AMINO ACID TRANSPORT AND RELEASE IN NEURAL TISSUE

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ABSTRACT

Cerebrocortical synaptosomes prepared from rat brains were frozen to -70^{o}C using glycerol (10% v/v) as a cryoprotectant. Although the frozen and thawed tissue retained major metabolic characteristics of oxygen uptake, K^{+} ion content, and LDH activity, the nerve ending showed no ability to release enhanced levels of candidate amino acid transmitters when stimulated in vitro.

The existence of a continuous uptake/release "couple" in the presynaptic membrane for the candidate amino acid neurotransmitters was determined using two in vitro experimental techniques. In particular, the uptake blocker PCMPS was found to induce a net enhancement to the flask synaptosome incubation medium of exogenous GABA (200%) and glutamate (225%) but not for leucine. Also, when synaptosomes were superfused so that the uptake/release couple of homoexchange could be observed, it was noted that only the short chain glutamate analogues e.g. homocysteic acid (containing 3 or less CH groups) evoked an exogenous ^{3}H glutamate signal to the superfusion medium, compared to the longer chain analogues (more than 3 CH groups) of DAP and adipic acid.

Evidence is presented that rat cortex synaptosomes synthesize prostaglandin PGD_2 (10.9ng/mg protein). Depolarization caused release of physiologically important amino acids but not prostaglandins.

Two independent ^{3}H glutamate binding sites were characterized in the cerebral cortex: a high affinity site with a K_D of 117nM, B_{max} 280pmoles/mg protein, and a low affinity site with a K_D of 23μM, B_{max} 280pmoles/mg protein. Inhibition of specific binding by glutamic acid analogues was also investigated.

The action of various glutamic acid analogues on spontaneous mepps detected at the locust neuromuscular junction were investigated. Most interestingly the compound (-)APB (500μM) was found to induce a reversible decrease (95%) in mepp frequency, similar to the effect produced by DL-pyroglutamate, the cyclic form of glutamate. However, the enantiomer (+)APB (500μM) had no detectable influence on either mepp frequency or muscle membrane potential.

Injections of GDEE and GDME into rats prior to the injection of the convulsant drug strychnine delayed the time of onset of convulsion by 335% and 221% respectively. Differential amino acid release and storage, as possible neurochemical correlates of these experiments,were investigated.

Attempts were also made to investigate the neurotransmitter(s) of the cortical striatal pathway in the rat. Using a stereotaxic lesioning technique, a specific decrease (25%) of evoked glutamate and aspartate release was observed from crude synaptosomes.

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PUBLICATIONS

Some of the work presented in this thesis has been incorporated in the following publications:

(1) Effects of Anticonvulsants on the In Vivo and In Vitro release of GABA.
Abdul-Ghani AS, Coutinho-Netto JC, Druce D, and Bradford HF. (1981).
Biochem. Pharmacology 30, 363-368.

(2) Suppression of Stimulus-Evoked Release of Neurotransmitters from Synaptosomes by Verapamil, a Supposed Ca^{2+} Antagonist.
Norris PJ, Dahliwal D, Druce D, and Bradford HF. (1981).
J. Neurochem. (submitted).

(3) Differential Amino Acid Neurotransmitter Release in Rat Neostriatum Following Lesions of the Cortical Striatal Pathway.
Druce D, Peterson D, and Bradford HF. (1981).
Brain Research (in press).

(4) The Action of Various Glutamate Analogues at the Locust Neuromuscular Junction.
Druce D, Collins JF, and Bradford HF. (1982).
Biochem. Pharmacology (submitted).

(5) Prostaglandins D_2 and E_2 are not Regulators of Amino Acid Release from Rat Cortical Synaptosomes.
Druce D, MacDermot J, Waddell KA, Bradford HF, and Blair IA. (1981).
Biochem. Pharmacology (in press).

(6) The effect of delaying the onset of strychnine induced seizure in rats, by the administration of glutamate diethyl ester and glutamate dimethyl ester. Abdul-Ghani AS, Druce D, and Bradford HF. (1982).
Biochem. Pharmacology (in press).

ABBREVIATIONS

Acetylcholine	ACh
γ-acetylinic GABA	GAG
γ-amino butyric acid	GABA
2-amino-4-phosphono butyric acid	APB
2-amino-5-phosphono valeric acid	APV
Central Nervous System	CNS
p-chloromercuriphenyl sulphonic acid	PCMPS
di-amino pimmeleic acid	DAP
dihydrokainic acid	DHK
kainic acid	KA
lactate dehydrogenase	LDH
miniature end plate potentials	mepps
Neuromuscular Junction	NMJ
Prostaglandin D_2	PGD_2
Prostaglandin E_2	PGE_2
Prostaglandin $F_{2\alpha}$	$PGF_{2\alpha}$
Prostaglandin I_2	PGI_2
DL-pyroglutamatic acid	DL-PG
Tetrodotoxin	TTX
Thyroxane B_2	TXB_2
γ-vinyl GABA	GVG

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CHAPTER 1

General Introduction

1.1 Introduction

At the turn of this century, Cajal and Golgi (1906) established that the brain is made up in part of isolated independent units, separated by a system of clefts about 150Å in width. Sherrington (1897) termed these junctions as synapses. It is now well established that the majority of inter-neural signalling occurs across the synapse, by release of specific small molecules (neurotransmitters) from the pre-synaptic site of a neuron, to interact with specific localized receptors on a post-synaptic neuron. Since thousands of synaptic contacts converge upon each of the millions of central neurons, and since each neuron in turn projects thousands of terminals upon other cells, it is clear that neuronal interactions are extraordinarily complex. The mechanisms concerning how neurons communicate with each other form the basis of all brain action. Thus events occurring at the synapse, concerning neurotransmitter biosynthesis, storage, release, receptor binding and its inactivation, will be of functional significance.

Certain simple amino acids may well be operating the majority of the synapses of the mammalian brain. This proposition follows as a possible corrollary from the dramatic advances of the past two decades, in our knowledge and understanding of synaptic organisation at the chemical level. Glutamate, aspartate γ-aminobutyrate (GABA) and glycine are the principal amino acids currently considered to act as neurotransmitters throughout the central nervous system. The assignment of these amino acids for neurotransmitter roles has been based upon the compliance with which

these compounds fulfill certain criteria. These criteria are based on the evidence obtained for the transmitter function of acetylcholine (ACh) at vertebrate neuromuscular and ganglionic synapses (see Paton, 1958, for review article). The general characteristics that suspected neurotransmitters should exhibit in transmission processes are listed below;

(1) The presence of a presynaptic biosynthetic pathway.

(2) The presence of a transmitter storage mechanism at the presynaptic site.

(3) The release of the candidate transmitter in quantitative amounts following physiologically-induced stimulation.

(4) The identity of a postsynaptic action.

(5) The presence of specific mechanism(s) to rapidly inactivate the action of the transmitter.

Neurochemistry has proved to be an essential discipline in the investigation of these criteria in relation to the putative amino acid transmitters. The neurochemical evidence, as well as the data from electrophysiological studies and other disciplines, is presented below.

1.2 Amino Acids as Neurotransmitters

On the whole, there has been slow acceptance by neurobiologists of a neurotransmitter role for amino acids, and this has been partly due to an understandable reluctance to see critical synaptic functions controlled by such simple ubiquitous compounds. The amino acids in question exist in most cytoplasmic compartments of the brain and other tissues in concentrations (10^{-3}M) several orders of magnitude higher than those required for transmitter action, and they are, of course, widely involved in biosynthesis and other metabolism.

(a) Excitatory Amino Acids, Glutamate and Aspartate

The naturally occurring nonessential amino acids, L-aspartate and L-glutamate may have major roles as excitatory neurotransmitters. Although the direct evidence for the role of L-aspartate is limited to data from the spinal cord, much of the evidence pointing to a neurotransmitter function for L-glutamate may apply equally well to L-aspartate. L-glutamate is a somewhat more powerful excitant and,of course, it is found in much larger amounts in the central nervous system, and therefore seems likely to play the more important role. Hence the main emphasis here in this review is on glutamate.

(1) Distribution and Metabolism

Glutamate is well known as the amino acid found in highest concentrations (5-10 μmol/gm) in the brain and in the spinal cord, the highest being in the forebrain and in the cerebellum. In parts of the brainstem, such as the medulla and the pons, the concentration is about 50% less (Berl and Waelsch, 1958; Perry et. al., 1971; Shaw and Heine, 1965). When a virus was used to selectively deplete 95% of cerebellar granule cells, the concentration of glutamate in the cerebellum was markedly decreased. No decrease in other amino acids including aspartate was observed (Young et. al., 1974).

Subcellular fractionation studies on rat and guinea pig brain homogenates do not indicate a selective localization of L-glutamate or L-aspartate with nerve-ending particles (Mangan and Whittaker, 1966), although the content is thought adequate for substantial excitatory effects on nerve cells

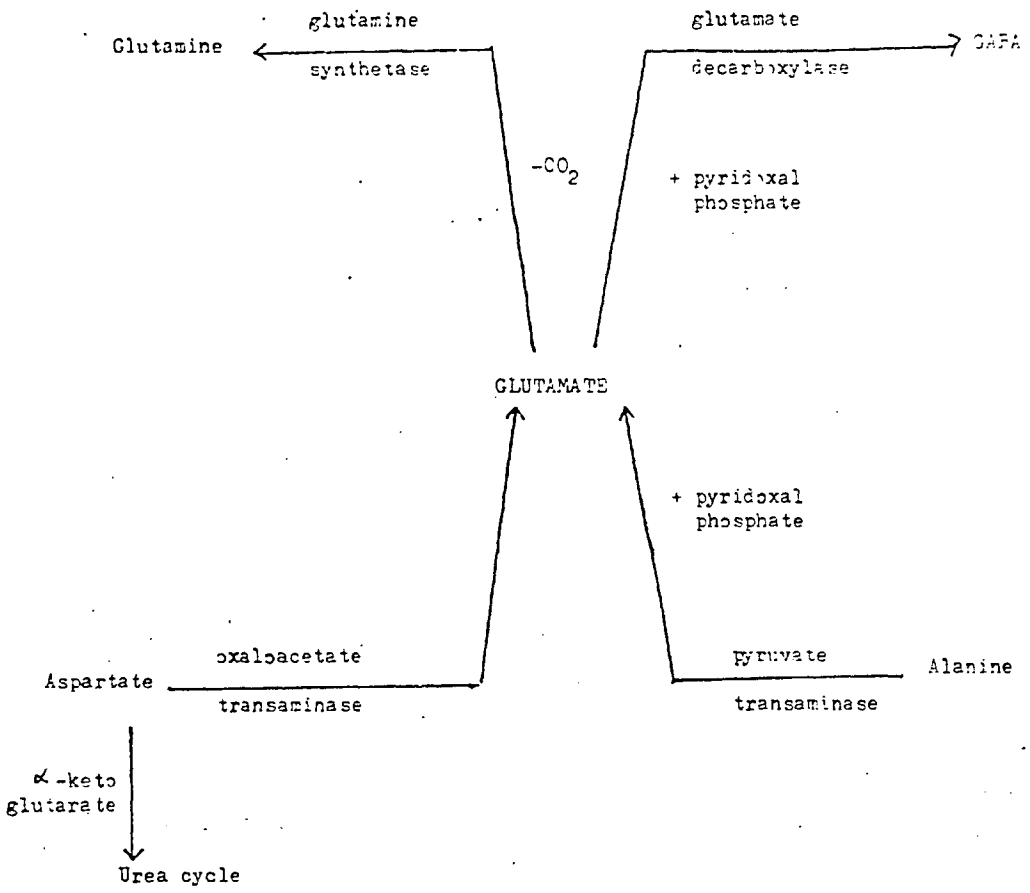
(Krrjevic and Whittaker, 1965).

The possibility of separate pools of amino acids for synthesis and storage has received growing acceptance during the past decade. Glutamate appears to be present in at least two different compartments of the brain. One compartment (a large pool) is very quickly and effectively labelled with systemic glucose and glucogenic precursors, whereas another glutamate compartment (a small pool) is labelled more effectively by injections of radioactive pyruvate acetate and by exogenous glutamate; also it is particularly closely associated with glutamine (Berl and Frigyesi, 1969; Lajtha et. al., 1960; Rose, 1970; Tarkowski and Cremer, 1972; O'Neal and Koeppe, 1966; Van den Berg, 1970). It is tempting to assign metabolic compartments of L-glutamate in terms of morphological components such as neurons, glial cells and of course synapses, but these attempts have met with little success to date.

(2) Release

The release of L-glutamate and L-aspartate has been studied by techniques capable of inducing neuronal depolarisation in mammalian brain preparations e.g. electrical stimulation (Katz et. al., 1969) and high potassium concentrations (Bradford, 1970). Roberts and Mitchell (1972) observed a calcium-dependent increased rate of L-glutamate, L-aspartate, glycine and GABA (but not of other amino acids) from isolated frog and toad hemicords when they directly stimulated the spinal cord. Similarly, electrical stimulation of the amphibian sciatic nerves increased the rate of release of both glutamate and aspartate (Wheeler et. al., 1966;

Figure 1.1 Metabolism of Glutamate, Alanine and Aspartate



De Feudis, 1971). However, the most convincing evidence that glutamate is released by the activity of nerve endings in the central nervous system is the in vivo work of Jasper and Koyama (1969). These workers showed that electrical stimulation of the cat reticular formation induced an increase in leakage of endogenous glutamate and to a lesser extent aspartate into fluid superfusing the cerebral cortex of the animal. Thus they were able to show a correlation of cortical activity with glutamate release. Similarly, Crawford and Conner (1973) detected endogenous glutamate efflux from the hippocampus in response to local or topical stimulation. A more sophisticated cannulation method for studying endogenous amino acid release has been developed by Dodd and Bradford (1974, 1976). There is also evidence that electrical stimulation of cortical slices (Hammerstead and Cutler, 1972) and cortical synaptosomes (Bradford, 1970b) evokes release of labelled glutamate.

(3) Postsynaptic action

Small amounts of L-glutamate or L-aspartate exert a rapid and potent depolarization of neurons in virtually all areas of the vertebrate central nervous system. Studies by Duggan (1974), however, have demonstrated differential sensitivity to the two amino acids, between physiologically distinct types of spinal interneurons in the cat. These so-called "glutamate-type" and "aspartate-type" receptors are thought to have differential affinities for various glutamate analogues. Thus for example, it is considered that N-methyl-D-aspartate, DL homocysteate, ibotenate have more affinity for aspartate receptors than

for glutamate receptors; and L-glutamate, glutamate diethyl ester and possibly kainate have greater affinity for glutamate receptors than for aspartate receptors (Curtis et.al., 1972; De Plazas and De Robertis, 1976; De Robertis and De Plazas, 1976a, 1976b).

The observed postsynaptic depolarizing action of glutamate seems mainly dependent on an increase in the permeability of the membrane to sodium ions (Curtis et.al., 1972) as does the the conduction of action potentials. However, different sodium channels are involved in the two processes as tetrodotoxin (TTX) blocks sodium-mediated conduction but not amino acid induced depolarization.

(4) Inactivation

In contrast to the situation at ACh synapses, enzymic degradation of excitatory (or inhibitory) amino acids does not appear to be the major mechanism for termination of neurotransmitter action. The supposed inhibitors of glutamate decarboxylase metabolism were found to have no effect on the latency of glutamate-induced excitation at cat Renshaw interneurons (Curtis et.al., 1960). An alternative mechanism for the rapid removal of glutamate from the synaptic cleft is via the high affinity uptake system present in glia (Henn et.al., 1964) and in synaptosomes, isolated from the central nervous system (Snyder et.al., 1973). This phenomenon has been confirmed and studied under various conditions by many authors (Balcar and Johnstone, 1972; Logan and Snyder, 1971,1972; Tsukade et.al., 1963). It appears that the high affinity uptake system for glutamate is a sodium dependent process, in contrast to an additional sodium independent low affinity

uptake. Glutamate uptake is competitively inhibited by aspartate, suggesting that the transport receptor site can bind both amino acids. However, compounds such as DL homocysteate and N-methyl-DL-aspartate have no inhibitory action on the high affinity uptake of glutamate, indicating that receptor sites mediating excitation and transport are conformationally distinct (Duggan, 1974; Roberts and Watkins, 1975).

