome (i.e. rye grass staggers, Bermuda grass staggers, paspalum staggers). The syndrome has been reported in various parts of the world (Cunningham and Hartley, 1959; Aasen et al., 1969; Di Menna et al., 1975; Mantle et al., 1977) but appears to be particularly prevalent in New Zealand. The fungus Claviceps paspali has been implicated in the paspalum staggers syndrome which occurs when animals (e.g. cattle) ingest grasses of the genus paspalum infected with this fungus (Hopkirk, 1936; Grayson, 1941; Simms, 1945; Mantle et al., 1977). Similarly, Penicillium cyclopium is believed to be responsible for rye-grass staggers in ruminants, occurring in New Zealand (Di Menna et al., 1978; Mantle et al., 1977).

In this chapter the effects of Penicillium cyclopium and penitrem A on putative amino acid neurotransmitter release from synaptosomes obtained from rat and sheep, was determined. The effect of Penicillium cyclopium on amino acid uptake was also studied. In addition preliminary experiments on the effects of Penicillium estinogenum on amino acid transmitter release from rat cerebrocortical synaptosomes, were performed.

It was hoped that the data obtained from these experiments might go some way to explaining how these mycotoxins produce their dramatic effects.

RESULTS

Behavioural and Neurological changes induced in rats by penitrem A and Penicillium cyclopium

Prior to studying the effects of these two agents on the biochemical processes occurring in nerve-endings, observations were made of the behavioural and neurological changes induced in rats following an intraperitoneal injection.

Animals were injected with pure toxin (400µg/kg in Et.OH/H_2O, 1:1) or the mycelium (400mg/kg in H_2O), controls receiving an injection of the carrier.

Approximately 10 mins following injection the animals developed a whole body tremor which increased in severity up to about 45 mins. At this point the hind limbs became paralysed and balance was lost. After about 60 mins the animals motor activity had been almost totally disrupted and they lay on the bottom of the cage with their legs extended. Seventy minutes following injection the fore limbs exhibited some evidence of paralysis which developed into a violent peddling movement. In some animals this peddling movement persisted, usually being followed by convulsions.

Some animals retained the symptoms described up to 24 hrs after injection, exhibiting complete paralysis of the hind quarters of the body and poor control over their bowel and bladder movements. The eyes also appeared to be inflamed and swollen. Usually however, the animals recovered 6 - 10 hrs post-injection.

In our biochemical studies the animals were normally killed 45 mins after injection when tremoring was maximal.

The effect of penitrem A on the medium and tissue amino acid levels of rat spinal/medullary synaptosomes

When rat spinal/medullary synaptosomes were incubated in Krebs-phosphate medium containing penitrem A (final concentration 100μM), no significant action on the spontaneous or stimulus-induced (75μM veratrine) amino acid release was observed (Table 6.a). However, nerve-endings prepared from animals suffering severe tremor and paralysis of the hind limbs as a result of an intraperitoneal injection of penitrem A showed a reduction in the veratrine stimulated release of several amino acids.

Under these conditions the toxin reduced the stimulus-induced release of glycine by 58% and GABA by 33% (percentages obtained by considering the difference between stimulated and control values). The apparent decreases in the amounts of aspartate, threonine, serine and glutamate released to the medium were found to be insignificant (Figure 6.1).

The amino acid content of nerve-endings prepared from animals pretreated with the mycotoxin, showed no decrease when compared to those levels found in normal synaptosomes (Table 6.b). Similarly, combining the data from Figure 6.1 and Table 6.b, no significant alteration in the total synaptosomal amino acid levels was observed, the one exception perhaps being aspartate. Thus, in spinal/medullary synaptosomes penitrem A seems to produce its effect by inhibiting the response to stimulation and not by diminishing the endogenous amino acid pools and hence the amount of neurotransmitter available for release.

The lack of effect observed when synaptosomes were

TABLE 6.a

AMINO ACID RELEASE FROM SPINAL/MEDULLARY SYNAPTOSOMES INCUBATED IN MEDIUM CONTAINING PENITREM A (100uM)

Amino acid in incubation medium (nmol/100mg protein)

	No addition		Toxin in medium	
	Control (4)	Stimulated (4)	Control (4)	Stimulated (4)
Aspartate	1200±91	1469±239	1184±157	1231±125
Threonine	234±19	226±35	235±40	238±35
Serine	583±57	636±107	601±116	613±69
Glutamate	1865±138	2367±225	1887±234	2245±226
Glycine	816±85	1482±195	755±90	1327±168
Alanine	569±57	761±102	572±104	704±88
GABA	68±5	624±47	76±9	532±60

Synaptosomes were incubated for 45 mins (controls) in Krebs-phosphate medium or for 35 mins followed by 10 mins veratrine (final concentration, 75µM) stimulation. Values are means ± S.E.M. for the number of determinations in brackets.

FIGURE 6.1

EFFECT OF INTRAPERITONEALLY ADMINISTERED PENITREM A ON THE RELEASE OF AMINO ACIDS FROM RAT SPINAL/MEDULLARY SYNAPTOSOMES

Spinal/medullary nerve-endings were incubated at 37°C in Krebs-phosphate medium. Veratrine (final concentration, 75μM) was used for stimulation. Values represent the mean ± S.E.M. for 6 determinations. Change in stimulated release due to penitrem A action significant with $p < 0.01$* and $p < 0.05$**

1. - control
2. - veratrine stimulated
3. - control } pentirem A treated
4. - veratrine stimulated } pentirem A treated

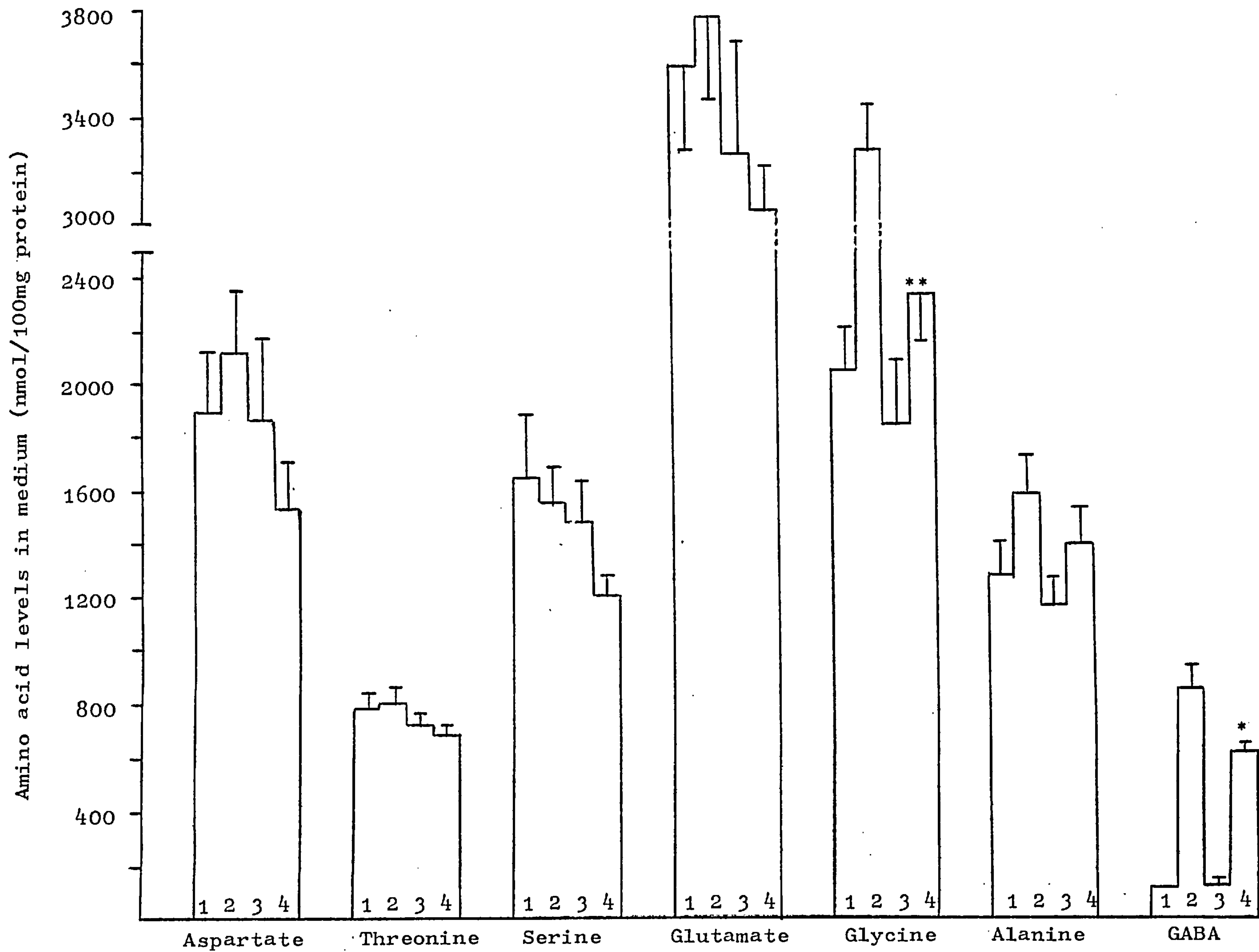


FIGURE 6.1

TABLE 6.b

AMINO ACID CONTENT OF SPINAL/MEDULLARY SYNAPTOSOMES AFTER TREATMENT WITH PENITREM A

Amino acid in tissue (nmol/100mg protein)

	No addition		Intraperitoneal toxin	
	Control (6)	Stimulated (6)	Control (4)	Stimulated (4)
Aspartate	2638±205	1869±195	2593±299	1355±180
Threonine	418±66	349±63	316±31	283±28
Serine	962±151	502±116	639±46	762±76
Glutamate	2140±231	1775±165	1934±379	1995±523
Glycine	2117±222	1605±246	2719±477	1895±579
Alanine	620±99	527±66	589±106	549±105
GABA	1514±270	1033±119	1363±253	1109±280

Synaptosomes were incubated for 45 mins in Krebs-phosphate medium. Stimulation was achieved using veratrine (final concentration, 75μM). Values are means ± S.E.M. for the number of samples in brackets.

incubated "in vitro" in medium containing the toxin, may have been due to the concentration used being too low. Alternatively, as for tetanus toxin (Osborne and Bradford, 1973), pretreatment of the animals with the mycotoxin may be necessary before an effect is produced.

The effect of penitrem A and Penicillium cyclopium on amino acid release from cerebrocortical synaptosomes prepared from rats given an i.p. injection of the pure toxin or mycelium

When cerebrocortical synaptosomes prepared from rats injected with penitrem A or Penicillium cyclopium were incubated in Krebs-phosphate medium for 45 mins, there was a 3 - 4 fold increase in the levels of aspartate and glutamate in the medium (Figures 6.2 and 6.3). The spontaneous efflux of GABA, however, was only raised in synaptosome suspensions prepared from animals treated with the pure toxin (2 fold increase). Veratrine stimulation produced the normal pattern of amino acid release.

Thus, penitrem A and its parent mycelium greatly reduce the response to stimulation in cerebrocortical synaptosomes. However, the mechanism used to achieve this result differs from that seen in spinal/medullary synaptosomes.

As with spinal/medullary synaptosomes, penitrem A did not appreciably alter the amino acid content of cerebrocortical synaptosomes (Table 6.c). Therefore, the total synaptosomal amino acid levels (sum of data from Figure 6.2 and Table 6.c) were not reduced, thus,

FIGURE 6.2

EFFECT OF INTRAPERITONEALLY ADMINISTERED PENITREM A ON THE RELEASE OF AMINO ACIDS FROM RAT CEREBROCORTICAL SYNAPTOSOMES

Synaptosomes were incubated in Krebs-phosphate medium for 45 mins (controls) or for 35 mins followed by 10 mins veratrine (final concentration, 75μM) stimulation. Values are means ± S.E.M. for 4 - 6 determinations. Change in spontaneous release due to penitrem A action significant with $p < 0.05$* or $p < 0.001$**.

1. - control
2. - veratrine stimulated
3. - control
4. - veratrine stimulated

(3 and 4: samples prepared from animals treated with penitrem A)

NB The spontaneous release of glycine was also significantly increased ($p < 0.01^{\dagger}$) in samples prepared from animals treated with the toxin. However, on the basis of electrophysiological observations made on cerebrocortical neurons, this result probably does not have any importance, i.e. glycine has been shown to be relatively weak as a depressant of cortical neuronal firing (see Chapter 1)

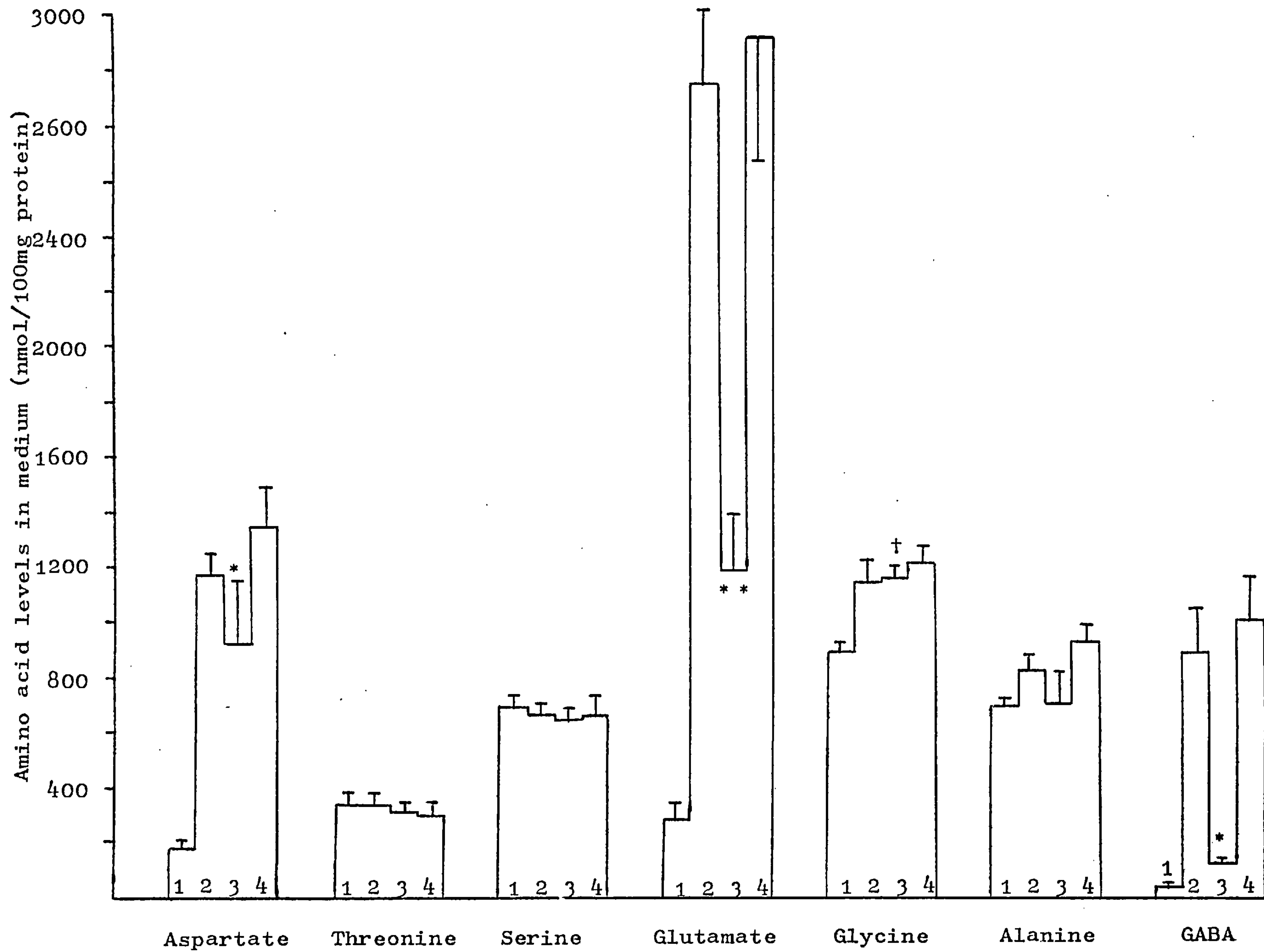


FIGURE 6.2

FIGURE 6.3

EFFECT OF INTRAPERITONEALLY ADMINISTERED PENICILLIUM CYCLOPIUM ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

Cerebrocortical nerve-endings were incubated in Krebs-phosphate medium at 37°C for 45 mins. Veratrine (final concentration, 75μM) was used for stimulation. Values are mean ± S.E.M. for 6 determinations. Change in control release due to presence of mycelium significant with $p < 0.001$*.

