ACTIONS OF TREMORGENIC MYCOTOXINS ON AMINO ACID NEUROTRANSMITTER RELEASE.

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THESIS ABSTRACT

The neurochemical effects of the tremorgenic mycotoxins Verruculogen and Penitrem A were studied using synaptosomes prepared from several CNS regions of sheep and rats previously injected with the toxins. Penitrem A (400 mg mycelium/kg) increased the spontaneous release of endogenous glutamate, GABA, and aspartate by 213%, 455%, and 277% respectively from cerebrocortical synaptosomes, Verruculogen (400 mg mycelium/kg) increased the spontaneous release of glutamate and aspartate by 1300% and 1200% from cerebrocortical synaptosomes. The spontaneous release of the transmitter amino acids or other amino acids was not increased by the tremorgens in spinal cord/medullary synaptosomes. Penitrem A pretreatment reduced the veratrine stimulated release of glutamate, aspartate, and GABA from cerebrocortical synaptosomes by 33%, 46%, and 11% respectively, and the stimulated release of glycine and GABA from spinal cord/medulla synaptosomes by 57% and 32% respectively. Verruculogen pretreatment did not alter the veratrine-induced release of transmitter amino acids from cerebrocortical or spinal cord/medulla synaptosomes. Penitrem A pretreatment increased the spontaneous release of aspartate, glutamate and GABA by 68%, 62%, and 100% from sheep corpus stratum synaptosomes, but did not alter the synthesis and release of dopamine.

Verruculogen was shown to cause a substantial increase (300-400%) in the miniature end plate potential frequency at the locust neuromuscular junction, and spontaneous spike frequency in the giant dopamine neuron of the water snail. The response was detectable within 1 min, rose to a maximum within 5 - 7 min, and declined to the control rate over a similar period.

Scorpion venom tityus toxin, and the alkaloids veratrine, caused specific release of the neurotransmitter amino acids glutamate, aspartate, and GABA in vitro from rat cortical synaptosomes by a depolarising action. Synaptosomal potassium levels were depleted by both agents, and their actions were blocked by tetrodotoxin. The calcium dependency of their

effects is discussed with reference to the blocking action by the "calcium antagonist" verapamil, which at 200μM inhibited evoked transmitter amino acid release due to veratrine and tityustoxin, but not that due to elevated potassium levels.

The effects of the irreversible inhibitors of GABA-transaminase, γ-acetylenic GABA (GAG) and γ-vinyl GABA (GVG) on the release and uptake of endogenous and exogenous GABA by rat cerebral cortical synaptosomes were studied. GAG increase the release of preloaded [U- ^{14}C] -GABA by 12 to 46% over concentration range 0.5 to 5mM and GVG at 0.25-5mM caused an increase of 22-95% above controls. The effect of both drugs on [V - ^{14}C] - GABA release was inhibited significantly by tetrodotoxin and blocked by verapamil.

When rats were pretreated with GAG (100 mg/kg) for 8h or GVG (100 mg/kg) for 14h, the control release of GABA from synaptosomes prepared subsequently was increased, as was GABA content of the tissue.

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CHAPTER ONE.

GENERAL INTRODUCTION

1) NEURONS AND GLIA.

2) SYNAPTOSOMES AS A TOOL OF NEUROBIOLOGY.

3) AMINO ACIDS AS TRANSMITTERS.
   a) GLUTAMATE AND ASPARTATE.
   b) GABA AND GLYCINE.

4) THE SCOPE AND PURPOSE OF THIS STUDY.
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GENERAL INTRODUCTION.

A specific role of amino acids and derivatives in the central nervous system is as chemical transmitters at synapses. Neuronal transmission is electrical, associated with transient changes in membrane polarisation, but between neurones in the central nervous system, and at the junctions between nerves and muscles in the peripheral motor system, there is no electrical conduction. The nerve impulse is carried from one neuron to another by a chemical transmitter, which is synthesised and compartmentalised in the presynaptic neuron. When there is electrical stimulation of this neuron, the stored transmitter is released into the synaptic cleft. Postsynaptically, it interacts with a receptor on the membrane, causing stimulation of electrical transmission in this post - synaptic neuron.

The present study considers alterations in the activity of the central nervous system associated with changed levels of certain amino acids induced by a variety of neuroactive agents, and to facilitate an understanding of the consequences of such modulations, a brief survey of the complexity of the CNS, and the evidence for a role in synaptic transmission for amino acids with particular emphasis on their involvement in recently proposed neural circuitry will be introduced in the preceding text.

1) NEURONS AND GLIA.

The central nervous system is extremely heterogeneous in both its gross anatomy and its histology. The anatomical complexity does not impede experimental investigations to a major degree, but the histological heterogeneity represents an enormous obstacle for experimental work. In addition, evidence is accumulating that the different cell types in grey matter of the CNS have widely different metabolic properties (HERTZ & SCHOUSBOE, 1975), and also that the biophysical characteristics of their membranes show fundamental differences (SOMJEN, 1975). A knowledge of this heterogeniety is essential for the understanding of, for instance, ion distribution in the brain.

Tissue from the CNS contains an interstitial space and two widely different types of cells, i.e. neurons and glial cells, which on morphological grounds have been subdivided into different types. The

rapidly increasing amount of information about function at the cellular level may, however, lead to a much more refined classification based upon neurochemical, neurophysiological, and neuropharmacological characteristics. The neurons are (with few exceptions (SOMJEN, 1975)) excitable cells in that they react to a certain depolarisation of their membranes with an all or none action potential, whereas no such response is given by glial cells (e.g. HILD & TASAKI, 1962; KUFFLER & NICHOLLS, 1966). Potential changes may however, spread between individual, electrically coupled glial cells over a distance of more than 1mm., (KUFFLER & NICHOLLS, 1966; WALKER & HILD, 1969).

No unequivocal answers are found to the fundamental questions of what fraction of the total volume of different regions is occupied, by respectively, neurons and glial cells, or to what extent each of the different cell types contributes to, for instance, the total energy expenditure or the total content of potassium and other constituents in brain cortical tissue. It is also not known with certainty which features of the complex electrical activity of the CNS reflect the phenomena occurring in each of the two cell types (SOMJEN, 1975). Suffice to say for the present that the neuronal perikarya in the brain cortex of larger mammals account for only about 5% of the total volume though the volume occupied by their processes is a couple of times higher. Nerve endings constitute another 15 - 25% of the brain volume and glial cells outnumber nerve cells by a factor varying between 2 and 10, depending on species . A large volume of the grey matter is made up by the neuropil, an intricately interwoven network of minute neuronal and neuroglial processes, tapering into lamellar sheets of 200 - 1000 Å thickness (PAPPAS & PURPURA , 1961). The ratio between neuronal and glial components in the neuropil is uncertain but it seems reasonable to suggest that up to one - half of the total brain cortical volume may be occupied by glial cells, and evidence has accumulated that these cells are not at all so functionally inert as they were previously considered to be. Glial cells may thus account for at least one third of the total oxygen consumption, about one half to three quarters of the total glutamate content (BENJAMIN & QUASTEL, 1976; SCHOUSBOE et al., 1975), and the majority of the Na^+- K^+- ATPase activity (NAGATA et al., 1974) in brain cortex. The brain specific S - 100 protein is mainly localised in glial cells (HYDEN & MCEWEN, 1966) and the primary lesion in certain pathological conditions may be glial rather than neuronal (GOMIRATO & HYDEN, 1968). Maturation of neurons

and glial cells seems to occur hand in hand as indicated by the identical developmental pattern of the neuronal specific protein 14 - 3 - 2 and the glial specific protein S - 100 (CICERO et al, 1972).

SOMJEN, (1975), reviews the field of glial and neuronal cell biology to great depth, but as the subsequent text is concerned more specifically with neuronal processes, the information presented above serves only as a reminder of the relevance of glia to the complexity of the nervous system.

The importance of the question of the magnitudes of the extra - and intra - cellular fluid spaces in the brain becomes apparent in a later chapter. The magnitudes of these parameters are of crucial importance for exact estimates of intracellular concentrations, of fluxes across cell membranes, and of synaptic cleft concentrations of for instance, experimental drugs. Furthermore, certain drugs affect the magnitude of the fluid spaces, and estimates must be made to avoid gross errors. KUFFLER & POTTER (1964) estimate that the extracellular space in the brain cortex in vivo accounts for only about 5% of the total volume, and is restricted to interstitial clefts of about 15nm. width. VAN HARREVELD (1962) estimated the extracellular space to be larger in vivo , but that it shrinks during the period between arrest of circulation and conventional fixation. Extracellular marker determinations estimate an extracellular space of 15 - 20% (FENSTERMACHER et al., 1970).

During incubation of brain slices in vitro , the extracellular space increases during the first half an hour (PATEL et al., 1971), and similarly the intracellular space in brain slices increases during incubation. Electron micrographic studies have shown that the intracellular swelling during incubation in an oxygenated physiological medium mainly occurs in glial cells (IBATA et al., 1971).

SYNAPTOSOMES AS A TOOL OF NEUROBIOLOGY.

The terminations of neuronal axons and dendrites represent the presynaptic portion of the synapse, or nerve terminal, and depolarisation of the membrane of this structure leads to a release of chemical transmitter from the nerve terminal. Subsequent to traversal of the 20nm synaptic cleft, the transmitter evokes a response in the post synaptic cell by combining with a specific site on the post synaptic membrane and causing a change in membrane polarisation. An action potential is generated if the depolarisation threshold is achieved, and the impulse

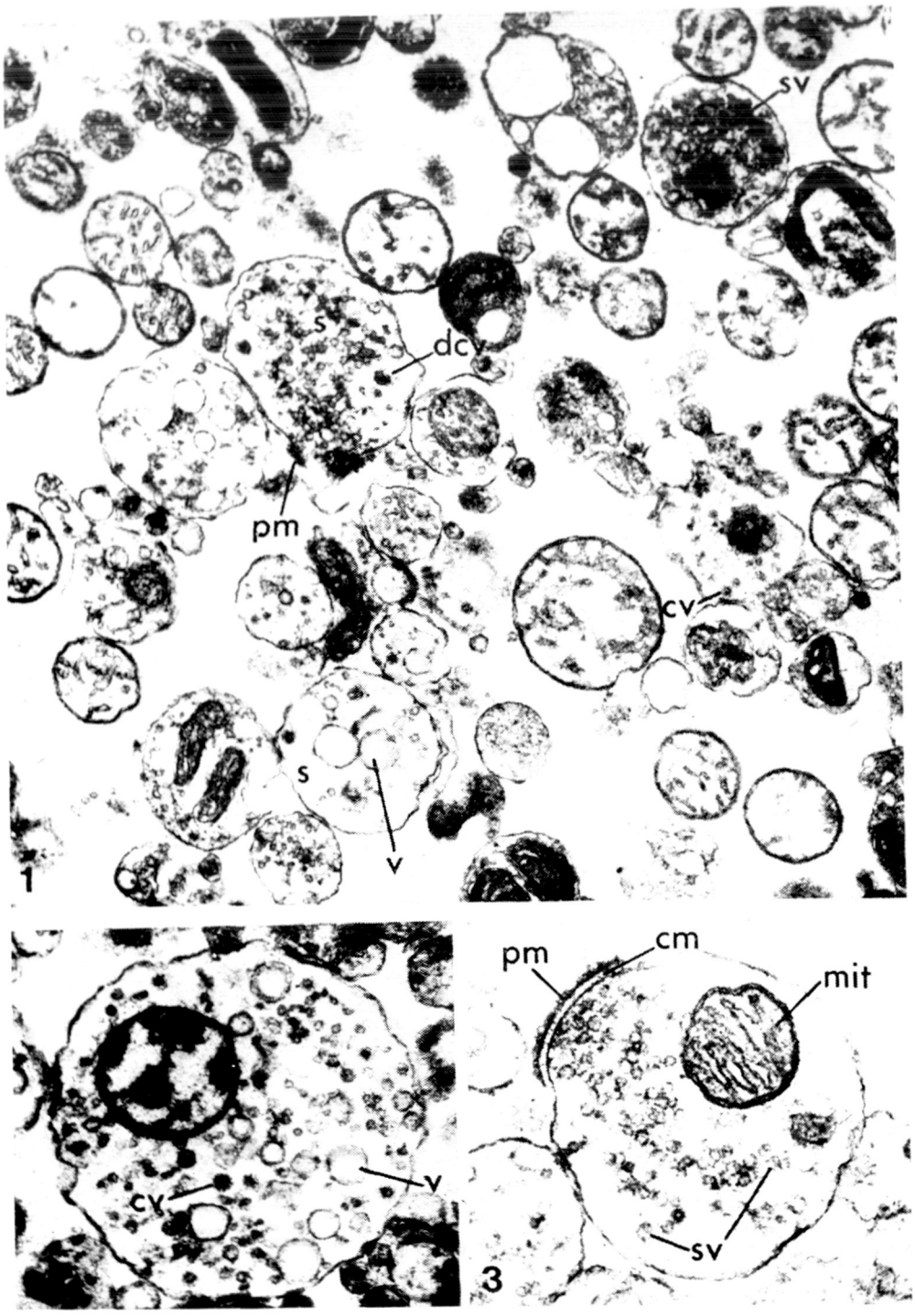


FIG. 1. Electron micrograph of cerebral cortex synaptosomes after 1 hr incubation in Krebs–phosphate medium at 37°C. Intact synaptosomes (s) containing synaptic vesicles (sv), mitochondria (mit) and a few vacuoles (v). Other items are dense cored vesicles (dcv), complex vesicles (cv), post-synaptic membranes (pm) and cleft material (cm). (Magnification, 1; ×30,000, 2; ×36,820, 3; ×57,680.)

passes further along the neural pathway. During this process, the terminal membranes undergo transient change in ionic potential, which is rapidly restored to its original state by activation of compensatory enzymes. As discussed above, the cytoplasmic volume of the synapse makes it an extremely difficult task to study the biochemical processes of the nerve terminal in situ , and consequently neurobiologists have devised methods for the quantitative preparation of nerve terminals (GRAY & WHITTAKER, 1962; DE ROBERTIS et al., 1962). The particles thus obtained were termed synaptosomes by WHITTAKER et al., (1964) but are also referred to as nerve ending particles by other workers.

The early studies of these workers showed that nerve terminals of mammalian brain areas not only survive the liquid shear forces which disrupt the whole tissue during homogenisation, but can also be isolated by sedimentation in hypertonic sucrose density gradients. BRADFORD (1969) published evidence that these enriched fractions of nerve terminals are metabolically active, despite their separation from the cell body, and showed further that the functional characteristics of the presynapse can be studied in isolation from the rest of the cellular structures normally associated with it. In fact, an approximation can be made of the intrinsic synthetic capacity versus the contribution from axoplasmic phenomena. Thus, an in vivo study can often mask the comparatively small changes occurring within the nerve ending within the larger activities of the entire neuron and surrounding cells. An in vitro control of the environment enables relatively easy observation of the effects of various physical parameters and of pharmacologically active substances on a very specialised and important part of the nervous system. Isolated synaptosomes exhibit very similar physical constituents to those found in the nerve ending in situ and perform for wholly adequate periods as viable living cells.

The major disadvantage of the preparation (irrespective of the purity argument) is that synaptosomes from any brain region are heterogeneous because of their derivation from terminals containing different transmitters. De Robertis has claimed separation into subfractions of cholinergic and non - cholinergic populations, but generally circumvention of the problem is achieved by preparing synaptosomes from brain regions predominantly rich in a single type of transmitter, e.g. glycine in spinal cord (OSBORNE et al., 1973), dopamine in substantia nigra, and peptides in hypothalamus (EDWARDSON et al., 1972). ISRAEL et al., (1976) have purified a synaptosome

containing only cholinergic nerve endings from the electric organ of Torpedo.

Subsequent to tissue rupture, the damaged membrane of the nerve terminal re - seals to form a continuous surface, and occassional photo - micrographs indicate that a small portion of the post synaptic thickening remains associated with the newly formed synaptosome. The presence of a continuous membrane is indicated by the osmotic changes of synaptosomes in media of differing tonicity, and synaptosomes retain their soluble components such as lactate dehydrogenase (JOHNSON & WHITTAKER, 1963), pyridine nucleotides (LINDALL & FRANZ 1967), potassium (MARCHBANKS, 1967), and amino acids (BRADFORD & THOMAS, 1969). The presence of significant quantities of metabolites retained within organised structures enables them to perform a variety of metabolic and synthetic activities.

Brain slices similarly maintain a fairly high membrane potential (BRADFORD & MCILWAIN, 1966) and respond to depolarisation with the generation of action potentials (RICHARDS & SERCOMBE, 1968), but they suffer from the disadvantage of even greater heterogeneity than do synaptosome preparations.

Synaptosomal ultrastructure under the lens of an electron microscope resembles the morphology of the intact nerve terminal, despite the fact that, for example, cytoplasmic filaments are slightly distorted during severance from the axon (JONES & BREARLY, 1972). Numerous small vesicles and usually 1 - 2 mitochondria can be seen, and also, occassionally, the odd post synaptic thickening. Synaptosomal diameter is approximately 1µm.. Subsequent to incubation in physiological media, synaptosomes become clearer and more spherical than those fixed directly after removal from the preparative gradient. The morphology of synaptosomes in culture is preserved for up to 24h., (BRADFORD et al., 1975).

A comprehensive review by BRADFORD (1977) describes the linear rate of oxygen uptake by suspensions of cerebrocortical synaptosomes in Krebs medium buffered with phosphate, Tris - HCl, or glycyl glycine, containing 10mM glucose when incubated at 37°C, and this uptake rate is similar to that of cortical slices, continuing linearly for four or more hours. BRADFORD & THOMAS (1969) describe how the full enzyme and co - enzyme complement for glycolysis and oxidative phosphorylation is present in synaptosomes, enabling considerable lactate production concomitant with conversion of isotopic glucose carbon into intermediary keto - acids,

giving rise to labeled glutamate, aspartate, GABA, and alanine. The glycolytic enzymes are distributed in the cytoplasmic matrix and the tricarboxylic acid cycle and oxidative phosphorylation capacities are confined within the intrasynaptosomal mitochondria. The mitochondria play an essential role in the function of synaptosomes, and contribute approximately 30% of total protein, and 25% volume (MOORE & LINDALL, 1970).

In spite of the wide range of enzymes found within synaptosomes, (DE BELLEROCHE & BRADFORD, 1973), some may be contaminants. HAJOS & KERPEL-FRONIUS (1971) were unable to detect succinate dehydrogenase in intra - terminal mitochondria in situ by electron microscopic histochemistry although in 1965, WHITTAKER reported such activity in intrasynaptosomal mitochondria.

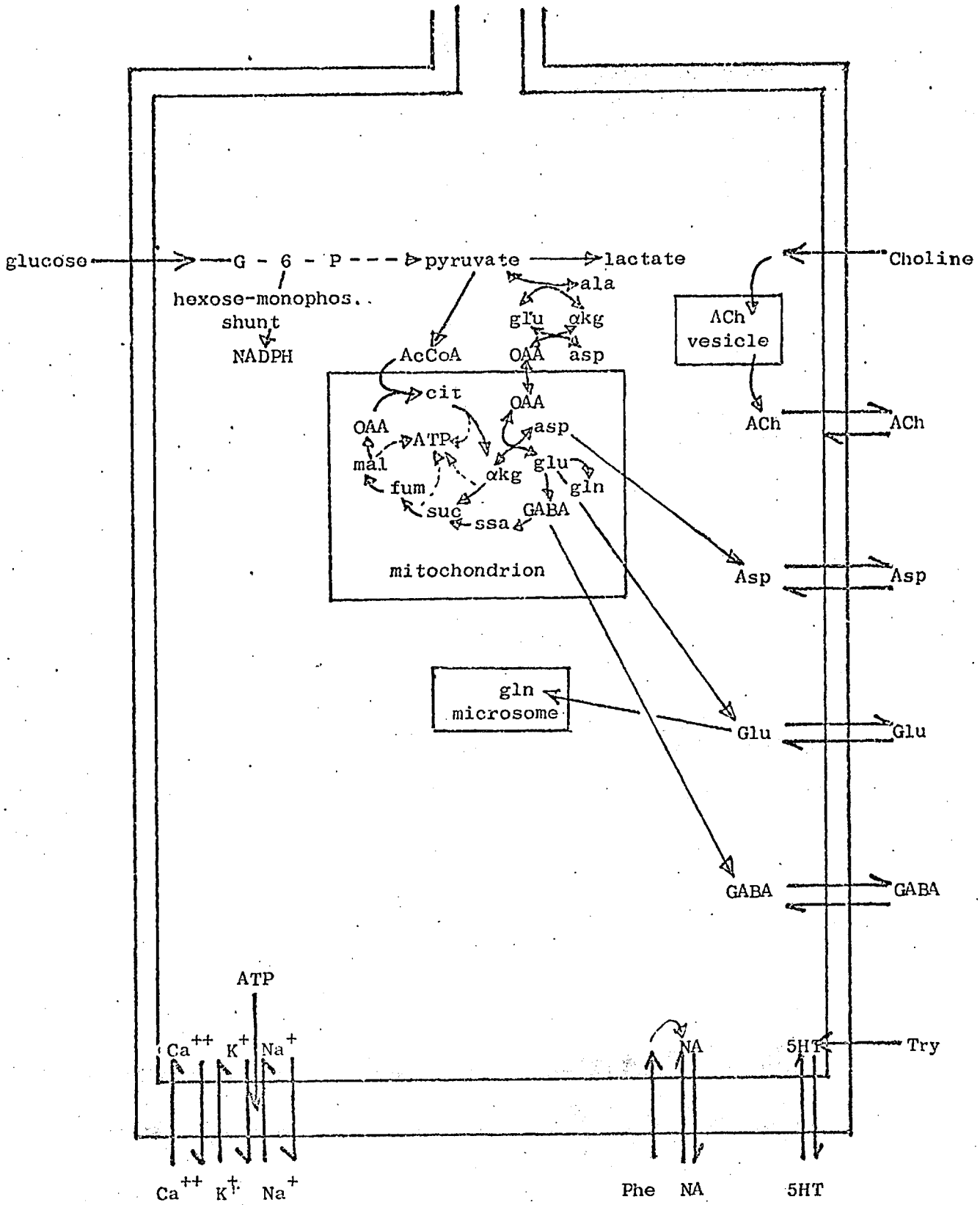
A number of pharmacologically and physiologically active substances have been found in synaptosomes, e.g., acetylcholine (GRAY & WHITTAKER, 1962), noradrenaline and serotonin (MICHAELSON & WHITTAKER, 1963; SNYDER et al., 1965). The localisation of serotonin in some presynaptic endings in situ has been shown using fluorescent histochemistry (DAHLSTROM & FUXE, 1965), and a large proportion (60 - 90%) of the total brain acetylcholine has been recovered in the synaptosomal fraction (WHITTAKER, 1959; KUROKAWA et al., 1965), as well as most of the bound dopamine (LAVERTY et al., 1963), which represents 40% of total brain dopamine. Amino acids, especially glutamate, aspartate, GABA , and taurine (AGRAWAL et al., 1971) and N - acetyl glutamate (REICHETT & FONNUM, 1969) are a dominant compositional feature, both of synaptosomes and of whole brain.

Synthetic enzymes present in synaptosomes have been demonstrated for acetylcholine (SCHUBERT et al., 1972), noradrenaline (BALDESSARINI & KAROBATH, 1973), serotonin (GRAHAME - SMITH , 1967), and amino acids (BRADFORD & THOMAS, 1969; review, CURTIS & JOHNSTON, 1974). NADPH is produced by the phosphogluconate pathway (APPEL & PARROT, 1970) for the synthesis of nucleotides, phosphoproteins, and phospholipids (ABDEL - LATIF et al., 1968), and LUNT & LAPETINA (1970) have demonstrated a high rate of formation of phosphatidyl inositol.

The presence of an osmotically sensitive barrier surrounding synaptosomes is indicated by their retention of LDH when incubated under physiological conditions (BRADFORD, 1971) which, like the potassium and sodium, is lost from association with the particulate fraction by water elution of synaptosomes from a Sephadex column (MARCHBANKS, 1967).

FIG. 1.2

SCHEMATIC DIAGRAM OF SOME BIOCHEMICAL EVENTS AT THE PRESYNAPSE.



Similarly, specific permeability to certain ions results in swelling, causing light scattering changes (KEEN & WHITE, 1970) which follow the laws of Boyle and Van't Hoff, and are reversible. Furthermore, the presence of a membrane potential indicated also by the presence of an active Na^+- K^+- ATPase (HOSIE , 1965; BRADFORD et al., 1966) provides corroborative evidence for this feature. Substitution of the K^+ and Na^+ concentrations, and the volume of the non - inulin space, into the modified Nernst equation (HODGKIN, 1958) gives a minimum value of -27mV (BRADFORD, 1971) rising to -7mV subsequent to electrical stimulation. Thus, if 'o' = extracellular concentration, 'i' = intracellular concentration, and 'b' = apparent permeability of the membrane to Na^+ relative to K^+ (0.04) then the membrane potential (E_m)

$$= \frac{RT}{F} \frac{(K^+)_o - b(Na^+)o}{(K^+)_i - (Na^+)_i}$$

Of course the value of the membrane potential calculated in this manner is less than one half that of nerve terminals <u>in situ</u> and this discrepancy is generally explained by the membranous contamination of synaptosomal preparations, and the large proportion of the total volume that is occupied by synaptic vesicles and mitochondria.

Active transport systems for Na^+ (LING & ABDEL - LATIF, 1968), K^+ (ESCUETA & APPEL, 1969), and Ca^{++} (LUST & ROBINSON, 1970) have also been described. There is also Na^+ - dependent uptake of choline (MARCHBANKS, 1968), GABA (KURIYAMA et al., 1969), noradrenaline (BODGANSKI et al., 1968), tryptophan (GRAHAME - SMITH & PARFITT, 1970) and serotonin (MARCHBANKS, 1966), and of glutamate and aspartate by a high affinity process (LOGAN & SNYDER, 1971).

3) AMINO ACIDS AS TRANSMITTERS.

Increasing evidence that amino acids are involved in chemical synaptic transmission has been obtained by subjecting them to the criteria established for neurotransmitters like acetylcholine and the catecholamines. Their concentration relative to other regions of the nervous system should be high, their synthesising enzyme(s) must show a high activity and the nerve endings should possess a selective high affinity uptake mechanism. All those presynaptic characteristics would then diminish or disappear when the terminals degenerate following a

lesion of the afferent neuron or axon. Further, the transmitter candidate should be released from the nerve terminals, either through the influence of high potassium concentration, or drugs like veratrine, or through electrical stimulation, diffuse or, better, applied to the corresponding afferent pathway. Microiontophoretic application of the amino acid should mimic the effect of the physiological synaptic action of the transmitter on the post synaptic element and specific antagonists should prevent this action. This implies the presence of specific post synaptic receptors, and transmitters and antagonists should bind to the receptors. Ideally, these characteristics should allow a localisation of specific pre - or post - synaptic elements at the light and electron microscopic levels using either autoradiography, or immunohistochemistry. The amino acids are limited in this respect, by some difficulties not present with the more classical transmitters. They and their synthesising enzymes are usually present not only as a 'transmitter' pool, but also as a 'metabolic' pool. Neither the pools nor their respective enzymes can be isolated separately at the present time. An exception is GABA, where only a transmitter pool is present, so GAD can be used as the marker enzyme. (Perhaps this may explain why some of the preceding data appear anomalous in respect of GABA when compared with similarly obtained data for the other amino acid putative transmitters.) While some post synaptic receptors can be blocked by rather specific antagonists, like bicucculine for the GABA receptor, or strychnine for the glycine receptor, others must still be studied without this very useful tool.

There can be no purpose in generating a thorough review of the enormous accumulation of data evidencing the roles for amino acids as transmitter substances and instead a couple of tables have been included to illustrate the neural circuitry in which amino acids are considered to be intimately involved. Reviews to be consulted for greater detail include CURTIS & JOHNSTON (1974), JOHNSTON (1976), and GERSCHENFELD (1973). However, an overview of the subject, and in particular regard to recent additions to the literature in respect of specific circuitry, e.g. the pigeon visual system, is of interest if not of value.

3a) GLUTAMATE AND ASPARTATE.

The early work of Aprison and his colleagues (DAVIDOFF et al., 1967; GRAHAM et al., 1967) showed the association of these amino acids with

defined regions of spinal cord and roots, and led to the suggestions that glutamate is an excitatory transmitter released by primary afferents, and aspartate is a transmitter associated with excitatory interneurons. At a similar time, SHAW & HEINE (1965) were commenting upon the high levels of glutamate in the cerebral cortex and cerebellum of rats when compared with the midbrain and pons - medulla, while aspartate is more evenly distributed. No workers to date have indicated a selective localisation of glutamate or aspartate with particular nerve ending particles. The synthesis and metabolism of those amino acids is well documented, and many outstanding reviews are to be found in the book edited by BALAZS & CREMER (1973).

In vivo studies involving perfusate collection from exposed brain regions have shown release of glutamate and a little aspartate from cerebral cortex following brain stem stimulation (JASPER & KOYAMA, 1969), from hippocampus following local stimulation (CRAWFORD & CONNOR, 1973) , and workers in this laboratory have performed acute and chronic cannulation studies on amino acid release from rat cerebral cortex (DODD & BRADFORD, 1974; 1976).

A role for glutamate in the cortico - striatal pathway has been well studied (e.g. WEBSTER, 1961). Cortical lesion induces a decrease in the striatal glutamate pool (KIM et al., 1977) and uptake (DIVAC et al., 1977; MCGEER et al., 1977), and activation of striatal units by cortical stimulation is blocked by glutamic acid diethyl ester, a putative glutamate receptor antagonist (SPENCER, 1976). Furthermore, the toxicity of the glutamate analogue kainic acid in the striatum is abolished after degeneration of the cortico - striatal pathway (BIZIERE & COYLE, 1978; MCGEER & MCGEER, 1978), and restored by glutamate application (BIZIERE & COYLE, 1978). Since glutamine has been shown to be a good precursor for the glutamate (BRADFORD & WARD, 1976; BRADFORD et al., 1978; WEILER et al., 1979) and GABA (TAPIA & GONZALEZ, 1978) releasable pools in the brain, REUBI & CUENOD (1979) investigated the release of isotopic glutamate and GABA in striatal slices, preincubated with isotopic glutamine, from rats with and without cortical ablation. The degeneration of the cortico - striatal pathway induced in the striatum a large (65%) decrease in K^+ - evoked release of newly synthesised glutamate without changes in GABA.

In the frog optic tectum, enucleation induces within 14 - 24 days a 30% decrease in the concentration of glutamate and a 20 - 40% decrease in that of GABA (YATES & ROBERTS, 1974). In the pigeon optic lobe,

TABLE 1.1

SOME PROPOSED GLUTAMATE AND / OR ASPARTATE NEURAL PATHWAYS.

PATHWAY.	PROPOSED TRANSMITTER / EVIDENCE.
Corticostriate	Glutamate (a), (b).
Entorhinal - hippocampal	Glutamate (c), (d).
Retinotectal	Glutamate (e).
Cerebellar granule cells	Glutamate (f).
Primary sensory afferents	Glutamate.
Spinal interneurons	Aspartate.
Hippocampal commisural	Aspartate (c).
Hippocampal ipsilateral	Aspartate and/or glutamate (d), (g).
Dentatohippocampal (mossy fibres)	Aspartate and/or glutamate (g).
Hippocampo/subiculoseptal/mammilary	Aspartate and/or glutamate (h).
Corticocortical	Aspartate and/or glutamate (i).
Olfactory bulb - olfactory cortex	Aspartate and/or glutamate (j), (k).
Visual corticotectal	Aspartate and/or glutamate (l).
Visual corticogeniculate	Aspartate and/or glutamate (l).
Corticothalamic	Aspartate and/or glutamate (m).

TABLE 1.1 (cont'd)

REFERENCES.

(a) DIVAC et al., 1977

(b) P. L. MCGEER et al., 1977

(c) NADLER et al., 1976

(d) STORM - MATHISEN, 1977

(e) HENKE, 1976

(f) YOUNG et al., 1974

(g) TAXT et al., 1977

(h) STORM - MATHISEN, 1978

(i) REIFFENSTEIN & NEAL, 1974

(j) HARVEY et al., 1975

(k) BRADFORD & RICHARDS, 1976

(l) LUND - KARLSEN & FONNUM, 1978

(m) FONNUM, 1978

cholinoacetyltransferase and glutamate decarboxylase activities decreased by 5 - 20% and 12% respectively after retinal ablation (CHAKRABARTI & DAGINAWALA, 1976). It can consequently be speculated that glutamate is or mimics the physiological excitatory transmitter of at least part of the avian optic nerve fibres. Furthermore, when (^{14}C) proline is injected into the vitreous body, the proteins of the optic nerve terminals are labeled by fast axoplasmic transport, and the isotope appears in synaptosomal fractions. When these fractions are incubated with (^{3}H) glutamate, the label is taken up by high affinity systems (HENKE et al., 1976). Unfortunately, double labeling of identical terminals has not yet succeeded.

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, 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), and locust Schistocerca gregaria (USHERWOOD et al., 1968)

Synaptosomes sequester radiolabeled glutamate and aspartate by high affinity (low K_m) and low affinity (high K_m) transport systems (LOGAN & SNYDER, 1971; LEVI & RAITERI, 1974). The high affinity transport systems are Na^+ and temperature sensitive. Each exhibits narrow specifity for its neuroactive substrate with a K_m of about 10^{-5}M. In contrast, the low affinity transport systems are associated with both neuroactive and inactive amino acids, exhibit broader specificity, are Na^+ and temperature independent, and exhibit a K_m of about 10^{-3}M (LOGAN & SNYDER, 1971). The high affinity systems have been suggested to function in terminating transmitter action by removing their specific substrates from the synaptic region (IVERSEN, 1971). 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 et al., 1971; ROBERTS & KEEN, 1973).

3b) GABA AND GLYCINE.

Several excellent reviews exist on this subject, and a comprehensive introduction is not required here (WERMAN & APRISON, 1968; CURTIS & WATKINS, 1965; CURTIS & JOHNSTON, 1974). The accumulation of evidence supports the view that glycine and GABA are inhibitory transmitters in the central nervous system. Low and high affinity

uptake systems have been described for these amino acids and there is evidence to indicate that uptake is important in the inactivation of glycine and GABA in the synaptic environment (SMITH, 1967; IVERSEN & NEAL, 1968; NEAL, 1971; LOGAN & SNYDER, 1972; BALCAR & JOHNSTON, 1973).

Abnormalities in the GABA system may be associated with such disorders as Parkinson's didease and Huntington's chorea (ENNA et al., 1977; WALTERS & CHASE, 1977). Agents active at GABA synapses may be useful for alleviating the clinical manifestations of these, and of other disorders such as schizophrenia (CURTIS, 1977; SMYTHIES, 1977), and epilepsy (MELDRUM, 1975). The interactions of GABA with other transmitter systems such as the dopamine nigrostriatal system are also being investigated (e.g., JAVOY et al., 1977).

Electrophysiological and biochemical investigations leave little doubt that GABA is a transmitter in the striato - nigral pathway (e.g., FONNUM et al., 1974; BUNNEY & AGHAJANIAN, 1976). Heavily isotopically labeled clusters of relatively small sized neuronal perikarya are observed in a restricted area of the caudoputamen's body following nigral (^{3}H) GABA application (STREIT et al., 1979). They propose that the connectivity and chemospecificity of neural pathways can be established in this way by transmitter - related retrograde tracing (HUNT et al., 1977) and can be based on specific terminal uptake after the administration of radiolabeled transmitter, retrograde axonal migration, and retention of labeled material. Their hypothesis of selective tracing originated from the observation of perikaryal labeling of neurons in the pigeon optic lobe after tritiated glycine was applied to the terminal area (HUNT et al., 1977) and this will be mentioned again briefly later.

Synaptosomes prepared from avian optic tectum take up GABA with high affinity (HENKE et al., 1976). The synthesising enzyme glutamate decarboxylase is present in the tectum, particularly in the superficial layers (HENKE & FONNUM, 1976). Microiontophoretic application of GABA inhibits large numbers of neurons in the superficial half of the tectum (BARTH & FELIX, 1974). The intertectal inhibition is antagonised by bicucculline, a specific blocker of GABA receptors (BARTH & FELIX, 1974). Light microscopic autoradiography has revealed two types of intrinsic tectal neurons, and two extratectal connections, one afferent, one efferent (HUNT & KUNZLE, 1976). The intrinsic tectal neurons labeled by GABA injection into the optic tectum are long, horizontal dendrites of Golgi type II neurons and the dendrites contain a large number of

TABLE 1.2

PROPOSED NEURONAL PATHWAYS INVOLVING GABA.

PATHWAY.	EVIDENCE.
Purkinje cells	Inhibitory action (a) GABA release (b) Pharmacological correlation (a) Lesion (c) Axoplasmic flow (d) Immunohistochem (e)
Cerebellar Golgi cells	Uptake (f) Inhibitory action (a) Immunohistochemistry (e)
Cerebellar basket cells	Uptake (f) Inhibitory action (a) Immunohistochemistry (e)
Cerebellar stellate cells	Uptake (f) Inhibitory action (a)
Hippocampal basket cells	Localisation (g) Uptake (h) Inhibitory action (i) Lesion (g) Immunohistochem (q)
Neostriatal interneurons	Uptake (j) Lesion (k)
Striatonigral neurons	Lesion (l)
Pallidonigral neurons	Uptake (j) Lesion (j) axoplasmic flow (m)
Retinal interneurons	Localisation (r) Uptake (h)
Spinal cord interneurons	Uptake (o) Inhibitory action (p) Localisation (p) Immunohistochemistry (q) Physiological action (r)
Cortical interneurons	Uptake (s) Inhibitory (t)
Olfactory bulb interneurons	Immunohistochemistry (u) Physiological action (v)

TABLE 1.2 cont'd

REFERENCES

(a) ITO & YOSHIDA, 1964
(b) OBATA & TAKEDA, 1969
(c) FONNUM et al., 1970
(d) E. G. MCGEER et al., 1975
(e) MCLAUGHLIN et al., 1974
(f) HOKFELT & LJUNGDAHL, 1972
(g) STORM - MATHISEN & FONNUM, 1971
(h) IVERSEN & SCHON, 1973
(i) ANDERSON et al.,1964
(j) HATTORI et al., 1973
(k) P.L. MCGEER & MCGEER, 1975
(l) OKADA et al., 1971
(m) P. L. MCGEER et al.,1975
(n) KURIYAMA et al., 1968
(o) IVERSEN & BLOOM, 1972
(p) MIYATA & OTSUKA, 1972
(q) WOOD et al.,1976
(r) ECCLES et al., 1963
(s) BLOOM & IVERSEN, 1971
(t) KRNJEVIC & SCHWARTZ, 1966
(u) RIBAK et al., 1977
(v) NICOLL, 1969

prosynaptic profiles. The fact that there is a strong inhibition following an incoming volley in the optic nerve (HOLDEN, 1968), and that dendritic spikes have been recorded in the neuropil of the pigeon tectum (STONE & FREEMAN, 1971) corroborate such a system of horizontal inhibitory GABA neurons - with dendrites both post-synaptic to optic nerve terminals and presynaptic to radial dendrites - playing an essential role in the local circuits of the tectum.

The concept of glycine acting as a major inhibitory transmitter in the spinal cord and brain stem owes much to the work of Aprison and his colleagues (e.g., APRISON et al., 1975). Glycine appears to be a purely vertebrate transmitter which makes it unique among the known synaptic transmitters. Abnormalities in the glycine system may be associated with spinal spasticity (HALL et al., 1976). Glycine metabolism is still poorly understood, and only non - specific effects of centrally active drugs have been described on glycine uptake systems (UHR & JOHNSTON , 1975). Strychnine sensitive synaptic inhibitions which may be mediated by glycine continue to be discovered e.g. inhibition of intralaminar thalamic neurons following stimulation of cortical neurones (ISHIDA, 1977), and there is increasing evidence that glycine may be a transmitter in the retina (NEAL, 1976). Not all neuronal effects of glycine are antagonised by strychnine. Glycine has a bimodal action on frog spinal motoneurons; the hyperpolarising response is blocked by strychnine but the depolarising action which is usually the major effect is not influenced by strychnine (EVANS et al., 1976).

Studies on the binding of strychnine to spinal membranes have indicated that glycine and strychnine bind to distinct sites that interact in a cooperative fashion, the strychnine binding site being possibly associated with the glycine ionophore (SNYDER & BENNETT, 1976). The displacement of bound strychnine by a series of strychnine analogues has been correlated with the convulsant and lethal effects of these alkaloids (MACKERER et al., 1977). A wide variety of substances are now known to act as glycine antagonists (JOHNSTON, 1977). Tetanus toxin, which blocks glycine release, has been shown to bind like glycine to synaptic terminals containing flattened and pleomorphic vesicles (PRICE et al., 1977).

In the pigeon optic tectum there is evidence that glycine plays a special role. Glycine is taken up with high affinity by tectal synaptosomes (HENKE et al., 1976). Microiontophoretic application of

glycine inhibits a large number of units throughout the tectum and this effect is antagonised by strychnine (BARTH & FELIX, 1974). Strychnine binding displaced by glycine, as a measure of glycine receptors, is present in the chick optic lobe (ZUKIN et al., 1975) and in the pigeon optic tectum (LE FORT et al., 1978). Moreover, exogenous glycine is released in the tectum during electrical stimulation of an afferent pathway (REUBI & CUENOD, 1976).

Following isotopic glycine (or serine or α-alanine) injection into the lateral region of the optic tectum there is, in addition to some neuropil labeling, a covering by silver grains of the perikarya of neurons within the subtectal nucleus isthmi, pars parvocellularis (Ipc nucleus). The theory behind this type of retrograde labeling was described briefly above for GABA. Re - investigation of the uptake characteristics of these three amino acids confirmed that they were all taken up with high affinity by tectal synaptosomes (HENKE & CUENOD, 1978). The serine or alanine uptake was competitively inhibited by glycine, but glycine uptake was only partially inhibited by serine or alanine, thus suggesting the existence of two separate glycine compartments - a mixed one, accessible to both serine and alanine, and one for glycine only. In the pigeon spinal cord, only the glycine compartment could be detected; in the telencephalon the glycine - serine - alanine compartment was mainly present; and the tectum possessed both compartments. Finally, some glycine derivates inhibited glycine uptake more than serine or alanine, while cysteine had the opposite effect (CUENOD & HENKE, 1978). The glycine compartment seems to correspond to nerve endings, and the localisation of the mixed compartments are assumed to represent another population of nerve terminals to which the Ipc - tectal neurons might contribute.

4) THE SCOPE AND PURPOSE OF THIS STUDY.

Two decades ago, insufficient information was available for amino acids to be seriously considered as synaptic transmitters in the central nervous system. Even in 1962, ELLIOT et al., were regarding amino acids as mainly concerned in the brain with intermediary metabolism, although considerable significance had already been attached to GABA as a possible vertebrate and invertebrate inhibitory transmitter. At the present time, the wealth of data accumulated and described in the reviews cited above, and the complex amino acid

transmittor involvement in neuronal circuitry, a short introduction to which was given above, may justify the use of terms such as glycinergic, GABAergic, aspartergic, and glutamatergic to indicate neurons and terminals which release these amino acids, and synaptic processes in which they participate. Hence it was natural for the purpose of the present study to examine the role of amino acid transmitters and synaptic processes in the aetiology of drug induced phenomena, but to concomitantly learn more about the factors controlling the synaptic process.

The principal compounds which were investigated in an attempt to describe the aetiology of tremor comprised Verruculogen and Penitrem A, two tremor inducing toxins produced by Penicillium fungi. The role of the novel anti - convulsant drugs γ - acetylenic GABA and γ - vinyl GABA in antagonising the tremor associated with the fungal toxins was simultaneously investigated, and concomitantly, the ion fluxes associated with these phenomena at the synapse were studied by means of experiments made using tetrodotoxin and the Ca^{++} antagonist verapamil.

4a) THE PRECEDENTS

That synaptosomes should be prepared from animals previously dosed with the tremorgenic mycotoxins or the GABA analogues (and this is a method used widely in the accompanying studies) is not without precedent. For instance, OSBORNE et al., (1973), when investigating the action of tetanus toxin, injected the toxin intramuscularly to rats 15h., before killing and removing the spinal cord and medulla, and under these conditions, they observed that the electrically stimulated release of glycine, GABA, and aspartate from synaptosomes subsequently prepared and incubated was inhibited by 70%, 44%, and 52% respectively. Interestingly, when tetanus toxin was added to the synaptosome incubation originating from control rats, no changes in amino acid accumulations were observed. The foresight of these authors is illustrated in a quote from their discussion : "The fact that pretreatment of animals with the (tetanus) toxin was required before any action could be displayed in vitro , shows that a similar experimental design may greatly enhance the value of synaptosome preparations in the study of other centrally acting substances." They concluded that the in vivo period probably allows a greater

number of synapses to interact, and a greater extent of interaction with the toxin. In their case, this correlated well with the established slow onset of tetanus following intramuscular injection, which of course was unlikely to be due to slow penetration of the spinal cord. In the case of the tremorgens, however, a very rapid onset of tremor occurs and the method of animal pre - injection was used here principally because of the aqueous insolubility of the toxins which complicated their direct addition to synaptosome suspensions.

That the study of the modes of action of tremorgenic mycotoxins and their in vivo antagonists, the novel anti - convulsants γ - vinyl GABA and γ - acetylenic GABA, should comprise a central theme of amino acid neurotransmission was not an arbitrary choice, despite the validity of alternative transmitter systems. Since from the reviews quoted above, it seems fairly certain that synapses carrying receptors for GABA or for glutamate - and which mediate inhibition and excitation respectively, and occur widely in the central nervous system together with a more restricted distribution of equivalent sites for glycine and aspartate - then the synapses which are operated by these amino acids are likely to constitute a large proportion of the total in the central nervous system, which means that the high concentrations and ubiquitous presence of these compounds, particularly glutamate, aspartate, and GABA, leads to a high risk of anomalous contact between the amino acids and their synapses. A few examples of some consequences are particularly worthy of note here.

The three enzymes, glutamate decarboxylase (GAD), GABA - transaminase (GABA - T ; and this enzyme features directly in a later chapter), and succinic semialdehyde dehydrogenase (SSDH) are responsible for the production and removal of GABA. Agents which reduce GAD activity often markedly lower brain GABA concentrations and in parallel cause seizures, e.g., hydrazides, allylglycine, 3 - mercapto propionic acid (KILLAM & BAIM, 1957; DE LOREZ ARNAIZ et al., 1971; 1972; 1973). Inhibition of GABA - T or SSDH activities leads to a rise in brain GABA level, and simultaneously protects against seizures, examples being the effects of α - amino oxyacetic acid (AAOA) (SNODGRASS & IVERSEN, 1973; COLLINS, 1973; PERRY et al., 1974) and n - dipropyl acetate (SIMLER et al. 1973; HARVEY et al., 1975). Whilst AAOA is toxic when administered to humans, and has convulsant action above a certain dosage (PERRY et al., 1974; TAPIA at al., 1969), n - dipropyl acetate, whose passage across th blood brain barrier is facilitated by its lipophilicity, is a useful

anti - convulsant (JEAVONS & CLARK, 1974). The anti - convulsants could also produce their effects by blocking GABA uptake and increasing extracellular GABA levels in regions containing GABA - ergic synapses. Since such evidence is available for an inhibition of GABA uptake, this feature of their action might at least assist any effect due to raised cellular levels of GABA (SNODGRASS & IVERSEN, 1973; JOHNSTON & BAKER, 1974; HARVEY et al., 1975). The potent inhibition of neuronal discharge produced by extracellular GABA can be seen following its iontophoresis locally in cerebral cortex and elsewhere in vivo (KRNJEVIK & PHILLIS, 1963), or when it is present in salines bathing slices of cerebellar cortex in vitro (OKAMOTO & QUASTEL, 1973).

In contrast then to the likely modes of action of the anti - convulsants (and a later chapter provides novel data on this point), a candidate for involvement in the tremor and occassional convulsions observed as a consequence of tremorgenic mycotoxin administration to animals is glutamate, and the considerations brought to bear on GABA apply equally to glutamate, though its more direct relationship with the Krebs cycle makes distinction between its metabolic and transmitter involvement much more difficult. However, glutamate is a potent excitatory agent and readily causes firing of most neurones when iontophoresed onto cerebral cortex and other central nervous system regions (see CURTIS & JOHNSTON, 1974, for review). It increases the firing rate of neurones present in cerebellar slices in vitro (OKAMOTO & QUASTEL, 1973), and can be shown to depolarise and increase the sodium permeability of cells in cerebral cortex slices (GIBSON & MCILWAIN, 1965; BRADFORD & MCILWAIN, 1966). Small amounts of glutamate introduced intracerebrally readily produce convulsions (HAYASHI, 1954; 1956; WEICHERT & GOLLNITZ, 1968; HENNECKE & WEICHERT, 1970). Systemically administered glutamate produces seizures in 10 - day old rats (JOHNSTON, 1973) and also, at higher doses, in adult rats (BHAGARAN et al., 1971; BRADFORD & DODD, 1977). Raised whole brain levels of glutamate and of glutamate dehydrogenase have been reported in the preconvulsive state before onset of seizures initiated by a range of agents (WEICHERT & GOLLNITZ, 1968; 1969) and a release of glutamate has been observed to occur from cobalt - foci during the onset of seizures as observed either by EEG or as motor response (KOYAMA, 1972; DODD & BRADFORD, 1977). MEYER et al., (1970) reported an elevation in cerebrospinal fluid potassium levels during convulsions in monkeys and this could be linked with concomitant rises in cerebrospinal fluid glutamate levels.

If glutamate release were to occur as readily from central nerve fibre tracts as is reported for peripheral nerves and spinal nerve roots (WHEELER et al., 1966; DE FEUDIS, 1971; WEINREICH & HAMMERSCHLAG, 1975) and its accumulation were to ensue to the appropriate extent, then some participation of glutamate in the mechanisms of tremor and seizure propagation could be envisaged. In addition, extracellular glutamate may mediate the considerable oedema which accompanies convulsions (DE LOREZ ARNAIZ, 1972; 1973) since incubating neural tissue with this amino acid has long been known to promote tissue swelling (TERNER et al., 1960; BRADFORD & MCILWAIN, 1966; VAN HARREVELD & FIFKOVA, 1971). Such an effect may, however, be exerted through the excitatory property of glutamate, with convulsive hypoxia being the final common mechanism in seizure induced oedema.

These transmitter systems are intimately involved in the dyskinesias and excellent reviews on these topics have been published (e.g. STRAUGHAN & DRAY, 1978; BRADFORD & MARSDEN, 1976; BRIMBLECOMBE & PINDER, 1972). Briefly, however, in Parkinson's disease, loss of GAD activity together with degeneration of the substantia nigra and concomitant loss of dopamine, causes deletion of the inhibitory nigrostriatal dopamine pathway. The inhibitory GABA terminals, which also impinge on caudate neurons, and striatonigral GABA terminals mediating inhibition in the substantia nigra are also absent, together with receptors for GABA. The result of this systems breakdown is hyperactivity of excitatory cholinergic neurons modifying the output of the caudate neurons. Conversely, in Huntington's chorea, degeneration of the caudate, supersensitivity of caudate neurons to dopamine, and loss of GAD activity causes supersensitivity of caudate neurones to dopaminergic inputs. There is parallel loss of the inhibitory GABA operated pathways impinging on those nigral neurones which provide the dopaminergic pathway to the caudate. Also there is a doubling of the GABA receptor concentration in the substantia nigra. The result of this systems failure is hyperactivity of inhibitory dopaminergic input to caudate neurones modifying their output.

Thus, our knowledge of the organisation of these transmitter systems predicts neurological consequences of specific defects and indicates particular vulnerable aspects. It is, therefore, compelling even on a priori grounds to look for a link between the localised neuronal hyperactivity presumably associated with tremor and convulsive states and failure of glutamate or GABA transmitter systems. Such

failure might involve decreased activity of GABA operated inhibitory synapses, due to decreased GABA content or release, or involve increased activity of glutamate operated synapses due to increased concentrations in the extracellular space in contact with glutamate receptors.

4b) WRITE - UP LAY - OUT.

As a start to the pursual of such a project, the experiments to be described here were embarked upon. The study does not pretend to have answered definitively any of the questions which arise, and indeed, certain parts are highly speculative, with many arguments in existence to contradict such speculations. What the study has tried to achieve, however, is to provide evidence for amino acid transmitter involvement in a very novel but practically important area of neurological dysfunction. Inevitably, the work has established arborisations to other topics, but as far as has been possible, the study has concentrated upon the neurological basis for the tremor consequent upon administration to animals of the tremorgenic mycotoxins and, of equal importance, the possible mechanism of action of neuroactive agents (the γ - analogues of GABA) which antagonise the tremor produced by the tremorgenic compounds.

The present description of the work has been split into several parts, but nevertheless, the degree of cross - referencing within each part attempts to develop a continuity throughout the thesis. Each chapter has an individual introduction which concentrates on providing a background to the work described in that particular chapter - hence repetition has been avoided in this GENERAL INTRODUCTION, which has attempted to validate the methods used and the reasons for investigating a role for amino acids in the observed phenomena. Similarly, each chapter has an individual discussion referring directly to the data presented in that particular chapter. Again, where possible, cross - references with other chapters have been made, and an attempt has been made also to tie the discussions more closely together with a short SUMMARY section at the end of the write - up.

CHAPTER TWO

MATERIALS AND METHODS

(1) PREPARATION OF SYNAPTOSOMES.

(2) SYNAPTOSOME SUSPENSIONS.

a) Incubation procedure.

b) Termination of incubation.

c) Isotopic amino acid preloading of synaptosomes.

(3) TISSUE AND SUPERNATANT EXTRACTION FOR AMINO ACID ANALYSIS AND RADIOLABEL COUNTING.

(4) AMINO ACID ANALYSIS.

(5) RADIOLABEL COUNTING.

(6) POTASSIUM DETERMINATION.

(7) PROTEIN ASSAY.

(8) LACTATE ASSAY.

(9) ISOLATION AND PURIFICATION OF NEUROACTIVE AGENTS

a) Tremorgenic mycotoxins.

b) Tityus toxin.

c) γ-Acetylenic GABA & γ-Vinyl GABA.

d) T_{46}

(10) ADMINISTRATION TO ANIMALS OF NEUROACTIVE AGENTS.

(11) ELECTROPHYSIOLOGICAL EXPERIMENTS

a) Locust neuromuscular preparation.

b) Freshwater snail giant dopaminergic neuron preparation.

(12) TECHNIQUES FOR PRESENTING AQUEOUS INSOLUBLE NEUROACTIVE AGENTS TO SYNAPTOSOME SUSPENSIONS

a) Emulsifying agents.

b) Nylon gauze.

c) Liposomes.

(1) PREPARATION OF SYNAPTOSOMES.

Synaptosomes were prepared by the method of GRAY & WHITTAKER (1962) as modified by BRADFORD et al (1973). Whole cerebal cortex or medulla/spinal cord tissue was removed from female Sprague-Dawley rats (250g) previously stunned and exsanguinated and was placed in ice-cold 0.32M sucrose. A 10% weight for volume homogenate was prepared by means of twelve up and down strokes of a Perspex and glass homogeniser with a difference in pestle and mortar diameter of 500µm, the pestle rotating at 900rev./min. This homogenate was centrifuged in a Beckman 30 rotor at 1000g for 10min. in a Beckman L2-65 B ultracentrifuge. Two fractions were obtained, the pellet (P1) and the supernatant (S1). The P1 from tissues other than cerebal cortex was resuspended in 0.32M sucrose and recentrifuged. The S1 subsequently obtained (single or combined with the P1 wash) was centrifuged in the 30 rotor for 20min. at 20 000gto yield the P2 and S2 fractions. The crude mitochondrial pellet (P2) was resuspended in 0.32M sucrose and 15ml of this suspension was layered on to a discontinuous sucrose density gradient composed of 20ml of 0.8M sucrose above 20ml of 1.2M sucrose. The sucrose gradients were centrifuged at 76 000g in a Beckman swing-out SW 25 rotor for 60min. to yield the three fractions P2A, P2B, and P2C. The P2B (synaptosomal) fraction was collected, ensuring that equal amounts of sucrose above and below it were taken to give a final sucrose concentration of approximately 1M. From a burette, cold de-ionised water was added dropwise to the P2B fraction in a conical flask, which was swirled continuously on ice, until the sucrose concentration dropped to approximately 0.45M-a level iso-osmotic with the contents of the synaptosomes. This suspension was centrifuged at 54 000g for 20min. in a Beckman 30 rotor to yield the final synaptosomal pellet.

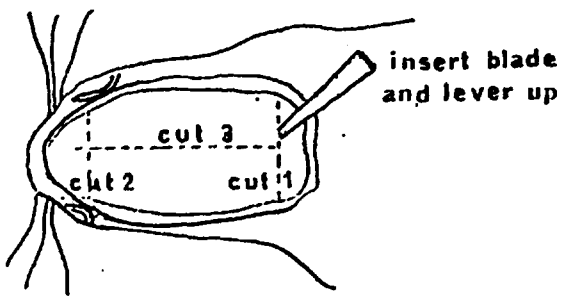
(2) SYNAPTOSOME SUSPENSIONS.

a) Incubation procedure.

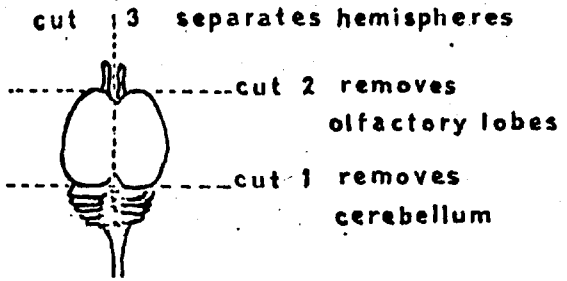
The pellets prepared as described above were gently suspended in the metabolic medium at room temperature with two or three strokes of a loose fitting Teflon pestle and glass mortar with a difference in diameter of pestle and mortar of 350-400µm. The metabolic media had the following composition: NaCl, 124mM; KCl, 5mM; Na_2HPO_4, 20mM; KH_2PO_4, 1.2mM; $MgSO_4$, 1.3mM; $CaCl_2$, 0.75mM; glucose, 10mM; and titrated to pH 7.4, or the same composition with the exception that tris-HCl (26mM) or $NaHCO_3$ (26mM) were substituted for the Na_2HPO_4. The media were gassed with O_2 or O_2/CO_2

Dissection of cerebral cortex

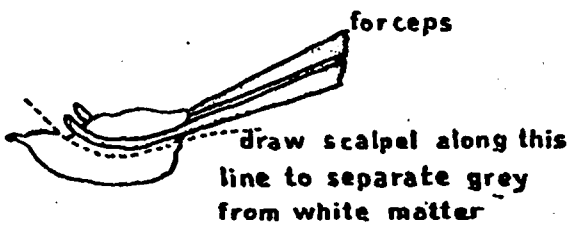
A. dorsal view



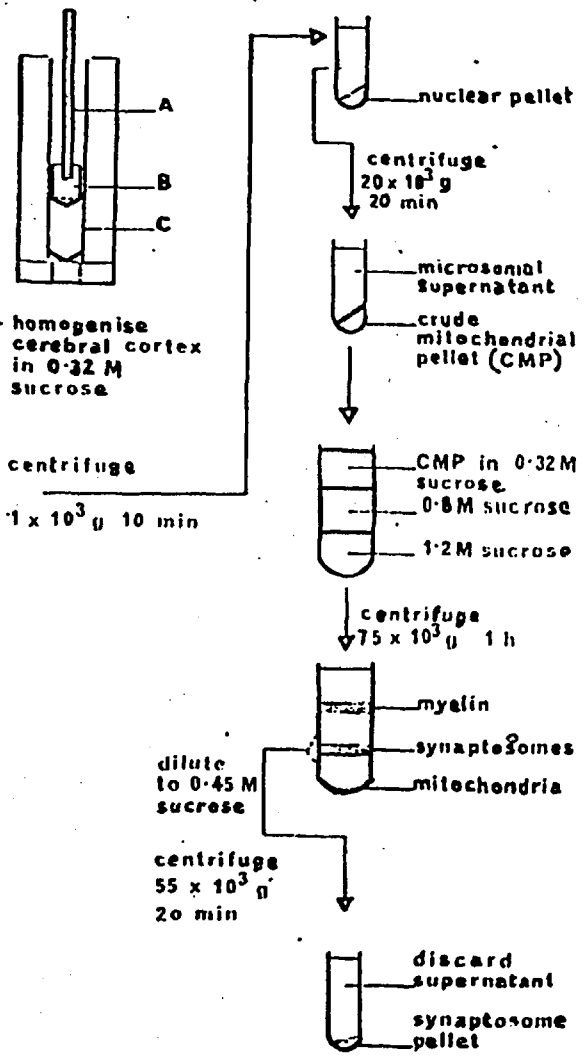
B. dorsal view



C. ventral surface uppermost



PREPARATION OF SYNAPTOSOMES



($NaHCO_3$).

Incubations were conducted either in 5ml conical vessels or in Warburg respirometer flasks, the centre well of each flask being fitted with an NaOH-soaked filter paper wick. The respirometer vessels were gassed with O_2 for some minutes at the start of incubation prior to closing of the system and allowing an equilibration period. The open conical vessels were gassed continuously throughout the incubation (but avoiding bubbling through the suspension).

Synaptosomes were incubated at 37°C in a shaking waterbath together with a variety of agents, at the concentrations and for the lengths of time, specified in the Results section. In most cases, stock solutions of the compound to be tested were made (in the relevant metabolic medium) at 10 or 100 times the final concentration presented to synaptosomes. After an equilibration time, an aliquot of the stock solution was added to the incubation. In every case, a control addition of medium alone was added to a parallel incubation.

b) Termination of incubation.

At the end of the incubation period the suspensions were sedimented in a bench top ultracentrifuge in order to separate the tissue from the incubating medium. The clear supernatant was taken for amino acid and lactate analysis, whilst the pellet was analysed for protein content, amino acids, and potassium ion levels.

c) Isotopic preloading (U-^{14}C amino acid) of synaptosomes.

A number of experiments were performed on synaptosome suspensions which had been preloaded with an isotopic amino acid. In these cases, the synaptosomes were suspended in metabolic medium containing 10μM isotopic amino acid (approximately 200nCi/ml) and incubated for 15min.. Subsequently, the synaptosomes were sedimented, washed, and resuspended in fresh medium. The experiment continued in the usual fashion.

That the bulk of the radioactivity released during these experiments was associated with the original amino acid was established by means of amino acid autoanalysis linked to scintillation counting of amino acid peaks.

(3) TISSUE AND SUPERNATANT EXTRACTION FOR AMINO ACID ANALYSIS AND RADIOLABEL COUNTING.

To both fractions was added a convenient amount of internal standard immediately at the end of the experiment. The internal standard was norleucine or (U-^{14}C) valine at stock concentrations of 1μmol/μl in 20% formic acid. The synaptosomal pellets were extracted with ice-cold methanol. Both the aqueous and the methanolic supernatants were taken

to dryness in a vacuum dessicator driven by a rotary piston vacuum pump. A card-ice/propanol condensor with solvent reservoir was placed between the pump and the dessicator. The solutes from the aqueous supernatant were extracted with methanol, and the methanolic supernatant was taken to dryness. Samples were subsequently dissolved in 0.025M HCl and analysed.

(4) AMINO ACID ANALYSIS.

Measurement of the amino acids in 150 µl of prepared sample was made with a Chromaspek J180 with fluorimeter J143 (Rank-Hilger, Margate Kent, U.K.). .Programmed gradient elution was used with an acid buffer (10.5g citric acid, 11.7g NaCl, 7ml Brij 35 (10%) , distilled water to 1000ml). The run time was 75min.. Fluorimetric analysis used orthophthalaldehyde with mercaptoethanol as the fluorogenic agent (800mg orthophthalaldehyde, 3ml mercaptoethanol, distilled water to 1000ml) with a borate buffer (30.5g $K_2B_4O_7.4H_2O$) which was added to the reagent stream before fluorogenic agent. This produced a fluorescence yield for proline of 62% of the norleucine fluorescence with a coefficient of variation at the 1nmol level of 2.4% compared with 2.1% for glutamic acid. The range for the other amino acids was from 1.2% for isoleucine (lowest) to 3.6% for arginine (highest) . The recovery of amino acid from the purification procedure used on samples was between 98-99% Peak areas were calculated on line with a Digico Micro 16V computer using standard routines.

(5) RADIOLABEL COUNTING.

Aliquots of the 0.025M HCl solution containing amino acid samples were dissolved in 20ml of liquid scintillant (toluene, 1l; methoxyethanol, 800ml; 2(4-tert-butyl phenyl)-5 (4-biphenyl)-1,3,4-oxodiazol, 6g) prior to counting, employing a Packard Tricarb scintillation counter.