(b) Inhibitor amino acids, GABA, Glycine and Taurine

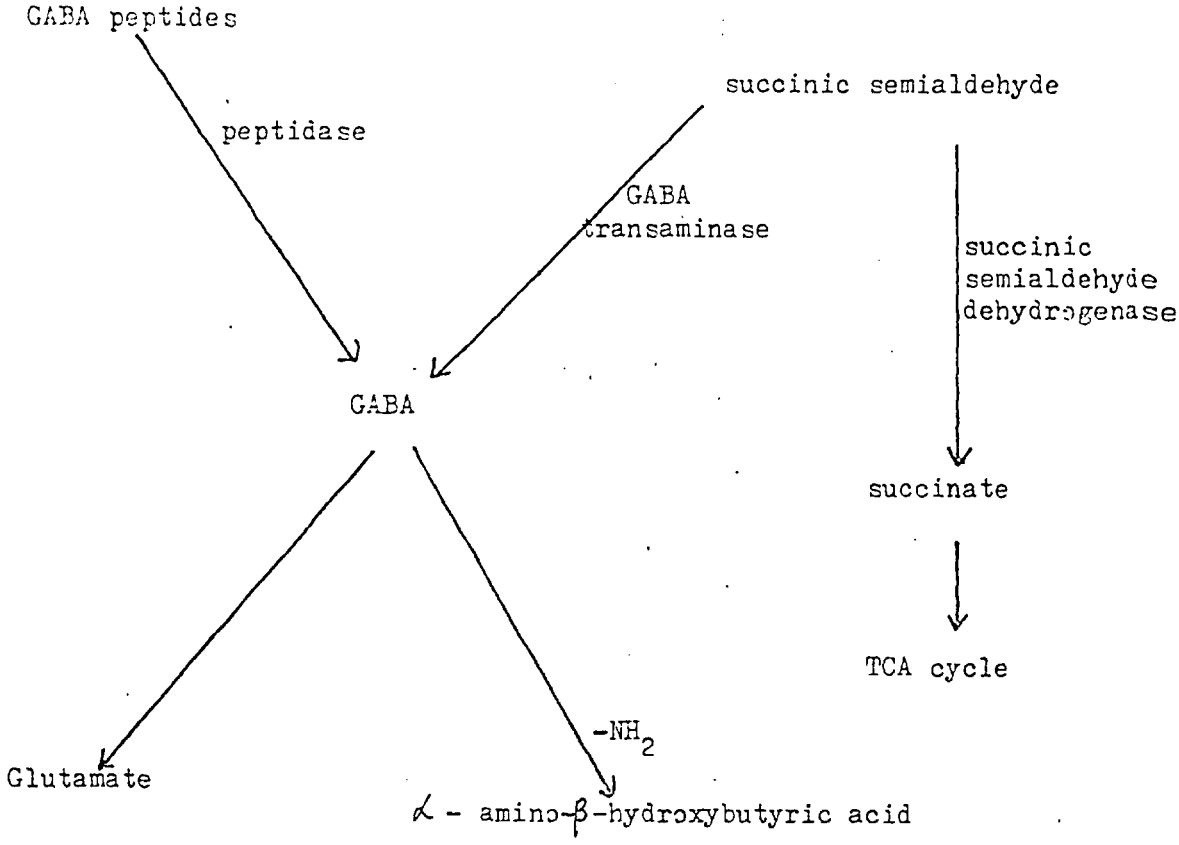
As with the excitatory amino acids, iontophoretic studies have played a major role in determining their mode of action. Thus, GABA and glycine have been shown to have a depressant effect on many mammalian neurones (reviewed by Werman and Aprison, 1968; Curtis and Watkins, 1965; Curtis and Johnston, 1974).

The amino acid, taurine, has also been shown to inhibit neuronal firing; however, in general, it has a weaker depressant action than GABA (Crawford and Curtis, 1964; Krnjevic and Phyllips 1963). Hence, since there is still considerable doubt as to taurine's proposed role as a neurotransmitter, I shall in the main consider in this review GABA and glycine.

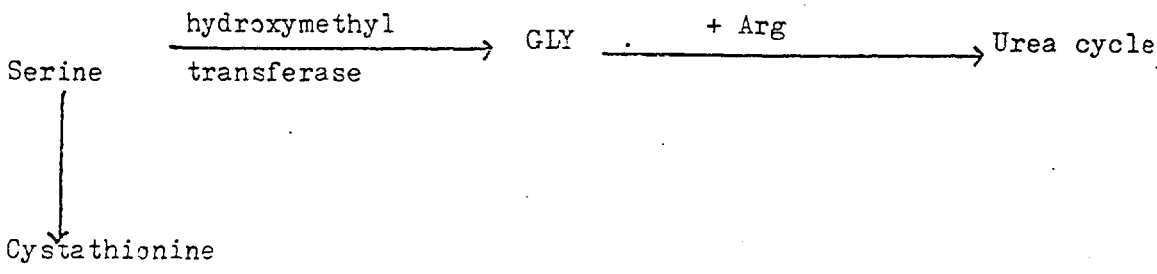
(1) Distribution and metabolism

The discovery of GABA in the mammalian brain in the early fifties initiated numerous studies on its distribution (Roberts and Frankel, 1950; Awapara et.al., 1950). It has been found in particularly high concentration (10^{-3}M) in the spinal cord and substantia nigra and in the striatum (Fahn and Cote, 1968; Okada et.al., 1971). According to some fractionation studies, substantial amounts of GABA

Figure 1.2 Metabolism of GABA and glycine



(a) Metabolism of GABA



(b) Metabolism of Glycine

appear to be situated in nerve terminals (Mangan and Whittaker, 1966; Weinstein et.al., 1963), but most of the brain GABA content appears to be in a relatively free form in cell cytoplasm rather than bound to some subcellular particles (Elliot et.al., 1965; Ryall, 1964). However, the amount present in cortical synaptosomes has been found to produce significant inhibition postsynaptically (Krnjevic and Whittaker, 1965).

Studies on the GABA content of the cerebellum have shown that the highest concentrations are to be found associated with the inhibitory Purkinje cells (Hirsch and Robins, 1962; Kuriyama et.al., 1966).

Glycine is found in highest concentration in the ventral grey matter of the spinal cord (Aprison and Werman, 1965; Aprison et.al., 1968), the dorsal root ganglia and some of the peripheral nerves, e.g. sural nerve and gastro-nemius nerve (Duggan and Johnston, 1970).

GABA is biosynthesized from glutamic acid by a specific cytoplasmic decarboxylase. Glucose has been found to be rapidly converted into glutamate and GABA in rat brain studies (Gaitonde et.al., 1965; Yoshimo and Elliot, 1970) via the tri-carboxylic acid cycle (Roberts and Eidelberg, 1960), and this relationship between GABA metabolism and the TCA cycle - the so-called GABA shunt, is now well understood. A specific transaminase associated with mitochondria accelerates the catabolism of GABA to

succinic semialdehyde (Roberts and Bregoff, 1953; Salvador and Albers, 1959).

Glycine appears to be biosynthesized by glucose and ribose metabolism, with glyoxylate and probably serine as immediate precursors in the CNS (Shank et. al., 1973). Glyoxylate can be transaminated to glycine in the presence of various amino group donors (Johnston et.al., 1970; Benuck et.al., 1971). The enzyme serine hydroxymethyl transferase interconverts serine and glycine and has been purified from brain by Broderick et.al. (1972).

(2) Release

Further evidence that GABA may be a physiological mediator of inhibition is that in vivo release of endogenous GABA in cats has been demonstrated. The principal studies concern the release of GABA into the fourth ventricle of the cat following stimulation of Purkinje cells (Obata and Takeda, 1969), and secondly the detection of calcium-dependent GABA release from the cerebral cortex, following thalamic and brainstem stimulation (Iversen et.al., 1968; Jasper and Koyama, 1969).

Exogenous GABA can also be released from the cerebral cortex in vivo (Mitchell and Srinivasen, 1969) and in vitro (Srinivasen et.al., 1969) by electrial stimulation. These results though suggestive, are less convincing evidence, since many compounds can be accumulated and released by a variety of cells (Ng et.al., 1971; Ng et.al., 1972; Thoa et.al., 1969).

The release of endogenous glycine in vivo, following stimulation of inhibitory pathways, has to be demonstrated,

although the stimulus-induced release of exogenous glycine has been shown both in vivo and in vitro. Jordan and Webster (1971) demonstrated ^{14}C-glycine release from the spinal cord of anaesthetized rats, when the uptake blocker p-hydroxymercuribenzoate was induced in the medium.

Calcium-dependent evoked release of exogenous glycine, by electrical and potassium-induced stimulation, has been demonstrated in slices of rat spinal cord (Hopkin and Neal, 1970; Hammerstad et.al., 1971). Osborne and Bradford (1973) have reported evoked release of endogenous glycine from spinal cord synaptosomes.

(3) Postsynaptic action

GABA-induced hyperpolarization can be demonstrated in every part of the vertebrate CNS, including cerebellar Purkinje cells (Siggins et.al., 1971), spinal interneurons (Bruggencate and Engberg, 1968), cerebral cortex (Krnjevic and Shwartz, 1967), and Deiters nucleus in the medulla (Obate et.al., 1967). The ionic basis of this hyperpolarization is attributed to an increased permeability of the membrane to chloride ions and perhaps potassium ions.

The postsynaptic effects of GABA seem to be antagonized by at least three classes of compounds - picrotoxin, bicuculline, and benzyl penicillin. This has been taken as evidence for multiple GABA receptor sites. Thus the fact that two molecules of GABA are required for receptor activation at the crayfish neuromuscular junction (Takeuchi, 1976) and three molecules seem to be required at a receptor site on locust muscle (Brookes and

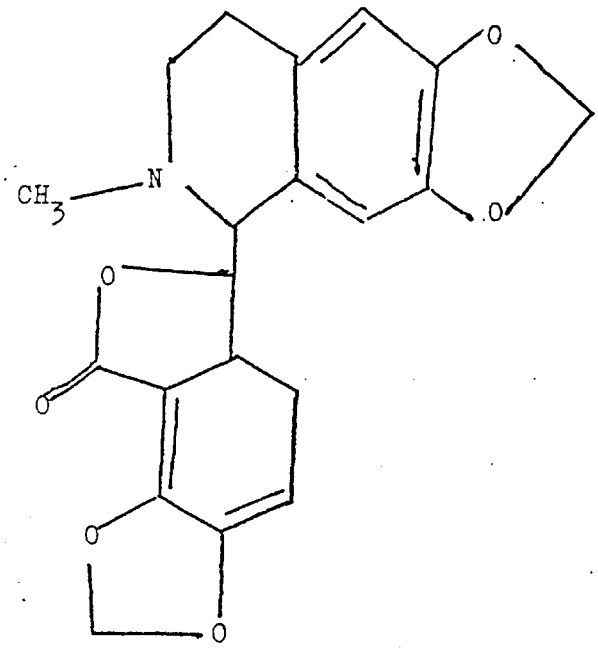
Werman, 1973), would suggest that several receptor sites must be activated by GABA, prior to increases in chloride conductance through the ionophore. Such elegant studies have not been attempted in the vertebrate CNS, because of the inaccessibility of GABA synapses. However, picrotoxin is known to antagonize a number of inhibitions in the mammalian CNS including the cuneate nucleus (Galindo, 1969), Deiters' nucleus (Bruggencate and Engberg, 1971), the spinal cord (Engberg and Thaller, 1970) and the cerebral cortex (Hill et.al., 1972).

The alkaloid bicuculline has been proposed as a specific antagonist of GABA in all regions of the mammalian CNS (Curtis et.al., 1970). The results by other workers (Tebecis et.al., 1971; Nicoll, 1971; Mclennan, 1971; Kelly and Renand, 1973) support the idea that bicuculline antagonizes GABA relatively specifically. However, Curtis and Felix's (1971) findings, and the work of Godfraind et. al. (1970), Biscoe et.al. (1963), Gardner and Gartside (1972),suggest that bicuculline is not a universally reliable antagonist of GABA-mediated inhibitions. Also, both picrotoxin and bicuculline may have a direct excitatory effect on many neurons, which greatly complicates the analysis (Godfraind et.al., 1970; Krnjevic et.al., 1966).

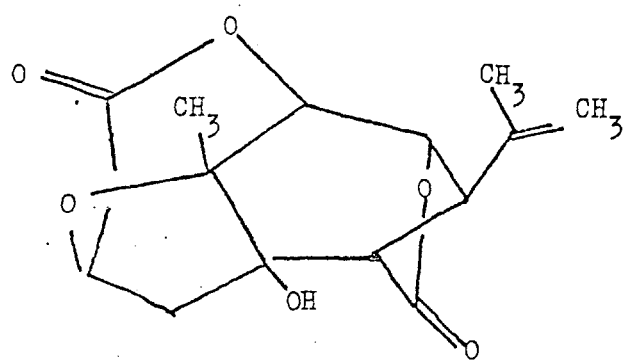
Benzyl penicillin is a convulsant that has been reported to antagonize GABA inhibitions in the mammalian cerebral cortex (Clarke and Hill, 1972) and in the amphibian spinal cord (Curtis, Game and Johnston, 1972; Davidoff, 1972). Bicuculline and penicillin are thought to act at the same receptors because of their similar