1. - control
2. - veratrine stimulated
3. - control } samples prepared from animals treated with mycelium
4. - stimulated } samples prepared from animals treated with mycelium

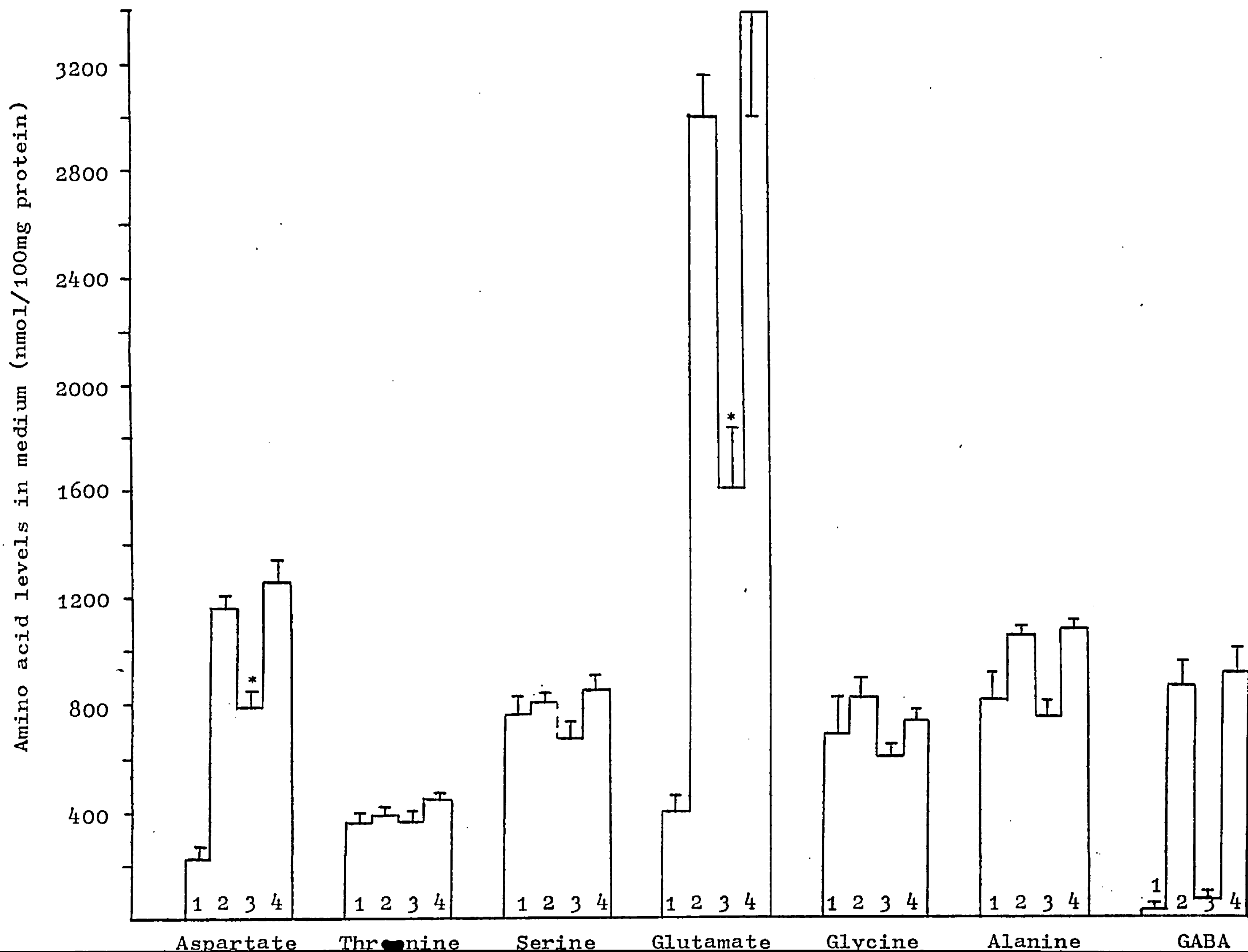


FIGURE 6.3

TABLE 6.c

AMINO ACID CONTENT OF CEREBROCORTICAL SYNAPTOSOMES AFTER TREATMENT WITH PENITREM A

Amino acid in tissue (nmol/100mg protein)

	No addition		Intraperitoneal toxin	
	Control (4)	Stimulated (4)	Control (4)	Stimulated (4)
Aspartate	2791±588	1021±159	2640±600	1135±207
Threonine	159±14	144±13	164±28	122±12
Serine	471±84	508±47	329±34	311±59
Glutamate	4143±673	2507±292	4766± 790	2671±408
Glycine	962±40	576±109	916±108	433±72
Alanine	617±49	597±96	779±130	398±72
GABA	1124±146	405±44	1343±233	450±77

Nerve-endings were incubated at 37°C in Krebs-phosphate medium for 45 mins. Stimulation was achieved using veratrine (final concentration, 75μM). Values are means ± S.E.M. for the number of samples in brackets.

invalidating Stern's hypothesis that penitrem action results from an inhibition of inhibitory neurotransmitter synthesis.

Finally, it must be mentioned that the total amount of amino acid released in this series of experiments far exceeded that observed previously, using cerebrocortical synaptosomes (see Chapter 5). This is probably due to the fact that incubations were done in 3mls of medium instead of 1.5mls as before. Hence, this provides further evidence for the dilution effect discussed in Chapter 3.

The effect of Penicillium cyclopium on synaptosomal respiration

Cerebrocortical synaptosomes prepared from rats previously dosed with Penicillium cyclopium exhibited respiratory rates similar to those shown by control synaptosomes.

Veratrine (final concentration 75μM) produced the expected sharp increase (approx 80%) in oxygen uptake (Table 6.d).

Effect of penitrem A on the K^+ content of rat cerebrocortical and spinal/medullary synaptosomes

Synaptosomes prepared from animals treated with penitrem A showed no significant alteration in the normal synaptosomal K^+ content (Table 6.e).

This is perhaps surprising in view of the fact that in both cerebrocortical and spinal/medullary synaptosomes, penitrem A effectively reduced the response to veratrine stimulation as evidenced by amino acid release. For

TABLE 6.d

THE EFFECT OF PENICILLIUM CYCLOPIUM ON RESPIRATION IN RAT CEREBROCORTICAL SYNAPTOSOMES

Respiratory Rate (μmol O_2/100mg protein/h)

	No addition	Intraperitoneal toxin
CONTROL	46.7±4.3 (7)	51.7±3.5 (8)
STIMULATED	121.7±12.0 (7)	93.5±10.5 (4)

Synaptosomes were incubated in Krebs-phosphate medium for 45 mins. Veratrine (final concentration, 75μM) was used for stimulation. Values are mean ± S.E.M. for the number of samples in brackets.

TABLE 6.e

POTASSIUM CONTENT OF RAT CEREBROCORTICAL AND SPINAL/ MEDULLARY SYNAPTOSOMES TREATED WITH PENITREM A

Potassium Content (μequiv/100mg protein)

Cerebrocortical Synaptosomes			
No addition		Intraperitoneal toxin	
Control	Stimulated	Control	Stimulated
28±3 (4)	16±1 (4)	27±3 (4)	18±2 (4)

Medulla/Spinal Cord Synaptosomes			
No addition		Intraperitoneal toxin	
Control	Stimulated	Control	Stimulated
22±1 (8)	16±1 (8)	22±2 (4)	15±2 (4)

Synaptosomes were incubated for 45 mins in Krebs-phosphate medium at 37°C. Stimulation was achieved using veratrine (final concentration, 75μM). Values are mean ± S.E.M. for the number of samples in brackets.

example, with spinal/medullary synaptosomes one might have expected this effect to have been accompanied by higher K^+ levels in those samples treated with veratrine.

The effect of Penicillium cyclopium on putative amino acid neurotransmitter uptake into rat cerebrocortical synaptosomes

Figures 6.4 and 6.5 show how pretreating animals with Penicillium cyclopium has no effect on the amino acid uptake properties of cerebrocortical synaptosomes. It is conceivable (taking into account the effect observed on release) that in this tissue the toxin could have been functioning as an uptake blocker for the putative transmitters.

Synaptosomal suspensions were incubated at 37°C for 5 mins, whereupon radioactively labelled amino acid was added (for concentrations see legends to figures) and the incubation continued for a further 5 mins. The reaction was then halted and TCA extracts counted.

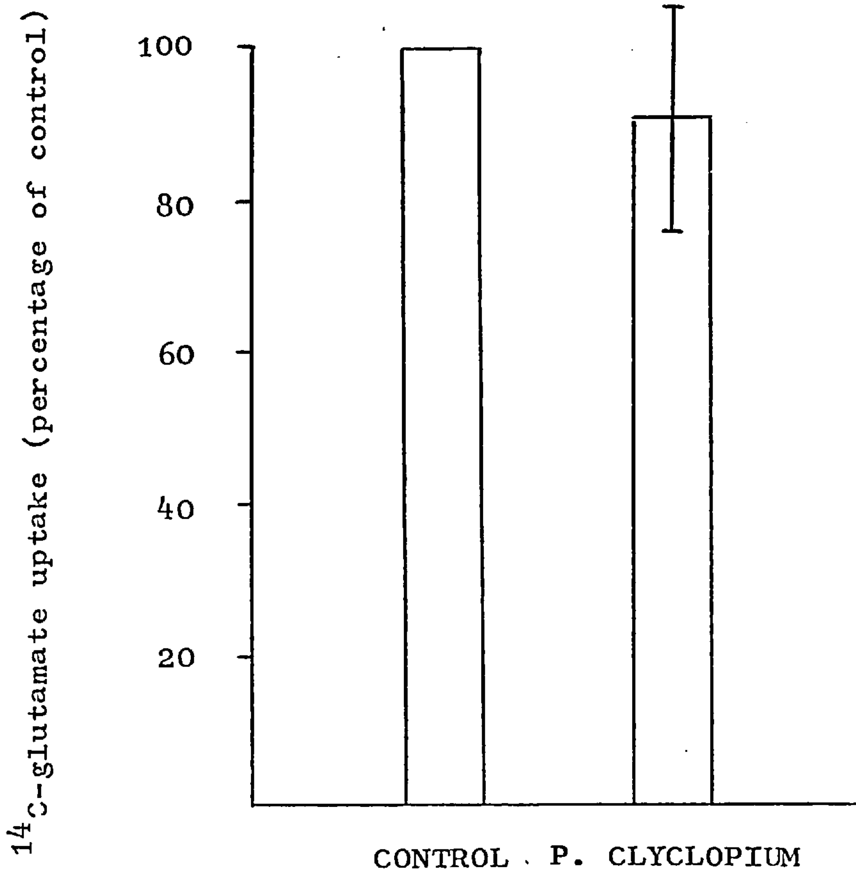
Using this method, the uptake of ^{14}C-labelled glutamate and GABA were studied.

The effect of orally administered Penicillium cyclopium on amino acid release from synaptosomes prepared from sheep CNS

Preliminary studies on the effect of orally administered Penicillium cyclopium on amino acid release from synaptosomes prepared from three neural regions (i.e. corpus striatum, cerebral cortex and spinal cord), were conducted. In addition the effect of the mycelium on dopamine release

FIGURE 6.4

THE EFFECT OF PENICILLIUM CYCLOPIUM ON ^{14}C-GLUTAMATE UPTAKE INTO RAT CEREBROCORTICAL SYNAPTOSOMES

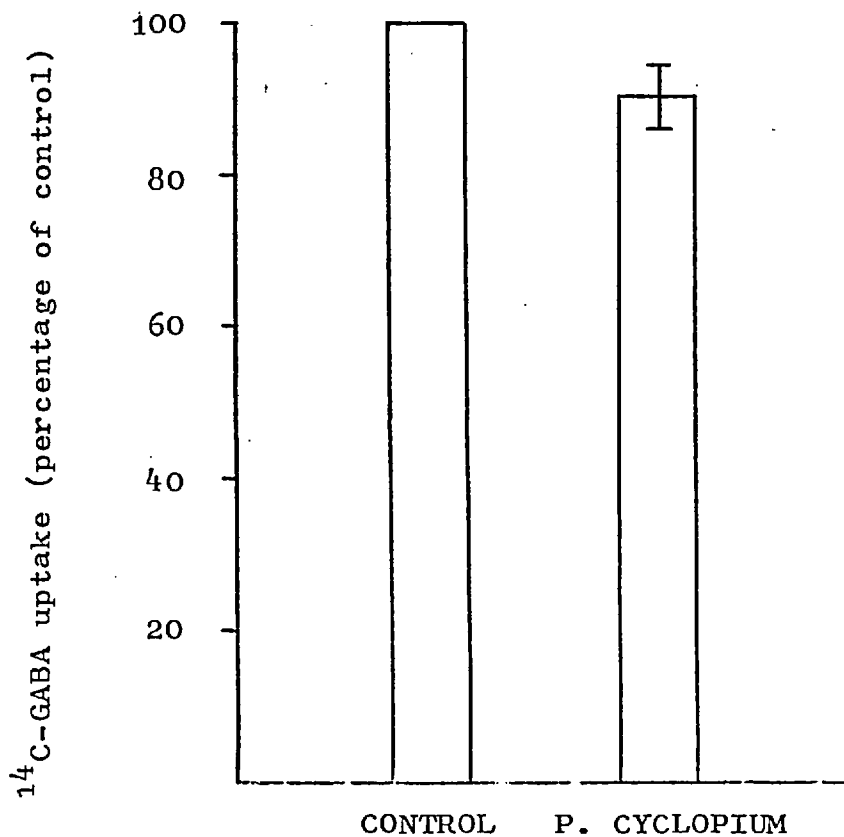


Synaptosomes were preincubated in Krebs-phosphate medium for 5 mins at 37°C, whereupon ^{14}C-glutamate (final concentration, 30µM) was added and incubation continued for a further 5 mins. TCA extracts were counted by liquid scintillation counting.

Values are mean ± S.E.M. for 4 determinations.

FIGURE 6.5

THE EFFECT OF PENICILLIUM CYCLOPIUM ON ^{14}C - GABA UPTAKE INTO RAT CEREBROCORTICAL SYNAPTOSOMES



Nerve endings were incubated in Krebs-phosphate medium for 5 mins at 37°C, whereupon ^{14}C-GABA (final concentration 20μM) was added and incubation continued for a further 5 mins.

The reaction was then halted and TCA extracts counted by liquid scintillation counting.

Values are mean ± S.E.M. for 4 experiments

from striatal synaptosomes was determined.

Sheep (30 - 35kg) were given daily doses of the mycelium (0.5g) equivalent to 10 - 15mg of penitrem A, over periods of 2 - 3 months. They exhibited symptoms similar to those seen in rats, i.e. general body tremor and paralysis of the hind limbs. Also, when "chased" around the paddock the animals collapsed, the hind limbs becoming completely rigid.

<u>The effect of Penicillium cyclopium on striatal neurotransmitter release</u>

Striatal synaptosomes prepared from sheep orally dosed with Penicillium cyclopium were incubated in Krebs-phosphate medium, resulting in a 66 - 91% increase in the spontaneous efflux of aspartate, glutamate and GABA (Figure 6.6).

This increase was sufficient to completely block the response to veratrine stimulation. Therefore, this result was very similar to that obtained with rat cerebrocortical synaptosomes.

Penicillium cyclopium was found to have no effect on the synaptosomal release of dopamine (Figure 6.7). The release data was mirrored by the dopamine levels measured in the intrasynaptosomal fractions (Figure 6.8).

<u>The effect of Penicillium cyclopium on amino acid release from sheep cerebrocortical synaptosomes</u>

Synaptosomes prepared from sheep cerebral cortex were incubated in Krebs-bicarbonate medium of composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 0.75; $MgSO_4$, 1.3;

FIGURE 6.6

EFFECT OF PENICILLIUM CYCLOPIUM ON AMINO ACID RELEASE FROM SHEEP STRIATAL SYNAPTOSOMES

Sheep striatal synaptosomes were incubated in Krebs-phosphate medium at 37°C. Stimulation was achieved using veratrine (final concentration, 75µM). Values are mean ± S.E.M. for 3-7 determinations. Change in control release due to toxin action significant with $p < 0.001$*, $p < 0.01$** or $p < 0.05$†.