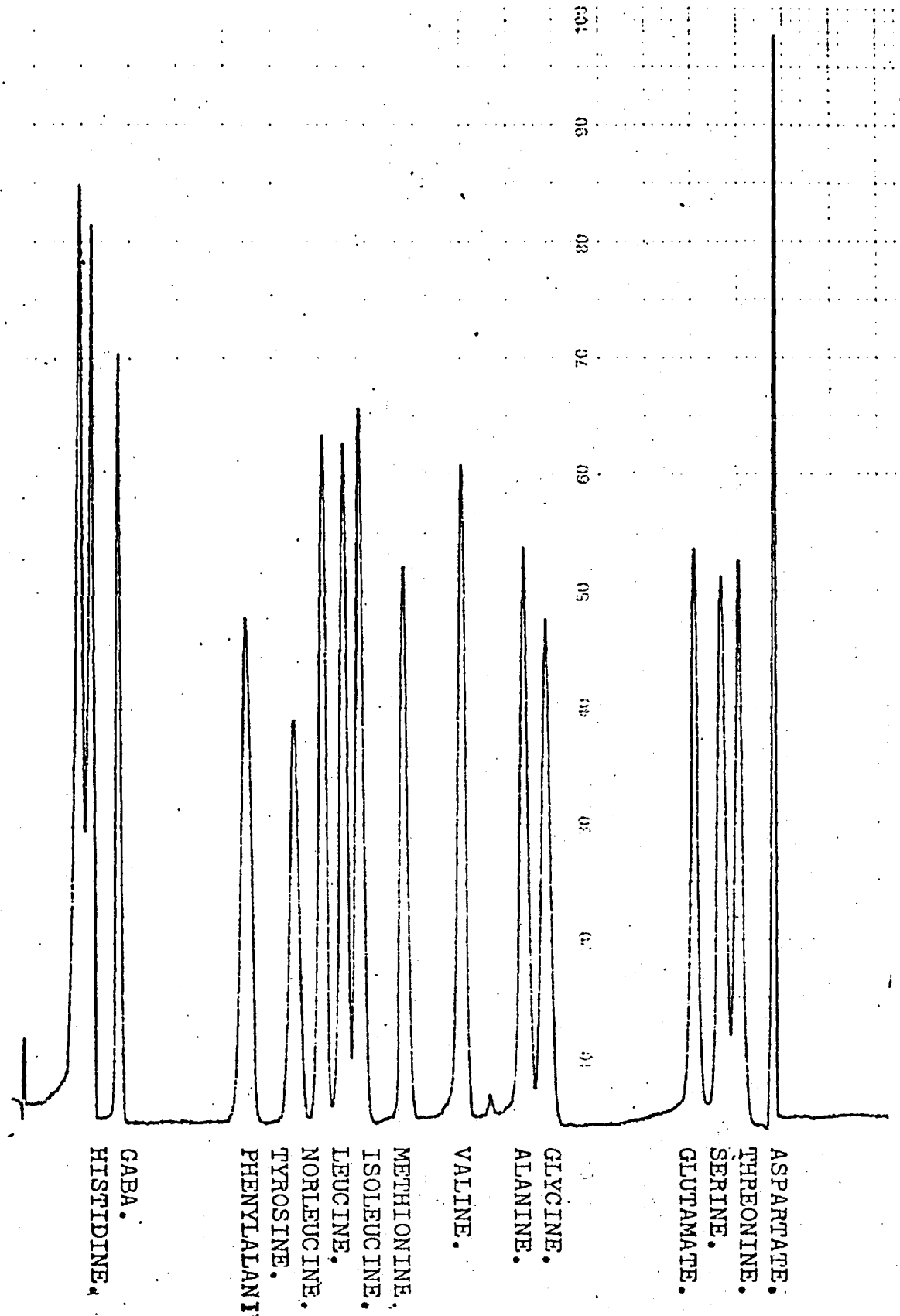
(6) POTASSIUM DETERMINATION.

Aliquots of the 0.025M HCl solution obtained from the methanolic extraction of the synaptosomal pellet (hence one of the reasons for using formic acid in this extraction becomes apparent) were measured for K^+ by means of a flame photometer (Evans Electroselenium Ltd., Halstead, Essex) with a K^+ filter (730-850nm).. Standards were dissolved in 0.025M HCl containing Na^+ at twice the concentration of the standard K^+.

(7) PROTEIN ASSAY.

FIG. 2

A 10NMOL MIXTURE OF AMINO ACIDS ANALYSED BY THE CHROMASPEK.



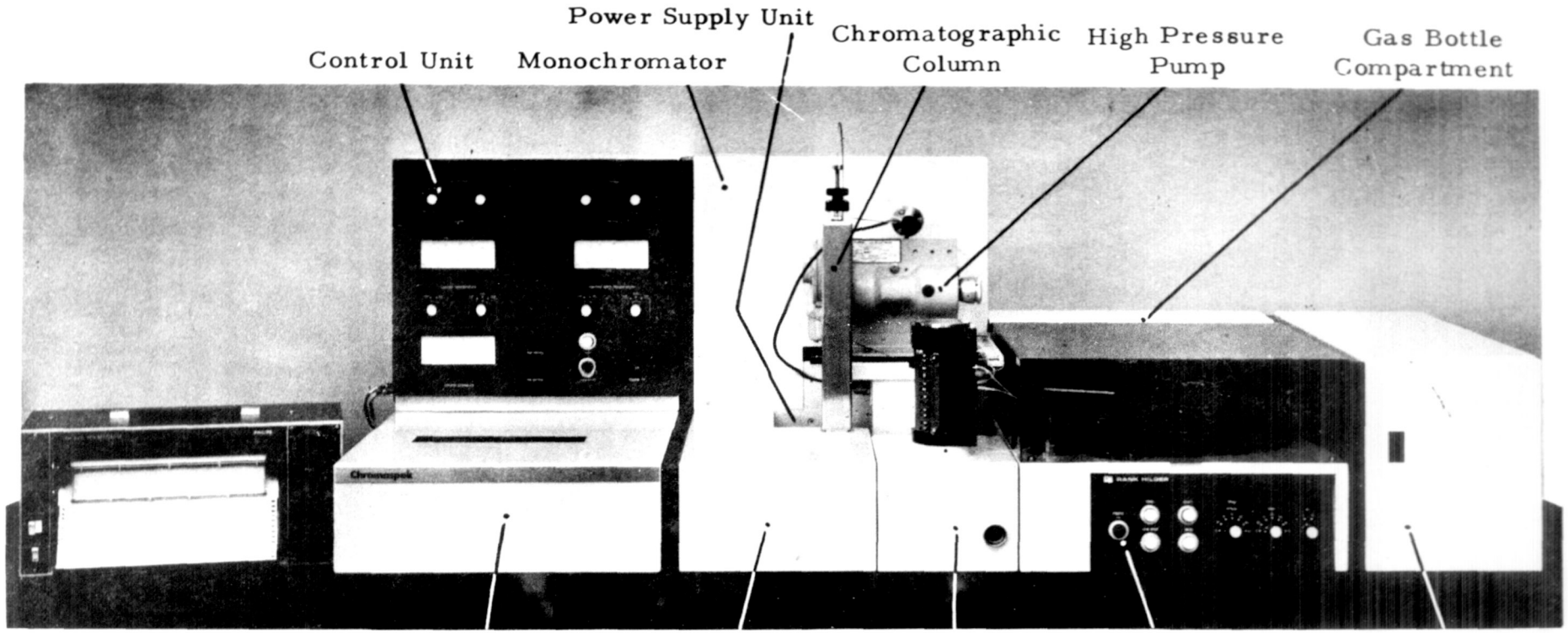


FIG. 3 CHROMASPEK J180

The precipitates remaining after the synaptosomal pellets were extracted with formic acid/methanol were assayed for protein according to the method of LOWRY et al (1951). The precipitates were digested for one hour in 3M NaOH, with frequent 'whirlimixing'. Bovine serum albumin (Sigma Grade V) was used as a standard. The alkaline copper reagent comprised 2% Na_2CO_3, 0.01% $CuSO_4$, and 0.02% Na-K-tartrate which, for the BSA standard assay, was made 0.1M NaOH. Folin-Ciocalteau reagent (BDH) was used diluted 1:1 with water. Volumes of 100 µl of sample digest were analysed. The absorbance after 40min. was determined at 500nm in a Unicam SP 600 , fitted with a flow cell.

(8) LACTATE ASSAY.

Lactate was assayed according to the method of BERGMEYER (1965) . Lactate dehydrogenase (Boehringer, pig heart LDH) converts lactate to pyruvate with a concomitant reduction of NAD^+. All absorbances were measured at 350nm in a Unicam SP500.

(9) ISOLATION AND PURIFICATION OF NEUROACTIVE AGENTS.

a)

Tremorgenic isolates of Penicillium estinogenum and Penicillium cyclopium were grown in surface culture (MANTLE et al.,1978; DI MENNA & MANTLE, 1978) and the respective tremorgenic metabolites, Verruculogen and Penitrem A, were isolated and purified by J.B.DAY at Imperial College using TLC and HPLC.

b)

The basic polypeptide Tityus toxin (mol. wt. 6995; pI 8.25) was purified from the venom of the Brazilian yellow scorpion Tityus serrulatus by the method of COUTINHO-NETTO & DINIZ (1976) by J.S.COUTINHO-NETTO working at Imperial College. The procedure involved extraction and chromatographic separation on Sephadex G-50 and CM-Cellulose-52. The toxin was homogenous as judged by electrophoresis in polyacrylamide gels with or without added sodium dodecyl sulphate. Only one NH_2-terminal (l-lysine) was detected in the preparation, which showed an LD_{50} of 18µg/kg in mice when administered intraperitoneally.

c)

The irreversible inhibitors of GABA transaminase, γ-acetylenic GABA (4-amino-hex-5-ynoic acid, RMI 71645) and γ—vinyl GABA (4-amino-hex-5-enoic acid, RMI 71754) were a gift from Dr.J.Wilkins and his colleagues at the Centre de Recherche Merrel International, Strasbourg, ———

(see METCALFE & CASARA, 1975).

d)

The agent responsible for sporadic fish kills during outbreaks of red tides on the Gulf of Mexico, T_{46}, was isolated from cultured Gymnodinium breve cells by M.RISK (RISK et al., 1979). The cells were extracted from an acidified ether fraction and fractioned by elution dry column chromatography. The most toxic fraction was further separated by preparative followed by analytical TLC. Purity of the T_{46} was demonstrated by rechromatography in analytical HPLC with both adsorptive and reverse phase packings.

(10) ADMINISTRATION TO ANIMALS OF NEUROACTIVE AGENTS.

a)

Dose response studies on the clinical effects of tremorgenic mycotoxins were made using rats. These involved the intraperitoneal injection of ethanolic solutions (10% (v/v) ethanol in water) of pure toxin (Verruculogen at 0.08-0.8mg/kg and Penitrem A at 0.1-2mg/kg), or aqueous suspensions of tremorgen containing mycelium (Penicillium estinogenum at 80-800mg/kg or Penicillium cyclopium at 60-600mg/kg). Control animals received the carrier alone.

b)

Studies on the actions of the tremorgenic mycotoxins on central synapses followed the intraperitoneal injection of rats with aqueous suspensions of tremorgenic mycelium (400mg/kg). Various regions of the CNS of the rats were removed at 45min. after injection, when clinical effects were at their maximum. Synaptosomes were subsequently prepared from these and relevant control regions of undosed rats.

c)

Three sheep were dosed by stomach tube with aqueous suspensions of Penicillium cyclopium mycelium containing Penitrem A, initially daily with 450mg (≡15mg Penitrem A), and then as required to maintain a constant level of general tremoring. The treatment periods were 50, 51, and 52 days, the number of doses 37, 38, and 43, and total Penitrem A 515, 580, and 650mg, respectively. If excited or made to run, the sheep would collapse but speedily recovered. A control for each Penitrem dosed sheep received water. Both dosed and control sheep were stunned and exsanguinated and their brains and spinal cords were removed within 30min.

d)

For the purpose of the endogenous GABA release from synaptosomes studies investigating the roles of GABA-analogues, rats were injected intraperitoneally with γ-acetylenic GABA (100mg/kg) or γ-vinyl GABA (1500mg/kg) in water, or with a similar volume of water in control animals at 6 hours or 14 hours respectively prior to preparation of cerebrocortical synaptosomes.

When studying the gross antagonism by γ-acetylenic GABA of Verruculogen induced tremor, rats were injected intraperitoneally with either γ-acetylenic GABA (100mg/kg) half an hour prior to injection of Verruculogen (mycelium, 400mg/kg), or half an hour subsequent to the onset of tremor.

(11) ELECTROPHYSIOLOGICAL EXPERIMENTS.

a)

Extensor tibialis muscle preparations from Schistocerca gregaria locust were exposed intact in the tibial segment of the hind leg and incubated at room temperature in Perspex chambers as described by USHERWOOD & MACHILI (1968) and in Locust-Ringer of composition (mM): NaCl, 140; KCl, 10; $CaCl_2$, 2; NaH_2PO_4, 4; Na_2HPO_4, 6; and at pH 6.8. Glass micro-electrodes (1M-KCl, tip diameter $<0.5\mu m$) were used to penetrate individual muscle fibres. Their d.c. resistance was 10-50Ω and this was checked during experiments to monitor tip breakage. The electrode was fitted into a non-polarisable electrode which consisted of AgCl-coated silver wire sealed into a 4cm length of glass tubing filled with 1% agar in 1M-KCl. This non-polarisable electrode was connected to one grid of a cathode follower probe by a short flexible lead and formed the input stage of a d.c. preamplifier whose output was displayed on a cathode ray oscilloscope and simultaneously fed to a pulse counter with a pen-recorder output which had a facility for collecting pulses of different amplitudes ranges by selection of the threshold levels. The counter was designed and built by A.J.THOMAS of Imperial College to measure the rate of discharge of miniature end plate potentials.

Three barrelled microinjection pipettes were drawn by hand from microelectrode glass fused together before drawing, and were made rigid with rubber sheathing and sealing wax applied between the main stems of the barrels. Tip diameters were approximately 150 μm. The injection pipette was attached to a micromanipulator positioned close to the incubation chamber and each of its barrels was connected by means of a

polyvinyl chloride tubing to a Hamilton syringe mounted near the chamber. The positions of the microinjection pipette and microelectrode were viewed through a binocular microscope. After selecting and penetrating a muscle with a high and stable membrane potential (50-80mV), and detecting miniature end plate potentials, the addition pipette was moved into position at a standard distance of 2-3mm from the recording electrode (4 fibres distance). Ten µl of solutionsto be tested were ejected onto the fibre and their effects on m.e.p,p. discharge rate and on muscle fibre membrane potential were recorded. Locust-Ringer was added first as control, then KCl to test fibre response. Subsequent to the m.e.p.p. rate returning to normal, the effect of absolute alcohol administration was studied. Finally, Verruculogen at a final concentrationof approx. 25µg/ml was added and the muscle response recorded.

b)

Investigations were also performed on the isolated circumoesophageal ganglia of the freshwater snail, Planorbis corneus. The excised ring of ganglia was placed in physiological solution (BERRY, 1972), the connective tissue between the two ganglia was cut and the ring was pinned onto a supporting pellet with the ventral side upwards in order to expose the giant dopamine neuron. The ganglia were placed in a 12ml chamber and superfused with physiological solution gassed with 95% O_2-5% CO_2 at 20'C. The supporting pellet was held on the bottom of the chamber by 3 pins. In this way, a stable fixation for electrophysiological recordings was obtained. For intracellular recordings, the giant dopaminergic neuron was impaled with single micropipettes filled with 2M KCl. The tip diameters of the electrodes were about 0.1-1.0µm and their resistances 5-20MΩ. Neuronal activity was continuously recorded on an U.V.-oscillograph (Bell & Howell 5-137) using Kodak Linagraph direct print paper. The effect of Verruculogen, on this neuron, applied in ethanol, was studied, and also the abolition by Verruculogen of the depolarising actions of microiontophoretically administered acetylcholine and dopamine.

(12) TECHNIQUES FOR PRESENTING AQUEOUS INSOLUBLE NEUROACTIVE AGENTS TO SYNAPTOSOME-SUSPENSIONS.

Penitrem A, Verruculogen, and the T_{46} toxin of Gymnodinium breve all proved to be insufficiently soluble in metabolic media to enable normal methods of administration to synaptosome suspensions to be used. In order to generate in vitro data for any modulation of amino acid accumulation as a consequence of the addition of these agents to synaptosome suspensions, various methods were utilised. Laboratory

solvent additions are described in the appropriate RESULTS section.

a) Emulsifying agents.

Mulgofen EL (G.A.F. (Great Britain) Ltd.) surfactants are polyoxyethylated vegetable oils that emulsify oils, fats, waxes, polyester resins, vinyl resins, fatty acids, Verruculogen, and T_{46}. Mulgofen EL-620 and EL-719 are closely related homologues and are predominantly hydrophilic, although EL-719 is the more water soluble of the two, due to a larger percentage of combined ethylene oxide. Also, EL -620 is anhydrous, while EL -719 contains approximately 3% water. Neither of these agents show dermatological or oral toxicity. Various weights of Verruculogen or T_{46} were dissolved in µl volumes of either of the surfactants and metabolic medium was subsequently added to give a final surfactant contribution of 0.2% v/v. The agents emulsified well, and injection intraperitoneally of 80 µg/kg of Verruculogen solubilised in this way resulted in tremor. A stock solution was made, and aliquots were added to synaptosome suspensions.

b) Nylon gauze.

Verruculogen was dissolved in chloroform, and T_{46} was dissolved in aceto-nitrile, to give known concentrations of the agents. Both solvents were maintained at 0°C to slow their rate of evaporation, and over a period of time, 5µl aliquots were pipetted onto $1cm^2$ pieces of nylon gauze (mesh 130µm) , and allowed to evaporate to dryness. Thus a series of gauzes were produced, each containing crystalline and known quantities of the agent. After the equilibration time had elapsed for the synaptosome incubation, each incubation vessel had added to it a piece of gauze containing a known quantity of the agent. Control gauzes were also added to other incubations, where only the solvent had been pipetted onto the gauze. Isotopic and bioassay techniques were used to estimate whether any of the agent, and if so, how much, had found its way into solution during the period of the incubation (although it is possible that direct contact might occur between the synaptosomes and the crystalline material on the gauze as a consequence of shaking during the experiment).

c) Liposomes.

Lipid vesicles have been employed to deliver a variety of materials (POSTE & PAPAHADJOPOULOS, 1976; GREGORIADAS, 1977; RAHAMIMOFF et al., 1978) as a consequence of vesicle fusion with the cell membrane. Unilamellar lipid vesicles of reasonably homogenous dimensions have been prepared by sonication of aqueous-lipid dispersions (HUANG , 1969;

BARENHOLZ et al., 1977). An important prerequisite for the effective use of liposomes as a drug carrier is control over their stability (GREGORIADAS , 1979). Defined here as the extent to which the carrier retains its drug contents in vitro and in vivo , stability is influenced not only by the biological environment with which liposomes come into contact, but also by their structural characteristics as well as those of the associated drugs.

Total cerebral lipids were extracted from rat brain by the method of FOLCH et al., (1957). Of the tissue, 1g was extracted with 19ml of chloroform-methanol (2:1 v/v) in a test tube homogeniser and allowed to sit for 10min.. Solids were filtered off using sintered glass, and the chloroform-methanol extract was partitioned with 0.2 vol. of 0.1M KCl in order to remove water soluble materials. The upper phase was removed using a capillary pipette with an upturned end, and the lower phase was washed several times with synthetic upper phase ($CHCl_3$; MeOH : 0.1M KCl, 3: 48: 47 v/v). The lower phase extract was taken to dryness in a stream of N_2. A portion of the total brain lipid thus obtained was retained, whilst the remainder was redissolved in 1ml of pet. ether (40°-60°), and to this was added 10ml acetone and 5 drops of saturated $MgCl_2$ in ethanol. After allowing to stand, the extract was centrifuged at 1000 rpm for 5min.. The precipitate was washed of cholesterol with a small volume of acetone, and the phospholipid extract was retained.

Into each of two vials was placed about 50mg of total lipid, and into each of another two vials was placed about 50mg of phospholipid. Into one of each pair of vials was placed known quantities of Verruculogen, and finally into all four vials was added 2ml of metabolic medium (without Mg or Ca). The suspensions were sonicated for a total of 10min., (1min. sonication with 30s cooling periods) at 4'C using a medium sized titanium probe, and with a continuous stream of nitrogen passing over the surface.

The liposomes thus prepared were added to synaptosome suspensions, and were also injected intraperitoneally to rats.

CHAPTER THREE.

IN VITRO APPROACHES TO BRAIN METABOLISM (I).

(1) INTRODUCTION.

(2) RESULTS.

1) Basal and stimulated synaptosomal respiration.
2) Basal and stimulated synaptosomal potassium retention.
3) Basal and stimulated levels of synaptosomal lactate.
4) Basal and stimulated levels of intra- and extra- synaptosomal amino acids.
5) Effect of synaptosomal protein concentration on amino acid release from synaptosomes.
6) The time course of basal and evoked amino acid accumulation in the incubation medium.
7) T_{46} : A toxin with the capacity to modulate the excitable sodium channel ?

(3) DISCUSSION.

(4) SUMMARY.

INTRODUCTION.

Much of the currently available information about energy metabolism and ion contents in the central nervous system in the presence or absence of drugs originates from in vitro studies of brain tissue. Such data supplement those obtained in vivo, since quantitative determinations are easily obtained and since different cell types can be studied separately under more or less physiological conditions. In view of the increased extracellular potassium concentration during excitation, seizures, and spreading depression, the fact is important that exposure of brain slices or synaptosomes to high concentrations of potassium, depolarising electrical pulses, or labilising drugs, (SHANES, 1958a, b; review-see BRADFORD, 1972) e.g. protoveratine (WOLLENBERGER, 1955; QUASTEL, 1965) leads to pronounced increases in the rates of oxygen uptake, aerobic glycolysis, and carbon dioxide production (ASHFORD & DIXON-K^+ on glycolysis-1935; MCILWAIN-brain slice electrical stimulation-1951; GONDA & QUASTEL - cerebal metabolism/transport mechanisms - 1962; HERTZ & SCHOU - cations and brain slice respiration - 1962; MCILWAIN & JOANNY - ion movements/electrical stimulation - 1963; DE PIRAS & ZADUNAISKY - glucose metabolism - 1965; BRADFORD - synaptosomal respiration - 1969; WHITTAKER - raised K^+- 1969; TAKAGAKI - brain slice glycolysis/pyruvate kinase - 1972; DE BELLEROCHE & BRADFORD -synaptosome review - 1973). Simultaneously, there is an accelerated turnover of the terminal phosphate in ATP (BROSSARD & QUASTEL, 1963; WALLGREEN, 1963), and the concentration of energy rich phosphates is decreased (YOSHIDA et al., 1963). Nerve fibres have been shown histochemically to be enriched in Na^+- K^+- ATP - ase activity (STAHL et al., 1977). The substrate requirement for the drug induced and the electrical stimulation is the same as that for potassium induced increase in oxygen uptake (WOLLENBERGER, 1955), and the stimulation is dependent upon the presence of a certain concentration of potassium in the medium (BILODEAU & ELLIOT, 1963). However the K^+ induced stimulation of oxygen consumption is accompanied by an increased oxidation of NAD(P)H, whereas the electrically induced increase in metabolic rate is accompanied by an increase in the reduced component (CUMMINS & BULL, 1971).

A threshold K^+ concentration of about 20mM is required to exert the K^+ induced respiratory stimulation (HERTZ & SCHOU, 1962) as well as the increase as well as the increase in aerobic glycolysis (TAKAGAKI, 1972) and the decrease in the level of ATP. The maximum effect on oxygen consumption (almost a doubling - n.b. cortical slices) occurs at about

50mM K^+ (HERTZ & SCHOU , 1962). Little or no stimulation of the oxygen uptake by addition of potassium to rat cortical synaptosomes was observed by BRADFORD et al., (1973) , but it caused increased metabolism and release of physiologically active amino acids. Sheep and rat hypothalamic synaptosomes, and rabbit, sheep, and rat cortical synaptosomes showed respiratory and glycolytic responses to electrical stimulation and also a parallel calcium dependent release of physiologically active amino acids (BRADFORD et al., 1973).

Veratridine is known to depolarise certain excitable cells by blocking the inactivation of the active Na^+ conductance (OHTA et al., 1973), and thus increasing Na^+ permeability. It has been shown that isolated nerve endings can be depolarised by micromolar concentrations of the alkaloid (BLAUSTEIN & GOLDRING, 1975) , which causes a large influx of Na^+ and a concomitant influx of Ca^+ and efflux of K^+. The cationic fluxes elicited by veratrine resemble more the fluxes occurring during physiological depolarisation than those taking place during depolarisation with high extracellular K^+ concentrations (GODDARD & ROBINSON, 1976). For this reason, and also because media containing high K^+ are either hypertonic or partly Na^+ deficient, the depolarisation caused by veratrine might be considered more physiological than that obtained with high K^+.

The ion fluxes which result from electrical stimulation and veratine stimulation of synaptosomes, and natural transmission <u>in vivo</u> , appear to be similar, in that an initial transient Na^+ influx is followed by a slower energy dependent extrusion of Na^+ in exchange for K^+. The increased Na^+ conductance across the membrane (KEESEY et al., 1965) can be blocked by low concentrations of tetrodotoxin (TTX) which therefore provides an excellent tool for investigating stimulus induced phenomena. At very low concentrations (0.3 - 1μM) , TTX is effective in abolishing the generation of action potentials and the associated influx of Na^+ into a variety of excitable neural tissues (NARAHASHI et al., 1964; RICHARDS & TERKEURS , 1971) , without affecting K^+ fluxes (GOLDMAN & BLAUSTEIN, 1966). It has been used in frog neuromuscular junction and squid stellate ganglion (KATZ & MILEDI, 1966; 1967; 1969) to block nerve impulses and hence allow the recording of miniature end plate potentials, and the effects of depolarising pulses applied locally on the presynaptic terminals. TTX also inhibits electrically and veratrine stimulated respiration, lactate production , and acetate oxidation (MCILWAIN, 1967; CHAN &QUASTEL , 1967; OKAMATO & QUASTEL , 1970) in

cerebral cortex slices, and prevents stimulus induced release of acetylcholine and noradrenaline from synaptosomes (BLAUSTEIN & GOLDRING, 1975) and of amino acids from cortical slices. It has no effect on changes due to elevated K^+ (CHAN & QUASTEL, 1967) thus perhaps supporting that which was said above in respect of "physiological" depolarisation. TTX can exist either as a zwitterion or as one of two cations, the latter being the more potent (CAMOUGIS et al., 1967). From measurements of the amount of TTX taken up by lobster nerve trunk during inhibition of action potentials, MOORE et al., (1966) reported an estimate of less than 13 channels for Na^+ per square micron of axon.

The present chapter describes data indicating that mammalian synaptosomes are fully capable of supporting the metabolic activities described above, when incubated in appropriate media, and are able to respond to depolarising conditions with concomitant release of neuroactive amino acids.

The synaptosome suspension technique was then used as a tool to elucidate the mechanism of action of the agent responsible for sporadic fish convulsions and death during the outbreaks of "red tides" on the Gulf of Mexico. The toxin is designated as T_{46} and was extracted from cultured Gymnodinium breve cells. The use of nylon gauzes as carriers of aqueous insoluble compounds is investigated in regard to the hydrophobic compound T_{46}. The importance of this question will become apparent in later chapters.

RESULTS.

1) Basal and stimulated synaptosomal respiration.

When cerebrocortical or spinal cord/medullary synaptosomes were incubated in Krebs phosphate medium containing 10mM glucose in Warburg respirometer vessels, they showed linear rates of respiration over the 30min. of experimentation (FIG. 3.1; TABLE 3.1).

In the presence of potassium (56mM) and veratrine (75µM), administered for a period of 10min. subsequent to an equilibration period of 20min., there was a significant increase in oxygen uptake. Veratrine was the more effective in eliciting this response, in that cerebo-cortical synaptosomal respiration increased by 177% over control, and spinal cord/ medullary respiration by 102% over control. Potassium evoked an increase of only 20% over control levels of respiration in cerebrocortical synaptosomes. In both cerebrocortical and spinal cord/medullary synaptosomes, the increase in respiration as a consequence of veratrine stimulation was inhibited by 1µM TTX, which was added to the synaptosome suspension in the Warburg vessel at the beginning of the experiment. The small increase in respiration as a consequence of potassium stimulation was not inhibited by TTX.

2) Basal and stimulated synaptosomal potassium retention

Stimulation of cerebrocortical synaptosomes with 75µM veratrine resulted in a loss of potassium from the synaptosomal pellet. This 44% loss of potassium was totally inhibited when 1µM TTX was present during the incubation. Square wave pulses of 10V amplitude, 0.4msec. duration, and alternating in polarity, applied to cerebrocortical synaptosome suspensions at a frequency of 100 per sec., for 10min., caused a loss of potassium from the pellet of magnitude similar to that caused by veratrine. Spinal cord/medullary synaptosomes lost 28% of their potassium content as a consequence of 75µM veratrine , and this loss was inhibited by the presence of 1µM TTX in the incubation medium (TABLE 3.1).

DATA LEGEND.

RE :- FIG. 3.1, and TABLE 3.1.

Synaptosomes were prepared from rat cerebral cortex and spinal cord + medulla tissues by means of the standard procedure described in METHODS. Pellet resuspension was into Kreb's phosphate buffered medium to give synaptosomal protein concentrations of approximately 3mg / ml..

1) Basal and stimulated synaptosomal respiration.

Into Warburg respirometer vessels (15ml) was pipetted 3ml., of synaptosome suspension. The vessels were oxygenated, with shaking, at 37'C for about 8min., prior to making the system airtight. Incubation continued for a further 8min., prior to the start of measurement of manometer levels. Readings were taken every 5min., for 20min., at which time relevant additions were made from the side arm. Readings were taken every 2.5min., for a further 10min.. At the end of the incubation, suspensions were sedimented, and the pellets were taken for protein estimation, which was established by the Lowry method, and for measurement of internal potassium and lactate contents.

2) Basal and stimulated synaptosomal potassium and lactate levels.

In addition to the datum points obtained above, synaptosome (vol.1ml) suspensions were more usually incubated in 5ml., volume conical flasks for 30min., the last 10min. being in the presence of any additions. Stimulation was effected by means of 56mM K^+, 75µM veratrine, or square wave pulses (10V amplitude; 0.4msec. duration; alternating polarity; frequency 100/sec.). At the end of the incubation, the suspensions were sedimented in a bench top ultracentrifuge, and pellets were taken for analysis.

The pellets were extracted and assayed, as described in METHODS, for lactate (Bergmeyer procedure) and K^+ (flame photometry), and protein content (Lowry).

The data are presented as the mean values $\pm$ standard error of the mean (S.E.M.), and were obtained from, in all cases, a number of experiments in excess of 20.

FIG. 3.1.

RESPIRATION RATE OF CEREBROCORTICAL SYNAPTOSOMES.

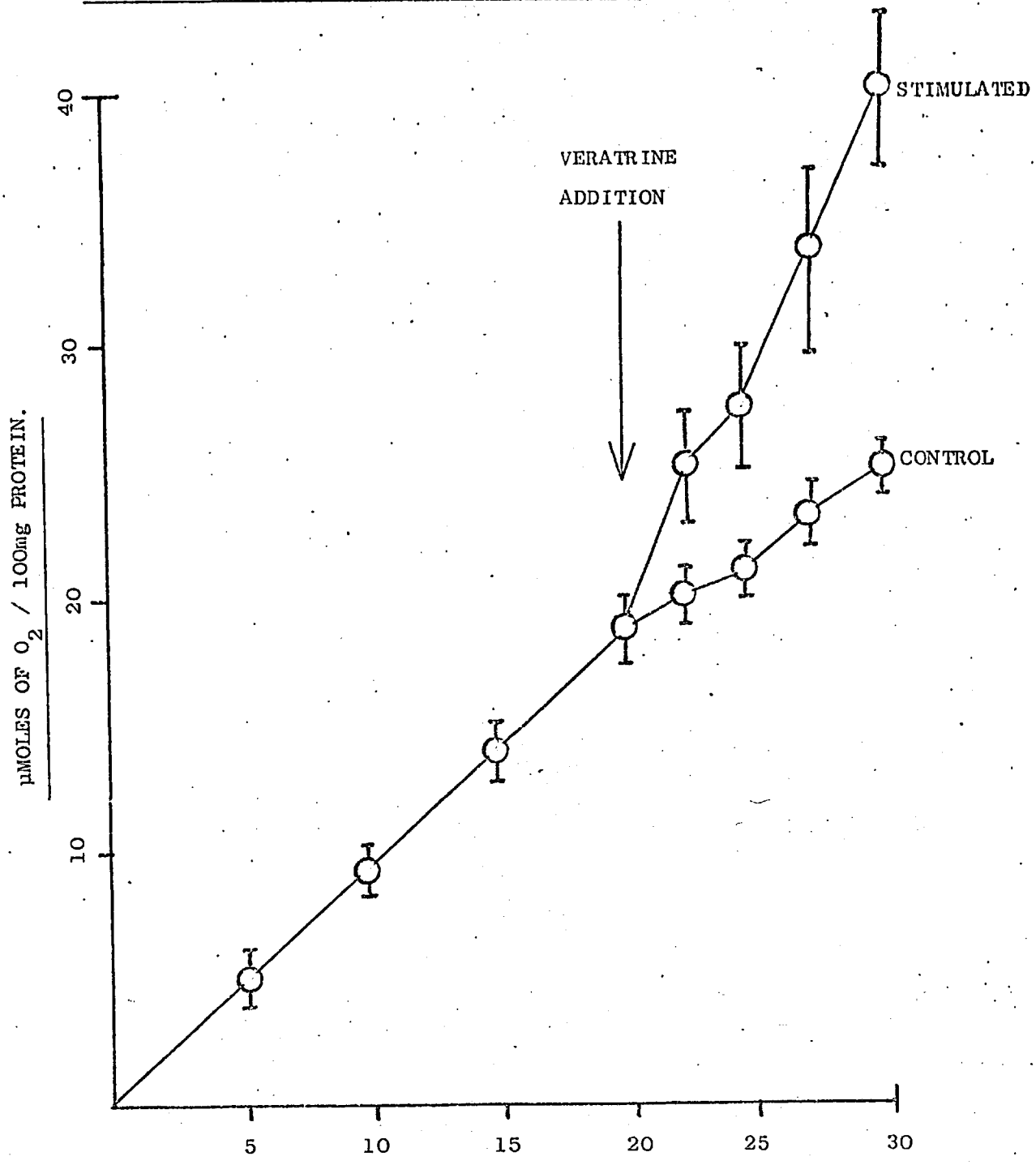


TABLE 3.1

BASAL AND STIMULATED SYNAPTOSOMAL RESPIRATION, K^+ RETENTION AND LACTATE PRODUCTION.

	µmol O_2	µ equiv.	µmol.
	100mg prot./ h.	100mg protein	100mg prot./ h.
	RESPIRATION	K^+ RETENTION	LACTATE PROD'N.
CEREBAL CORTEX			
Control	56.5 ± 3	34.2 ± 2.2	15.5± 2.5
K^+	67.5 ± 4.4	-	24.3 ± 2.8
K^+ + TTX	65.7 ± 3.9	-	n.d.
Veratrine	156.7 ± 6.2	19.1 ± 1.1	24.9 ± 1.3
Veratrine + TTX	46.7 ± 4.3	35.8 ± 1.5	n.d.
Electrical pulses	n.d.	19.2 ± 2.3	25 ± 2
SPINAL CORD / MEDULLA			
Control	60.3 ± 6.6	22.1 ± 1	11.8 ± 0.9
Veratrine	121.7 ± 12	16 ± 1	15.9 ± 1.1
Veratrine + TTX	54.8 ± 6.2	22.7 ± 1.2	n.d.

3) Basal and stimulated levels of synaptosomal lactate.

A large glycolytic response, measured as accumulated lactate was shown in parallel to the respiratory response by synaptosomes. Cerebrocortical synaptosomes increased their accumulation of lactate in response to 75μM veratrine stimulation, 56mM potassium stimulation, and electrical pulses, by 60% over control levels, and veratrine elicited a rather smaller response (35%) in spinal cord/medullary synaptosomes (TABLE 3.1)

4) Basal and stimulated levels of intra- and extra- synaptosomal amino acids

In addition to the changes evoked in respiration and glycolysis, potassium, veratrine, and electrical pulses applied to the nerve endings caused loss of amino acids to the incubation medium. Chromatograms of amino acids which accumulated in the incubation medium of synaptosomes in the presence of, and the absence of, depolarising stimuli (FIGS. 3.2, 3.3, 3.4, and 3.5) clearly show that from cerebrocortical synaptosomes, glutamate, aspartate, and GABA are the amino acids exhibiting greatest release, and that from spinal cord/medullary synaptosomes , glycine, and GABA exhibited the greatest release.

TABLE 3.2 presents data comparing the release of amino acids from cerebrocortical synaptosomes evoked by alternative methods of stimulation. Included with the data on neuroactive amino acid release are data for the accumulation of threonine, serine, glycine, and alanine in the incubation medium. For these amino acids, data on which are presented as representative of all cerebrocortical non-neuroactive amino acids measured, any changes in stimulated from control are significant $p < 0.05$. Changes in stimulated from control for the neuroactive amino acids, aspartate, glutamate, and GABA are significant $p < 0.001$.

Potassium levels of 56mM evoked the release of aspartate, glutamate and GABA from cerebrocortical synaptosomes. The subsequent accumulation of these amino acids in the incubation medium was increased by 179% (aspartate), 235% (glutamate), and 257% (GABA). The accumulation of these amino acids in the medium was also increased by 75μM veratrine administration, by 776% (aspartate), 1526% (glutamate), and 3910% (GABA). In addition, electrical pulses increased the accumulation in the medium of aspartate by 559%, glutamate by 292%, and GABA by 271%. These amino acid accumulations as a consequence of veratrine addition and to a

DATA LEGEND.

RE :- FIG. 3.2, 3.3, 3.4, 3.5, TABLE 3.2, 3.3, 3.4.

Synaptosomes were prepared from rat cerebral cortex and spinal cord + modulla tissues by the standard procedure described in METHODS. The final pellets were suspended in appropriate media (FIG. 3.2 - 3.5 in Kreb's medium buffered with Tris - HCl ; TABLE 3.2 - 3.4 in Kreb's phosphate buffered medium, of composition described in METHODS.) Incubation of 1ml. volumes of suspension took place in 5ml conical flasks on a shaking waterbath at 37'C, with continous oxygenation over the surface, but avoiding excessive evaporation. Incubation was for a total of 30min., the last 10min. being with any additions. Stimulation was effected by administration of (final concentrations) 75µM veratrine, 56mM K^+ , or square wave pulses (10V amplitude, 0.4msec. duration, alternating polarity, frequency 100/sec.). TTX concentration was 1µM.

At the end of the incubation, suspensions were sedimented on a bench top ultracentrifuge, and the fractions were separated. To each fraction was added a suitable quantity of norleucine in formic acid and the synaptosomal pellet was extracted with ice cold methanol. Protein was resedimented, and taken for analysis (Lowry method). The methanol and aqueous supernatants were taken to dryness under vacuum. The solute from the aqueous supernatant was extracted with methanol, which was taken to dryness. Suitable volumes of 0.025M HCl were added to the two methanol extracts, and aliquots were analysed for amino acid content by means of a Chromaspek auto - analyser.

Amino acid data are expressed as nmol. amino acid / 100mg. of synaptosomal protein. These values are presented as mean $\pm$ S.E.M.. The data were obtained from a number of experiments in excess of 20.

FIG. 3.2

AMINO ACID ACCUMULATION TO THE MEDIUM FROM CEREBROCORTICAL SYNAPTOSOMES

A CONTROL INCUBATION, 30min., Krebs - tris HCl incubation medium.

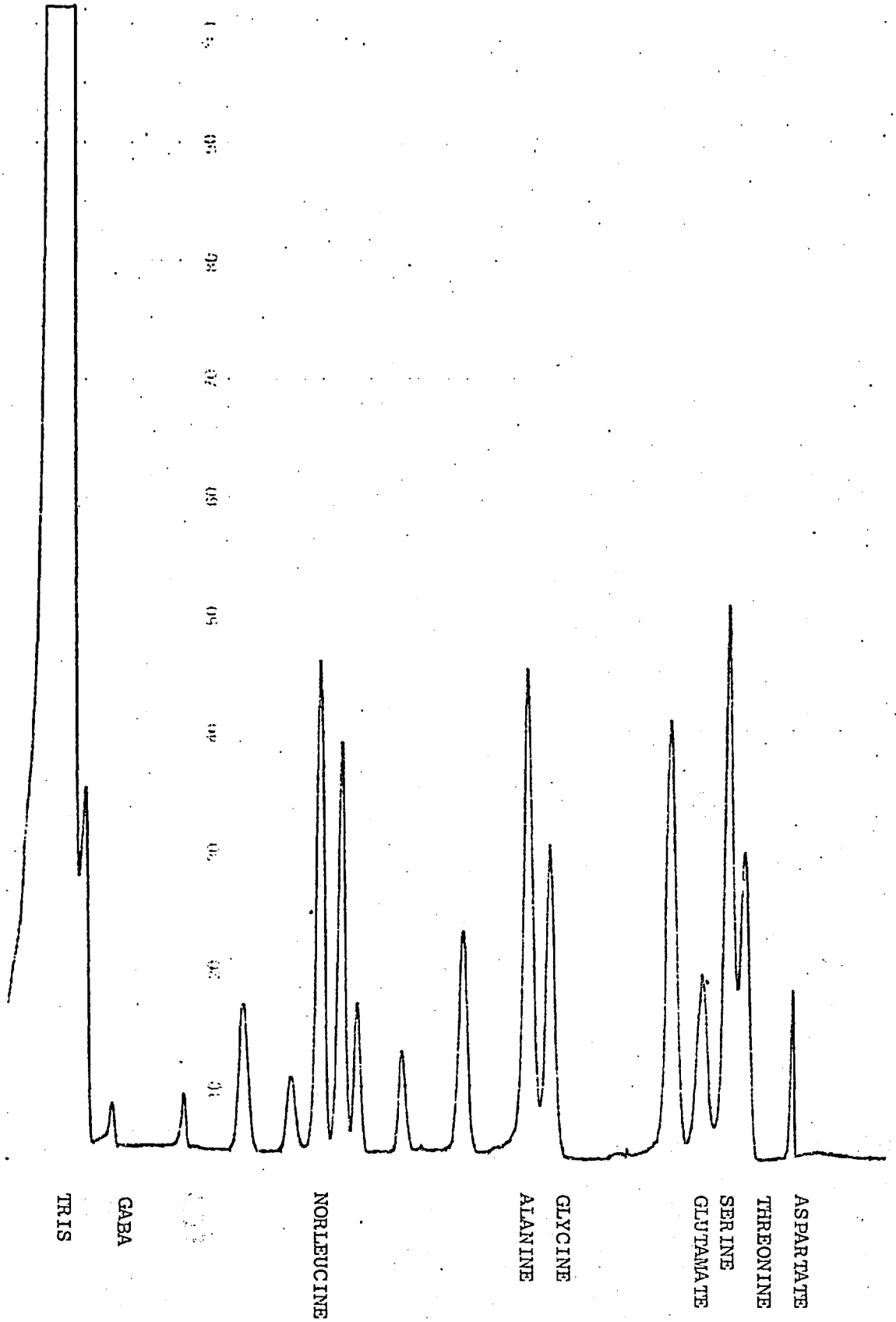


FIG. 3.3

AMINO ACID ACCUMULATION TO THE MEDIUM FROM CEREBROCORTICAL SYNAPTOSOMES

A STIMULATED INCUBATION, 75μM veratrine for last 10min. of 30min. incubation

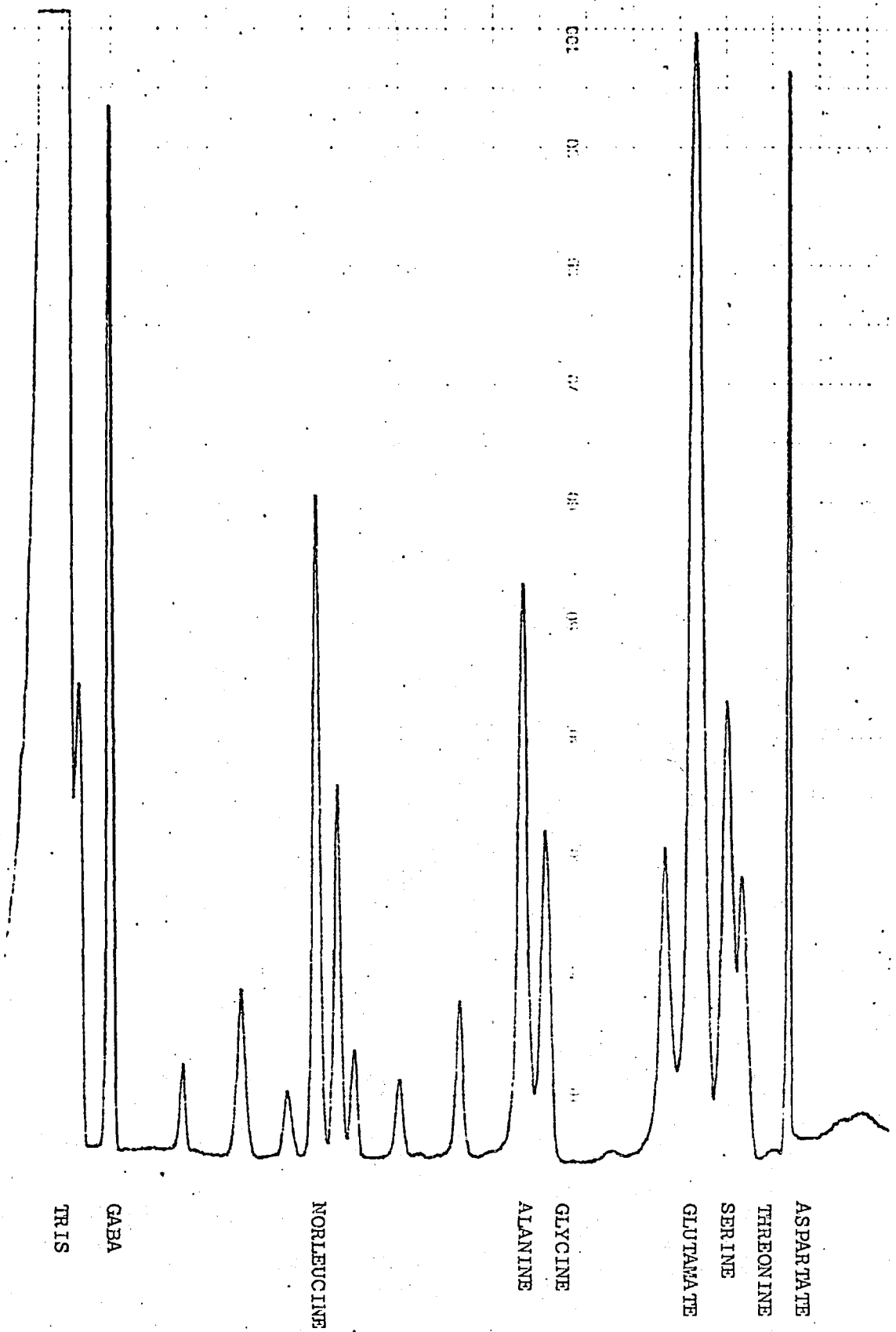


FIG. 3.4

AMINO ACID ACCUMULATION TO THE MEDIUM FROM SPINAL / MEDULLARY SYNAPTOSOMES

A CONTROL INCUBATION, 30min., Krebs - tris HCl incubation medium.

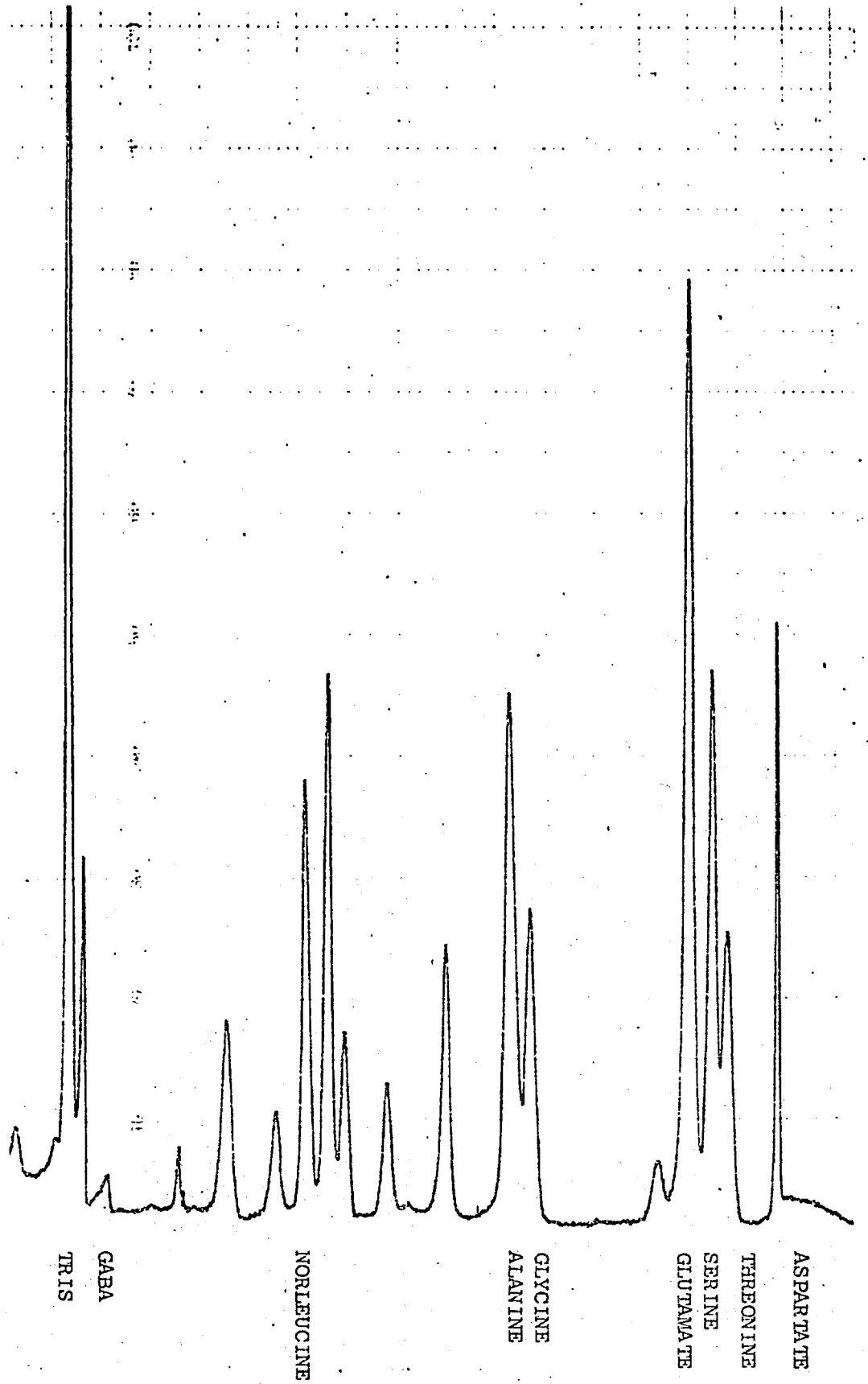


FIG. 3.5

AMINO ACID ACCUMULATION TO THE MEDIUM FROM SPINAL / MEDULLARY SYNAPTOSOMES

A STIMULATED INCUBATION, 75μM veratrine for last 10min. of 30min incubation

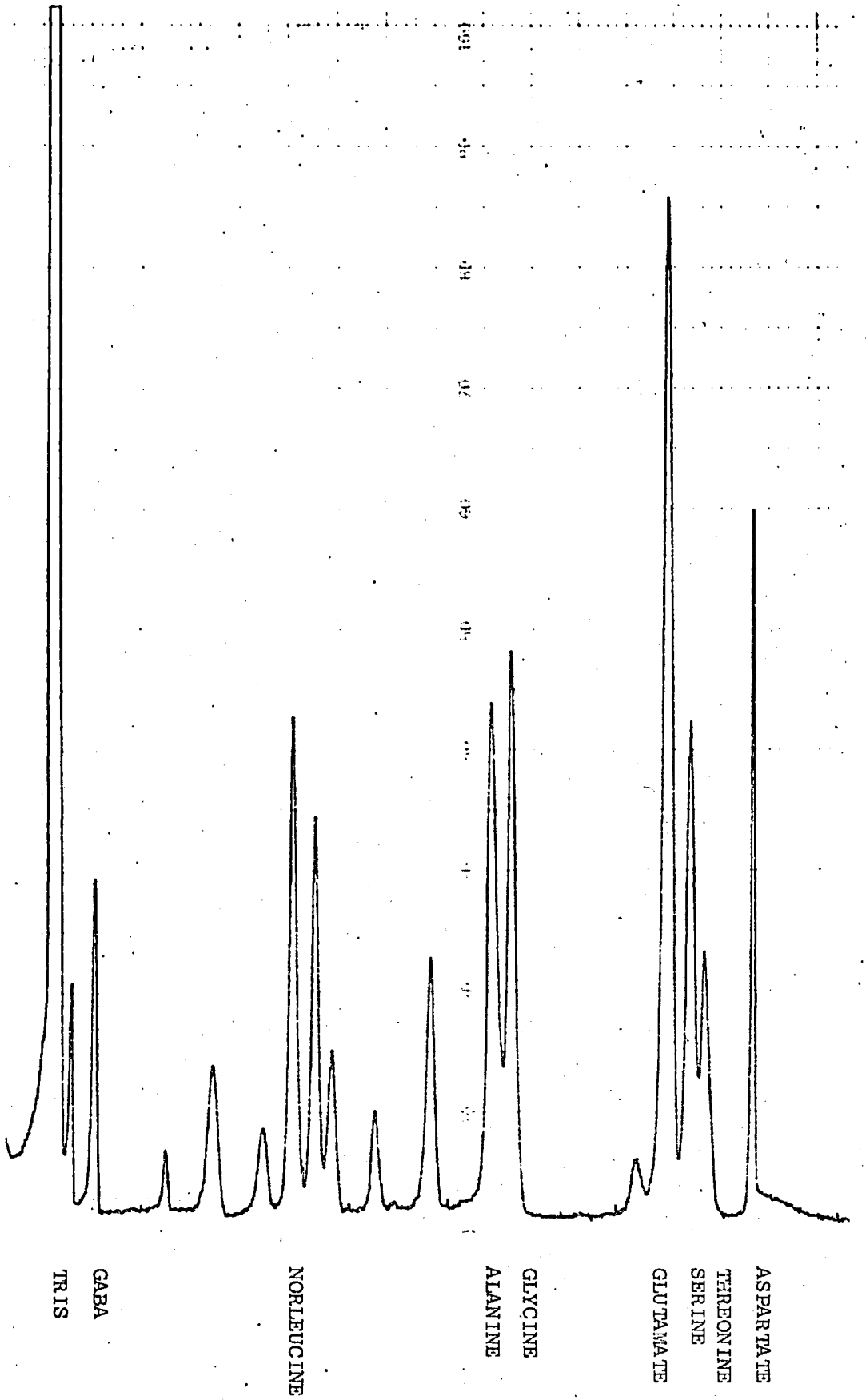


TABLE 3.2

THE EVOKED RELEASE OF AMINO ACIDS FROM CEREBROCORTICAL SYNAPTOSOMES

	nmol amino acid / 100mg protein (accumulation in medium)			
AMINO ACID	CONTROL	K^+ STIMULATED	VERATRINE STIMULATED	ELECTRICAL PULSES
ASPARTATE	63 ± 2	176 ± 17	552 ± 49	415 ± 38
THREONINE	254 ±11	212 ± 25	307 ± 7	328 ± 29
SERINE	347 ± 13	470 ± 34	442 ± 16	378 ± 32
GLUTAMATE	93 ± 3	312 ± 40	1512 ± 76	365 ± 29
GLYCINE	217 ± 13	434 ± 30	316 ± 13	273 ± 24
ALANINE	437 ± 24	462 ± 30	678 ± 45	472 ± 41
GABA	21 ± 2	75 ± 12	842 ± 19	78 ± 13

TABLE 3.3

THE EVOKED RELEASE OF AMINO ACIDS FROM SPINAL CORD / MEDULLA SYNAPTOSOMES.

	nmol amino acid (accumulation in medium) / 100mg protein.		
AMINO ACID	CONTROL	VERATRINE STIMULATED	VERATRINE + TTX
ASPARTATE	728 ± 129	808 ± 81	872 ± 90
THREONINE	653 ± 11	633 ± 90	654 ± 50
SERINE	1202 ± 28	1216 ± 40	1196 ± 30
GLUTAMATE	2075 ± 26	2326 ± 54	2195 ± 69
GLYCINE	800 ± 80	1390 ± 25	796 ± 52
ALANINE	1393 ± 15	1431 ± 25	1394 ± 170
GABA	80 ± 16	620 ± 37	117 ± 24

TABLE 3.4

TOTAL AMINO ACID CONTENT OF CEREBROCORTICAL AND SPINAL CORD / MEDULLA SYNAPTOSOMES SUBSEQUENT TO INCUBATION (CONTROL).

$$\frac{\text{nmol amino acid (total intra- + extra- synaptosomal)}}{\text{100mg protein.}}$$

AMINO ACID	CEREBROCORTICAL	SPINAL CORD / MEDULLA
ASPARTATE	4419 ± 880	4676 ± 858
THREONINE	780 ± 138	912 ± 131
SERINE	1242 ± 153	1819 ± 150
GLUTAMATE	7115 ± 1156	5904 ± 815
GLYCINE	1232 ± 103	4869 ± 755
ALANINE	1894 ± 281	2537 ± 267
GABA	1683 ± 131	2758 ± 534

lesser extent, electrical pulses were blocked by prior administration of 1μM TTX.

TABLE 3.3 presents data for the evoked release of amino acids from spinal cord/medulla synaptosomes. Similar conventions apply here to those for TABLE 3.2, and the changes for stimulated from control for glutamate and aspartate are significant $p < 0.01$. The changes for stimulated from control for glycine and GABA are significant $p < 0.001$. Threonine, serine, and alanine data are presented as representative of non-neuroactive amino acids measured.

The evoked release of glycine and GABA from spinal cord/medulla synaptosomes as a consequence of 75μM veratrine administration does not extend to either glutamate or aspartate. The release of these amino acids is not elevated over control values. The veratrine evoked release of glycine was increased by 74% over control, and of GABA is increased by 675% over control. This evoked release was inhibited in total by prior addition of 1μM TTX.

TABLE 3.4 presents data for the total (intra- + extra- synaptosomal) amino acid content of cerebrocortical and spinal cord/medullary synaptosomes at the end of a 30min. incubation. Particularly noteworthy is the fact that levels of glycine were 294% higher in spinal cord/medullary synaptosomes than they were in cerebrocortical synaptosomes.

5) Effect of synaptosomal protein concentration on amino acid release from synaptosomes.

Several workers (e.g. ROSKOSKI, 1978) have expressed concern about the role of synaptosomal protein concentrations in suspensions on their behaviour under basal or stimulating conditions. In view of the importance of this problem to work to be discussed in a later chapter, data are presented here (FIG. 3.6) comparing the basal and 75μM veratrine evoked release of amino acids from sheep cerebrocortical synaptosomes incubated at protein concentrations of 5mg/ml and 0.5mg/ml in suspension in different incubation media.

When sheep cerebrocortical synaptosomes were incubated at 5mg/ml protein concentrations in Krebs phosphate medium, the addition of 75μM veratrine evoked an increase in the accumulation in the medium of 281% for aspartate, 110% for glutamate, and 767% for GABA; and in Krebs bicarbonate medium, an increase in accumulation in the medium of 246% for aspartate, 1002% for glutamate, and 1480% for GABA was observed.

DATA LEGEND.

RE :- FIG. 3.6a, 3.6b.

EFFECT OF SYNAPTOSOMAL PROTEIN CONCENTRATION ON AMINO ACID RELEASE FROM SHEEP CEREBRAL CORTICAL SYNAPTOSOMES.

Synaptosomes were prepared from sheep cerebral cortex tissue obtained earlier in the day from an abottoir (approx. 3h. between death and the start of the preparation). At the point of dilution of the P_2B gradient fraction, the sucrose suspension was divided into four volumes, which, on the basis of previous estimations, contained quantities of synaptosomal protein that after final sedimentation and resuspension in the appropriate media would give synaptosomal protein concentrations of 5mg./ml., and 0.5mg./ ml. Resuspension was into Kreb's phosphate or Kreb's bicarbonate as indicated on the figure. Incubation was for 30min, the last 10min., being in the presence of additions. Final veretrine concentration was 75µM.

At the end of incubation, the synaptosomes were sedimented in a bench top ultracentrifuge. Relevant quantities of norleucine internal standard were added to both fractions. The figures present data for amino acids extracted from the aqueous supernatant, by means of drying in a vacuum dessicator, extracting with methanol which was also dryed down, and amino acids were dissolved in 0.025M HCl ready for auto - analysis. Protein estimations were performed on the methanol extracted synaptosomal pellets.

The data are the mean amino acid accumulation in the medium, presented as nmol.amino acid /.100mg. protein. The blacked portion of each histobar represents the background accumulation, and the white portion represents the additional signal attributable to veratrine administration. Three separate experiments were performed on three individual sheep cerebral cortices, and single values varied from the mean within 15 - 20% limits.

FIG. 3.6a

EFFECT OF SYNAPTOSOMAL PROTEIN CONCENTRATION ON AMINO ACID RELEASE FROM SYNAPTOSOMES (SHEEP CEREBRAL CORTEX). CONCENTRATION = 5mg protein / ml.

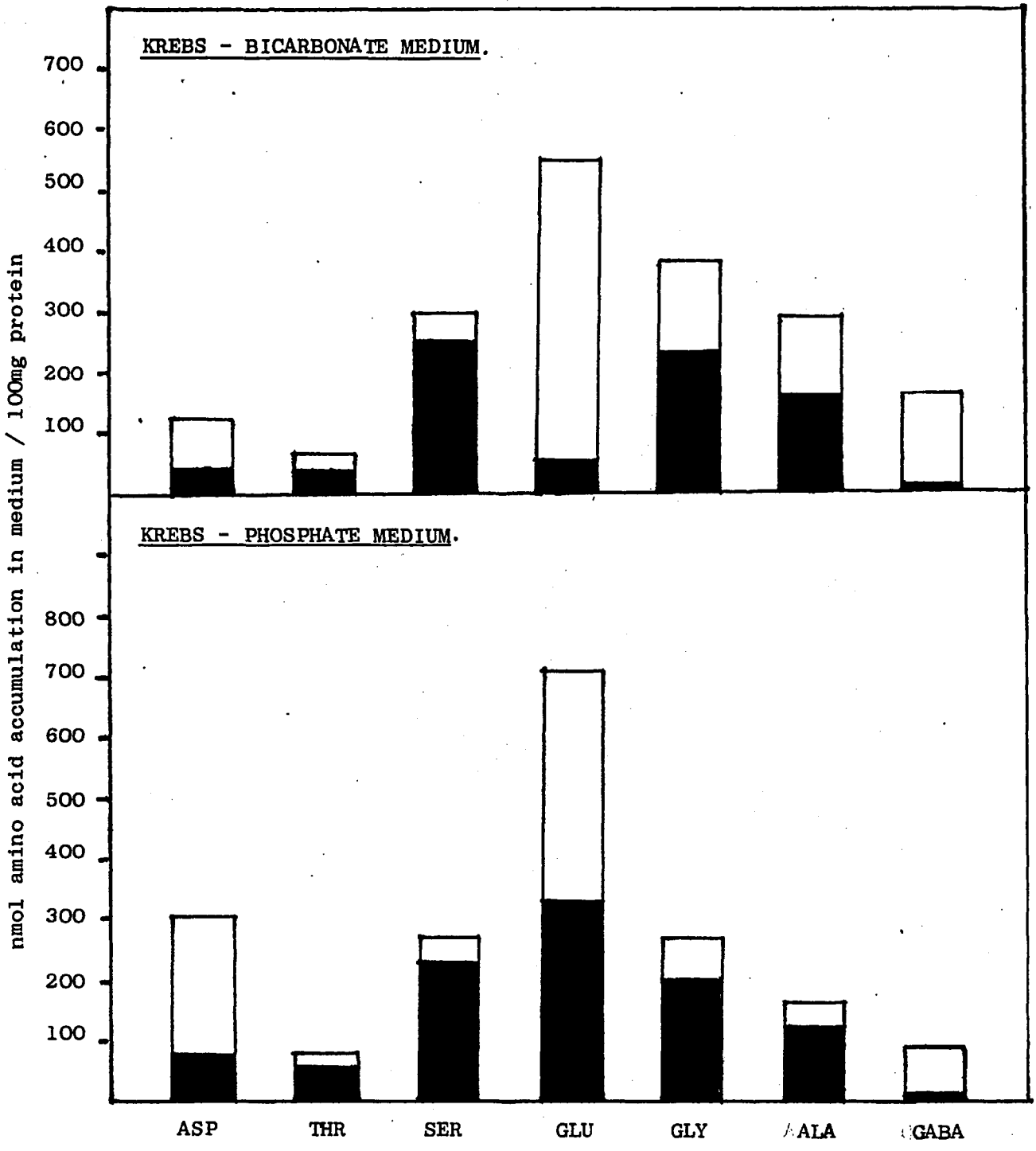
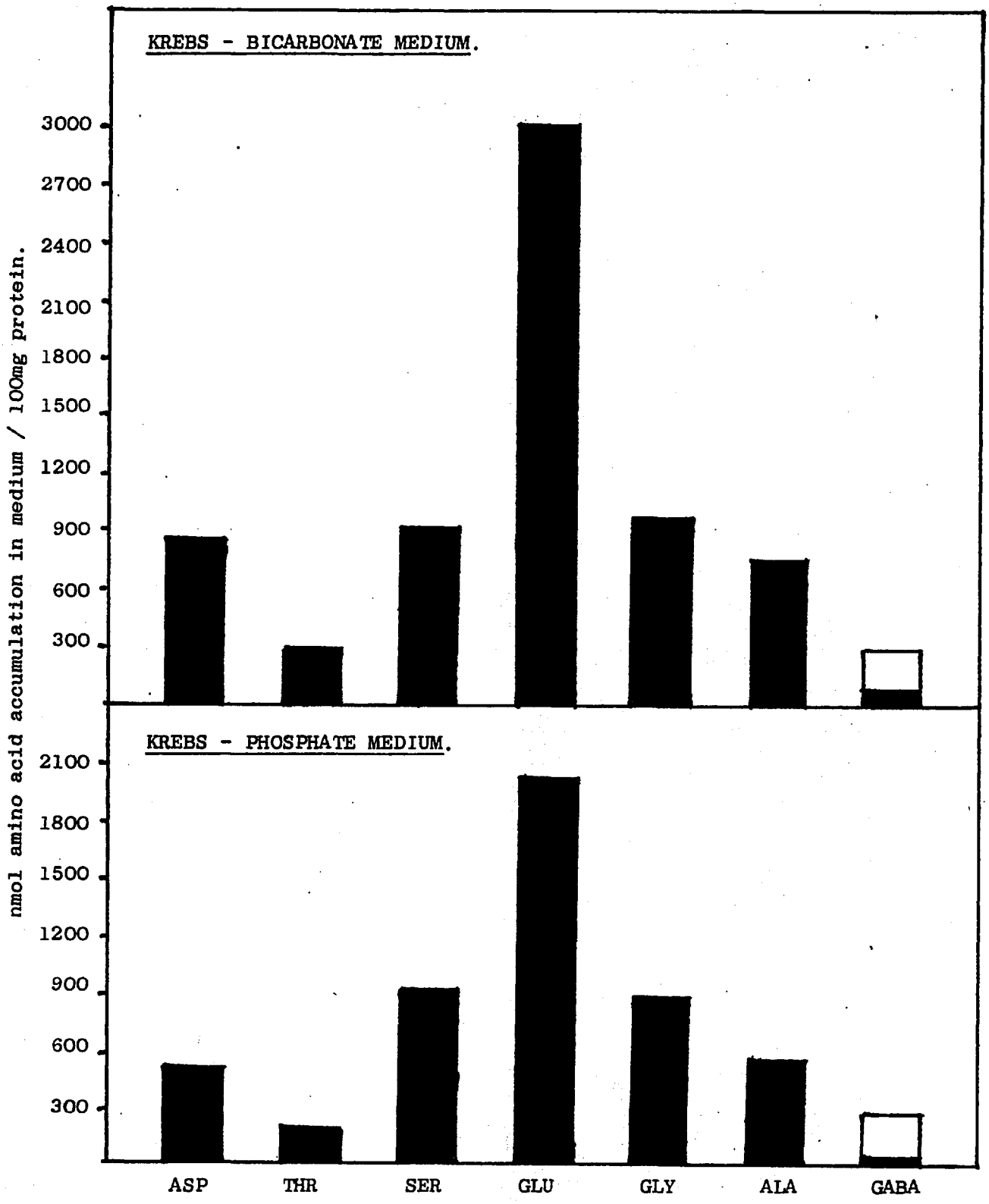


FIG 3.6b

EFFECT OF SYNAPTOSOMAL PROTEIN CONCENTRATION ON AMINO ACID RELEASE FROM SYNAPTOSOMES (SHEEP CEREBRAL CORTEX). CONCENTRATION = 0.5mg protein / ml.