Figure 1.3 Chemical structures of some GABA antagonists

BICUCULLINE



PICROTOXININ



BENZYL
PENICILLIN

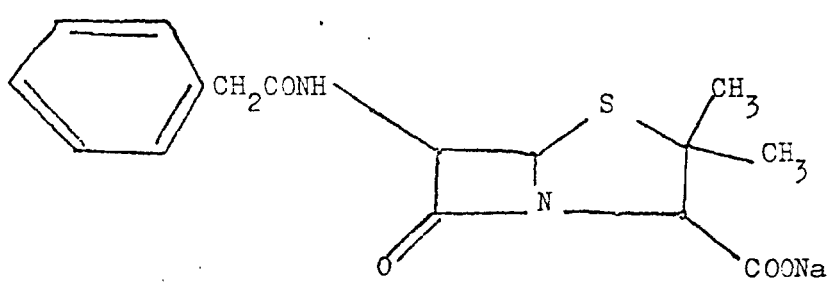
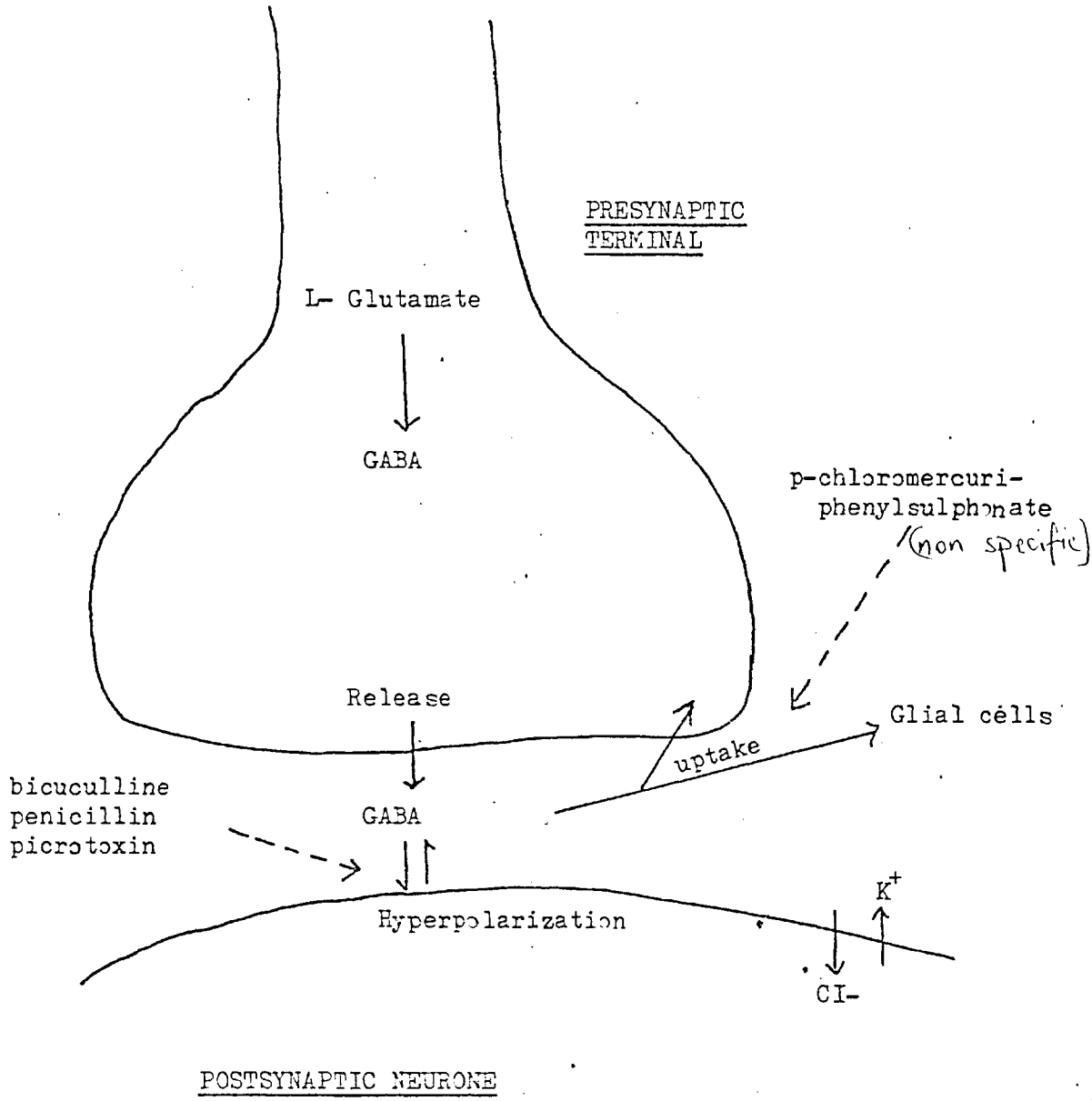


Figure 1.4 The GABA synapse



structure (Fig. 1.3).

Like GABA, glycine inhibits the firing of neurons throughout the mammalian CNS. Its hyperpolarizing action is similarly attributed to increasing membrane permeability to chloride and perhaps potassium ions.

Strychnine blocks the depressant action of glycine on neurons whilst having little or no action on GABA-induced depression in the CNS (Curtis et.al., 1968a).

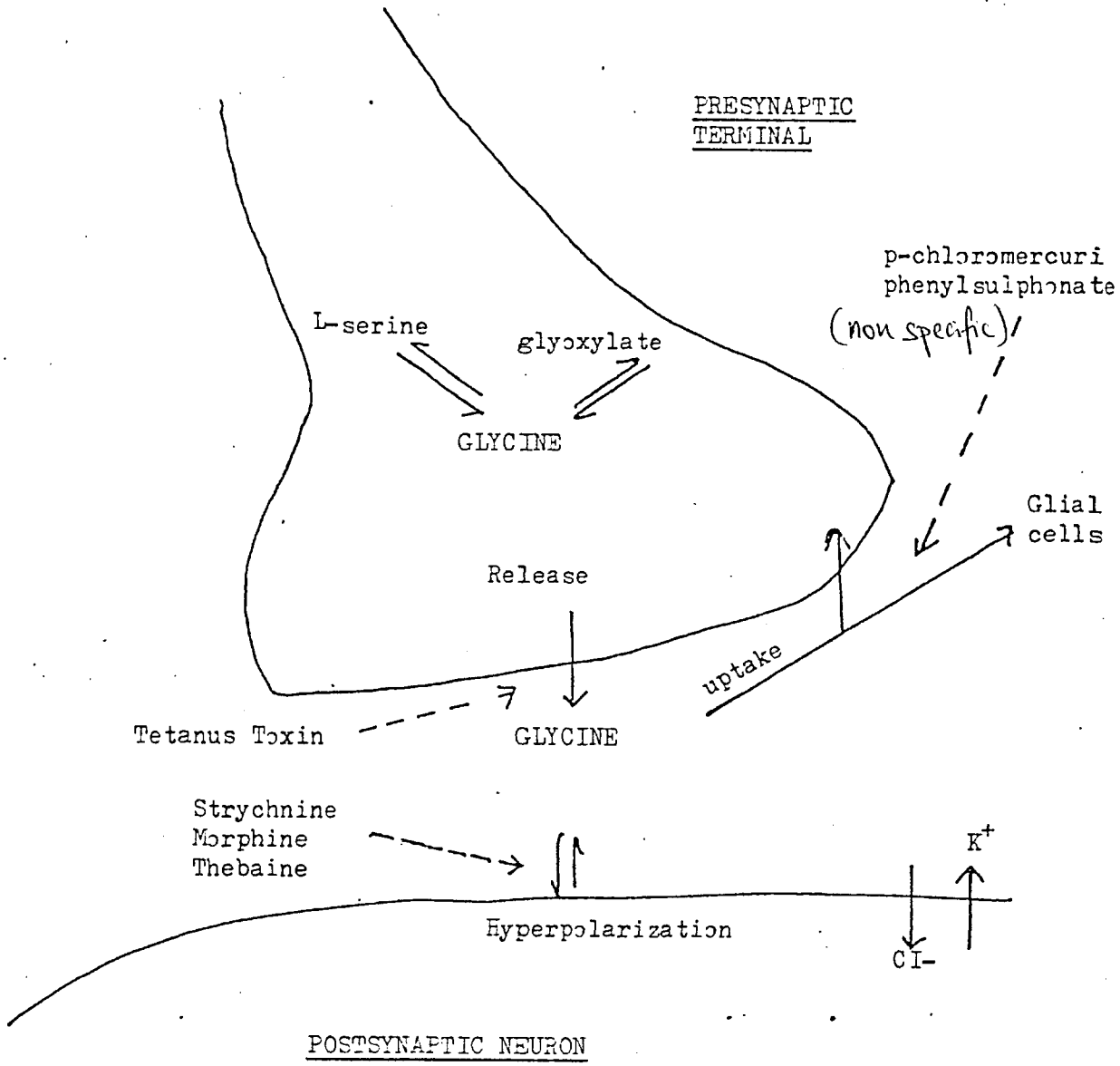
This remarkably clearcut division of amino acids on the basis of strychnine antagonism is also supported on the selective basis of bicuculline antagonism of GABA.

(4) GABA uptake

The most significant method of removal of GABA from extracellular space in the brain is the Na^+-dependent high affinity uptake process (Bennett et.al., 1972). Studies by Iversen and Neal (1968), and Beart et.al. (1972), have shown that some closely related amino acids can compete with GABA in this uptake system, although glutamate, aspartate and glycine do not appear to be active.

The GABA high affinity uptake system appears to operate in both nerve endings (Iversen and Bloom, 1972), and in neuroglia (Henn and Hamberger, 1971; Gottesfeld et.al., 1973). The GABA analogue β-alanine appears to inhibit GABA uptake into neuroglia, whereas 2,4 di-amino-butyric acid (DABA) appears to inhibit the uptake into nerve endings (Iversen and Kelly, 1975; Schon and Kelly, 1975).

Figure 1.5 The Glycine Synapse



A sodium-dependent and temperature-sensitive high affinity uptake system for glycine has been defined by Neal and Pickles (1969) and Neal (1971) in mammalian spinal cord slices. In the cerebral cortex, however, glycine is accumulated by low affinity uptake only (Johnston and Iversen, 1971; Logan and Snyder, 1971). This fact provides further evidence for glycine's role as an inhibitory neurotransmitter in the spinal cord.

1.3 Synaptosomes as a Tool in Neurobiology

The biochemical study of synaptic events occurring in the brain has been under investigation for some thirty years. Several experimental approaches have been used including:

(1) Cytochemical analysis of stain-fixed section of brain tissue.

(2) The use of the intracellular electrode to provide knowledge about impulse conduction and postsynaptic events associated with transmitter action.

(3) The biochemical analysis of enriched fractions of axon terminals, obtained by means of tissue dispersion with gradient centrifugation.

The latter approach was the method most commonly used for the work presented in this thesis, since the mass isolation of synaptosomes provides the scientist with an *in vitro* preparation for the routine study of chemical mechanisms underlying neurotransmission in the mammalian CNS. Being enriched in the biochemical agents and morphological structure directly mediating synaptic transmission, synaptosomes overcome some of the limitations of the more intact and complex preparations.

However, gains in simplicity are necessarily losses in complexity, and the absence of the surrounding glial cells, the axon and the postsynaptic cell, may also mean the absence of important modulating influences. Also preparations which necessarily involve tissue damage followed by membrane re-sealing obviously have limitations. This is particularly so where putative amino acids are concerned since because they are in such high concentration, any experimental effects could be due to artefactual release of neurotransmitter by tissue damage or to diffusion processes down concentration gradients. This is particularly so in synaptosomal flask incubations, where high fluid/tissue ratios are present. However, when depolarized-induced transmitter release from synaptosomes is found to be calcium-dependent , or tetrodotoxin (TTX) sensitive, greater confidence can be placed upon the preparation and the validity of the experimental approach. The viability of synaptosomes, which are metabolically active and respond to various depolarizing influences, with enhanced glycolysis and respiration, appropriate ion flux changes and calcium-dependent transmitter release, is reviewed below.

(a) Preparative aspects

Tissue rupture, by liquid shear force techniques, is usually carried out in isotonic sucrose (0.32 M) although the sucrose polymer, Ficoll, is also popular. Because of its large molecular weight it gives dense colloidal solutions of zero osmotic pressure, and so isotonicity is usually maintained through the inclusion of 0.32 M sucrose. A homogenizer consisting of a rotating pestle and stationary mortar is the instrument commonly employed to

produce tissue rupture, with internal clearances of 80-250μm, and rotating pestle speeds of 900-2500rpm (Kurokawa et.al., 1965; Fahn et.al., 1969; Whittaker et.al., 1959; and Gray and Whittaker, 1962).

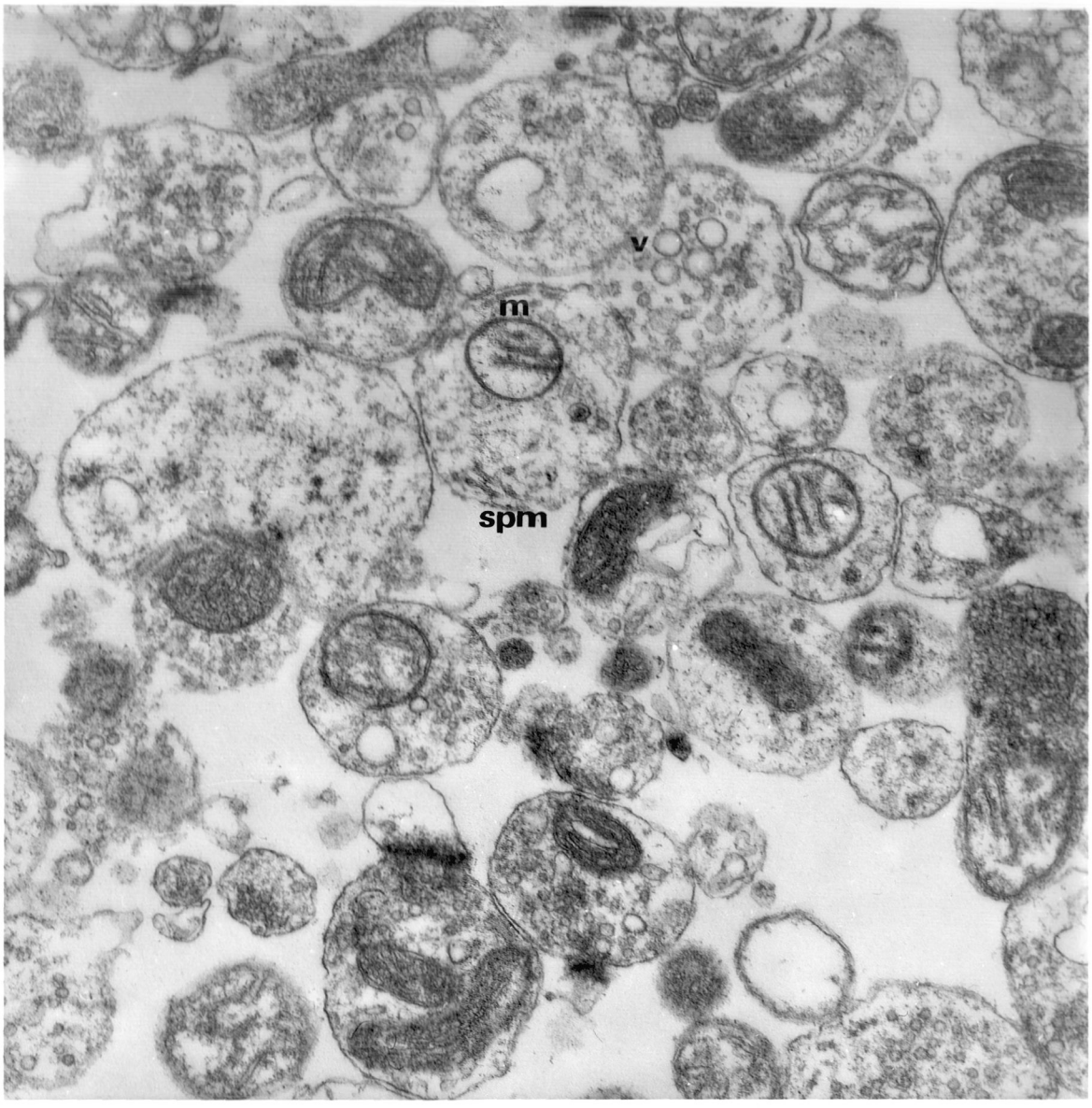
Discontinuous gradient centrifugation methods have been found to be adequate for synaptosome separation from tissue dispersions, since continuous gradients and zonal rotors do not appear to produce purer synaptosomes, free from membrane and mitochondrial contamination. However, in attempts to purify subpopulations of synaptosomes, which have specific neurotransmitter uptake, and perhaps differ slightly in their density and sedimentation property, continuous sucrose gradients may prove useful (Wofrey et.al., 1971).

Synaptosomes can now be prepared from many areas of the brain, e.g. spinal cord (Ross et.al., 1971; Osbourne et.al.; 1973), hypothalamus (Iversen and Snyder, 1968; Bradford et.al., 1972; Edwardson et.al., 1972), basal ganglia (Laverty et.al., 1963; de Belleroche et.al., 1975) and the cerebellum (Balazs et.al., 1972; 1974).

(b) Morphology

The characteristic structures of synaptosomes are revealed by electron microscopy (Fig. 1.6). These profiles of osmium-glutaraldehyde-fixed synaptosomes show a completely sealed structure containing mitochondria (m) and many small vesicles (v). The synaptic plasma membrane (spm) appears to be continuous. Frequently, in juxtaposition to the plasma membrane, the dark thickening of a postsynaptic membrane can be seen. This is

Figure 1.6 (opposite) Electron micrograph of freshly prepared rat cerebrocortical synaptosomes, showing mitochondria (m), vesicles (v), and synaptic plasma membrane (spm).


v
m
spm
1um

considered to be the junctional region, where the postsynaptic receptors are localized.