1. - control
2. - veratrine stimulated
3. - control } samples prepared from animals orally dosed with mycelium
4. - veratrine stimulated } samples prepared from animals orally dosed with mycelium

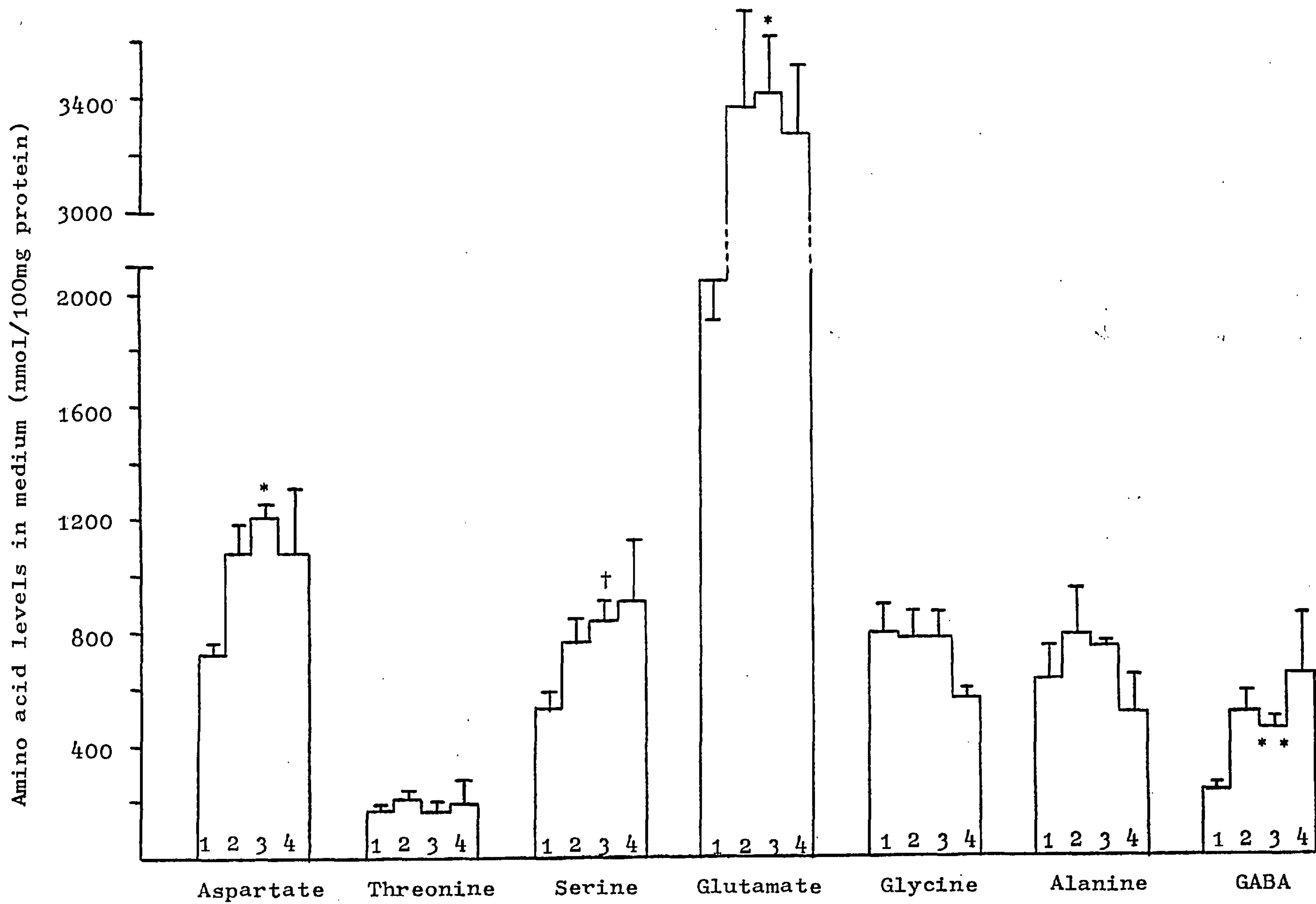
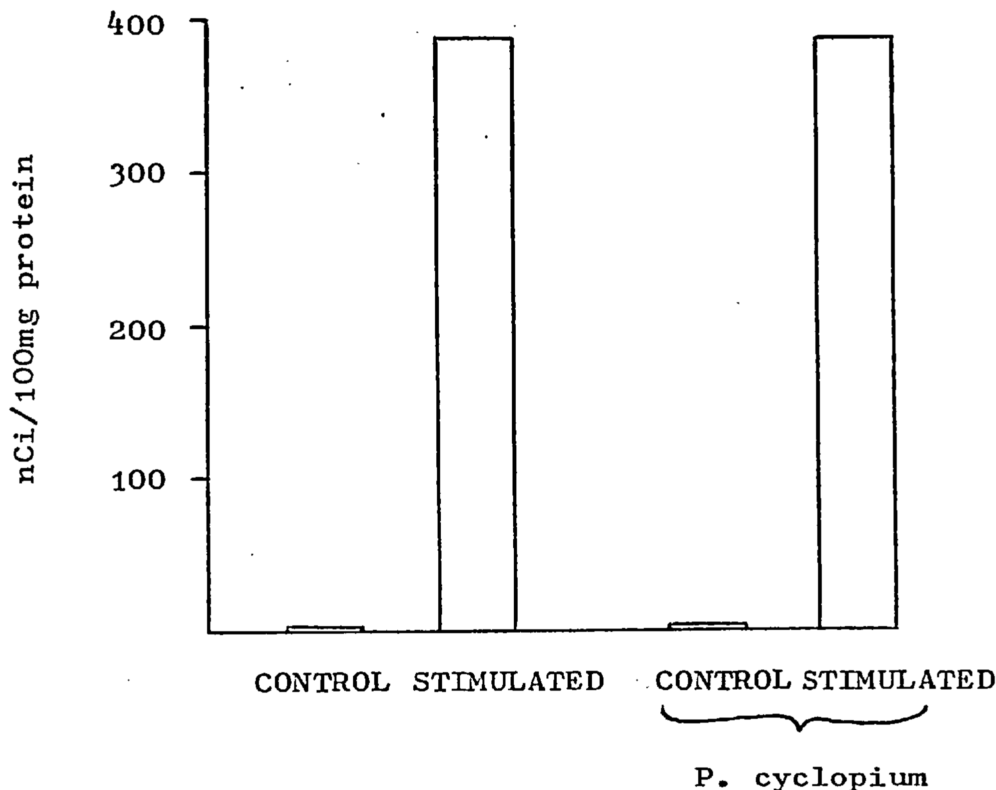


FIGURE 6.6

FIGURE 6.7

THE EFFECT OF PENICILLIUM CYCLOPIUM ON DOPAMINE RELEASE FROM SHEEP STRIATAL SYNAPTOSOMES - MEDIUM LEVELS



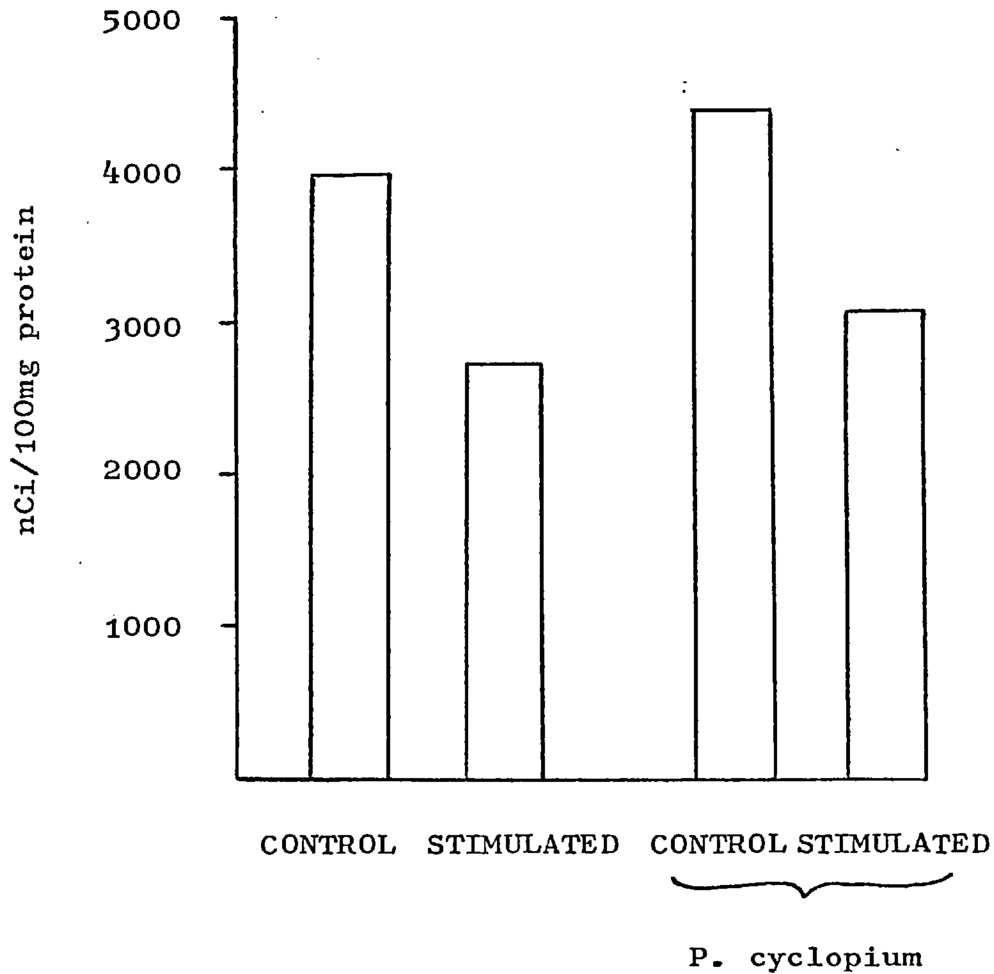
Striatal synaptosomes were incubated at 37°C as suspensions in Krebs-bicarbonate medium containing 10mM glucose, 0.5mM L-ascorbate, 0.1mM nialamide and 2.5μCi L - ^{14}C tyrosine (495mCi/mmole, gassed with 95% O_2/5% CO_2, according to the method of de Belleroche and Bradford (1978). Veratrine (final concentration, 75μM) was used to achieve stimulation.

The levels of dopamine in the tissue and medium extracts were determined as described by de Belleroche et al. (1976b).

Values are mean for 2 experiments.

FIGURE 6.8

THE EFFECT OF PENICILLIUM CYCLOPIUM ON DOPAMINE RELEASE FROM SHEEP STRIATAL SYNAPTOSOMES - TISSUE LEVELS



For details of experimental procedure, see Figure 6.7.

Values are mean for 2 determinations.

$NaHCO_3$, 26; pH 7.4 containing 10mM glucose. This medium was chosen as Bradford et al., (1973) have shown that under these conditions they exhibit properties similar to those of rat and rabbit cerebrocortical synaptosomes, responding to stimulation with augmented glycolysis (lactate production) and differential release of aspartate, glutamate and GABA. With Krebs-phosphate medium this is not the case. Penicillium cyclopium was found to exert no influence on amino acid release from sheep cerebrocortical synaptosomes (Table 6.f).

The effect of Penicillium cyclopium on amino acid release from sheep spinal/medullary synaptosomes

Incubations were performed in both Krebs-phosphate and Krebs-bicarbonate medium, satisfactory results only being obtained with the latter. Again, the data represents the result of only one experiment, however, the individual values were closely allied.

Synaptosomes prepared from animals pretreated with the toxin exhibited a reduced release of all the amino acids assayed in both the control and veratrine stimulated situations. Amino acids particularly affected were aspartate, serine, glutamate and glycine (Figure 6.9).

The effect of Penicillium estinogenum on rat cerebrocortical synaptosomes

Introduction

As a pure sample of the mycotoxin, verruculogen was not available, rats were given an i.p. injection of the fungal mycelium Penicillium estinogenum which is known to

TABLE 6.f

THE EFFECT OF ORALLY ADMINISTERED PENICILLIUM CYCLOPIUM ON AMINO ACID RELEASE FROM SHEEP CEREBROCORTICAL SYNAPTOSOMES

Amino acid in incubation medium (nmol/100mg protein)

	Control	Stimulated	Control	Stimulated
Aspartate	25	106	45	135
Threonine	24	50	41	61
Serine	285	326	215	257
Glutamate	65	508	34	593
Glycine	202	387	266	378
Alanine	148	311	173	284
GABA	4	144	0	171

Synaptosomes were incubated at 37°C, for 45 mins in Krebs-bicarbonate medium.

Values are the means for two determinations.

FIGURE 6.9

EFFECT OF PENICILLIUM CYCLOPIUM ON AMINO ACID RELEASE FROM SHEEP SPINAL/MEDULLARY SYNAPTOSOMES

Sheep spinal/medullary nerve-endings were incubated in Krebs-bicarbonate medium at 37°C, for 45 mins. Veratrine (final concentration, 75μM) was used to achieve stimulation. Values are the mean for 2 determinations.

1. - control
2. - veratrine stimulated
3. - control
4. - veratrine stimulated

(3 and 4: samples prepared from animals given mycelium)

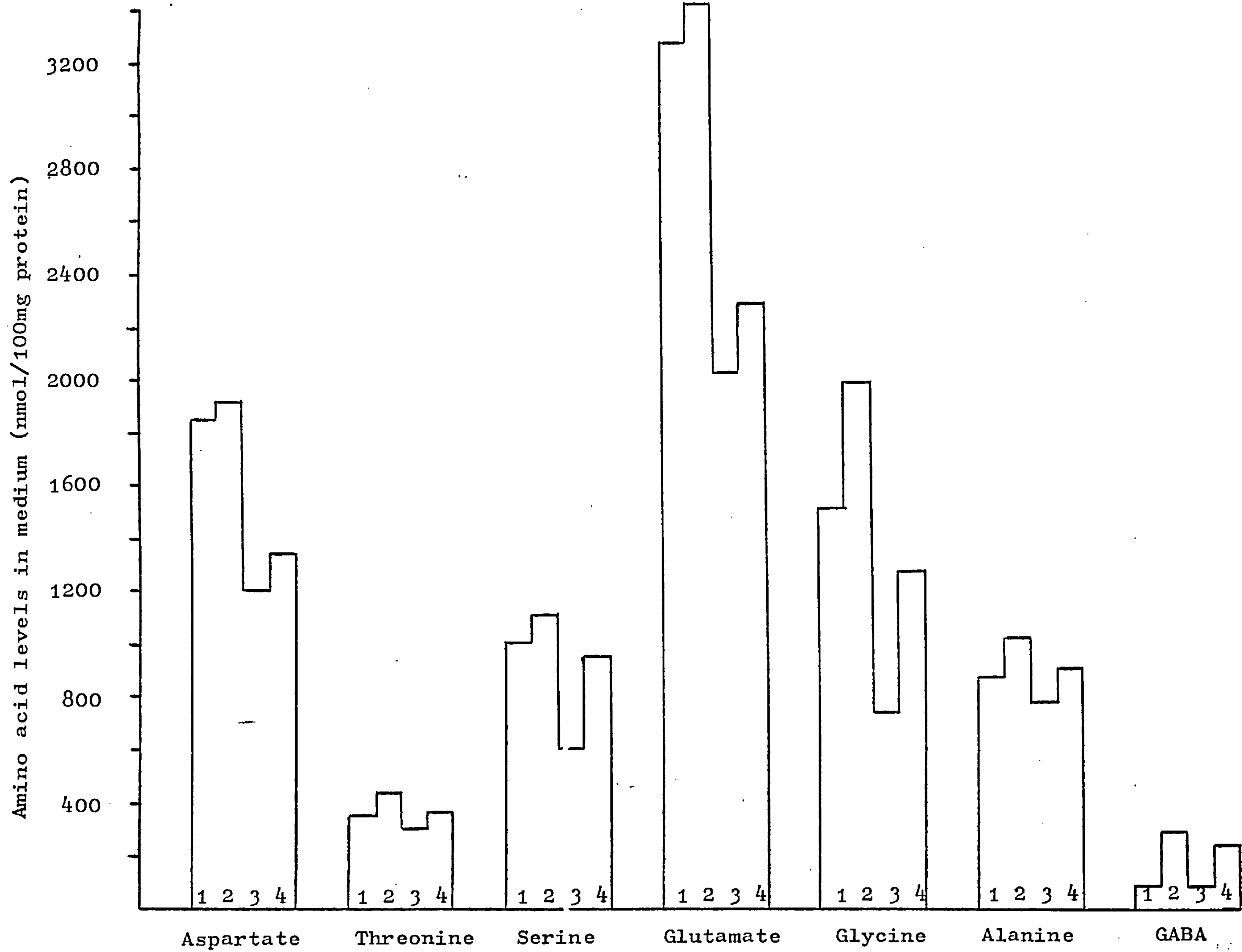


FIGURE 6.9

produce this substance.

Penicillium estinogenum was found to produce symptoms very similar to those of Penicillium cyclopium and its mycotoxin, penitrem A, i.e. severe tremor and paralysis of the hind limbs. However, the onset of the symptoms was found to be more rapid than for Penicillium cyclopium or penitrem A.

The effect of Penicillium estinogenum on amino acid release

When cerebrocortical synaptosomes prepared from animals previously dosed with Penicillium estinogenum, were incubated in Krebs-phosphate medium for 45 mins, both the spontaneous and stimulus-induced release of aspartate and glutamate were enhanced (Figure 6.10). However, the differences between control and stimulated values remained relatively unchanged being similar to those obtained using nerve-endings prepared from untreated animals.

The effect of Penicillium estinogenum on synaptosomal respiration

Penicillium estinogenum did not appear to significantly influence respiration in rat cerebrocortical synaptosomes.

Veratrine elicited the usual response, i.e. a sharp increase in the respiratory rate (Table 6.g).

FIGURE 6.10

EFFECT OF PENICILLIUM ESTINOGENUM ON AMINO ACID RELEASE FROM RAT CEREBROCORTICAL SYNAPTOSOMES

Cerebrocortical nerve-endings were incubated in Krebs-phosphate medium at 37°C, for 45 mins. Veratrine (final concentration, 75µM) was used to achieve stimulation. Values are the mean ± S.E.M. for 4 determinations. Change in release due to mycelial action significant with $p < 0.05$*, $p < 0.01$** or $p < 0.001^{+}$ when comparing relevant stimulated samples and control samples.