When sheep cerebrocortical synaptosomes were incubated at protein concentrations of 0.5mg/ml, increased basal levels of amino acids were observed in the incubation medium, and the capacity to respond, by specifically releasing aspartate, glutamate, and GABA, to stimulating concentrations of veratrine was lost.

These data are particularly relevant when it is remembered that Levi and co-workers (LEVI & RAITERI, 1974; LEVI et al., 1974) reported that the high affinity L-glutamate system mediates exchange of endogenous and external glutamate, but little or no net uptake. Their experiments were performed with 0.25 - 0.5mg/ml of synaptosomal protein. ROSKOSKI (1977) repeated this effect, but when the synaptosomal protein concentration was raised, net glutamate uptake was observed.

Consequently, the data in FIG. 3.6 would appear to provide corroborative evidence for the lack of net uptake of neuroactive amino acids, resulting in depleted intra-synaptosomal releasable pools of these amino acids at low protein concentrations. Consequently, the administration of a depolarising agent will not elicit a further response. It should be noted, however, that GABA is anomalous in this respect.

6) The time course of basal and evoked amino acid accumulation in the incubation medium.

When freshly prepared rat cerebrocortical synaptosomes were first resuspended in Krebs phosphate medium, they lost a large proportion of their amino acid content to the incubation medium. However, within 15min. of the start of incubation, aspartate, glutamate, and GABA had been very nearly removed from the medium by the synaptosomes (FIGS. 3.7a, b. Data prior to 10min. of incubation not shown.). Under the experimental conditions, the concentrations of all three of these amino acids were less than 40μM and consequently in the high affinity range (LOGAN & SNYDER, 1971). The concentrations of the non-neuroactive amino acids, and the data for serine is shown as representative of this group (FIG. 3.7b), did not show any similar diminishing effect in the incubation medium.

Addition of 75μM veratrine to the incubation medium (FIGS. 3.7a, b) resulted in a large and rapid accumulation of aspartate, glutamate, and GABA in the medium, and this accumulation was very rapidly reversed on addition of 1μM TTX. Levels of these amino acids in the medium had returned to those of the control incubations within 15min. subsequent

DATA LEGEND.

RE : FIG. 3.7a, 3.7b, 3.8a, 3.8b.

THE TIME COURSE OF BASAL AND EVOKED AMINO ACID ACCUMULATION.

Rat cerebrocortical synaptosomes were incubated as suspensions of 3mg/ml in Krebs phosphate medium in 25ml conical flasks in a shaking water bath at 37'C. Samples of volume 0.5ml were taken at the relevant times indicated, and the supernatants were separated from the pellets by centrifugation in a bench top ultracentrifuge. Fractions were placed in cardice/propanol to await extraction at the end of the experiment. Veratrine (final concentration 75µM) and TTX (final concentration 1µM) were added at times indicated by (1) and (2) respectively.

At the end of the experiment, the separated fractions were extracted. The supernatants were taken to dryness and extracted with methanol, and the pellets were extracted with acidified methanol and taken to dryness, all by means of a vacuum dessicator. Samples were dissolved in appropriate volumes of 0.025 HCl and analysed for amino acid content by means of an autoanalyser. Pellet potassium levels were analysed by means of a flame photometer.

The datum points represent the means for three separate experiments and individual values fell within 10% of the mean. Control data are represented by (*) and data obtained from stimulated incubations are represented by (O). Protein estimations were performed on the methanol extracted synaptosomal pellets, and all data are presented as nmol. amino acid / 100mg protein and µequiv. K^+ / 100mg protein.

FIG. 3.7a

THE TIME COURSE OF BASAL AND EVOKED AMINO.ACID ACCUMULATION.

EXTRA - SYNAPTOSOMAL AMINO ACID LEVELS.

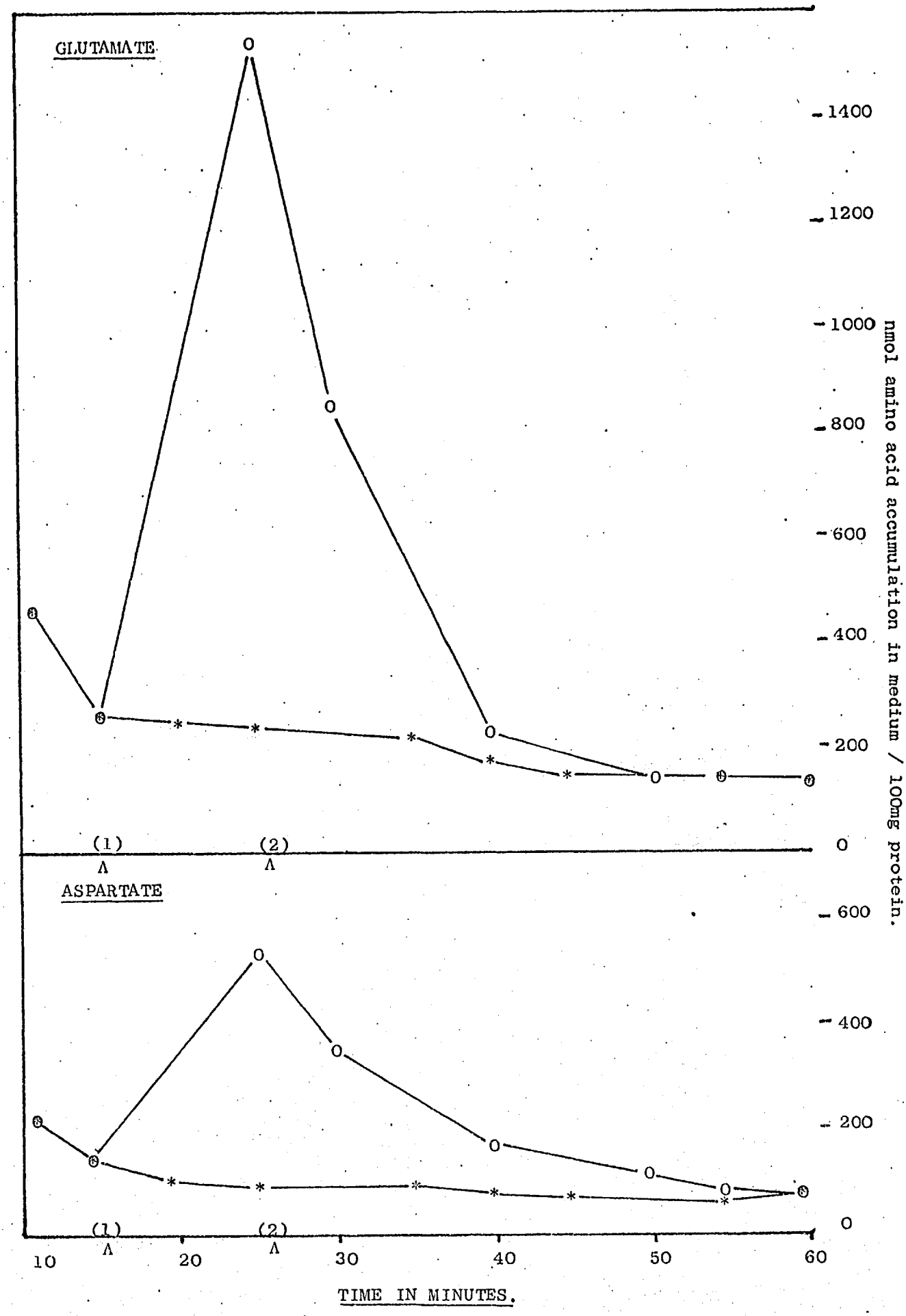
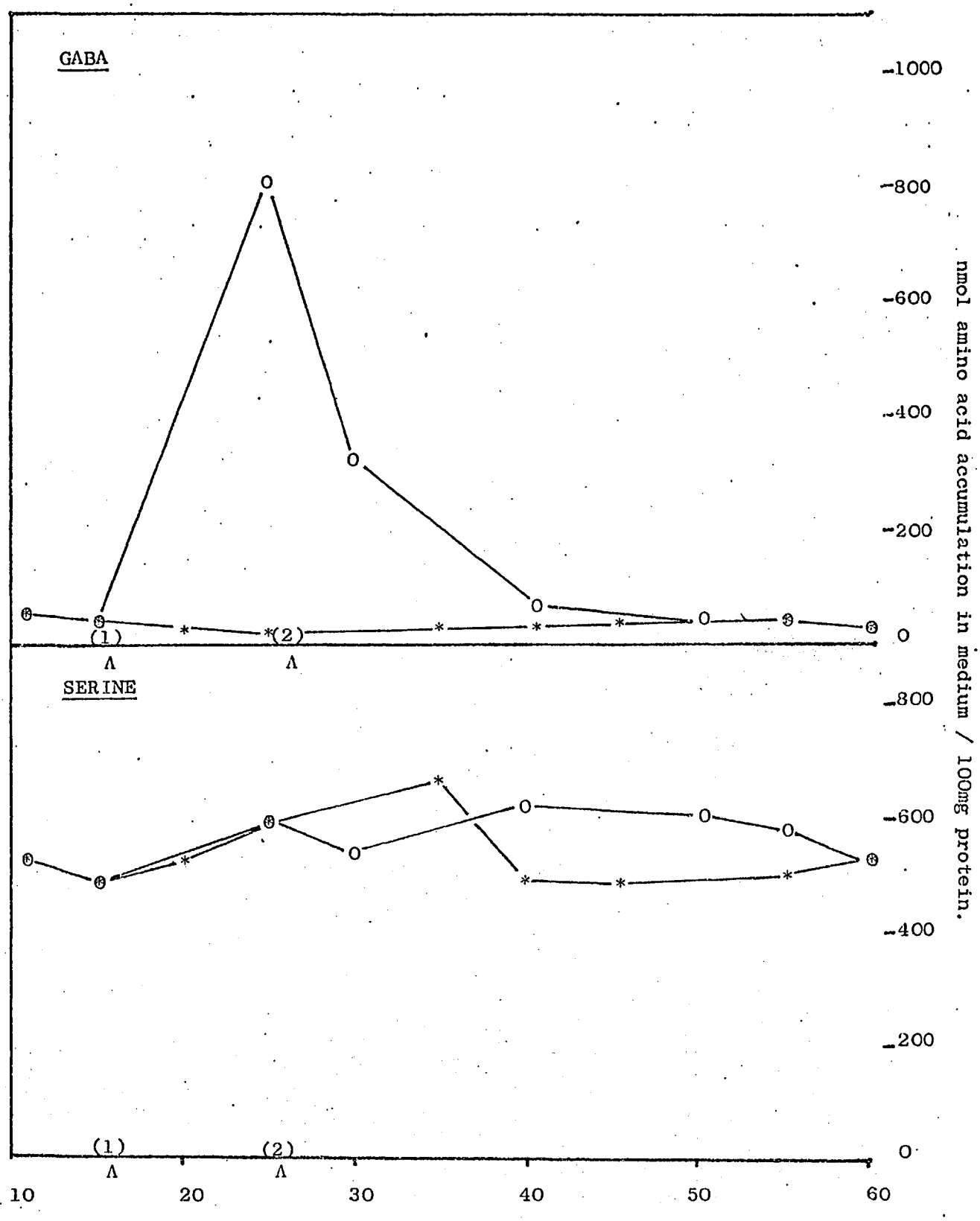


FIG. 3.7b

THE TIME COURSE OF BASAL AND EVOKED AMINO ACID ACCUMULATION.

EXTRA - SYNAPTOSOMAL AMINO ACID LEVELS.



to the addition of TTX. Estimation of the rates of uptake (glutamate, 5200; GABA , 3000 ; and aspartate , 1600 nmol / 100mg protein / hour) give values which are low compared with those quoted for uptake by cortical slices, e.g. 50μmol / g.wet weight / hour (TSUKADA et al., 1963). Since uptake rates into synaptosomes are particularly dependent upon external concentrations in the high affinity range, these rates must be regarded as minimal. Serine data are provided (FIG. 3.7b) as representative of a lack of evoked release of non-neuroactive amino acids from synaptosomes.

FIG. 3.8a, b, provide data for intra-synaptosomal amino acid pool sizes during the period of the time course study. Even in the presence of glucose, the tissue pool sizes of glutamate, GABA, and serine diminished in size over the period of incubation. Aspartate was anomalous in this respect, and the pool size of this amino acid actually increased during the 60min. period. This is most likely due to the use of endogenous amino acids as respiratory substrates, despite the presence of glucose, and synaptosomes are able to oxidise both added glutamate (BRADFORD & THOMAS, 1969), and added GABA. The 'lost' amino acids did not appear in the incubation (FIG. 3.7a, b). The potassium content of the unstimulated synaptosomes fell by only about 16% over the duration of the experiment, providing strong evidence that substrate oxidation was coupled to ATP formation for the period studied (FIG. 3.8).

When evoking concentrations of veratrine were added to the incubation, there was a large and sudden fall in the levels of intra-synaptosomal pools of aspartate, glutamate, and GABA. Subsequent to the addition of TTX, the levels began to increase, particularly in the case of GABA, where there was a clear stimulation of synthesis of this amino acid (FIG. 3.8). Unfortunately, as a consequence of the magnitude of the standard errors associated with data for intra-synaptosomal pool size, it is difficult to calculate with any degree of accuracy the extent to which synthesis of amino acid occured, particularly in respect of glutamate and aspartate.

Extensive loss of potassium from the synaptosomes occured on addition of veratrine, but on addition of 1μM TTX, intra-synaptosomal potassium levels rose again very rapidly (at a rate of 216μequiv. K^+ / h / 100mg protein). The energy dependent extrusion of Na^+ in exchange for K^+ uptake to the synaptosome is clearly not TTX sensitive. Subsequently,

FIG. 3.8a

THE TIME COURSE OF BASAL AND EVOKED AMINO ACID ACCUMULATION.

INTRA - SYNAPTOSOMAL AMINO ACID LEVELS.

Y - COORDINATE VALUES = nmol. amino acid / 100mg protein.

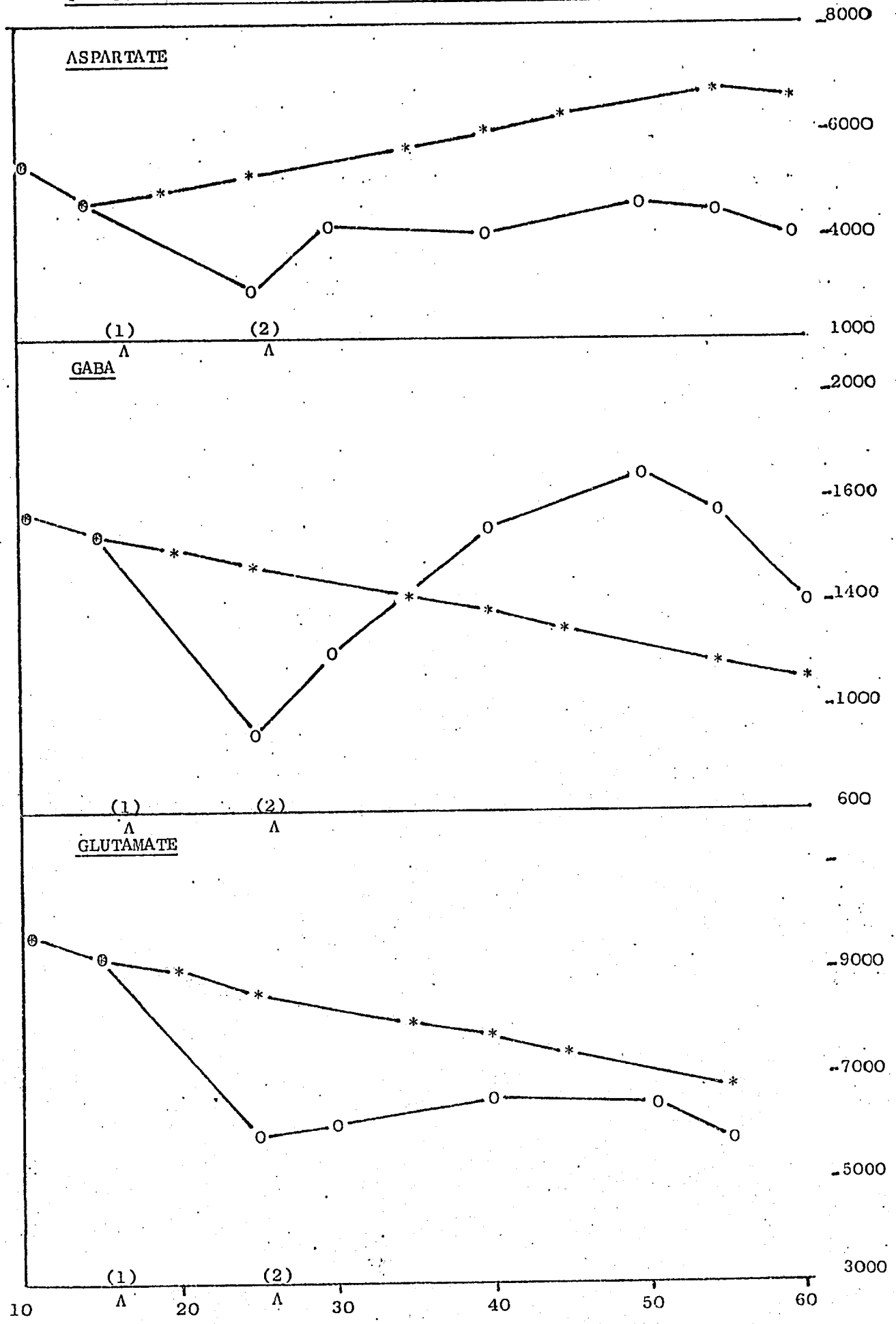
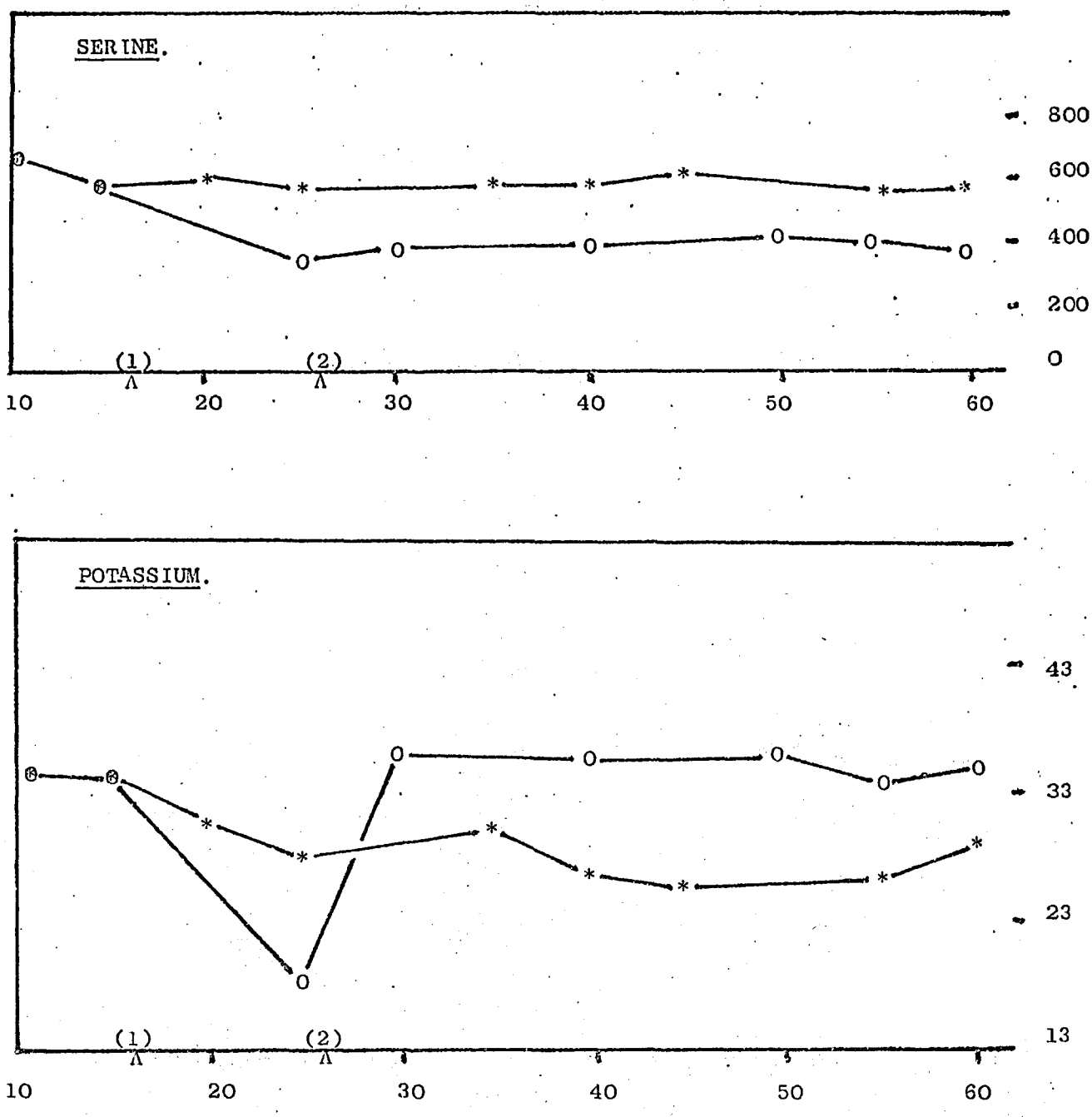


FIG. 3.8b.

THE TIME COURSE OF BASAL AND EVOKED AMINO ACID AND K^+ ACCUMULATION.
INTRA - SYNAPTOSOMAL AMINO ACID AND POTASSIUM LEVELS.
Y - COORDINATE VALUES = nmol. amino acid / 100mg protein. (serine)
Y - COORDINATE VALUES = µequiv. K^+ / 100mg protein. (potassium)



TIME IN MINUTES.

intra-synaptosomal K^+ levels were maintained for the remainder of the incubation.

7) T_{46} : A toxin with the capacity to modulate the excitable sodium channel ?

Gymnodinium breve is a unicellular alga (Pyrrophyta) endemic to the southern Gulf of Mexico, and is associated with massive fish convulsions and death during its sporadic blooms. Fractionation of the lipid extract from cultured cells yielded an active component T_{46} (M.W. 894).

Due to its aqueous insolubility, addition of the toxin to synaptosome suspensions was made by means of $1cm.^2$ nylon gauzes which had previously been impregnated with known quantities of crystalline T_{46} (see METHODS).

T_{46} toxin elicited an increase (at 700ng / ml. maximum) in rat cerebrocortical synaptosome respiration of 30% over the relevant controls. The nylon gauze had been placed in the suspension in Warburg respirometer vessels at the beginning of the experiments (it was impracticable to add from the side arms as normal protocol demands), and consequently was presumably eliciting an effect for the 15min. oxygenation and equilibration period prior to the start of measurement of oxygen utilisation. The value of 30% increase increase in respiration must therefore be taken as a minimum value. At the end of the experiment, measurement of internal potassium levels revealed a fall of 36% against controls.

T_{46} administration to synaptosome suspensions evoked an accumulation of aspartate (521%), glutamate (1150%) , and GABA (418%) in the incubation (FIG. 3.9). The evoked release of these amino acids was inhibited by 1μM TTX, added to the suspension prior to the addition of T_{46} (FIG. 3,9). Intra-synaptosomal amino acid pool levels did not change significantly from control values.

DATA LEGEND.

RE : FIG. 3.9.

THE EFFECT OF T_{46} TOXIN ON AMINO ACID RELEASE FROM CEREBROCORTICAL SYNAPTOSOMES.

Cerebrocortical synaptosome suspensions in Krebs phosphate medium were incubated in 5ml. conical vesicles on a shaking waterbath at 37'C. Present in appropriate vessels were $1cm^2$ pieces of nylon gauze, some of which had previously been impregnated with 700ng T_{46} toxin as described under Methods, the remainder being present as a control feature only. The suspensions were incubated for a total of 40min., the gauzes being present for the last 30min only. Where TTX was present in the incubation, this was added 1min. prior to the addition of the T_{46} to a final concentration of 1µM.

The histobars are grouped for each amino acid.

1) is a control incubation containing nylon gauze only.

2) is the data obtained from incubations to which gauze previously impregnated with 700ng T_{46} was added.

3) is the data obtained from incubations to which TTX and T_{46} gauze were added.

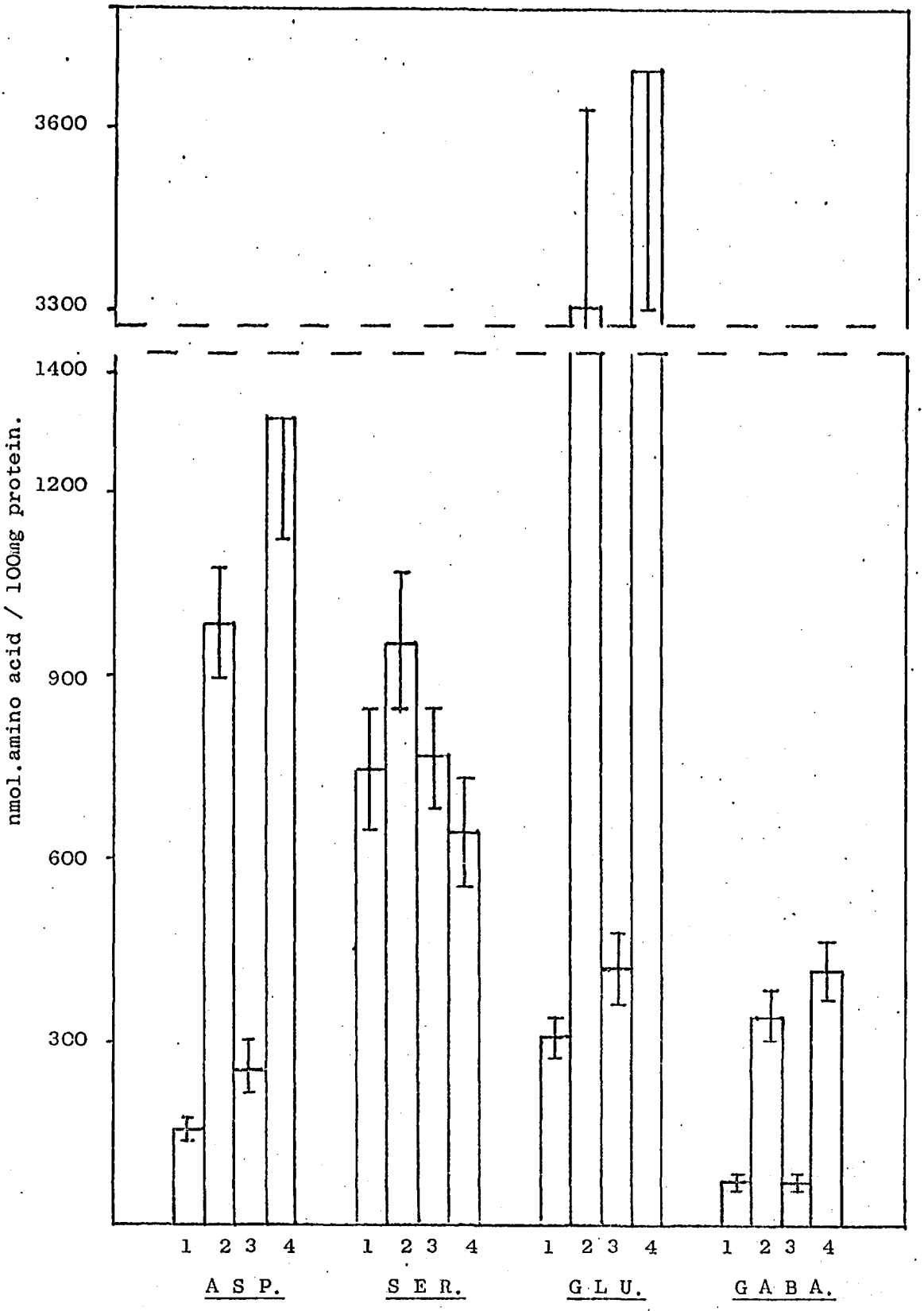
4) is the data obtained from incubations to which T_{46} impregnated gauze was added, but the Krebs phosphate medium was made in the absence of Ca^{++}.

At the end of the experiment, the suspensions were sedimented, and the supernatants and pellets were extracted with methanol. Samples were analysed for amino acids by means of an autoanalyser, and pellet protein was analysed by the Folin reagent.

The data represent the means $\pm$ SEM for four experiments. Data are included for serine as representative of all non - neuroactive amino acids measured.

FIG. 3.9

THE EFFECT OF T_{46} TOXIN ON AMINO ACID RELEASE FROM CEREBROCORTICAL SYNAPTOSOMES



1 = CONTROL : 2 = T_{46} (700ng / ml) : 3 = T_{46} + TTX : 4 = T_{46} - Ca^{++}

DISCUSSION.

It is clear from the parameters which have been measured that synaptosomes are able to maintain their key metabolic activities and their capacity to respond to high external potassium, veratrine, and electrical pulses. The presence of the full enzyme and co-factor complement necessary for complete glycolysis and oxidation of glucose linked to ATP production is clearly indicated by the considerable lactate production. The accelerated respiration and glycolysis observed under stimulating conditions may be seen as reflections of the increased ATP turnover involved in restoring the ion balance following the loss of potassium and gain in sodium (VARON & MCILWAIN, 1961). These factors are strongly indicative of the existence of a synaptosomal transmembrane potential, which, through sodium influx, is lowered by stimulation.

The almost complete suppression of stimulated release by TTX (not elevated K^+) indicates that neuronal depolarisation via fluxes through voltage sensitive Na^+ - channels is an obligatory prerequisite of increased amino acid release evoked by stimulation. The very small residual release of amino acids argues that, although electrical pulses might still depolarise neuronal elements despite the presence of TTX (KATZ & MILEDI, 1967; MCILWAIN, 1967; NARAHASHI, 1974), the pulses are most effective in opening active Na^+ channels.

Veratrine increases negative after potentials and can initiate repetitive discharges in nerve (SHANE, 1952; ULBRICHT, 1969; OHTA et al., 1973), and ULBRICHT (1969) showed that small amounts ($2.5 * 10^{-6}$ g / ml.) may only slightly depolarise nerves, but larger concentrations (10^{-5} g / ml) produce a strong and persistant depolarisation within 1min. of exposure. Veratrine triggers a Ca^{++} dependent release of noradrenaline from synaptosomes (BLAUSTEIN et al., 1972) and blocks the uptake of dopamine (HOLZ & COYLE, 1974) and noradrenaline (WHITE, 1975) into synaptosomes by a Ca^{++} independent process. Depolarising concentrations of veratrine stimulate synaptosomal dopamine synthesis (PATRICK & BARCHES 1974), and BLAUSTEIN & GOLDRING (1975) have presented indirect evidence that veratrine does in fact depolarise synaptosomes. In connection with this depolarisation, veratrine also stimulates Ca^{++} uptake by synaptosomes (BLAUSTEIN, 1975).

The absence of the effect of veratrine in the presence of TTX, or in Na^+ - free - media, suggests that the observed decrease in synaptosomal K^+ content by veratrine was probably secondary to the drug's primary

action on Na^+ influx. This association between Na^+ and K^+ permeabilities is consistent with the ionic events during depolarisation of excitable membranes when an increased permeamibility to Na^+ is rapidly followed by an increased permeability to K^+ (HODGKIN & HUXLEY, 1952a; b).

The rapid reversal of K^+ and Na^+ contents of synaptosomes together with the evidence of BLAUSTEIN & GOLDRING (1975) that the drug depolarises synaptosomes could account for observations that veratrine inhibits the Na^+ coupled uptake of neurotransmitters (HOLZ & COYLE, 1974; WHITE, 1975) by synaptosomes, since both of the former sffects would diminish the magnitude of the inward directed electrochemical gradient for Na^+ across the synaptosomal membrane (WHITE , 1976).

BLAUSTEIN (1975) suggested that the increase in Ca^{++} influx induced by veratrine was probably a direct consequence of the drug's depolarising action rather than due to an increase in $Na^+ - Ca^{++}$ exchange. He doubted that the Na^+ contents of synaptosomes would be altered during 30sec. exposure to veratrine. However, in the light of the finding (LI & WHITE, 1977) that veratrine increased the Na^+ contents of synaptosomes within 10sec. exposure, it would seem that $Na^+ - Ca^{++}$ exchange could play a part in the increased influx of Ca^{++} observed by Blaustein, and consequently in the Ca^{++} mediated release of noradrenaline from synaptosomes described by BLAUSTEIN et al., in 1972. The importance of this concept will arise again in a later chapter.

The amino acid release from cerebrocortical synaptosomes in response to elevated levels of external potassium did not increase to similar levels to those evidenced as a consequence of veratrine administration, and, as will be seen in the following chapter, the effect of high levels of potassium is not inhibited entirely by the absence of Ca^{++} from the incubation medium; and as was seen in the present chapter, is not inhibited at all by administration of TTX. Elevated potassium facilitates Ca^+ dependent secretion of accumulated transmitters from synaptosomes, presumably as a result of depolarisation induced increases in calcium permeation through voltage sensitive ionophores (BLAISTEIN, 1975; COTMAN et al., 1976). Cotman has also evidenced (HAYCOCK et al., 1978) K^+ dependent, Ca^{++} independent GABA efflux derived from a distinct pool from Ca^{++} dependent efflux, and this has an absolute requirement for Na^+ whereas Ca^{++} dependent efflux does not require Na^+ at the time of release (COTMAN et al., 1976). Potassium dependent, Ca^{++} independent efflux of accumulated GABA was associated with all

subcellular fractions exhibiting GABA accumulation, whereas K^+ facilitated, Ca^{++} dependent efflux was restricted to fractions containing synaptosomes (HAYCOCK et al., 1978) and they concluded that Ca^{++} dependent efflux reflects stimulus secretion processes, whereas Ca^{++} independent efflux may reflect membrane transport processes. Further discussion of these phenomena will be found in the following chapter.

Evidence was provided to illustrate the sequestration by synaptosomes of extra - synaptosomal aspartate, glutamate, and GABA, and mention was made of high affinity (low Km) and low affinity (high Km) transport systems (LOGAN & SNYDER , 1971; IVERSEN & JOHNSTON , 1971; WEINSTEIN et al., 1965; LEVI & RAITERI , 1974; MARTIN, 1974). LEVI & RAITERI, (1974) , incubated synaptosomes (0.25mg protein / ml) in 10µM ^{14}C-L - glutamate. Although the synaptosomes took up radio - isotope, the medium L - glutamate concentration remained the same, and the authors postulated a homoexchange of synaptosomal and medium L - glutamate to account for the results. However, ROSKOSKI, (1977), using synaptosomal protein concentrations of at least 1mg / ml found that radio - isotope and L - glutamate uptake are equivalent. The mechanism of this effect is unknown, but the process is temperature and Na^+ dependent and the concentration of glutamate is in the range expected of high affinity uptake systems. The high levels of glutamate accumulating in the medium in low protein concentrations of sheep cerebrocortical synaptosomes can presumably be explained in terms of this phenomenon, and consequently it would seem reasonable that, at these protein concentrations, the lack of a veratrine evoked release of neuroactive amino acids is a result of depleted releasable pools intrasynaptosomally. Furthermore, the fact that the evoked release of glutamate is affected to a greater extent at the low protein concentrations than is that of GABA could be explained by the more general role of glutamate in the metabolism of all neuronal and glial cells, whereas that of GABA is thought to be associated with more specialised neurotransmitter function.

Purified toxin T_{46} extracted from Gymnodinium breve red tide organisms elicited in rat cerebrocortical synaptosome suspensions an increase in respiration, a loss from the synaptosomes of potassium, and a release of neuroactive amino acids which was inhibited by the administration of TTX. In 1975, KIM et al., showed that a partially

purified toxin from the organism elicited a spontaneous train of action potentials in the giant axon of the squid. The spikes had a shape similar to that in normal seawater except for an increase in the rate of recovery from the afterhyperpolarisation. With this more rapid recovery, the membrane potential overshoots the resting potential and threshold, triggering another spike and thus producing repetitive firing. Voltage clamp studies (WESTERFIELD et al., 1977) revealed that the toxin had no effect on the normal sodium or potassium conductance changes produced by step depolarisation. However, consistent with the faster recovery after an action potential, the toxin speeds recovery of the 'shut - off' currents to their steady state values after a depolarisation. The most likely mechanism by which the toxin accelerates recovery after an action potential (leading to repetitive firing) is the induction of a small additional inward current which was found to be reduced by prehyperpolarisation. This toxin induced current which speeds recovery was blocked by tetrodotoxin (WESTERFIELD et al., 1977) and hence presumably flows through the sodium channel. Similar currents have been observed in myelinated nerves where they were induced by scorpion venom (CAHALAN, 1975), aconitine (SCHMIDT & SCHMITT , 1974) , and veratrine (ULBRICHT, 1965), and in squid axon when induced by veratrine (MOORE et al., 1968).

SUMMARY.

The main thrust of these experiments has been to examine some of the physiological properties of synaptosomes and particularly in terms of the data generated in the hands of the present experimenter. It should now be apparent that the isolated nerve endings appear to retain a considerable degree of functional integrity. Perhaps most important from the physiological viewpoint, is the evidence that synaptosomes exhibit some of the special properties of intact nerve terminals: depolarising agents reversibly increase the Na^+, K^+, and Ca^{++} permeability of synaptosomes, and the ensuing Ca^{++} entry can be correlated with the release of neuraltransmitter substances. This sequence of events has been well documented at periphal nerve terminals, and the data presented here indicate that similar processes exist at mammalian central endings which have been subjected to the rigours of homogenisations and fractionations.

That the integrity of synaptosomes is well maintained is made very clear by their response not only to depolarising agents , but also by

their rate of recovery in terms of measured internal K^+ and rate of uptake of released neuroactive amino acids subsequent to termination of the stimulating response by TTX. The roles of Na^+ and K^+ are made abundantly obvious.

The movement across the synaptosomal membrane of Na^+ and K^+ has been the particular theme of the present chapter. A natural progression is a study of Ca^{++} involvement and the following chapter investigates the usefulness of a particular Ca^{++} antagonist on transmitter release studies in relation to the Ca^{++} dependency of a variety of depolarising agents

CHAPTER FOUR.

IN VITRO APPROACHES TO BRAIN METABOLISM II.

ON THE THEME OF THE ROLE OF Ca^{++}.

(1) INTRODUCTION.

(2) RESULTS.

1) Rates of oxygen utilisation, and levels of potassium retention achieved by synaptosomes under depolarising conditions, and in the presence of, or absence of, Ca^{++}.

2) The spontaneous release of amino acids from synaptosomes incubated in the presence and absence of Ca^{++}.

3) The evoked release of amino acids from synaptosomes incubated in the presence and absence of Ca^{++}.

4) The effect of verapamil concentration on the inhibition of veratrine evoked amino acid release.

5) The effect of increasing Ca^{++} concentrations on the inhibition by verapamil of the veratrine evoked release of amino acids.

6) The inhibition by verapamil of the tityus toxin evoked release of amino acids from rat cerebrocortical and spinal cord / medulla synaptosomes.

7) The effect of verapamil on the elevated potassium stimulation of cerebrocortical synaptosomes.

(3) DISCUSSION.

1) Spontaneous amino acid release.

2) Evoked amino acid release.

3) Contrasting the neurotoxic actions of tityus toxin and veratrine.

4) Summary.

INTRODUCTION.

It is now well known that Ca^{++} plays a critical role in the release of neurotransmitters at synapses, and in the secretion of hormones (e.g. MILEDI , 1973). Release occurs spontaneously at a rate proportional to the level of resting intracellular Ca^{++} (DUNCAN & STATHAM , 1977; ERULKAR et al., 1978; HUBBARD et al., 1968; MILEDI , 1973) $(Ca^{++})_{in}$, while evoked release due to nerve action potentials is a function of a transient increase in $(Ca^{++})_{in}$ resulting from Ca^{++} influx from extracellular space during the action potential (DODGE & RAHAMIMOFF, 1967; HODGKIN & KEYNES , 1957; KATZ , 1969). Presumably the intracellular site at which Ca^{++} acts to permit transmitter release is the same whether release is spontaneous or evoked (HUBBARD et al., 1968). The electrophysiological studies of KATZ & MILEDI (1967, 1969) on squid giant axon synapses have demonstrated that presynaptic terminals show a depolarisation dependent increase in Ca^{++} permeability and the subsequent entry of Ca^{++} ions has been shown to be essential in the stimulus - coupled release of neurotransmitter (KATZ & MILEDI , 1967). There is also evidence that Ca^{++} may regulate the activities of various enzymes in the nerve terminals (KAKIUCHI & YAMAZAKI , 1970 ; OHASHI et al., 1970). The studies of NICHOLLS & SCOTT (1980) demonstrate that the mitochondria, possessing a sophisticated mechanism for regulating the distribution of Ca^{++} ions across their inner membrane, based on a steady state cycling of Ca^{++} between independent Ca^{++} uptake and efflux pathways, play a predominant role within the cytosol of the isolated synaptosome; whereas previous studies (KENDRICK et al., 1977; BLAUSTEIN et al., 1978; RAHAMIMOFF & ABRAMOVITZ , 1978) emphasise the role of ATP - dependent Ca^{++} accumulation by intrasynaptosomal reticular membranes. ICHIDA et al., (1980) , provide data on high potassium depolarised synaptosomes indicating that Ca^{++} binding on the synaptic plasma membrane may be as important as a Ca^{++} reservoir or regulator of Ca^{++} in the nerve terminals as the mitochondria.

The stimulated Ca^{++} uptake by synaptosomes shares many of the properties of the Ca^{++} conductance mechanisms in a variety of tissues including vertebrate cardiac muscle, invertebrate muscle, and squid axons (see reviews by BAKER , 1972, and REUTER , 1973) , and BLAUSTEIN , (1975) provides evidence to show that the depolarising agents potassium, veratrine, and scorpion venom stimulate Ca^{++} uptake and illustrates a Ca^{++} dependent high K^{+} stimulated release of acetylcholine (see also

DE BELLEROCHE & BRADFORD , 1972) and noradrenaline, and a partially Ca^{++} dependent veratrine and scorpion venom stimulated release of noradrenalin.

The dependence of the release of GABA on extracellular Ca^{++} has not been uniformly observed in brain slices or synaptosomes; the effect of omitting Ca^{++} from the incubation medium has depended on the nature of the releasing stimulus. The release of exogenously labeled GABA evoked by elevated K^{+} from brain slices was consistently reduced in the absence of Ca^{++} (SRINIVASON et al., 1969; MULDER & SNYDER, 1974; VARGAS et al., 1977; REUBI et al., 1977; NEAL & BOWERY, 1979) as was the release of endogenous GABA (NADLER et al., 1977). Elevated K^{+} also released exogenously or endogenously labelled and endogenous GABA from synaptosomes in a Ca^{++}dependentmanner (DE BELLEROCHE & BRADFORD, 1972; REDBURN et al., 1976; COTMAN et al., 1976; HAYCOCK et al.,1978). In contrast, the release of exogenous, labelled GABA from brain slices induced by electrical stimulation was not reduced by omitting Ca^{++} (KATZ et al.,1969; SRINIVASAN et al., 1969; HAMMERSTAD et al., OKADA & HASSLER, 1973; ORREGO & MIRANDA, 1976), although partial Ca^{++} dependence of electrically released endogenously formed (^{14}C) GABA was also reported(POTASHER , 1978). Valdo and Orrego (1978) reported that the release of endogenous GABA evoked by sine-wave stimulation was suppressed if the Ca^{++} content of the medium was reduced from 1 to 0.1mM, but increased again when Ca^{++} was entirely eliminated. Short exposure to veratridine induced a release of both exogenously labeled and endogenous GABA from brain slices, which was partially dependent on extracellular Ca^{++} (NADLER et al., 1977), but Benjamin & Quastel (1972) have found that the absence of Ca^{++} potentiated endogenous GABA release from brain slices induced by 1 hour exposure to protoveratridine. The release of GABA from the brain in vivo, whether induced by electrical stimulation (IVERSEN et al., 1971) or by elevated K^{+} (CLARK & COLLINS , 1976) was suppressed in the absence of Ca^{++} .

All manner of explanations have been contrived to account for the less consistent dependence of neuroactive amino acid release on external Ca^{++} than tnat of other transmitters such as acetyl choline or noradrenaline. SRINIVASAN et al., (1969) suggested that sufficient Ca^{++} was left in the tissue even when Ca^{++} was omitted from the incubation medium to support release by electrical stimulation. MULDER& SNYDER (1974) having found a greater Ca^{++} dependence of the release of transmitter amino acids from brain slices induced by high K^{+} than for electrically induced release, concluded that release

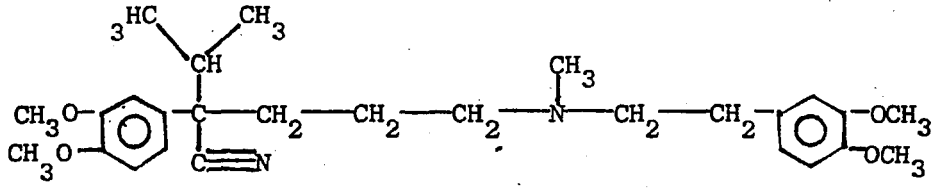
induced by electrical stimulation is not as physiological as that induced by depolarisation with high K^+ (c.f. LEVI et al.,1980.). COTMAN et al., (1976) observed a residual , Ca^{++} dependent, high K^+ induced release of GABA from synaptosomes. and tentatively suggested that this might have originated from other subcellular elements than synaptosomes. VARGAS et al., (1977) compared the release of exogenously labeled noradrenaline and GABA from cortex slices induced by electrical stimulation and by elevated K^+ in the presence of different concentration of Ca^{++}. They concluded that exogenous GABA is not taken up into synaptic vesicles but is located in the cytoplasm of terminals, as is noradrenaline subsequent to reserpine administration , and the release of transmitters from the cytoplasm requires more intensive stimulation and is less dependent on Ca^{++} than that from synaptic vesicles.

The purpose of the present experiments was to look further at the whole question of Ca^{++} dependency of both basal and evoked amino acid transmitter release from synaptosomes, and concentrating particularly on the validity of a role for Verapamil (iproveratril) as a Ca^{++} antagonist which blocked the evoked release of neuroactive amino acids caused by veratrine and scorpion venom toxin tityus toxin, the active component of the venom of the Brazilian yellow scorpion Tityus serrulatus.

Sufficient has been said, for the moment, about veratrine. Scorpion venom has been shown to increase the release of acetylcholine from slices of rat brain (GOMEZ et al., 1973) and segments of guinea pig ileum (DINIZ & TORRES, 1968) . It enhances the release of preloaded (^{14}C) glutamate and (^{14}C) GABA from rat cortical synaptosomes (TSE et al., 1980) and there is evidence that it releases catecholamines from rat myenteric plexus (TAFUN et al., 1971). Tityus toxin, the active component of the venom which has been used in these studies, appears to work by depolarising nerve terminals through an action at the active Na^{++} channel (LINDEN & RAFTERY,,1976). Thus, its actions are prevented by added tetrodotoxin. Application of tityus toxin directly onto the sensori-motor cortex of awake rats (COUTINHO NETTO et al., 1980) produced contralateral myoclonic forelimb jerks, which is the charateristic response to similar application of veratrine or electrical pulses (DODD & BRADFORD, 1976) thus supporting a depolarising action for tityus toxin. The in vivo release of aspartate, glutamate and GABA activated by the toxin is prevented by TTX, which also prevented the in vivo limb jerking response from occuring and

indicates that Na^+ channel activation is the mode of action. (COUTINHO NETTO et al, 1980). The Ca^{++} dependence of this release , and the release measured as a consequence of in vitro administration was far from absolute(COUTINHO NETTO et al., 1980) and was similar in extent to that found by TSE et al., (1980) for exogenous (^{14}C) glutamate and (^{14}C) GABA release from synaptosmes.

In recent years, verapamil has become an increasingly important agent both as a tool for studying movements of Ca^{++} and as a potentially useful therapeutic agent for treatment of certain cardiac arrythmias (SINGH et al., 1978) . Evidence has also been gathered suggesting that it may be useful for the treatment of hypertension (LEWIS et al., 1978). The actions of verapamil have been studied extensively on the cardiac action potential. The drug has little effect on the fast depolarisation phase of the action potential (TTX sensitive Na^+ conductance) but blocks the prolonged depolarisation phase (ROSEN et al.,1974; BAYER et al., 1975), which generally has been thought to be carried by Ca^{++} ions. Thus the conclusion was reached that the actions of verapamil are due to a rather specific blockade of "slow" Ca^{++} channels (SINGH et al., 1978). This blockade of Ca^{++} channels has also been suggested as the explanation for the ability of verapamil to block vascular smooth muscle contraction, thus causing peripheral and coronary vasodilation (MC MURTRY et al., 1976). In addition to its action of blocking slow Ca^{++} currents, Shigenobu (MC LEAN et al., 1974; SHIGENOBU et al., 1974) has shown that verapamil can block "slow" Na^+ currents. These slow Na^+ currents (distinct from TTX sensitive action potential currents) are generally small under most conditions but they can be easily observed in certain systems (MC LEAN et al., 1974; SHINGENOBU et al., 1974).



VERAPAMIL.

ISOPTIN.

IPROVERATRIL.

It is perhaps worth drawing attention, at this point, to the fact that the data presented in this chapter has little medical significance, since the few studies that have been located (e.g. MANGIARDI et al., 1978 SHOMERUS et al., 1976) indicate that the serum concentration of verapamil achieved clinically probably is somewhere in the range of 1μM: However, the purpose of the present investigations is more concerned with the potential for utilisation of verapamil as a tool for the study of Ca^{++} dependence of synaptosomal amino acid release mechanism than with its clinical mode of action.

RESULTS.

1) Rates of oxygen utilisation, and levels of potassium retention achieved by synaptosomes under depolarising conditions, and in the presence of, or absence of, Ca^{++}.

TABLE 4.1 summarises data obtained subsequent to incubation of synaptosomes in high external potassium (56mM) , veratrine (75µM) , and tityus toxin (50nM) , and the extent to which these data were altered in the presence of, or absence of, Ca^{++}. The rate of oxygen utilisation by cerebrocortical synaptosomes increased by 34% in the absence of Ca^{++} from the incubation medium and this was reduced as a consequence of administration of the Ca^{++} antagonist verapamil (200µM) to the incubation medium. Intrasynaptosomal potassium levels remained unchanged from the Ca^{++} containing control under these conditions. Veratrine (75µM) addition increased the respiration of cerebrocortical synaptosomes in Ca^{++} free medium by 176% (a level comparable to that achieved in the presence of 0.75mM Ca^{++} (see previous chapter)). A more extensive loss of internal potassium occurred, however. Potassium loss from veratrine stimulated synaptosomes in the presence of Ca^{++} was 44% , whereas loss in the absence of Ca^{++} as a result of stimulation was 61%. Prior addition of verapamil (200µM) to the suspension substantially reduced the increase in synaptosomal respiration resulting from the administration of veratrine, and the loss of potassium was also markedly reduced in the presence of the Ca^{++} antagonist.

Elevated levels of external potassium (56mM) did not increase cerebrocortical synaptosomal oxygen utilisation to the extent described by some authors, and this was probably a consequence of the use of a more 'physiological' level of Ca^{++} in the medium (0.75mM). Other authors have used external Ca^{++} concentrations of 1.2mM, and some as high as 3mM Ca^{++}. However, an increase of 20% in rates of respiration were recorded under these conditions.

Tityus toxin (50nM) evoked a 276% increase in cerebrocortical synaptosomal respiration and thus had a substantially greater effect on this parameter than did the administration of 75µM veratrine, although potassium loss was not as extensive (36%). Tityus toxin also caused a loss of potassium in spinal cord / medulla synaptosomes, and this loss was diminished by prior administration of 1µM TTX or 200µM verapamil.

DATA LEGEND.

RE : TABLE 4.1.

EFFECT OF ELEVATED POTASSIUM, VERATRINE, AND TITYUS TOXIN ON THE RESPIRATORY RATE AND POTASSIUM CONTENT OF RAT CEREBRAL CORTICAL AND SPINAL CORD / MEDULLARY SYNAPTOSOMES IN THE PRESENCE AND ABSENCE OF CALCIUM AND VERAPAMIL.

Rat cerebrocortical synaptosomes were incubated in Krebs phosphate medium at 3mg / ml. in Warburg respirometer vessels, and spinal cord / medullary synaptosomes were incubated in Krebs phosphate medium at 1mg / ml. in 5ml. conical flasks, at 37'C. All incubations were for a total of 40min.. Calcium containing medium was 0.75mM in respect of Ca^{++} ions.

Verapamil (final conc. 200µM) was present for the entire duration of the experiment. Veratrine (75µM), Tityus toxin (50nM), and potassium (56mM) were added for the last 10min of incubation.

At the end of the experiment, the suspensions were sedimented in a bench top ultracentrifuge. The pellets were extracted with acidified methanol, and after being taken to dryness, potassium levels were measured by means of a flame photometer. Protein contents were assayed by means of the Folin reagent.

Respiratory measurements were calculated to give the µmol O_2 utilised / 100mg protein / h., and potassium contents were calculated as µequiv. K^+ / 100mg protein. Data can be compared directly with the data presented in Table 3.1. Data is presented as means $\pm$ SEM for a minimum of six experiments.

CONTROL ($-Ca^{++}$) RESPIRATION is significantly different ($p < 0.05$) from VERAPAMIL ($-Ca^{++}$). CONTROL ($+Ca^{++}$) RESPIRATION is not significantly different from VERAPAMIL ($-Ca^{++}$).

TABLE 4.1

EFFECT OF ELEVATED POTASSIUM, VERATRINE, AND TITYUS TOXIN ON THE RESPIRATORY RATE AND POTASSIUM CONTENT OF RAT CEREBRAL CORTICAL AND SPINAL CORD / MEDULLARY SYNAPTOSOMES IN THE PRESENCE AND ABSENCE OF CALCIUM AND VERAPAMIL.

	POTASSIUM $\mu equiv.K^+/100mg$	RESPIRATION $\mu mol.O_2/100mg./h.$
CEREBROCORTICAL.		
CONTROL ($+Ca^{++}$)	34.2 ± 2.2	56.5 ± 3
CONTROL ($-Ca^{++}$)	31.4 ± 2.7	76 ± 6.2
200µM VERAPAMIL ($-Ca^{++}$)	31.7 ± 2.4	62.5 ± 6.6
75µM VERATRINE ($-Ca^{++}$)	13.2 ± 1.9	156 ± 15
56mM POTASSIUM ($+Ca^{++}$)	-	67.5 ± 4.4
50nM TITYUS TOXIN ($+Ca^{++}$)	22 ± 3	213 ± 18.2
75µM VERATRINE + 200µM VERAPAMIL	25.6 ± 3	69 ± 8
56mM K^+ + 200µM VERAPAMIL ($+Ca^{++}$)	-	59.2 ± 4.7
Ca^{++} / VERAPAMIL (5:1) + VERATRINE	20.1 (2)	n.d.
SPINAL CORD / MEDULLARY.		
CONTROL ($+Ca^{++}$)	22.1 ± 1.2	n.d.
50nM TITYUS TOXIN ($+Ca^{++}$)	15.8 ± 1.6	n.d.
50nM TS-TX + 75µM VERATRINE ($+Ca^{++}$)	14.2 ± 0.7	n.d.
50nM TS-TX + 1µM TTX ($+Ca^{++}$)	16.5 ± 1.3	n.d.
50nM TS-TX + 200µM VERAPAMIL ($-Ca^{++}$)	17.2 ± 1	n.d.

The parameters measured here (TABLE 4.1) indicate that the depolarising agents initiated a normal sequence of events, and therefore the extent to which these values were altered by the absence of Ca^{++} and / or the presence of verapamil are particularly intriguing. These changes will be discussed, in addition to the changes observed in other parameters measured, below.

2) The spontaneous release of amino acids from synaptosomes incubated in the presence and absence of Ca^{++}.

Cerebrocortical synaptosomes were incubated in the presence (0.75mM) or absence of Ca^{++} in the incubation fluid. Analysis of the amino acids accumulating extrasynaptosomally revealed that not only did synaptosomes incubated in the absence of Ca^{++} tend to be 'leaky', in that a small accumulation of non - neuroactive amino acid occurred (FIG. 4.1a; b), but also a significant rise in neuroactive amino acids accumulation was observed. The well documented presence of high affinity transporters for aspartate, glutamate, and GABA in cerebrocortical synaptosomes would be expected to reduce such an accumulation even if a degree of leakiness was a contributing factor to this. In the absence of Ca^{++}, GABA accumulation in the medium was increased by 138% , and that of glutamate and aspartate by 100% and 117% respectively. This additional release of all amino acids was prevented by prior administration of 200µM verapamil (FIG. 4.1a; b).

Incubation of synaptosomes in Ca^{++} - free medium containing 0.5mM EGTA resulted in an amino acid accumulation in the medium, the profile of which was identical to the accumulation observed in Ca^{++} - free medium alone (FIG. 4.1a; b). However incubation in 5mM EGTA evoked a very substantial release of aspartate and glutamate, but no additional accumulation of any of the other 14 amino acids measured. Aspartate and glutamate release increased by 408% and 795% respectively over the Ca^{++} - free controls (FIG. 4.1a; b) and a substantial loss of potassium occurred. It is intriguing that 5mM EGTA did not release any additional GABA over and above the accumulation observed in the absence of Ca^{++} alone.

3) The evoked release of amino acids from synaptosomes incubated in the presence and absence of Ca^{++}.

Elevated levels of external potassium specifically evoked the release of aspartate, glutamate, and GABA from cerebrocortical synaptosomes incubated in the presence of Ca^{++}. This release was to a degree Ca^{++}-

DATA LEGEND.

RE : FIG. 4.1a, 4.1b.

THE SPONTANEOUS RELEASE OF AMINO ACIDS FROM CEREBROCORTICAL SYNAPTOSOMES INCUBATED IN THE PRESENCE AND ABSENCE OF CALCIUM.

Cerebrocortical synaptosomes were incubated in the presence (0.75mM) or absence of calcium in Krebs phosphate medium for a total of 30min.. Also present, as appropriate, for the duration of the experiment were Verapamil (200μM), or EGTA (0.5mM or 5mM).

At the end of the experiment, the suspensions were sedimented in a bench top ultracentrifuge. The supernatants were taken to dryness in a vacuum dessicator. Samples were extracted with methanol, and dissolved in appropriate volumes of 0.025 HCl. Aliquots were taken to measure amino acid levels using an autoanalytical system.

The data presented is the mean ± SEM obtained from a minimum of six experiments. Amino acid levels are presented as nmol / 100mg protein, the latter being measured in the extracted pellet by means of the Folin reagent.

FIG. 4.1a

THE SPONTANEOUS RELEASE OF AMINO ACIDS FROM CEREBROCORTICAL SYNAPTOSOMES INCUBATED IN THE PRESENCE AND ABSENCE OF CALCIUM.