Very few free mitochondria are visible in synaptosome preparations, using the Whittaker preparation technique, although spherical cytoplasmic bodies, containing no synaptic structures are visible . Whether or not these can be ascribed to as gliosomes (pinched off glial processes), or pinched of regions of unmyelinated axon (Lemkey-Johnston and Dekirmenjian, 1970) is doubtful since many planes of section through synaptosomes are possible, some of which may not include the characterisic synaptic structures.

Finally, Jones and Brealy (1972a,b) have recently made a detailed morphological comparison of synaptosomes and the nerve terminal in situ , and found that the differences between them were minimal.

(c) Oxygen consumption and glucose metabolism

Bradford (1969), Whittaker (1969), and Balfour and Gilbert (1970), have all detected cortical synaptosomal respiration in the region of 60µmol O_2/100mg synaptosome protein/hour, in the presence of 5mM glucose. The results of Bradford et.al. (1974) demonstrate that oxygen uptake for a period of at least 3hrs can be maintained, indicating that the glycoltic mechanism can supply the mitochondria with sufficient pyruvate over this time period.

(d) Active transport

Analysis of the composition of synaptosomes after incubation in glucose-containing medium shows high concentration of potassium, ATP and phosphocreatine

(Bradford, 1969; de Belleroche and Bradford, 1972). Pyruvate, malate and glutamate also serve as substrates for these energy consuming processes (Bradford, 1967, 1969; Verity, 1972). The fact that ion gradients can be supported across the synaptic plasma membrane, and that complex responses are seen when synaptosomes are depolarized (see section (g)) suggests the presence of a membrane potential. Calculation based on synaptosomal K^+ and Na^+ ion concentrations predict the membrane potential to be -27mV (Bradford, 1971). This somewhat low value arises since it is assumed that K^+ ion equilibration occurs between the cytoplasm and the subcellular organelles. In situ, the cytoplasmic K^+ ion concentration may be much higher than the experimentally determined mean values for the whole of the non-inulin space.

(e) Synthesis and presence of neurotransmitters

Since the presynaptic terminal is the region believed to synthesize, store and release neurotransmitter substances, it has been an important task to show that synaptosomes are able to synthesize and concentrate putative transmitters. In fact, acetylcholine, noradrenaline, serotonin, and dopamine have all been found in high concentrations in synaptosomes (Fry and Whittaker, 1962; de Robertis et.al., 1962; Inouge et.al., 1963; Levi and Maynert, 1964; Laverty et.al., 1963). For example, several groups of workers (Whittaker, 1959; de Robertis et.al., 1962; Kurokawa et.al. , 1965) have shown that up to 90% of the total brain acetylcholine is recovered in the synaptosomal fractions.

The putative amino acid transmitters glutamate, aspartate,

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

Enzymes capable of synthesizing putative transmitters have been characterized in synaptosomal preparations using isotopically labelled precursors, such as ^{3}H-choline, ^{3}H-phenylalanine, ^{3}H-tyrosine, ^{14}C-tryptophan, and ^{14}C-glucose. In this way the synaptosomal synthesis of acetylcholine, noradrenaline, 5HT and the putative amino acid transmitters, has been demonstrated (Schuberth et.al., 1970; McGeer et.al., 1967; Karobuth and Baldessavini, 1972; Grahame-Smith, 1967).

(f) Uptake systems

Putative amino acid neurotransmitter uptake appears to be mediated by both high affinity (K_m 1-50μM) and low affinity (K_m 100μM-1mM) uptake systems (Logan and Snyder, 1971; Balcar and Johnston, 1972, 1973; Iversen and Johnston, 1971; Wofsey et.al., 1971; Curtis et.al. 1968b; Aprison and McBride, 1973). The high affinity systems appear to be absolutely Na^{+}-dependent, whereas the low affinity systems are not. Johnston and Iversen

(1971) detected a low affinity system for glycine both in the spinal cord and in the cerebral cortex. A high affinity system, however, was detected only in spinal cord for this amino acid, where glycine is considered an inhibitory transmitter. It is suggested therefore that high affinity uptake may play a role in the termination of amino acid transmitter action (Aprison and McBride, 1968). Some workers, however, have suggested that low affinity uptake may be of equivalent importance to high affinity uptake, in the termination of excitatory neurotransmitter action (Cox et.al., 1977), since the rise time to plateau of excitation by electrophoretically applied D-glutamate, L-glutamate, D-aspartate and L-aspartate are very similar, although D-glutamate is not being taken by a high-affinity carrier (Balcar and Johnston, 1972; Benjamin and Quartel, 1976; Davies and Johnston, 1976).

Active transport of glucose seems to play a vital role in maintaining normal function in the nervous system. Both a high affinity and a low affinity system for glucose uptake seem to exist in nerve endings (Diamond and Fishman, 1971, 1972; Fletcher and Bachelard, 1978). The high affinity system is carrier-mediated, Na^+-dependent and has a K_m of 0.24 mM. It appears to be unique to the nervous system and its function may be to support the high rate of oxidative metabolism that occurs in the nerve ending.

(g) Stimulation of synaptosomes

If synaptosomes are to have central features in common with the nerve terminal *in vivo*, they should exhibit augmented metabolism and release of synaptically active substances, when treated by depolarizing agents such as electrical pulses, high K^+ levels and veratrine.

The increased respiratory and glycolytic response to electrical pulses and raised potassium levels has been reported by Bradford (1970),Kuroda and McIlwain (1974), Bleasdale and Hawthorne (1974). Measurements of ion contents of synaptosomes indicate loss of potassium, but high sodium ion accumulation accompanied with either electrical- or veratrine-induced depolarization (Bradford, 1971). The increased sodium content would presumably accelerate the Na^+/K^+ ATPase pump, and thereby increase respiratory and glycolytic activity.

Synaptosomes from cortex, spinal cord and hypothalamus exhibit preferential release of glutamate, aspartate and GABA when stimulated with electrical pulses, veratrine,or high K^+ levels (Bradford, 1970; de Belleroche and Bradford, 1972a; Levy et.al., 1973; Nicklas et.al.,1973; Redman and Cotman, 1974). The spinal cord preparation shows release of glycine (Osbourne et.al., 1973). In common with the nerve terminal *in vivo* several workers have shown that stimulated amino acid neurotransmitter release *in vitro* is a calcium dependent process (de Belleroche and Bradford, 1972a; Blaustein and Wiseman, 1970).

Stimulated release of other neurotransmitters from synaptosome preparations has been well documented. Thus, using cerebral cortical synaptosomes , acetylcholine release by both raised potassium levels and electrical

pulses has been demonstrated by Haga (1972), de Belleroche and Bradford (1972b). Noradrenaline release has been shown by Blaustein et.al. (1972) and dopamine release from striatal synaptosomes by Ferris et.al. (1972), de Belleroche et.al. (1976). Mulder et.al. (1975) have demonstrated calcium-dependent serotonin release from superfused rat brain synaptosomes.

1.4 Aims of this study

Since the site of origin of release of neurotransmitters is still uncertain, investigations using synaptosomes were carried out to see if there were any connections between transmitter release/uptake cycles and homoexchange mechanisms (Chapter 4). It was considered that such studies might prove useful with regards to a possible cytoplasmic component to neurotransmitter release in the mammalian brain. It was decided that these mechanisms of candidate amino acid neurotransmitter transport across the presynaptic membrane could be probed by the selective use of various structurally related GABA and glutamic acid analogues, eg. nipecotic acid, 2-amino-4-phosphono-butyric acid. Also in this part of the study prostaglandins, which are structurally unrelated compounds to amino acids, but nonetheless are agents which are known to be physiologically active in other organs, were tested for their effect on neurotransmitter release in vitro.

Alternatives to synaptosomal preparation techniques for the study of neurotransmitter transport were sought for by investigating the utility of spontaneous miniature end plate potential frequency in a locust leg muscle (Chapter 6). Such a bioassay system it was thought, would provide a convenient and a relatively physiological analysis for detecting modulated glutamate release following the application of various receptor agonists/antagonists. In addition, it

was hoped that this system would provide an additional and complementary body of evidence for cytoplasmic transmitter uptake/release cycles, to the synaptosomal studies mentioned earlier.

For the final part of this study, it was decided to examine whether glutamic acid receptor antagonists could be used therapeutically as anticonvulsants (Chapter 7). In order to do this, it was considered necessary to look at the criteria of glutamate as a candidate transmitter in the CNS. Initially, glutamic acid receptor populations were characterized by the kinetics of glutamic acid binding to synaptic membranes (Chapter 5). Then, following on from this, glutamic acid release was monitored after lesioning of the rat cortical/striatal pathway. Hence it was hoped that some of the in vitro studies of brain metabolism mentioned above ie. analysis of neurotransmitter transport could be applied to studies of animal models of brain malfunction in vivo; for example, (a) the demonstration that an injection of a glutamate antagonist prior to the administration of a chemical convulsant delays the onset of convulsions, could possibly provide neurochemical correlates to chemically induced convulsions; and eg. (b) the demonstration of differential transmitter release following decortication of the cortical-striatal pathway would provide further evidence that this pathway is glutamatergic, and in addition that decortication was perhaps a useful model for the study of Huntington's chorea. Thus broadly speaking, the ambitious aim of this project was to elucidate some fundamental neurochemical mechanisms and to apply them to the medical problems of brain seizure and Huntington's chorea.

CHAPTER 2

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CHAPTER 2

Materials and Methods

2.1 Animal Sacrifice and Brain Dissections

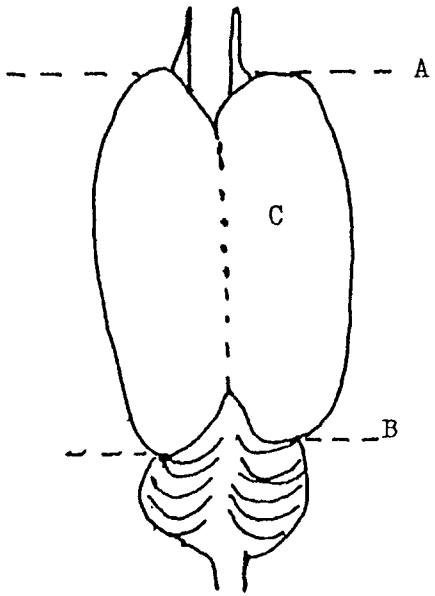
Adult female Sprague-Dawley rats (200-250g) were sacrificed by stunning followed by exsanguination. The brains were removed and dissected in the following manner.

(a) Cerebral Cortex dissection (Figure 2.1)

Firstly, a cut (A) was made to remove the olfactory bulbs; secondly, a cut (B) was made to remove the cerebellum. Next a saggital cut (C) was made along the midline of the brain to separate the two hemispheres. Each hemisphere was then inverted on the dissection block to reveal the ventral aspects of the cortex. Finally, the white of the corpus callosum and other internal organs of the brain were removed by making a cut (D) along the edge of the dissection forceps. The cortex was then transferred from the dissection block to a preweighed flask containing 10 volumes of ice-cold 0.32M sucrose.

(b) Corpus Striatum dissection (Figure 2.2)

Animals were sacrificed and the brain removed and placed on a dissection block as described previously. The striatum was removed by the following method. Firstly, a cut (A) was made along the midline of the brain but only extending posteriorly to the anterior section of the cerebellum and ventrally to the corpus callosum. Next, the cerebral cortex hemispheres were gently peeled back until each ventral surface was exposed. Finally the striatum was removed by a cut (B) along the edge of the dissection forceps. The fresh striatum



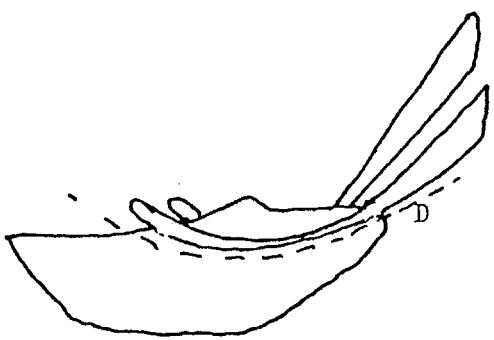


Figure 2.1 Dissection of rat cerebral cortex.

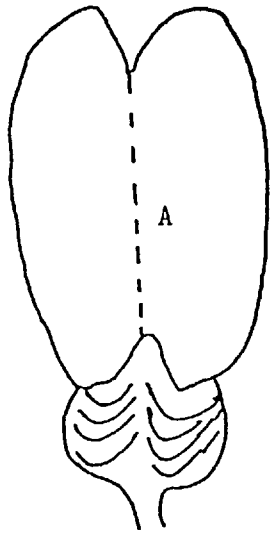


Figure 2.2 Dissection of rat corpus striatum

was weighed on a torsion balance and placed in 10 volumes of ice-cold 0.32M sucrose.

2.2 Preparation of synaptosomes

Synaptosomes were prepared by the method of Gray and Whittaker (1962) as modified by Bradford et.al. (1973). Whole cerebral cortex was rapidly removed from female adult Sprague-Dawley rats (200-250g) previously sacrificed by stunning followed by exsanguination. A 10% (w/v) homogenate was prepared in ice-cold sucrose (0.32M) and centrifuged in a Beckmann type 30 rotor at 1000g for 10 minutes. The supernatant was retained while the nuclear pellet (P_1) was resuspended in sucrose (0.32M) and centrifuged at 1000g for 10 minutes. The supernatants were combined and centrifuged for 20 minutes at 20,000g to yield the P_2 and S_2 fractions. The microsomal supernatant (S_2) was discarded and the crude mitochondrial pellet (P_2) was resuspended in sucrose (0.32M). An aliquot (15ml) of this suspension was layered onto a discontinuous sucrose density gradient consisting of 20ml sucrose (0.8M) on top of 20ml 1.2M sucrose. The sucrose gradients were then centrifuged at 75,000g for 70 minutes in a Beckmann swing-out rotor and three fractions were obtained. The synaptosomal enriched fraction P_2B was collected from the 0.8M-1.2M interface. Cold deionised distilled water was added dropwise to the synaptosomal fraction, the suspension being continuously stirred until a sucrose concentration of 0.45M was reached. The suspension was then centrifuged at 55,000g for 20 minutes in a type 30 rotor to give a synaptosomal pellet (P_2B). The pellet was resuspended in an oxygenated Krebs phosphate medium (NaCl 124mM, $NaHPO_4$ 20mM, KCl 5.0mM, $MgSO_4$ 1.3mM, $CaCl_2$ 0.75mM, KH_2PO_4 1.2mM, glucose 10mM) pH 7.4, the final concentration being