1. - control
2. - veratrine stimulated
3. - control } samples prepared from animals treated with mycelium
4. - veratrine stimulated } samples prepared from animals treated with mycelium

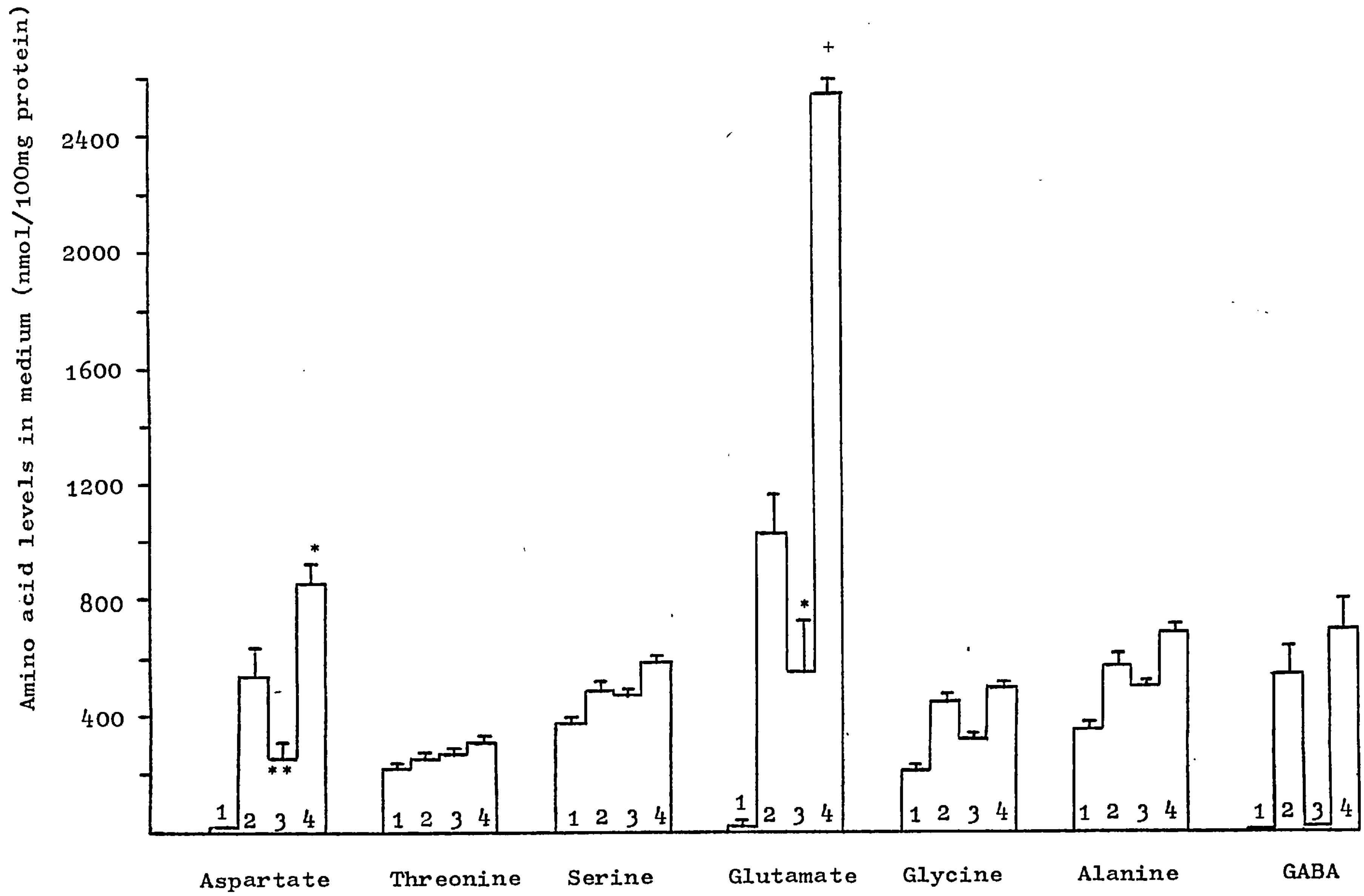


FIGURE 6.10

TABLE 6.g

THE EFFECT OF PENICILLIUM ESTINOGENUM ON RESPIRATION IN RAT CEREBROCORTICAL SYNAPTOSOMES

Respiratory Rate (μmol O_2/100mg protein/h)

	No addition	Intraperitoneal toxin
CONTROL	60	65
STIMULATED	113	126

Nerve-endings were incubated in Krebs-phosphate medium for 45 mins. Veratrine (final concentration, 75μM) was used as the stimulatory agent. Values are means for two determinations.

DISCUSSION

The effect of penitrem A and Penicillium cyclopium on rat synaptosomes

Stern (1971) has shown that a number of substances known to inhibit spinal interneurones, including glycine and GABA, prevent penitrem A induced tremors in mice, whilst administration of glutamic acid, an excitant of spinal interneurones, enhances penitrem action. He has suggested that penitrem A causes its effects by inhibiting glycine and GABA production, however, the data presented in this chapter does not appear to support this hypothesis.

When rat spinal/medullary synaptosomes were prepared from animals pretreated with the mycotoxin a marked reduction in the stimulated release of glycine and GABA was obtained. Initially, this might lead one to suppose that Stern's hypothesis was correct. However, penitrem A was not found to significantly decrease the total synaptosomal amino acid content. Hence, it may be concluded that the toxin has no inhibitory action on the synthesis of any of the amino acids <u>in vitro</u> and therefore could not be reducing release of the inhibitory transmitters by interfering with their synthesis. The data would therefore suggest that penitrem A produces its effect through an action on the presynaptic membrane, preventing the release of these inhibitory substances. The findings of Wilson et al., (1972), who demonstrated that penitrem A increased miniature end-plate potential activity in a rat phrenic nerve-diaphragm preparation, supports the idea of a presynaptic mode of action for penitrem A. However, increased m.e.p.p. frequency is thought to be associated

with increased spontaneous neurotransmitter release and this would appear to conflict with the pattern of amino acid release observed for spinal/medullary synaptosomes. Nevertheless, in subsequent experiments performed on cerebrocortical nerve-endings, an increase in putative amino acid transmitter efflux was observed.

It is interesting to note that Osborne and Bradford (1973) studying the effects of tetanus toxin on synaptosomal amino acid release, obtained results similar to those described above for spinal nerve-endings. Their findings combined with the observation reported by various other researchers (Brooks et al., 1957; Curtis and de Groat, 1968; Kryzhanovsky, 1973) led them to conclude that tetanus toxin was also presynaptic in action. Penitrem A given in high doses (2.5mg/kg) to mice has been shown to produce "tetanic-like" convulsions, perhaps providing further evidence for its proposed presynaptic mechanism of action.

Measurement of the synaptosomal K^+ content revealed no differences between nerve-endings prepared from treated and untreated animals. Therefore, we may conclude that penitrem A does not influence depolarization to any degree.

As mentioned above, the toxin had a different effect on amino acid release from cerebrocortical synaptosomes to that observed with spinal/medullary nerve-endings. The stimulated release of the physiologically active amino acids was unaffected by either the pure toxin or mycelium, however, both agents increased the spontaneous efflux of aspartate and glutamate and in the case of penitrem A, the release of GABA.

Despite the differing effects produced by the toxin in

these two neural regions, the net result of the toxin's action appears to be the same, this being a reduced response to veratrine stimulation.

As for spinal/medullary synaptosomes, no discernible changes in the endogenous amino acid or K^+ levels, were observed. Similarly, no deviation from the normal synaptosomal respiratory pattern was evident, providing further proof that penitrem A does not produce its action by intefering with synaptosomal metabolism e.g. amino acid synthesis. Also, any depolarizing action of the drug would have been manifested through increased respiratory rates.

As described in the introduction, Stern (1971) has shown that administration of glycine and GABA to animals suffering from penitrem A induced tremors alleviates the symptoms, whilst glutamate enhances penitrem A action. It is interesting that results consistent with abnormalities in both excitatory and inhibitory transmitter release were obtained, however, in different neural regions.

The effect of Penicillium cyclopium on sheep synaptosomes

The data obtained using sheep cerebrocortical and spinal/medullary nerve-endings represent the sum total of two experiments. Therefore, as previously mentioned these results must be treated with some caution and not too much importance may be attached to them. However, it is interesting that in the cerebral cortex Penicillium cyclopium appeared to be ineffective, thus disagreeing with the results obtained using rat cerebrocortical synaptosomes.

In the spinal cord the toxin's action also differed

from that observed in rat spinal/medullary synaptosomes. Spinal nerve-endings prepared from sheep orally dosed with the mycelium showed a reduced stimulus-induced release of aspartate, glutamate and glycine, however, unlike rat spinal medullary synaptosomes the spontaneous efflux was also inhibited. In addition, the levels of serine in the medium were substantially depleted.

Measurements of the endogenous amino acid levels gave inconsistent results, however, the data obtained with sheep spinal/medullary synaptosomes was in close agreement with that obtained with rat nerve-endings. Therefore, the assumption that Penicillium cyclopium produces its effect by an action on the presynaptic membrane is probably correct.

Striatal amino acid release presented a picture very similar to that observed with rat cerebrocortical synaptosomes, i.e. an increase in the spontaneous efflux of aspartate, glutamate and GABA. However, the extent of the efflux was much greater, the response to veratrine stimulation being effectively blocked.

The data obtained regarding dopamine release from striatal synaptosomes would tend to discount this neurotransmitter as being involved in the penitrem-induced "staggers" syndrome. This is in agreement with the findings of Stern (1971), who demonstrated that γ-hydroxybutyrate, an agent increasing brain dopamine levels (Gessa et al., 1968), does not exert any action, the same being true for the dopamine precursor L-DOPA. Selective dopamine depletion by means of α-methyl-m-tyrosine (Udenfriend and Zalzman-Nirenberg, 1962) has also been shown to remain without effect on the tremor. This lends support to the hypothesis that

there is no similarity between penitrem A induced tremor and Parkinsonian tremor with respect to biochemical changes in the corpus striatum.

The effect of Penicillium estinogenum on rat cerebrocortical synaptosomes

Nerve-endings prepared from animals pretreated with Penicillium estinogenum exhibited an enhanced spontaneous and veratrine stimulated release of aspartate and glutamate. These observed increases would tend to substantiate reports that verruculogen increases miniature end-plate potential discharge at the neuromuscular junction. As mentioned previously, an increase in m.e.p.p. frequency is believed to reflect an increased spontaneous neurotransmitter release.

Hotujac et al. (1973) have reported that verruculogen behaves as a GABA antagonist in the central nervous system and claims that brain GABA levels are substantially reduced in mice treated with the toxin, however, our data does not appear to substantiate these claims. No action was observed on synaptosomal GABA release and it may be assumed that any effect on GABA synthesis would have been reflected in an alteration in this process. Unfortunately, time did not permit the measurement of the intrasynaptosomal amino acid content. This is a pity as these measurements would have provided a better index of synaptosomal amino acid synthesis.

The data presented in this thesis could be interpreted as indicating an action on synaptosomal amino acid uptake, verruculogen functioning as an inhibitor of this process. Uptake studies using radioactively labelled amino acids will

provide the answer to this question.

How an alteration in transmitter release may cause tremor

Tremor may be interpreted as resulting from an increase in excitatory activity at the synapse. Unfortunately there is no physiological basis for this suggestion as yet. Nevertheless, the fact that in spinal/medullary synaptosomes penitrem A caused a reduction in the release of the inhibitory amino acids, thus upsetting the balance between excitation and inhibition in favour of excitation, is consistent with this view.

Similarly, in cerebrocortical synaptosomes prepared from animals treated with Penicillium cyclopium further evidence for increased excitatory funtion was obtained, the release of the excitants aspartate and glutamate being shown to increase. This was also found to be the case with animals injected with Penicillium estinogenum.

The only factor which would appear to disagree with the above hypothesis is that in the presence of the pure toxin (penitrem A), the spontaneous release of the inhibitory amino acid, GABA, was also enhanced. However, the action produced by the excitatory amino acids may well have exceeded the contribution made by GABA.

Further remarks

The primary conclusion that can be drawn from this study is that the tremorgenic agents tested produce their effects through an action on the presynaptic terminal. They do not appear to modify synaptosomal amino acid synthesis and hence influence neurotransmitter release via this

mechanism. Respiratory rates measured for nerve-endings prepared from animals pretreated with the mycotoxins provided confirmatory evidence for their ineffectiveness as modulators of synaptosomal metabolism.

In the future, Na^{+} and Ca^{2+} dependence studies will have to be conducted in order to determine whether either of these ions influence the toxins' actions on synaptosomal amino acid release. Thus, it is possible to discover if one is dealing with a purely release or uptake phenomenon.

In the case of Penicillium estinogenum amino acid uptake studies may provide a further insight into the mode of action of verruculogen. Confirmatory evidence of its lack of effect on synaptosomal amino acid synthesis and depolarization (determined by measuring the synaptosomal content of amino acid and K^{+}) will also have to be obtained.

Finally, it would have been interesting to have tested some of the drugs used by Stern (1971) to relieve the symptoms of penitrem action, to see whether the effects observed on amino acid release were preventable.

CHAPTER SEVEN

SOME STUDIES ON A LOCUST NERVE-MUSCLE PREPARATION

INTRODUCTION

METHODS AND MATERIALS

Treatment of haemolymph samples for amino acid analysis.

Intracellular recording.

RESULTS

Measurement of the amino acid content of locust haemolymph.

Spontaneous miniature potentials from locust extensor tibiae muscle fibres.

Miniature end-plate potential activity in relation to the volume of locust saline added.

The action of a known depolarizing agent (K^+) on miniature end-plate potential discharge.

The effect of L-glutamate and kainic acid on miniature end-plate potential activity.

The effect of β-bungarotoxin on miniature end-plate potential

discharge.

DISCUSSION

The amino acid composition of locust haemolymph.

The effect of potassium on miniature end-plate potential discharge.

The effect of L-glutamate and kainic acid on miniature end-plate potential discharge.

The effect of β-bungarotoxin on miniature end-plate potential discharge.

Further Remarks

INTRODUCTION

Some of the agents used in synaptosomal studies on amino acid release were tested for their effects on miniature end-plate potential activity in a neuromuscular junction preparation. It was thought that such a preparation would provide a more biologically meaningful system and it was hoped that a further insight into the workings of these agents might be forthcoming.

Depending upon the response elicited in the muscle fibre it is possible to determine an agents site of action. An increase or decrease in miniature end-plate potential frequency would tend to suggest an action on the presynaptic nerve terminal, alternatively, a change in the amplitude of spontaneous potentials indicates an action on the post-synaptic (muscle fibre) membrane.

Spontaneous potential changes have been observed in a variety of invertebrate and vertebrate preparations. For example, Fatt and Katz (1952) have described miniature end-plate potentials occurring at the frog neuromuscular junction and it has been suggested that quantal "packets" of acetylcholine released from the presynaptic terminals of the motor nerve are responsible for the transient depolarizations produced at the postsynaptic muscle membrane. Similarly, Dudel and Orkand (1960) reported the occurrence of spontaneous potential changes at crustacean neuro-muscular junctions and found that these closely resembled vertebrate miniature end-plate potentials.

It was Usherwood (1961) who first demonstrated spontaneous miniature end-plate potential activity at the insect neuromuscular junction. Using the giant South American cockroach, Blaberus giganteus, he recorded randomly

occurring spontaneous potentials of up to 1mV, from single muscle fibres of the extensor tibia. He observed considerable variations in both their amplitude and rise-times, however, these findings are consistent with the multi-terminal nerve innervations known to be present in insects (Hoyle, 1955a, b).

Miniature end-plate potentials have also been recorded from the metathoracic extensor tibia muscle fibres of the locust Schistocerca gregaria (Usherwood, 1961, 1963) and it was this preparation that was chosen for this study, having been particularly well characterized by Usherwood and his colleagues.

It is now well established that glutamic acid is the excitatory transmitter at insect nerve-muscle synapses. Usherwood et al. (1968), using a perfusion system, have demonstrated the electrically-induced release of this amino acid from an isolated retractor unguis nerve-muscle preparation. Under their conditions two ions, Ca^{2+} and Mg^{2+}, known to influence neuromuscular transmission were found to affect glutamate release. Ca^{2+} which potentiates neuromuscular transmission was shown to increase glutamate release, whereas Mg^{2+} which inhibits transmission, markedly reduced glutamate release. This information therefore provides evidence for a strong link between neurotransmission and neurotransmitter release.

Another interesting observation made by Usherwood and his coworkers (1968) was that 5HT, which is known to block transmission at locust excitatory nerve-muscle synapses, potentiated the release of glutamate two fold during stimulation. It has been proposed that 5HT may produce this effect by competing with the transmitter for receptor

sites on the post-synaptic membrane (Hill and Usherwood, 1961).