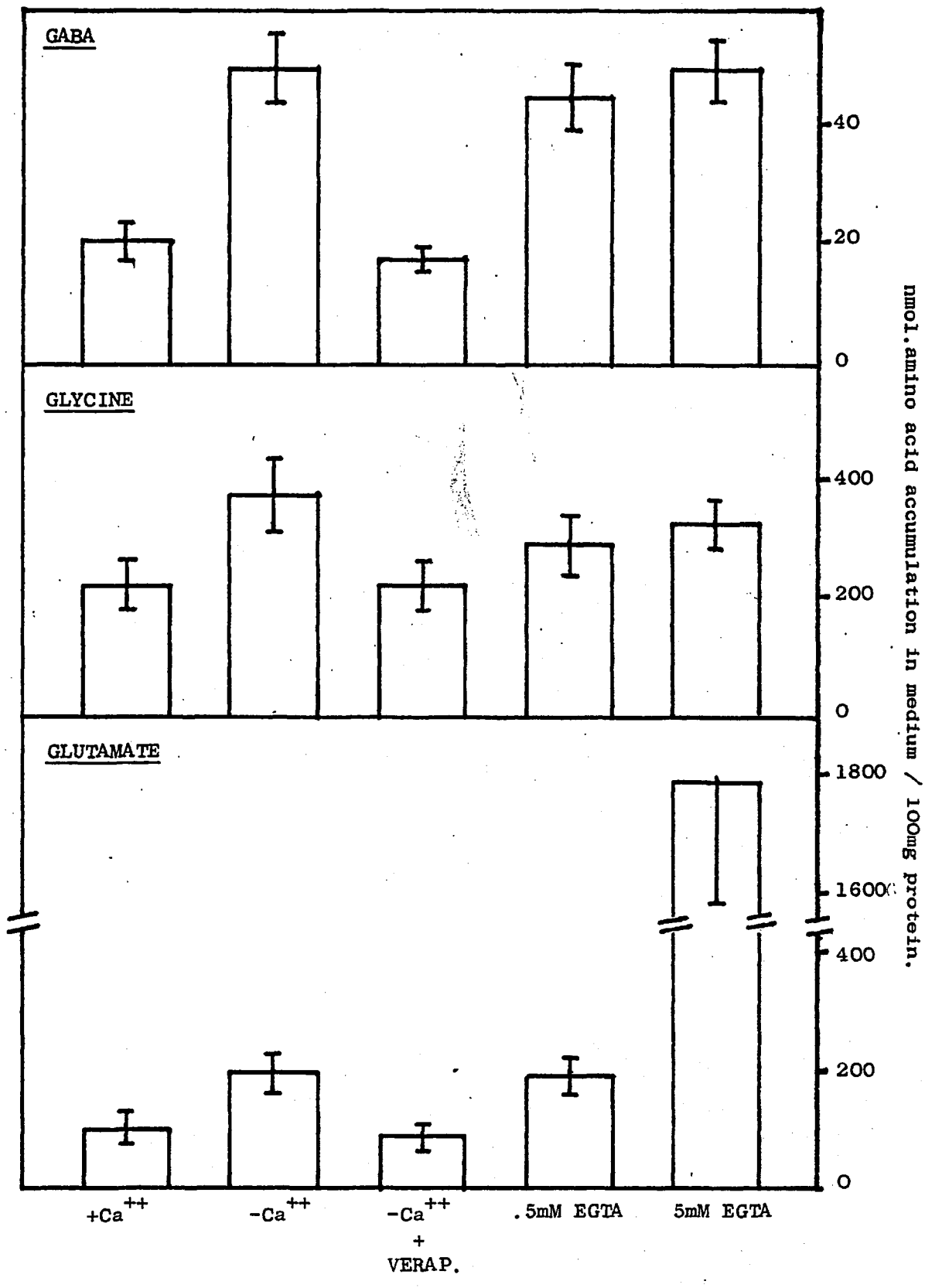
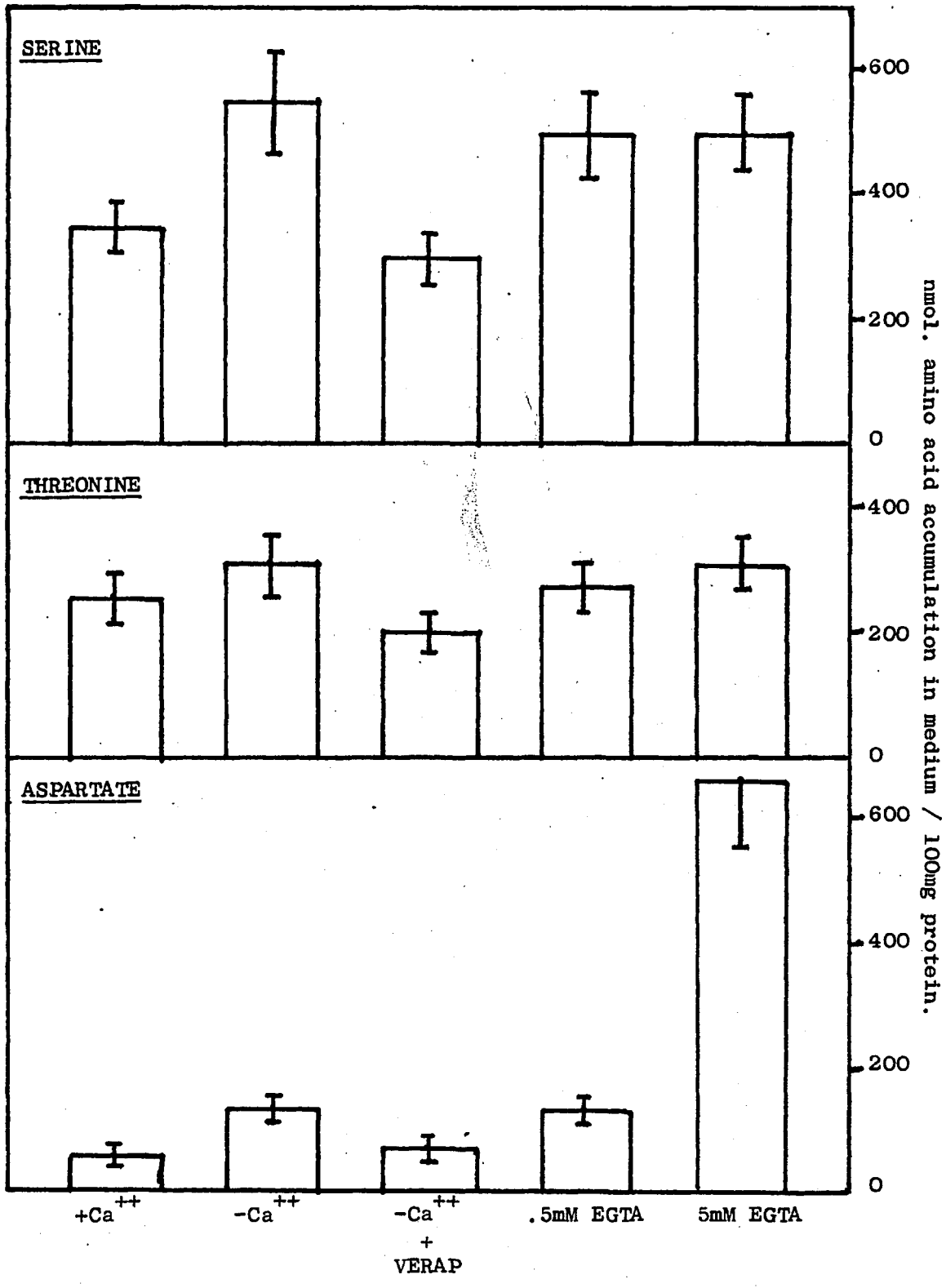


FIG. 4.1b

THE SPONTANEOUS RELEASE OF AMINO ACIDS FROM CEREBROCORTICAL SYNAPTOSOMES INCUBATED IN THE PRESENCE AND ABSENCE OF CALCIUM.



dependent, (FIG. 4.2a; b), in that in the absence of Ca^{++} in the incubation medium, the evoked release was reduced by 28% for aspartate, 20% for glutamate, and 38% for GABA. Since in the absence of both Ca^{++} and elevated K^{+} (FIG. 4.1a; b), there is an accumulation of these amino acids in the medium, it is perhaps not surprising that extrasynaptosomal amino acid levels do not return to the levels obtained in Ca^{++} containing controls when the effect of Ca^{++}- free K^{+} stimulation is examined. More relevant to the speculations presented below, however, is the slight increase in ^{23}Na uptake by synaptosomes under elevated K^{+} stimulation observed by GODDARD & ROBINSON (1976). The non - Ca^{++}- dependent component of K^{+} stimulated release may be attributable to this Na^{+}- influx by a mechanism to be described below.

The evoked release of neuroactive amino acids from synaptosomes in the presence of 75µM veratrine and in Ca^{++} containing medium produced the expected response (FIG. 4.2a; b). In the absence of Ca^{++}, however, the veratrine evoked accumulation of glutamate was increased by a further 56% over the level obtained in the presence of Ca^{++}. It is perhaps worth noting at this point that veratrine in the presence of Ca^{++} evokes a 1400% increase in the release of glutamate <u>when compared to a Ca^{++} containing control</u>, and veratrine in the absence of Ca^{++} evokes a 1000% increase in the release of glutamate <u>when compared with a Ca^{++} - free control</u>. Thus, although the absence of Ca^{++} from the incubation medium <u>increases</u> the level to which glutamate accumulates extrasynaptosomally, in fact the percentage increase against the Ca^{++} - free control is <u>smaller</u> than that achieved in the presence of Ca^{++}. (This illustrates the importance not only of examining the relevant control (which is obvious), but also of defining clearly the control against which a change is compared. This is particularly important in regard to the literature describing preloaded amino acid data, since actual levels of accumulation are rarely recorded, and only the "% increase against 'control'" is discussed). Using a similar argument, the percentage increase in aspartate and GABA release evoked by veratrine <u>in the absence of Ca^{++} when compared with a Ca^{++} - free control</u> is also reduced against the percentage increase in release evoked by veratrine <u>in the presence of Ca^{++} and compared with a Ca^{++} containing control</u>.

Tityus toxin evoked a 1380% increase in release of aspartate, 1800% for glutamate, and 2140% increase in the release of GABA from cerebrocortical synaptosomes incubated in the presence of Ca^{++}. The accumulation of the other 13 amino acids measured was unchanged. In the

DATA LEGEND.

RE : FIG. 4.2a, 4.2b.

THE EVOKED RELEASE OF AMINO ACIDS FROM CEREBROCORTICAL SYNAPTOSOMES INCUBATED IN THE PRESENCE AND ABSENCE OF CALCIUM.

Cerebrocortical synaptosomes were incubated in Krebs phosphate medium at 3mg / ml in 5ml. conical flasks in a shaking water bath at 37'C for a total of 30min.. Calcium containing medium was made (0.75mM) in respect of Ca^{++}. Stimulating agents were added for the last 10min. of incubation as appropriate, at final concentrations, potassium (56mM), veratrine (75µM), and tityus toxin (50nM). EGTA concentration was 0.5mM.

At the end of the experiment, the suspensions were sedimented in a bench top ultracentrifuge, and the supernatants were taken to dryness, and extracted with methanol. Samples were autoanalysed for amino acid content. Pellet protein contents were measured by means of the Folin reagent.. Amino acid levels are expressed as nmol / 100mg protein.

Histobars represent data collected from a minimum of six experiments and are the mean $\pm$ SEM. Control data were obtained from Ca^{++} - containing incubations. Data for threonine and serine are presented as representative of non - neuroactive amino acid levels measured.

For aspartate and glutamate, K^{+}–Ca^{++} data are significantly different ($p < 0.05$) from both K^{+}+ Ca^{++} and CONTROL. Similarly, TS - TX + Ca^{++} data are significantly different ($p < 0.01$) from TS - TX (-Ca^{++} + EGTA).

FIG. 4.2a

THE EVOKED RELEASE OF AMINO ACIDS FROM CEREBROCORTICAL SYNAPTOSOMES INCUBATED IN THE PRESENCE AND ABSENCE OF CALCIUM.

Y-COORDINATE VALUES = nmol amino acid accumulation in medium / 100mg prot.

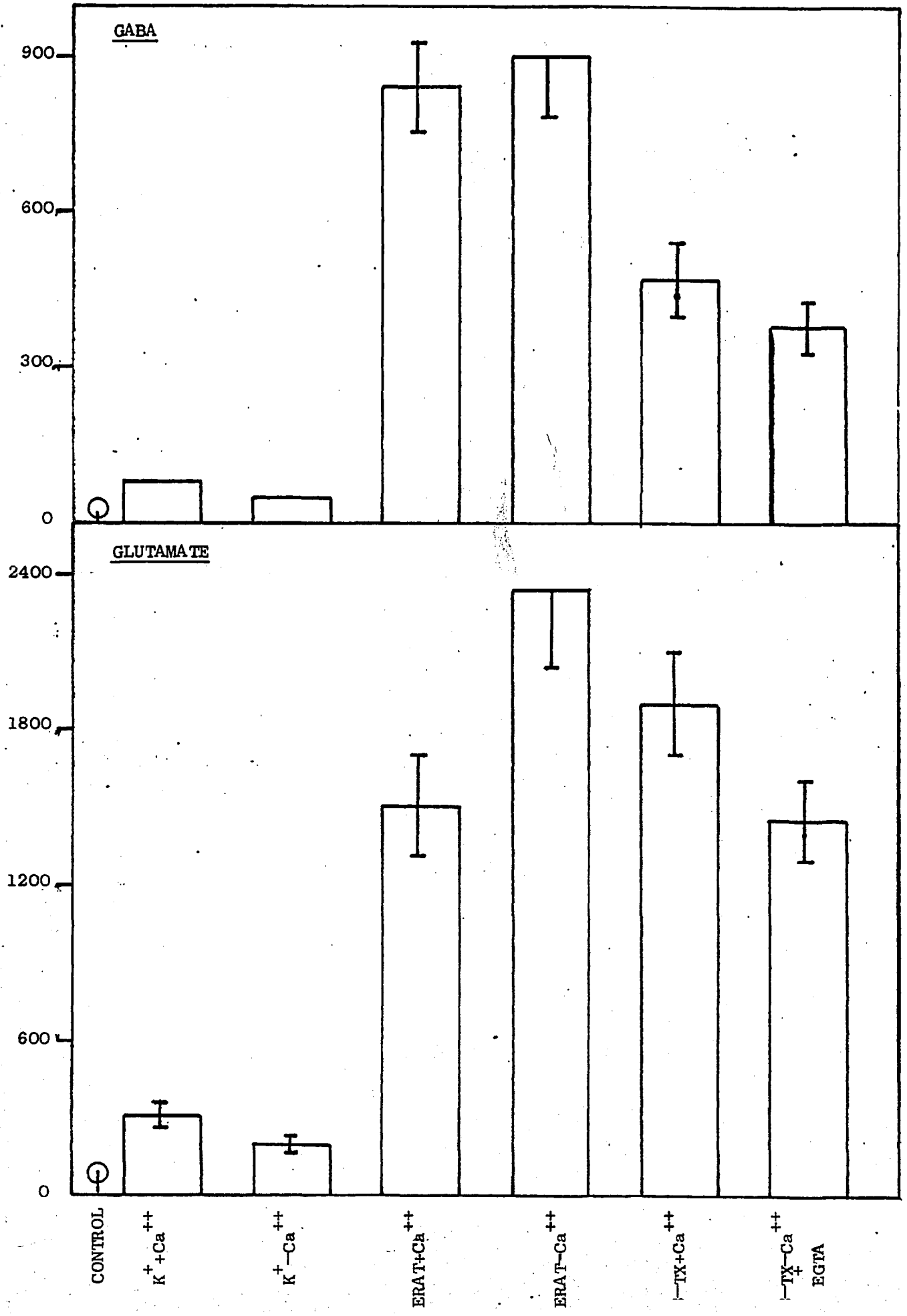
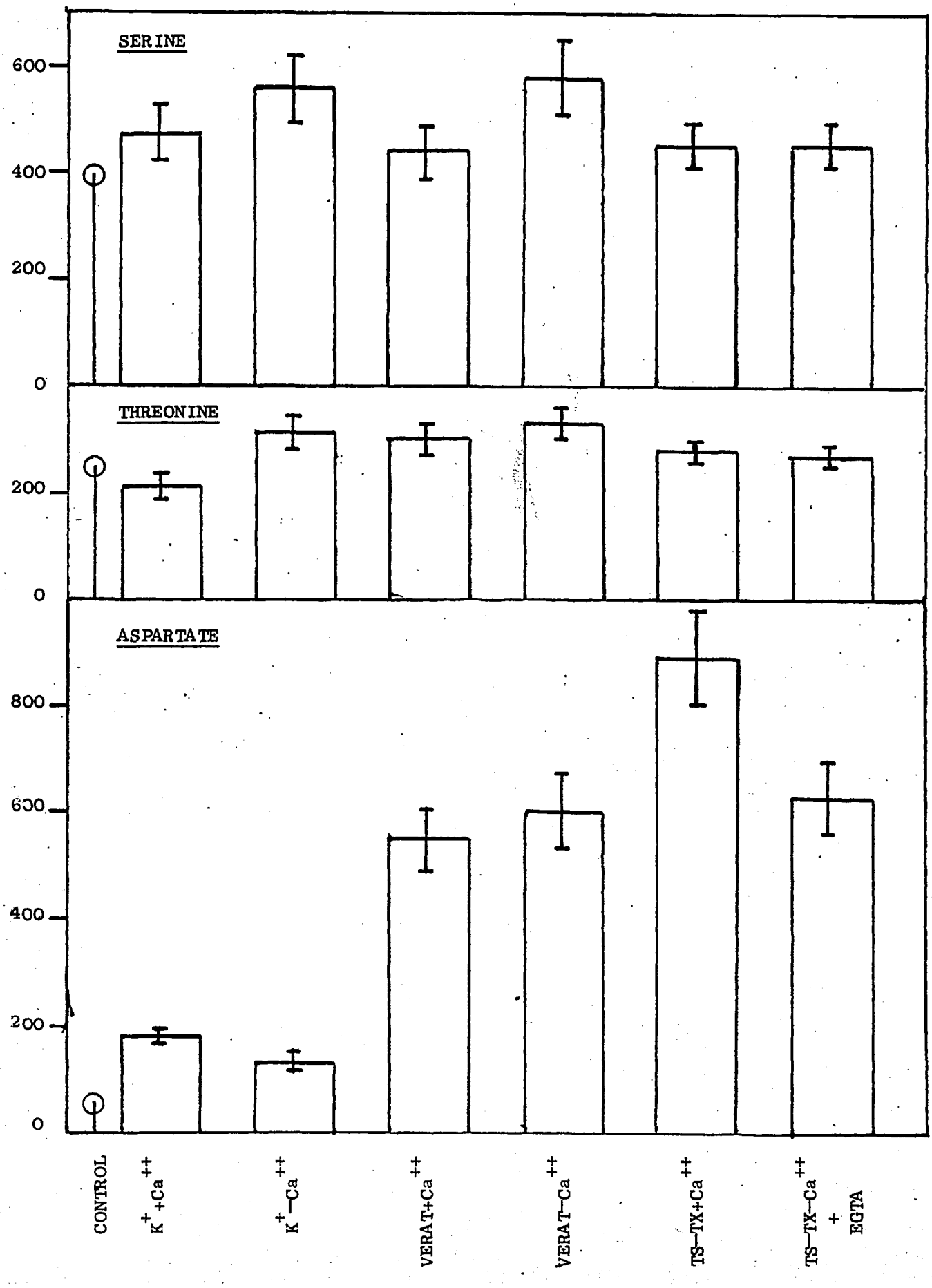


FIG. 4.2b

THE EVOKED RELEASE OF AMINO ACIDS FROM CEREBROCORTICAL SYNAPTOSOMES INCUBATED IN THE PRESENCE AND ABSENCE OF CALCIUM.

Y-COORDINATE = nmol. amino acid accumulation/100mg protein.



absence of Ca^{++} and the presence of 0.5mM EGTA , the release of aspartate, glutamate and GABA evoked by tityus toxin (50nM) was reduced to the extent illustrated in FIG. 4.2a; b.

4) The effect of verapamil concentration on the inhibition of veratrine evoked amino acid release.

The data reported above were obtained from experiments made using verapamil at a concentration of 200μM. FIG. 4.3a; b, illustrates the inhibition of veratrine evoked endogenous amino acid release from cerebrocortical synaptosomes incubated in the absence of Ca^{++}, and increasing verapamil concentrations (30μM - 200μM) and FIG. 4.3c illustrates the inhibition of veratrine evoked exogenous (preloaded) amino acid release under similar conditions. Verapamil at 30μM was the lowest concentration which elicited a significant inhibition of evoked release by 75μM veratrine and the maximum inhibition was achieved using a verapamil concentration of 200μM. Thus to take the release of endogenous glutamate as an example , 30μM verapamil reduced the evoked release by 21%, 75μM verapamil by 46%, 150μM verapamil by 75%, and 200μM verapamil by 86%. FIG. 4.3b illustrates a lack of an effect of increasing verapamil concentrations on the accumulation of threonine and serine, and these exemplify the lack of an effect on the accumulation of all non - neuroactive amino acids measured.

When a stock solution of Ca^{++} and verapamil at relative concentrations 5 : 1 was added to a cerebrocortical synaptosome suspension to give final concentrations of 1mM Ca^{++} / 200μM verapamil, the veratrine evoked release of aspartate and glutamate was uninhibited in contrast to the release of GABA, which was inhibited to the same extent as the addition of 200μM verapamil alone (FIG. 4.3a; b). The purpose of making this experiment was to investigate whether verapamil was likely to be exerting an effect by means of a simple Ca^{++} chelation, and an estimate was made from the structural formula that five moles of Ca^{++} could theoretically be chelated by one mole of verapamil. The data presented for GABA here, and data to be presented below, tend to argue against such a mechanism of action, at least for the major part of the action of verapamil. However, the lack of any large inhibition of the evoked release of glutamate and aspartate under this protocol remains anomalous, and consequently the question requires further

DATA LEGEND.

RE : FIG. 4.3a, 4.3b, 4.3c.

THE EFFECT OF VERAPAMIL CONCENTRATION ON THE INHIBITION OF VERATRINE EVOKED AMINO ACID RELEASE.

Rat cerebrocortical synaptosomes were suspended in Krebs phosphate medium at 3mg / ml. in 5ml. conical flasks in a shaking waterbath at 37'C.

For preloaded (U - ^{14}C) GABA release studies (FIG. 4.3c), suspensions were incubated for 15min. with 10µM of the GABA isotope (200nCi / ml.) prior to washing and resuspension.

Suspensions were incubated for a total of 30min.. Verapamil was present for the last 15min. and veratrine for the last 10min. of incubation, as appropriate. VERAP / Ca^{++} comprised the addition of a stock solution of verapamil and calcium to give final concentrations of 200µM and 1.2mM respectively. Calcium was absent in all other incubations. The final veratrine concentration was 75µM.

At the end of the experiment, the suspensions were sedimented in a bench top ultracentrifuge. For isotope counting, the supernatants were decanted into 20ml of liquid scintillant and taken for counting. For amino acid determinations, the supernatants were taken to dryness and extracted with methanol, and aliquots in 0.025 HCl were autoanalysed. Protein determinations were performed on extracted pellets using the Folin reagent. Amino acid release is expressed as nmol amino acid in incubation fluid / 100mg. protein. Isotopic GABA release is expressed as % increase in label in incubation fluid over Ca^{++} - containing controls.

Histobars represent the data obtained from a minimum of six experiments and are the mean $\pm$ SEM. Data for threonine and serine are included as representative of all non - neuroactive amino acids measured.

FIG. 4.3a

THE EFFECT OF VERAPAMIL CONCENTRATION ON THE INHIBITION OF VERATRINE EVOKED AMINO ACID RELEASE.

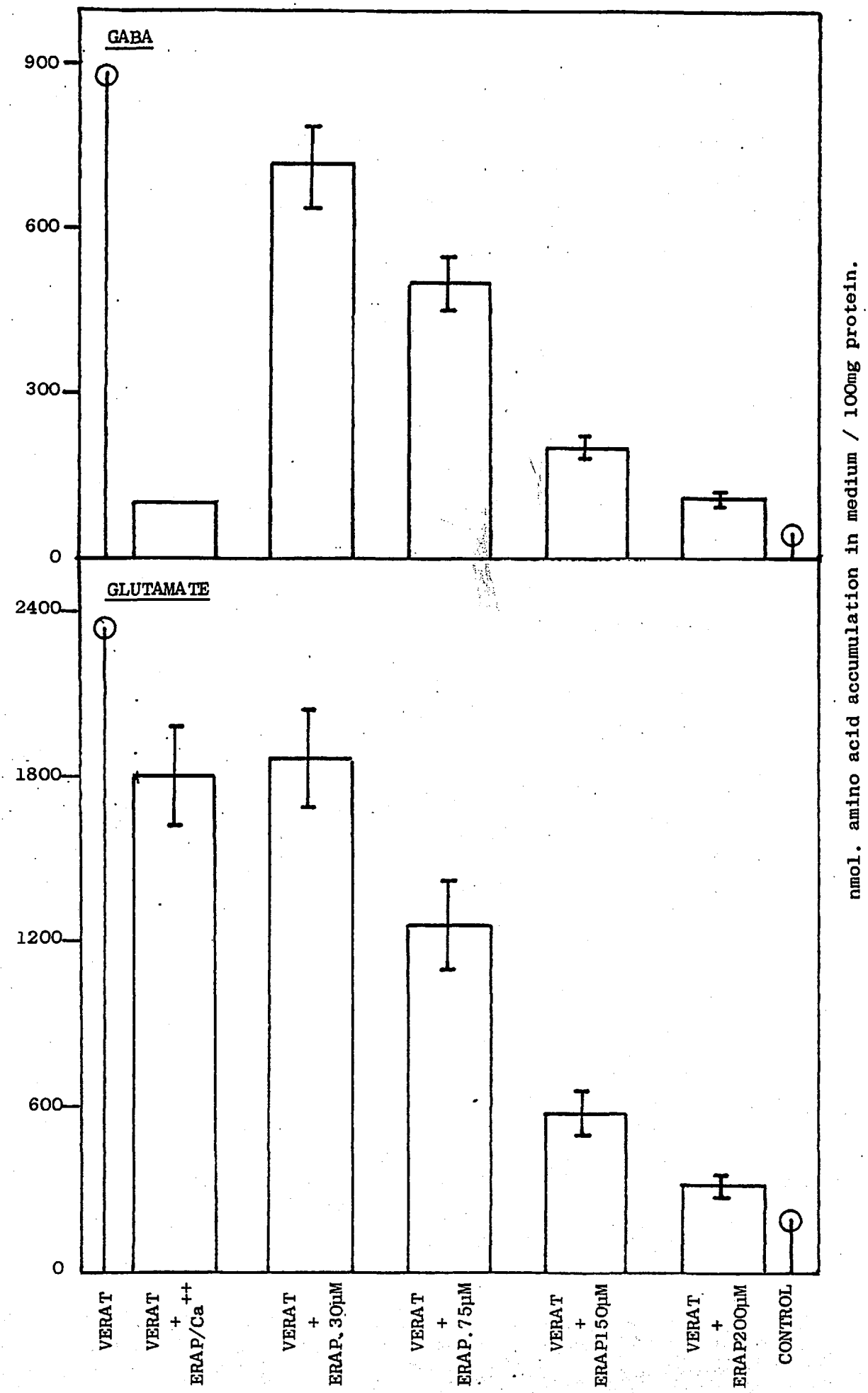


FIG. 4.3b

THE EFFECT OF VERAPAMIL CONCENTRATION ON THE INHIBITION OF VERATRINE EVOKED AMINO ACID RELEASE.

Y-COORDINATE VALUES = nmol amino acid accumulation in medium / 100mg prot.

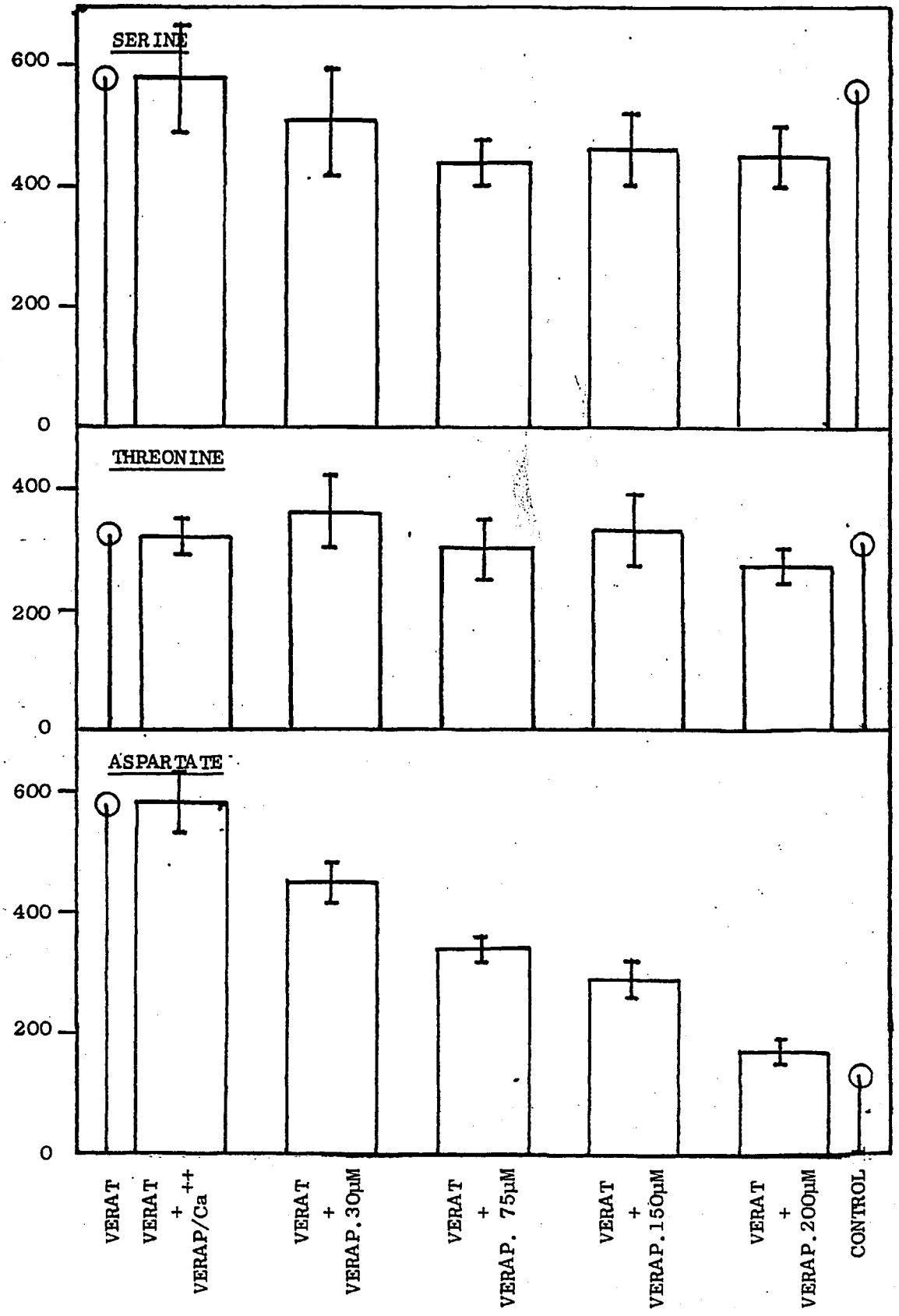
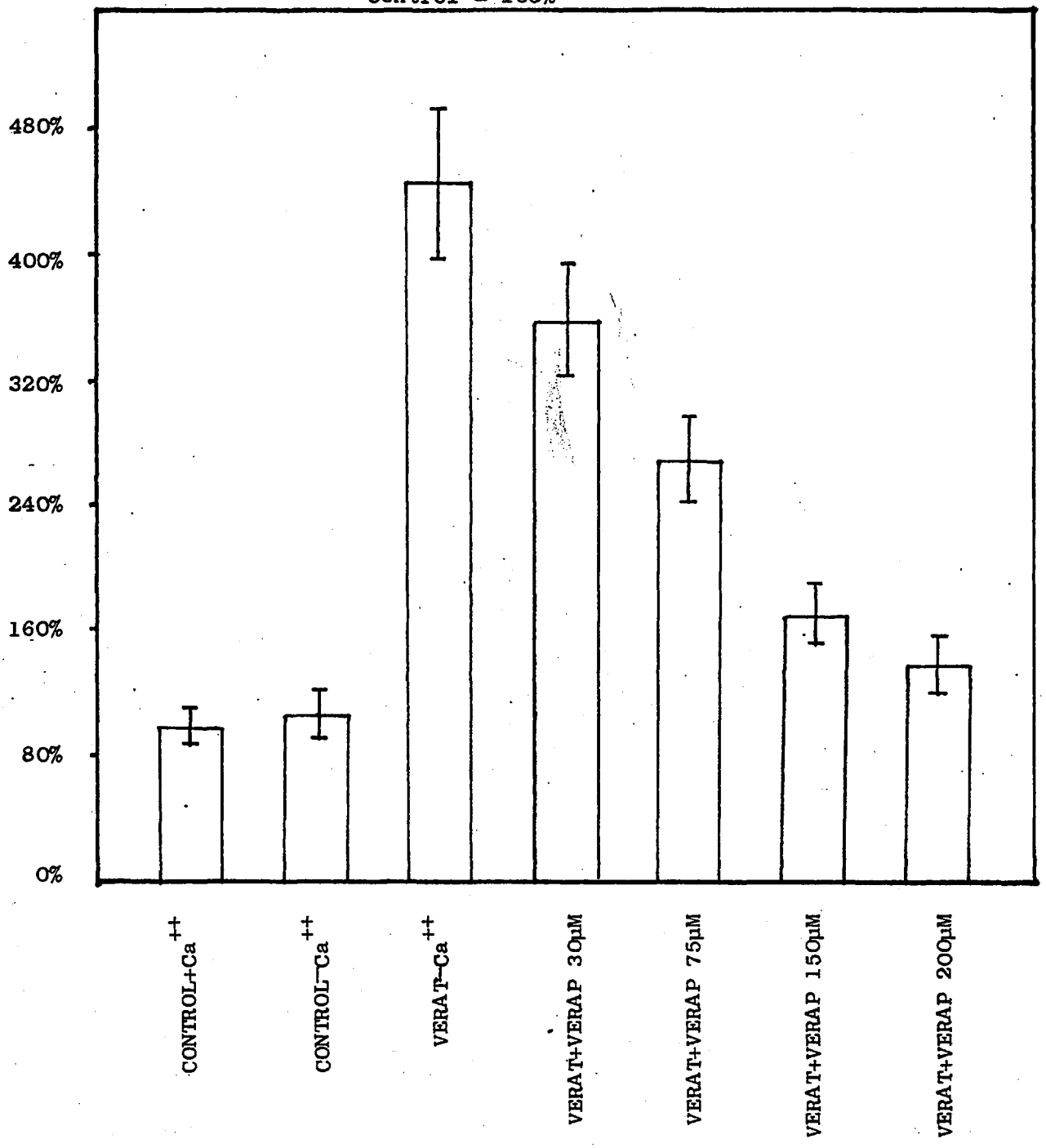


FIG. 4.3c

THE EFFECT OF VERAPAMIL CONCENTRATION ON THE INHIBITION OF VERATRINE EVOKED EXOGENOUS ($U-^{14}C$) GABA RELEASE FROM SYNAPTOSOMES.

Y-COORDINATE VALUES = % increase in medium label over Ca^{++}- containing controls, adjusted for protein.

Control = 100%



study.

FIG. 4.3c illustrates the extent to which exogenous GABA accumulated in the incubation medium in the presence and absence of Ca^{++}, and when synaptosomal release was evoked by veratrine (75μM) and inhibited by increasing concentrations of verapamil. Endogenous GABA accumulation in the incubation medium in the absence of Ca^{++} was significantly elevated, but no such rise in accumulation of exogenous (U - ^{14}C) GABA was recorded. This contrast was not a consequence of the preloading procedure since amino acid analysis of such an incubation revealed an amino acid profile of a nature identical to that recorded in FIG. 4.1a; b. Similarly, the evoked release of exogenous (U-^{14}C) GABA by 75μM veratrine was small (35O%) when compared with the release of endogenous GABA evoked by the same means. This was despite the fact that the preloading concentration for (U - ^{14}C) GABA was in the high affinity range for the transporter. Either a great proportion of the evoked accumulation of endogenous GABA was released from non - transmitter pools, or a large proportion of exogenous GABA was not taken up into transmitter pools. Both forms of transmitter were released by similar mechanisms and FIG. 4.3c emphasises the relationship by the fact that increasing concentrations of verapamil progressively inhibited the veratrine evoked release of (U - ^{14}C) GABA from synaptosomes to an extent which was similar to that observed for endogenous amino acids.

5) The effect of increasing Ca^{++} concentrations on the inhibition by verapamil of the veratrine evoked release of amino acids.

Experiments were made to determine whether the inhibition by verapamil (200μM) of the veratrine (75μM) evoked release of amino acids from cerebrocortical synaptosomes was antagonised by increasing concentrations of Ca^{++} (0.25mM - 4mM). FIG. 4.4a; b illustrate that an increase in accumulation in the medium of all amino acids occurred as the Ca^{++} concentration was increased from 0.25mM to 0.75mM , but that increasing the Ca^{++} concentration further to 4mM was ineffective in antagonising the inhibition attributable to verapamil (200μM). These results are in contrast to those obtained when a stock solution of Ca^{++} / verapamil (1mM / 200μM) was added to the suspension (FIG. 4.3a and b) of synaptosomes, and this dilemma is discussed below.

6) The inhibition by verapamil of the tityus toxin evoked release of

DATA LEGEND.

RE : FIG. 4.4a, 4.4b.

THE EFFECT OF INCREASING CALCIUM CONCENTRATIONS ON THE INHIBITION BY VERAPAMIL OF THE VERATRINE EVOKED RELEASE OF AMINO ACIDS.

Rat cerebrocortical synaptosomes were incubated in Krebs bicarbonate medium at 3mg / ml. in 5ml conical flasks in a shaking waterbath at 37'C. Incubation was for a total of 30min.. Calcium, at relevant concentrations,was present from the start of the incubation. Verapamil (200µM) was present for the last 15min., and veratrine (75µM) was present for the last 10min. of incubation.

At the end of the experiment, the suspensions were sedimented in a bench top ultracentrifuge. The supernatants were taken to dryness, and extracted with methanol...Protein estimations were made of the extracted pellet. Amino acid release to the medium was calculated as nmol / 100mg. protein.

Histobars represent data obtained from six experiments and are means ± SEM.

FIG. 4.4a

THE EFFECT OF INCREASING Ca^{++} CONCENTRATIONS ON THE INHIBITION BY VERAPAMIL OF THE VERATRINE EVOKED RELEASE OF AMINO ACIDS.

Y-COORDINATE VALUES = nmol amino acid accumulation in medium / 100mg prot.

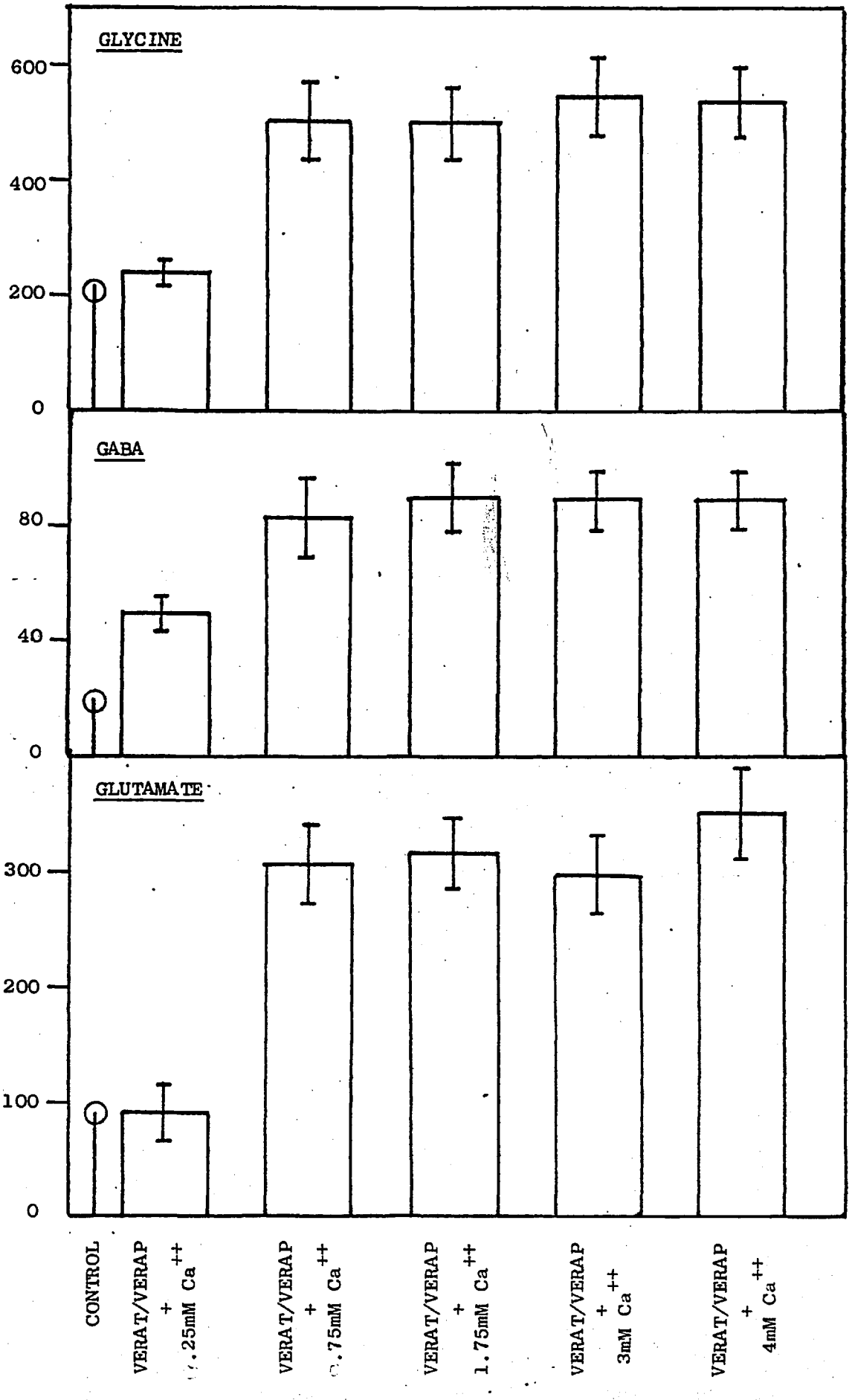
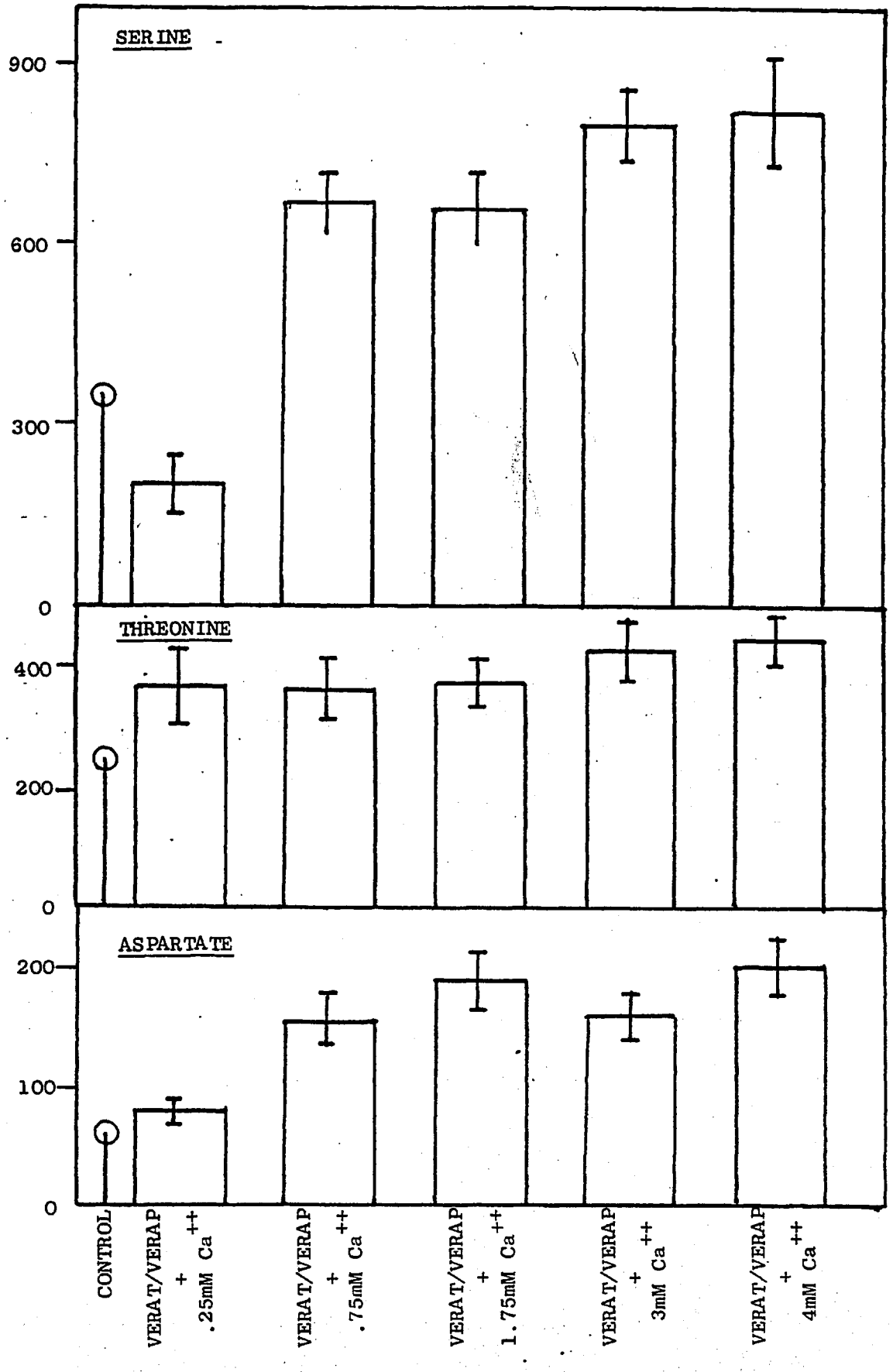


FIG. 4.4b

THE EFFECT OF INCREASING Ca^{++} CONCENTRATIONS ON THE INHIBITION BY VERAPAMIL OF THE VERATRINE EVOKED RELEASE OF AMINO ACIDS.

Y-COORDINATE VALUES = nmol amino acid accumulation in medium / 100mg prot.



amino acids from rat cerebrocortical and spinal cord / medullary synaptosomes.

Tityus toxin (50nM) evoked a significant release of aspartate, glutamate, and GABA from cerebrocortical synaptosomes, and of glycine and GABA from spinal cord / medullary synaptosomes. The release of glycine and GABA from spinal cord / medullary synaptosomes was further enhanced by the addition 1min. subsequently of 75µM veratrine (FIG. 4.5a; b; c). No significant accumulations of any other amino acids were observed in the incubation medium, and threonine is illustrated as an example (FIG. 4.5b). The tityus toxin-evoked release of the neuroactive amino acids was substantially reduced in the presence of 1µM TTX , and further reduced by addition of 200µM verapamil. Thus , in cerebrocortical synaptosomes, the evoked release of aspartate , glutamate, and GABA was reduced by TTX by 58% , 64% , and 79% , respectively ; and by verapamil by 87% , 85% , and 89% , respectively. Similarly , in spinal cord / medullary synaptosomes, the tityus toxin evoked release of glycine and GABA was reduced by TTX by 24% , and 60% , respectively ; and by verapamil by 53% , and 86% , respectively.

Clearly, two distinct binding sites exist for veratrine and tityus toxin respectively on the components that modulate Na^+ flux through the filter of the Na^+ channel of synaptosomes as evidenced by their differential response to TTX (i.e. veratrine evoked release is completely blocked, whereas tityus toxin evoked release is not completely blocked), and the enhancement of the tityus toxin-evoked release by veratrine added subsequently (50nM tityus toxin is on the plateau of a concentration curve for evoking release of amino acids from synaptosomes - data not shown). Furthermore, it is clear that the response of tityus toxin and veratrine-evoked release respectively to verapamil administration is very similar as evidenced by FIG. 4.5a; b; c; and FIG. 4.3a; b.

7) The effect of verapamil on the elevated potassium stimulation of cerebrocortical synaptosomes.

In view of the data presented above showing that 200µM concentrations of verapamil inhibit the evoked release of amino acids from synaptosomes depolarised by 75µM veratrine in spite of the presence of 0.75mM Ca^{++} in the incubation medium, a natural progression of this

DATA LEGEND.

RE : FIG. 4.5a, 4.5b, 4.5c.

THE INHIBITION BY VERAPAMIL OF THE TITYUS TOXIN EVOKED RELEASE OF AMINO ACIDS FROM RAT CEREBROCORTICAL AND SPINAL CORD / MEDULLARY SYNAPTOSOMES.

Rat cerebrocortical and spinal cord / medullary synaptosomes were suspended in Krebs phosphate medium at protein concentrations of 3mg / ml. and 1mg / ml., respectively, in 5ml. conical flasks in a shaking water bath at 37'C. Incubation lasted for a total of 30min.. Verapamil (200µM) was added 15min. after the start of incubation, TTX (1µM) after 19min., and veratrine (75µM) and / or tityus toxin (50nM) after 20min.

Histobars labelled SC contain data obtained from spinal cord derived synaptosomes, and histobars labelled CC contain data obtained from cerebrocortical synaptosomes.

At the end of the experiment, the suspensions were sedimented in a bench top ultracentrifuge. The supernatants were taken to dryness, and extracted with methanol. Aliquots were taken in 0.025M HCl for autoanalysis of amino acids. Protein content of the methanol extracted pellets were assayed by means of the Folin reagent. Amino acid levels in the incubation fluid are expressed as nmol / 100mg. protein.

Histobars represent the data as mean $\pm$ SEM obtained from six experiments.

FIG. 4.5a

THE INHIBITION BY VERAPAMIL OF THE TITYUS TOXIN EVOKED RELEASE OF AMINO ACIDS FROM RAT CEREBROCORTICAL AND SPINAL CORD / MEDULLARY SYNAPTOSOMES.

Y-COORDINATE VALUES = nmol amino acid accumulation in medium / 100mg prot.

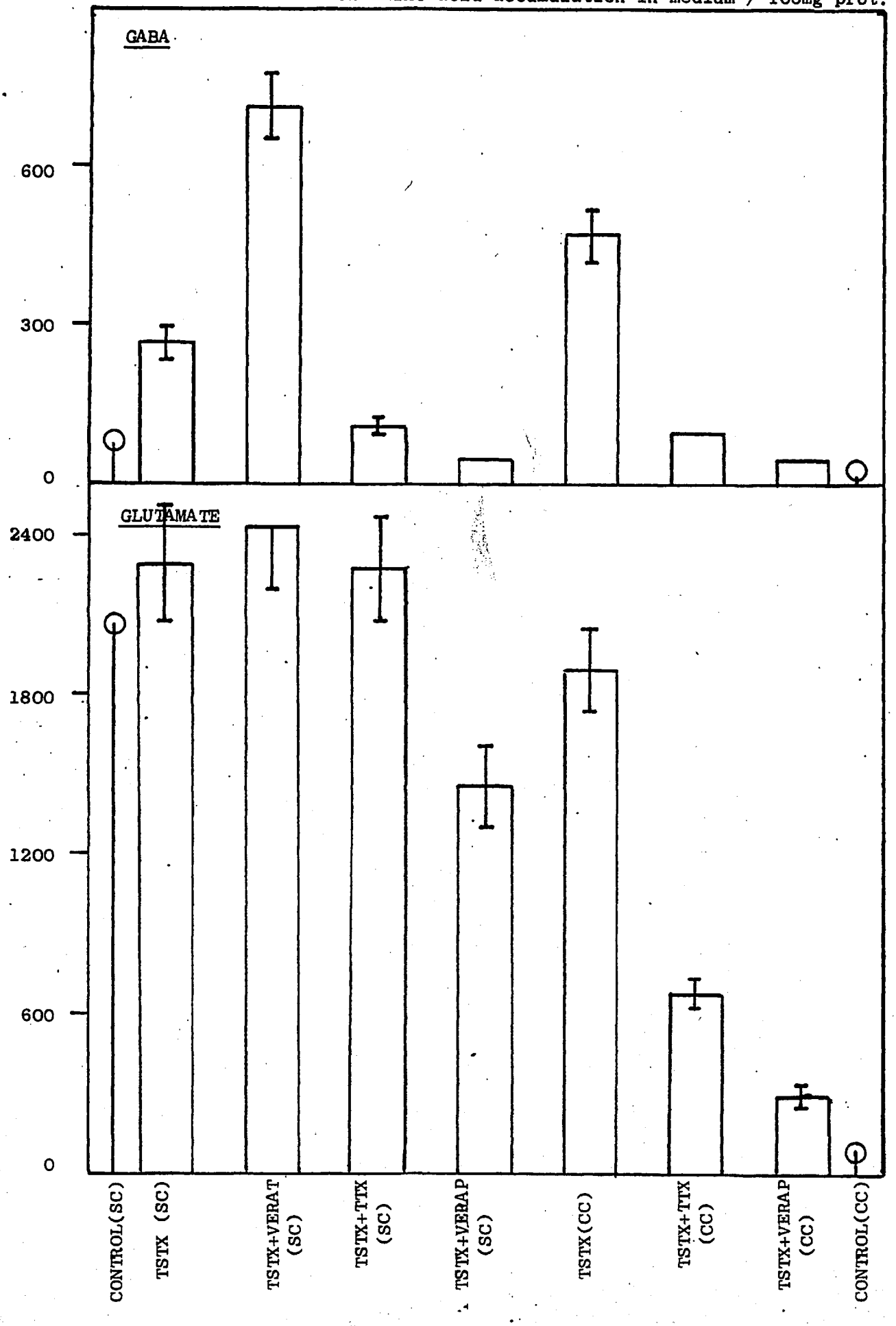


FIG. 4.5b

THE INHIBITION BY VERAPAMIL OF THE TITYUS TOXIN EVOKED RELEASE OF AMINO ACIDS FROM RAT CEREBROCORTICAL AND SPINAL CORD / MEDULLARY SYNAPTOSOMES.

Y-COORDINATE VALUES = nmol amino acid accumulation in medium / 100mg prot.

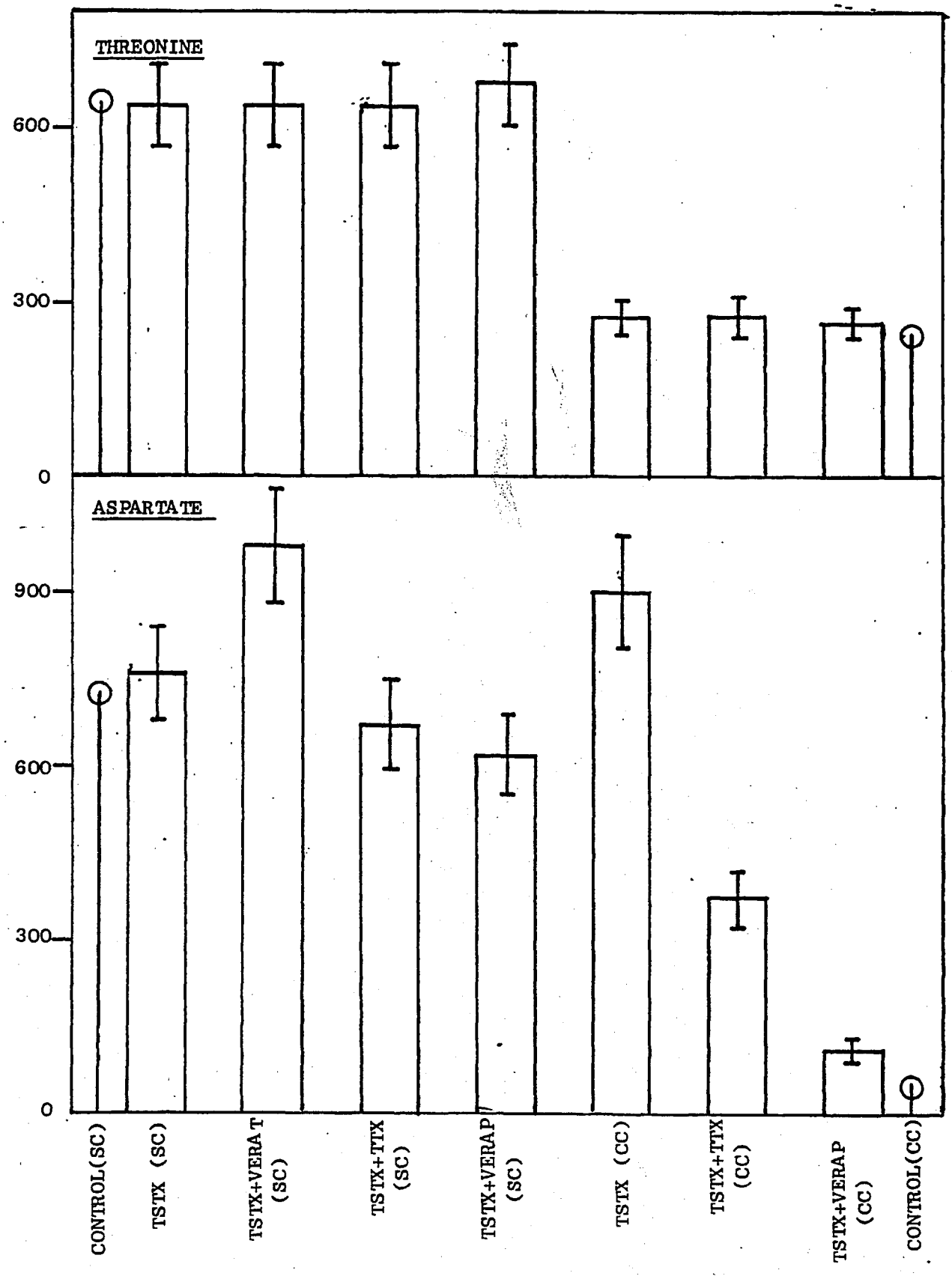
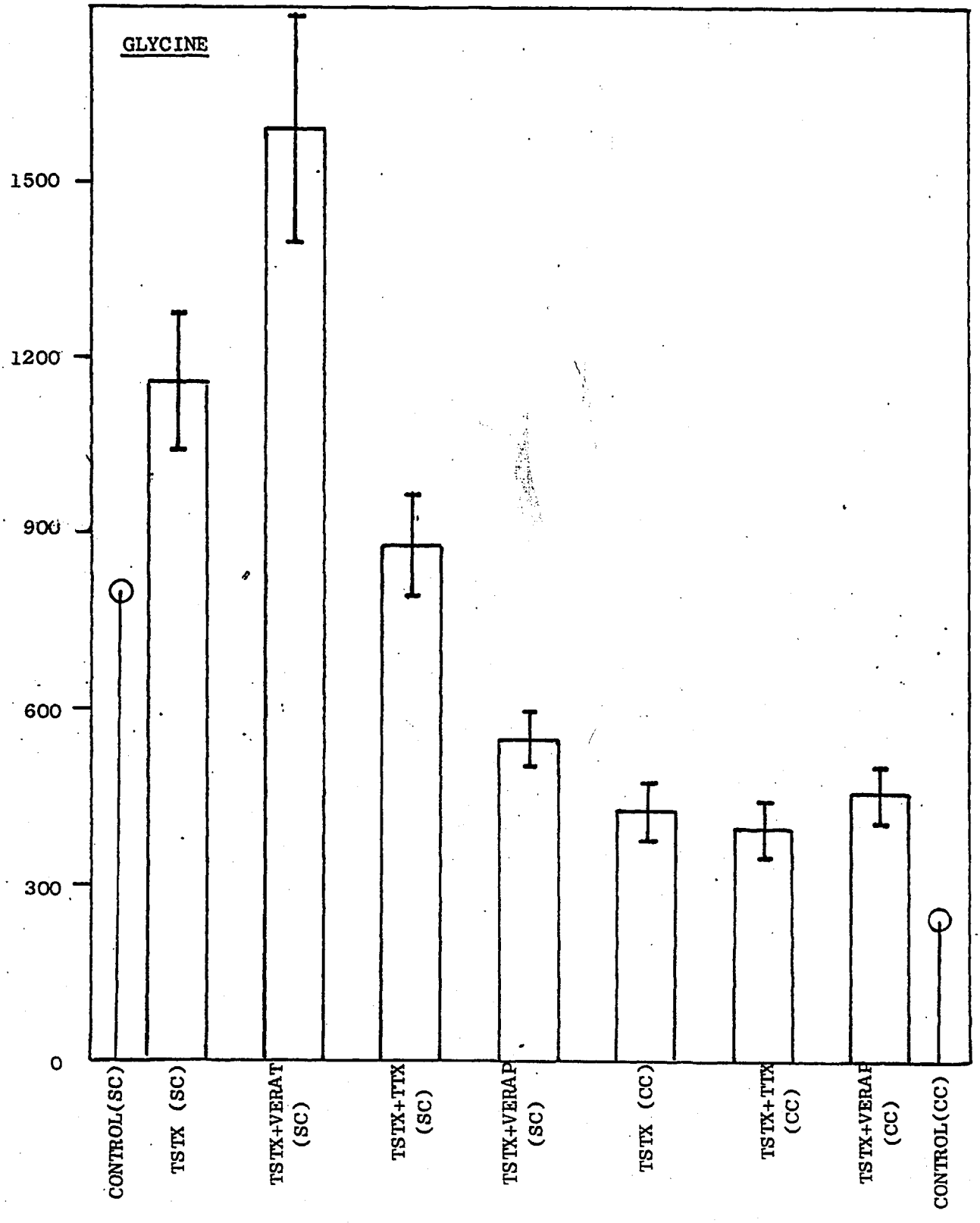


FIG. 4.5c

THE INHIBITION BY VERAPAMIL OF THE TITYUS TOXIN EVOKED RELEASE OF AMINO ACIDS FROM RAT CEREBROCORTICAL AND SPINAL CORD / MEDULLARY SYNAPTOSOMES.

Y-COORDINATE VALUES = nmol amino acid accumulation in medium / 100mg prot.



observation was to investigate the effect of verapamil on the depolarisation of synaptosomes evoked by 56mM potassium when the synaptosomes were incubated in the presence of 0.75mM Ca^{++}. TABLE 4.1 shows that no significant effect of the verapamil administration was observed on the slighty elevated rate of oxygen utilisation caused as a consequence of the elevated K^{+} levels. Similarly, 200µM verapamil did not influence the extent to which aspartate, glutamate, and GABA were released from cerebrocortical synaptosomes in the presence of both Ca^{++} and elevated K^{+}.

DISCUSSION.

The basic assumption in this discussion is that calcium concentration inside the nerve terminal controls transmitter release (e.g. KATZ & MILEDI , 1967). The exact location of the action of intracellular ionised Ca^{++} is still unknown, but for simplicity it can be assumed that the free Ca^{++} ion concentration near the inside of the presynaptic membrane determines transmitter release, and this will depend on a balance between several processes : (a) the resting Ca^{++} influx and the Ca^{++} influx following depolarisation (BAKER et al., 1971) which raises $(Ca^{++})_{in}$; (b) the opposing Ca^{++} efflux, which lowers $(Ca^{++})_{in}$ (BAKER & BLAUSTEIN , 1968; BLAUSTEIN & HODGKIN , 1969; BAKER et al., 1969; DI POLO , 1973; MULLINS & BRINLEY , 1975) ; (c) intracellular Ca^{++} buffers such as the mitochondria (LEHNINGER , 1970; NICHOLLS & SCOTT, 1980), and other Ca^{++} binding systems such as ATP - dependent Ca^{++} accumulation by intrasynaptosomal reticular membranes (KENDRICK et al., 1977; BLAUSTEIN et al., 1978; RAHAMIMOFF & ABRAMOVITZ, 1978). Thus the regulation of the intracellular Ca^{++} concentration is a complex process depending on the contributions of the various components.

1) Spontaneous amino acid release.

The partial inhibition by TTX of the spontaneous release of neuroactive amino acids from synaptosome suspensions (data not shown) incubated in the presence of Ca^{++} during unstimulated periods suggests that, like cerebellar slices (OKAMATO & QUASTEL, 1973), there may be spontaneous electrical activity that results in the release of amino acids. Undoubtedly also, the oscillations of the incubation flasks and the relatively large fluid - tissue ratio in the synaptosome suspensions may provide mechanical agitation and continuous washing of the synaptosomes, resulting in a proportion of synaptosomes breaking up and releasing their contents to the medium, and this too will contribute to the accumulation of amino acids during unstimulated periods., but would not be TTX sensitive.

The larger spontaneous release observed with Ca^{++} deprivation was attributed by JOHNSTONE (1975) to an inhibition of residual uptake as

the rate of Na^+ - dependent transport of amino acids is probably a function of the magnitude of the Na^+ - K^+ gradient and the membrane potential, both of which are reduced by Ca^{++} deprivation (SHANES, 1958). However, it seems simplest to assume that the increase in the spontaneous release of transmitter results from an increase in the intrasynaptosomal Ca^{++} concentration and the fact that an increase in spontaneous release of transmitter was observed in the absence of appreciable amounts of Ca^{++} in the incubation fluid (there may or may not be small amounts of Ca^{++} remaining associated with the membrane of the synaptosomes) suggests that the main source of Ca^{++} for this elevated spontaneous release is intrasynaptosomal and, according to NICHOLLS & SCOTT, (1980) , derived from mitochondrial stores.

Generally, verapamil is thought to block TTX insensitive late phase Ca^{++} channels (SINGH et al., 1978) . However, it might be tentatively suggested that verapamil abolished the increased spontaneous activity in the absence of external Ca^{++} by interfering with Ca^{++} translocation within the synaptosomes leading to a fall in $(Ca^{++})_{in}$, or that verapamil blocks a presumed internal binding site for Ca^{++} or receptor obligatory for some sort of quantal release. Possibly this could be the same class of receptor activated with intracellular injection of Ca^{++} (MILEDI, 1973). That verapamil should enter the synaptosome is possible as a consequence of its highly lipophilic property and verapamil is known to inter fere with mitochondrial Ca^{++} translocation, albeit at higher concentrations than those used in the present studies (FREY & JANKE, 1975).

If verapamil were to be blocking an internal Ca^{++} binding site and the $(Ca^{++})_{in}$ increase is an absolute prerequisite for transmitter amino acid release, and it is assumed that in Ca^{++}- free incubation fluid the increased spontaneous release of these amino acids is a consequence of an elevated $(Ca^{++})_{in}$ derived from internal stores, it is interesting to invoke the data of CARAFOLI et al., (1974) evidencing a Na^+ promoted release of Ca^{++} from mitochondria. In addition, BAKER et al., (1969) showed that the absence of Ca^{++} from the bathing medium inhibited Na^+ efflux from squid axon and that the entry of one Ca^{++} ion is linked to the efflux of two to five Na^+ ions depending on the medium. The fact that TTX inhibits the spontaneous release of neuroactive amino acids from synaptosome suspensions indicates that Na^+ influx is occurring, and if, in the absence of external Ca^{++} , the internal Na^+ concentration were to rise sufficiently to promote Ca^{++} release from mitochondria,

the end result would be an increase in spontaneous release of amino acid which would be susceptible to blocking by verapamil.

That such a mechanism is worthy of some small consideration prior to dismissal is enhanced by the fact that in squid axons, internal levels of EGTA inhibit Ca^{++}- dependent Na^{+} efflux, and this would assist in the explanation of the data illustrating the lack of an inhibitory effect of 0.5mM EGTA on elevated spontaneous amino acid release from Ca^{++} deprived synaptosomes, and since the reduction in extracellular Ca^{++} reduces the depolarisation required to open regenerative Na^{+} channels and delays their inactivation (FRANKENHAEUSER & HODGKIN, 1957; HILLE, 1968), the depolarisation elicited by 5mM EGTA could thus be explained, since the Ca^{++} required to elevate $(Ca^{++})_{in}$ must come from somewhere if this elevation is to be an absolute prerequisite for transmitter amino acid release.

Inevitably, the principal argument to be cited against the proposed role for Na^{+} promoting Ca^{++} efflux from synaptosomal mitochondria is that in intact neurons , even a single action potential rarely increases the internal sodium concentration by more than 10^{-6}%. That this is true is not in question, but in view of the mounting body of evidence supporting specialised mechanisms of subcellular compartmentation, there is no evidence to support a notion that ionic channels will not also be subjected to the rigours of being compartmentalised.

2) Evoked amino acid release.