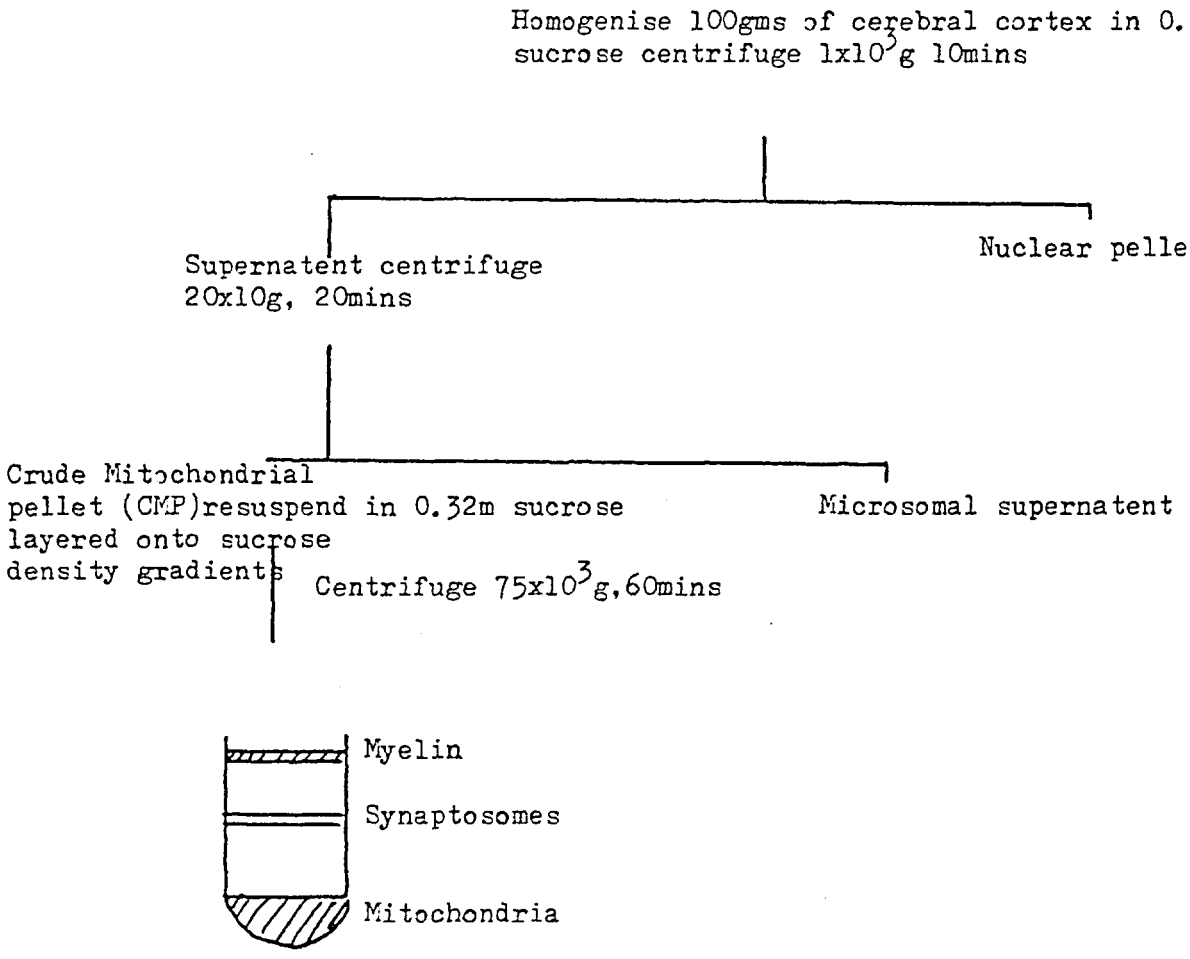


Figure 2.3 Preparation of synaptosomes

1-3mg protein/ml. This procedure is summarized in Figure 2.3.

2.3 Incubation of synaptosomes

Incubations of synaptosome suspensions were carried out at 37°C for a period of 30 minutes in all cases. On resuspension of synaptosomes, amino acid loss to the medium may occur which can then be accumulated, either by synaptosomal reuptake or by diffusion. Thus the time period for incubation allows stabilization and equilibration of the nerve terminals with the medium.

(a) Respiration Measurements of synaptosomes

Respiration of synaptosomes was measured using Warburg flasks and manometers. Synaptosome suspensions in Krebs phosphate medium (3mg/ml) were aliquoted into Warburg flasks ($1\frac{1}{2}$ -3ml) (Figure 2.4). The centre well of each flask was fitted with a NaOH soaked filtre paper wick. The flasks were then connected to manometers using greased ground glass joints, and to an oxygen supply. Pre-equilibration and oxygenation were carried out for a period of 15 minutes on a shaking water bath at 37°C. After this time period, the O_2 gas supply was closed, and the Warburg flasks sealed from the atmosphere by means of glass taps on the manometers, and on the flasks themselves. After a further 5 minute equilibration period with the closed atmosphere, manometric readings reflecting internal oxygen pressure changes, were taken at five minute intervals for a period of 30 minutes. A thermal barometer was also set up for each experiment to enable variations in external temperature and pressure to be taken into account. If the effect of any drug or depolarizing agent on synaptosome respiration was to be determined, the agent was added from the Warburg flask side arm. Hence, after the 30 minute control period, the manometers and flasks were

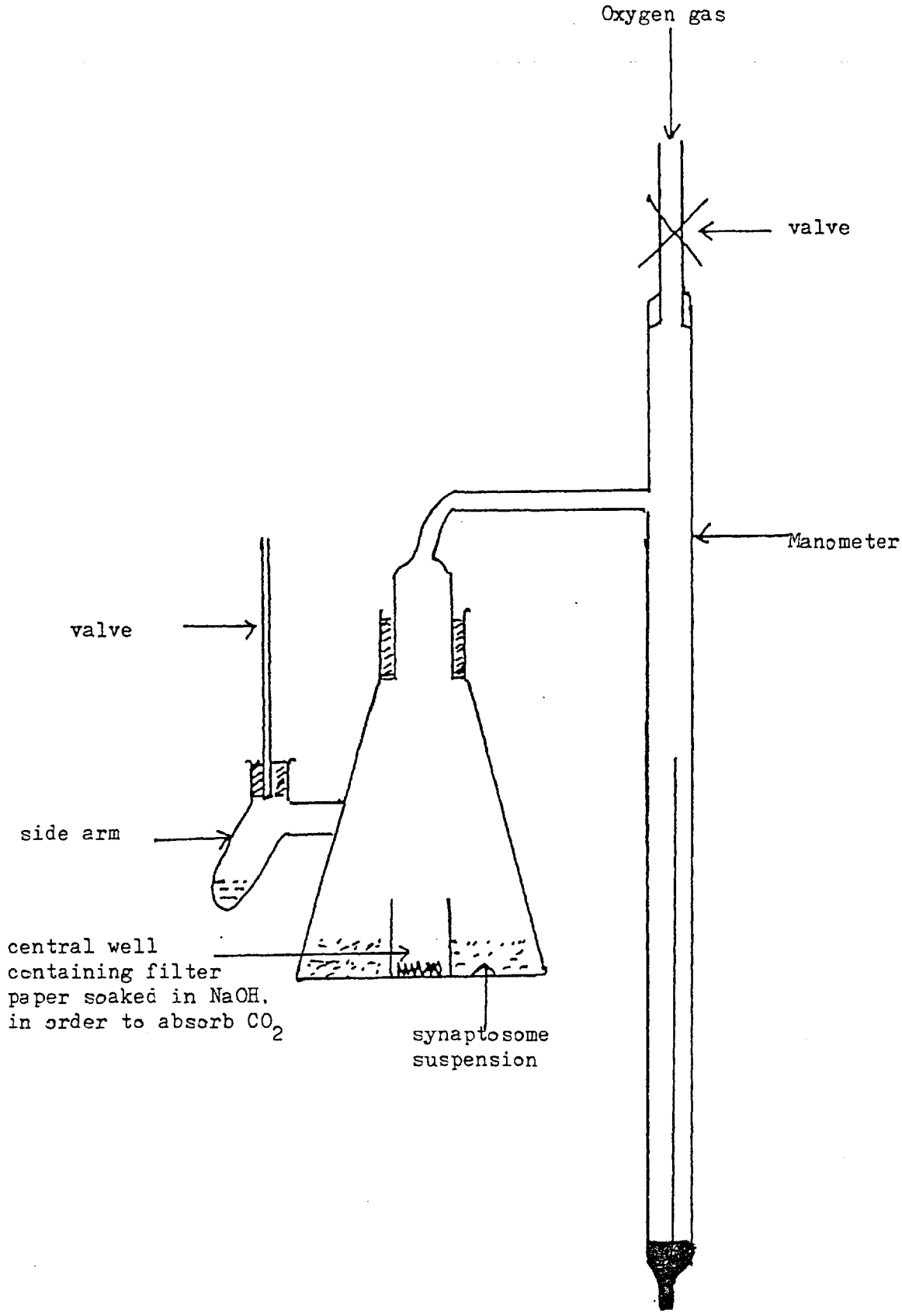


Figure 2.4 Warburg flask and manometer.

tilted, so that the incubate could run into the side arm, and mix efficiently with the test solution. Manometric readings were continued for a further 30 minutes after this addition. At the end of this time period, incubation was terminated by subjecting the suspensions to bench centrifugation at 3,000-10,000g for 2 minutes. The clear supernatant was then taken for amino acid and LDH analysis whilst the pellet was analysed for protein, amino acids, K^+ levels and LDH activity.

(b) Amino acid uptake measurements of synaptosomes

This type of incubation was carried out using conventional flasks (20ml beakers). After the synaptosome suspensions (1-3mg/ml, 1-3ml) had been pre-equilibrated for 30 minutes, at 37°C on a shaking water bath, a small spike of the test solution (20µl) was added to the incubates. One minute later, a small volume of radiolabelled amino acid (10µl, 2µM) was also added and the incubation continued for a further 10 minutes. Uptake into the tissue was then terminated by centrifugation, (3,000-10,000g, 2 minutes). The pellet was then superficially rinsed with fresh medium and then washed by homogenisation and centrifugation. Radiolabel from inside the synaptosomal pellet was extracted with TCA 10%, 1mg/ml protein, on ice for 30 minutes. Aliquots of the supernatant after centrifugation (20,000g, 10 minutes) were then subjected to liquid scintillation counting.

(c) Amino acid release experiments

After the synaptosome suspension had been preincubated as before (1-3mg/ml, 1-3ml, 37°C, 30mins), a small volume of the test solution was added to the medium, and the incubation

allowed to proceed for a further 10 minutes. Release of amino acids was terminated by centrifugation, and separation of the clear supernatant from the pellet was achieved. For endogenous amino acid release studies, aliquots of the supernatants were treated for autoanalysis as outlined overleaf, whilst the pellet was lysed with Tris-HCl pH 7.4 (10mM, 1ml/mg protein). In order to extract the synaptosol, this Tris suspension was centrifuged (20,000g, 10 mins) and the supernatant decanted off. The clear synaptosol was used for endogenous amino acid analysis or LDH, K^+ level determination. The pelleted tissue would be used for protein analysis. For exogenous amino acid release studies, radiolabel in the supernatant was extracted with 10% TCA on ice for 30 minutes. Aliquots of the supernatant after centrifugation (20,000g, 10mins) were then subjected to liquid scintillation counting.

(d) Experiments to detect a "Transport Shuttle" in nerve endings

After preparation and incubation/prelabelling as described earlier, nerve terminals were subjected to the scheme indicated in Figure 2.5. Note that group I uses prelabelled synaptosomes, whereas group 2 uses non- prelabelled nerve terminals. The uptake blockers in the experiment were dissolved in Krebs phophate medium pH 7.4. The centrifugations at the end of the experiment were performed with a bench centrifuge at 10,000g for 2 minutes.

2.4 Superfusion of synaptosomes

Synaptosome superfusion was based upon the method of Raiteri et.al. (1974). 0.5 ml aliquots of radiolabelled synaptosome suspensions were placed on Millipore filtres (pore size 0.8μm),

	0min	30mins	10mins	
group I	Incubation 30mins of ^{14}C prelabelled synaptosomes	Addition of uptake blocker and 2 M cold amino acid		Spin suspension, detect radiolabel in tissue and medium
group I control	Incubation 30mins of ^{14}C prelabelled synaptosomes	Addition of 2 M cold amino acid		Spin suspension, detect radiolabel in tissue and medium
group II	Incubation 30 mins of synaptosomes	Addition of uptake blocker and 2 M hot amino acid		Spin suspension, detect radiolabel
group II control	Incubation 30 mins of synaptosomes	Addition of 2 M hot transmitter		Spin suspension, detect radiolabel

Figure 2.5. Summary of method to detect the presence of a "transport shuttle" in nerve endings.

situated at the bottom of five parallel superfusion chambers (Fig. 2.6) thermostatically maintained at 37^{o}C. The filtres were washed twice (2 x 5mls) using fresh Krebs phosphate medium under moderate vacuum to remove excess radioactivity. After washing, 8mls of standard superfusion medium was added to the chambers, which were connected to a multichannel peristaltic pump. The superfusion rate was set at 0.5mls/minute and 1 minute fractions were collected. Superfusion was continued for a period of 5-10 minutes (or as stated in the results) to establish a baseline rate of efflux. After this control period, the test medium was introduced to the chamber to give the required concentration. The differences in this superfusion technique to that of Raiteri's and Levi's method is that in their system, a resevoir above the chamber contained the medium which flowed into the perfusion chambers at a constant rate of 0.5mls/minute, thereby maintaining a constant volume of fluid in the chamber. In the method described here, however, a fixed volume of medium was added to the chambers and this volume decreased as superfusion proceeded.

At the end of an experiment, radioactivity in the fraction (0.5mls) and in the pellet was determined by liquid scintillation counting. Results are expressed as:

$$\% \text{ radioactivity per fraction} = \frac{\text{radiolabel in fraction}}{\text{radiolabel in tissue}} \times 100\%$$

2.5 Storage of synaptosomes

Freshly prepared synaptosomes were suspended in Krebs phosphate medium (at room temperature) containing 10% (v/v) glycerol. The suspension was transferred to polystyrene ampoules (volume 3mls) and placed on ice for 30 mins. before being put in a polystyrene box having 20 cm thick walls. The box was sealed and placed in a deep freeze at

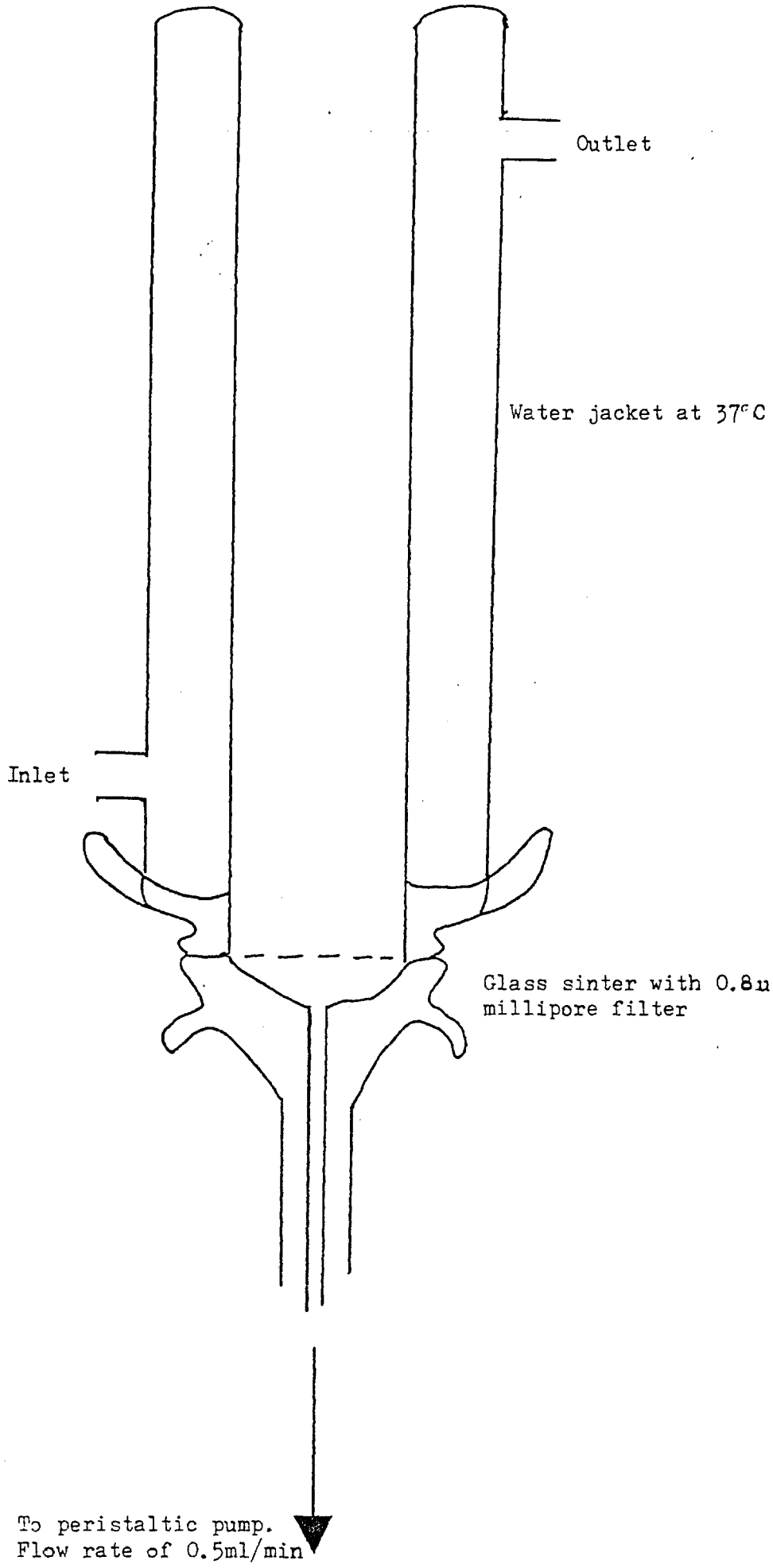


Figure 2.6 Synaptosome superfusion chamber.

either -10° or -70°C for the required length of time. Before use, the ampoule containing the stored synaptosomes was left at room temperature for 1½ hours in order to defrost the cells. The synaptosomes were then treated as in the normal experimental procedure for freshly resuspended synaptosomes.