Topical application of L-glutamate to muscles of the locust has been shown to elicit an excitatory response (Usherwood et al., 1968; Usherwood and Grundfest, 1965; Beranek and Miller, 1968). Similar results have been obtained with the muscles of the grasshopper (Usherwood and Grundfest, 1965) and cockroach (Usherwood and Grundfest, 1965; Kerkut et al., 1965), thus providing further evidence for glutamate's proposed role as the excitatory transmitter at insect neuromuscular synapses. Usherwood et al. (1968) took these iontophoretic studies a stage further and showed that only very circumscribed areas on the muscle fibres were sensitive to glutamate, the magnitude of the glutamate response at these sites being directly proportional to the amount of glutamate applied. The distance between the glutamate and recording electrodes was also found to be critical (Usherwood, 1969). These glutamate sensitive areas were shown to coincide with excitatory synaptic sites (Beranek and Miller, 1968; Usherwood and Machili, 1968).

For this study the distal fibres of the extensor tibialis muscle of the locust (Schistocerca gregaria and Locusta gregaria) metathoracic leg were used. This muscle has been shown to be particularly rich in glutamate receptors. In fact, evidence has been obtained suggesting the existence of two separate populations of receptor referred to as junctional and extrajunctional receptors (Cull-Candy and Usherwood, 1973; Usherwood and Cull-Candy, 1974). In these studies the glutamate analogue ibotenic acid was used as a specific probe for the non-synaptic receptors.

The presence of extrajunctional acetylcholine receptors on vertebrate skeletal muscle fibres is well established (Miledi, 1960; Katz and Miledi, 1964). However, in the locust these receptors were subsequently found to be of two types which Cull-Candy (1975, 1976) designated D and H. The biphasic response (depolarization preceeding hyper-polarization) seen when glutamate is applied iontophoretically to extrajunctional regions of locust muscle results from simultaneous activation of these two types of receptor (Cull-Candy, 1975, 1976). The D receptor (which mediates a depolarization) is activated by L-glutamate but insensitive to the glutamate analogue DL-ibotenate, while the H receptor (which mediates a hyperpolarization) is activated by both L-glutamate and DL-ibotenate.

Following denervation locust muscle was found to develop a high degree of extrajunctional sensitivity to glutamate (Usherwood, 1969). This phenomenon may be compared with the situation encountered in vertebrate muscle where following denervation the extrajunctional regions become more sensitive to acetylcholine (Axelsson and Thesleff, 1959; Miledi, 1960).

Rees (1974) has shown that application of a number of metabolic inhibitors to nerve-muscle synapses on 'white' and 'red' fibres in the retractor unguis muscles of P. americana and B. giganteus results in a dramatic increase in the spontaneous miniature potential discharge, this being accompanied by a summation of the miniature potentials to form 'composite' potentials.

A brief mention must now be made of GABA, which is believed to be the inhibitory transmitter at synapses on

insect skeletal muscle fibres (Usherwood and Grundfest, 1964, 1965; Kerkut et al., 1965; Usherwood, 1968). Using an iontophoretic technique Usherwood (1973) has demonstrated the existence of GABA sensitive sites on locust metathoracic coxal adductor muscle fibres. These GABA sensitive sites have been shown to correspond with the inhibitory synapses on these muscles.

Initial studies for the chapter involved investigating the amino acid composition of locust haemolymph (which bathes the muscles) in order to determine the concentrations of the putative transmitters present. In a similar study, Miller and his coworkers (1973) found that in the crab (Carcinus maenas) 80 per cent of blood glutamate was associated with the haemocytes, whilst in the locust (Schistocerca gregaria) most of the glutamate occurred in the plasma. This plasma borne glutamate was subsequently shown to be pharmacologically inactive *in vivo*, although it was found to develop pharmacological activity 5 - 60 mins *ex situ*. Miller et al., (1973) concluded that in both the crab and locust, the high blood glutamate levels did not affect excitatory neuromuscular transmission.

Studies on miniature end-plate potential activity in the extensor tibialis nerve-muscle preparation were initiated by determining the volume of locust saline that could be added without altering potential frequency or amplitude. This was followed by an examination of the effects of a known depolarizing agent, K^+. Using a range of K^+ concentrations the influence on depolarization and m.e.p.p. frequency was investigated. In a similar study using the neuromuscular junction of the rat, Liley (1956) demonstrated

a strong relationship between K^+ concentration and m.e.p.p. frequency.

The actions of the excitants glutamate and kainate were also tested in this study.

Finally, an attempt was made to elucidate the mechanism of action of the polypeptide neurotoxin, β-bungarotoxin. It has been suggested that β-bungarotoxin may function as a depolarizing agent (Sen and Cooper, 1978) and as mentioned in Chapter 5 using vertebrate neuromuscular preparations, several groups (Chang et al., 1973; Kelly and Brown, 1974) have shown this agent to increase m.e.p.p. frequency. However, this increase in spontaneous potential frequency is subsequently followed by a complete inhibition of neuromuscular transmission.

METHODS AND MATERIALS

Treatment of haemolymph samples for amino acid analysis

Haemolymph was collected as it ran from an incision in the thorax of the animal. Aliquots were diluted with an equal volume of 10% TCA, in order to precipitate blood protein, and centrifuged. After centrifugation the supernatants were purified and analysed as previously described (Chapter 2).

The results obtained were expressed as concentrations for comparison with the data of Miller et al (1973).

Intracellular recording

All experiments in this study were performed on fibres from the metathoracic extensor tibiae muscles of the locusts Schistocera gregaria and Locusta migratoria. Figure 7.1 shows the anatomy and innervation of the metathoracic leg as described by Hoyle (1955a, b).

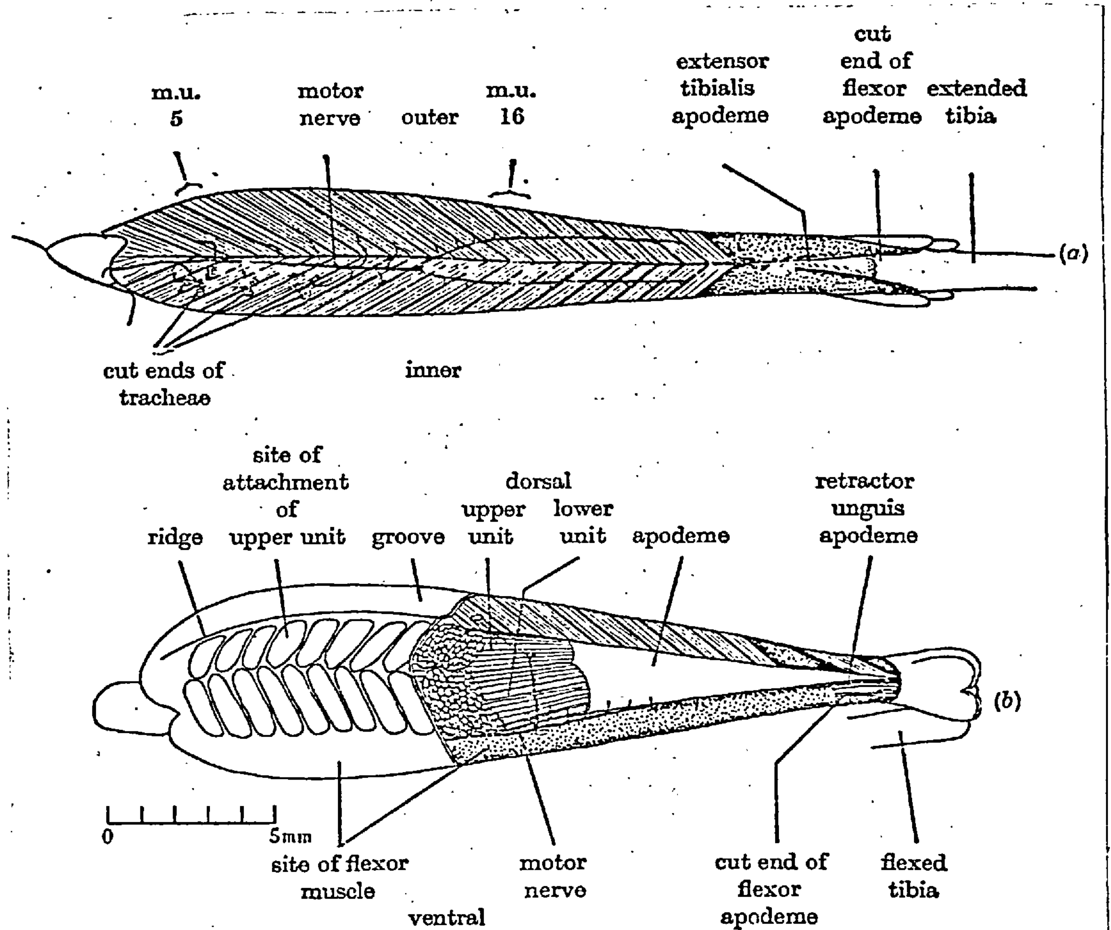
Hind legs were removed from adult insects and the femoral segments mounted horizontally in a perfusion bath by attaching the external surface to the wall of the bath with Tackiwax (Figure 7.2). A large proportion of the exoskeleton was then removed and the underlying tissue dissected away to expose the extensor tibialis muscle (as described by Usherwood, 1968).

The dissection and setting up procedures were performed in locust saline of the following composition: NaCl, 140; KCl, 10; $CaCl_2$, 2; NaH_2PO_4, 4; Na_2HPO_4, 6mM, buffered at pH 6.8.

Recordings were made using glass microelectrodes filled

FIGURE 7.1

ANATOMY AND INNERVATION OF THE LOCUST METATHORACIC LEG

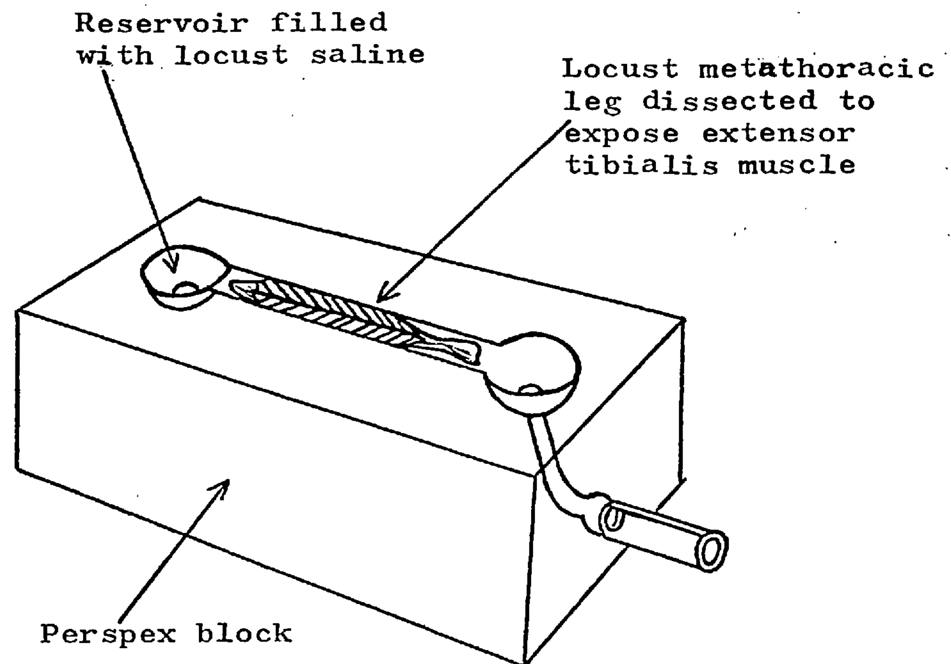


(*a*) Ventral view of the extensor tibialis muscle of the jumping leg of the left side after removal of all other muscles, tracheal and nerve trunks and the diaphragm. Only a few of the cut ends of tracheae are shown. The margins of two muscle units of the ventral row, outer side are indicated. (*b*) Lateral view of the extensor muscle of the jumping leg of the right side after partial dissection, to show the mode of attachment of the muscle units to the apodeme. Note the additional row of units on the upper margin of the inner side. These can be exposed in the intact femur by removing the strip of cuticle on the upper crest on the outer side.

Taken from Hoyle, 1955.

FIGURE 7.2

LOCUST PERFUSION BATH



Diagrammatic representation of perfusion bath and isolated extensor tibialis muscle. NB Muscle not drawn to scale.

with 1M KCl and of 5 - 20 MΩ resistance, together with conventional recording apparatus (Katz, 1966). Permanent records of spontaneous miniature end-plate potential activity were made using a multichannel pen recorder set at 6 amplitude thresholds (50µV, 75µV, 100µV, 150µV, 200µV and 250µV) and a cassette tape recorder. The sensitivity of the pen recorder was adjusted from experiment to experiment, depending upon the miniature end-plate potential activity encountered.

Drugs dissolved in locust saline and buffered at pH 6.8, were applied to the neuromuscular preparation from a microsyringe via a multibarreled micropipette. In each experiment the micropipette and recording electrode tips were positioned 3 - 4 muscle fibres apart, in order to provide consistent recording conditions between experiments. As outlined previously, Usherwood (1969) studying the effects of iontophoretically applied glutamate on muscle fibre sensitivity, discovered that the distance between the glutamate and recording electrodes strongly influenced the type of result obtained.

Between drug additions the preparation was thoroughly washed with fresh locust saline.

All experiments were conducted at room temperature (approx 18 - 20°C), and the muscle was allowed 1hr at the start of each experiment to equilibrate with the saline.

Finally, only muscle fibres exhibiting membrane potentials of-40mV and above were used for these studies.

RESULTS

Measurement of the amino acid content of locust haemolymph

Table 7.a shows the amino acid levels that were detected in deproteinized locust haemolymph samples. The results obtained by Miller et al. (1973) are presented in the same table for comparative purposes.

Spontaneous miniature potentials from locust extensor tibiae muscle fibres

Figure 7.3 shows a typical population of miniature end-plate potentials recorded from a distal fibre of the locust extensor tibialis muscle. These potentials were found to vary considerably in both their amplitudes and rise-times (c.f. Usherwood, 1961, 1963). The smaller amplitude potentials were by far the most numerous nevertheless, potentials of up to 1mV were recorded.

The frequency and amplitude of miniature end-plate potentials were also found to vary greatly between preparations. Figure 7.4 gives miniature end-plate potential frequency profiles for all the amplitude thresholds measured, from three separate preparations.

Miniature end-plate potential activity in relation to the volume of locust saline added

Varying the volume of locust saline applied to the neuromuscular preparation from a multibarreled pipette, was found to strongly influence m.e.p.p. frequency.

Figure 7.5 illustrates how a saline addition of as little as 25µl causes a substantial increase in the

TABLE 7.a

AMINO ACID CONTENT OF HAEMOLYMPH OBTAINED FROM SCHISTOCERCA GREGARIA

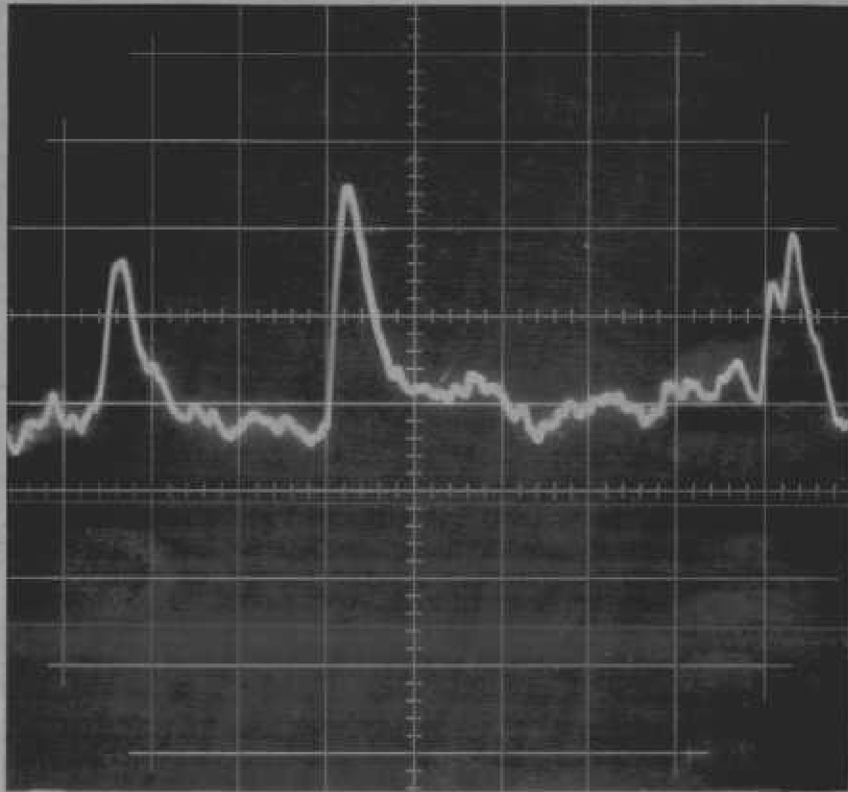
Amino acid	Total Concentration in haemolymph (mM)	Total Concentration in haemolymph (mM) - calculated from the data of Miller et. al., 1973
Aspartate	0.38±0.05	0.30
Threonine	2.16±0.32	3.12
Serine	6.80±0.58	} 14.20
Glutamine	5.18±0.89	
Glutamate	3.94±0.20	0.59
Glycine	20.34±0.86	13.70
Alanine	4.79±1.01	6.60
Valine	2.53±0.5	3.18
Isoleucine	1.39±0.33	1.35
Leucine	1.89±0.68	1.78
Tyrosine	4.89±0.66	2.22
Phenylalanine	3.27±0.68	0.67
Histidine	4.39±0.73	1.73
Lysine	5.11±0.40	2.57
Arginine	1.41±0.24	2.09
	*	

Amino acid levels were determined in purified TCA extracts, prepared from haemolymph, collected from an incision made in the locust thorax.