The evoked release by veratrine and tityus toxin of transmitter amino acids was blocked by the presence of TTX or verapamil. In view of the data illustrating that separate addition of Ca^{++} (at least for the concentrations examined) did not reverse the antagonism by verapamil of veratrine stimulated release, it was decided to examine the effect of verapamil on amino acid release from synaptosomes incubated in elevated K^{+} concentrations and in the presence of 0.75mM Ca^{++}. Verapamil did not reduce the level of K^{+} stimulated release of aspartate, glutamate, or GABA from synaptosomes, in spite of the fact that Ca^{++} deprivation did inhibit the releasing effect of elevated K^{+}. Consequently, if external Ca^{++} is a prerequisite of elevated K^{+}-stimulated amino acid release, and Ca^{++} mobilisation internal to the synaptosome is a prerequisite to veratrine and tityus toxin stimulated

amino acid release, and the latter is verapamil - sensitive while the former is not, then it is surely necessary to invoke the idea that separate Ca^{++} receptors are utilised by K^+ stimulated processes, and by veratrine or tityus toxin stimulated processes, and furthermore, that the subsequently released transmitters originate from separate synaptosomal pools. Several lines of evidence lead to such an hypothesis :

a) An increase in $(Ca^{++})_{in}$ is essential in stimulus coupled release of transmitter (KATZ & MILEDI, 1967; MILEDI, 1973).

b) Elevated potassium stimulated, Ca^{++} - dependent transmitter efflux primarily derives from bound (possibly vesicular) pools (e.g. CARROLL & GOLDBERG, 1975; 1976).

c) Elevated potassium stimulated, Ca^{++} - dependent transmitter efflux does not require Na^+ at the time of release (COTMAN et al., 1976) and conforms to characteristics of stimulus - secretion coupling.

d) Ca^{++} promotes the release of catecholamines in the presence of veratrine and in the case of biogenic amines, which are largely stored in synaptic vesicles, the influx of Ca^{++} elicited by veratrine (BLAUSTEIN, 1975) triggers an exocytotic release(RAITERI & LEVI, 1978).

e) Isolated brain mitochondria possess an active Ca^{++} uniporter and an Na^+- dependent Ca^{++} efflux pathway, a mitochondrial membrane potential sufficiently high to prevent Ca^{++} efflux occurring by reversal of the Ca^{++} uniporter, a sufficiently high matrix Ca^{++} content to saturate the Ca^{++} efflux pathway, and can be shown to regulate the extramitochondrial free Ca^{++} concentration within the cytosol of intact synaptosomes (NICHOLLS & SCOTT, 1980).

f) There is evidence for Ca^{++}- dependent Na^+ efflux in synaptosomes from mammalian brain (BLAUSTEIN & WIESMANN, 1970).

It is thus tempting to propose that K^+ stimulated, Ca^{++}- dependent transmitter efflux is effected by a Ca^{++} receptor (I) which utilises extrasynaptosomal Ca^{++} and triggers the secretion of transmitter from bound (possibly vesicular) pools. Contrastingly, it might be proposed that agents such as veratrine and tityus toxin, whose primary action is at the site of the active Na^+ channel but whose action in stimulating the release of neuroactive amino-acids is not dependent upon an external source of Ca^{++}, and whose evoking stimulus is inhibited by the Ca^{++} antagonist verapamil, utilise the rapid influx of Na^+ to mobilise mitochondrial stores of Ca^{++} via the mitochondrial Na^+- dependent Ca^{++} efflux pathway, and the subsequently elevated cytosolic Ca^{++} triggers the release of sequestered but relatively unbound transmitter through

an action at a Ca^{++} receptor (II). Verapamil could then be thought of as antagonising a component of receptor (II) , but not receptor (I).

If these suggestions were to be utilised as a working hypothesis, it becomes necessary to explain the data contained in TABLE 4.1, which describes the inhibition by verapamil of stimulated respiration caused by the addition of veratrine. That the veratrine induced stimulation of respiration is due only to enhanced ion cycling across the plasma membrane is shown by the evidence that it is inhibited by the addition of either oligomycin or ouabain as is the case for basal respiration (SCOTT & NICHOLLS, 1980). This demonstrates that the changes in respiratory rates are not secondary consequences of an altered cytosolic ionic composition on adding veratrine. The working hypothesis requires that verapamil does not interfere with the active movements of Na^+ and K^+ across the synaptosomal membrane, and consequently the inhibition of stimulated respiration is required to be explained in terms of a mechanism other than an inhibition of TTX - sensitive Na^+ influx.

The surprising feature of the verapamil action in terms of its ability to block, or at least interfere with, the responses which usually follow depolarisation of synaptosomes, i.e. decreased potassium accumulation and acceleration of respiration, are of fundamental importance. This suggests that the agent was preventing Na^+ channel activation by veratrine and tityus toxin. Such non - specificity would, of course, destroy the value of verapamil as a tool for measuring Ca^{++} involvement in neurotransmission processes. Concern over this question of specificity has been expressed in the literature (FAIRHURST et al., 1980), and high concentrations have been reported to block the fast transient Na^+ current. VAN DER KLOOT & KITA, (1975), reported that mM range concentrations block the active sodium current in crustacean muscle, although μM range concentrations do not appear to affect sodium channels (KOHLHARDT et al., 1972).

The possibility that Na^+, once entered into the nerve endings as a result of veratrine addition, mobilises an amount of intraterminal Ca^{++} sufficient to trigger neuroactive amino acid release appeared remote to Levi and Raiteri (LEVI & RAITERI, 1980), and indeed a major criticism of whether an appreciable Ca^{++} transport could occur across the mitochondrial inner membrane during the 1msec. of the initiation of membrane depolarisation and neurosecretion. Similarly, the point mentioned above with respect to the extent to which an action potential will increase the internal Na^+ concentration is relevant to their argument.

LEVI et al., (1980) believe that the large release of neuroactive amino acids in response to veratrine, occurs from the cytoplasm by the depolarising action of veratrine independently of Ca^{++}. However, the data presented here would appear to show that a mobilisation of Ca^{++} which is antagonised by verapamil is a necessary prerequisite to amino acid transmitter release, whatever the source of the transmitter pool, although clearly a great deal of additional work is required on aspects such as the the degree of compartmentation between Ca^{++} efflux sites on the mitochondria, and Ca^{++} receptor sites on the internal presynaptic membrane.

3) Contrasting the neurotoxic actions of tityus toxin and veratrine.

It is thought that there are three separate receptor sites for neurotoxins associated with voltage sensitive sodium channels in excitable cells (e.g. CATTERAL , 1979) , and that each of these resides on a separate subunit of a multi - subunit complex comprising the functional sodium channel. These subunits may undergo conformational changes that are dependent on voltage and are coupled through protein - protein interactions such that the conformational state of one subunit influences the conformational states of the others. HODGKIN & HUXLEY, (1952c) showed that there was a delay in activation of sodium channels following a depolarisation. This delay has been interpreted in terms of a requirement for multiple changes of state to occur before activation of the ion channel, and these multiple events may be conformational changes in each of the three subunits.

Receptor I binds the inhibitors saxitoxin and tetrodotoxin, and occupancy of this site inhibits ion transport by the sodium channel. Receptor site II binds veratrine and the activators batrachotoxin, aconitine, and grayanotoxin, and occupancy of this site causes a shift in the membrane potential dependence of activation and an inhibition of inactivation resulting in a persistant activation of a fraction of sodium channels at the resting membrane potential. The toxins act through an allosteric mechanism by binding selectively to the active state of the sodium channel and causing a shift of ion channels to the active state according to the law of mass action. Since binding of toxins to this site dramatically alters the voltage dependent regulation of ion channel activity, it is likely that this receptor site resides on an important regulatory component of the sodium channel.

Receptor site III binds the polypeptide toxins scorpion toxin and sea anemone toxin. Occupancy of this site inhibits inactivation of sodium channels in voltage - clamp experiments and enhances persistant activation of sodium channels by veratrine. This heterotropic co - operative interaction between the polypeptide toxins acting at receptor site III and the activating toxins acting at receptor site II indicates that these two receptor sites undergo conformational changes that are strongly coupled.

The data presented in FIG. 4.5a; b; c, indicate that tityus toxin affects synaptosomes in a manner which would be anticipated from the information presented above, and the enhancement by veratrine of GABA and glycine release evoked by tityus toxin from spinal cord / medullary synaptosomes is predictable. TSE et al., (1980) showed that the release of preloaded ($U-^{14}C$) GABA and glutamate evoked by 1μM tityus toxin from cerebrocortical synaptosomes was completely blocked by prior addition of 15μM TTX. In the present experiments the release of endogenous aspartate, glutamate, and GABA evoked by 50nM tityus toxin from cerebrocortical synaptosomes , and glycine and GABA from spinal cord / medullary synaptosomes were incompletely inhibited by prior addition of 1μM TTX. Contrastingly, the evoked release of these amino acids by veratrine was completely blocked by prior addition of 1μM TTX. Presumably then, tityus toxin is also exerting an effect on another component of the ion channels, or the toxin has an additional action of blocking transmitter reuptake. Separate experiments (data not shown) indicated that uptake of neuroactive amino acids was not modified by tityus toxin.

Returning now to the role of verapamil, it is intriguing to see that verapamil inhibited the release evoked by veratrine or tityus toxin of aspartate, glutamate, GABA, and glycine from cerebrocortical and spinal cord / medullary synaptosomes to the same extent, despite the fact that not only do veratrine and tityus toxin act on separate components i.e. site II and site III respectively, of the active Na^+ but also the degree to which their response is inhibited by TTX is different for each toxin.

If LEVI et al., (1980) were correct in their assertion that the release of neuroactive amino acids from the cytoplasm is elicited by the depolarising action (i.e. that the large influx of Na^+ makes the nerve ending membrane more permeable to neurotransmitters free in the

cytoplasm) of veratrine - and presumably also of scorpion venom - independently of Ca^{++} , then the question must arise as to why the release caused by the two depolarising agents is unequally inhibited by TTX, yet equally blocked by verapamil. If the hypothesis is invoked that Na^{+} influx caused by the two agents mobilises intrasynaptosomal Ca^{++} then clearly the antagonism by verapamil of an internal Ca^{++} receptor will block the evoked release to the same degree for both toxins

The theme of the previous two chapters has been the roles of ionic channels in nerve cells, and the extent to which these channels are modulated by administered agents. The information gained as a consequence of putting the synaptosome preparation to use in this manner will be called upon again in the proceding chapters which will investigate the manner in which tremorgenic compounds, and anticonvulsant compounds that antagonise the <u>in vivo</u> tremor, exert their actions through modulation of the concepts discussed already.

CHAPTER FIVE

ACTIONS OF IN VIVO ADMINISTERED TREMORGENIC FUNGAL TOXINS ON NEUROTRANSMITTER RELEASE FROM SYNAPTOSOMES.

(1) INTRODUCTION.

(2) RESULTS.

1) Tremorgenic and other effects of the mycotoxins.

a) Sheep.

b) Rats.

2) Effects of tremorgens on respiratory rates and potassium content of rat brain synaptosomes.

3) Effects of tremorgens on amino acid release from rat brain synaptosomes.

4) Effects of tremorgens on intrasynaptosomal amino acid levels.

5) Effects of tremorgens on amino acid uptake by synaptosomes.

6) Recovery of rats from mycotoxin induced tremor.

7) The effect of orally administered Penitrem A mycelium on amino acid and dopamine release from synaptosomes subsequently prepared from sheep.

(3) DISCUSSION.

INTRODUCTION.

Over the past decade, several indolic mycotoxins, metabolites of both soprophytic and plant parasitic fungi, have been shown to cause tremor, incoordination, and convulsions in laboratory and farm animals (WYLLIE & MOREHOUSE, 1977; COLE et al.,1977; MANTLE et al.,1977). Some tremorgenic mycotoxins are extremely potent, and the possible involvement of these substances in certain naturally occurring neurological disorders of animals has been reviewed (MANTLE et al., 1978). Several pharmacalogical studies on the tremorgens Penitrem A and Verruculogen have been published (STERN , 1971; WILSON et al., 1972; HOTUJAC & STERN , 1974; HOTUJAC etal., 1976; SOBOTKA et al., 1978) , and the present study describes biochemical changes in neurotransmission in animals affected by these tremorgenic mycotoxins.

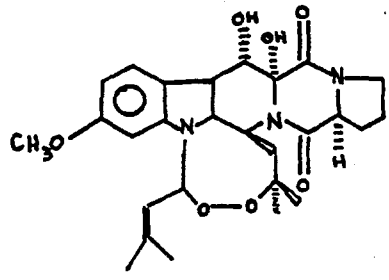
Of the 15 or so tremorgenic mycotoxins that have been isolated to date, four groupings are outstanding based on chemical similarity. They are the Penitrem group, the Fumitremorgen - Verruculogen group, the Paspaline group, and the Tryptoquivaline group. The first two of these groups form the basis of this study.

The chemical structures of none of the members of the Penitrem group have been elucidated to date, although Penitrem A was first reported in the literature in 1968 (WILSON et al., 1968). These studies have been hampered by insufficient amounts of material, their extremely complex chemical nature, and the lack of adequate crystals for use in single crystal X-ray diffraction studies. Penitrem A, however, is known to be a chlorine containing metabolite isolated principally from Penicillium cyclopium , that, with the exception of an indole NH , has has a fully substituted indole nucleus containing at least 2 - OH groups; one which readily acetylates and one which does not (CIEGLER et al., 1976) , and H^1 nuclear magnetic resonance and infra red spectra indicate an H - bonded carbonyl function and at least 5 methyl groups. It has a molecular formula of $C_{37}H_{44}NO_6Cl$ (WILSON, 1971). Fungi known to produce the Penitrems are P. crustosum, P. palitans, P. puberulum, and P. cyclopium, all members of the subsection Fasiculata, section Asymetrica (CIEGLER & PITT , 1970).

There have been several reported cases where fungi capable of producing Penitrems have been implicated in toxic syndromes of cattle, horses, and sheep (e.g. WILSON et al., 1968; CIEGLER , 1969).

Seven tremorgenic metabolites belonging to the Fumitremorgen - Verruculogen group have been isolated and identified, including, in most cases, their stereochemistry. This family of compounds is characterised by 6 - O - methyl indole, one or more mevalonate units, and a diketopiperazine ring system derived from tryptophan and proline or hydroxyproline. Verruculogen, the first reported member of this group (COLE et al., 1972) , was originally isolated from a mold identified as P. verruculossum, but has also been found to be produced by Aspergillus caespitosus, A. fumigatus, and P. estinogenum. Verruculogen contains a novel 8 - membered non - endo peroxide ring.

VERRUCULOGEN



Substances preventing intentional tremor (penicillamine and α - methyl DOPA) have not shown any effect on Penitrem A induced tremor in mice, and high doses of anticholinergic drugs snhanced the tremor (STERN, 1971) , possibly as a consequence of reduced brain acetylcholine. Similarly, chlorophenoxamine, an antihistaminic agent and anti-Parkinsonism drug enhanced the tremor, while mepyramine, an antihistaminic drug inactive against Parkinsonism, was inactive in its influence on Penitrem A induced tremor. Glycine, GABA, mephenesine, meprobamate, and diazepam all prevented the tremor (STERN, 1971) , and these substances are thought to inhibit interneurons in the spinal cord (CURTIS & WATKINS, 1965; GOODMAN & GILMAN , 1966; CURTIS et al., 1968). Amino-oxyacetic acid, a drug raising brain GABA levels (CURTIS et al., 1968) by inhibiting GABA transaminase (POPOV & MATTHIES , 1969) prevented Penitrem A induced tremor in mice. STERN (1971) concluded that Penitrem A produces tremor by inhibiting the interneurons which inhibit the α - motor cells of the anterior horn.

WILSON et al., (1972) studied the effects of Penitrem A on

transmission in rat phrenic nerve - diaphragm preparations, where acetylcholine is the transmitter. They concluded that the tremorgen may act at pre - and post - junctional sites and thus produce an increased sensitivity of the post - junctional membrane and also an increase in the release of transmitter. Their observed increase in miniature end plate potential frequency indicated that the toxin stimulated or facilitated spontaneous release of acetylcholine, and the observed increase in amplitude could be due to increased "packet size" , or increased sensitivity of the acetylcholine receptor to normal "packet sizes" of acetylcholine.

CYSEWISKI et al., (1975) studied the effects of Penitrem A in orally dosed calves and observed marked tremor, muscular rigidity, and tonic - clonic convulsions. Marked increases in concentrations of plasma lactic acid, pyruvic acid, glucose, and creatine phosphokinase activity were found coincident with the development of severe tremors. The changes in lactic and pyruvic acids were explained by a shift to anaerobic glycolysis in skeletal muscle, and the glucose concentration increase as a result of 8 - 9 fold increase in plasma catecholamines, which are known to have a hyperglycaemic effect. No alterations in muscles were observed by gross examination and light microscopy, and the increases in plasma enzyme activities were apparently the result of leakages of enzymes from muscle in response to the muscular activity. A direct effect of the toxin on the muscle by increasing the permeability of the cell membrane was not excluded. The only gross tissue change observed was an accumulation of lipid content in the liver concomitant with depletion of liver glycogen, explained by the release of catecholamines and other hormones that stimulate lipolysis and mobilisation of free fatty acids.

In studies on Verruculogen induced tremors in mice and 3 - 7 day old chicks, HOTUJAC et al., (1976) 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. They also found that brain GABA levels were substantially reduced in mice treated with the toxin, substantiating the suggestion that Verruculogen induced tremor is mediated by a loss of inhibitory function. The effects of Verruculogen on neuromuscular transmission and contractile activity of directly and indirectly stimulated rat phrenic nerve - hemidiaphragm

preparations indicate that post - tetanic potentiation is enhanced by Verruculogen, suggesting a post junctional action. Reduction in contractile activity resulted from both post junctional and musculotropic inhibitory effects. SOBOTKA et al., (1978) observed that even at very high doses of Verruculogen there were no changes in acetylcholine, noradrenaline, or dopamine levels, or on acetylcholine esterase activity in mouse whole brain.

That the present chapter should concentrate on amino acid accumulation consequent upon Verruculogen and Penitrem A induced tremor and convulsions is not without precedent in the literature. The roles of glycine, GABA, and glutamate in particular in tremor and convulsive symptoms are fairly well substantiated and documented.

CURTIS & JOHNSTON (1974) have shown that antagonism of the inhibitory action of glycine explains the convulsive action of strychnine and a number of alkaloids and other substances of diverse structure.

A convulsant of clinical interest in relation to glycine is the toxin of Clostridium tetanus. Similarities between the effects of tetanus toxin and strychnine were first noted by Sherrington and subsequently the toxin was found to block strychnine sensitive inhibitions of spinal moto - neurones. The poisoned neurones were still however sensitive to glycine and it has been shown that spinal and supra - spinal inhibitions mediated by GABA are also affected , and again the post - synaptic sensitivity of neurones to this amino acid is unchanged (CURTIS & al ., 1973). Tetanus toxin thus appears to have a presynaptic action at central synapses which release glycine and GABA and such an effect can account for many clinical features.

Although the dramatic clinical improvements achieved in certain forms of Parkinsons syndrome by the use of L - DOPA have focussed attention on the importance of a nigro - striatal pathway (LLOYD et al., 1975) , there is increasing evidence that GABA also has a significant role as an inhibitory transmitter of interneurones in the extrapyramidal system. From experiments in cats and rats, GABA appears to be the transmitter of caudonigral (OKADA, 1976), pallido - nigral (MCGEER & MCGEER , 1976) ., and caudopallidal pathways (OBATA & YOSHIDA , 1973). High levels of GABA and GAD occur in the various components of the human basal ganglia, and in Huntington's chorea, there appears to be a selective loss of GABA levels with reduced levels of both the amino acid and the enzyme (MCGEER & MCGEER , 1976; BIRD & IVERSEN , 1974; URQUHART et al., 1975). In contrast, in Parkinson's disease, although

GAD levels are reduced, there appears to be no corresponding reduction in either GABA levels or the number of cells in the striatum (HORNYKIEWICZ et al., 1976). The restoration of GAD levels to near normal values after prolonged therapy with L - DOPA has suggested that the reduced enzyme levels are secondary to the loss of nigro - striatal dopamine releasing neurones which results in reduced function of the GABA system. As yet, no therapeutic measures have become available for either Parkinson's disease or Huntington's chorea which might influence GABA synapses.

The convulsant action of benzyl penicillin is of clinical interest and antagonism of the inhibitory action of GABA (CURTIS et al., 1972) appears to be the best explanation for the effects of this compound when administered topically to the cerebral cortex or after massive systemic doses , especially when blood brain barriers are lowered by hypoxia. Ampicillin, a derivative of low consulvant potency, is a very weak GABA antagonist.

The convulsant action of a number of compounds has been interpreted in terms of GAD inhibition. Defective GABA production could account for convulsions arising from pyridoxine deficiency, as well as for those which follow administration of pyridoxine anti - metabolites, the hydrazides, and other carbonyl trapping agents (TAPIA, 1975).

A number of amino acids of plant and fungal origin are structurally related to glutamic acid, and also excite neurones (JOHNSTON, 1974). These include β - N - oxalyl - L - α , β diamino propionic acid, isolated from the seeds of the chick pea Lathyrus sativus; ibotenic acid, an isoxazole related to muscimol and isolated from Amanita muscaria; kainic acid, a pyrrolidone derivative from the seaweed Digenia simplex and used as an anthelmintic; domoic acid, from certain marine algae, and again with anthelmintic properties (BISCOE et al., 1975) and quisqualic acid, an ascaricidal from the seeds of the plant genus Quisqualis. Compounds of this type are convulsants when administered to young rodents, but not in adults (JOHNSTON , 1973). Hence, under normal conditions, blood brain barriers presumably limit the access of amino acids such as these to neurones, but under conditions where such barriers are of lowered effectiveness, convulsant activity might be expected.

It is the amino acids rather than acetylcholine or the monoamines which are currently emerging as the compounds of special interest to epilepsy. Glutamate increases the firing rate of neurones present in

cerebellar slices in vitro (OKAMATO & QUASTEL , 1973) , and causes sharp and massive depolarisation of cells in cerebral cortex slices (BRADFORD & MCILWAIN, 1966). Small amounts of glutamate introduced intracerebrally readily cause convulsions (HAYASHI, 1954; HENNECKE & WEICHERT , 1970) , and even systemically administered glutamate produces seizures in 10 - day old rats (JOHNSTON , 1973) , and in adult rats at higher concentrations (BHAGARAN , 1971; BRADFORD & DODD , 1977) , and and release of glutamate from experimentally produced epileptic foci has been observed (KOYAMA , 1972; DODD & BRADFORD , 1976) and the release showed a close correlation with the onset of seizures as indicated by EEG or muscular hyperactivity. The complementary change was observed to occur in the epileptic focus produced in cat cortex by local freezing and there was a reduction in glutamate content which correlated with the time of onset of epileptiform EEG activity (BERL et al., 1959).

With these fairly well defined roles for neuroactive amino acids in mind, and also the previous literature mentioned above, describing interactions between the tremorgenic mycotoxins and amino acids, the experiments to be described in the following section were performed to investigate whether, subsequent to administration of the mycotoxins to animals, the parameters which have been discussed in previous chapters were altered in a way that might assist in the formulation of a biochemical explanation for the observed disorder.

RESULTS.

1) Tremorgenic and other effects of the mycotoxins.

a) Sheep.

Three sheep at the Royal Veterinary College Field Centre were dosed by stomach tube with aqueous suspensions of P. cylopium mycelium containing Penitrem A, initially daily with 450mg (equivalent to approximately 15mg Penitrem A), and then as required to maintain a constant level of general tremoring. The treatment periods were 50, 51, and 52 days; and the number of doses 37, 38, and 43; and total Penitrem A 515, 580, and 650mg respectively. Tremors of the head were the first signs of an effect, followed by more generalised body tremors associated with incoordination, hind leg abduction, and a staggering gait. When made to run, the sheep showed the typical unison action of the hind limbs and eventually collapsed on most occassions. After a few minutes they would regain their feet and walk away somewhat improved. Stress, such as being forced to run, always exacerbated the degree of tremor. The doses were regulated so that protracted recumbency was not a feature.

Tremoring was well established in all sheep after three doses of Penitrem A, although a variation in the speed of response was seen which was not related to body weight. When not disturbed, sheep continued to eat readily and they maintained body weight despite the severe whole body tremoring and occassional convulsions which were elicited. Body temperature was not found to vary. Subsequent to culling, gross observation and light microscopy of samples of most tissues showed no changes in character from sheep dosed with water alone.

b) Rats.

Aqueous suspensions of P. estinogenum containing Verruculogen were administered to female (250g) Sprague Dawley rats by the intraperitoneal route (80 - 800mg mycelium / kg). Characteristically, neurotoxic doses induced a sequence of hyper - responsiveness to stimuli (eyes closed, ears pinned back, and occassional vocalisation), general immobility, and a whole body tremor best described as an 'intention' tremor, since it generally occurred when the animals moved. The rear legs were splayed outwards and backwards. Tremor occurred within 5 - 30min. following injection and subsided within 4 - 5h. at doses of between

80 - 500mg mycelium / kg. At the highest doses of Verruculogen, most of the rats exhibited intermittent bilateral clonic - tonic seizures that terminated in death within two hours. At the lower doses of Verruculogen, convulsions occurred rarely and for short periods, and mortalities were not observed.

Penitrem A administered in the same fashion to rats at doses of 0.15 - 2mg / kg for pure toxin, and 80 - 500mg / kg for Penitrem A - containing mycelium (P. cyclopium) induced a similar pattern of response to that described above for Verruculogen although the symptoms were additionally characterised by a rigid outstretched tail which either lay flat on the cage base, or rose in the air. However, at higher doses of Penitrem A (above 500mg mycelium / kg) seizures were seen less often, and these rarely resulted in death.

Application of several crystals of Penitrem A to the sensorimotor cortex of rats exposed at one side resulted in contra - lateral body tremor within half an hour of the animal recovering from the nitrous oxide anaesthesia. Within 1 - 2h. this tremor spread to the ipsilateral side, perhaps as a consequence either of Penitrem A reaching the blood stream in sufficient concentrations, or by diffusion over the surface of the cortex.

Effects of tremorgens on aspects of central nervous system biochemistry.

The data presented in the proceding sections of this chapter were obtained from synaptosomes which were prepared from animals (rats or sheep as appropriate) which were dosed with Verruculogen or Penitrem A prior to culling. Rats were in most cases injected intra - peritoneally with 400mg mycelium / kg. unless otherwise specified, and were culled at about 45min. subsequent to onset of tremor, as described in METHODS. The sheep data were obtained from those animals described in a previous sections of this chapter.

Data obtained from experiments made under conditions of in vitro application of toxins are described in the following chapter.

2) Effects of tremorgens on respiratory rates and potassium content of rat synaptosomes.

Following in vivo administration of the mycotoxins, synaptosomes which were prepared from cerebral cortex were incubated in Krebs -

DATA LEGEND.

RE : TABLE 5.1.

RESPIRATION AND POTASSIUM CONTENT OF RAT CEREBROCORTICAL AND SPINAL / MEDULLARY SYNAPTOSOMES PREPARED FROM ANIMALS PREVIOUSLY DOSED WITH MYCOTOXINS.

Synaptosomes prepared from rats previously injected intra - peritoneally with 400mg / kg mycelial mycotoxin were incubated in Krebs phosphate medium containing 10mM glucose in Warburg respirometer vessels at 37'C. Stimulation began at 35min. and lasted 10min.. Veratrine was added to a final concentration of 75µM where appropriate. Subsequent to incubation, the synaptosomal pellet, obtained by sedimentation of the suspension in a bench top ultracentrifuge, was extracted with acidified methanol, which was taken to dryness. The solute was dissolved in an appropriate volume of 0.025M HCl. This extract was analysed for potassium by means of a flame photometer.

The values presented here were obtained from six or more experiments and represent the mean value $\pm$ SEM. No determinations were made in those instances labelled nd. The respiration data obtained from synaptosomes prepared from rats dosed with Penicillium estinogenum mycelia were from only two experiments.

TABLE 5.1

RESPIRATION AND POTASSIUM CONTENT OF RAT CEREBROCORTICAL AND SPINAL CORD / MEDULLARY SYNAPTOSOMES PREPARED FROM ANIMALS PREVIOUSLY DOSED WITH THE TREMORGENIC MYCOTOXINS PENITREM A (P. CYCLOPIUM) AND VERRUCULOGEN (P. ESTINOGENUM)

	POTASSIUM CONTENT (μequiv.K^+ / 100mg protein)		RESPIRATION (μmol.O_2 / 100mg protein / h.)	
	CONTROL	STIMULATED	CONTROL	STIMULATED
UNTREATED				
CEREBROCORTICAL	28 ± 3	16 ± 1	46.7 ± 4.3	121.7 ± 12
SPINAL / MEDULLARY	22 ± 1	16 ± 1	n.d.	n.d.
P. CYCLOPIUM				
CEREBROCORTICAL	27 ± 3	18 ± 2	57.7 ± 3.5	93.5 ± 10.5
SPINAL / MEDULLARY	22 ± 2	15 ± 1	n.d.	n.d.
P. ESTINOGENUM				
CEREBROCORTICAL	27 ± 3	17 ± 2	65 (2)	126 (2)
SPINAL / MEDULLARY	22 ± 2	16 ± 1	n.d.	n.d.

phosphate medium containing 10mM glucose. They showed linear rates of respiration over at least 60min. In the presence of depolarising concentrations of veratrine (75µM) , there was a significant increase in oxygen uptake and in lactate production. The effect of veratrine was to cause an increase (161%) in oxygen taken up in control synaptosomes. The respiratory rates of synaptosomes prepared from animals injected with Penitrem A or Verruculogen were increased by a small, but significant amount, and by 81% and 94% respectively by veratrine addition.

Potassium loss during veratrine stimulation was extensive, although no significant differences were observed cerebrocortical synaptosomes prepared from control or tremorgen treated animals. Similar results were obtained from spinal cord / medullary synaptosomes (TABLE 5.1)

3) Effects of tremorgens on amino acid release from synaptosomes.

Penitrem A (pure toxin for three experiments, remainder with mycelium) administered to rats increased the release of endogenous glutamate (213%), GABA (455%), and aspartate (277%) from cerebrocortical synaptosomes subsequently prepared and incubated (FIG. 5.1), but was without effect on these or other amino acids in spinal cord / medulla synaptosomes (FIG. 5.2). Verruculogen given in an amount designed to elicit a similar degree of tremoring produced (in cerebrocortical synaptosomes) a much larger increase in glutamate (1300%), and aspartate, but was apparently without effect on GABA (FIG. 5.3). The other nine amino acids measured were not changed by these treatments, and values for alanine, threonine, and serine are included (FIG. 5.1 - 5.3) as representative of this group.

The effect of Verruculogen administered to rats on amino acids release from spinal cord / medullary and cereballa synaptosomes subsequently prepared is illustrated in FIG. 5.4 ; 5.5.

Administration in vivo of these two agents also affected the extent of any further release of neuroactive amino acids induced by the depolarising agent veratrine (75µM) (i.e. the depolarisation induced signal). Thus Penitrem A caused a reduction in the stimulated release signal of putative amino acid transmitters compared with cerebrocortical synaptosomes prepared from control animals. The glutamate signal was reduced by 33%, and those of aspartate and GABA

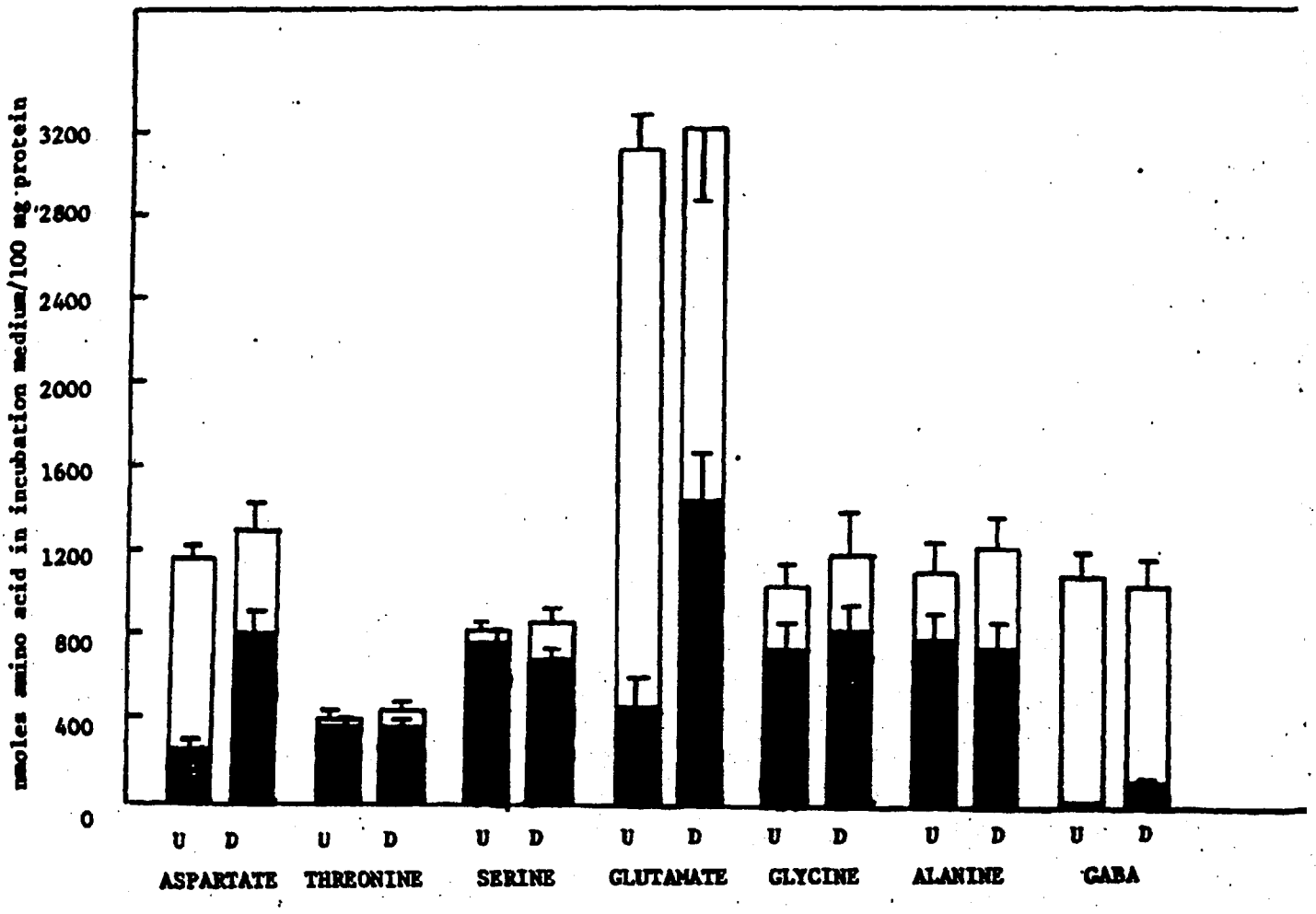


FIG.1. EFFECT OF P.CYCLOPIUM (PENITREM A) ADMINISTERED IN VIVO (400 MG/KG) ON AMINO ACID RELEASE BY RAT CEREBROCORTICAL SYNAPTOSOMES. SYNAPTOSOMES WERE INCUBATED AT 37°C IN KREBS-PHOSPHATE MEDIUM CONTAINING 10 MM GLUCOSE FOR 45 MIN., THE LAST 10 MIN. OF INCUBATION BEING IN THE PRESENCE OF VERATRINE, TO A FINAL CONCENTRATION OF 75 UM, WHERE APPROPRIATE. SUBSEQUENT TO INCUBATION, THE SYNAPTOSOMES WERE SEDIMENTED IN A BENCH ULTRACENTRIFUGE AND THE CLEAR SUPERNATANT WAS PURIFIED AND ANALYSED EMPLOYING AN AMINO ACID ANALYSER. HISTOBARS REPRESENTING VALUES OBTAINED FROM UNTREATED RATS (U) AND DOSED RATS (D) ARE THE MEANS ± S.E.M. FOR SIX OR MORE EXPERIMENTS. THE BLACKED PORTION OF EACH BAR INDICATES THE VALUE OBTAINED FOR AMINO ACID RELEASE TO THE INCUBATION MEDIUM IN THE ABSENCE OF VERATRINE, AND THE WHITE PORTION, THE VALUE OBTAINED IN THE PRESENCE OF THE DEPOLARISING AGENT.

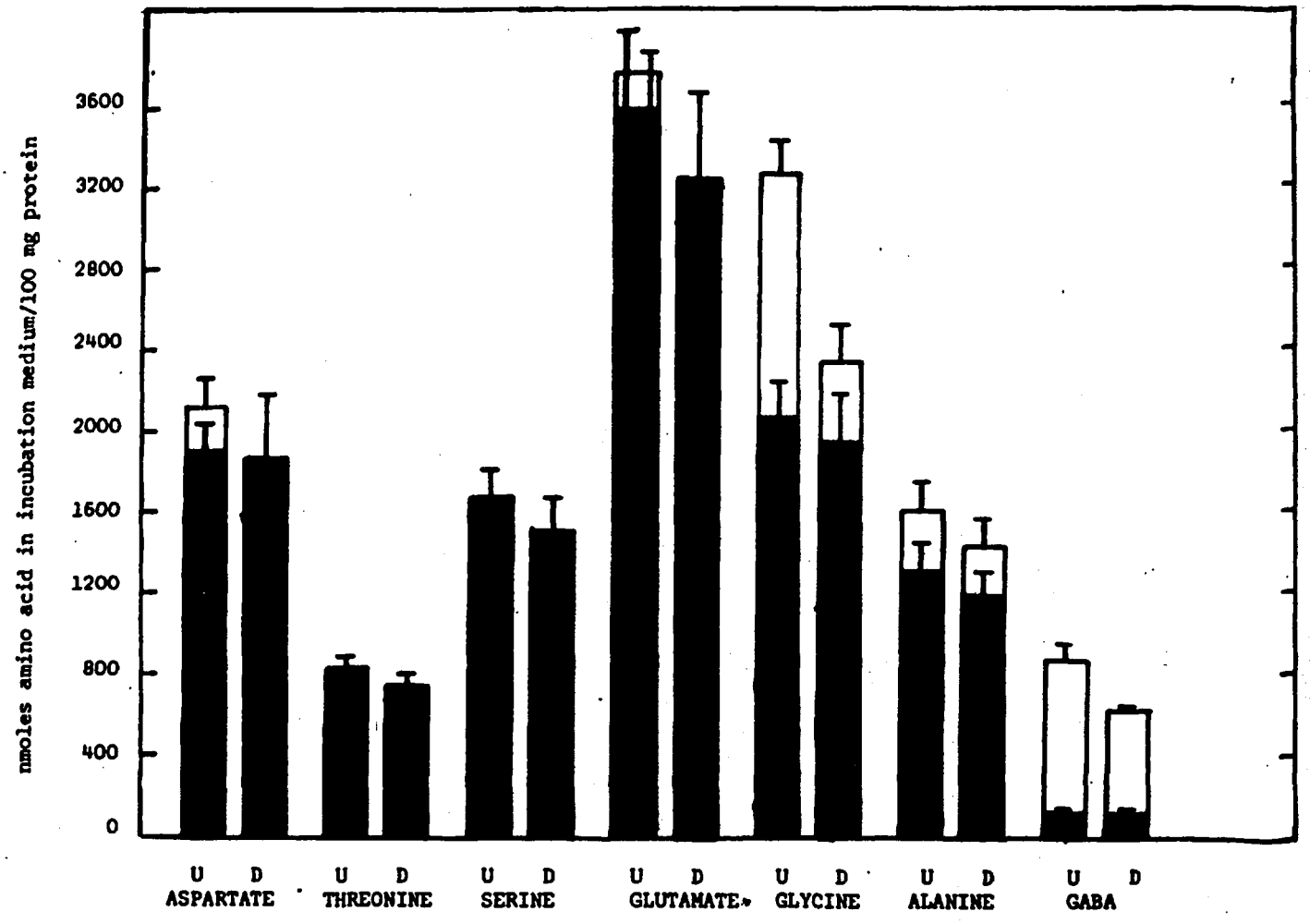


FIG. 2. EFFECT OF P. CLYCLOPIUM (PENITREM A) ADMINISTERED IN VIVO (400 MG/KG) ON AMINO ACID RELEASE BY RAT SPINAL CORD/MEDULLARY SYNAPTOSOMES. SYNAPTOSOMES WERE INCUBATED AT 37°C IN KREBS-PHOSPHATE MEDIUM CONTAINING 10 mM GLUCOSE FOR 45 MIN., THE LAST 10 MIN. OF INCUBATION BEING IN THE PRESENCE OF VERATRINE, TO A FINAL CONCENTRATION OF 75 µM, WHERE APPROPRIATE. SUBSEQUENT TO INCUBATION, THE SYNAPTOSOMES WERE SEDIMENTED IN A BENCH ULTRACENTRIFUGE AND THE CLEAR SUPERNATANT WAS PURIFIED AND ANALYSED EMPLOYING AN AMINO ACID ANALYSER. HISTOBARS REPRESENTING VALUES OBTAINED FROM UNTREATED RATS (U) AND DOSED RATS (D) ARE THE MEANS ± S.E.M. FOR SIX OR MORE EXPERIMENTS. THE BLACKED PORTION OF EACH BAR INDICATES THE VALUE OBTAINED FOR AMINO ACID RELEASE TO THE INCUBATION MEDIUM IN THE ABSENCE OF VERATRINE, AND THE WHITE PORTION, THE VALUE OBTAINED IN THE PRESENCE OF THE DEPOLARISING AGENT.

by 46% and 11% respectively. These reductions in the effectiveness of veratrine to release transmitters must be seen in the light of the considerable increases in the background release of these compounds caused by the administration of Penitrem A prior to yhe addition of veratrine (FIG. 5.1)

Somewhat different results were obtained when the amino acid release from spinal cord / medullary synaptosomes was measured subsequent to the animals being doses with Penitrem A. For this preparation, the background levels of amino acid release from synaptosomes prepared from Penitrem A treated rats was similar to those for synaptosomes prepared from control animals. However, on addition of veratrine, the stimulus induced release of glycine and GABA was reduced by 67% and 32% respectively when compared with the signal obtained from control animal synaptosomes (FIG. 5.2)

Cerebrocortical synaptosomes prepared from animals injected with Verruculogen were found to produce high levels of glutamate and aspartate in the incubation medium as compared with those produced by control synaptosomes (FIG. 5.3). In this case, however, the additional amounts of transmitter amino acids released by veratrine stimulation remained approximately equivalent to those produced by veratrine in synaptosomes from rats not treated with the mycotoxin (FIG. 5.3).

FIG. 5.4 illustrated the results obtained from release studies on synaptosomes prepared from the spinal cord / medulla of rats treated with Verruculogen. It was observed that there was no significant difference between either spontaneous or stimulus evoked amino acid release from synaptosomes prepared from animals previously dosed with Verruculogen, and those animals dosed with water. FIG. 5.5 provides similar information for rat cereballa synaptosomes, which were prepared by the same procedure as for spinal cord / medullary synaptosomes. In this region, however, it was found that the evoked release of GABA was reduced by approximately 50% subsequent to Verruculogen pretreatment of rats.

The data contained in FIG. 5.6 shows the TTX and verapamil (200μM) dependency of the increased background aspartate and glutamate medium levels found in cerebrocortical synaptosomes prepared from rats dosed with Verruculogen. TTX (1μM) reduced these levels by 56% for both aspartate and glutate, and verapamil reduced the elevated background levels down to those observed for cerebrocortical synaptosomes prepared from control animals (FIG. 5.6). Calcium - free medium alone, or

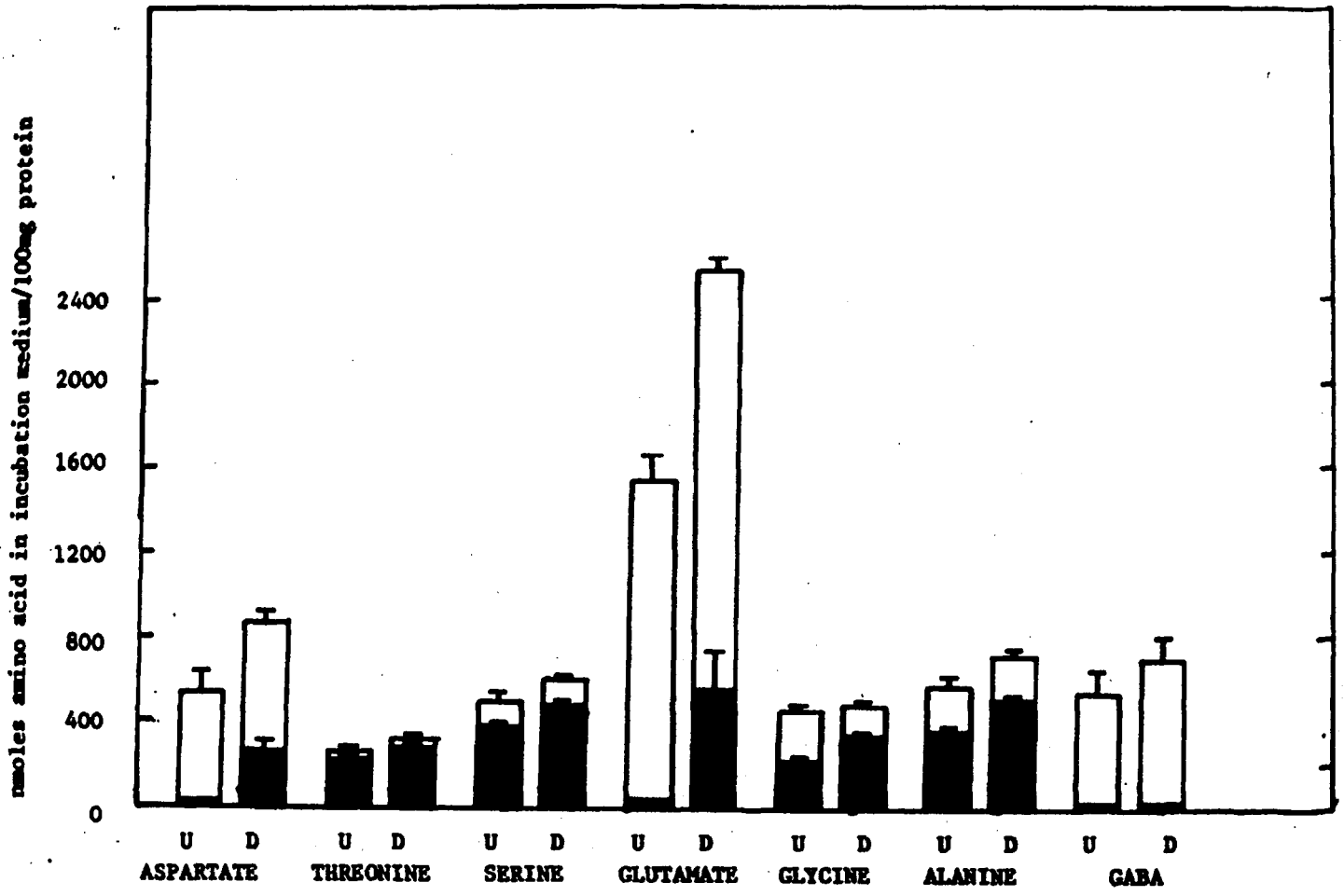


FIG.3. EFFECT OF P.ESTINOGEN (VERRUCULOGEN) ADMINISTERED IN VIVO (400 MG/KG) ON AMINO ACID RELEASE BY RAT CEREBROCORTICAL SYNAPTOSOMES. SYNAPTOSOMES WERE INCUBATED AT 37°C IN KREBS-PHOSPHATE MEDIUM CONTAINING 10 MM GLUCOSE FOR 45 MIN., THE LAST 10 MIN. OF INCUBATION BEING IN THE PRESENCE OF VERATRINE, TO A FINAL CONCENTRATION OF 75 UM, WHERE APPROPRIATE. SUBSEQUENT TO INCUBATION, THE SYNAPTOSOMES WERE SEDIMENTED IN A BENCH ULTRACENTRIFUGE AND THE CLEAR SUPERNATANT WAS PURIFIED AND ANALYSED EMPLOYING AN AMINO ACID ANALYSER. HISTOBARS REPRESENTING VALUES OBTAINED FROM UNTREATED RATS (U) AND DOSED RATS (D) ARE THE MEANS ± S.E.M. FOR SIX OR MORE EXPERIMENTS. THE BLACKED PORTION OF EACH BAR INDICATES THE VALUE OBTAINED FOR AMINO ACID RELEASE TO THE INCUBATION MEDIUM IN THE ABSENCE OF VERATRINE, AND THE WHITE PORTION, THE VALUE OBTAINED IN THE PRESENCE OF THE DEPOLARISING AGENT.

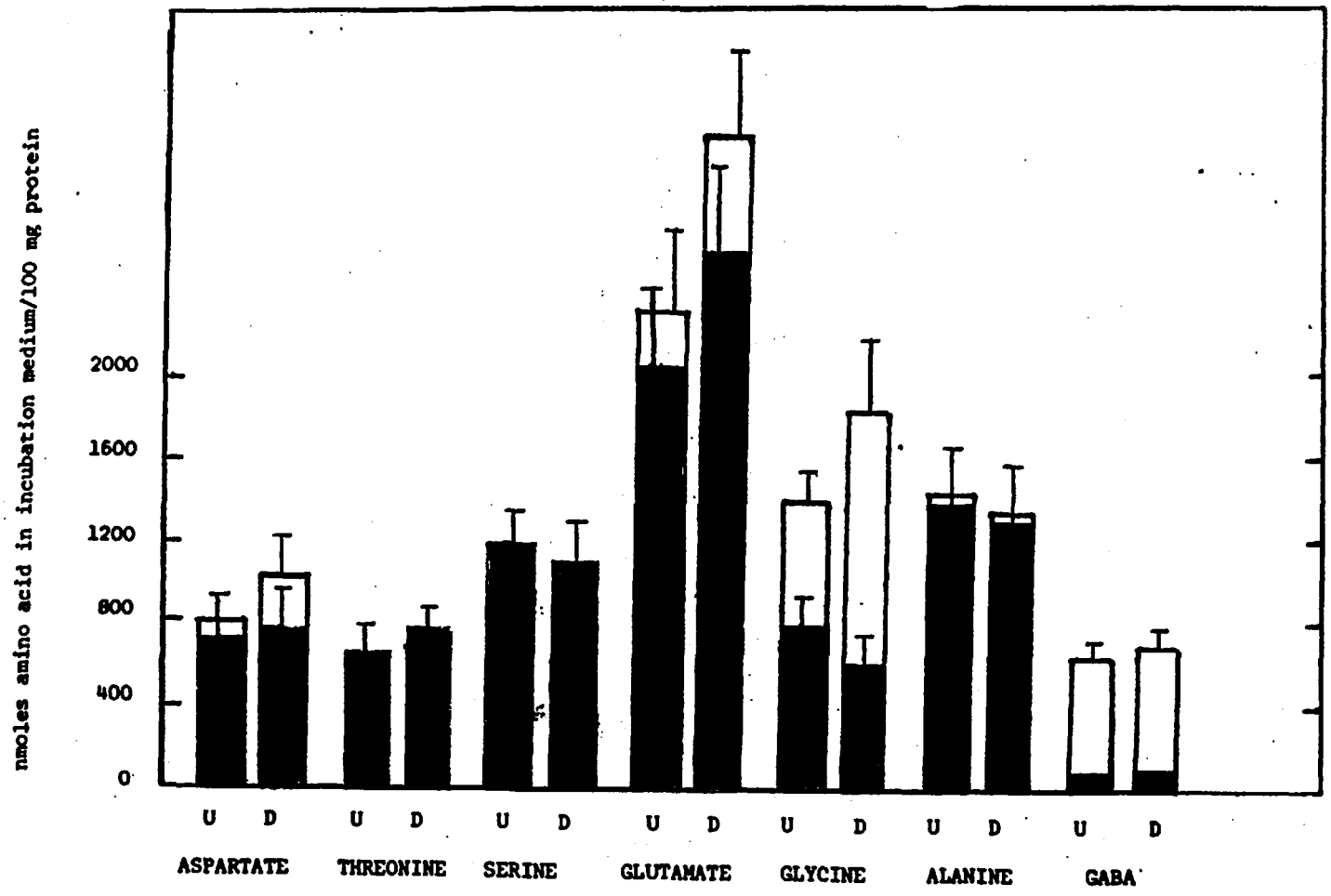


FIG.4. EFFECT OF P. ESTINOGENUM (VERRUCULOGEN) ADMINISTERED IN VIVO (400 MG/KG) ON AMINO ACID RELEASE BY RAT SPINAL CORD/MEDULLARY SYNAPTOSOMES. SYNAPTOSOMES WERE INCUBATED AT 37°C IN KREBS-PHOSPHATE MEDIUM CONTAINING 10 MM GLUCOSE FOR 45 MIN., THE LAST 10 MIN. BEING IN THE PRESENCE OF VERATRINE, TO A FINAL CONCENTRATION OF 75 UM, WHERE APPROPRIATE. SUBSEQUENT TO INCUBATION, THE SYNAPTOSOMES WERE SEDIMENTED IN A BENCH CENTRIFUGE AND THE CLEAR SUPERNATANT WAS PURIFIED AND ANALYSED EMPLOYING AN AMINO ACID ANALYSER. HISTOBARS REPRESENTING VALUES OBTAINED FROM UNTREATED RATS (U) AND DOSED RATS (D) ARE THE MEANS ± S.E.M. FOR SIX OR MORE EXPERIMENTS. THE BLACKED PORTION OF EACH BAR INDICATES THE VALUE OBTAINED FOR AMINO ACID RELEASE TO THE INCUBATION MEDIUM IN THE ABSENCE OF VERATRINE, AND THE WHITE PORTION, THE VALUE OBTAINED IN THE PRESENCE OF THE DEPOLARISING AGENT.

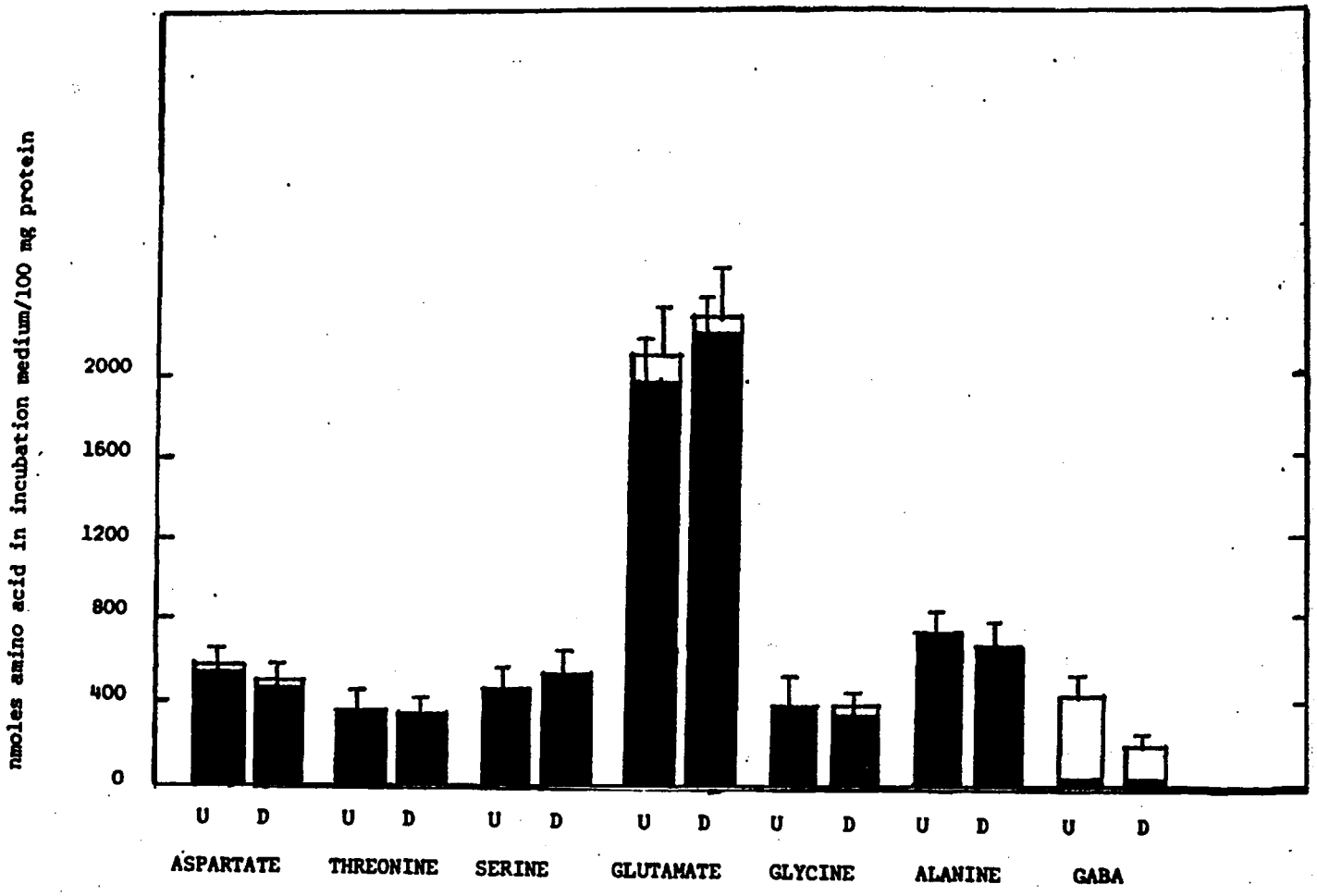


FIG.5. EFFECT OF P. ESTINOGENUM (VERRUCULOGEN) ADMINISTERED IN VIVO (400 MG/KG) ON AMINO ACID RELEASE BY RAT CEREBELLAR SYNAPTOSOMES. SYNAPTOSOMES WERE INCUBATED AT 37°C IN KREBS-PHOSPHATE MEDIUM CONTAINING 10 MM GLUCOSE FOR 45 MIN., THE LAST 10 MIN. BEING IN THE PRESENCE OF VERATRINE, TO A FINAL CONCENTRATION OF 75 UM, WHERE APPROPRIATE. SUBSEQUENT TO INCUBATION, THE SYNAPTOSOMES WERE SEDIMENTED IN A BENCH CENTRIFUGE AND THE CLEAR SUPERNATANT WAS PURIFIED AND ANALYSED EMPLOYING AN AMINO ACID ANALYSER. HISTOBARS REPRESENTING VALUES OBTAINED FROM UNTREATED RATS (U) AND DOSED RATS (D) ARE THE MEANS ± S.E.M. FOR SIX OR MORE EXPERIMENTS. THE BLACKED PORTION OF EACH BAR INDICATES THE VALUE OBTAINED FOR AMINO ACID RELEASE TO THE INCUBATION MEDIUM IN THE ABSENCE OF VERATRINE, AND THE WHITE PORTION, THE VALUE OBTAINED IN THE PRESENCE OF THE DEPOLARISING AGENT.

DATA LEGEND.

RE : FIG. 5.6.

THE EFFECT OF TTX AND VERAPAMIL ON THE RELEASE OF AMINO ACIDS FROM SYNAPTOSOMES SUBSEQUENT TO THE ADMINISTRATION OF VERRUCULOGEN TO RATS.

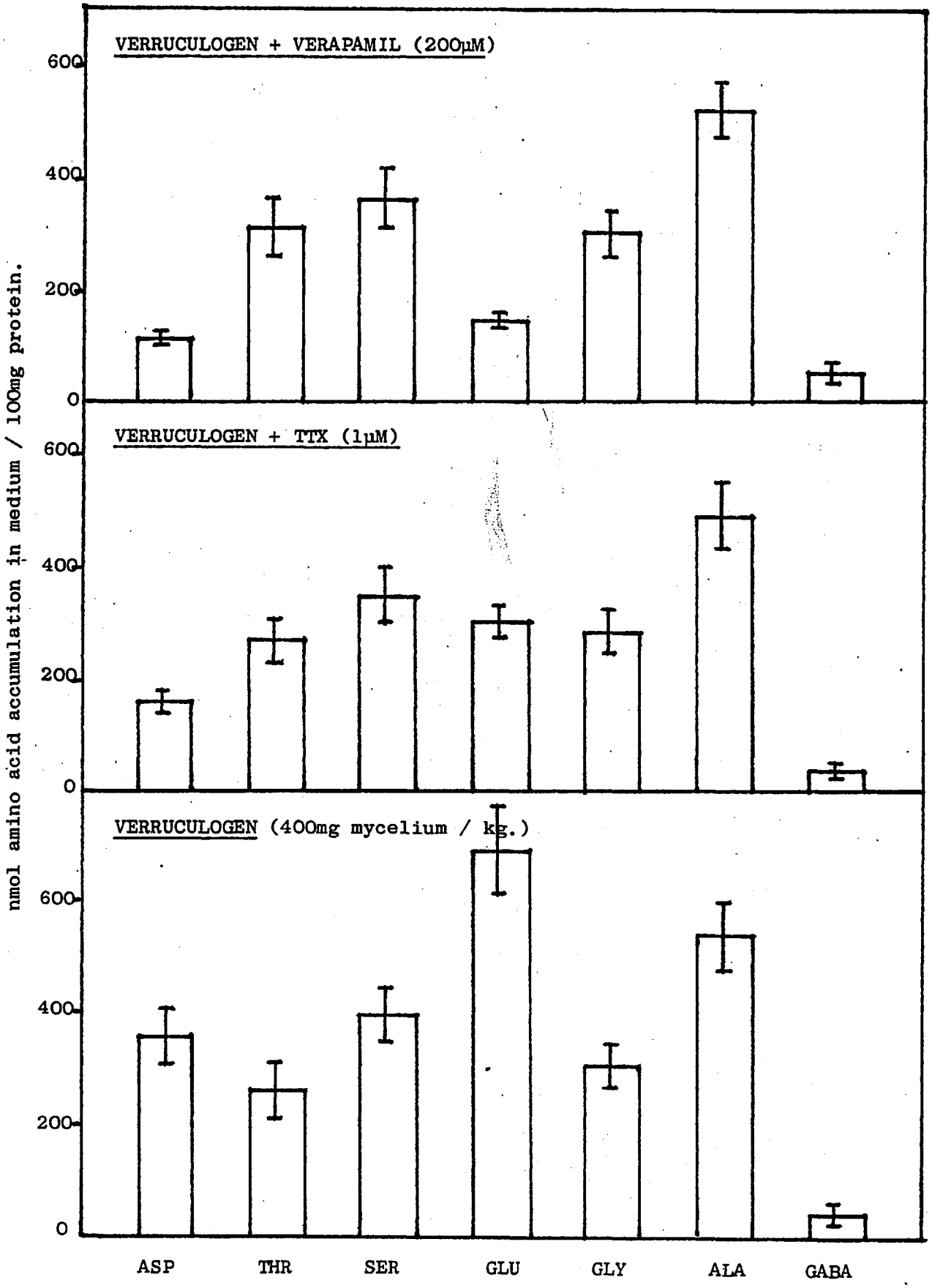
Cerebrocortical synaptosomes were prepared from rats previously injected intra - peritoneally with Verruculogen (400mg mycelium / kg). Synaptosomes were suspended in Krebs phosphate medium (Ca^{++}- free), at 3mg / ml., in 5ml conical flasks on a shaking waterbath at 37'C. Incubation was for a total of 30min. and in the presence of 1µM TTX or 200µM Verapamil as appropriate.

At the end of the experiment, the suspensions were sedimented in a bench top ultracentrifuge. The supernatants were taken to dryness and extracted with methanol. Aliquots were taken in 0.025M HCl for amino acid autoanalysis. Amino acid levels measured in the incubation fluid are expressed as nmol / 100mg synaptosomal protein, the latter being measured by means of the Folin reagent.

Histobars represent data obtained from six experiments, and are the means $\pm$ SEM.

FIG. 5.6

THE EFFECT OF TTX AND VERAPAMIL ON THE BACKGROUND ACCUMULATION OF AMINO ACIDS SUBSEQUENT TO THE ADMINISTRATION OF TREMORGENS TO RATS.



calcium - free medium plus 0.5mM EGTA did not influence the elevated background accumulation of amino acids as a consequence of Verruculogen administration. The accumulations of threonine, serine, glycine, and alanine levels are illustrated as an example of the lack of any effects of these treatments on non - neuroactive amino acids.

4) Effects of the tremorgens on intrasynaptosomal amino acid levels.

Measurement of the total amino acid content of cerebrocortical and spinal cord / medullary synaptosomes prepared from rats treated with Penitrem A in vivo as the mycelium (TABLE 5.2), and of cerebrocortical, spinal cord / medullary, and cerebellar synaptosomes prepared from rats treated with Verruculogen administered in vivo as the mycelium (TABLE 5.3), did not produce a detectable lowering of the tissue content of these compounds with the exception of cerebellar synaptosomes, which exhibited a significant lowering in their content of GABA. The cerebrocortical and spinal cord / medullary tissue data were observed in spite of the action of the tremorgenic mycotoxins in releasing glutamate and aspartate to the incubation medium. However, as the additional amounts of these amino acids released were of the same order of magnitude to the S.E.M. values for the mean tissue contents, then detecting a significant decrement or more possibly an elevation in the tissue contents would be difficult (the former particularly so, if any substantial re - synthesis of the amino acids occurred following their loss to the medium). It is worthy of note, however, that the content of GABA is not reduced in cerebrocortical synaptosomes prepared from rats treated with Penitrem A or Verruculogen, and consequently diminished GABA contents cannot explain the lack of a releasing effect for this amino acid. It is nevertheless possible that an interference with the distribution of GABA between releasable and non - releasable pools may occur.

5) Effects of tremorgens on amino acid uptake by synaptosomes.

Since it was conceivable that the extrasynaptosomal aspartate and glutamate accumulation from cerebrocortical synaptosomes prepared from rats injected with the mycotoxins could have been due to an inhibition of re - uptake by the tremorgens of these amino acids, the high affinity

DATA LEGEND.

RE : TABLE 5.2, 5.3.

THE EFFECT OF TREMORGENIC MYCOTOXINS ON INTRASYNAPTOSOMAL AMINO ACID CONTENTS.