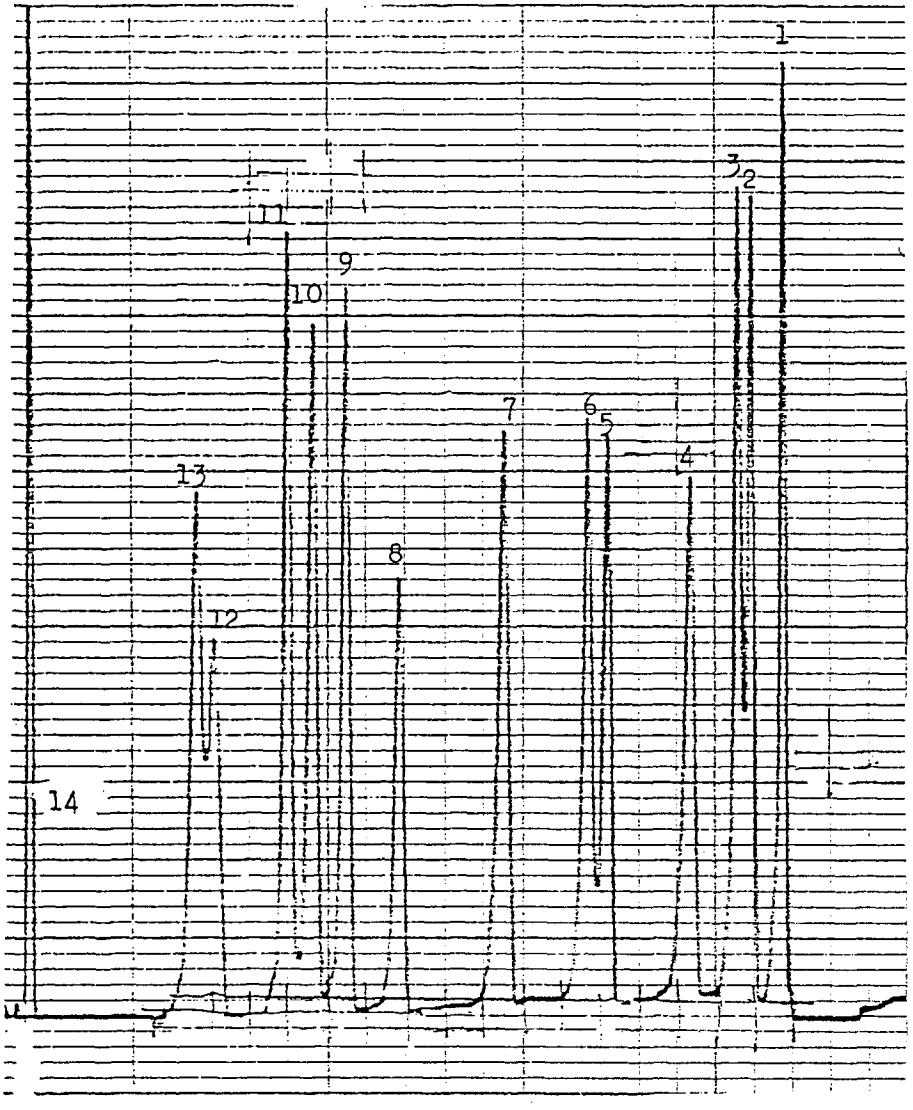
2.6 Endogenous amino acid analysis

Amino acid levels were measured by autoanalyser as described by Bradford and Thomas (1969). Briefly, nor-leucine (10-20nmoles/ml) was added to 1ml portions of either synaptosome cytosol or incubation medium to act as an internal standard. The samples were then taken to dryness by vacuum dessication. Free amino acids were then extracted from the dry salts and protein by the addition of ice-cold methanol (1ml). The samples were then bench-centrifuged (10,000g, 10 minutes), methanol supernatants were decanted off, and taken to dryness. The dried extracts were prepared for amino acid analysis by dissolution in 1.0ml of 0.025N HCl. Results were calculated by peck area ratios of a calibration amino acid standard using a small online computer (Digico 165) with reference to the standard nor-leucine signal in the sample. The calibration amino acid standard chromatogram is shown in Figure 2.7.

2.7 Exogenous amino acid analysis by liquid scintillation counting

Radioactive U^{14}C-gaba, U^{14}C-glutamate, L^{3}H-glutamate, U^{14}-dopamine levels in protein free samples (0.1-1ml) were determined using a toluene-PBD based scintillant (5-20mls) and a Beckman scintillation spectrometer. The cpm data was quench corrected to machine efficiency, which was typically 90% for ^{14}C, and 25% for the tritium label.

Figure 2.7 Amino Acid Standard Reference Chromatagram.



1. Aspartate
2. Threonine
3. Serine
4. Glutamate
5. Glycine
6. Alanine
7. Methionine
8. Valine
9. leucine
10. Isoleucine
11. Nor-leucine
12. Tyrosine
13. Phenyl-alanine
14. GABA

2.8 Protein determination

Tissue samples from synaptosomes or crude mitochondrial pellets were dissolved in small volumes of NaOH (0.1M, 0.25-1ml) at 60^{o}. Aliquots of these solutions were then analysed for protein concentration using an automatic Technicon autoanalyser (see Figure 2.8). The method was based upon that of Lowry et.al. (1951) using bovine serum albumin as a standard. A typical standard reference curve is shown in Figure 2.9.

2.9 Potassium determination

Potassium ions were extracted from pelleted synaptosomes by vortex mixing with 10% TCA. After removal of the particulate fraction by centrifugation, the potassium content of the supernatant was measured using an Evans Electroselenium flame photometer fitted with a potassium filtre (730-850nm). Samples were run routinely along with standard solutions of potassium chloride (0.1-0.5mM), the concentration of potassium ions in the samples being calculated from a standard curve (Figure 2.10).

2.10 Lactate Dehydrogenase assay

The activity of this enzyme was determined using the method of Bergmeyer (1965) in which the consumption of NaDH is measured at 340nm according to the reaction:

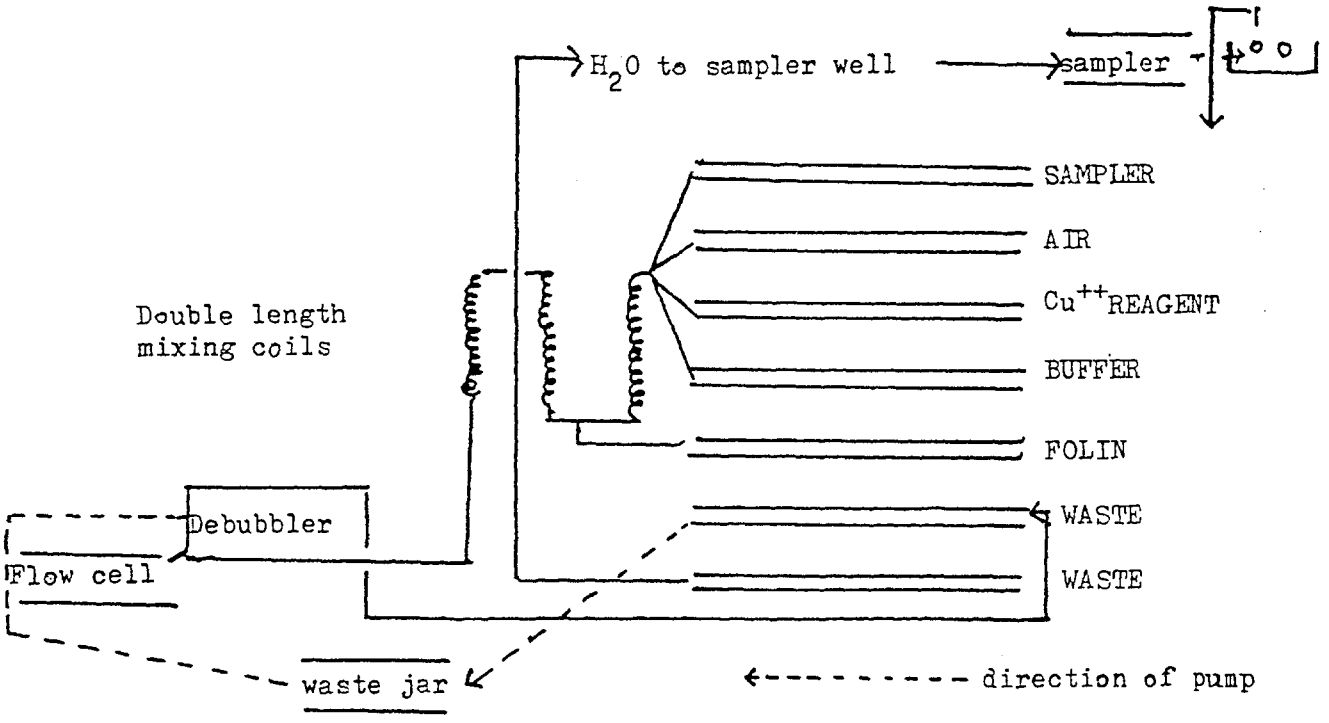
$$\text{pyruvate} + \text{NaDH} + H^{+} \dashrightarrow \text{lactate} + NAD^{+}.$$

Samples (0.1ml) to be analysed were mixed in a cuvette with sodium pyruvate phosphate buffer (2.85mls, pH7.5) and NADH solution (0.67% w/v, 0.05ml). Absorbances were then read at 340nm at 30 second intervals in a Varian CARY 210 spectrophotometer and LDH activity was calculated using the equation:

change in OD per minute=LDH activity (International units/min).

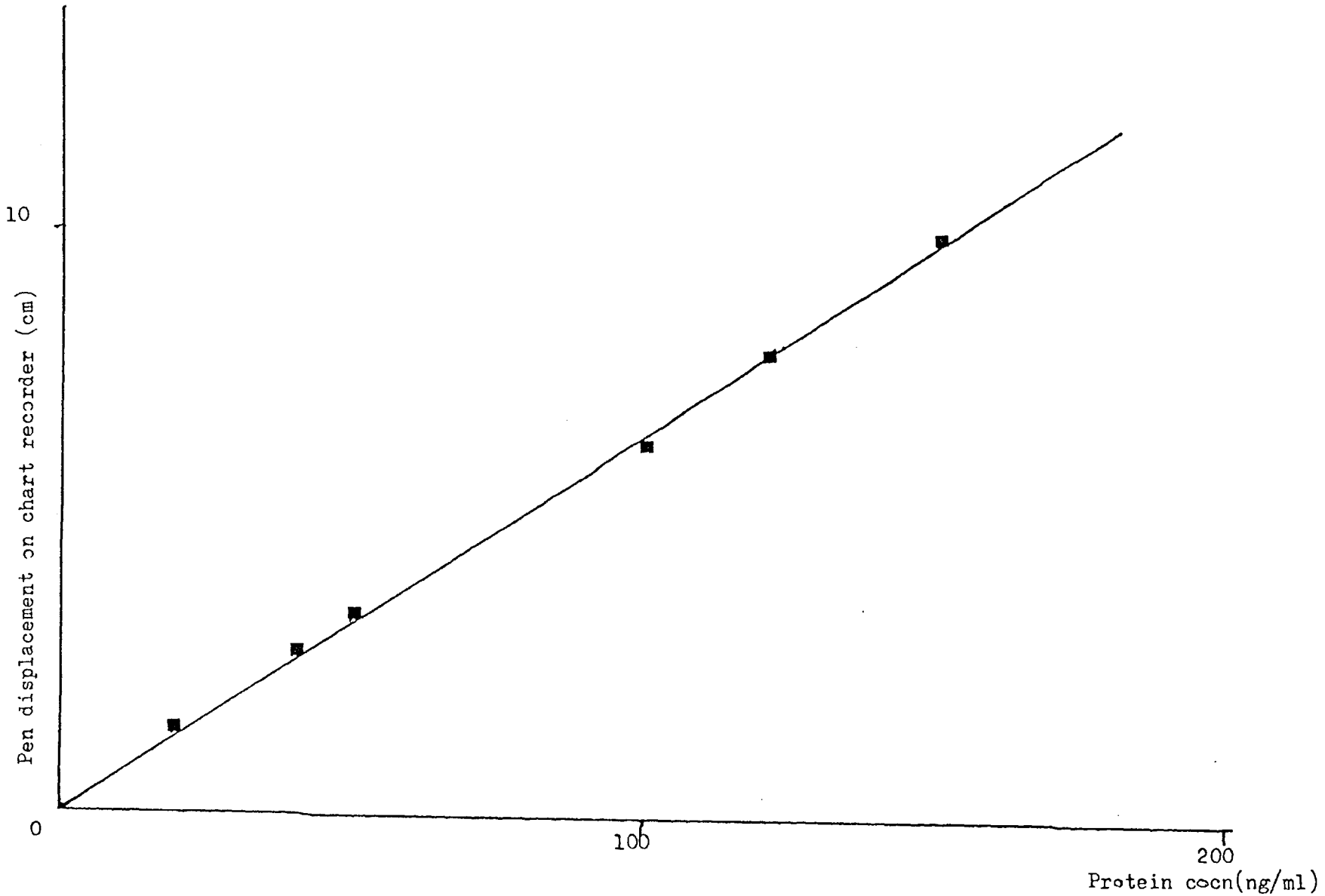
Figure 2.8 Flow chart for the Protein Auto-Analyser.

Protein samples were dissolved in 0.3m NaOH and diluted to a final concentration of 0.1m NaOH. The samples generally contained in the range of 50-100mg Protein/ml. BSA standards were run in the same way to cover the range 10-200 mg BSA/ml.



40 samples/hr sampling volume 0.13ml, read at 547nm

Figure 2.9 Protein Standard Curve.