* Values are means ± S.E.M. for 4 determinations.

FIGURE 7.3

MINIATURE END-PLATE POTENTIALS RECORDED FROM A DISTAL FIBRE OF THE LOCUST EXTENSOR TIBIALIS MUSCLE



The above is a photograph of a typical population of spontaneous miniature potentials registered on the screen of an oscilloscope.

Each division on the photograph is equivalent to 1cm on the oscilloscope screen. The vertical calibration is equal to 100μV/cm and the horizontal (time) calibration was set at 50msec/cm.

FIGURE 7.4

SPONTANEOUS MINIATURE POTENTIALS RECORDED FROM THREE LOCUST EXTENSOR TIBIAE NEUROMUSCULAR PREPARATIONS

Potentials were recorded at 6 amplitude thresholds (50µV, 75µV, 100µV, 150µV, 200µV and 250µV) from three separate neuromuscular preparations. The purpose of this study was to examine the variation in m.e.p.p. frequency and amplitude occurring between muscle fibres.

● - 50µV threshold

○ - 75µV threshold

▲ - 100µV threshold

△ - 150µV threshold

■ - 200µV threshold

□ - 250µV threshold

FIGURE 7.4

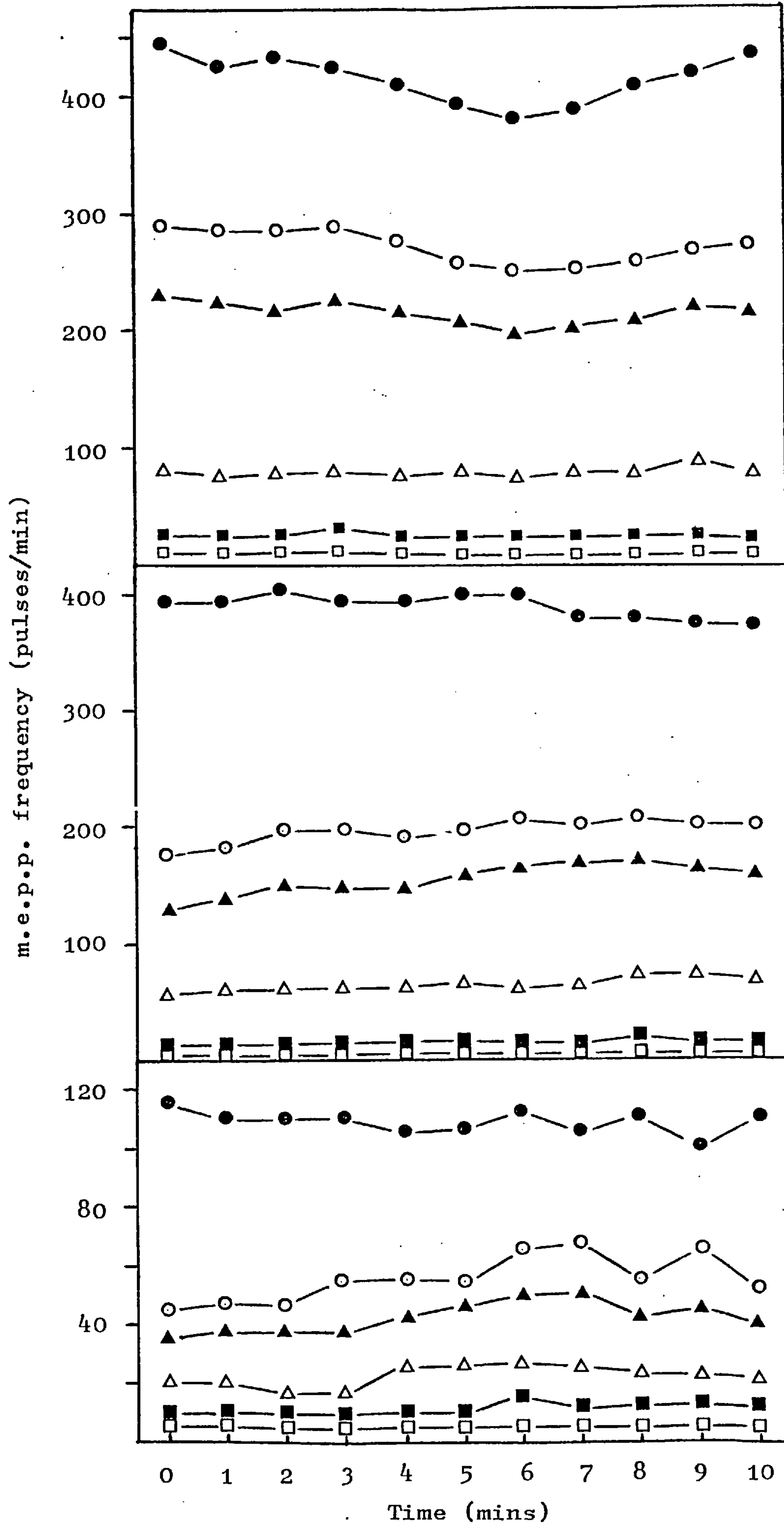


FIGURE 7.5

MINIATURE END-PLATE POTENTIAL ACTIVITY IN RELATION TO THE VOLUME OF LOCUST SALINE ADDED

Spontaneous potentials were recorded for a control period, whereupon, 25µl of locust saline was added (as indicated by the arrow) from a multibarrelled pipette and recording continued. The figure shows the response elicited by this addition on potentials at the 50, 75 and 100µV thresholds.

The data shown is representative of three experiments.

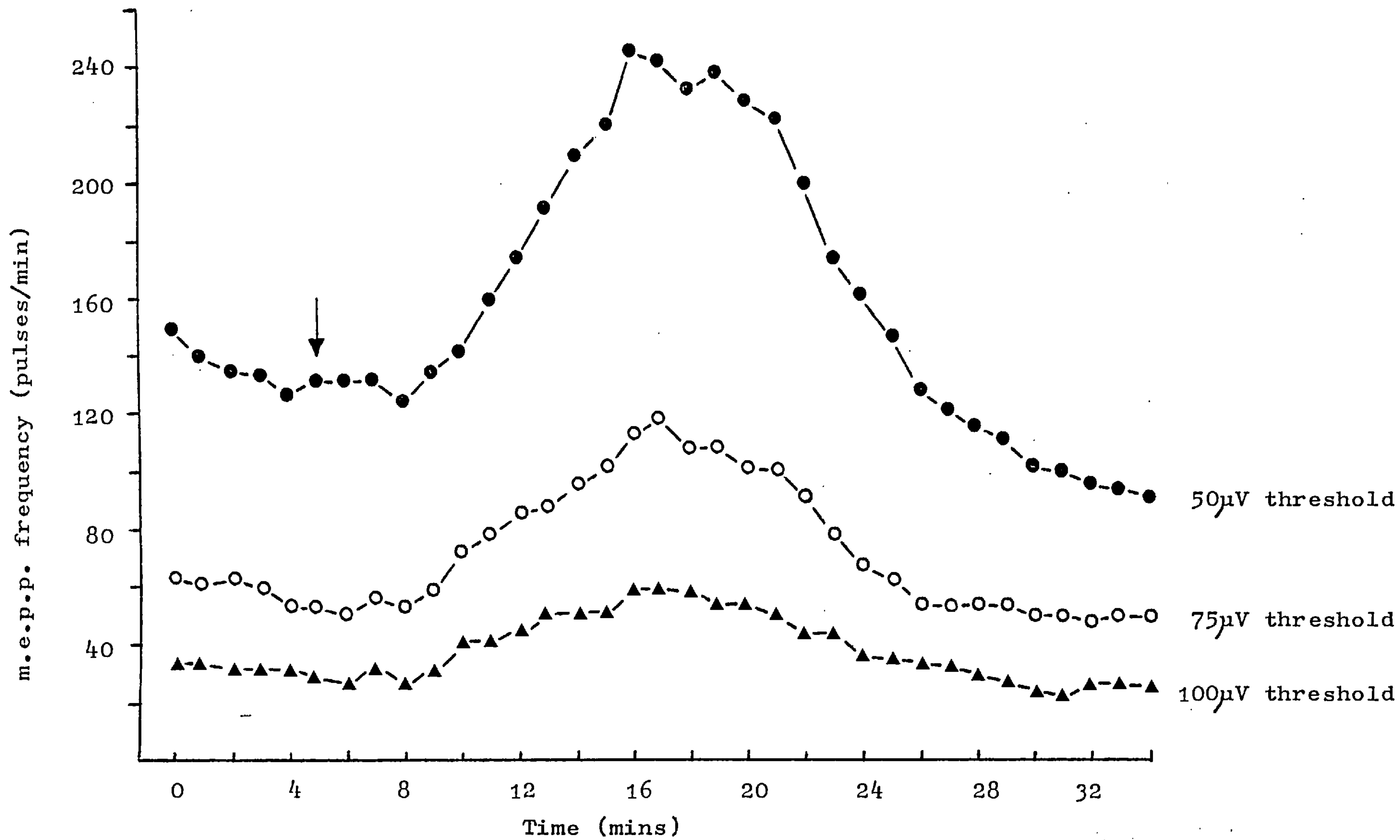


FIGURE 7.5

frequency of spontaneous potentials in the lower (50 - 100μV) amplitude ranges. However, no change in the frequency of higher amplitude potentials was observed.

A 10μl addition was found to have no effect on m.e.p.p. frequency at any of the amplitude threshold levels measured. Therefore, in all subsequent experiments in which the effects of various agents were tested, 10μl additions were made.

<u>The action of a known depolarizing agent (K^+) on miniature end-plate potential discharge</u>

Altering the potassium concentration of the bathing medium affected both the amplitude and frequency of miniature end-plate potential discharge.

A range of K^+ concentrations (10, 25, 75, 150 and 300mM - i.e. K^+ concentration in the micropipette) were applied to the neuromuscular preparation. Ten and 25mM KCl were found to exert no influence on m.e.p.p. activity when ejected from the micropipette, however, concentrations of 75, 150 and 300mM increased the frequency and amplitude of all potentials dramatically. This result is partly in agreement with that of Usherwood (1963), however instead of an increase in m.e.p.p. amplitude, he reported a reduction.

Unfortunately, it was not possible to determine the peak frequency of these pulses as the discharges became too numerous. Despite this problem figure 7.6 clearly illustrates the nature of the response produced by this agent and as regards a link between K^+ concentration and m.e.p.p. frequency, 300mM KCl certainly appears to have a

FIGURE 7.6

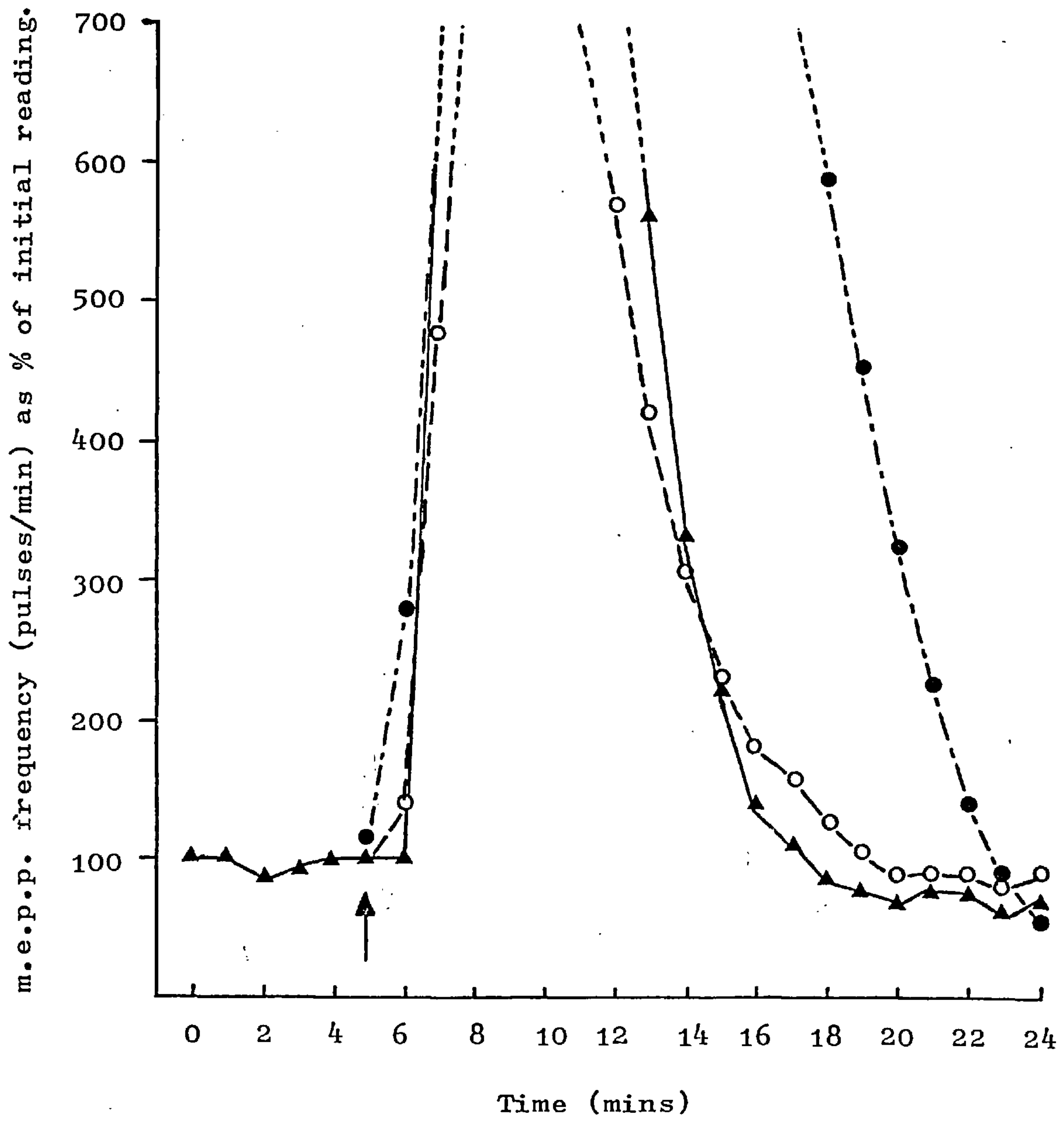
THE EFFECT OF KCl ON MINIATURE END-PLATE POTENTIAL DISCHARGE

Spontaneous potentials were recorded for a control period, whereupon, 10µl of a concentrated KCl solution was added from a micropipette (indicated by arrow) and recording continued.

The figure shows the effect produced by three concentrations of KCl on m.e.p.p. frequency at the 100µV threshold.

The data represents typical results from 4-6 experiments.

FIGURE 7.6



▲ — 75 mM KCl (micropipette concentration)

○ — 150 mM KCl (micropipette concentration)

● — 300 mM KCl (micropipette concentration)

more dramatic and longer lasting action than the other two concentrations.

The relationship between K^+ concentration in the pipette and depolarization as determined by measuring the decrease in the membrane potential of the muscle fibre is shown in Figure 7.7. Usherwood (1963) claims that doubling the K^+ content of locust saline (i.e. from 10 to 20mM) produces a mean depolarization of the muscle fibre of approximately 12.5mV.

The concentration quoted by Usherwood (1963) represents the local concentration of K^+ surrounding the muscle fibre. In contrast, our K^+ levels refer to the concentration in the barrel of the micropipette positioned approximately 6mm from the fibre. In order to relate our data to that of Usherwood's (1963) use was made of the observation (Usherwood, 1963) that a 10mM increase in the bath K^+ concentration produces a 12.5mV change in the membrane potential. It was assumed that depolarization is linearly related to bath K^+ concentration and the curve of best fit (seen in Figure 7.7) was constructed, using quadratic regression analysis of depolarization versus micropipette concentration. Using this curve it was possible to determine the local concentration at the muscle fibre for a given concentration of KCl ejected from the micropipette.

The same curve was used to calculate the local concentrations of some of the other (small molecular weight) agents used in this study, by assuming that the rate of diffusion of these substances from the pipette tip was approximately the same as that for K^+.