Cerebrocortical and spinal cord /medullary synaptosomes were prepared from rats previously injected intraperitoneally with Penitrem A mycelia (400mg /kg), TABLE 5.2, and cerebrocortical, spinal cord / medullary, and cerebellar synaptosomes were prepared from rats previously dosed with Verruculogen mycelia (400mg / kg), TABLE 5.3. Synaptosomes were suspended in Krebs phosphate medium in 5ml conical flasks in a shaking waterbath at 37'C. Incubation lasted for a total of 30min, the last 10min. being in the presence of veratrine (75µM) as appropriate.

At the end of the experiment, the suspensions were sedimented in a bench top ultracentrifuge. The synaptosomal pellets were extracted with acidified methanol, which was taken to dryness. Aliquots were taken in 0.025M HCl for amino acid autoanalysis. Pellet protein was measured by means of the Folin reagent.

All data are expressed as nmol amino acid / 100mg protein, and are the mean ± SEM for six experiments.

TABLE 5.2

THE EFFECT OF PENITREM A (P. CYCLOPIUM) ON INTRASYNAPTOSOMAL AMINO ACID CONTENT.

	ASPARTATE	THREONINE	SERINE	GLUTAMATE	GLYCINE	ALANINE	GABA
CEREBROCORTICAL							
CONTROL (U)	2791 ± 588	159 ± 14	471 ± 84	4143 ± 673	962 ± 40	617 ± 49	1124 ± 146
STIMULATED (U)	1021 ± 159	144 ± 13	508 ± 13	2507 ± 292	576 ± 109	597 ± 96	405 ± 44
CONTROL (D)	2640 ± 600	164 ± 28	329 ± 34	4766 ± 1790	916 ± 108	779 ± 130	1343 ± 233
STIMULATED (D)	1135 ± 207	122 ± 12	311 ± 59	2671 ± 408	433 ± 72	398 ± 72	450 ± 77
SPINAL / MEDULLARY							
CONTROL (U)	2638 ± 205	418 ± 66	962 ± 151	2140 ± 231	2117 ± 222	620 ± 99	1514 ± 270
STIMULATED (U)	1869 ± 195	349 ± 63	502 ± 116	1775 ± 165	1605 ± 246	527 ± 66	1033 ± 119
CONTROL (D)	2593 ± 299	316 ± 31	639 ± 46	1934 ± 379	2719 ± 477	589 ± 106	1363 ± 253
STIMULATED (D)	1355 ± 180	283 ± 28	702 ± 76	1995 ± 523	1895 ± 579	549 ± 105	1109 ± 280

TABLE 5.3

THE EFFECT OF VERRUCULOGEN (P. ESTINOGENUM) ON INTRASYNAPTOSOMAL AMINO ACID CONTENT.

	ASPARTATE	THREONINE	SERINE	GLUTAMATE	GLYCINE	ALANINE	GABA
CEREBROCORTICAL							
CONTROL	4340 ± 840	340 ± 72	621 ± 73	7124 ± 1146	615 ± 59	1138 ± 174	1684 ± 131
VERRUCULOGEN	4179 ± 496	240 ± 54	548 ± 89	7453 ± 938	533 ± 96	942 ± 157	1864 ± 192
SPINAL / MEDULLARY							
CONTROL	3255 ± 728	268 ± 39	660 ± 77	3549 ± 799	3424 ± 691	940 ± 183	2469 ± 423
VERRUCULOGEN	2519 ± 643	365 ± 72	545 ± 101	3890 ± 753	2744 ± 580	1006 ± 127	1888 ± 397
CEREBULLUM							
CONTROL	3006 ± 1987	216 ± 145	477 ± 164	4622 ± 1219	490 ± 157	739 ± 119	2261 ± 540
VERRUCULOGEN	1164 ± 1340	302 ± 157	427 ± 184	2604 ± 1890	507 ± 167	575 ± 98	597 ± 130

uptake rates of (U - ^{14}C) glutamate and also of (U - ^{14}C) GABA were studied (data not shown) over 1min. time periods, during which time significant re - release of radio - label would not be expected to occur. There was no significant difference between the uptakes of (U - ^{14}C) glutamate or (U - ^{14}C) aspartate by synaptosomes prepared from predosed or untreated animals.

6) Recovery of rats from mycotoxin induced tremor.

At the doses of tremorgens administered to rats in these exoeriments, the time taken for them to lose all signs of tremor varied between four and six hours. When rats were allowed to recover in this manner, and were then sacrificed, the amino acid accumulation in the incubation fluid of cerebrocortical synaptosomes subsequently prepared was not significantly different from the amino acid profiles observed for control rats (data not shown). Consequently, it may be anticipated that the amino acid accumulations as a result of mycotoxin administration were intimately associated with the observed tremor, and that the dysfunction of releasing mechanisms correlated with the time during which tremoring was apparent.

7) The effect of orally administered Penitrem A mycelium on amino acid and dopamine release from synaptosomes subsequently prepared from sheep.

Synaptosomes prepared from the corpus striatum of sheep dosed orally over a period of several weeks with Penitrem A, when incubated in Krebs - phosphate medium containing 10mM glucose showed (FIG. 7) an increase in the background levels of aspartate (68%), glutamate (62%), and GABA (100%). In the cases of aspartate and glutamate, these increases were sufficiently large that 75µM veratrine administration did not release any further quantities amino acids. However, the veratrine stimulated GABA signal was not significantly changed by the pretreatment from veratrine stimulated signals obtained from control animal signals.

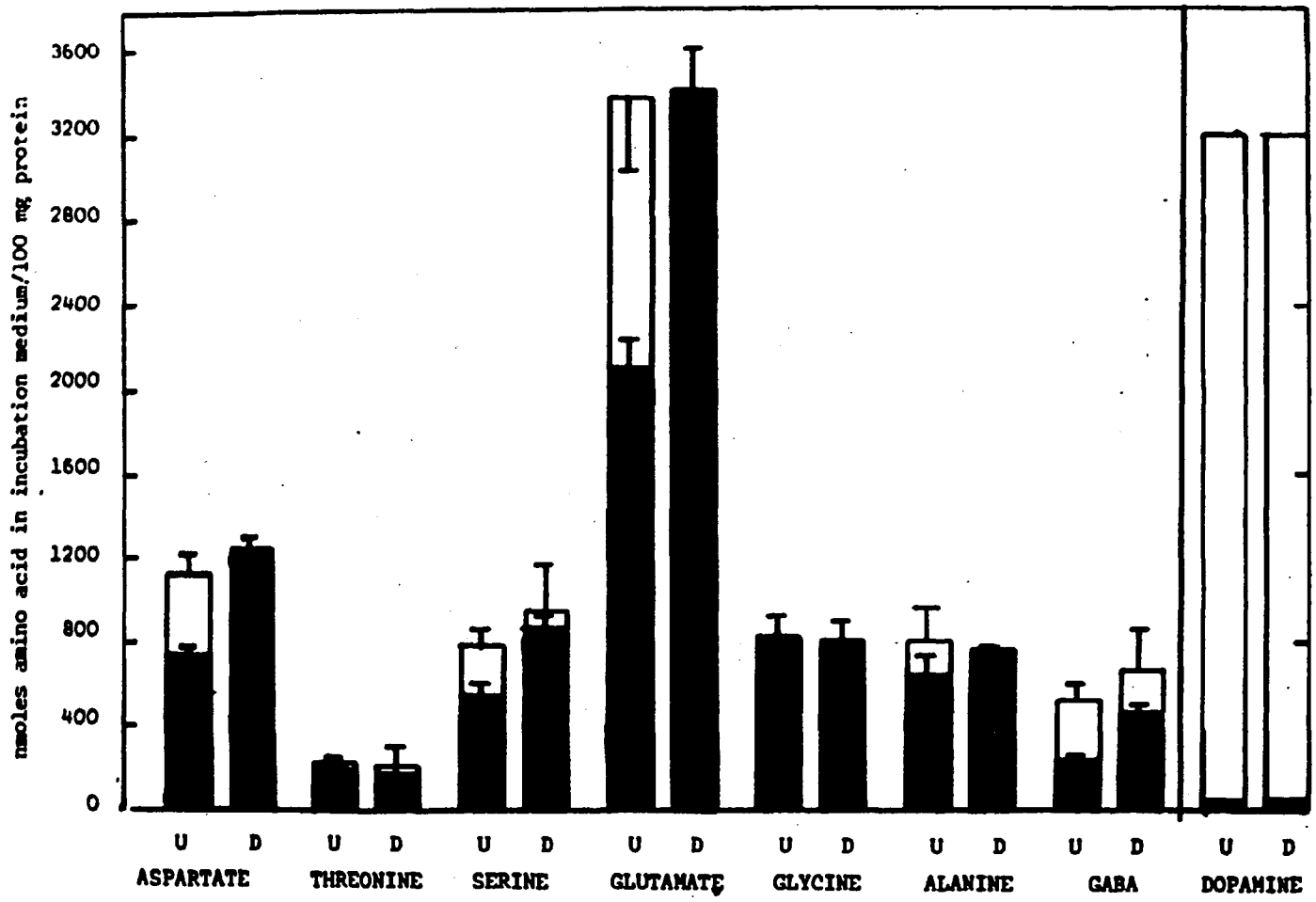
The spontaneous and stimulus induced release of dopamine from synaptosomes of sheep corpus striatum were measured (FIG. 5.7), employing preparations from control and Penitrem A treated sheep. There was found to be no significant difference in the extent of dopamine release by the separate synaptosome preparations.

FIG.7. EFFECT OF P.CYCLOPIUM (PENITREM A) ADMINISTERED IN VIVO TO SHEEP, ON AMINO ACID AND DOPAMINE RELEASE BY SHEEP CORPUS STRIATUM SYNAPTOSOMES SUBSEQUENTLY PREPARED. AMINO ACID RELEASE WAS MEASURED FROM SYNAPTOSOMES INCUBATED AT 37°C IN KREBS-PHOSPHATE MEDIUM CONTAINING 10 mM GLUCOSE FOR 30 MIN., THE LAST 10 MIN. OF INCUBATION BEING IN THE PRESENCE OF VERATRINE, TO A FINAL CONCENTRATION OF 75 μM, WHERE APPROPRIATE.

DOPAMINE RELEASE WAS MEASURED FROM SYNAPTOSOMES INCUBATED AT 37°C IN KREBS-PHOSPHATE MEDIUM CONTAINING 10 mM GLUCOSE, 1.8 mM L-ASCORBATE, 0.54 mM NIALAMIDE, AND 10 μM L- U-^{14}C -TYROSINE (513 mCi/mmol) FOR 30 MIN., THE LAST 10 MIN. OF INCUBATION BEING IN THE PRESENCE OF THE DEPOLARISING AGENT, TO A FINAL CONCENTRATION OF 75 μM, WHERE APPROPRIATE.

AT THE END OF INCUBATION, BOTH EXPERIMENTAL GROUPS WERE SEDIMENTED AT ROOM TEMPERATURE IN A BENCH ULTRACENTRIFUGE, AND THE CLEAR SUPERNATANTS WERE ANALYSED FOR AMINO ACIDS OR DOPAMINE AS APPROPRIATE. THE AMINO ACID SAMPLES WERE PURIFIED AND ANALYSED EMPLOYING AN AMINO ACID ANALYSER. THE DOPAMINE SAMPLES WERE EXTRACTED WITH PERCHLORIC ACID (FINAL CONC. 0.4 M) CONTAINING SODIUM METABISULPHITE (15 mM), CARRIER DOPAMINE (0.5 μmol) AND INTERNAL STANDARDS OF L- U-^{14}C -VALINE (7.5-12.5 nCi) AND NORLEUCINE (30-100 nmol). THE EXTRACTS WERE FRACTIONATED AND ANALYSED.

HISTOBARS REPRESENTING VALUES OBTAINED FROM UNTREATED SHEEP (U) AND DOSED SHEEP (D) ARE THE MEANS ± S.E.M. FOR THREE SHEEP IN EACH CLASS. THE BLACKED PORTION OF EACH BAR INDICATES THE VALUE OBTAINED FOR RELEASE TO THE INCUBATION MEDIUM IN THE ABSENCE OF VERATRINE, AND THE WHITE PORTION, THE VALUE OBTAINED IN THE PRESENCE OF THE DEPOLARISING AGENT.


nmoles amino acid in incubation medium/100 mg protein
3600
3200
2800
2400
2000
1600
1200
800
400
0
U D
ASPARTATE
THREONINE
SERINE
GLUTAMATE
GLYCINE
ALANINE
GABA
DOPAMINE

Incubations of sheep spinal cord /medullary synaptosomes were performed in both Krebs - phosphate and Krebs - bicarbonate media, and satisfactory results in that glycine and GABA release were evoked by veratrine administration, were found only in the latter. The data presented in FIG. 8 for amino acid release from spinal cord / medullary synaptosomes prepared from Penitrem A treated sheep are notable only in that background glycine levels were decreased. The data was obtained from only one sheep, and is the mean of results obtained from incubations performed in triplicate. Similarly unfortunate incubations of sheep cerebrocortical synaptosomes yielded no information of any validity to further a comprehension of amino acid accumulations as a consequence of Penitrem A administration to sheep. Possible reasons for this lack of success have been discussed in a previous chapter.

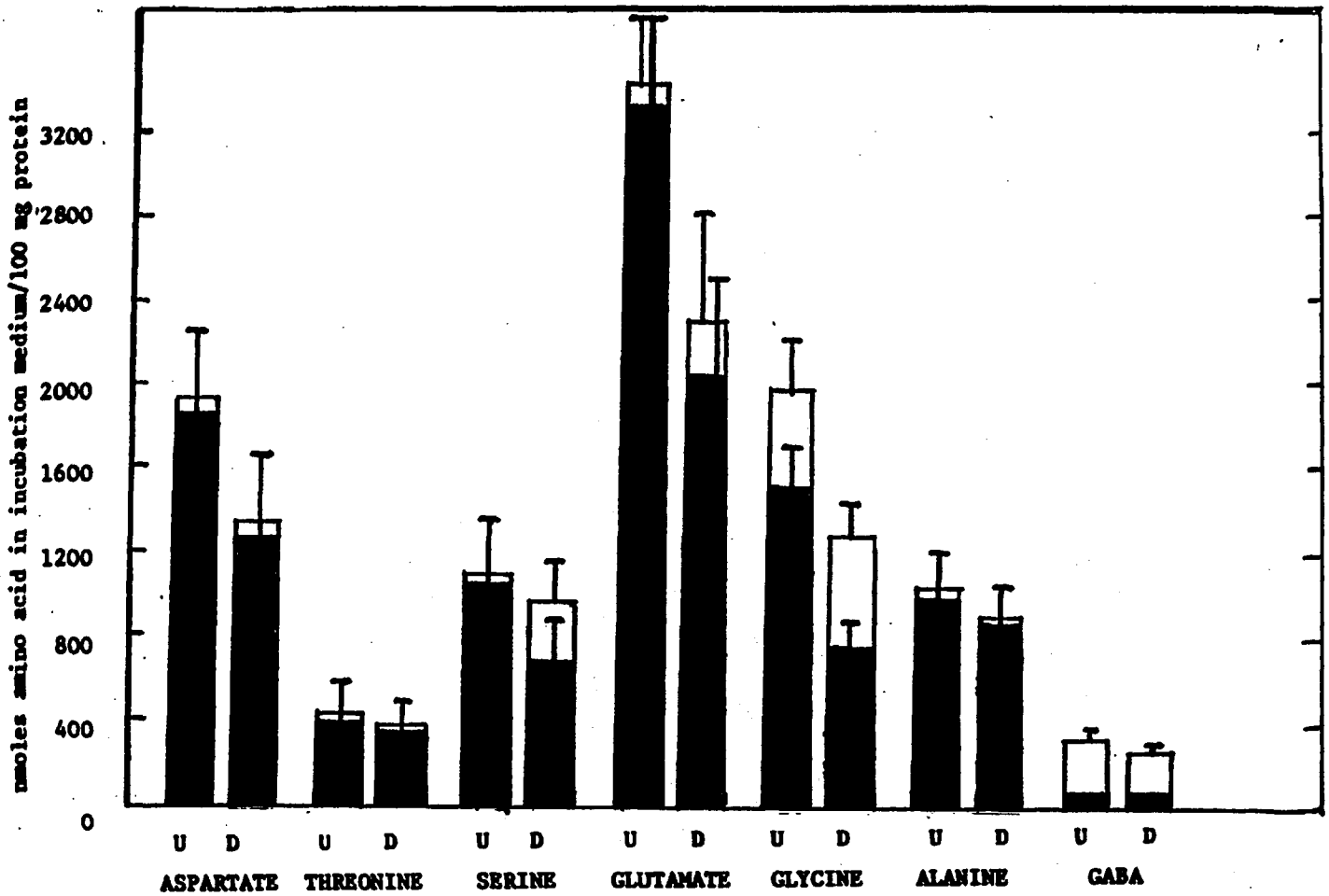


FIG 8. EFFECT OF P.CYCLOPIUM (PENITREM A) ADMINISTERED IN VIVO (SHEEP) ON AMINO ACID RELEASE BY SPINAL/MEDULLA SYNAPTOSOMES. SYNAPTOSOMES WERE INCUBATED AT 37°C IN KREBS-PHOSPHATE MEDIUM CONTAINING 10 mM GLUCOSE FOR 45 MIN., THE LAST 10 MIN. OF INCUBATION BEING IN THE PRESENCE OF VERATRINE, TO A FINAL CONCENTRATION OF 75 μM, WHERE APPROPRIATE. SUBSEQUENT TO INCUBATION, THE SYNAPTOSOMES WERE SEDIMENTED IN A BENCH ULTRACENTRIFUGE AND THE CLEAR SUPERNATANT WAS PURIFIED AND ANALYSED EMPLOYING AN AMINO ACID ANALYSER. HISTOBARS REPRESENTING VALUES OBTAINED FROM UNTREATED RATS (U) AND DOSED RATS (D) ARE THE MEANS ± S.E.M. FOR THREE INCUBATIONS. THE BLACKED PORTION OF EACH BAR INDICATES THE VALUE OBTAINED FOR AMINO ACID RELEASE TO THE INCUBATION MEDIUM IN THE ABSENCE OF VERATRINE, AND THE WHITE PORTION, THE VALUE OBTAINED IN THE PRESENCE OF THE DEPOLARISING AGENT.

DISCUSSION.

No consistent changes could be detected by light microscopy in the structure of neural tissue taken from many brain regions of sheep previously chronically dosed with Penitrem A and showing severe symptoms of tremor over an 8- week period. As these same sheep were used in the present studies, this sugggests that the primary site of action of the tremorgens is at the biochemical level and even over long periods is not reflected at the histological level. Thus, studies at the neurochemical level are most likely to provide a description of the mode of action of these agents.

Previous studies have provided evidence that central amino acid transmitter systems may be affected by the fungal mycotoxins Penitrem A and Verruculogen (STERNet al., 1971; HOTUJAC et al., 1974; 1976), and an action on periphal cholinergic synapses was indicated by the enhancement by Verruculogen of miniature end plate potential frequency and post-tetanic potentiation in a phrenic nerve-diaphragm perparation. Mechanisms of action involving central dopaminergic, nor-adrenergic, or cholinergic transmitter systems seem to be unlikely since both mycotoxins administered at high doses were without influence on the levels of catecholamines and had no effect on acetylcholinesterase activity (SOBOTKA et al., 1978; WILSON, 1971; WILSON et al., 1972).

The results reported here support a mechanism in which a primary site of action of both Penitrem A and Verruculogen is presynaptic and involves central synapses operated by amino acid transmitters. Since the total contents of amino acid transmitters were unchanged in synaptosomes from tremoring animals, interference with transmitter synthesis seems an unlikely mode of action. This question is, however, of particular importance, especially in respect of the data obtained for intra-cerebellar synaptosomal GABA levels. A knowledge of the distribution and compartmentation of amino acids in brain tissue at the subcellular level and the effect of drugs on this distribution are critical to a complete understanding of the roles of neuroactive amino acids in brain. Unfortunately, real or putative technical difficulties have hindered research in this area. Specifically, it has been claimed that the observed distribution of GABA after subcellular fractionation does not reflect the *in vivo* situation, due to the loss of, for instance, GABA from the organelles during either the homogenisation process or centrifugation procedures, or due to the extensive redistribution of

GABA during fractionation (NEAL & IVERSON, 1969; TACHIKA et al, 1972)

Synaptosomes from sheep corpus striatum showed effects of the mycotoxin on amino acid transmitters but none on dopamine synthesis or release, indicating that the mycotoxins show some degree of specificity in their action.

Although both mycotoxins had an action specifically on amino acid transmitters rather than other other amino acids, the qualitative differences in their actions are sufficient to indicate differences in their precise mechanisms of action. Thus, whilst Penitrem A pretreatment increased the release of transmitter amino acids from incubated synaptosomes, it reduced the release signal due to veratrine. This reduction was particularly well illustrated in the sheep corpus striatum. This reduction does not appear to be related to the high background amino acid levels in the incubation medium caused by the mycotoxin, since a similar reduction in the veratrine induced signal occurred with spinal cord / medullary synaptosomes, where, interestingly, no such background release was evoked.

Verruculogen, on the other hand, produced high background levels of transmitter amino acids from cortical synaptosomes without causing a reduction in the signal due to added veratrine. Thus, whilst both agents showed the capacity to cause specific accumulation of transmitter amino acids in the incubation medium, only Penitrem A modified the amplitude of the veratrine-induced signal. As no effect of the two toxins on (U -^{14}C) glutamate or (U -^{14}C) GABA uptake could be demonstrated, it is unlikely that the increases in background levels in the medium are due to inhibition of amino acid transmitter uptake. Instead, a direct enhancement of release is indicated.

The low level of intrasynaptosomal GABA in synaptosomes prepared from the cerebella of Verruculogen dosed animals is intriguing. There is no evidence to indicate that GABA synthesis or metabolism in the cerebellum (although for example, two multimolecular forms of GABA-T exist (TARDY et al., 1976))is any different from other brain regions, and the low levels found here are inconsistent with the unchanged levels observed for the other brain regions studied. The response to veratrine stimulation, although perhaps smaller than might have been expected, was unchanged from the release observed in control cerebellar synaptosomes. HOTUJAC et al., (1976) reported that Verruculogen behaves as a GABA antagonist in the CNS and that whole brain GABA levels are substantially reduced in mice treated with the toxin. Cerebellar synaptosomes exhibit fairly low levels of respiration in

suspension (data not shown), and the respiration rate does not increase on veratrine stimulation. It would thus seem plausible that cerebellar synaptosomes prepared by the methods used here do not perform as satisfactorily as cerebral cortical synaptosomes for example, as in vitro aids to the study of nerve terminal action in situ. Nevertheless, the low level of GABA observed is of great importance. GABA is an inhibitory transmitter released on to granule cells from Golgi axon terminals (BISTI et al., 1971), and golgi cells readily accumulate radiolabelled GABA (HOKFELT & LJUNGDAHL, 1972). GABA is likely to be an inhibitory transmitter on those basket and golgi cells which receive collaterals from Purkinje axons, and is the transmitter released onto Purkinje cells from basket and stellate axons. (e.g, CURTIS et al., 1970; BISH et al., 1971). Consequently, any disturbance of GABA levels in this brain region is likely to result in severe dysfunction of these neural pathways, particularly in respect of the well known function of the cerebellum enabling smooth, coordinated, effective locomotor responses to occur.

The data obtained for dopamine release from sheep corpus striatum is in agreement with the work of STERN, (1971) who demonstrated that γ-hydroxy butyrate, an agent increasing brain dopamine levels, and also L-dopa, the dopamine precursor, were without an effect on Penitrem A induced tumour. Similarly, α-methyl-m-tyrosine, which selectively depletes dopamine levels, was without any effect; and consequently it may be anticipated that there is no similarity between Penitrem A induced tremor and Parkinsonian tremor with respect to biochemical changes in the corpus striatum.

The unfortunate fact that both Penitrem A and Verruculogen lose their biological activity with storage in terms of tremor observed subsequent to adminstration and also the biochemical changes observed concomitant with this tremor, whether the tremorgens are stored as the mycelium or as the purified compounds at 4°C and even at -20°C, has hindered the study of any effects of the mycotoxins on serotonin, another indole compound, accumulation in synaptosomes. Clearly such a study is highly relvant to the problem, and data will be collected in the future. Activity loss problems have also prevented the study of the dependency of amino acid accumulation upon TTX and verapamil in synaptosomes prepared from animals injected with Penitrem A. Such a correlation with Verruculogen is of great importance when the actions of the mycotoxins are compared and contrasted. This question will also be resolved in the future.

The small elevation in respiration of synaptosomes prepared from rats previously injected with one of the two mycotoxins, and the absence of any loss of potassium from these synaptosomes are intriguing when it is considered that the subsequent amino acid accumulation in the medium of synaptosome suspensions is to some extent TTX dependent, and totally verapamil dependent (at least for Verruculogen). These data corroborate the findings that the mycotoxins do not influence the uptake of transmitter amino acids, and that their action is specifically one of release effecting.

Since the amino acid accumulation from synaptosomes resulting from mycotoxin pretreatment is so much smaller than observed when synaptosomes are depolarised by means of , for instance, veratrine administration, it may not be surprising that alterations in respiration rate and intrasynaptosomal potassium levels are absent or at least only small. Potassium (56mM) stimulation produced only a very small increase in respiration rate for control synaptosomes, and indeed the evoked release of transmitter amino acids by this means is very much smaller than the levels evoked by veratrine. Similarly, the levels of amino acid accumulation subsequent to tityus toxin stimulation are different from those observed with veratrine stimulation, despite the fact that both agents act via the active sodium channel. Consequently, the data point towards a mechanism of action of the mycotoxins as an active one at the TTX dependent sodium channel, and that the subsequent amino acid release is Ca^{++}- channel - or at least verapamil - sensitive.

The tremor observed when rats are injected with either Penitrem A or Verruculogen is so nearly indistinguishable that it is probably a consequence of those biochemical changes described above which are similar. The contrasting changes , such as the differing response to veratrine stimulation, or the evidence that biochemical changes are observed at the spinal cord subsequent to Penitrem A administration, but not subsequent to Verruculogen, may be additional refinements which illustrate the activity of the compounds at sites distant from a possible common modulator site. The fact that the structure of Penitrem A is not yet known makes it difficult to speculate further on this point. Furthermore, the data showing that GABA accumulation is elevated, albeit only slightly , from synaptosomes prepared from Penitrem A pretreated animals do not correlate well with the lack of an effect of Verruculogen on extrasynaptosomal GABA accumulation.

The notion that glutamate and aspartate should be specifically

released when GABA is not might appear to be verging on the heretical, but this indeed seems to be the case. There is no obvious reason why GABA should not be released also, i.e. with the exception of the cerebellum, intrasynaptosomal levels of GABA are not reduced. These data are particularly intriguing, and perhaps it might be worth warning the reader that a similar situation arises in a later chapter, except that on that occassion, GABA is released specifically in cerebrocortical synaptosomes subsequent to the administration of two GABA γ - analogues, whilst aspartate and glutamate are not.

The biochemical observation common to both of the tremorgenic mycotoxins is the elevation in extrasynaptosomal accumulation of aspartate and glutamate from the cerebral cortex. Work is in progress at the present time on an investigation of amino acids in the effluent of a cannula implanted on the sensorimotor cortex of tremoring rats, and corroboration of the above data from in vivo experiments will be of great value. Clearly, further work may provide evidence of other similarities e.g.,serotonin (as mentioned above), but for the present, the accumulation of glutamate and aspartate stands out to be the most likely parameter to be associated directly with the tremor producing mycotoxins. That this effect is the actual source of the tremor syndrome, or merely a consequence of it , is difficult at this stage to decide, but it is present only for the duration of the tremor and subsides with the animals' recovery from the observed tremor.

The data contained in, and discussed in, a later chapter concerning the biochemistry of two novel anti - convulsants would appear to substantiate the relationship between tremor and the anomalous release of excitatory and inhibitory transmitters. Thus, when tremorgenic moulds are ingested in sufficient quantity by sheep in the field, or administered experimentally, a subsequent loss of coordination of neural mechanisms controlling muscle action would be the expected result. The non - polar properties of the mycotoxins would enable them to pass the blood brain barrier and gain access to many of the synapses present. The pattern of clinical response observed to follow administration to rats and sheep fits in well with such proposed sites of action for these tremorgens.

This reversible biochemical pathology and the absence of histopathology is in accord with the course of certain naturally occurring neurological disorders in sheep and cattle, concerning

which, evidence for the involvement of tremorgenic mycotoxins has recently been proposed (MANTLE et al., 1977; DI MENNA & MANTLE, 1978; MANTLE et al., 1978).

The present chapter has comprised data and discussion which has concentrated upon the consequences of the administration to sheep and rats of the tremorgenic mycotoxins. Clearly, the next step is to investigate whether similar data is obtained when the compounds are administered directly to in vitro preparations. This question is addressed in the proceding chapter.

CHAPTER SIX.

ACTIONS OF IN VITRO ADMINISTERED

TREMORGENIC FUNGAL TOXINS.

(1) INTRODUCTION.

(2) RESULTS.

1) In vitro additions of the purified tremorgenic mycotoxins to synaptosome suspensions.

a) Penitrem A.

b) Verruculogen.

b1) Ethanol.

b2) Mulgofen EL - 620 and EL - 719 emulsifiers.

b3) Nylon gauze administration of Verruculogen.

b4) Liposomal administration of Verruculogen.

2) In vitro additions of the purified tremorgenic mycotoxins to synapses in situ.

a) Effects of the tremorgenic mycotoxins at the locust neuromuscular junction.

b) Effects of Verruculogen on the giant dopamine neuron of the water snail.

(3) DISCUSSION.

INTRODUCTION.

The non - polar character of the purified tremorgenic compounds Verruculogen and Penitrem A has been described in the previous chapter. Consequently, that particular chapter described data obtained by means of administration of the tremorgens to animals in the form of mycelial aqueous suspensions, or the pure compound solubilised in organic solvents and saline, and synaptosomes were subsequently prepared.

The present chapter describes experiments made to investigate the action of the tremorgens when applied in vitro to neurological preparations. In an earlier chapter, the problems of working with non - aqueous soluble substances were described in relation to the toxin T_{46} extracted from Gymnodinium breve red tide organisms. In that case, the toxin was soluble in a limited number of organic solvents only, and attempts to generate even low aqueous concentrations proved to be unsuccessful. In order to manoeuvre around this problem, an idea of dissolving the toxin in organic solvent, measured amounts of which were layered onto small nylon gauzes and dried, was employed. The nylon gauze was then submerged in the shaking synaptosome suspension. Presumably, the shaking action of the flask enabled synaptosomes, or clumps of synaptosomes to make direct contact with the toxin covered gauze, or possibly very small but sufficient quantities of the toxin may have become solubilised during the course of the experiment. In any event, a very specific action of of the toxin on cerebrocortical synaptosome suspensions was observed, although it was difficult to subsequently identify the precise quantity of toxin which elicited the observed effects.

Similar problems have been found with other compounds which show a high degree of hydrophobic character. DE BELLEROCHE & BRADFORD (1977) dissolved veratrine in 30% ethanol, and added an aliquot of this to their synaptosome suspension (final ethanol concentration approximately 0.3% v/v). Such ethanol concentrations appeared to have little or no effect on the parameters measured in their subsequent experiments, but nevertheless, a procedure which requires the addition of fairly high concentrations of neuroactive compounds clearly necessitates additional study of parameters not under direct examination in order to impart sufficient confidence in the data obtained in such a manner.

In addition to the use of ethanol to assist in the administration of the tremorgenic compounds Verruculogen and Penitrem A to synaptosome suspensions, the present chapter describes novel procedures whose use

has been investigated. One such method has already been referred to above, and described in a previous chapter, i.e. the idea of dissolving the substance under study in a convenient solvent and pipetting aliquots onto nylon gauze which is submerged in the synaptosome suspension. Despite the crudeness of this manner of administration, results were nevertheless forthcoming.

A method also used in the present studies required that the tremorgenic compounds be administered to synaptosome suspensions in the form of an emulsion. These were made by means of the use of commercial non - ionic surfactants : Mulgofen EL - 620 and Mulgofen EL - 719 (GAF, Great Britain, Ltd.). These surfactants are polyoxyethylated vegetable oils that that are generally recommended for the emulsification of oils, fats, waxes, polyester resins, vinyl resins, organic solvents, and fatty acids. The Mulgofen surfactants are predominantly hydrophilic, though EL - 719 is the more water soluble of the two, due to the larger percentage (by weight) of combined ethylene oxide. The other significant difference is that EL - 620 is anhydrous, while EL - 719 contains approximately 3% water, since the hydrocarbon solubility of many non - ionics is increased by the presence of a small amount of water.

A further method used in the present studies required that the tremorgenic compounds be administered to synaptosome suspensions by way of incorporation of the toxins in a liposome preparation. Such an approach is not entirely without precedent, since, for instance, several areas of cell membrane research have fruitfully employed artificial lipid vesicles. Such vesicles have been useful as reconstitution vehicles for the identification (RACKER, 1972; RACKER & EYTAN, 1975; GOLDIN, 1977) and purification (KASAHARA & HINHLE, 1977; GOLDIN & RHODEN, 1978) of proteins that catalyse transmembrane transport processes and in studies of the permeability of the lipid bilayer (BANGHAM et al., 1965; PAPAHADJOPOULOS et al., 1971; TOYOSHIMA & THOMPSON, 1975). Liposomes have recently been employed to deliver a variety of materials to cells (GREGORIADAS, 1977; POSTE & PAPAHADJOPOULOS, 1976; RAHAMIMOFF et al., 1978) as a consequence of vesicle fusion with the cell membrane, and this technique can cause specific modification of various aspects of the physiological or metabolic behaviour of the cell.

Unilamellar liposomes of reasonably homogenous dimensions have been prepared by sonication of aqueous lipid dispersions (HUANG, 1969; BARENHOLZ et al., 1977), and by injection of solutions of lipid in organic solvent into aqueous medium (KREMER et al., 1977), and by removal of

detergent from detergent - solubilised lipid suspensions (GOLDIN, 1977; MILSMANN et al., 1978). The use of mild detergents, such as the bile salt sodium cholate, makes this latter technique particularly useful when it is necessary to reconstitute membrane components into the bilayer or to entrap soluble proteins for delivery to cells; the possible denaturing effect of intense sonication or of organic solvents on the biological activity of the protein of interest is avoided.

The extent to which the liposome retains its drug contents in vitro or in vivo is influenced not only by the biological environment with which liposomes come into contact, but also by their structural characteristics as well as those of the associated drugs (GREGORIADIS, 1979; KIMELBERG & MAYHEW, 1978). For instance, liposomes containing charged lipids retain certain drugs through electrostatic bonding (GREGORIADIS et al., 1977) and lipophilic drugs can be anchored into the liposomal lipid phase (GREGORIADIS, 1973; JULIANO & STAMP, 1978; DINGLE et al., 1978). On the other hand, entrapped macromolecules, such as enzymes (GREGORIADIS & RYMAN, 1972; WEISSMANN et al., 1975), nucleic acids (DIMITRIADIS, 1978; OSTRO et al., 1978), polymers (DAPERGOLAS et al., 1976), and viruses (WILSON et al., 1977) or viral components (MANESIS et al., 1979), are unable to cross the lipid bilayer because of their large size.

One variant in the liposomal structure that could cause retention of drugs is cholesterol content. Indeed, studies on the use of liposomes as membrane models have shown that cholesterol, by increasing the packing of phospholipid molecules (LADBROOKE et al., 1968; DEMEL & DE KRUYFF, 1976) reduces bilayer permeability to non - electrolyte and electrolyte solutes (DE GIER et al., 1970; DEMEL et al., 1972; PAPAHADJOPOULOS et al. 1973). Consequently, in the present studies whole brain lipids were extracted from rat brain, with or without cholesterol, and liposomes associated with Verruculogen were prepared and administered to rat cerebrocortical synaptosomes.

Studies of the response of synaptosomes to various incubations are highly relevant, and this point has been discussed in previous sections. It is important nevertheless to pursue parallel studies when possible on the response of in vitro preparations of synapses as close to an in situ condition as possible. Thus, parallel studies of the response to in vitro administration of the tremorgenic compounds were carried out on synapses in situ, and to this end were used the giant dopamine neuron of the water snail Planorbis corneus, and the distal fibres of

the extensor tibialis muscle of the locust (Schistocerca gregaria) metathoracic leg.

The European water snail Planorbis corneus possesses a giant dopamine containing neuron in its left pedal ganglion (MARSDEN & KERKUT, 1970; OSBORNE et al., 1975; POWELL & COTTRELL, 1974). It can be reliability identified in an in vitro preparation, so that electrophysiological recordings and subsequent quantitative histochemical or biochemical analyses can be performed on the same cell. The typical unipolar structure of molluscan neurons allows selective investigations of the cell body which can be completely separated from dendritic and axonal arborisations. This neuron thus appeared to provide ideal conditions for a further analysis of the mechanism of action of the tremorgenic toxins. Since the giant dopaminergic neuron has been found to respond to cholinergic substances administered either to the medium, or iontophoretically (GER & ZEIMAL, 1976; LOGAN & COTTRELL, 1975; ZEIMAL et al., 1975), experiments were made to examine these responses.

The giant dopaminergic neuron provides two kinds of information : On the one hand, it can be considered as a model system for more selective analyses at the cellular level, while on the other hand, it is of interest to investigate whether invertebrate dopamine neurons react to changes of activity in a way similar to mammalian dopaminergic neurons. The latter were examined in the previous chapter, but in spite of common features in dopamine metabolism (OSBORNE et al., 1975), a similarity in the response to activation could not be expected with certainty because, for instance, the molluscan nerve cell body occupies a different position with respect to the afferent input to the neuron.

It was Usherwood (see USHERWOOD, 1974) 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, and these findings are consistent with the multi - terminal nerve innervations known to be present in insects (HOYLE, 1955).

Miniature end plate potentials have also been recorded from the metathoracic extensor tibia muscle fibres of the locust Schistcerca gregaria (see USHERWOOD, 1974) and it is now well established that glutamate is the excitatory transmitter at these synapses. USHERWOOD, (see 1974), using a perfusion system, demonstrated the electrically

induced release of this amino acid from the isolated retractor unquis nerve muscle preparation. Topical application of glutamate to locust muscle has been shown to elicit an excitatory response (BERANEK & MILLER, 1968; *see* USHERWOOD, 1974), and similar results have been obtained with grasshopper muscle (USHERWOOD & GRUNDFEST, 1965) and cockroach muscle (KERKUT et al., 1965), thus providing further evidence for the role of glutamate as the excitatory transmitter at insect neuromuscular junctions.

Usherwood has also shown 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 quantity of glutamate applied. The distance between the application site and recording electrodes was also found to be critical (USHERWOOD, 1974). The glutamate sensitive areas were shown to coincide with excitatory synaptic sites (BARENEK & MILLER, 1968; USHERWOOD & MACHILI, 1968).

Depending on the response elicited in the muscle fibre, it is possible to determine the site of action of any added agent. An increase or decrease in the miniature end plate potential frequency suggests an action on the presynaptic terminal, and conversely a change in the amplitude of spontaneous potentials indicates an action on the post synaptic membrane. Thus the system provided another ideal preparation to study the site and mode of action of the tremorgenic mycotoxins.

RESULTS.

1) In vitro additions of the purified tremorgenic mycotoxins to synaptosome suspensions.

a) Penitrem A.

Attempts were made to dissolve Penitrem A in Kreb's phosphate medium, but these were in all cases unsuccessful to the extent that analysis of the medium for Penitrem A (3ml. methanol + 180µl. conc. sulphuric acid, heated at 70'C for 20min., and read at 630nm.) subsequent to prolonged centrifugation to remove suspended material, proved to be negative. Penitrem A was, however, soluble in absolute ethanol to an extent that final medium concentrations of Penitrem A were obtainable up to approximately 30µg / ml., when the Penitrem A was taken up initially in absolute ethanol and aliquots were added to the synaptosome suspension to give a maximum final ethanol concentration of 0.1% v / v. Synaptosomes were prepared from sheep cerebral cortex, rat spinal cord, cerebellum, and cerebral cortex. As mentioned in the previous chapter cerebellar synaptosomes did not perform well in incubation, in that their veratrine response was very poor, and large background levels of all amino acids were observed when synaptosomes from this region were prepared in the manner described for spinal cord / medulla tissue. As mentioned in an earlier chapter, the problems associated with sheep cerebral cortex had been overcome by raising the synaptosomal protein concentration in the incubation medium.

When Penitrem A was added to these incubations (as an ethanolic solution) subsequent to a 20min. equilibration period, and for 40min., the amino acid accumulations both intra - and extra - synaptosomally were not significantly different from those of the relevant controls. Data for such control accumulations have been presented in earlier chapters and need not be duplicated here, but TABLE 6.1 illustrates as an example, the data obtained for the extra - synaptosomal accumulation of amino acids from rat cerebral cortex synaptosomes incubated in the presence of increasing concentrations of Penitrem A.

Also included in TABLE 6.1 are the data obtained from two experiments where synaptosomes were incubated in the presence of the acetylcholine esterase inhibitor, eserine. Acetylcholine was subsequently measured by means of the guinea pig ileum bioassay. The data are the mean of two

DATA LEGEND.

RE : TABLE 6.1.

EFFECT OF ETHANOLIC ADMINISTRATION OF PENITREM A TO SYNAPTOSOMES SUSPENSIONS ON AMINO ACID AND ACETYLCHOLINE RELEASE.

Rat cerebrocortical synaptosomes were suspended in Krebs phosphate medium in 5ml conical flasks in a shaking water bath at 37'C. Subsequent to a 20min. equilibration period, aliquots of ethanol alone, or ethanolic solutions of Penitrem A were added as appropriate, to give a final ethanol concentration of 0.1% v/v, and Penitrem A concentrations ranging between 1µg/ml and 30µg/ml, as indicated. Incubation continued for a further 40min..

At the end of the experiment, the suspensions were sedimented in a bench top ultracentrifuge, and fractions were extracted and analysed for amino acid content. Pellet protein was estimated by means of the Folin reagent.

The amino acid data are the means ± SEM for values obtained from six experiments.

In experiments made to determine the effect of Penitrem A on acetylcholine levels, the Krebs phosphate medium was made 1mg/ml in respect of eserine sulphate. At the end of the experiments, the pH of the two fractions was brought down to 3 - 4 with HCl. Acetylcholine levels were determined by means of the guinea pig ileum bioassay, (PATON, W.D.M. (1957), Br. J. Pharmac. Chemother., 12, 119). The data presented here are the means of two experiments.

The amino acid data is presented as nmol amino acid / 100mg protein, and the acetylcholine data as ng ACh / 100mg protein.

TABLE 6.1.

EFFECT OF ETHANOLIC ADMINISTRATION OF PENITREM A TO SYNAPTOSOMES SUSPENSIONS ON AMINO ACID AND ACETYLCHOLINE RELEASE.

	ASPARTATE	THREONINE	SERINE	GLUTAMATE	GLYCINE	ALANINE	GABA	ACH (MED.)	ACH (PELL.)
	(nmol amino acid accumulation in medium / 100mg. protein.)							(ng ACh / 100mg protein)	
CONTROL.	63 ± 2	254 ± 11	347 ± 13	93 ± 3	217 ± 13	437 ± 24	21 ± 2	200	260
CONT + ETOH.	105 ± 16	243 ± 45	292 ± 18	213 ± 70	185 ± 21	368 ± 47	15 ± 7	194	267
1µg. PEN. A.	114 ± 24	233 ± 40	272 ± 19	165 ± 33	166 ± 13	409 ± 29	65 ± 22	244	265
5µg. PEN. A.	148 ± 36	255 ± 12	319 ± 47	209 ± 48	166 ± 25	444 ± 68	68 ± 22	265	330
10µg. PEN. A.	104 ± 20	236 ± 35	285 ± 23	160 ± 24	189 ± 37	465 ± 68	70 ± 22	292	338
15µg. PEN. A.	174 ± 51	364 ± 89	443 ± 91	295 ± 88	264 ± 70	602 ± 128	93 ± 24	323	330
30µg. PEN. A.	117 ± 24	302 ± 16	351 ± 19	214 ± 46	205 ± 28	546 ± 43	61 ± 21	254	275

estimations, and it would appear that as a proportion of the total acetylcholine for each experimental condition, Penitrem A also had no effect on intra - or extra - synaptosomal acetylcholine levels.

It is perhaps worthy of emphasis that the maximum Penitrem A concentration used in these experiments (30µg / ml.) produced an observable tremor when injected intraperimentally to 250g rats within 5 - 15min. of injection, and this compares with the 40min. incubation time during which synaptosomes were in contact with Penitrem A in the experiments described.

b) Verruculogen.

1) Ethanol

A scheme similar to that described for Penitrem A was applied to Verruculogen. Verruculogen also proved to be insoluble in Kreb's phosphate medium to the extent evidenced by the assay performed (3ml. ethanol + 2ml. conc. sulphuric acid, incubated for 40min., at 70'C, and read on a Farrand spectrofluorimeter) on the medium subsequent to prolonged centrifugation and pelleting of suspended material. It was possible to dissolve Verruculogen in absolute ethanol and make an ethanol addition to the incubation fluid to the extent of 40µg. Verruculogen / ml. Kreb's phosphate medium (final ethanol concentration 0.1% v/v).

Experiments within this range of Verruculogen concentration were performed on suspensions of sheep cerebrocortical, and rat cerebrocortical cerebella, and spinal cord synaptosomes. The data obtained for sheep cerebrocortical, rat cerebella, and spinal cord synaptosomes incubated in the presence of 40µg / ml. Verruculogen were not significantly different from those values obtained from the respective control incubations, data for which have been presented in previous chapters.

When additions of Verruculogen dissolved in ethanol were made to rat cerebrocortical synaptosomes after a 20min. equilibration period, and for a total of 40min., in the range 1µg - 40µg Verruculogen / ml. incubation fluid, a rather anomalous situation arose. The data for the six experiments performed are presented in FIG. 6.1. Aspartate accumulation in the medium was elevated by 125% at Verruculogen concentration of 20µg / ml., and by 215% at Verruculogen concentration 40µg / ml.. Similarly, glutamate accumulation to the incubation fluid was elevated by 173% and 291% at Verruculogen concentrations 20µg / ml and 40µg / ml respectively. GABA accumulation, and indeed the accumulation of all the other amino acids measured, of which data is presented for threonine, serine, glycine, and alanine, were not

DATA LEGEND.

RE : FIG. 6.1a, 6.1b.

AMINO ACID RELEASE FROM SYNAPTOSOMES INCUBATED IN THE PRESENCE OF VERRUCULOGEN, ADMINISTERED AS AN ETHANOLIC SOLUTION.

Rat cerebrocortical synaptosomes were suspended in Krebs phosphate medium in 5ml conical flasks in a shaking waterbath at 37'C. Subsequent to an equilibration period of 20min., aliquots of ethanol alone, or ethanolic solutions of Verruculogen were added as appropriate, to give a final ethanol concentration of 0.1% v/v, and Verruculogen concentrations ranging between 1µg/ml and 40µg/ml, as indicated. Incubation continued for a further 40min..

At the end of the experiment, the suspensions were sedimented in a bench top ultracentrifuge, and fractions were extracted and analysed for amino acid content. Pellet protein was estimated by means of the Folin reagent.

The histobars represent the data obtained from six experiments, and are the means $\pm$ SEM. Values are expressed as nmol amino acid / 100mg protein.

FIG. 6.1a.

AMINO ACID RELEASE FROM SYNAPTOSOMES INCUBATED IN THE PRESENCE OF VERRUCULOGEN, ADMINISTERED AS AN ETHANOLIC SOLUTION.

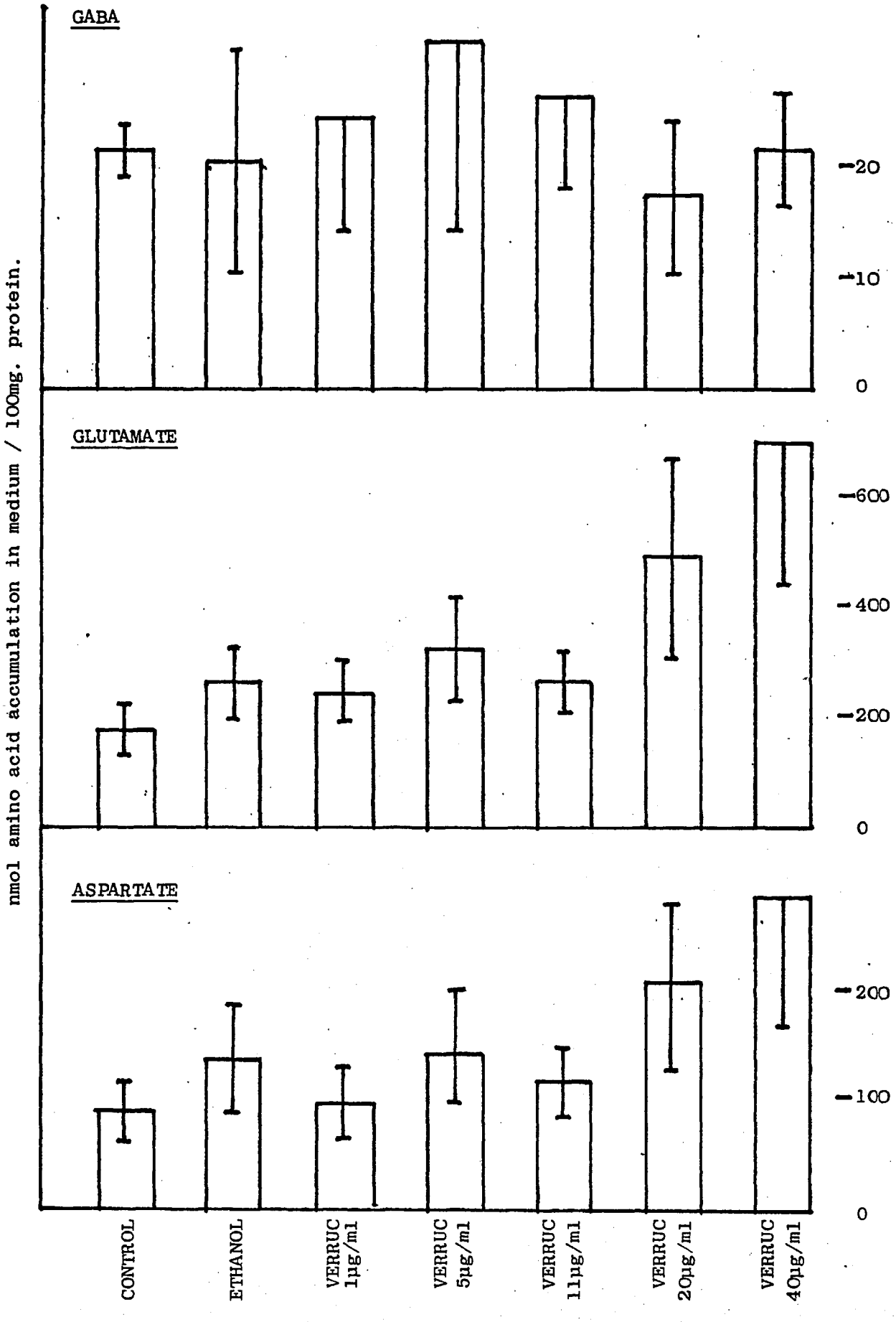
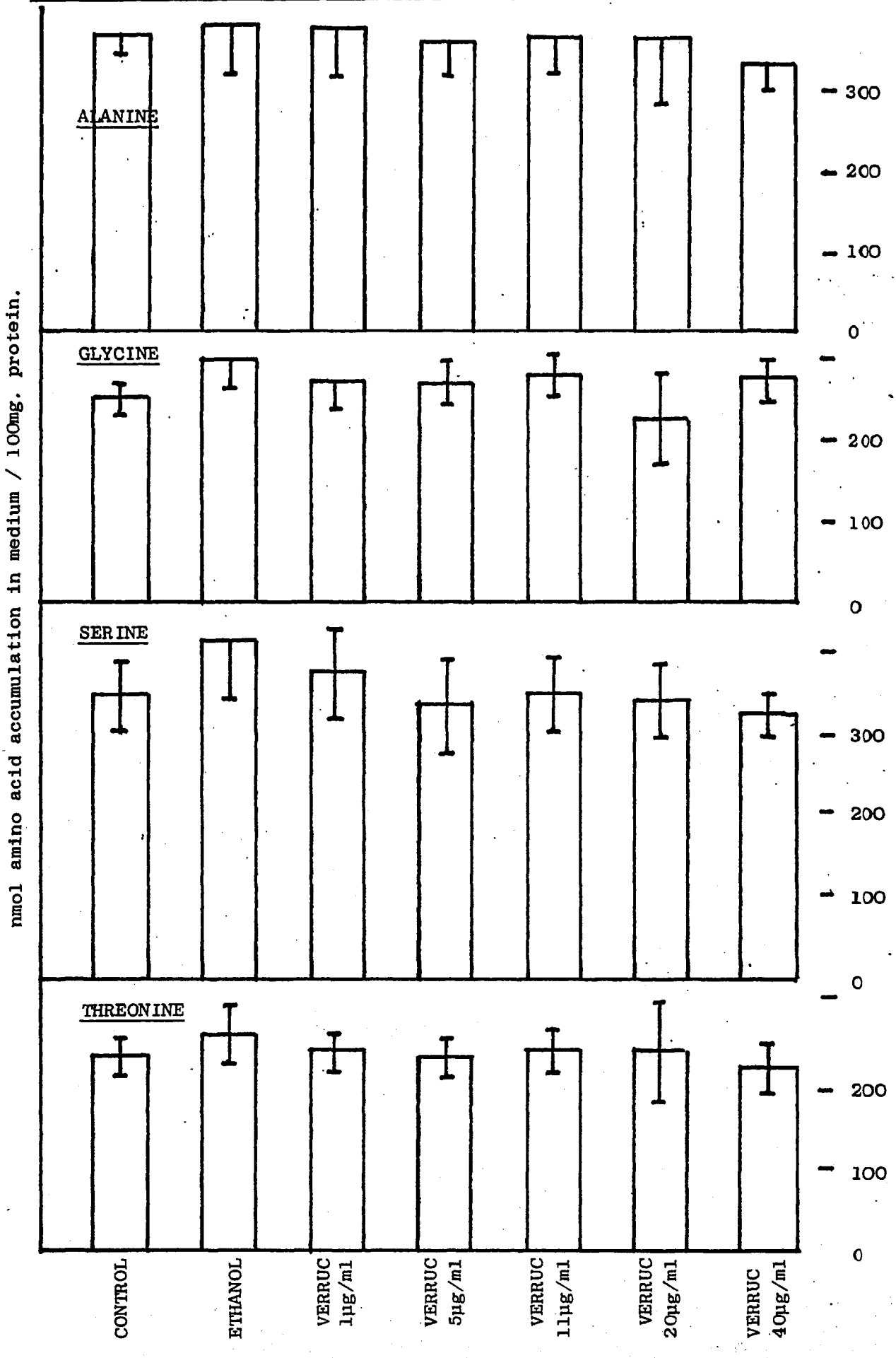


FIG. 6.1b.

AMINO ACID RELEASE FROM SYNAPTOSOMES INCUBATED IN THE PRESENCE OF VERRUCULOGEN, ADMINISTERED AS AN ETHANOLIC SOLUTION.



significantly changed from those observed in the relevant control incubations. The anomalous aspect of these data existed in the observation that elevations of aspartate and glutamate occurred in only three of the six experiments performed - hence the larger than usual values for S.E.M.. In fact, when the data were extracted from the three experiments in which elevations of glutamate and aspartate were observed, from those in which elevations were not observed, the mean glutamate level increased to 2039nmol glu. / 100mg. protein (+ 1020%), and the mean aspartate level increased to 855nmol. asp. / 100mg. protein (+ 829%), when rat cerebrocortical synaptosomes were incubated in 40µg Verruculogen / ml incubation fluid. Intrasynaptosomal potassium levels were not altered from those measured in control synaptosomes. As can be seen from the data presented in FIG. 6.1 (the data being obtained from all six experiments performed), the levels of ethanol used for administration of the toxin did not significantly alter the parameters which have been measured here.

The experiments give a clear impression that the Verruculogen molecule does exert an influence on synaptosomes, and that influence is of a nature similar to that obtained and measured in synaptosomes prepared from animals previously dosed intraperitoneally with Verruculogen. However, the fact that any influence of Verruculogen on aspartate and glutamate release from synaptosomes exhibited only a 50% 'success rate' was an intolerable situation. Indeed, when experiments were subsequently performed to investigate whether the in vitro addition of Verruculogen at these concentrations and administered in this manner caused an extrasynaptosomal accumulation of glutamate and aspartate, the release of which was Ca^{++}, verapamil, or TTX dependent, the data obtained was so variable, and the S.E.M.'s were so large, that quantitative, or even qualitative conclusions could not be reached.

Consequently, a search was made for alternative methods for the administration to synaptosomes suspensions of the purified tremorgenic compounds, such that reliable, quantitative measurements of the effects of these compounds, and also modulation of these effects by other agents in an in vitro capacity, could be obtained.

2) Mulgofen EL - 620 and EL - 719 emulsifiers.

Verruculogen was solubilised easily into both of these surfactants. The final surfactant concentration subsequent to the preparation of the emulsions with Kreb's phosphate medium to produce stock solutions,

and further dilution on addition of an aliquot of these stock solutions to the synaptosome suspensions was approximately 0.2% v/v in each case. It proved impracticable to reduce this concentration further because not only were very small quantities of Verruculogen available, but also the highly viscous nature of these surfactants made it impossible to measure in a quantitative way smaller volumes than ten or so µl.. An alternative order of solubilisation was attempted in terms of making an emulsion with a smaller surfactant composition prior to adding this to the Verruculogen, but when the procedure was tried in this order, the Verruculogen did not solubilise to the same extent. When emulsions were made in the aforementioned manner, however, and aliquots were injected intraperitoneally to rats, the onset and degree of tremor observed subsequently were indistinguishable from the extent of occurrence of the same parameters when the toxin was injected in solvent only, and in the same quantity. Control animals injected with the surfactants alone showed no response.

When cerebrocortical synaptosomes were prepared from control animals and incubated in Kreb's phosphate medium for 20min. to equilibrate and aliquots of the surfactant emulsion alone (to a final surfactant concentration of 0.2% v/v) were added to the suspensions of synaptosomes for a period of 40min., all amino acids levels measured extra - synaptosomally were elevated (TABLE 6.2). However, by far the greatest accumulation increase in the medium occurred for aspartate, glutamate, and GABA, and the levels to which these amino acids accumulated were in some respects reminiscent of a veratrine evoked release of these amino acids. The same data were obtained when Verruculogen was included in the emulsion to a final concentration of 50µg / ml..

It was concluded with every good reason that the Mulgofen emulsification method was unlikely to prove fruitfull in terms of assisting in the administration of Verruculogen to synaptosome suspensions Whether or not the emulsifiers contained some component which was evoking the release of these neuroactive amino acids to the extent to which they accumulated was beyond the scope of the present study. It is, of course, more feasible that the synaptosome membrane became leaky as a consequence of micelle / membrane interaction, as evidenced by the accumulation extrasynaptosomally of all the other amino acids measured, and as examples of these, data for threonine, serine, glycine, and alanine are included in TABLE 6.2.

DATA LEGEND.

RE : TABLE 6.2.

EFFECT OF MULGOFEN SURFACTANTS ON THE INCUBATION FLUID ACCUMULATION OF AMINO ACIDS FROM CEREBROCORTICAL SYNAPTOSOMES.

Rat cerebrocortical synaptosomes were suspended in Krebs phosphate medium in 5ml conical flasks in a shaking waterbath at 37'C. Subsequent to an equilibration period of 20min., aliquots of Mulgofen EL - 620, or EL - 719 were added to give final surfactant concentrations of 0.2% v/v. Incubation continued for a further 40min..

At the end of the experiment, the suspensions were sedimented in a bench top ultracentrifuge, and fractions were extracted and analysed for amino acid content. Pellet protein was estimated by means of the Folin reagent.

The data were obtained from four experiments, and are the mean values $\pm$ SEM. Values are expressed as nmol amino acid / 100mg protein.

TABLE 6.2.

EFFECT OF MULGOFEN SURFACTANTS ON THE INCUBATION FLUID ACCUMULATION OF AMINO ACIDS FROM CEREBROCORTICAL SYNAPTOSOMES.

	ASPARTATE	THREONINE	SERINE	GLUTAMATE	GLYCINE	ALANINE	GABA
	(nmol amino acid accumulation in medium / 100mg. protein)						
CONTROL.	97 ± 11	226 ± 31	299 ± 34	286 ± 71	198 ± 30	422 ± 43	40 ± 11
EL - 620	928 ± 103	326 ± 41	486 ± 72	3513 ± 743	354 ± 85	672 ± 146	515 ± 112
EL - 719	752 ± 101	274 ± 38	380 ± 66	2869 ± 672	285 ± 48	573 ± 73	484 ± 97

3) Nylon gauze administration of Verruculogen.

Chapter Three described a method by which another aqueous insoluble neuroactive agent (T_{46} toxin) was successfully administered to synaptosome suspensions. The toxin was initially dissolved in an appropriate solvent, and aliquots were spread over $1cm^2$ pieces of nylon gauze and allowed to evaporate to dryness. The nylon gauze containing crystalline material was then added to the incubation flask containing the synaptosome suspension. The data presented in that chapter illustrated the fact that the method, though very crude, was applicable when problems such as aqueous insolubility needed to be overcome, and the results obtained corroborated the data published by other laboratories using the same toxin, but on different biological systems.

This same method was used to administer Verruculogen to rat cerebrocortical synaptosome suspensions. Verruculogen was pipetted onto the nylon gauze from a cold chloroform solution at up to 200µg Verruculogen per nylon gauze. Subsequent to incubating the synaptosomes for 20min. to equilibrate, the gauzes were added to the incubation flasks, ensuring that they were submerged and moving freely with the shaking motion of the waterbath, for a further period of 40min.. At the end of the incubations, amino acid levels intra - and extra - synaptosomally were measured, as also were pellet potassium levels. The data thus obtained for these paramaters did not differ in profile from the data presented in previous sections for control synaptosome incubations.

When a small quantity of radiolabeled Verruculogen became available as a result of the Penicillium fungus being grown up on radiolabeled substrates, an estimation was made of the maximum amount of Verruculogen which would have become available to the synaptosome suspension under these conditions. When 200µg Verruculogen (radiolabeled) was pipetted onto a piece of nylon gauze and incubated in Kreb's phosphate medium at 37'C for 40min., only 12.7µg of Verruculogen could be measured (from radiolabel counting) in the medium after the gauze had been carefully removed. Some or all of these counts may well have originated from a small quantity of the material being dislodged from the gauze as a result of the shaking motion of the waterbath. Thus, unless synaptosomes had made direct contact between a proposed membraneous receptor, and a Verruculogen molecule, then taking the concentrations of Verruculogen required to evoke an effect when administered in ethanol, it comes as no surprise that effects were not observed when the mycotoxin was administered in this manner.

4) Liposomal administration of Verruculogen.

Preliminary experiments have been performed using liposomes as carrier preparations for Verruculogen, the intention being that if liposomes were prepared from lipids extracted from rat brain, there might be a greater chance of the liposomes becoming integrated within the synaptosomal membrane, thus improving the likelyhood of Verruculogen molecules coming into contact with proposed receptors on synaptosomes for this compound. Liposomes were prepared which either comprised cholesterol, or were made in the absence of cholesterol, which is thought to determine the lability/stability of the liposome structure.

When liposomes were prepared in the presence of Verruculogen, and aliquots were injected intraperitoneally to rats, the rate of onset and the degree of tremor conformed to the levels of those parameters observed when Verruculogen was injected in solvent at the same concentration.

Cholesterol containing liposomes were added to suspensions of rat cerebrocortical synaptosomes, the volume additions being 10µl., 40µl., 100µl., and 200µl., equivalent to approximately 0.25mg., 1mg., 2.5mg., and 5mg. of total brain lipid estimated by weight measurement only. The liposomes were added to the incubation for a period of 40min., subsequent to an equilibration period of 20min.. The data obtained from these experiments, and others performed when Verruculogen had been incorporated in to the liposome preparation procedure to give a final synaptosome incubation fluid content of 2.5µg., 10µg., 25µg., and 50µg. of Verruculogen per ml. are presented in TABLE 6.3. Neither increasing liposome content in the incubation fluid, nor the presence of increasing amounts of Verruculogen affected the accumulation in the medium of threonine, serine, glycine, or alanine to a significant extent when compared with control values in the absence of these additions. However, increasing addition volumes of liposome (100µl., 200µl.) did have a small but significant effect on the extrasynaptosomal accumulation of aspartate, glutamate, and GABA - these accumulations were elevated at the 200µl. addition level by 400%, 590%, and 150% respectively.

A similar trend was observed when Verruculogen was present in the liposome preparation, i.e. no significant effect on neuroactive amino acid accumulation at 10µl., or 40µl. (equivalent to 2.5µg. and 10µg. Verruculogen / ml. incubation fluid). However, at the level of 200µl. liposome addition (equivalent to 50µg. Verruculogen), the aspartate and

DATA LEGEND.

RE : TABLE 6.3.

AMINO ACID RELEASE FROM SYNAPTOSOMES INCUBATED WITH VERRUCULOGEN ADMINISTERED VIA A LIPOSOME CARRIER.

Rat brain lipids were extracted, and liposomes were prepared in the presence of, and in the absence of Verruculogen, as described under METHODS.