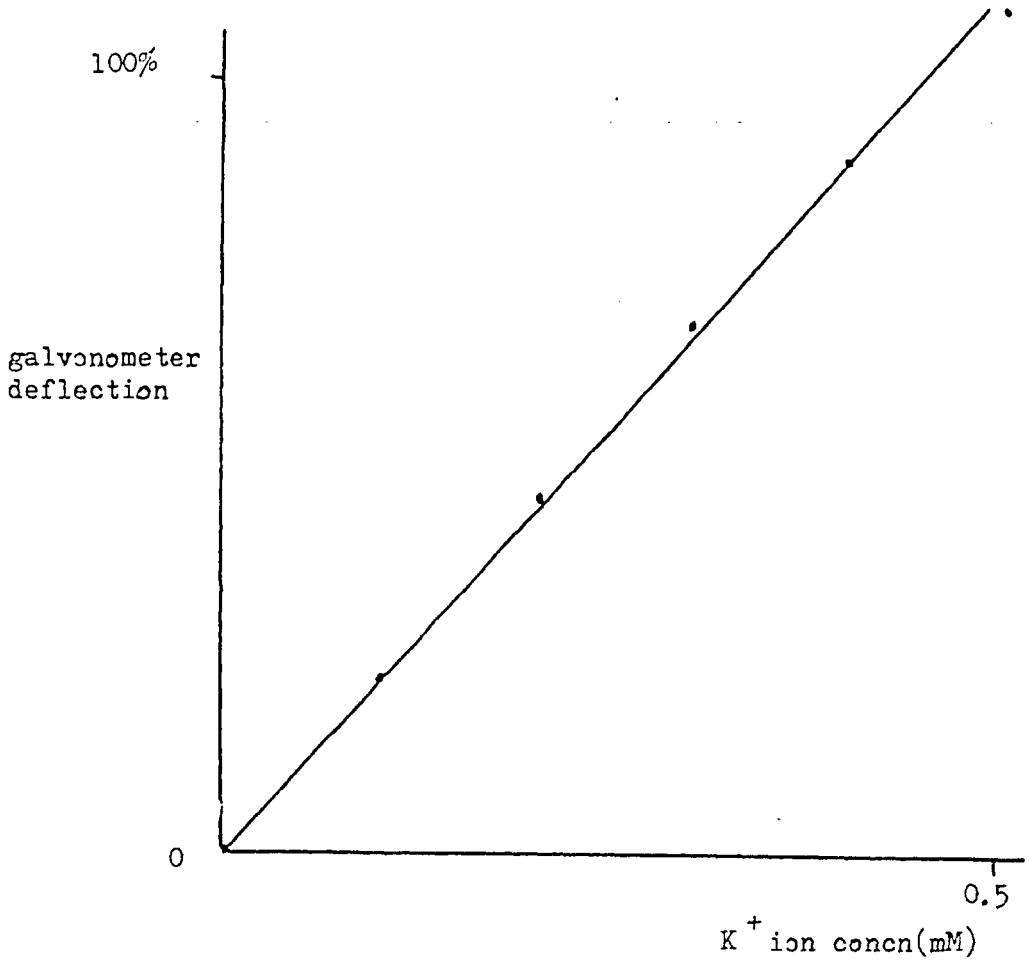


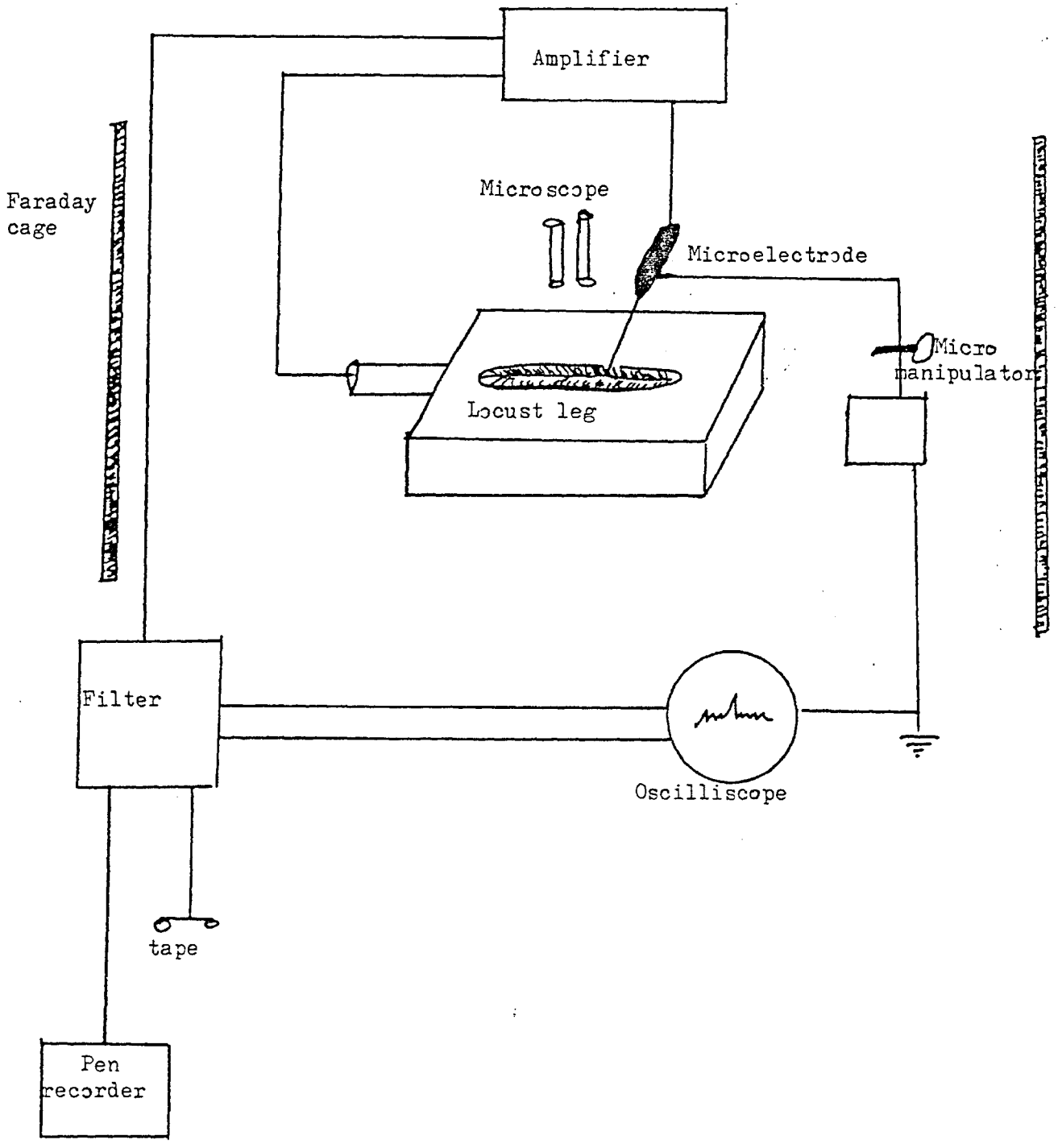
Figure 2.10 Potassium Standard Curve.

2.11 Miniature End Plate Potential Analysis at the Locust Neuromuscular Junction

Extensor tibialis muscle preparations from Schistocerca gregaria were used. The muscles were exposed whilst being left intact in the tibial segement of the hind leg. Preparations were incubated at room temperature in perspex chambers as described by Usherwood and 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 microelectrodes of tip diameter 0.5µm, filled with 1M-KCl, were used to penetrate individual muscle fibres. Their d.c. resistance was 10-50 Ω and this was checked during the 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. The cathode-follower probe 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. The latter had a facility for collecting pulses of different amplitude ranges by selection of the threshold levels. The counter was designed and built to measure the rate of discharge of miniature end-plate potentials (m.e.p.p.). The output of the preamplifier was also recorded on magnetic tape for later analysis. A schematic diagram of the equipment is shown in Figure 2.12.

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

Figure 2.12 The Locust NMJ apparatus.



150μm. The injection pipette was attached to a micromanipulator positioned close to the incubation chamber and each of its barrels was connected by means of polyvinyl chloride tubing to a Hamilton syringe mounted near the chamber. The positions of the microinjection pipette and the microelectrode were viewed through a binocular microsocope. After selecting and penetrating a muscle fibre 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. In practice, this was equivalent to 4 fibres distance. Small volumes (10μl) of the solutions to be tested were ejected onto the fibre and their effects on m.e.p.p. discharge rate and on muscle fibre membrane potential (observed on the cathode ray oscilloscope)were recorded. Locust-Ringer was always added first as control and was without effect in the 5-10μl range. Potassium chloride was then added to test the response of the fibre.

The maximal concentration of agent at the fibre surface (i.e. the dilution factor) was calculated from the relationship between K^+ concentration (in the addition pipette) and degree of membrane depolarisation observed on ejecting 10μl onto the fibre. This was then equated with the theoretical degree of depolarisation caused by standard external KCl solutions, to give the required dilution factor. This factor was then applied to drug solutions to give the maximal concentration at the muscle fibre.

2.12 Preparation of synaptosomal membranes

Membranes for binding assays were prepared in the following manner. A P_2 or P_2B pellet was resuspended in 50-100 volumes of Tris-HCl (50mM, pH 7.5) in order to lyse the synaptosomes. The suspension was then centrifuged for ten minutes at 17,000g. The

resulting pellet was then superficially rinsed with 3 x 5mls of Tris-HCl buffer, and then washed again by resuspension and centrifugation. The pellet obtained was rinsed superficially again and resuspended in this buffer to a final protein concentration of 1-2ng/ml. For binding studies carried out in Krebs phosphate buffer, the final pellet was resuspended in this medium, and not Tris.

Membranes were prepared fresh for binding assay studies on the same day.

2.13 L-^{3}H Glutamic Binding Assay

L-^{3}H glutamate binding to membranes was measured by adding 200µl of membrane suspension (0.2-0.4ng protein) to test tubes containing a total volume of 300µl of L-^{3}H glutamate, L-glutamate and Tris-HCl buffer (50mM, pH 7.4) as indicated in Table 2.1. The tubes were then incubated at 37^{o}C for 10 minutes. The reaction was terminated by filtration under reduced pressure; i.e. transferring the membrane suspension by pasteur pipette, and placing it on a Whatman DE 81 cellulose filtre paper situated in a Millipore filtration system. The tube and pipette were then rinsed with 3mls of ice cold Tris buffer pH 7.4, 50mM, which was then passed through the filtre system. The membranes on the filtre were then further rinsed with 3mls of ice cold Tris buffer before being subjected to liquid scintillation counting. This whole procedure took between 10 and 15 seconds to complete.

Table 2.1. Concentrations of L-^{3}H glutamate and L-glutamate used in various experiments.

Experiments for:	Concentration L-3H glutamate	Concentration glutamate
Indirect Scatchard plots	28nM	0-20μM
Direct Scatchard plots	0-10μM	0 or 2mM
Further Binding Studies	28nM	0 or 2mM

The concentration shown is the final concentration in the assay. Stock L-^{3}H glutamate 35 Ci/mmole, 1Ci/ml was diluted 1 in 10 in Tris HCl buffer pH7.4, and a spike of 100μl, 14 picomoles was added to the relevant tube. The unlabelled L-glutamate used was made up from the hydrochloride salt.

2.14 ^{3}H-prostacyclin binding assay

The assay was carried out by a modification of previously described Millipore filtration techniques (Blair and MacDermot, 1981). Synaptosomes were incubated with 20nM ^{3}H-PGI_2 in 50mM Tris-HCl buffer pH 8.5 containing 10mM $MgSO_4$. Incubations were terminated after 15min at 20^{o}C, specific binding was taken to be that displaced by 25μM PGI_2 in parallel incubations.

2.15 Prosataglandin analysis

Aliquots (1ml) of the supernatants from synaptosome incubations were equilibrated with 25ng each of 3,3',4,4'-$^{2}H_4$-$PGF_{2\alpha}$, 3,3',4,4'-$^{2}H_4$-PGE_2 and 3,3',4,4'-$^{2}H_4$-6-oxo-$PGF_{1\alpha}$ and acidified to pH3 with 0.1M HCl. Ethyl acetate extraction (2 x 5ml) was followed by back extraction into 2M borax buffer (2ml) pH8.5. The borax layer wasextracted with ethyl acetate, the organic phase discarded and the aqueous phase adjusted to pH3 with 0.1M HCl. The prostaglandins were extracted into ethyl acetate (2 x 3ml), the solvent evaporated under nitrogen at 40^{o} and the residue transferred in methanol to glass vials. The methanol was removed under nitrogen, methoxyamine hydrochloride in dry pyridine (100μl, 5mg/ml) was added and the derivatisation allowed to proceed overnight. The pyridine was removed in vacuo, methanol (0.25ml) added, followed by freshly distilled iazomethane in ether (0.5ml). The solvent was removed in a stream of nitrogen and the residue re-dissolved in N,O-bis-trimethylsilyl-trifluoroacetamide (25μl). The samples were heated to 60^{o} for 1hr to complete the derivatisation, and then analysed by gas chromatography-mass spectrometry (GC-MS). Standards were carried through the same procedure and standard curves constructed to the range 1 ng-80 ng/ml. Selected ions monitored were: $PGF_{2\alpha}$ 494 (M-TMSOH), $^{2}H_4PGF_{2\alpha}$ 498(M-TMSOH), PGD_2 508