Calculated local concentrations are given in parentheses

FIGURE 7.7

RELATIONSHIP BETWEEN K^+ CONCENTRATION AND DEPOLARIZATION OF THE MUSCLE FIBRE

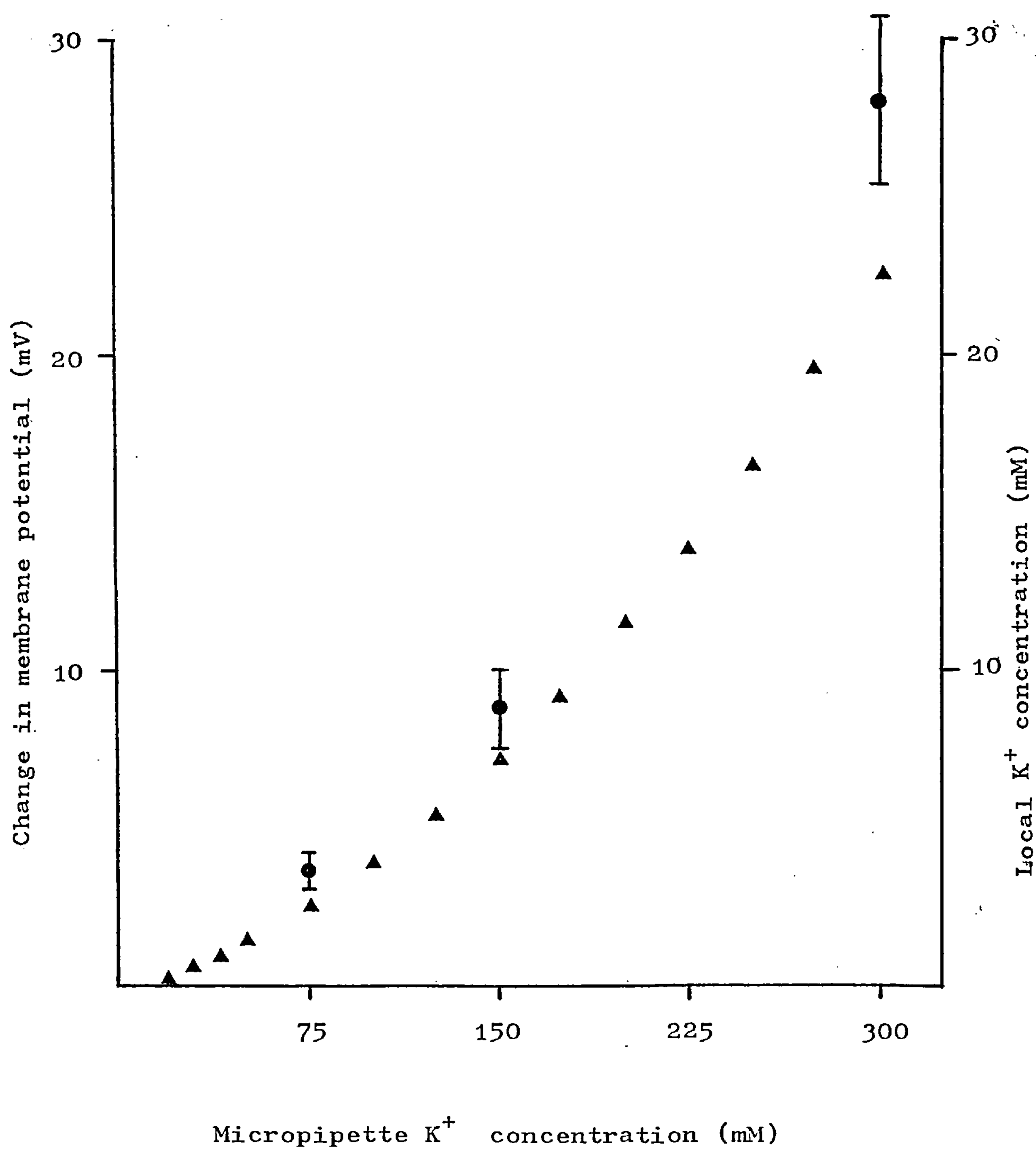
Muscle fibres were impaled with a microelectrode, special note of the membrane potential being taken. The tissue was then allowed to equilibrate for a short period, whereupon, 10µl of a concentrated KCl solution was added from a micropipette. The change (decrease) in the membrane potential was then noted.

● - change in the membrane potential produced by 75, 150 and 300mM (barrel concentration) KCl (represents data from 4-6 experiments)

▲ - calculated local K^+ concentration surrounding the muscle fibre, under our experimental conditions.

On the basis of the quadratic regression analysis method used, the calculated local K^+ concentration s seen by the muscle fibre upon ejection of 75, 150 and 300mM KCl from the micropipette,were 2.4, 7.1 and 22.5mM,respectively.

FIGURE 7.7



after the quoted concentration of substance ejected from the barrel.

Usherwood (1961) has shown that altering the tonicity of the bathing medium influences m.e.p.p. activity. Therefore, in order to confirm that the results obtained with KCl were specific to this agent, the effects of the corresponding concentrations of NaCl were tested. Certainly at the higher concentrations, application of NaCl produced a small increase in m.e.p.p. frequency in the lower amplitude threshold range. However, nothing of the order of the effects observed with KCl were obtained and no action on the membrane potential was detected.

Figure 7.8 shows the effect the application of 150mM (7.1mM - calculated local concentration) NaCl had on pulse frequency at the 100μV threshold.

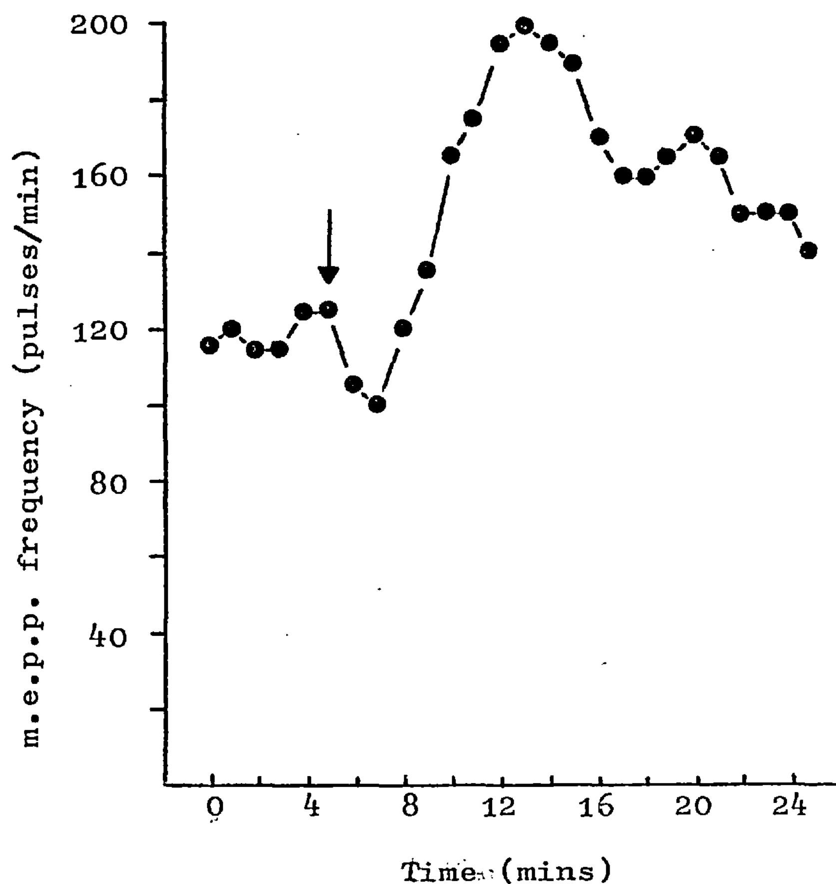
The effect of L-glutamate and kainic acid on miniature end-plate potential activity

50mM (1.3mM - calculated local concentration) glutamate and kainate were found to produce very similar effects on miniature end-plate potential activity. Both agents drastically reduced the incidence of potentials at all the threshold levels measured. Figures 7.9 and 7.10 illustrate typical responses elicitedat the 100μV threshold.

In view of the fact that both agents are believed to function as excitants, acting through the same receptor the afore mentioned result is perhaps surprising. Since both glutamate and kainate depolarized the muscle membrane it seems likely that both agents are interacting with the postsynaptic receptor but that desensitisation follows (see

FIGURE 7.8

THE EFFECT OF NaCl ON MINIATURE END-PLATE POTENTIAL DISCHARGE

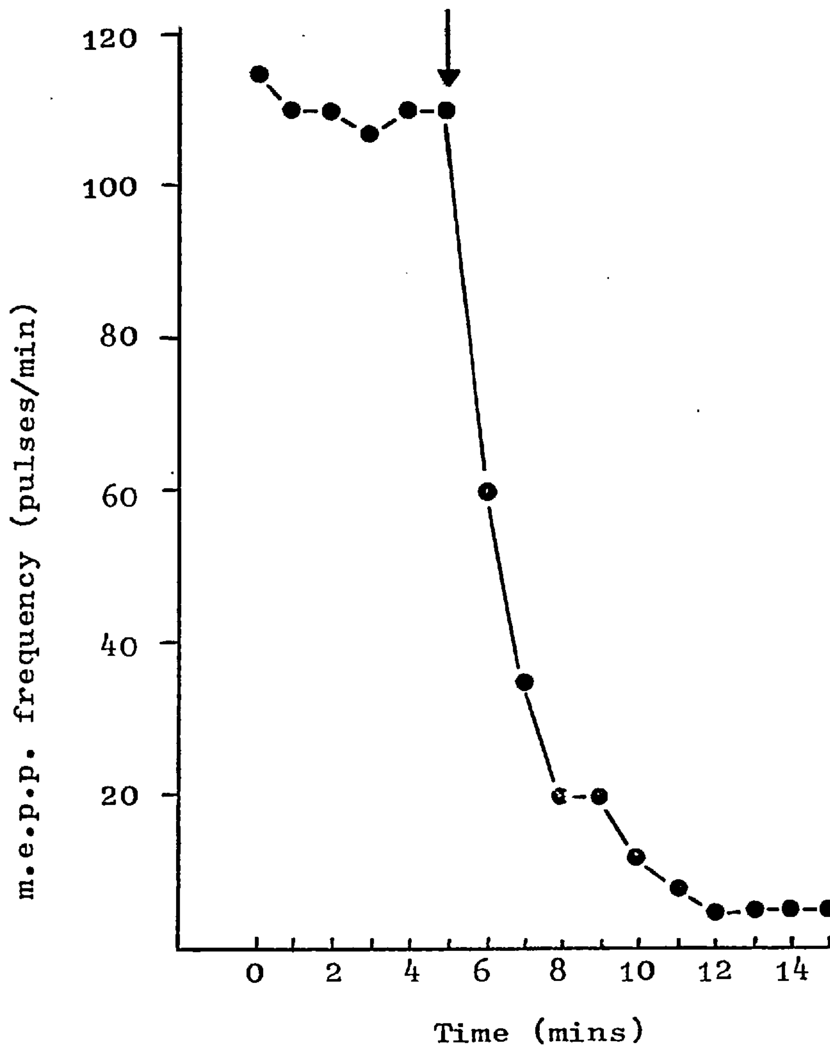


M.e.p.p.'s were recorded for a control period, whereupon 10µl of 150mM NaCl (7.1mM - calculated local concentration) was added (as indicated by arrow) and recording continued.

The above shows the response elicited by NaCl on m.e.p.p. discharge at the 100µV threshold and is representative of 2 experiments.

FIGURE 7.9

THE EFFECT OF L-GLUTAMATE ON MINIATURE END-PLATE POTENTIAL ACTIVITY



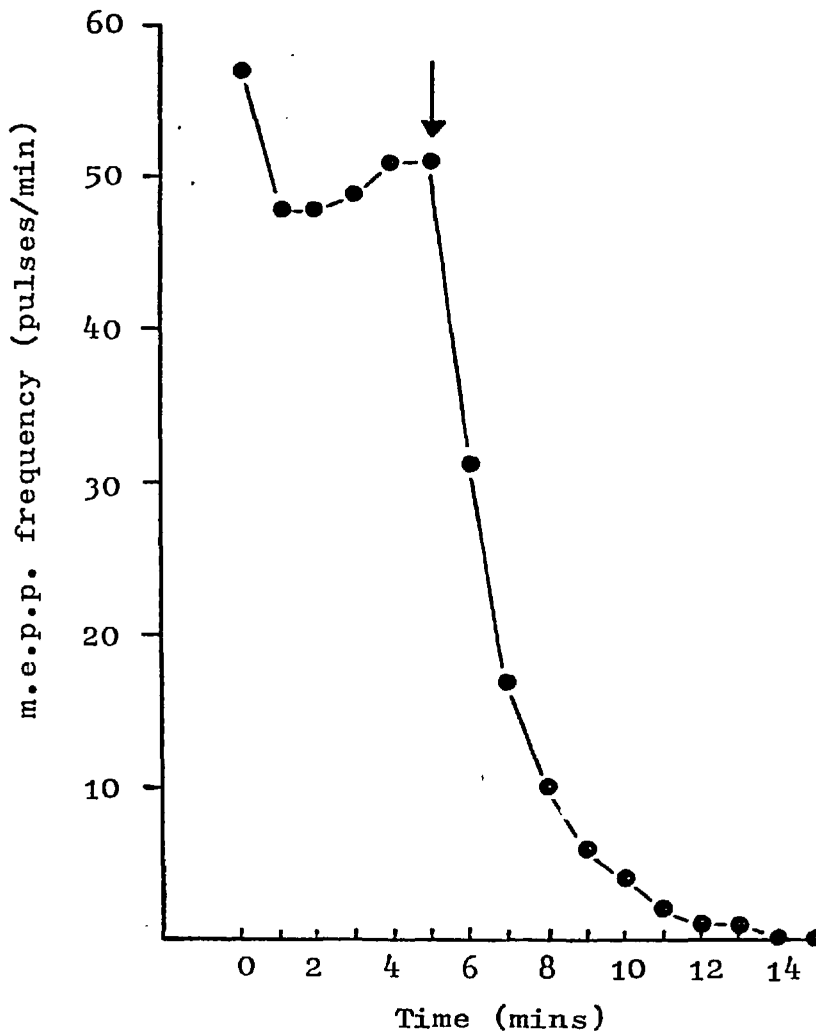
Spontaneous potentials were recorded for a control period, whereupon 10µl of 50mM L-glutamate (1.3mM - calculated load concentration) was added (at point indicated by arrow) from a multibarreled pipette and recording continued.

The above shows the response elicited by this agent on potentials at the 100µV threshold.

Data represents a typical result from 6 experiments.

FIGURE 7.10

THE EFFECT OF KAINIC ACID ON MINIATURE END-PLATE POTENTIAL ACTIVITY



Miniature end-plate potentials were recorded for a control period. 10µl of 50mM kainate (1.3mM calculated local concentration) was then added and recording continued.

The above illustrates the effect produced by kainate on m.e.p.p. discharge at the 100µV threshold.

The figure represents a typical result from 4 experiments.

Cull-Candy, 1976). The results obtained using K^+ shows that depolarization is otherwise associated with an increase in m.e.p.p. frequency.

Finally, it is interesting to note that whereas glutamate decreased the membrane potential by 2-3mV, kainate produced a 40 - 50mV reduction. This observation may be a reflection of the relative effectiveness of these two excitants in their interaction with the receptor and activation of the ionophore. Biscoe et al. (1975) claim to have shown that kainic acid is 40 - >200 times more potent than L-glutamate in rat spinal interneurones.

The effect of β-bungarotoxin on miniature end-plate potential discharge

Figure 7.11 shows the effect of β-bungarotoxin (100μg/ml pipette concentration, 5.6 μg/ml - approximate local concentration) on m.e.p.p. discharge in the 100μV range.

It was found to substantially and reversibly increase m.e.p.p. frequency at all the amplitude thresholds determined. However, unlike the reports of Chang et al. (1973) and Kelly and Brown (1974) this increase in m.e.p.p. frequency was not followed by a complete block of neuromuscular transmission.

At this concentrations β-bungarotoxin was also found to produce a 1.5 - 2.5mV decrease in the membrane potential. 10μg/ml (approximate local concentration - 0.6 μg/ml). β -bungarotoxin had no effect on m.e.p.p. activity.