Rat cerebrocortical synaptosomes were suspended in Krebs phosphate medium in 5ml conical flasks in a shaking waterbath at 37'C. Subsequent to an equilibration period of 20min, aliquots of liposome suspension with or without Verruculogen were added to the flasks, as appropriate. The volume additions were 10µl, 40µl, 100µl, and 200µl. In those flasks to which Verruculogen was added, the incubation fluid content ranged between 2.5µg/ml and 50µg/ml..

At the end of the experiment, suspensions were sedimented in a bench ultracentrifuge, and fractions were extracted and analysed for amino acid content. Pellet protein content was determined by means of the Folin reagent.

The data were obtained from four experiments, and represent the means $\pm$ SEM. Units are nmol amino acid / 100mg protein.

TABLE 6.3.

AMINO ACID RELEASE FROM SYNAPTOSOMES INCUBATED WITH VERRUCULOGEN ADMINISTERED VIA A LIPOSOME (+CHOLESTEROL) CARRIER.

	ASPARTATE	THREONINE	SERINE	GLUTAMATE	GLYCINE	ALANINE	GABA
	(nmol amino acid accumulation in medium / 100mg protein)						
50µg VERR/200µl LIP.	536 ± 79	281 ± 42	470 ± 70	721 ± 108	307 ± 45	612 ± 101	26 ± 5
25µg VERR/100µl LIP.	239 ± 39	211 ± 35	358 ± 56	306 ± 61	239 ± 36	448 ± 64	15 ± 4
10µg VERR/40µl LIP.	158 ± 22	226 ± 29	372 ± 58	153 ± 24	258 ± 39	460 ± 69	14 ± 4
2.5µg VERR/10µl LIP.	111 ± 18	167 ± 25	332 ± 51	107 ± 16	215 ± 34	399 ± 60	14 ± 4
200µl LIP.	389 ± 58	221 ± 33	368 ± 56	528 ± 74	250 ± 38	510 ± 92	25 ± 6
100µl LIP.	229 ± 35	215 ± 33	347 ± 53	262 ± 39	228 ± 34	437 ± 69	16 ± 5
40µl LIP.	154 ± 27	237 ± 36	397 ± 61	182 ± 42	251 ± 37	499 ± 76	16 ± 5
10µl LIP.	77 ± 13	165 ± 26	270 ± 43	75 ± 14	154 ± 24	313 ± 63	10 ± 4

glutamate extrasynaptosomal accumulations were significantly higher than the accumulations observed when a 200µl. addition of liposomes alone was made. Thus, aspartate levels in the medium were elevated by 38%, and glutamate levels by 37%. These were preliminary experiments, and further experiments presently under way indicate that a practicable improvement to the technique involving raising the Verruculogen content of the liposome stock suspension will underline the usefullness of this particular technique.

When liposomes were prepared from cholesterol free brain lipid extracts, some rather extraordinary results were obtained which related very closely with the data shown in TABLE 6.2. Very high extrasynaptosomal levels of aspartate, glutamate, and GABA were measured, but no changes were observed in the accumulation of other amino acids. These data are of course of great interest but of no direct relevance to the project in hand, and further investigation of this phenomenon has been left for another time.

2) In vitro administration of the purified tremorgenic compounds to synapses in situ.

a) Effects of the tremorgenic mycotoxins at the locust neuromuscular junction.

The action of Verruculogen on miniature end plate potentials was studied using a system which allowed small volumes of the toxin solution (in absolute ethanol) to be applied to locust neuromuscular junctions. The locust muscle was chosen as it appears to employ glutamate as its neurotransmitter (USHERWOOD, 1974).

The recording electrode was positioned in the muscle fibre post - synaptic to the junction, and the resting potentials of the fibres whose responses were to be studied were selected to be in the range -50 to -80mV. The resting rate of spontaneous miniature end plate potential production was recorded on a multichannel pulse analyser, which allowed pulses of different amplitudes to be sorted and integrated and their frequency displayed on a multichannel pen recorder. When Verruculogen (500µg/ml of absolute ethanol) was applied in 10µl. amounts to the muscle surface, a substantial rise in miniature end plate potential frequency occurred which was detectable within 1min., and rose to a maximum response (300% - 400% increase in rate) within 5 - 7min., before falling to the original

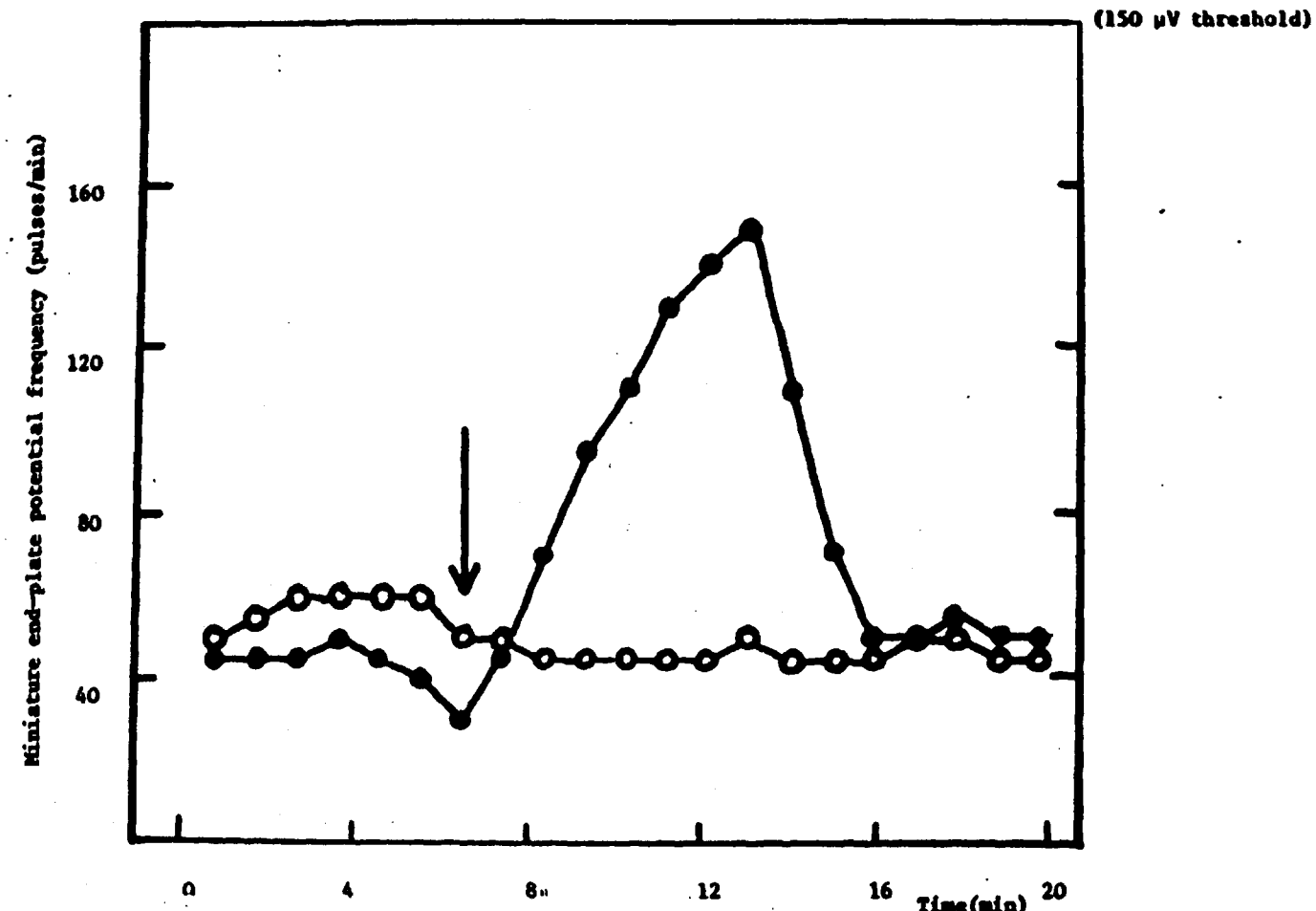


FIG.6. THE EFFECT OF VERRUCULOGEN ON THE RATE OF DISCHARGE OF MINIATURE-END-PLATE POTENTIALS MEASURED ON EXTENSOR TIBIALIS MUSCLE PREPARATIONS FROM SCHISTOCERCA GREGARIA. AFTER PENETRATION OF A MUSCLE FIBRE WITH A HIGH AND STABLE MEMBRANE POTENTIAL (50-80 mV), AND DETECTING MINIATURE-END-PLATE POTENTIALS, THE ADDITION PIPETTE WAS MOVED INTO POSITION AT A STANDARD DISTANCE OF 2-3 MM. FROM THE RECORDING ELECTRODE. SMALL VOLUMES (10 µL) OF ABSOLUTE ETHANOL, OR VERRUCULOGEN (500 µG/ML IN ABSOLUTE ETHANOL) WERE EJECTED ON TO THE FIBRE AND THEIR EFFECTS ON MINATURE-END-PLATE POTENTIALS WERE RECORDED ON A PULSE COUNTER WITH A PEN RECORDER OUTPUT. THE FIGURE PRESENTED HERE INCLUDES THE RESULTS FROM ONE EXPERIMENT WHICH WAS TYPICAL OF THE SIX EXPERIMENTS PERFORMED.

control rate over a similar period (FIG. 6.3). The concentration of the mycotoxin at the muscle surface was likely to be about 25µg / ml.. Subsequent additions produced almost identical responses, and after periods of 12 - 18min. following the first application of Verruculogen, control rates of miniature end plate potential production and transmembrane potentials in the fibre were unchanged showing that there was no long term effect of the mycotoxin on these parameters. In addition, the magnitude of the response to Verruculogen at this time was unchanged. Addition of KCl (75 - 300mM) in locust - Ringer produced its expected membrane depolarisation in proportion to concentration, and this was accompanied by a reversible rise in miniature end plate potential frequency.

Penitrem A additions were without effect on either membrane potential or miniature end plate potential frequency. Although, as mentioned above, this mycotoxin is also up to a point soluble in absolute ethanol, the compound came out of solution when ejected from the addition pipette and probably did not make proper contact with the muscle fibre.

b) Effects of Verruculogen on the giant dopamine neuron of the water snail.

The giant dopamine neuron of Planorbis corneus was reliably located on the ventral surface of the left pedal ganglion on the basis of its characteristic position between the statocyst and the medial edge of the ganglion, and of its size. In addition, a yellowish pigment spot was often detectable.

In the absence of any additions, the neurons of different snails had membrane potentials between -90 and -30mV.. In different neurons, the spontaneously active spikes ranged in size between 40 and 80mV. with a duration of about 10msec.. Spontaneous firing rates varied from one cell to another up to a maximum of 10Hz., and with few exceptions, the neurons fired at a regular rate over prolonged periods of time.

The data obtained when Verruculogen (500µg / ml. absolute ethanol) was administered to preparations at final concentrations of approximately 25µg / ml incubation fluid, of the water snail giant dopaminergic neuron are presented in FIG. 6.4. The mycotoxin was administered to five neurons, labeled GDN 1 to GDN 5.

Verruculogen had no direct depolarising action, but induced a characteristic bursting pattern of firing. This was seen in neurons

DATA LEGEND.

RE : FIG. 6.4 / GDN - 1 to FIG. 6.4 / GDN - 5.

EFFECTS OF VERRUCULOGEN ON THE GIANT DOPAMINE NEURON OF THE WATER SNAIL, PLANORBIS CORNEUS.

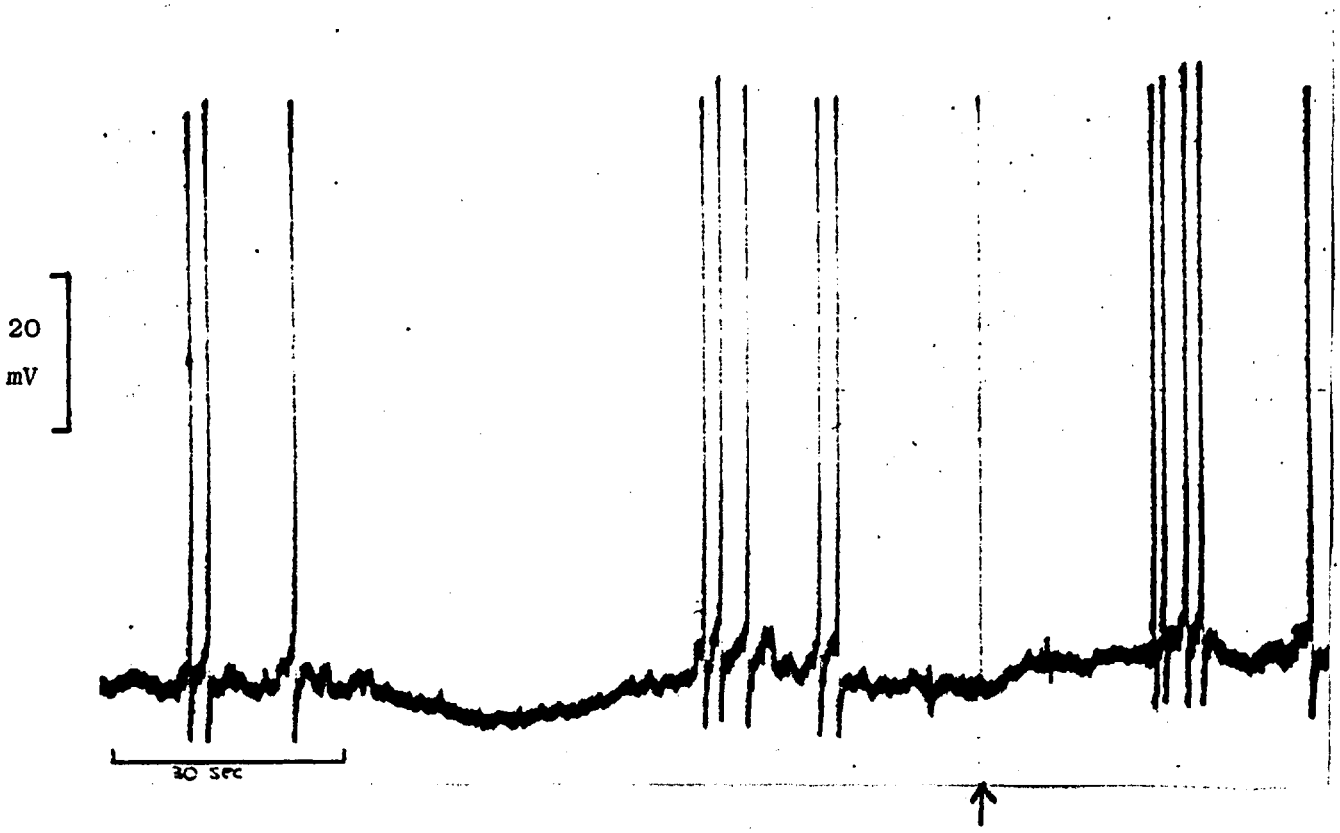
The giant dopamine neuron of the water snail was reliably located on the ventral surface of the left pedal ganglion. Membrane potentials were measured in the absence of any additions, and were found to be between -90mV and -30mV.

Verruculogen (500µg / ml absolute ethanol) was administered to preparations to give final concentrations of approximately 25µg / ml. incubation fluid. The mycotoxin was administered to five neurons, labelled GDN - 1 to GDN - 5.

FIG. 6.4 / GDN - 1

EFFECTS OF VERRUCULOGEN ON THE GIANT DOPAMINE NEURON OF THE WATER SNAIL.

Membrane potential = -75mV : Low spontaneous activity.



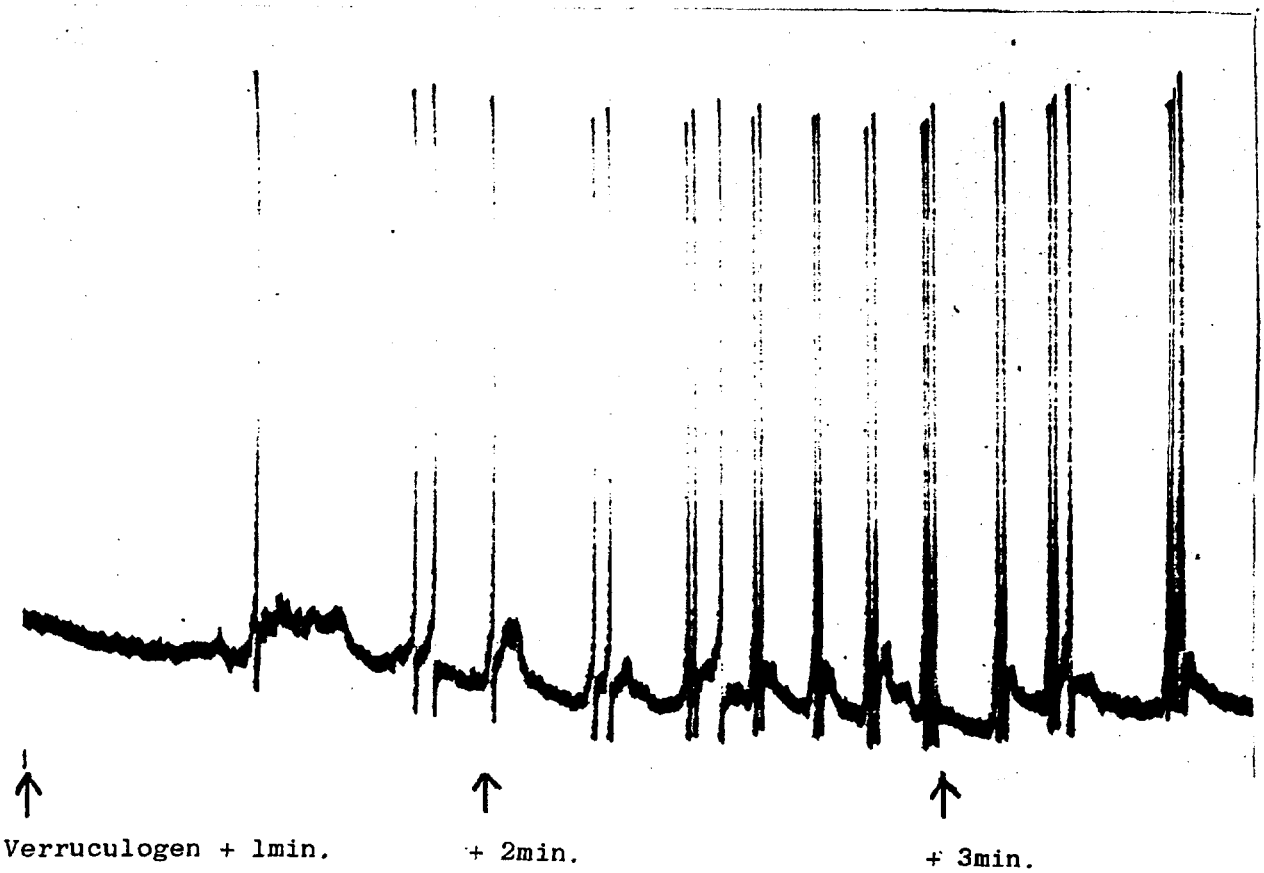


FIG. 6.4 / GDN - 2

EFFECTS OF VERRUCULOGEN ON THE GIANT DOPAMINE NEURON OF THE WATER SNAIL.

Membrane potential = -60mV.

Regular spontaneous firing pattern.

Constant current pulses (1nA, 500msec duration) through recording electrode.

Control. (See overleaf for Verruculogen addition.)

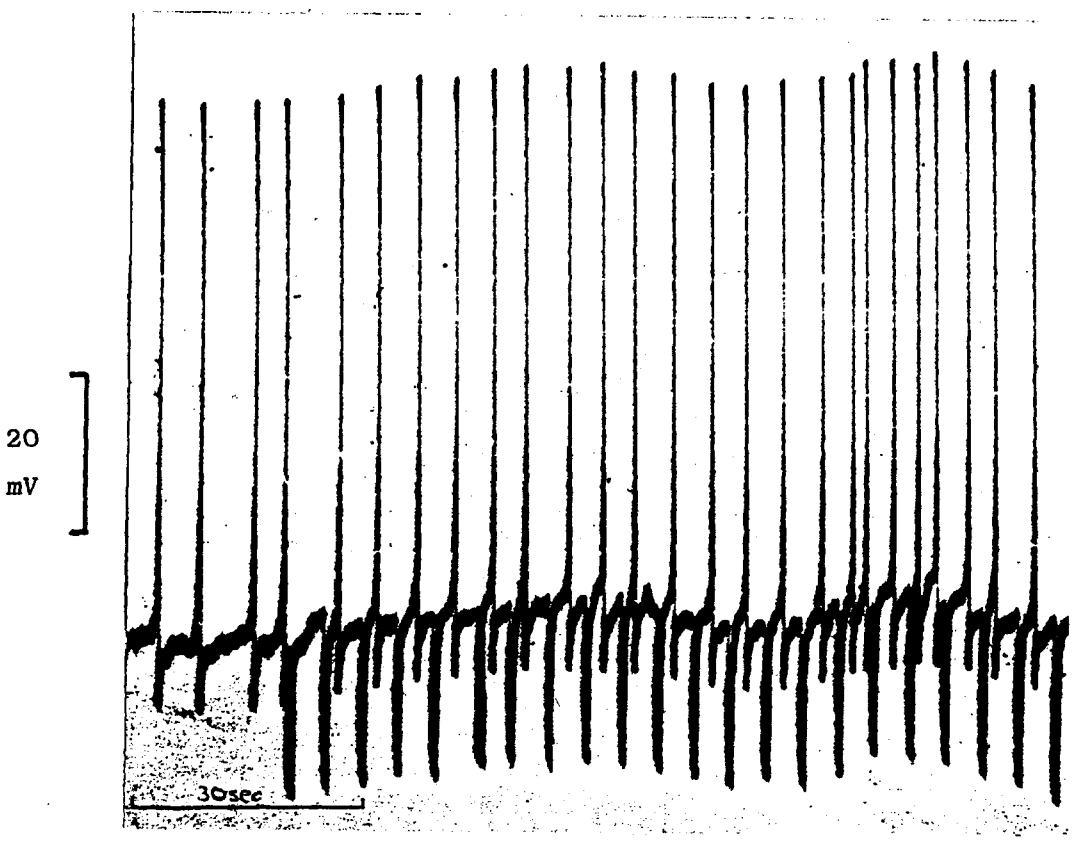
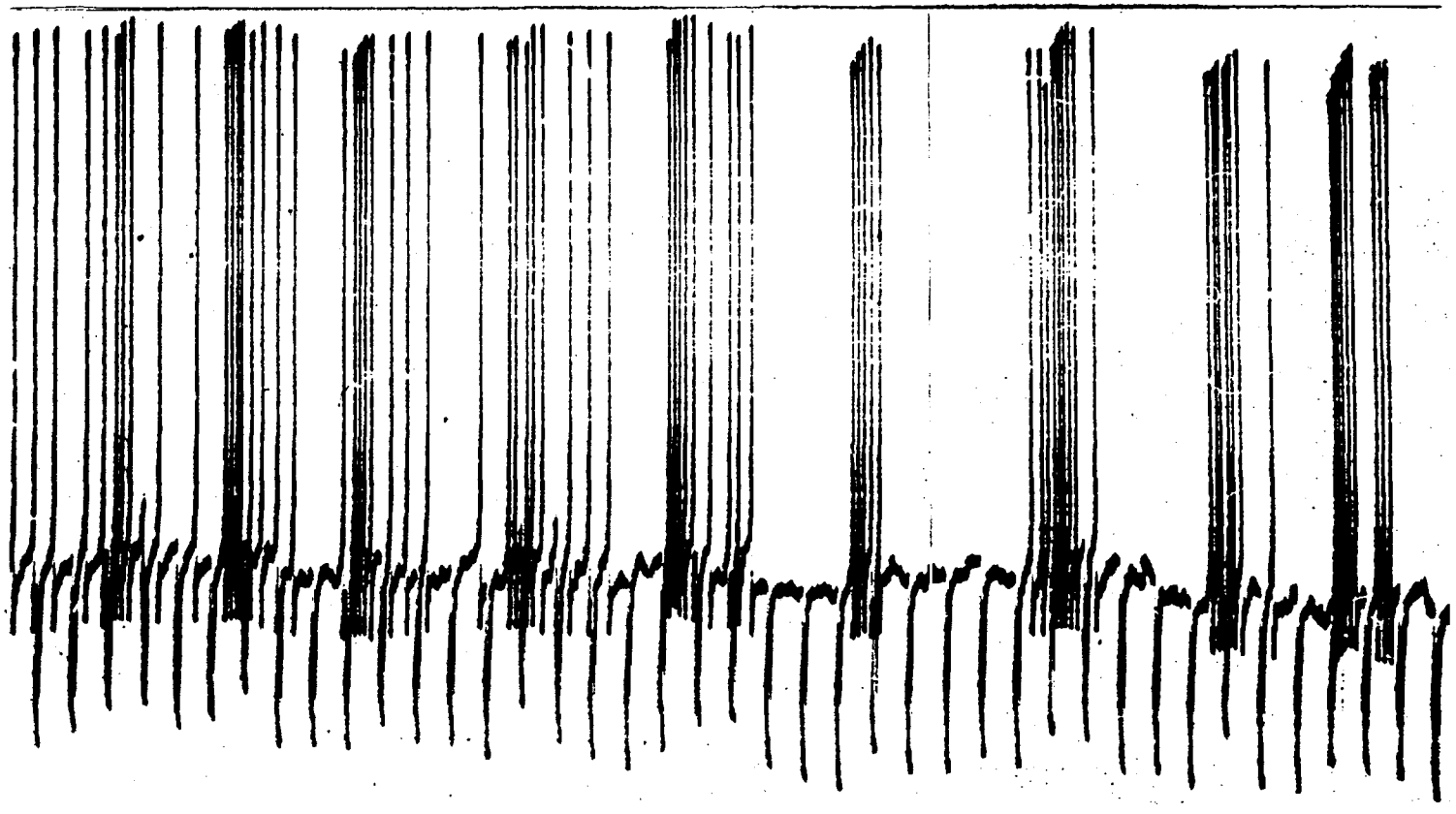


FIG. 6.4 / GDN - 2 (continued).

EFFECTS OF VERRUCULOGEN ON THE GIANT DOPAMINE NEURON OF THE WATER SNAIL.



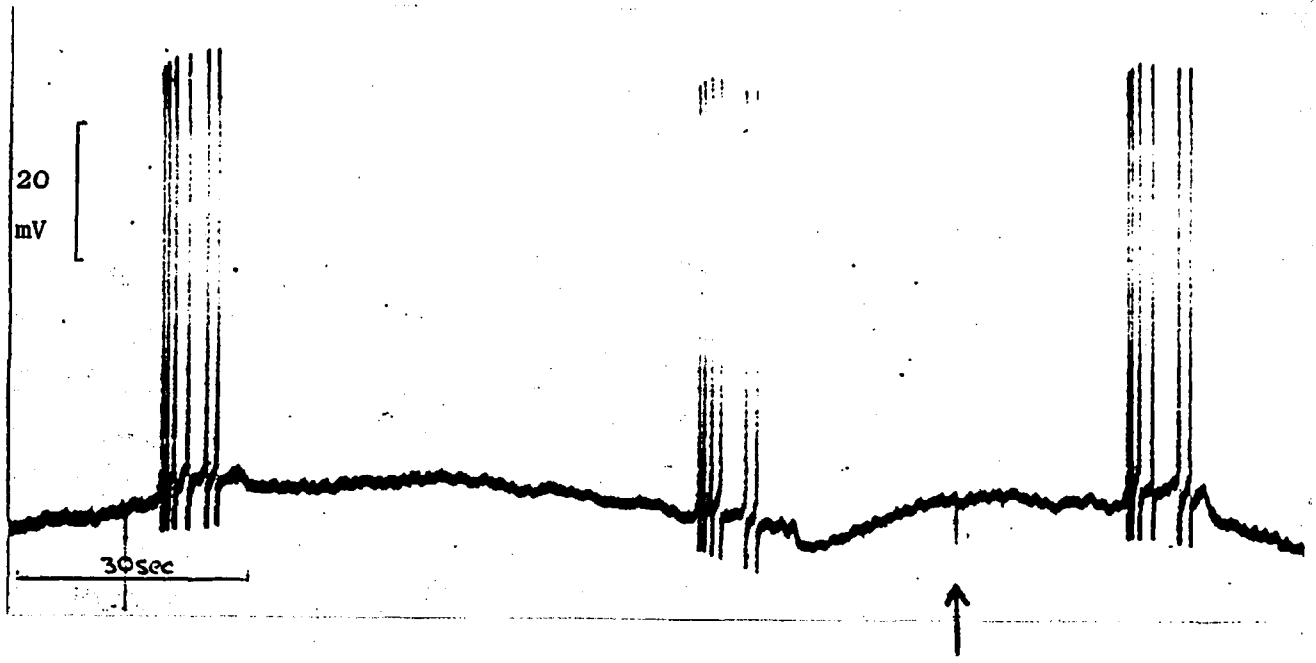
↑
4min. after Verruculogen (40µl).

FIG. 6.4 / GDN - 3.

EFFECTS OF VERRUCULOGEN ON THE GIANT DOPAMINE NEURON OF THE WATER SNAIL.

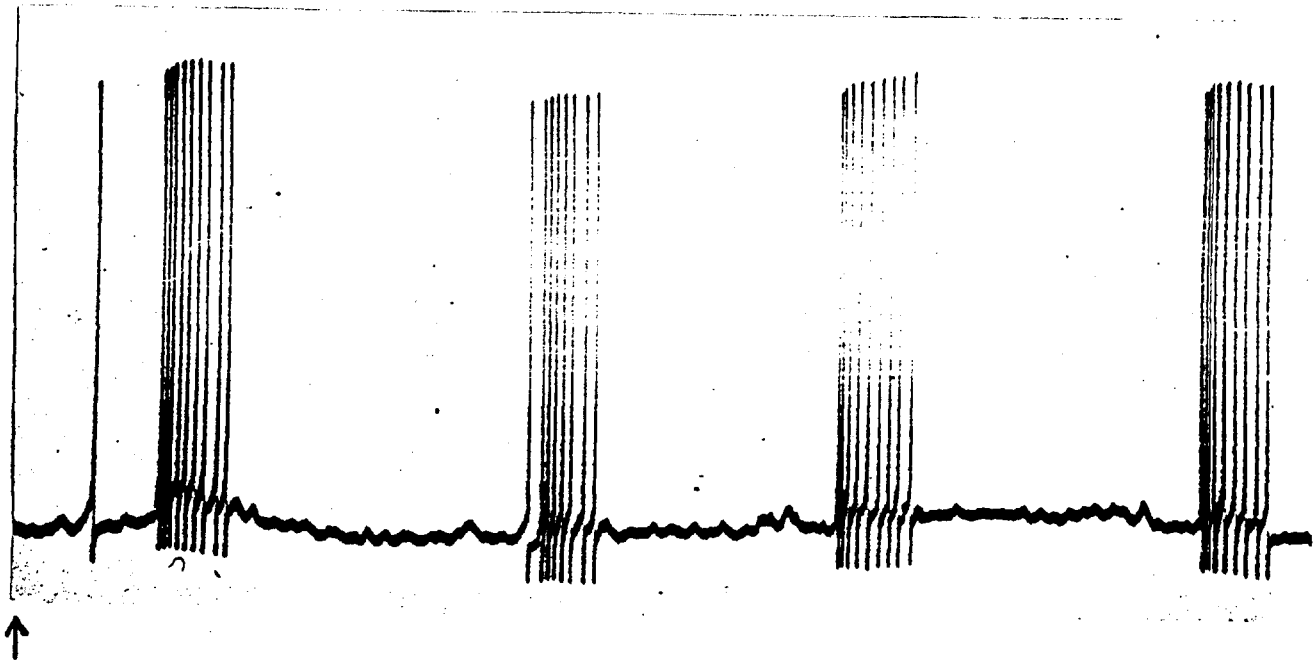
Membrane potential = -65mV

Low spontaneous bursting pattern.



Control

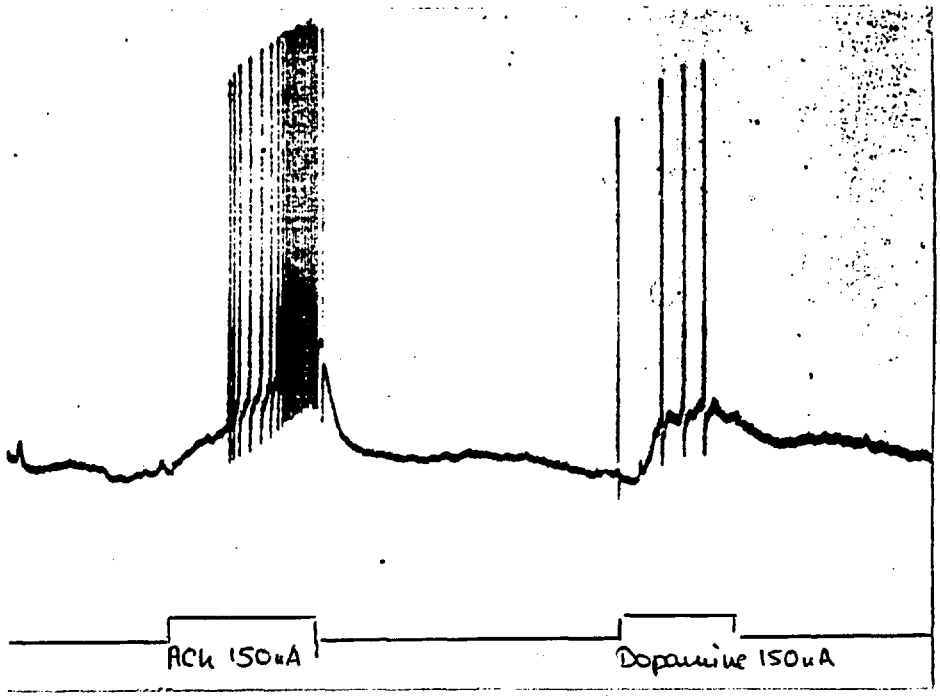
Verruculogen (20µl)



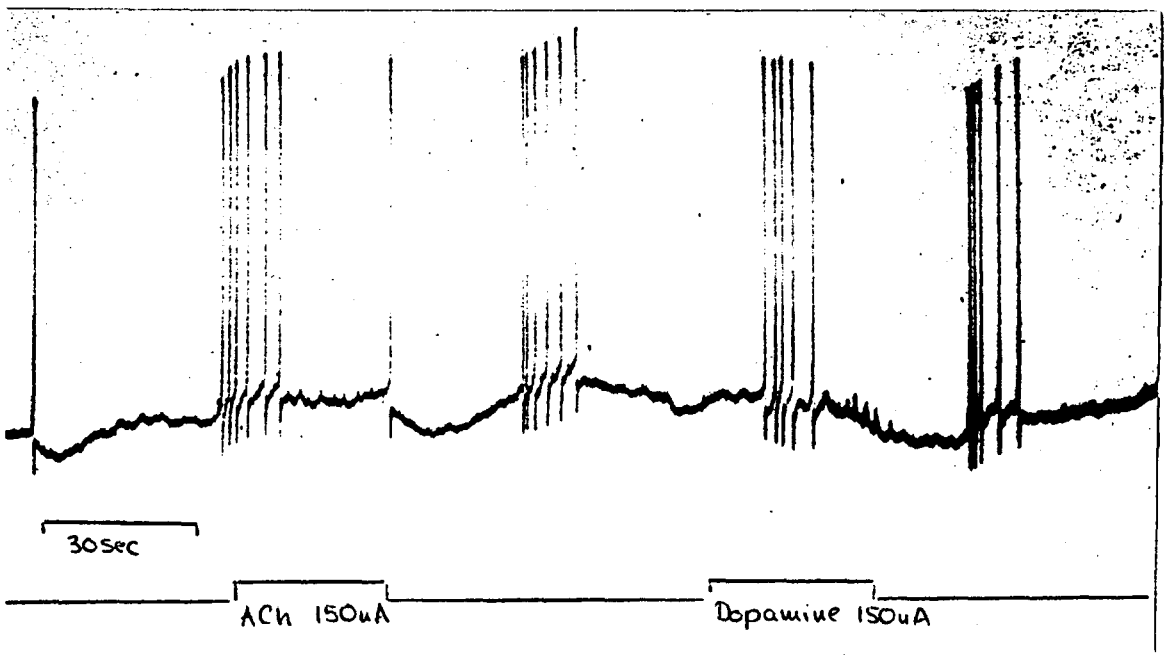
Verruculogen + 8min.

FIG. 6.4 / GDN - 4.

EFFECTS OF VERRUCULOGEN ON THE GIANT DOPAMINE NEURON OF THE WATER SNAIL.



Controls : Effects of iontophoretically administered Acetylcholine and Dopamine.



Verruculogen + 4min.(30µl).

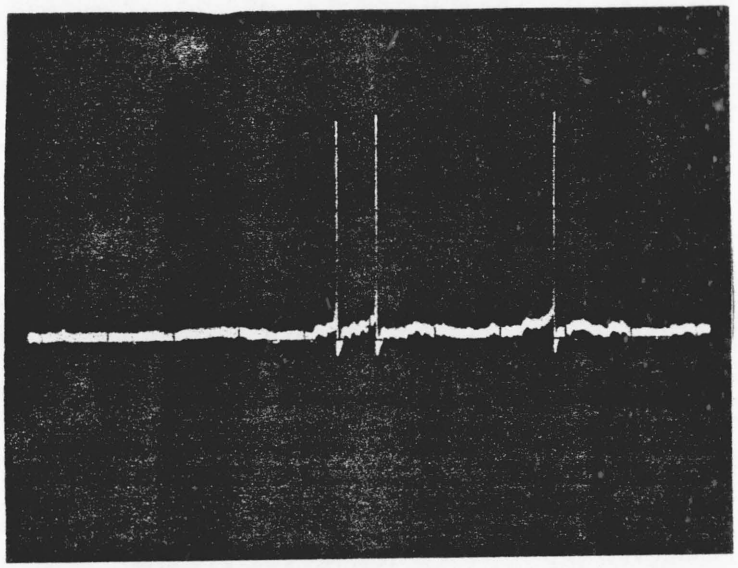
Recovery observed 8min. after wash.

FIG. 6.4 / GDN - 5.

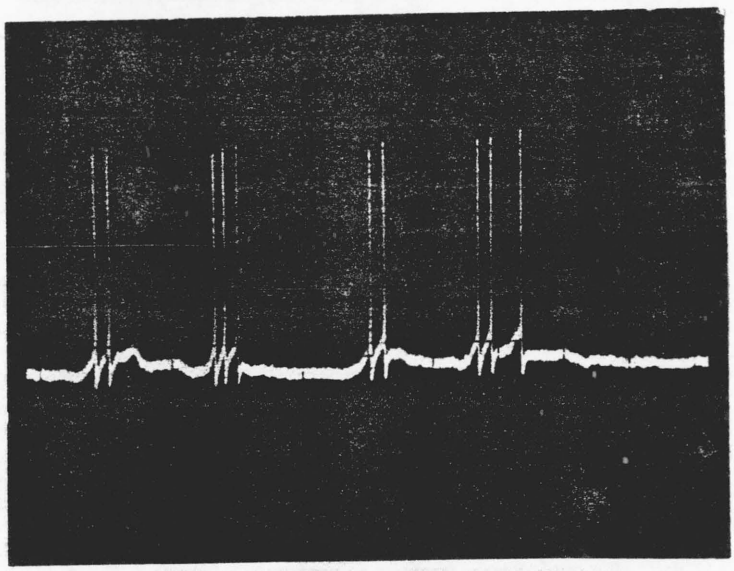
EFFECTS OF VERRUCULOGEN ON THE GIANT DOPAMINE NEURON OF THE WATER SNAIL.

Membrane potential = -80mV. Low spontaneous activity.

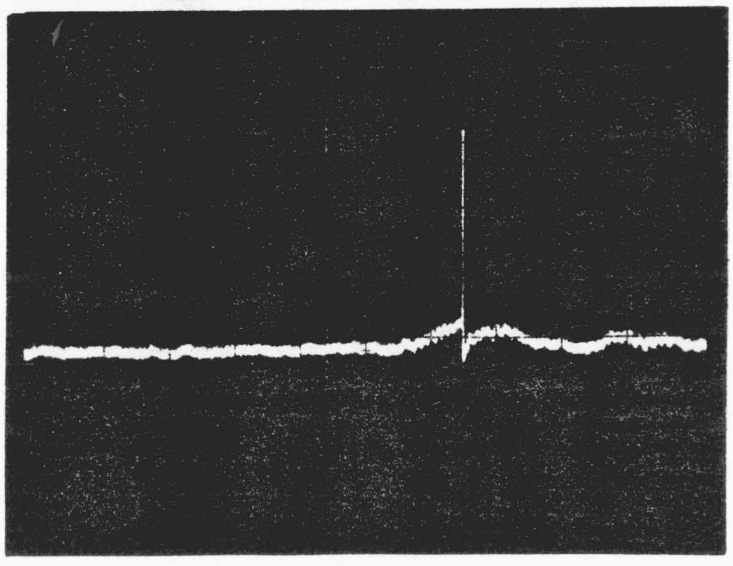
Control, 5sec/cm.



3min. after Verruculogen, (20µl).



30min. after wash.



with very low spontaneous activity (GDN 1, GDN 5 in FIG. 6.4), and the effect was more pronounced on a neurone with regular spontaneous firing (GDN 2 in FIG. 6.4). In a neuron with spontaneous bursting patterns (GDN 3 in FIG. 6.4), Verruculogen induced more bursts at shorter intervals and with more spikes per burst.

Constant current pulses of 1nA. and 500msec. duration passed through the recording electrode indicated that the membrane resistance of the neuron was unchanged on addition of Verruculogen (GDN 2 in FIG. 6.4), and the reversibility of the action of Verruculogen is illustrated in GDN 5 (FIG. 6.4) where, 30min. after washing, the bursting pattern response to Verruculogen had disappeared.

The giant dopamine neuron GDN 4 was exposed to acetylcholine and dopamine. When dopamine or acetylcholine were administered microiontophoretically (150μA., final concentration approximately 10^{-5}M), a characteristic depolarisation of the neuron was observed. However, this was abolished by administration of Verruculogen 4min. prior to the addition of acetylcholine and dopamine, and recovery was observed within 8min. subsequent to a wash (GDN 4).

DISCUSSION.

Administrations of Penitrem A to synaptosomes prepared from sheep cerebral cortex, rat spinal cord, cerebellum, and cerebral cortex, and to the locust neuromuscular junction preparation, were without significant effect on the principal paramaters investigated in these experiments, even when the maximum concentration (approximately 30µg/ml) was presented to these preparations. In contrast to these data, WILSON et al., (1972), when they administered Penitrem A directly by perfusing isolated rat diaphragm with a 60µg/ml suspension in their modified Locke's solution, evidenced an elevation in miniature end plate potential frequency which lasted for 60 - 90min., even after washing, in addition to an increase both in end plate potential duration, and the mean miniature end plate potential amplitude.

Concepts were raised in the GENERAL INTRODUCTION in respect of approximations made of extra - cellular and intra - cellular fluid spaces, and the likely concentrations of neuroactive agents at the synaptic cleft. If it is assumed that, from Wilson's experiments, the Penitrem A concentration at the synaptic region approaches 60µg/ml (approximately 10^{-4}M - and this must be a maximum level in view of the fact that WILSON et al., (1972) presented the phrenic nerve - diaphragm preparation with a Penitrem A suspension), then it becomes anomalous that in the present experiments, no effects were observed when Penitrem A was administered at 30µg/ml..

Three explanations are outstanding for the lack of an effect of Penitrem A on the preparations studied here. (1) The alterations observed in amino acid accumulation in synaptosome suspensions prepared from the spinal cords and cerebral cortices of animals pre - injected with the mycotoxin were secondary effects, in that the primary action of the tremorgen was at another part of the nervous system, or that the amino acid accumulations observed in those experiments were merely consequences of the generated tremor rather than the cause. However, the spinal cord and cerebral cortex would appear to be, from the precedents which have been described earlier, likely candidates for a primary source of action for Penitrem A ; and secondly, if the primary source of the action were to lie at another nervous system region, it would be unlikely that the observed synaptosomal phenomena would survive the duration and rigours of the

preparation of cerebrocortical or spinal medullary synaptosomes.

Explanation (2) for the lack of aneffect when Penitrem A was administered in vitro would be that the mycotoxin was activated by metabolism subsequent to injection, but prior to action. Since the common feature of the tremorgenic mycotoxins are their nidole nuclei, then activation at this point might appear the more likely. The metabolism of indoles in the rat has been described by KING et al., (1966), and principally involves scission of the pyrrole ring. Such a required activation would, however, also be unlikely, since WILSON et al., (1972) reported direct effects of Penitrem A when administered in vitro.

Explanation (3), and probably the most likely one for the lack of any action of Penitrem A in the present experiments is that the concentration of dissolved mycotoxin which was available to the synaptic region was not sufficiently high.

The data obtained to date for Verruculogen, on the other hand, not only augers well for future experiments, but provides additional valuable information for the present. Unfortunately, Verruculogen administered as an ethanolic solution to synaptosome suspensions elicited no effect on the parameters measured in sheep cerebrocortical, or rat spinal medullary and cerebella synaptosomes. The release from synaptosomes of aspartate and glutamate which were observed when the mycotoxin was administered to rat cerebrocortical synaptosomes, however, compliments the data obtained from synaptosomes prepared from rats pre - injected with the mycotoxin. The individual datum points obtained from this manner of in vitro administration indicated that at the concentration of 40µg/ml., Verruculogen was highly effective in releasing aspartate and glutamate from rat cerebrocortical synaptosomes. Presumably, in the three experiments in which positive effects were not observed, the hydrophobic nature of the mycotoxin resulted in sufficient quantity falling out of solution, thus reducing the release evoking concentration below a threshold level. However, the degree of intolerability of this situation was evidenced by the lack of any significant effect of verapamil or TTX on synaptosomes incubated in the presence of Verruculogen.

The administration of Verruculogen in ethanol to the locust neuromuscular junction preparation, and the snail giant dopaminergic neuron, proved to elicit the responses described on every occassion.

The important point is the observation that the Verruculogen moiety as such is the active component eliciting these effects. Presumably, then, the alterations in synaptosomal release of amino acids were more likely to be the cause of the observed tremor in animals, than a consequence of it.

The low reproducibility of data obtained from experiments made to investigate the effects of ethanolic Verruculogen administration to synaptosome preparations required that alternative methods were examined. The Mulgofen emilsions, at the surfactant concentrations employed, proved to be of little value. ' Presumably, detergent type action which reduced the integrity of the synaptosome membrane was the major feature of the observed leakiness, but other actions cannot be excluded and are beyond the scope of the present study.

The experiments made using the nylon gauze technique for the administration of Verruculogen to synaptosome suspensions did not evidence any biochemical changes in the parameters measured. This was principally a consequence of the extremely small quantity of Verruculogen which may have dissolved in the incubation medium. The failure to observe any changes also illustrates the requirement for a solubilised administration technique to be utilised, since synaptosome - crystalline toxin interactions (which must inevitably have occurred with high incidence) did not activate presumed receptors or channels which may be available for activation when the toxin is solubilised.

It is not possible at this moment in time to be too optimistic over the value of the liposome preparation as a technique to administer Verruculogen to synaptosome suspensions. The lipid extraction from whole brain utilised a crude procedure, and rather than attempt to purify further the lipid extracts, it may in the future be preferable to prepare liposomes from commercially available lipids. In this manner, the liposome composition could be strictly controlled with regard to the degree of lipid packing and bilayer permeability. Furthermore, the absence of potential neuroactive compounds, which may have been present in the extraction procedure used here, could be relied upon (for instance, see BALCAR & MANDEL, 1976). Nevertheless, the technique is a simple one to perform and control, and warrants further investigation as a method for introducing aqueous insoluble compounds to aqueous biological systems.

The large and reversible increase in the rate of miniature end

plate potential generation at the locust neuromuscular junction preparation treated with Verruculogen strongly implies that the agent increases the output of glutamate at this synapse. The effect was of relatively short time course and was completely reversible. In contrast to what is observed subsequent to KCl additions, there was observed no displacement of muscle membrane potential, indicating that if the toxin causes enhanced miniature end plate potential frequencies by acting as a depolarising agent, the quantity of glutamate subsequently released was insufficient to depolarise the post - junctional site. The combined actions of Verruculogen and depolarising agents on the locust neuromuscular junction preparation have not been examined to date. However, when this question was examined in the giant dopamine neuron of the water snail, Verruculogen administered prior to acetylcholine and dopamine abolished the characteristic depolarisation of the neuron observed in the absence of the mycotoxin. This may have been a consequence of receptor desensitisation, but for the moment the mechanism of the effect must be left unexplained. As in the locust preparation, there was observed no membrane depolarisation, and the membrane resistance of the neuron was unchanged on addition of Verruculogen.

The common data to emerge from these two types of preparation are those showing elevated spontaneous activity at these neurons. Can the elevated spontaneous activity here be correlated with the elevation in aspartate and glutamate release from synaptosomes treated with Verruculogen? KATZ & MILEDI (1967) have shown that in the frog sartorius neuromuscular junction preparation, there was no significant change in the frequency or size of spontaneous potentials in the presence of tetrodotoxin. Consequently, it is not permissible to compare the elevated spontaneous activity recorded in the presence of Verruculogen in the locust neuromuscular junction and the water snail dopaminergic neuron with the elevated release of transmitter observed in synaptosomes, since in the latter case the response was at least partially inhibitted by tetrodotoxin.

It has not yet been possible to determine the TTX sensitivity, or indeed the verapamil sensitivity, of the responses observed in the neuronal preparations when Verruculogen is present in the superfusion fluid. Such experiments are necessary in order to compare or contrast the responses with those observed in synaptosome suspensions.

An additional anomaly exists in the responses of the two types of preparation to depolarising stimuli. In synaptosome suspensions prepared from animals dosed previously with Verruculogen, the transmitter signal observed in response to veratrine was not significantly different from the signal observed in synaptosomes prepared from control animals. This is in contrast to the inhibition by Verruculogen of depolarising stimuli in the water snail dopaminergic neuron. Such a contrast is unlikely to be a consequence only of the fact that in the one situation Verruculogen was administered '<u>in vivo</u>', and in the other, '<u>in vitro</u>'.

External calcium is not required for the appearance of miniature end plate potentials. However, tests at frog neuromuscular junctions in which various metabolic inhibitors, such as ruthenium red, cause mitochondria to release their stored calcium into the terminal cytoplasm. The result is an increased release of miniature potentials (ALNAES & RAHAMIMOFF, 1975). Spontaneous release, like the amount of evoked release, therefore, can be interpreted as in CHAPTER FOUR as a reflection of the calcium concentration within terminals, which is normally kept at a low and constant value. Thus, the total inhibition by verapamil of the release of transmitter from synaptosomes prepared from Verruculogen injected animals may not preclude a comparison between the spontaneous potentials observed in the present chapter, and the transmitter release observed in the previous chapter.

CHAPTER SEVEN.

EFFECTS OF γ - ACETYLENIC GABA AND γ - VINYL GABA SYNAPTOSOMAL GABA ACCUMULATION.

(1) INTRODUCTION.

(2) RESULTS.

1) Protection afforded to rats by γ - acetylenic GABA against whole body tremor induced by the tremorgenic mycotoxin Verruculogen.

2) Purity and stability of γ - vinyl GABA and γ - acetylenic GABA.

3) Extrasynaptosomal accumulation of preloaded (U - ^{14}C) GABA when incubated in the presence of γ - vinyl GABA and γ - acetylenic GABA.

4) Release of endogenous GABA from cerebrocortical synaptosomes incubated in the presence of γ - acetylenic or γ - vinyl GABA.

5) Effects of γ - acetylenic and γ - vinyl GABA on (U - ^{14}C) GABA uptake into cerebrocortical synaptosomes.

6) Time course of the accumulation of amino acids in synaptosome suspensions subsequent to the addition of γ - vinyl GABA.

(3) DISCUSSION.

INTRODUCTION

Neurobiologists have for some years explored the functional roles of GABA in the mammalian central nervous system by altering the brain GABA content of laboratory animals with drugs. Brain GABA content has been elevated by using inhibitors of 4-amino-butyrate; 2-oxoglutarate amino transferase (EC 2.6.1.19, GABA amino transferase, GABA-T), the first enzyme in the degradative pathway for GABA. Also, the ability to increase GABA content in human brain may eventually prove to have practical significance.

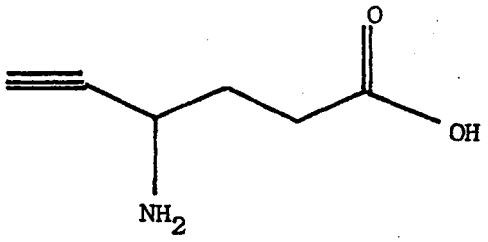
A deficiency of GABA has been demonstrated in certain regions of autopsied brain obtained from patients dying with Huntington's chorea (PERRY et al., 1973; BIRD & IVERSEN, 1974; URQUHART et al., 1975). In addition, activity of the enzyme that synthesises GABA, L-glutamate 1-carboxylase (EC 4.1.1.15, glutamic acid decarboxylase, GAD) is reduced in the same brain regions of these patients (BIRD & IVERSON, 1974; URQUHART et al., 1975). Efforts to elevate brain GABA content by administering high doses of the GABA-T inhibitor isonicotinic acid hydrazide (isoniazid) have produced limited clinical improvements in patients with Huntington's chorea (PERRY et al., 1977, 1979a). Elevation of brain GABA might have beneficial effects in some forms of human epilepsy (MELDRUM, 1978), and possibly also in schizophrenia. ROBERTS (1972) has suggested that abnormalities in brain GABA metabolism may be involved in schizophrenia. PERRY et al., (1976b) have found in autopsied brain from patients dying with schizophrenia a reduction in the mean GABA content of the nucleus accumbens and the thalamus as marked as that in Huntington's chorea. A deficiency of GABA function has also been demonstrated or implicated in tardive dyskinesia (PALFREYMANetal., 1978) and Parkinsonism (BARBEAU, 1973).

As GABA itself does not readily cross the blood brain barrier, its oral administration would not correct a deficiency of GABA in brain (ROBERTS & KURIYAMA, 1968). Hence the concept requiring that an inhibitor containing a latent reactive functionality which is liberated as a result of a target enzyme's own mechanism of action has been enunciated. Such inhibitors, which have been designated "K cat" inhibitors, or "suicide enzyme inactivators" are expected to be highly specific because they should only inhibit those enzymes which can accept them as substrates. Several inhibitors, which function by a mechanism requiring activation by GABA-T prior to that enzyme's irreversible inactivation have been designed and synthesised (FOWLER &

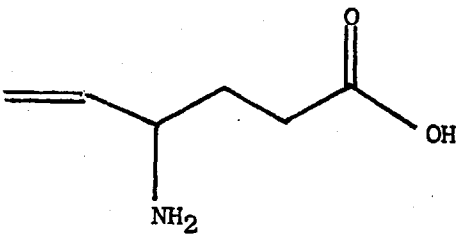
JOHN, 1972; JUNG & METCALF , 1975; LIPPERT et al., 1977), or isolated from natural sources (KOBAYASHI et al., 1976).

GABA-T, being a pyridoxal phosphate (Py CHO) - dependent enzyme, operates via a tautomerism of the Schiff base formed between GABA and PyCHO. The Schiff base function activates the adjacent C-H bond, so that proton abstraction is facilitated. Reprotonation leads to the tautomeric Schiff base which is hydrolysed to the products, succinic semialdehyde and pyridoxamine. In order for further transamination of GABA to proceed, the holoenzyme must be converted back to the pyridoxal form. This is achieved with the advent of another substrate, α-ketoglutarate, which undergoes Schiff base formation with the pyridoxamine. The sequence is then reversed with the amino group of pyridoxamine being transferred to α-ketoglutarate, thus regenerating PyCHO and glutamic acid. Two forms of brain enzyme have been characterised, one being associated with free mitochondria, the other with a synaptosomal fraction (BUU NGUYEN & VAN GELDER, 1973; TARDY et al 1976).

γ - acetylenic GABA and γ- vinyl GABA are two catalytic irreversile inhibitors of GABA - T (JUNG et al., 1977; JUNG & METCALF, 1975). They cause a sharp decrease of whole brain GABA-T activity and a dose dependent increase in brain GABA concentration. (JUNG et al., 1976; 1977). In vivo, both drugs elicit a complex response such as sedation and lowered body temperature (SCHECHTER & TRANIER, 1977a) and an increase in the control and stimulated release of GABA to fluid superfusing the rat sensorimotor cortex (ABDUL GHANI et al., 1980). LOSCHER (1979) and BOHLEN et al., (1979) have shown an increase in GABA levels in cerebrospinal fluid following intraperitoneal injection of γ - acetylenic GABA and γ—vinyl GABA.



γ - ACETYLENIC GABA



γ - VINYL GABA

At a dose of 100 mg / kg i. p. in mice, γ - acetylenic GABA induces a long - lasting inhibition of whole brain GABA - T activity, which drops to nearly zero within 2h. and is still only 15 - 20 % of control 24h. after administration. As a consequence brain GABA levels

rise to about 4 times control levels at 8h., then slowly return to control values.(JUNG et al., 1977a). γ - Vinyl GABA enters the brain less readily than does γ - Acetylenic GABA, hence higher doses are required to induce a comparable inhibition of GABA -T . Thus a single dose of 1500 mg / kg i.p. in mice reduces brain GABA -T activity to approximately 20 % of control, this level of inhibition being maintained for at least 48h. Concomitant with this inhibition of GABA - T , brain GABA concentrations rise to about 5 - 6 times control levels, and are still more than double control after four days (JUNG et al., 1977b)

Both γ - acetylenic GABA and γ - vinyl GABA protect mice against convulsions induced by audiogenic stimuli, electroshock , thiosemicarbazide, isoniazide and strychnine, the pattern of protection mirroring that of whole brain GABA elevation (SCHECHTER et al., 1977a,b) Neither compound protects against seizures induced by picrotoxin or bicuculline, suggesting that their anticonvulsant activity is not secondary to their sedative effect (SCHECHTER & TRANIER, 1977b).

In view of the protection afforded by γ - acetylenic GABA against the tremor induced in rats by tremorgenic mycotoxins, a study was made of the accumulation of GABA externally of synaptosomes as a consequence of γ - vinyl GABA and γ - acetylenic GABA administration, with particular emphasis on the relevance of the inhibition of GABA -T to this accumulation.

RESULTS.

1) Protection afforded to rats by γ - acetylenic GABA against whole body tremor induced by the tremorgenic mycotoxin Verruculogen.

When rats were injected intraperitoneally with Verruculogen (400mg mycelium / kg), whole body tremor was observed in 5 - 10 min., as described in a previous chapter. When γ - acetylenic GABA was injected intraperitoneally (100mg / kg) half an hour prior to the administration of Verruculogen, no tremor was observed for the subsequent 8h., when tremor induced by Verruculogen alone would have subsided. When γ - acetylenic GABA was injected intraperitoneally (100mg / kg) half an hour subsequent to the administration of Verruculogen, the tremor induced by Verruculogen subsided within a further half an hour / 40min., and was not observed to re - occur. These experiments were performed on six rats and in every instance, control animals were injected with one or other of the agents alone. The observed effects on individual rats occurred within ± 10min. of the mean time for the effect to be observed. The rats injected with γ - acetylenic GABA exhibited a tendency towards sedation, but were observed to feed and drink normally, and would climb the bars of the cage at regular intervals. Some of the rats injected with Verruculogen alone showed some convulsing about 2h., subsequent to dosing but this tended to subside by about the third hour.

2) Purity and stability of γ - vinyl GABA and γ - acetylenic GABA.

The possible breakdown of γ - vinyl GABA and γ - acetylenic GABA to GABA was examined, and for this purpose, cerebrocortical synaptosomes were incubated in Krebs - phosphate medium at 37'C for 20min.. Tissue was then sedimented and lysed in cold water. Incubation medium, membranes, and lysate were collected individually, and the drugs were then incubated (5mM) for 5min., at 37'C with each of the fractions. No detectable GABA was generated in this period. Also, γ - vinyl GABA was introduced directly onto the sensorimotor cortex of rats through a continuous superfusion cannula line at 100μM for 170min.. No larger GABA peak was observed (4pmol. / min. / sq.cm.) than followed i.p. injection of γ - vinyl GABA (9pmol. / min. / sq.cm.), when lower

DATA LEGEND.

RE : TABLE 7.1.

AMINO-ACID ACCUMULATION IN INCUBATION FLUID OF SUSPENSIONS OF RAT CEREBROCORTICAL SYNAPTOSOMES IN THE PRESENCE OF γ - VINYL GABA OR γ - ACETYLENIC GABA.

Cerebrocortical synaptosomes were suspended in Krebs phosphate medium in 5ml conical flasks in a shaking waterbath at 37'C. Subsequent to an equilibration period of 30min., γ - acetylenic GABA or γ - vinyl GABA were added as appropriate, to final concentrations of 5mM. Incubation continued for a further 5min..

At the end of the experiment, suspensions were sedimented in a bench top ultracentrifuge, and the supernatants were decanted and taken to dryness in a vacuum dessicator. After a methanol extraction, samples were dissolved in 0.025M HCl, and autoanalysed for amino acid levels.

The data were obtained from six experiments, and are the mean values ± SEM. Data is presented as nmol amino acid / 100mg pellet protein.

TABLE 7.1

AMINO ACID ACCUMULATION IN MEDIUM CONTAINING SUSPENSIONS OF RAT CEREBROCORTICAL SYNAPTOSOMES AND γ - VINYL GABA OR γ - ACETYLENIC GABA TO FINAL CONCENTRATIONS OF 5mM.

	CONTROL	γ - VINYL GABA (5mM)	γ - ACETYLENIC GABA
ASPARTATE	71 ± 25	77 ± 10	72 ± 12
THREONINE	134 ± 13	198 ± 25	154 ± 16
SERINE	293 ± 35	343 ± 19	324 ± 18
GLUTAMATE	207 ± 85	233 ± 60	246 ± 50
GLUTAMINE	34 ± 13	31 ± 8	37 ± 13
GLYCINE	159 ± 12	213 ± 26	174 ± 11
ALANINE	222 ± 15	231 ± 24	250 ± 11
GABA	22 ± 12	171 ± 17	n.d.

concentrations prevailed in tissue and superfusate. The possibility that GABA was a contaminant of the drug samples was dispelled by analysing 5mM samples of 5mM γ - vinyl GABA under conditions where 5 - 50pmol. GABA could be detected. This implies that any contamination was less than one in 10^5 parts of the drug.

3) Extrasynaptosomal accumulation of preloaded (U - ^{14}C) GABA when incubated in the presence of γ - vinyl GABA or γ - acetylenic GABA.

γ - Acetylenic GABA and γ - vinyl GABA at 1mM concentrations caused a significant increase in preloaded (U - ^{14}C) GABA release from cerebrocortical synaptosomes incubated in Krebs - phosphate medium for 30min. prior to administration of the drugs for 10min.. The accumulation in the medium of GABA associated radiolabel corresponded to 23% ($p<0.025$) and 57% ($p<0.005$) respectively over the levels found in control incubations (FIG. 7.1). This effect increased with drug concentration to 46% with γ - acetylenic GABA (5mM), and to 96% with γ - vinyl GABA (5mM). The minimum effective dose with γ - acetylenic GABA was 0.5mM (12%), and with γ - vinyl GABA, 0.25mM (22%).

Tetrodotoxin (1μM) added to the incubated synaptosomes 1min. prior to γ - acetylenic GABA (5mM) and γ - vinyl GABA (5mM and 0.5mM) significantly inhibited the accumulation in the medium of (U - ^{14}C) GABA by 46%, 35%, and 60% respectively (FIG. 7.2). The release of (U - ^{14}C) GABA from synaptosomes in the presence of either drug at 5mM concentration was totally inhibited in the absence of Ca^{++} when verapamil was added to 100μM.

4) Release of endogenous GABA from cerebrocortical synaptosomes incubated in the presence of γ - acetylenic or γ - vinyl GABA.

When γ - vinyl GABA at a final concentration of 5mM was added to suspensions of cerebrocortical synaptosomes for 5min. subsequent to a 30min. incubation in the absence of the drug, GABA concentration was greatly increased in the medium, from a control value of 22 ± 12nmol / 100mg protein to 171 ± 17 nmol / 100mg protein. However, no change occurred (TABLE 7.1) in the accumulation of other amino acids. Similar results were obtained with γ - acetylenic GABA (5mM), although it was not possible to separate γ - acetylenic GABA from GABA itself in the

DATA LEGEND.

RE : FIG. 7.1.

EFFECT OF γ - VINYL GABA AND γ - ACETYLENIC GABA ON THE RELEASE FROM SYNAPTOSOMES OF EXOGENOUS (U - ^{14}C) GABA.