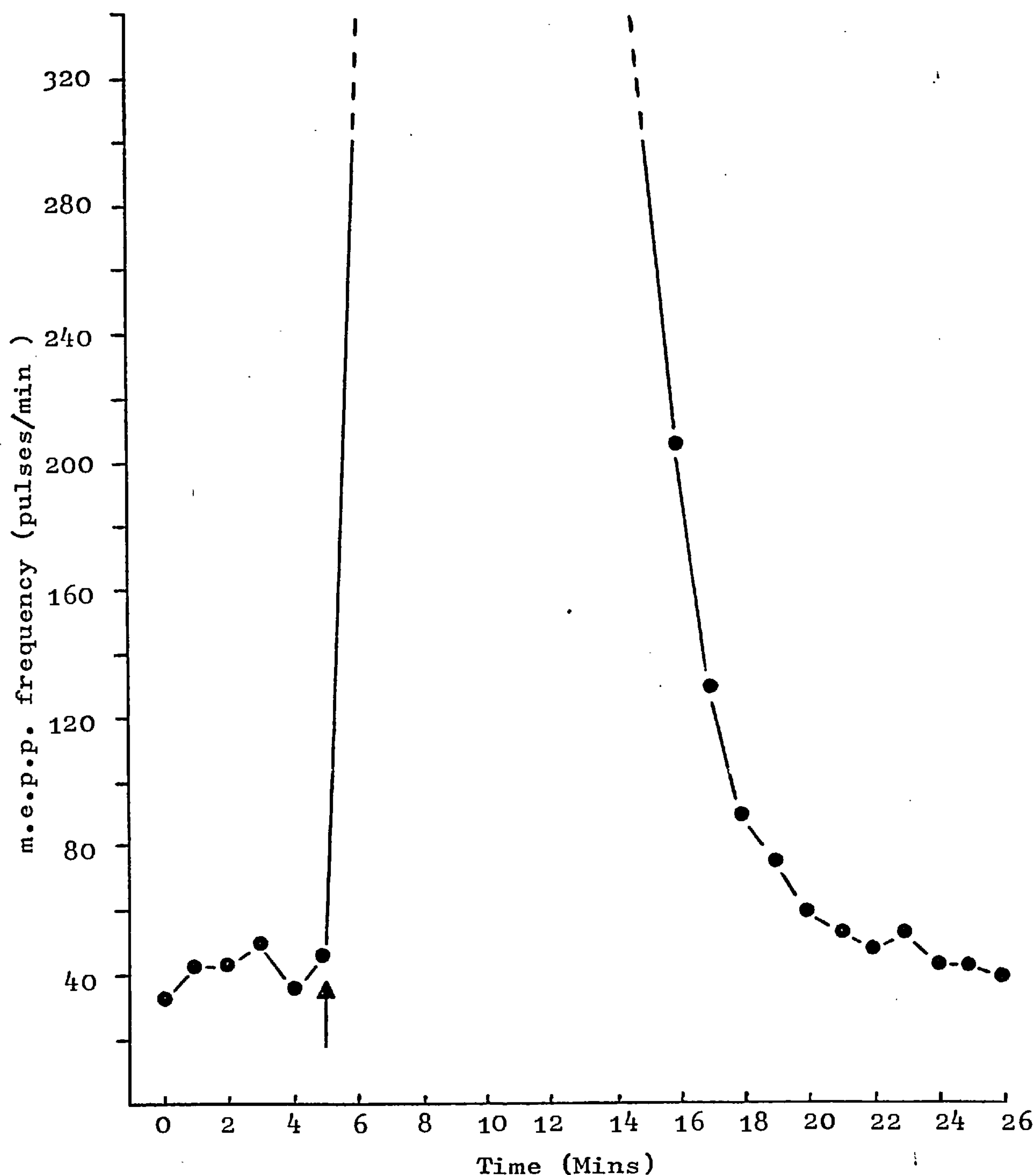
DISCUSSION

The amino acid composition of locust haemolymph

On the whole, the amino acid levels detected in our

FIGURE 7.11

THE EFFECT OF β-BUNGAROTOXIN ON MINIATURE END-PLATE POTENTIAL DISCHARGE



Potentials were recorded for a control period, whereupon β-bungarotoxin (5.6 µg/ml - approximate local concentration) was added and recording continued.

The figure shows the effect produced by β-bungarotoxin on m.e.p.p. discharge at the 100µV threshold and is a typical result from 4 experiments.

samples differed quite considerably from those recorded by Miller et al. (1973). This may have resulted from the cruder method of tissue extraction employed with our samples. Nevertheless, like Miller and his coworkers (1973), the levels of serine/glutamine and glycine were shown to predominate. Again as reported by Miller et al. (1973), GABA, which is believed to be the inhibitory transmitter at synapses on insect skeletal muscle fibres (Usherwood and Grundfest, 1964, 1965; Kerkut et al., 1965; Usherwood, 1968) was found to be totally absent.

Glutamate which is now generally accepted as being the excitatory transmitter at insect neuromuscular synapses, occurred in our samples at much higher concentrations than those reported by Miller et al (1973). It has been suggested that the presence of glutamate in arthropod blood, casts some doubt on glutamate's proposed role as an excitatory transmitter. Certainly, the high levels detected under our conditions, could be interpreted as adding further weight to this criticism.

As mentioned in the introduction, locust blood has been shown to be pharmacologically inactive (as determined by bioassay using the locust retractor unguis muscle) in vivo, although it develops activity 5 - 60 min ex situ. This increase in pharmacological activity has been demonstrated to parallel an increase in glutamate levels appearing in the plasma (Miller et al., 1973). It has therefore been proposed that this increase in the glutamate concentration is as a result of its release from some bound form in the plasma. The data obtained by Miller et al. (1973) discounted the blood cells as being

the carrier for glutamate and it has been suggested that a plasma protein may perform this carrier function.

It is worth noting that glutamine is particularly rich in locust haemolymph. Bradford and Ward (1975) have obtained evidence that glutamine, under certain conditions, is able to maintain the pool sizes of mammalian synaptosomal amino acids, which glucose alone does not. Glutaminase was found concentrated in these synaptosomes preparations. Arguing by analogy it seems possible that the high levels of glutamine detected in locust blood may be a pharmacologically inactive precursor for glutamate used at the neuromuscular junction.

The effect of potassium on miniature endplate potential discharge

Several groups (Liley, 1956; Takeuchi and Takeuchi, 1961; Usherwood, 1963) have shown that when isolated neuromuscular junction preparations are exposed to high potassium concentrations there is an increase in miniature endplate potential discharge that has been attributed to depolarization of the presynaptic nerve terminal. Using the locust neuromuscular preparation a very similar result was obtained. However, in our experiments the m.e.p.p. amplitude was also found to increase, Usherwood (1963) reported a decrease under their conditions. This discrepancy may result from the differing conditions under which the experiments were performed. Whereas, Usherwood bathed the whole neuromuscular preparation in medium containing a high concentration of K^+, our experiments involved loading small quantities (10µl) of KCl onto the individual muscle fibres, thus allowing the agent to rapidly diffuse away. Thus we

applied relatively brief periods of depolarization whilst Usherwood (1963) exposed the fibres to chronic depolarization.

Liley (1956) using a rat phrenic nerve diaphragm preparation has demonstrated a relationship between m.e.p.p. frequency and K^+ concentration. In our system maximal pulse discharges were far too frequent for accurate measurement with our equipment.

However, a clear correlation between K^+ concentration and muscle membrane potential change was obtained.

As outlined in the introduction an action on the presynaptic nerve terminal membrane potential would manifest itself as an increase or decrease in m.e.p.p. discharge due to a change in rate of quantal release of transmitter discharge. As K^+ is known to cause neurotransmitter release in a variety of neural preparations, the results obtained with this agent using neuromuscular preparations provide a good correlation between m.e.p.p. frequency and transmitter release.

The results obtained, using high concentrations of NaCl provided confirmatory evidence for the tonicity effects observed by Usherwood (1961). Lower concentrations of NaCl provided a useful control addition.

The effect of L-glutamate and kainic acid on miniature end-plate potential discharge

The retractor unguis muscle of the locust is known to respond to applied glutamate with depolarization (Usherwood 1969). The depolarizations have been shown to occur only at very circumscribed areas on the muscle fibres, the

magnitude of the glutamate response at these sites being directly proportional to the amount of amino acid ejected from the glutamate electrode (Usherwood et al., 1968).

Under our conditions both glutamate and kainate depolarized the muscle membrane, kainate being approximately twenty times more potent than glutamate, thus agreeing to some extent with the observations made by Biscoe et al. (1975) who studied the action of these agents on spinal interneurones. These observed depolarizations were however, associated with a complete inhibition of m.e.p.p. discharge, which contrasts with the result obtained using high K^{+} concentrations, where depolarization was accompanied by a substantial increase in m.e.p.p. frequency and amplitude. It was shown in Chapter 5 that kainate caused neurotransmitter (aspartate and glutamate) release from mammalian synaptosomes, suggesting that it was having a depolarizing action in this mammalian system as well. Thus, it seems likely that the locust NMJ released more transmitter in response to kainate. The lack of the expected correlated rise in m.e.p.p. frequency may be explained by (a) receptor desensitization by exposure to excessive amounts of glutamate or (b) the existence of presynaptic receptors, the level of neurotransmitter (glutamate) or agonist (kainate) present at the synapse being sufficient to combine with these receptors to inhibit further transmitter release i.e. m.e.p.p. discharge.

The effect of β-bungarotoxin on miniature endplate potential discharge

As discussed in the introduction and in Chapter 5,

β-bungarotoxin is known to have a presynaptic mode of action and has been shown to increase miniature endplate potential frequency in vertebrate neuromuscular preparations (Chang et al., 1973; Kelly and Brown, 1974). Using the locust NMJ preparation a similar effect on m.e.p.p. discharge was observed however, unlike the aforementioned workers a subsequent block in neuromuscular transmission was not obtained. However, it must be remembered that whereas we used a β-bungarotoxin local concentration of approximately 5.6 μg/ml, these workers used considerably higher concentrations (i.e. 3-20μg/ml). Also, as mentioned in connection with the results obtained using K^+, the conditions of their experiments were far more severe, involving chronic exposure of the muscle fibres to the toxin. In addition it must be noted that we are dealing with two completely different systems, on the one hand a mammalian cholinergic NMJ and on the other an invertebrate glutamatergic system.

An elevation in m.e.p.p. discharge is consistent with an increase in neuro-transmitter release as a result of an action on the presynaptic neuronal membrane, and as described in Chapter 5, in common with several other workers (Kelly et al., 1975; Dolly et al., 1978), β-bungarotoxin was found to increase the release of aspartate, glutamate and GABA from synaptosomes.

Recently, it has been suggested that β-bungarotoxin may function as a depolarizing agent (Sen and Cooper, 1978), depolarization of the nerve terminal being responsible for the increase in neurotransmitter release, mentioned above. It is unfortunate that we were unable to measure the

membrane potential change induced in the presynaptic nerve terminal as a result of β-bungarotoxin action, however, its ability to influence membrane ion conductance is evidenced by the membrane (muscle fibre) potential decrease (all be it very small) observed in the muscle fibre following toxin application. It is interesting to note that Chang et al., (1973) using the rat phrenic nerve diaphragm preparation detected no postsynaptic action on the membrane potential as a result of the addition of β-bungarotoxin.

Further evidence of β-bungarotoxin's action on ion transport is outlined in Chapter 5; synaptosomes exposed to β-bungarotoxin were found to have reduced intracellular K^+ levels.

Further Remarks

The locust NMJ preparation potentially offers a convenient, rapid and simple bioassay system for testing various drugs. The future will reveal how valuable this system proves to be.

It is unfortunate that time did not permit the testing of several other agents used previously in synaptosomal studies. For example, penitrem A and verruculogen require extensive further examination before a clear picture of their synaptic mode of action is elucidated. Obviously the locust neuromuscular preparation could be of some use in this connection.

Investigation of the effects produced by glutamine may also provide some interesting information, especially when one considers the high concentrations of this amino

acid found in locust blood.

CHAPTER 8

CONCLUDING REMARKS

One of the main aims of this research project was to obtain unequivocal evidence for the existence of presynaptic autoreceptors involved in the mediation of putative amino acid transmitter release. On the basis of the hypothesis propounded in Chapter 5, this study would not appear to have been very successful, however, observations were made suggesting a presynaptic mode of action for a number of the agents tested.

Isolated nerve-endings (synaptosomes) were chosen as the principal *in vitro* preparation for this study. Apart from their ease of preparation they offer one main advantage over techniques such as *in vivo* superfusion, this being the fact that presynaptic effects can be studied to the exclusion of other processes occurring at the synapse. As the purpose of this study was to demonstrate presynaptic modulation of neurotransmitter release, this preparation would appear to offer the ideal system.

The metabolic and transmitter releasing properties of synaptosomal suspensions are well established and these were confirmed in Chapter 3, measuring parameters such as O_2 consumption, lactate production, K^+ retention and the depolarization induced release of aspartate, glutamate, GABA and glycine. In the same chapter, the relationship between the concentration of a depolarizing agent (veratrine) applied to synaptosomal suspensions and the

extent of amino acid transmitter release was investigated. It was felt that using this protocol and measuring the total levels of these amino acids remaining in the tissue after veratrine treatment, some idea of the size of the releasable and non-releasable pools might be gained. If measurement of the synaptosomal membrane potential ever becomes possible it would be interesting to study the link between depolarization and neurotransmitter release more closely.

The action of a general anaesthetic, pentobarbital, was also examined (Chapter 3) and shown to inhibit the release of aspartate whilst increasing the release of GABA. This result is in agreement with some of the hypotheses that have been put forward to explain general anaesthesia.

In the future it may prove to be rewarding to extend these studies to other general anaesthetics in order to see whether they produce similar effects. The fact that a variety of chemically unrelated compounds should produce general anaesthesia in experimental animals, underlines the importance of determining whether they have a common mode of action.

Data consistent with the "transport shuttle" proposed by de Belleroche and Bradford (1976) was obtained (see Chapters 3 and 5). However, more detailed studies will have to be concluded before concrete evidence "for or against" this theory is gained. The demonstration of shared control mechanisms for uptake and release will probably achieve this purpose.

In Chapter 4 an attempt was made to study amino acid transmitter release using an alternative <u>in vitro</u> system,

i.e. superfusion of nerve-endings, thus avoiding the problem of synaptosomal reuptake of released transmitter. This method met with a limited amount of success, nevertheless the reproducibility of the results obtained between experiments was thought to be too poor to warrant further investigation. However, in the future, examination of the system described by Raiteri et al. (1974) may prove to be rewarding.

When synaptosomes were exposed to a number of amino acid analogues and uptake blockers (Chapter 5) a variety of effects on putative amino acid transmitter release were observed. The uptake blockers p-chloromercuriphenyl sulphonate and 3-threo-hydroxy aspartate were found to selectively increase the efflux of aspartate, glutamate and GABA. Similar results were obtained with the glutamate agonist, kainate, thus giving the opposite effect to that predicted by our hypothetical mechanism for presynaptic control of amino acid transmitter release, outlined in Chapter 5, This agent was subsequently shown to function to some extent as an uptake blocker for glutamate. The GABA antagonists bicuculline and picrotoxin produced variable effects on ^{14}C-GABA efflux, thus adding to the confusion that has been expressed in the literature regarding their modes of action.

Evidence was obtained suggesting that the polypeptide neurotoxin, β-bungarotoxin functions as a depolarizing agent (Chapters 5 and 7). Synaptosomes exposed to the toxin exhibited greatly enhanced release of aspartate, glutamate and GABA; the intrasynaptosomal levels of K^+ were also reduced. However, another parameter usually

associated with depolarizing action was not satisfied, i.e. no increase in synaptosomal respiration as a result of β-bungarotoxin action, was observed.

Cerebrocortical synaptosomes prepared from animals previously treated with the tremorgenic mycelia, Penicillium cyclopium and Penicillium estinogenum (Chapter 6) produced results consistent with some of the suggestions made by electrophysiologists. Similarly, spinal/medullary nerve-endings exposed to penitrem A yielded data compatible with its proposed role as a modulator of presynaptic function . Studies in which the Na^{+} and Ca^{2+} dependencies of the toxins' effects on amino acid release are tested, will probably provide a further insight into their modes of action. Similarly, some of the drugs used by Stern (1971) to alleviate the symptoms produced by penitrem A may offer another avenue of research.

Despite the importance of the synaptosomal preparation as a tool in neurochemical research, there comes a stage when an attempt needs to be made to study neurotransmission using a more intact and therefore more physiological system. The locust neuromuscular junction preparation was chosen for this purpose and several of the agents used in synaptosomal studies on amino acid release were tested for their effects on spontaneous miniature end-plate potential discharge. The depolarizing agent, potassium produced similar results to those reported previously (Usherwood, 1963; Liley, 1956), i.e. an increase in m.e.p.p. frequency. Similarly, β-bunarotoxin increased m.e.p.p. discharge thus providing further evidence

for its role as a mediator of presynaptic activity and its depolarizing action. Glutamate and the glutamate agonist, kainate, both reduced m.e.p.p. discharge and in the case of the latter agent results inconsistent with those obtained using synaptosomal preparations. However this result may provide some evidence for neurotransmission being mediated through the action of a presynaptic receptor.

The locust NMJ preparation offers a great deal of potential for studying factors influencing neurotransmission. It is unfortunate that time did not permit the testing of several other agents, e.g. penitrem A, verruculogen and glutamine, on this system. Similarly, it would have been interesting to have further characterized β-bungarotoxin's proposed depolarizing action by determining whether the effects produced by this agent were negated in Ca^{2+}-free medium or in the presence of tetrodotoxin.

In retrospect it would appear that, under the terms of this project, the locust neuromuscular preparation probably offered one of the greatest opportunities for obtaining evidence for the existence of presynaptic receptors. As regards synaptosomal studies, experiments along the lines of those described by Benjamin and Quastel (1977a, 1977b), de Belleroche and Bradford (1978) and Giorguieff et al. (1978) may also prove to be extremely useful.

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