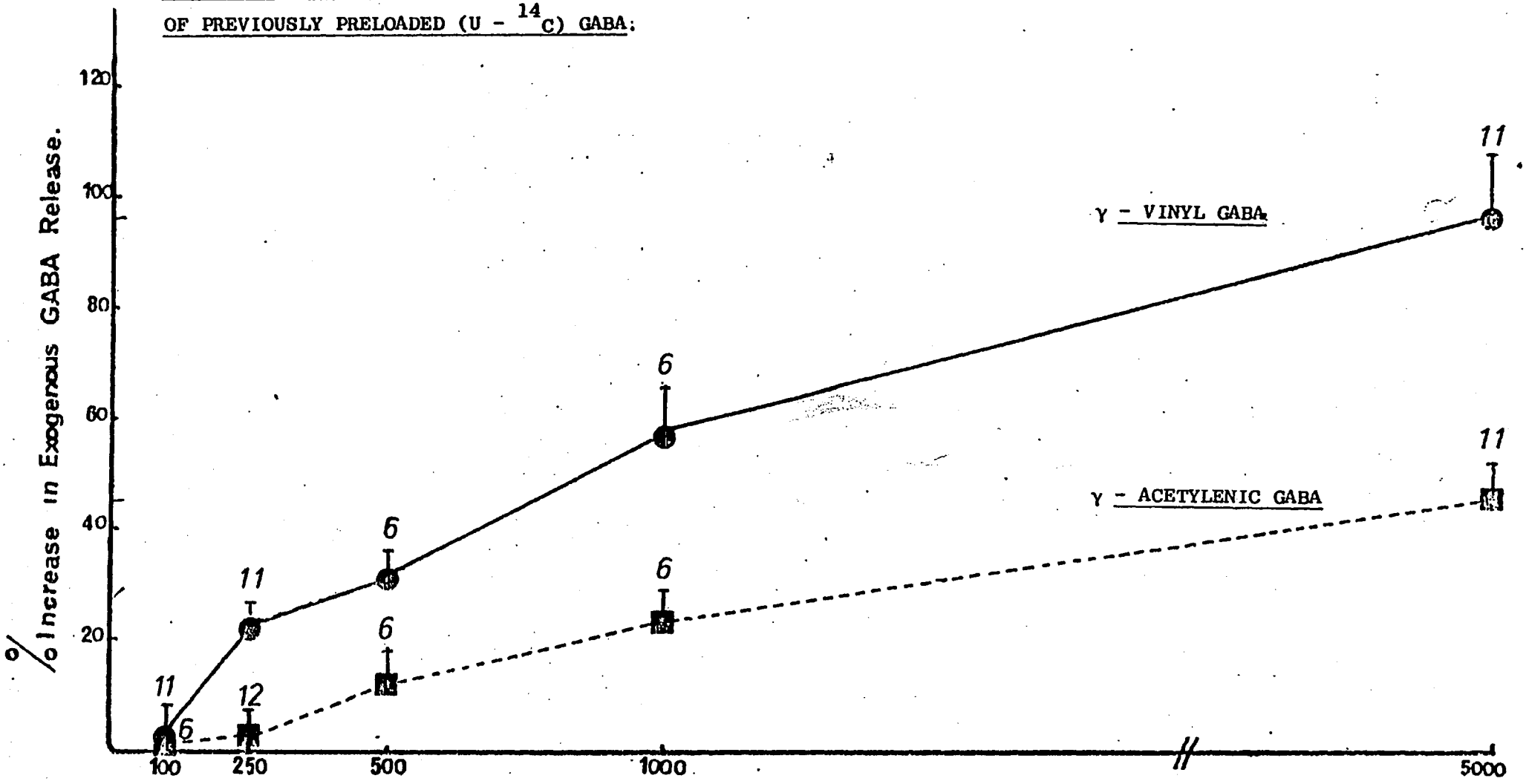
Rat cerebrocortical synaptosomes were suspended in Krebs phosphate medium in 5ml conical flasks in a shaking waterbath at 37'C. Also present was 10μM (U - ^{14}C) GABA (200nCi / ml). Incubation was for 15min.. Suspensions were then washed and resuspended in fresh medium. Subsequent to an equilibration period of 30min., appropriate concentrations of the two drugs were added and incubation continued for a further 10min..

At the end of the experiment, suspensions were sedimented in a bench top ultracentrifuge, and the supernatants were decanted into scintillation vials containing liquid scintillant. Radio - isotope was counted thereafter. Pellet protein content was determined by means of the Folin reagent.

The data presented are the means ± SEM for the number of experiments noted above datum points. Data are plotted as % increase in GABA measured in the incubation fluid in the presence of the drugs over those levels measured in control experiments.

FIG. 7.1

EFFECT OF γ - VINYL GABA AND γ - ACETYLENIC GABA ON THE EXTRASYNAPTOSOMAL ACCUMULATION OF PREVIOUSLY PRELOADED (U - ^{14}C) GABA.



DATA LEGEND.

RE : FIG. 7.2.

EFFECT OF TETRODOTOXIN AND VERAPAMIL ON THE RELEASE OF EXOGENOUS GABA FROM SYNAPTOSOMES INCUBATED IN THE PRESENCE OF γ - ACETYLENIC GABA OR γ - VINYL GABA.

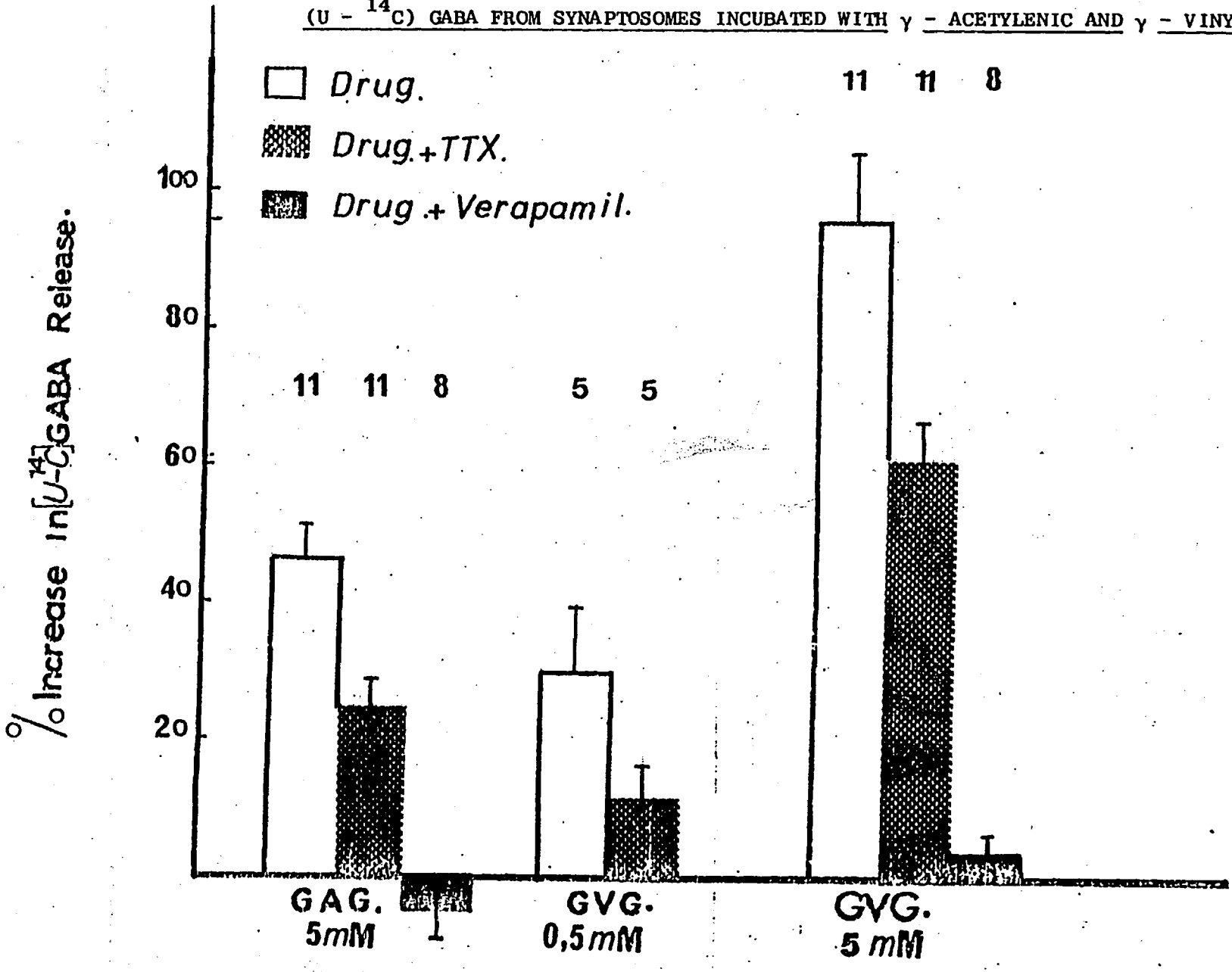
Rat cerebrocortical synaptosomes were suspended in Krebs phosphate medium in 5ml conical flasks in a shaking waterbath at 37'C. Also present was 10μM (U - ^{14}C) GABA (200nCi / ml). Incubation was for 15min.. Suspensions were then washed and resuspended in fresh medium.. Subsequent to an equilibration period of 29min., TTX (1μM), or verapamil (100μM) were added as appropriate. One minute later , one or other of the drugs were added at appropriate concentrations. Incubation continued for a further 10min..

At the end of the experiment, suspensions were sedimented in a bench top ultracentrifuge, and the supernatants were decanted into scintillation vials containing liquid scintillant. Radio - isotope was counted thereafter. Pellet protein content was determined by means of the Folin reagent.

The data presented are the means ± SEM for the number of experiments noted above datum points. Data are plotted as % increase over relevant controls.

FIG. 7.2

EFFECT OF 1μM TETRODOTOXIN AND 100μM VERAPAMIL ON THE EXTRASYNAPTOSOMAL ACCUMULATION OF PREVIOUSLY PRELOADED (U - ^{14}C) GABA FROM SYNAPTOSOMES INCUBATED WITH γ - ACETYLENIC AND γ - VINYL GABA.



analytical system employed.

Following intraperitoneal administration of γ - acetylenic GABA (100mg / kg) or γ - vinyl GABA (1000mg / kg) for periods of 6h., and 14h., respectively, animals were sacrified and cerebrocortical synaptosomes were prepared and incubated in Krebs phosphate medium for 30min., before addition of 75μM veratrine and / or 1μM TTX.

The release of endogenous GABA from synaptosomes prepared from animals pretreated with the drugs was much higher than from controls treated with saline. No change in release was observed in any of the other amino acids measured. In the presence of depolarising concentrations of veratrine (FIG. 7.3a; b; c) , a large increase in the release of glutamate, aspartate, and GABA occurred. Other amino acids remained unaffected. However, this well established veratrine stimulated release of putative amino acid neurotransmitters was much larger when the animals were pretreated with γ - acetylenic GABA and γ - vinyl GABA prior to the preparation of synaptosomes. The evoked release of endogenous aspartate, glutamate, and GABA in all cases was totally inhibited when the synaptosomes were incubated with TTX at a final concentration of 1μM (FIG. 7.3a; b; c).

Interestingly, when the veratrine data from FIG. 7.3 is expressed : medium amino acid / medium amino acid + intrasynaptosomal amino acid (from TABLE 7.2), the percent of total GABA released by veratrine following drug pretreatment is similar to that seen in the saline control situation.

No changes were observed in the accumulation intrasynaptosomally of any amino acid other than GABA, for which γ - acetylenic GABA and γ - vinyl GABA administered in vivo caused a increase of 3 - 4 fold in the content of this amino acid in the synaptosome preparation (TABLE 7.2).

5) Effects of γ - acetylenic GABA and γ - vinyl GABA on (U - ^{14}C) GABA uptake into cerebrocortical synaptosomes.

γ - acetylenic GABA and γ - vinyl GABA at 1mM concentrations caused a significant ($p<0.001$) inhibition of (U - ^{14}C) GABA high affinity uptake corresponding to 21%, and 36% respectively (FIG. 7.4). This inhibition was increased with rising drug concentration to a plateau of 70% with γ - vinyl GABA in the 3 - 5mM range, and up to 50% with γ - acetylenic GABA. No significant effects on uptake were obtained

DATA LEGEND.

RE : FIG. 7.3a, 7.3b, 7.3c, and TABLE 7.2.

ENDOGENOUS AMINO ACID RELEASE FROM SYNAPTOSOMES PREPARED FROM RATS PREVIOUSLY INJECTED WITH γ - ACETYLENIC GABA OR γ - VINYL GABA.

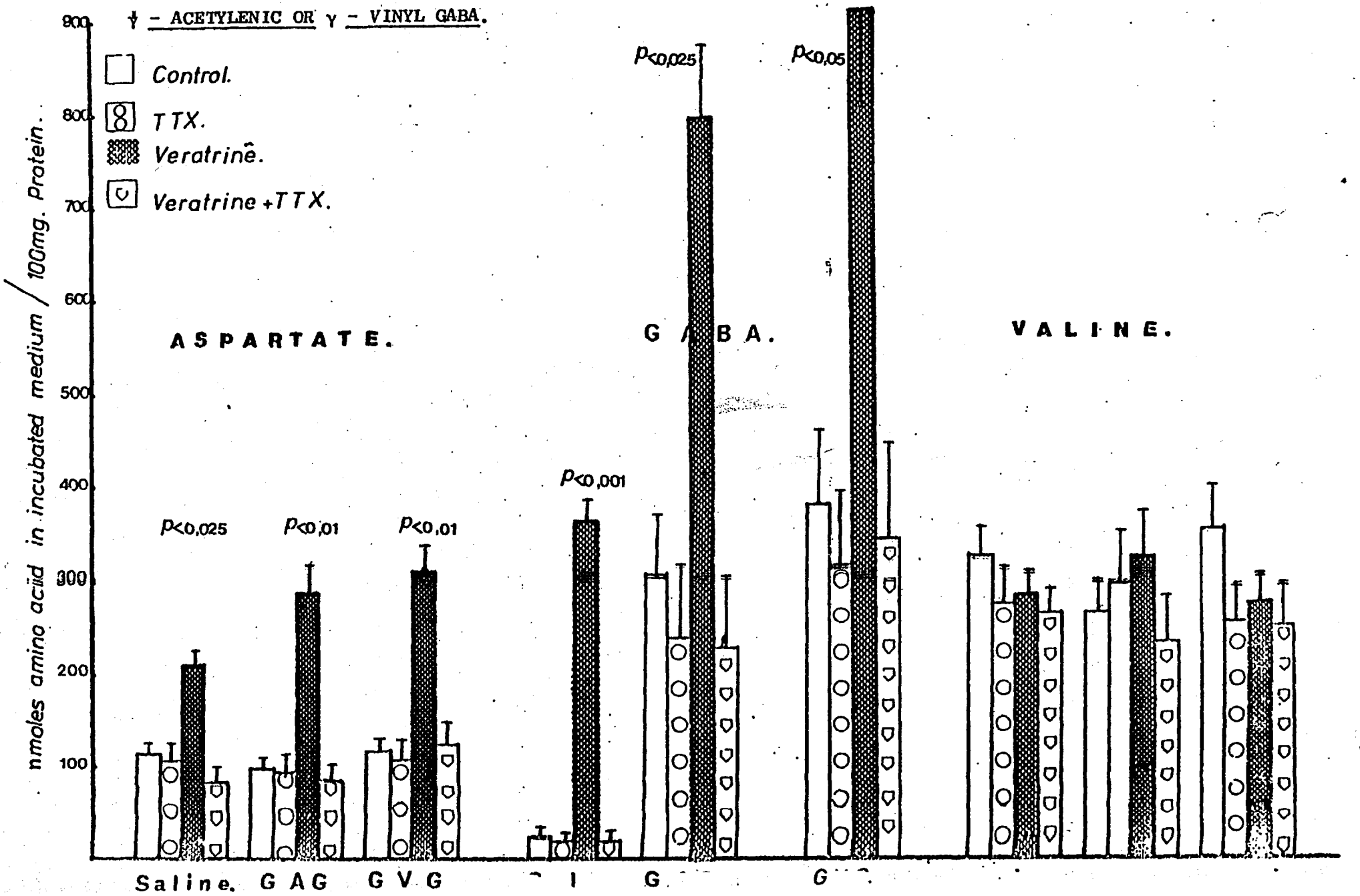
Following intraperitoneal injection of γ - acetylenic GABA (100mg/kg), or γ - vinyl GABA (1000mg/kg) for periods of 6h., and 14h., respectively, animals were killed and cerebrocortical synaptosomes were prepared and incubated in Krebs phosphate medium in 5ml conical flasks in a shaking waterbath at 37'C. Subsequent to an equilibration period of 30min., veratrine (75μM) and/or TTX (1μM) were added for a further 10min..

At the end of the experiment, suspensions were sedimented in a bench top ultracentrifuge, and fractions were extracted and autoanalysed for amino acid content. Pellet protein contents were determined by means of the Folin reagent.

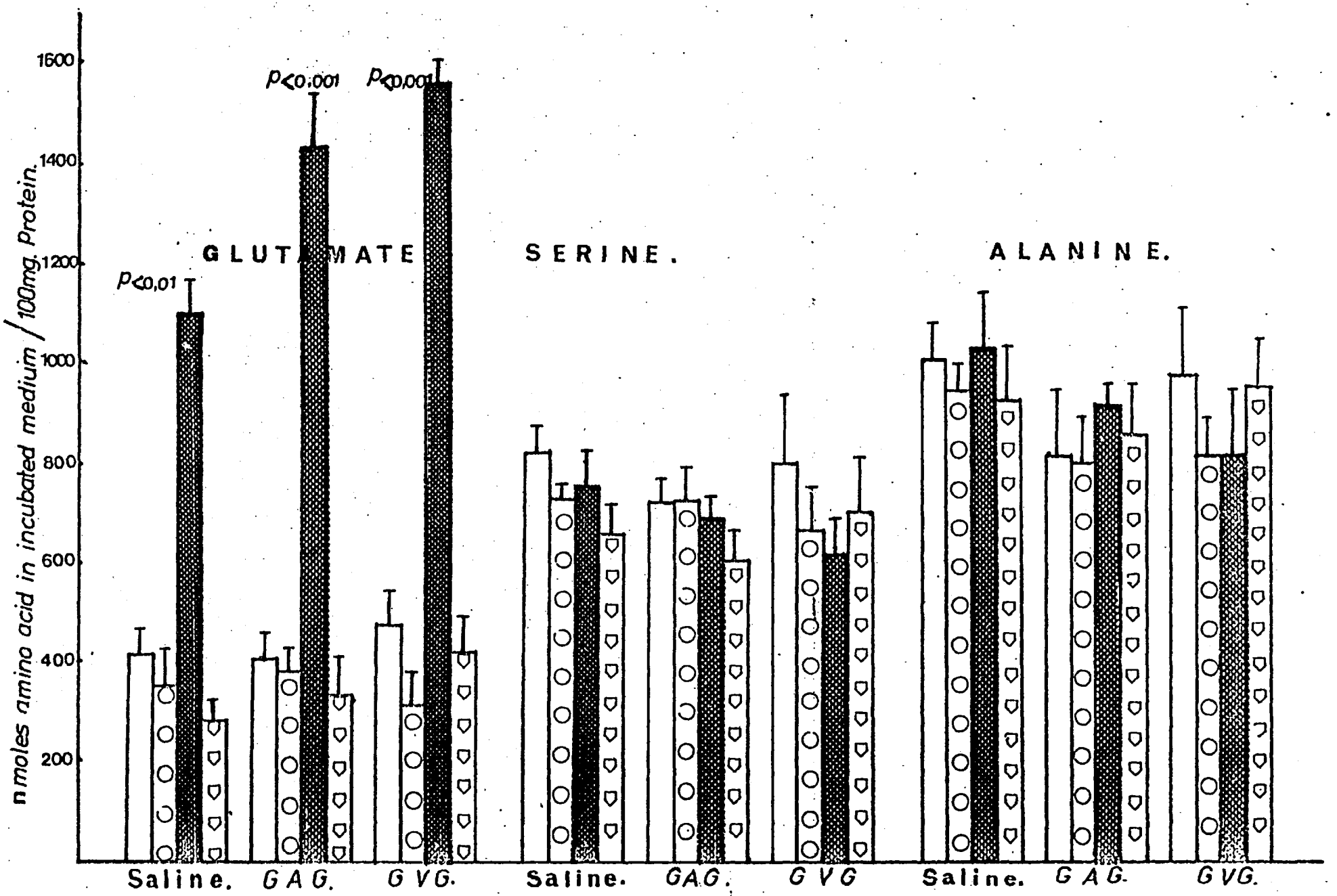
Data are the means ± SEM for values obtained from four experiments, and are expressed as nmol amino acid / 100mg protein.

FIG. 7.3a

ENDOGENOUS AMINO ACID RELEASE FROM SYNAPTOSOMES PREPARED FROM RATS PREVIOUSLY INJECTED (i.p.) WITH ǂ - ACETYLENIC OR γ - VINYL GABA.



B.


nmoles amino acid in incubated medium / 100mg. Protein.
1600
1400
1200
1000
800
600
400
200
p<0,01
p<0,001
p<0,001
GLUTAMATE
SERINE.
ALANINE.
Saline.
GAG.
GVG.
Saline.
GAG.
GVG
Saline.
GAG.
GVG.

C.

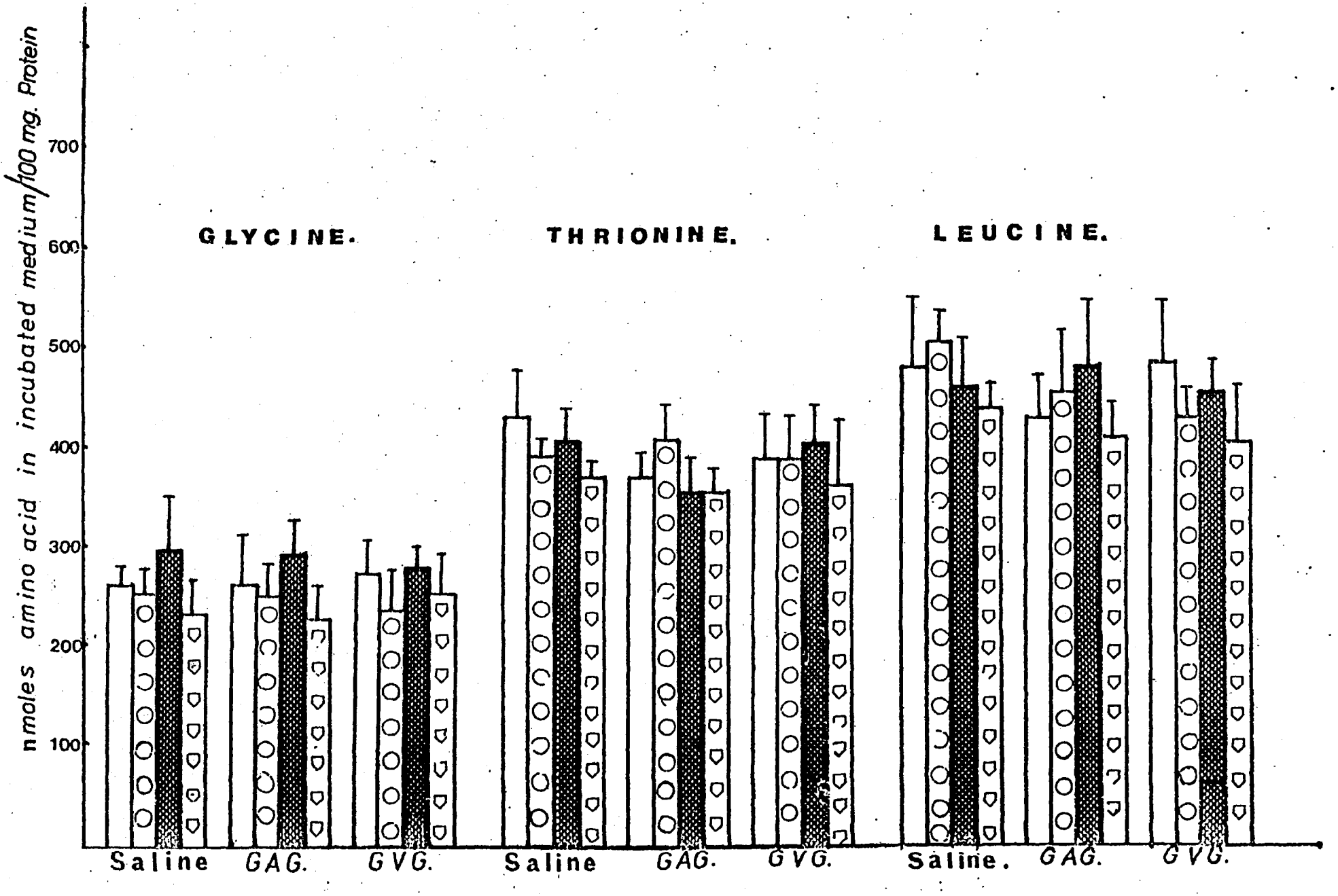

nmoles amino acid in incubated medium/100 mg. Protein
100
200
300
400
500
600
700
GLYCINE.
THRIONINE.
LEUCINE.
Saline
GAG.
GVG.
Saline
GAG.
GVG.
Saline.
GAG.
GVG.

TABLE 7.2

Effect of γ-Acetylenic GABA and γ-Vinyl GABA on intrasynaptosomal amino acid content

(nmoles/100 mg Protein)

Pre-treatment	Treatment	Aspartate	Glutamate	Glycine	Serine	Valine	Alanine	Leucine	GABA
Saline	Control	3754±248	4835±393	672±38	594±27	165±8	662±23	266±20	1164±45
	Tetrodo-toxin	3497±300	4750±661	615±96	575±22	174±8	595±54	238±46	1158±82
	Veratrine	1871±228	4408±381	487±106	441±26	143±9	485±42	197±11	743±15
	Verat.+TTX	3543±606	4393±418	602±95	588±53	180±14	642±53	223±17	1305±62
γ-Acetylenic GABA	Control	2877±164	4417±415	749±69	620±86	177±14	518±16	239±3	3698±748
	Tetrodo-toxin	2939±225	4010±168	772±99	611±52	204±33	567±30	282±46	3561±456
	Veratrine	1625±135	4307±564	535±123	571±74	166±10	439±72	224±26	2678±167
	Verat.+TTX	2841±222	4683±618	815±134	593±51	179±21	526±77	235±35	4168±252
γ-Vinyl GABA	Control	3396±399	4715±259	879±188	654±46	216±45	690±86	304±15	4628±396
	Tetrodo-toxin	2985±169	4227±424	981±224	468±72	203±22	714±36	291±10	4539±837
	Veratrine	1679±177	4176±143	549±100	562±94	183±5	445±25	230±12	3243±60
	Verat.+TTX	3259±322	4704±534	817±100	481±86	191±11	592±22	244±14	4751±691

DATA LEGEND.

RE : FIG. 7.4, 7.5, 7.6.

EFFECTS OF γ - ACETYLENIC GABA AND γ - VINYL GABA ON THE UPTAKE OF (U - ^{14}C) GABA INTO CEREBROCORTICAL SYNAPTOSOMES.

Cerebrocortical synaptosomes were incubated in Krebs phosphate medium in 5ml conical flasks in a shaking waterbath at 37'C for an equilibration period of 20min.. γ - Acetylenic GABA or γ - vinyl GABA at concentrations cited on the figures, were then added (in the case of FIG. 7.6, 1min subsequent to the addition of 1μM TTX). Incubation continued for a further 5min. prior to addition of the GABA radiolabel, 10μM, 200μCi / ml.. Except in the case of FIG. 7.5 (where incubations times are cited), incubation continued for a further 5min..

At the end of the experiment, suspensions were sedimented in a bench top ultracentrifuge, and the pellets were extracted. The extract was decanted into scintillation vials containing liquid scintillant. Samples were taken for radio - label counting.

Data are presented for the number of experiments noted above datum points (with the exception of FIG. 7.6, where n = 6), and are the means ± SEM.

FIG. 7.4

EFFECT OF γ - ACETYLENIC GABA AND γ - VINYL GABA ON THE UPTAKE BY SYNAPTOSOMES OF (U - ^{14}C) GABA.

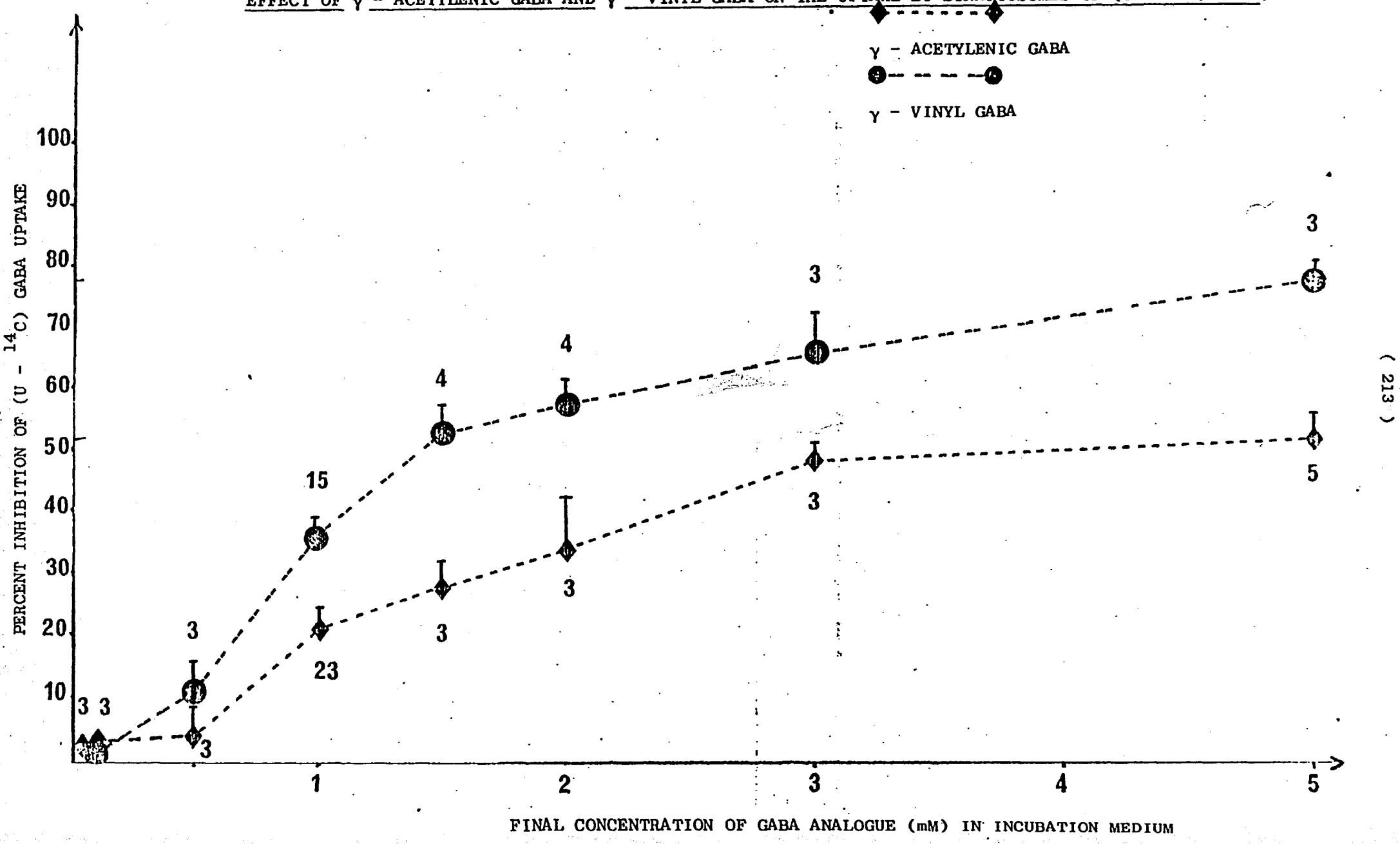


FIG. 7.5

EFFECT OF INCUBATION TIME ON THE INHIBITION OF UPTAKE OF (U - ^{14}C) GABA INTO SYNAPTOSOMES IN THE PRESENCE OF γ - VINYL AND γ - ACETYLENIC GABA.

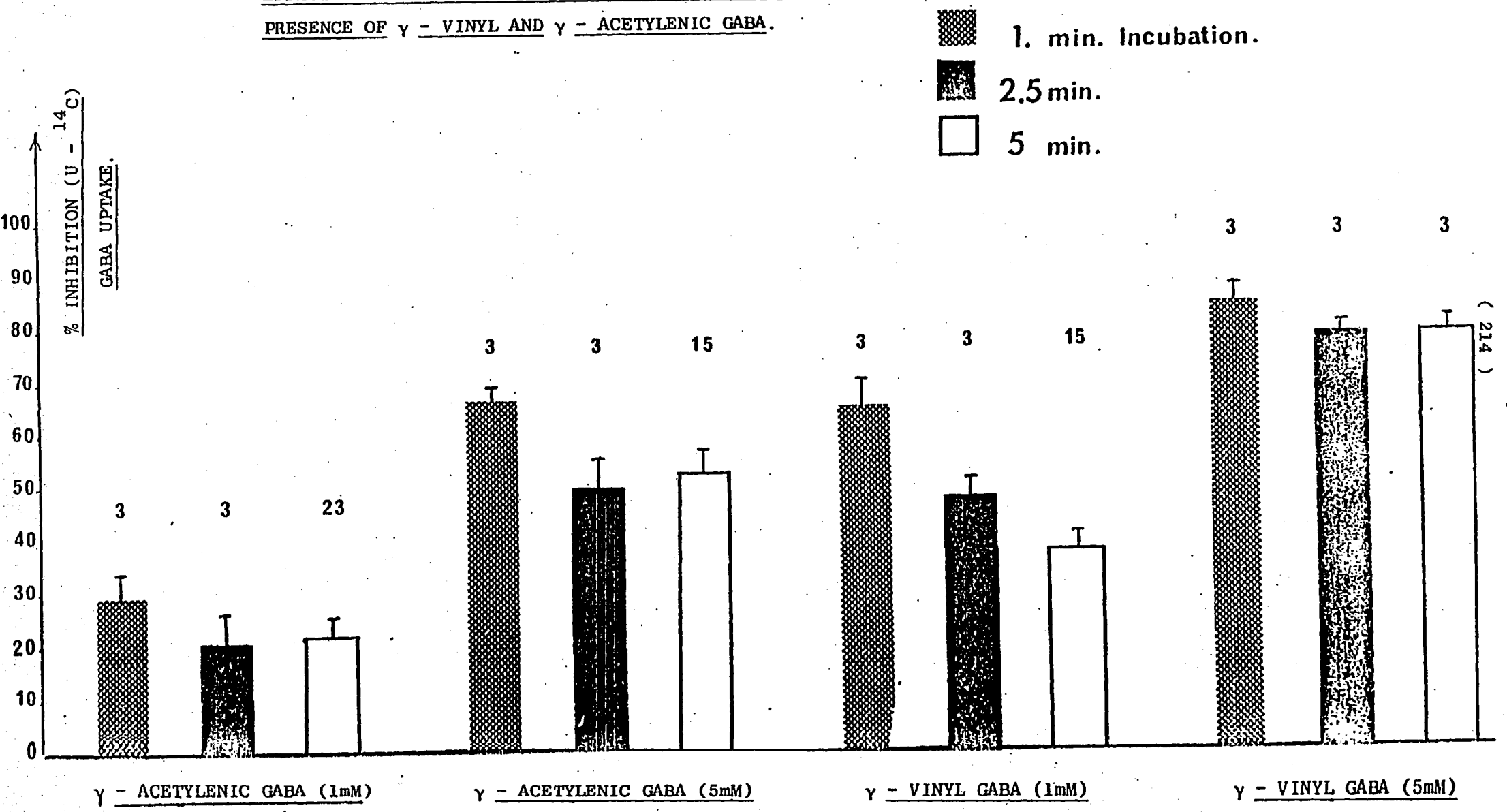
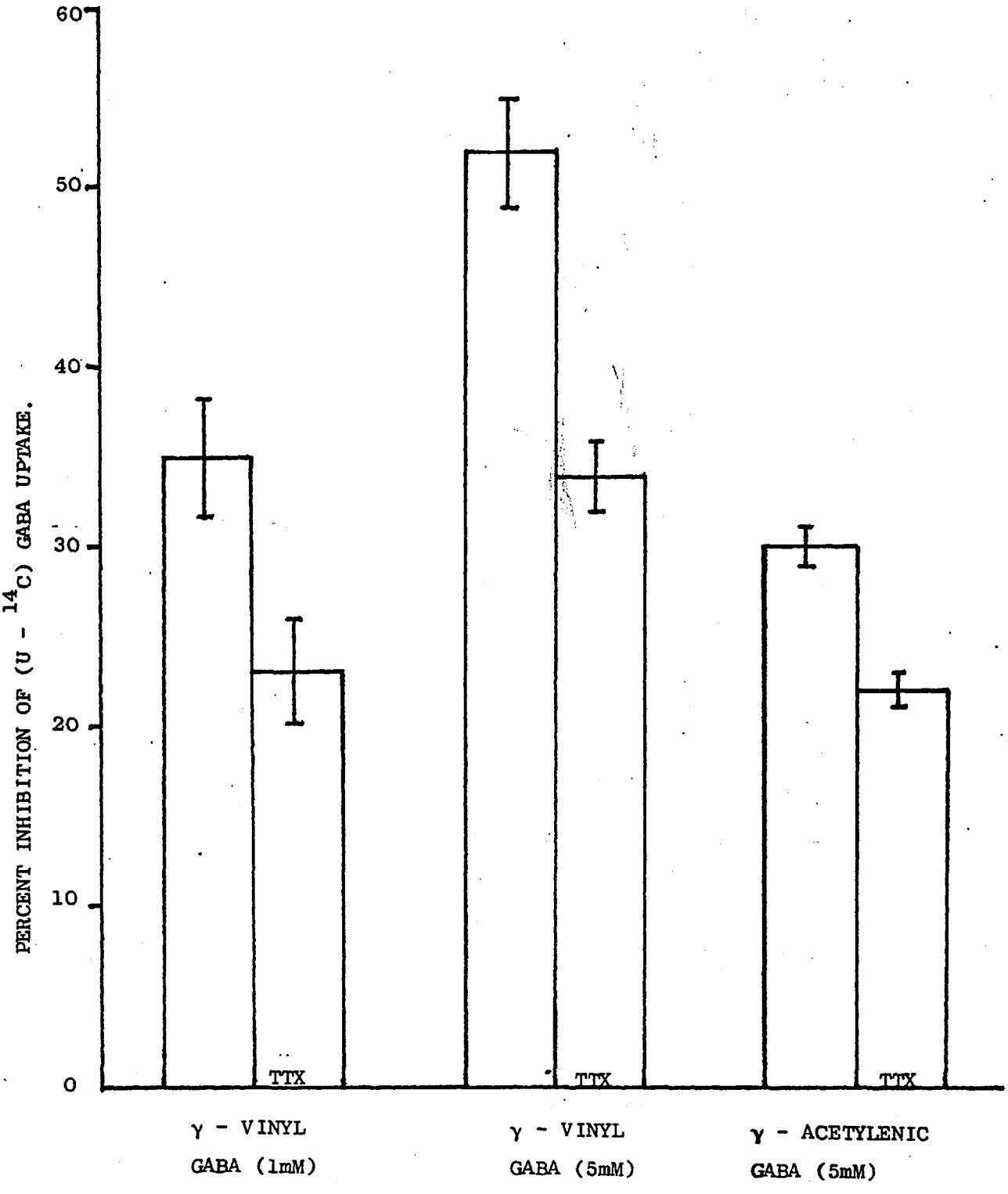


FIG. 7.6

EFFECT OF TTX ON THE INHIBITION BY γ - VINYL GABA AND γ - ACETYLENIC GABA OF (U - ^{14}C) GABA UPTAKE BY CEREBRAL CORTICAL SYNAPTOSOMES.



with concentrations below 0.5mM of either drug. Similar effects on uptake were obtained after 1min., and 2.5min., incubation with (U - ^{14}C) GABA using both drugs at both concentrations, in an attempt to ensure that significant quantities of GABA which had been taken up were not re - released (FIG. 7.5).

At this point , it was difficult to rule out that there was a real component of inhibition of GABA uptake by γ - acetylenic GABA and γ - vinyl GABA, which is a critical consideration for a valid interpretation of the release studies. These compounds are structural analogues of GABA, and at these concentrations, they may compete with GABA for the transporter. If the observed inhibition of uptake was due to the TTX - or verapamil - sensitive release process, then TTX or verapamil should prevent the apparent effect of the drugs on uptake. This was tested by means additional uptake experiments, and a time course experiment of the kind described in a previous chapter.

FIG. 7.6 illustrates the data obtained when the inhibition of (U - ^{14}C) GABA high affinity uptake as a consequence of γ - acetylenic GABA and γ - vinyl GABA administration was measured in the presence of 1μM TTX. TTX administration significantly abolished a proportion of the apparent inhibition of uptake, and the remaining component of the (U - ^{14}C) GABA accumulation appeared to be a real inhibition of GABA uptake by the agents.

When the release and uptake inhibition of (U - ^{14}C) GABA were measured under similar conditions, but using 1mM and 5mM GABA to replace γ - acetylenic GABA and γ - vinyl GABA in these experiments, the following results were obtained.

GABA at 1mM increased the degree to which previously preloaded (U - ^{14}C) GABA accumulated in the medium by 238%, and 5mM GABA by 234% over controls, to which 'cold' GABA was not added. Intriguingly, this efflux over the 5min. incubation period was not significantly TTX dependent, and the (U - ^{14}C) GABA accumulation in the presence of the active Na^+ channel blocker was 217%, and 187% respectively.

Similarly, when the uptake of (U - ^{14}C) GABA was measured into synaptosomes incubated in the presence of 1mM and 5mM GABA, the percent inhibition was 90%, and 95%, respectively , relative to the control synaptosomes, to which no cold GABA was added. Again, the inhibition of (U - ^{14}C) GABA uptake was not significantly dependent upon prior addition of 1μM TTX (89.5%, and 94.3% inhibition respectively)

DATA LEGEND.

RE : FIG. 7.7.

TIME COURSE OF THE ACCUMULATION OF AMINO ACIDS IN SYNAPTOSOME SUSPENSIONS SUBSEQUENT TO ADDITION OF γ - VINYL GABA.

Cerebrocortical synaptosomes were suspended in Krebs phosphate medium in 25ml conical flasks in a shaking waterbath at 37'C. Subsequent to an equilibration period of 10min., samples of volume 0.5ml. were taken at the relevant times shown. These samples were immediately sedimented in a bench top ultracentrifuge, and fractions were separated, and stored until the end of the experiment in cardice / propanol.

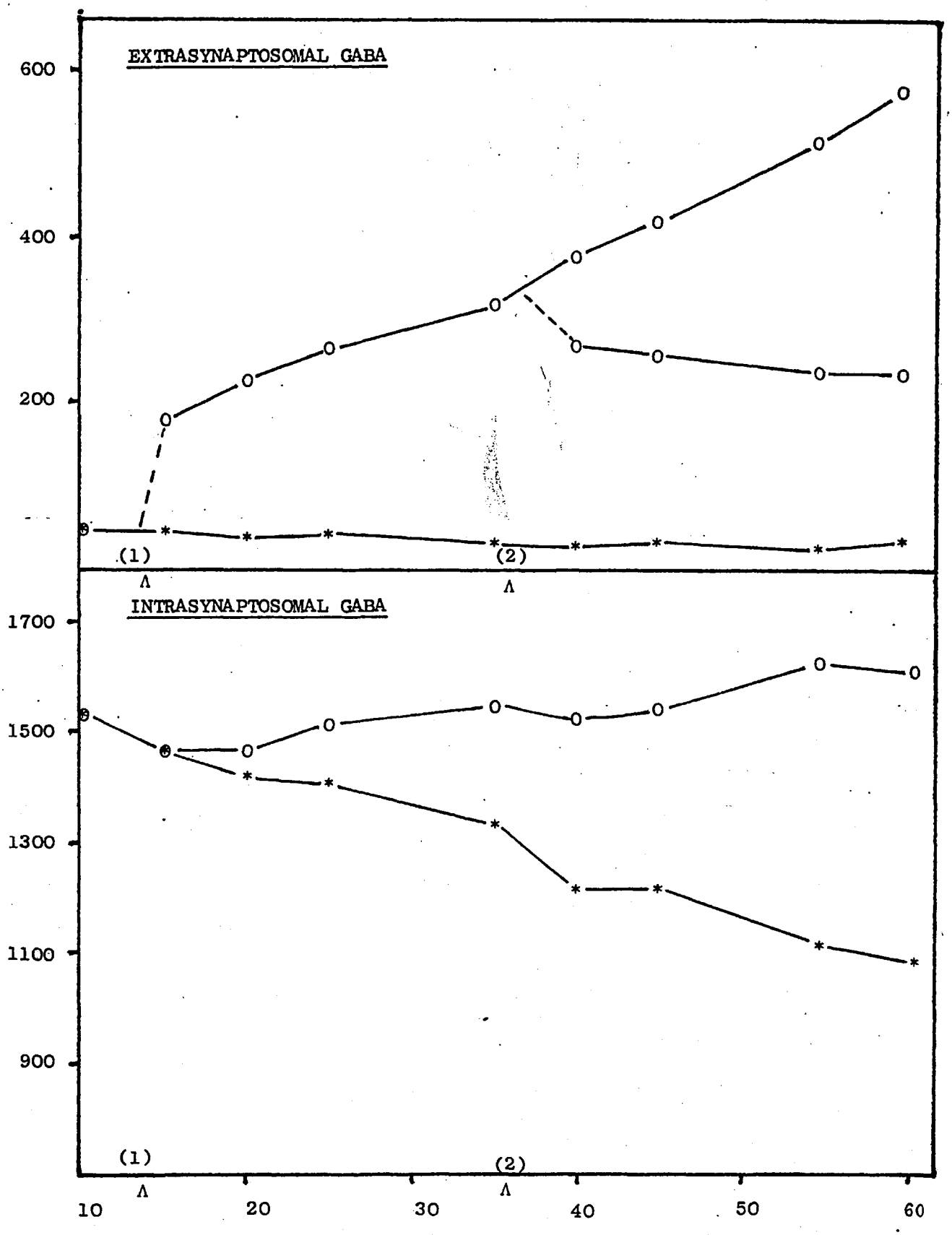
At the time indicated by (1), γ - vinyl GABA was added to a final concentration of 5mM, as appropriate. At the time indicated by (2), TTX was added to a final concentration of 1μM, as appropriate. Graphs with notation (*) are control experiments. Graphs with the notation (O) represent experiments to which γ - vinyl GABA was added.

At the end of the experiment, all fractions were extracted, and autoanalysed for amino acid content. Pellet protein contents were determined by means of the Folin reagent.

Each datum point is the mean of three experiments. Individual experimental values lay within 10% of their mean.

FIG. 7.7

TIME COURSE OF THE ACCUMULATION OF AMINO ACIDS IN SYNAPTOSOME SUSPENSIONS SUBSEQUENT TO ADDITION OF 5mM γ - VINYL GABA.
Y - COORDINATE = nmol amino acid accumulation / 100mg protein.



In spite of the difficulty of providing an explanation for these data, it is perhaps sufficient for the purposes of the present investigation to observe that the lack of TTX dependency of these effects indicates that γ - acetylenic GABA and γ - vinyl GABA are exerting their effects through a mechanism which cannot simply be described in terms of acting at presynaptic GABA receptors or being recognised as GABA.

6) Time course of the accumulation of amino acids in synaptosome suspensions subsequent to addition of γ - vinyl GABA.

On addition of γ - vinyl GABA to cerebrocortical synaptosome suspensions, there was a large and rapid accumulation of endogenous GABA in the incubation fluid, and this accumulation continued but at a slower rate for the remainder of the experiment (FIG. 7.7) when no further additions were made. At 60min., GABA accumulation had increased by nearly 2000% relative to the control incubated in the absence of γ - vinyl GABA. When 1μM TTX was added, there was a rapid fall in the level of endogenous GABA measured extrasynaptosomally, which quickly reached a steady state within 5min.. In the presence of TTX, GABA accumulation no longer increased, but then neither did it fall significantly below the plateau level. A similar profile was obtained when verapamil (100μM) was added at the same time, but independently of TTX (data not shown).

Measurement of synaptosomal pellet content of GABA over the period of the incubation revealed that the administration of γ - vinyl GABA (5mM) did not result in a significant increase in the levels of GABA - rather, γ - vinyl GABA maintained intrasynaptosomal GABA content at a steady level, in contrast to the control incubations, where internal GABA levels fell significantly during the experimental period.

The accumulation in the incubation medium and the intrasynaptosomal contents of all other amino acids measured - apart from GABA - showed no change as a consequence of γ - vinyl GABA administration, although β - alanine (also a substrate for GABA - transaminase (BEART & JOHNSTON, 1973)) levels were not measured. These amino acid profiles were similar to those described for other time course experiments in an earlier chapter.

DISCUSSION

In this study, γ - acetylenic GABA and γ - vinyl GABA, at concentrations above 0.5mM and 0.25 mM respectively, cause a synaptosomal release of GABA which is apparently dependent upon TTX - sensitive Na^+channels and verapamil sensitive Ca^{++}receptors or channels. These results suggest that the drugs may be depolarising the synaptosomes in some way that appears specific for GABA, since endogenous glutamate and aspartate are not released by these drugs in vitro (in contrast to what is seen with veratrine). While the mechanism for such an action is unclear, these findings are intriguing, providing they relate to the drug's action in vivo.

In vivo , γ - acetylenic GABA and γ- vinyl GABA were found to elevate GABA levels in the cortex superfusate (ABDUL GHANI et al.,1980). That this effect is TTX- or verapamil- sensitive has not been determined to date. It is possible that the in vivo results are explained by the data presented showing that pretreatment of animals with γ - acetylenic GABA or γ -vinyl GABA leads to elevated synaptosomal GABA levels and an elevated resting GABA release which was not significantly TTX - sensitive. The action of verapamil on this elevated release from synaptosomes prepared from rats pretreated with the drugs has not yet been determined. Thus it is difficult to reach the conclusion that levels of at least 0.5 mM (γ - acetylenic GABA) and 0.25 mM (γ - vinyl. GABA) are reached in vivo following intraperitoneal injection. It is not sufficient to measure total brain levels of the drugs following administration because substantial concentration of the drugs may occur for instance at nerve terminals.

PERRY et al., (1979) have shown evidence for reduced total brain GAD activity subsequent to chronic administration to rats of γ - vinyl GABA. The data presented indicate that some inhibition of GABA synthesis may be occuring, and for instance at the end of the time course incubation, total GABA levels (intra - plus extra - synaptosomal GABA levels) in the presence of γ - vinyl GABA were only 93 % higher than in the absence of γ - vinyl GABA. Similarly there was an increase of 300 % in GABA levels as a consequence of pretreating rats with a single dose of γ - vinyl GABA 14h., prior to sacrifice. FONNUM & WALBERG (1973) estimated that the intraterminal GABA concentration was approximately 60 mM, and that there is sufficient GAD present to synthesise this amount 5 - 6 times per hour. This is an important

question as there is much evidence that GAD activity can act as a regulator of cerebral excitability. An equation has been developed which relates excitablity to both GAD activity and GABA levels, with the former being the major factor (WOOD & PEESKER, 1974). There appears to be a threshold level of GAD activity below which convlsions occur (TAPIA et al., 1975). This implies that there is a relatively rapid turnover of transmitter GABA and that newly synthesised GABA is usually readily available for release from GABA terminals. 3 - Mercaptopropionic acid, a potent competitive inhibitor (WU & ROBERTS, 1974) of GAD, produces convulsions, decreased GAD activity, and decreased GABA levels in rats seven minutes after intraperitoneal injection (KARLSSON et al., 1974).

During short term incubations, changes in GABA content subsequent to drug administrations are negligible, and it may be concluded that blockade of GABA - T leading to an accumulation of GABA in a compartment from which it spills out into the medium is not a contributory factor in the release of GABA at least in these in vitro studies.

It is not possible to rule out that there is some inhibition of GABA uptake by γ - acetylenic GABA and γ - vinyl GABA, which is an important consideration for interpreting the release studies. The inhibition of uptake of ($U^{14}C$)- GABA during incubation times of 1 min., and 2.5 min.,(during which time only a very small proportion of GABA taken up could be subsequently released) shows that these compounds, which are after all, structural analogues of GABA, and at these concentrations, may compete with GABA for the transporter. However, a significant component of the observed inhibition of uptake is due to a TTX - sensitive release process, and indeed, in the presence of TTX, a proportion of the inhibition of GABA uptake was negated. It is intriguing nevertheless that verapamil blocks the entire accumulation of exogenous GABA in the medium when this compound is added prior to γ - acetylenic GABA or γ- vinyl GABA. (Fig 7.2)

The conclusions that can be reached at this stage are that

a)

γ - acetylenic GABA and γ - vinyl GABA cause an accumulation of GABA intrasynaptosomally and extrasynaptosomally.

b)

A component of the extrasynaptosomal GABA accumulation is caused by an inhibition of GABA uptake and may be a direct consequence of

structural analogues of GABA competing with GABA for the transporter.
c)

A component of the extrasynaptosomal GABA accumulation is caused by an action of the drugs at active Na^{+} channels and can be blocked by a Ca^{++} antagonist.

The question mark hanging over the precise role of evoked GABA release, inhibited GABA uptake, and total GABA accumulation for the action of γ - acetylenic GABA and γ - vinyl GABA as possible therapeutically relevant anticonvulsant agents and especially as antagonists of Verruculogen induced tremor is particularly important, and consequently it is interesting to note that many anticonvulsant agents are not only inhibitors of GABA - T, but also have effects on GAD and often on GABA uptake.

Aminooxyacetic acid is a potent competitive inhibitor of GABA - T (SCHOUSBOE et al., 1974), and is one of the most widely used agents for increasing the levels of GABA in the brain. Unfortunately, aminooxyacetic acid seems to have other effects that must be taken into account. The anticonvulsant action of aminooxyacetic acid appears to involve at least two mechanisms, only one of which is concerned with the GABA system (WOOD & PEESKER, 1975; MURAKAMI et al.,1976). The depressant action of aminooxyacetic acid on spinal reflexes does not appear to involve GABA. (BELL & ANDERSON, 1974). It is a carbonyl-trapping agent which is a quite potent competitive inhibitor of GAD (WU & ROBERTS, 1974) and which inhibits most if not all transaminases (SCHOUSBOE et al., 1974) and the transport of many amino acids and amines (JOHNSTON & BALCAR, 1974).

Sodium di -n propylacetate (sodium valproate) is used clinically in the treatment of epilepsy (SIMON & PENRY, 1975). Systemic administration of sodium valproate protects against seizures and increases brain levels of GABA (SIMLER et al., 1973). Although the agent has been reported to be a competitive inhibitor of GABA - T (SIMLER et al., 1973), it has a more potent actionon succinic semialdehyde dehydrogenase, the next enzyme in the metabolic sequence from GABA - T than on GABA - T itself. (HARVEY et al., 1975). Sodium valproate does not influence GABA uptake at 1 mM (BALCAR & MANDEL, 1976). A pharmacokinetic study on the anticonvulsant action of valproate in monkeys indicated that it has multiple effects (LOCKARD & LEVY, 1976) and studies using mice have shown a clear anticonvulsant action in the absence of significant changes in the levels of brain GABA (ANLEZARK

et al., 1976) and consequently it seems probable that sodium di -n-propyl acetate has other actions, besides effects on GABA degradation, that contribute to its anticonvulsant action.

The active site directed irreversible inhibitor of GABA - T ethanolamine - O - sulphate, introduced by FOWLER & JOHN(1972) is being used extensively to study the effects of increased levels of GABA. The agent forms an initial reversible complex with the enzyme followed by irreversible inactivation by a pseudo first order process in vitro Unlike aminooxyacetic acid, it does not inhibit GAD (FOWLER & JOHN 1972), but like aminooxyacetic acid, ethanolamine - O sulphate is a weak inhibitor of GABA uptake in rat brain slices.(BEART & JOHNSTON, 1973) and unlike sodium valproate, does not inhibit succinic semialdehyde dehydrogenase (ANLEZARK et al., 1976). Ethanolamine - O - sulphate does not elevate brain GABA after systemic administration and must be injected directly into the brain for unilateral injection into the substantia nigra to study the role of GABA in rotational behaviour (DRAY et al.,1975) and bilateral injection into the globus pallidus to assess the possible control by GABA of an accumbens - pallidal pathway (PYCOCK & HORTON, 1976).

Gabaculine, the active site inhibitor of GABA- T was discovered in a culture filtrate of Streptomyces toyocaemis subsp.,1039, and was characterised by KOBAYASHI et al., (1976) as (-) - 5 aminocyclohexa - 1, 3 - diene - 1 - carboxylic acid. Gabaculine is much more potent than ethanolamine - O - sulphate or γ - acetylenic GABA, (KOBAYASHI et al., 1977), and is a relatively weak inhibitor of GAD and a moderately potent inhibitor of GABA uptake. It does not act as a GABA agonist with respect either to the sodium independent binding of GABA to membranes from rat brain or to GABA receptors on cat spinal interneurons (ALLAN et al,. 1977). Systemically administered gabaculine increases brain levels of GABA and protects against picrotoxin induced convulsions.(MATSUI & DEGUCHI, 1977).

Although GABA is considered to be a major inhibitory transmitter in the brain, its precise role in modulating central nervous system excitability in general and in the mechanism of seizure inducing and seizure protecting drugs remains conjectural. However, it is clear that γ - acetylenic GABA and γ - vinyl GABA produce elevations in brain GABA concentration after peripheral administration, and this is associated with a differential anticonvulsant activity in animal systems. Similarly, both agents cause an accumulation of extrasynaptic GABA despite the fact that the precise mechanism and the relative roles

of accumulation components remains in question. Although suggestive, this association is not sufficient to ascribe a cause - effect relationship between protection against Verruculogen induced raised levels of excitatory transmitters extrasynaptically which are presumably eliciting the tremor symptoms, and increased brain GABA concentations. In addition to their inhibitory effects on GABA - T activity, γ - acetylenic GABA and γ - vinyl GABA have a less potent but significant irreversible action on brain GAD, and although less likely, these changes in GAD activity could play a part in tremor protection. Furthermore, being structural analogues of GABA , γ - acetylenic GABA and γ - vinyl GABA may act as direct GABA agonists at receptor sites, and also of course, an inhibition of GABA uptake mechanisms may prolong the action of this inhibitory transmitter. Elucidation of a mechanism for tremor protection at the neurochemical level must await the success of in vitro additions of tremorgenic mycotoxins or their active metabolites to synaptosomes with concomitant releasing effects similar to those observed subsequent to the preparation of synaptosomes from animals injected with the toxins. Such a mechanism may well involve presynaptic receptors and will surely further our understanding of the role for amino acids as neurotransmitters.

CONCLUDING REMARKS.

Nerve endings from many brain regions appear to survive the rigours of the preparative techniques with sufficient biochemical and morphological complexity to provide a functioning in vitro nerve terminal preparation. Chemical events which occur as hardly detectable trace signals in slices or other more intact systems become at once localised and amplified in the synaptosome preparation. The synthesis, storage, and depolarisation evoked release of transmitters become amenable to study, together with the actions of pharmacological agents on these processes. Comparing the in vivo actions of such agents with their effects on these in vitro preparations allows their level of action to be clarified - at least, for instance, whether primarily on cell body or on nerve ending. However, these possibilities are circumscribed by the degree of the damage, both biochemical and morphological, sustained by terminals and the extent to which they are degenerating and malfunctioning as a system in vitro. Nevertheless, synaptosomes prepared from mammalian brain show considerable biochemical versatility, performing in many ways like small whole cells when incubated in nutrient salines of appropriate composition. Evidence is presented to support the hypothesis that they are able to develop a membrane potential, an important property which would provide a good overall index of their capacity to accumulate certain ions and maintain their structure and metabolic integrity. On the basis of the evidence presented in the earlier chapters, and elsewhere, one may be confident about the performance of synaptosomes over at least several hours following isolation, during which time they are able to display a range of impressively sophisticated properties linked to the processes of neurotransmission and neurosecretion.

The project has been concerned with the function of amino acids of relatively simple chemical structure as excitatory and inhibitory transmitters regulating the activity of neurons throughout the mammalian central nervous system. Attention was drawn to the suggestion that these substances deserve consideration as major central transmitters involved with the relay of afferent information from the spinal to the cortical level, the control of cerebral pyramidal and cerebellar Purkinje cells, and of other cortical, subcortical, and spinal neurons.

The precise mechanism by which calcium exercises a role in the modulation of the release of the transmitter amino acids was brought

into question early in CHAPTER FOUR. The lack of conclusive evidence to define a solely intra - terminal source of the calcium required to elevate $(Ca^{++})_{in}$ (consequently evoking transmitter release from synaptosomes) does not detract from the validity of the suggestion. The finding of an answer to the question was not assisted by the proposition that at the concentrations used in those experiments, the calcium antagonist verapamil may also exert an action at active sodium channels. Nevertheless, the finding that extra - terminal sources of calcium were not necessary for the release of transmitter amino acids evoked by agents which act at the active sodium channel is an intriguing one. It will be interesting to investigate the dependence of the release of acetylcholine and dopamine (which are likely to be secreted by exocytosis) evoked by sodium channel active agents, on extra - terminal sources of calcium, and whether their release is also inhibited by verapamil.

Verapamil was used in addition to tetrodotoxin throughout the studies described here. The mechanism and specificity of action of tetrodotoxin has been documented here and elsewhere, and it is important to appreciate that the consequences of tetrodotoxin administration were by no means identical with those of verapamil administration. Thus, since the study of the mechanism of action of verapamil is still progressing, it is reasonable that the effects of this compound on the parameters measured throughout the studies, should have been included in the write - up. Even if these modulations cannot be explained with confidence at the present, the data will be of interest and importance in the near future, when the precise mechanism of action has been elucidated.

The data presented in CHAPTERS THREE and FOUR established the validity of using synaptosomal suspension techniques and procedures for the study of the effects and mechanism of action of the fungal tremorgenic mycotoxins. Two toxins, Penitrem A (extracted from Penicillium cyclopium), and Verruculogen (extracted from Penicillium estinogenum), were studied. The effects on amino acid release from synaptosomes prepared from animals dosed with each of the mycelia were in some ways comparable, and in other ways quite contrasting, despite the similarity in tremor observed in animals subsequent to injection to animals of the two toxins. The intriguing common feature of their effect was the very large elevation of aspartate and glutamate release

from rat cerebrocortical synaptosomes, and from sheep corpus striatum synaptosomes. P. cyclopium also elicited a small additional release of GABA from these preparations, but this effect was entirely absent in the case of P. estinogenum. That the raised levels of amino acid release were inhibited (by different degrees) by TTX and verapamil is, however, anomalous. (Recent experiments on sheep cerebrocortical synaptosomes have produced the same type of data - but not included here). Agents which depolarise by action at active sodium channels also elicit raised levels of synaptosomal respiration, which is required to provide additional ATP necessary to fuel the $Na^+ - K^+$ - ATPase in order to return to a status quo. Similarly, potassium loss is recorded from the synaptosome pellet. Neither of these parameters were significantly altered in the experiments performed. Perhaps depolarisation was occurring, but the change in membrane potential was not sufficiently large to alter these parameters at the sensitivity level at which they were measured. In such a case, why was GABA not released also by the treatments? These questions remain to be answered by work which is progressing on these features. It is valid to presume, however, that release, by whatever mechanism, of the excitatory amino acids in a dischordant manner, could well upset the delicate balance required for control of posture and movement of affected animals.

In order to study the mechanism of such actions, it was necessary to gain greater control of experimental conditions. It was at this point that enormous problems were encountered in respect of the aqueous insolubility of the purified tremorgenic compounds. Most solvents, even at very low concentrations, are likely to influence neural biochemistry in some manner, even if no obvious effect is observed on those parameters under study. However, extremely low solvent concentrations could not be used because the compounds under investigation then came out of solution. The example of ethanol was given in CHAPTER SIX, and of the six experiments performed, an effect was observed in only three. This effect that was observed was, however, highly compatible with data presented in the previous chapter. Of other techniques for presentation of the tremorgens to synaptosomes which were used, the liposome technique may well prove to be valuable. Work is progressing to improve this procedure.

Also presented in CHAPTER SIX was data describing the action of the

tremorgens at synapses in situ. Again, no evidence of depolarisation of these synapses was recorded, and the effect was limited to an elevation in the frequency of spontaneous potentials. Work is progressing on investigations of any TTX or verapamil sensitivity of this paramater. Nevertheless, an increase in spontaneous potential frequency essentially describes a situation analagous to the elevated release of transmitter from synaptosomes. The end result would be similar despite the fact that the mechanisms may not.

CHAPTER SEVEN describes the study of two irreversible inhibitors of GABA - transaminase. Since administration of these compounds, γ - acetylenic GABA and γ - vinyl GABA, alleviated the tremor associated with injection to animals of the fungal mycotoxins, it was necessary to explain why and how. The data evidenced a dual effect of the agents at the level of GABA accumulation. On the one hand, GABA uptake was inhibited, but on the other, strong evidence was presented which indicated that GABA release from synaptosomes was elevated in a manner which was not dependent on an inhibition of GABA - T. As in the case of Verruculogen, it was intriguing that, in this case, only GABA was released - and in a TTX and verapamil dependent manner.

It seems a little naive to assume that in a brain system which requires very delicate balance between inhibitory and excitatory circuitry, that an imbalance in excitatory transmitter release as in the case of the tremorgens, can be balanced by an elevation in inhibitory transmitter release using an essentially crude procedure. However, since the motor activity of the animals treated in this manner was not significantly depressed, such an explanation is an obvious one.

The project has enabled a description of the actions of tremorgenic mycotoxins on amino acid release. Unfortunately, very little progress has been made on a description of the precise mechanism by which these actions are produced. This is principally a consequence of the problems associated with the chemical nature of the compounds under study. Thus, further work will require approaching the problem in a different manner. One such path which will be pursued when the compounds have been produced with a sufficiently high specific radioactivity, will be a study of the binding characteristics of the toxins to brain and synaptic regions. A large amount of work is required to be done in this area.

The purpose of this project has been to study actions on amino acid transmitters in order to improve our knowledge of these systems. However,

it is also important to consider also, other transmitter systems. Data has been presented for a lack of any effect of the tremorgens on dopamine synthesis and release. Clearly, serotonin is a transmitter which should, and will, be investigated for a role in the tremor syndrome, particularly because of its chemical structure.

Much additional work now requires to be done.

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