Figure 2.13 PG F2A

CALIBRATION CURVE

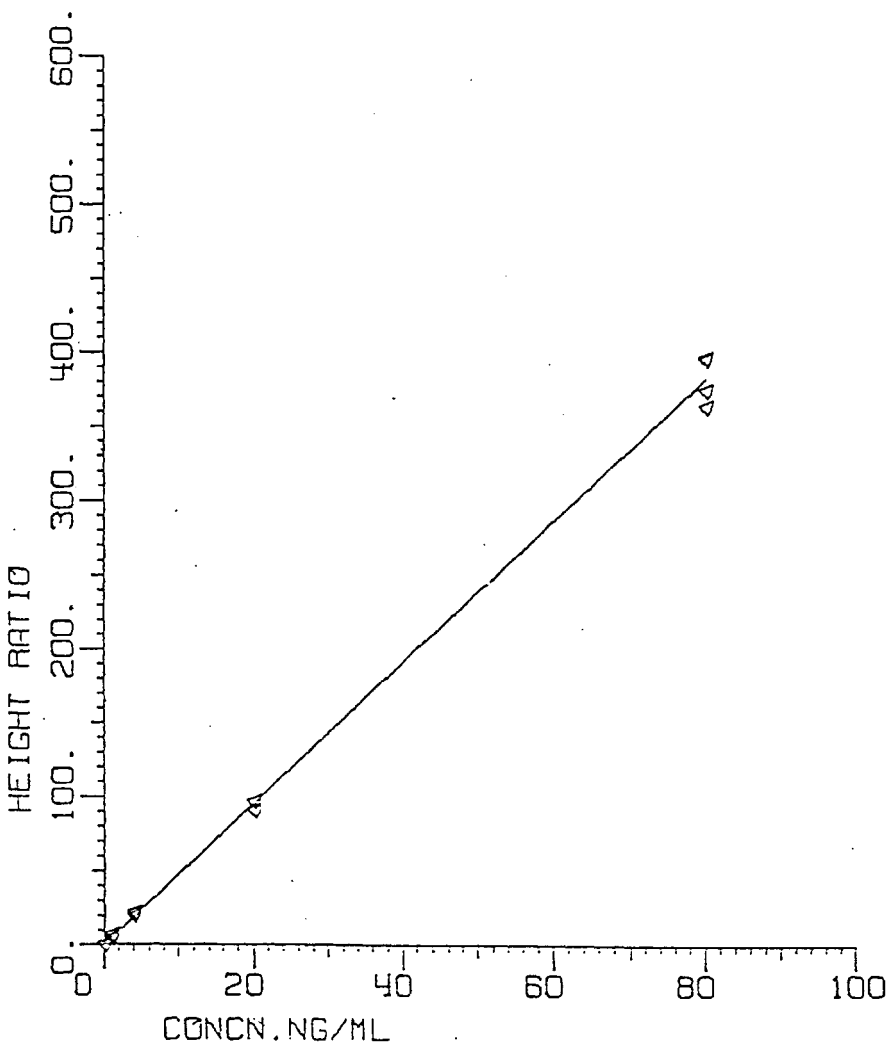


Figure 2.14 PG E2

CALIBRATION CURVE

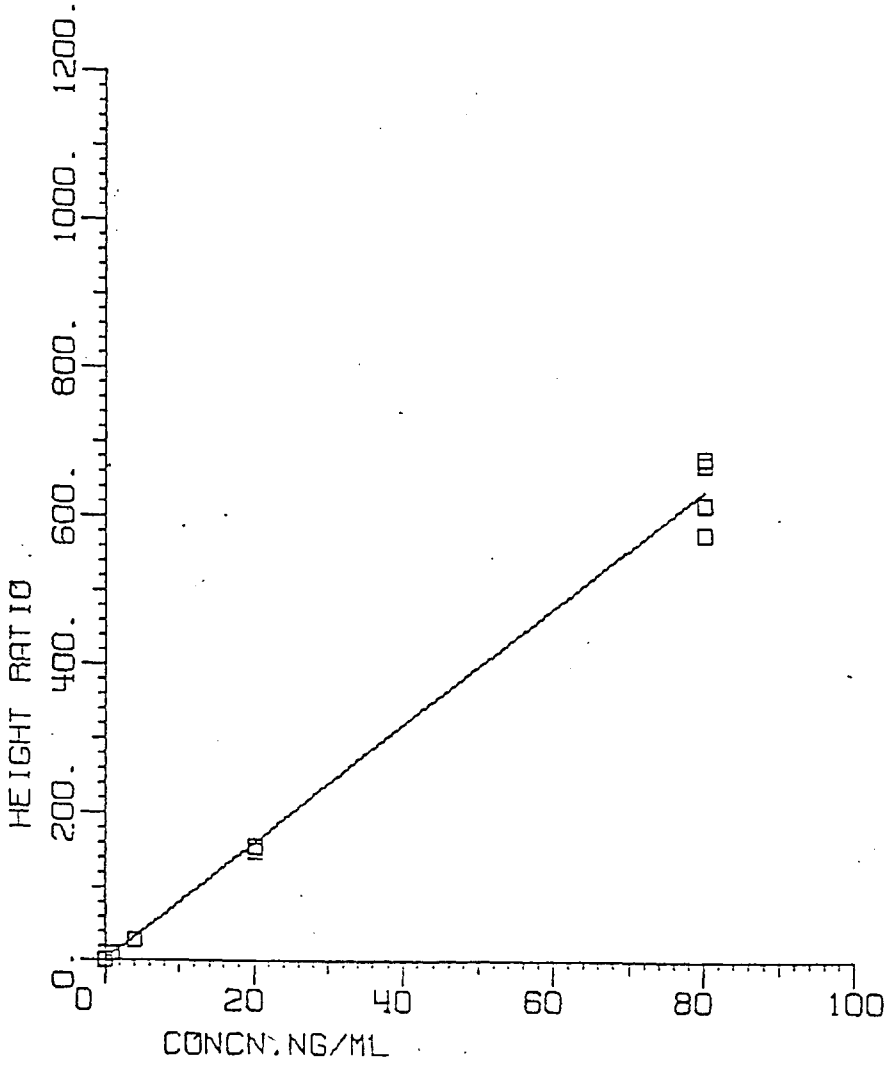
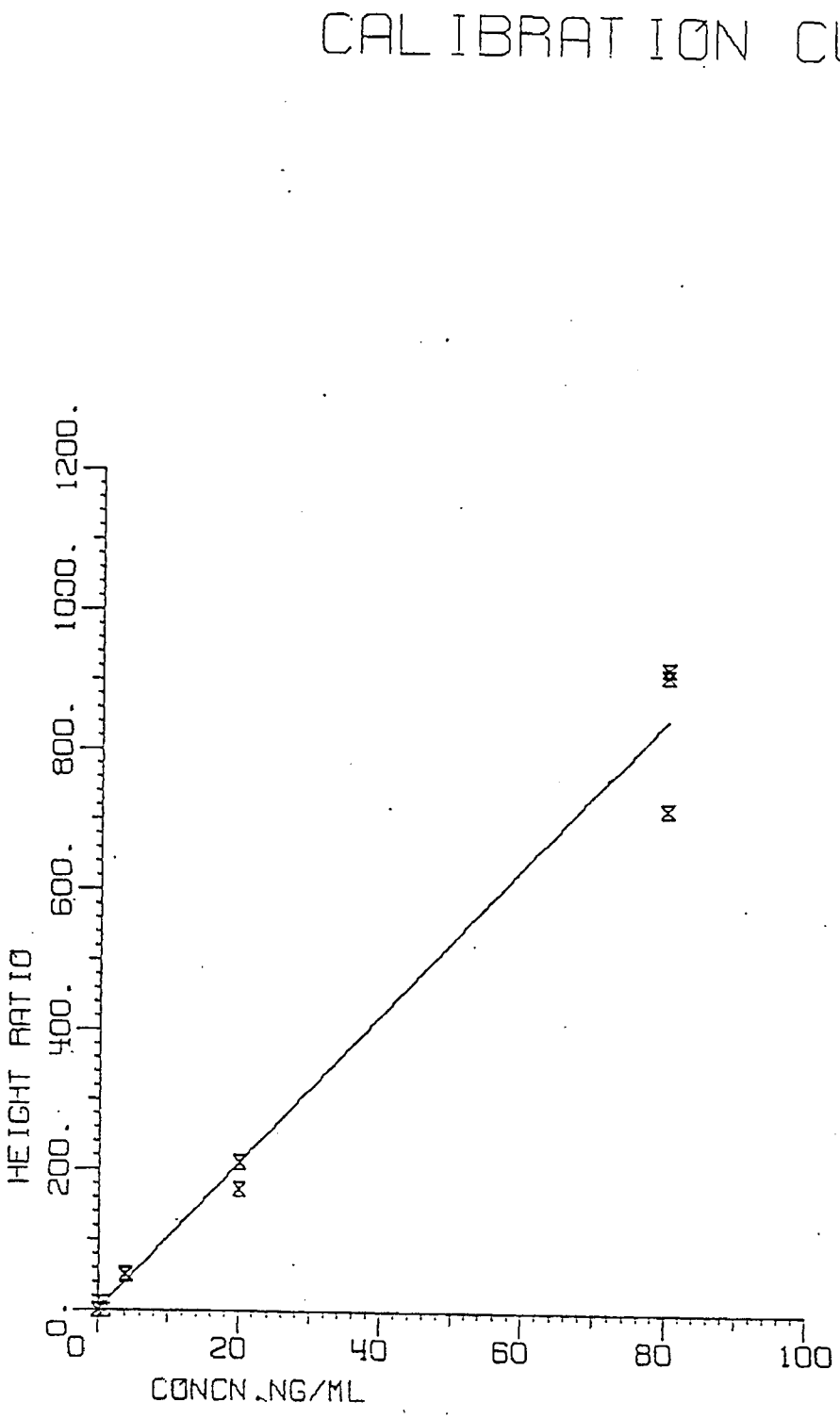


Figure 2.15


PG D2
CALIBRATION CURVE
HEIGHT RATIO
0.
200.
400.
600.
800.
1000.
1200.
0
20
40
60
80
100
CONCN.NG/ML

(M-OMe), $^{2}H_4$-PGE_2 512 (M-Me) TXB_2 508 (M-OMe-TMSOH), 6-oxo-PGF_{1_α} 508, (M-OMe-TMSOH), $^{2}H_4$-6-oxo-PGF_{1_α} 512 (M-OMe + TMSOH). Standard curves were constructed for PGF_{2_α} (Fig. 2.13), PGE_{2_α} (Fig. 2.14) and 6-oxo-PGF_{1_α} with reference to the corresponding heavy isotope internal standard, $^{2}H_4$-PGE_2 was employed as the standard for PGD_2 (Fig. 2.15) and $^{2}H_4$-6-oxo-PGF_{1_α} as the internal standard for TXB_2.

A Finnigan 4000 automated GC-MS system was operated at 25 eV. Separations were achieved by temperature programming a 2 m x 2mm id 3% OV1 packed column from 200-265°C at 10°C/min with a flow rate of 30ml/minute of helium carrier gas.

2.16 Lesioning of the Cortico-Striatal tract

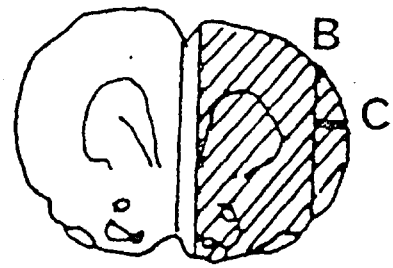
Decortication was carried out by the method of McGeer and McGeer (1977). Briefly, the right corpus striatum was isolated from the cortex by a transverse cut (A, Figure 2.16) ahead of the caudate, a parasaggital cut (B) lateral to the caudate, and a horizontal cut (C) through the cortex to exclude fibres from the cortex dorsal to the neostriatum.

Two weeks after lesioning the rats were sacrificed, the brains removed and dissected. The position of the lesion was verified visually by examination on dissection. The neostriatum was removed from both the lesioned and the intact sides of the brain, and the weights were recorded. The tissue was then homogenised in 10 volumes of ice cold sucrose (0.32M), and from this homogenate, crude striatal synaptosomes (P_2) were prepared.

2.17 Electron microscopy

Suspensions of synaptosomes in Krebs phosphate medium were fixed at 4°C with uranyl acetate buffered OsO_4 for 1 hour. The pellets of fixed synaptosomes were dehydrated with alcohol and embedded in Araldite. A Phillips 300 electron microscope was used

Figure 2.16 Lesioning of the Cortico-striatal tract.



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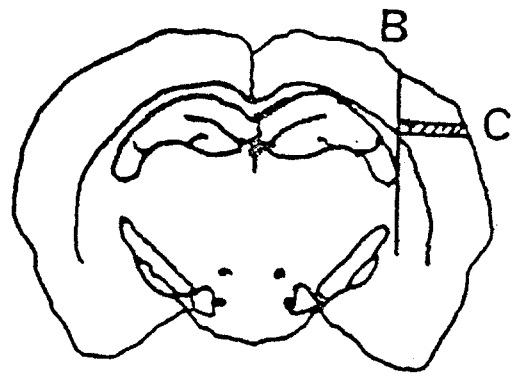
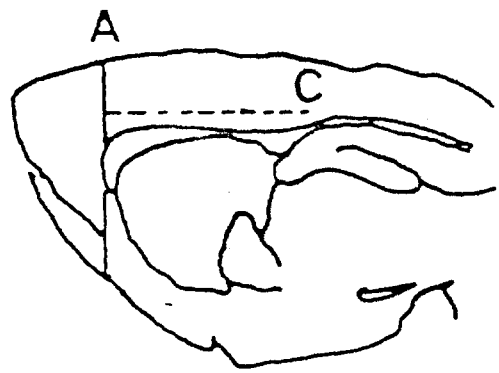


A 3990



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for examination of the specimens. Sections were stained on the grids with a 1% ethanol solution of uranyl acetate followed by lead citrate.

2.18 Source of Reagents and Compounds

Common laboratory chemicals used were of analar grade and were obtained from either BDH UK Ltd, or the Sigma Chemical Company UK Ltd. The following generous gifts were received: PGI_2 was supplied by the Wellcome Research Laboratories and PGE_2, PGD_2, $PGF_{2\alpha}$ supplied by the Upjohn Co. The glutamic acid analogues APB, APV, DAP, DL-PG, hydroxy-aspartate, kainic acid, dihydrokainate amino adipiate, homocysteic acid, nipecotic and isonipecotic acid were kindly donated by Dr J. Collins, City Polytechnic, London, England.

CHAPTER 3

Cryoprotection of Synaptosomes by Glycerol

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CHAPTER 3

Cryoprotection of cerebral-cortical synaptosomes by glycerol

3.1 Introduction

Investigations into CNS neurological disorders frequently rely on the use of animal models, since the availability of fresh human post-mortem brain is comparatively rare for most laboratories. Therefore it would be considered useful if synaptosomes prepared from rare bioptic material could be stored frozen in some way. The convenience of such frozen brain specimens would then mean that firstly, post-mortem brain nerve endings could be stored for later or repeat studies, and secondly that the transfer of specimens between laboratories would be possible. Also, banks of control and diseased brain could be established. As a means to this end, studies were carried out to maximise the viability of rat cerebrocortical synaptosomes following freeze-thaw manipulations.

The literature reveals that frozen brain specimens have been used repeatedly in the determination of enzymatic parameters (Bowen et.al., 1979; Puymirat et.al., 1979), and in the isolation of neuronal and glial perikarya and other subcellular fractions (Iqbal and Tellez-Nagel, 1972; Brammer and Carey, 1980; Ramsey et.al., 1974; Stahl and Swanson, 1975). However, little effort has so far been made regarding investigations into the dynamics of neurotransmitter release and reuptake occurring in nerve endings from frozen brain tissue. This is probably due to the morphological changes that are known to occur because of freeze-thaw injury (Larramendi and Wolosewich, 1971; Routtenberg and Tarrant, 1974; Swanson et.al., 1973), and this highlights the problem of developing a suitable technique for synaptosomal cryoprotection

since it is necessary to achieve a system whereby the organelle remains intact, complete with plasma membrane, mitochondrion, synaptic vesicles, etc.

In microbiology, bacterial cells are quite often protected from freeze-thaw injury by the inclusion of glycerol in the initial incubation medium. It is thought that the glycerol enters the cells so that when on freezing the ordering of water molecules into ice crystals is substantially reduced and hence disruption of the plasma membrane is also likely to be reduced. Thus cryoprotection of the culture is achieved (Farrocent, 1980).

A slow step freezing procedure using 10% (v/v) glycerol as a cryoprotective agent was adopted based on the ideas above. Recovery following freezing and thawing of the rat cerebrocortical synaptosomes was assessed by the characteristics of (1) respiration, (2) K^+ content, (3) presence of the intracellular enzyme marker LDH, (4) release and reuptake of physiologically active amino acids and (5) electron microscopy.

3.2 Results

(a) Respiration

Stored synaptosome preparations showed high linear rates of oxygen uptake when incubated in Krebs phosphate medium containing glucose (10mM) and in the presence of glycerol (10% v/v; see figures 3.1-3.4). After storage for 6 days, these rates varied from 56 to 53 μmoles O_2 / 100mg protein / hr at -70°C to -10°C respectively. This represents a decrease in oxygen consumption of approximately 25% from that optimal for freshly prepared synaptosomes. When synaptosomes were stored in the absence of glycerol in the medium, the rate of respiration was considerably reduced by approximately 60% (Figure 3.2).

Legend to Figures 3.1-3.4

Respiration of stored rat cerebrocortical synaptosomes

The details of how nerve terminal preparations were frozen are given in the Materials and Methods chapter of this thesis. Briefly, freshly prepared synaptosomes (3mgs/ml) suspended in a Krebs-phosphate-glycerol (10%v/v) medium were initially cooled in an ice bath at $0^{o}C$ in small capsules for 30 minutes. If final storage was to be at $-10^{o}C$ or $-70^{o}C$, the suspensions were insulated in the centre of a polystyrene cube (20 x 20 x 20cm) and placed in a cold store at the required temperature. The purpose of the insulating cube was to present a slow cooling gradient ($1^{o}C\ min^{-1}$) to the nerve endings. When the suspensions were required for experiments, the frozen synaptosomes were removed from the cold room and left to attain room temperature in the laboratory ($1\frac{1}{2}$ hours). Incubations were then carried out in Warburg respirometers at $37^{o}C$ in Krebs phosphate medium. The graphs in Figures 3.1-3.4 show typical results from six to nine experiments.

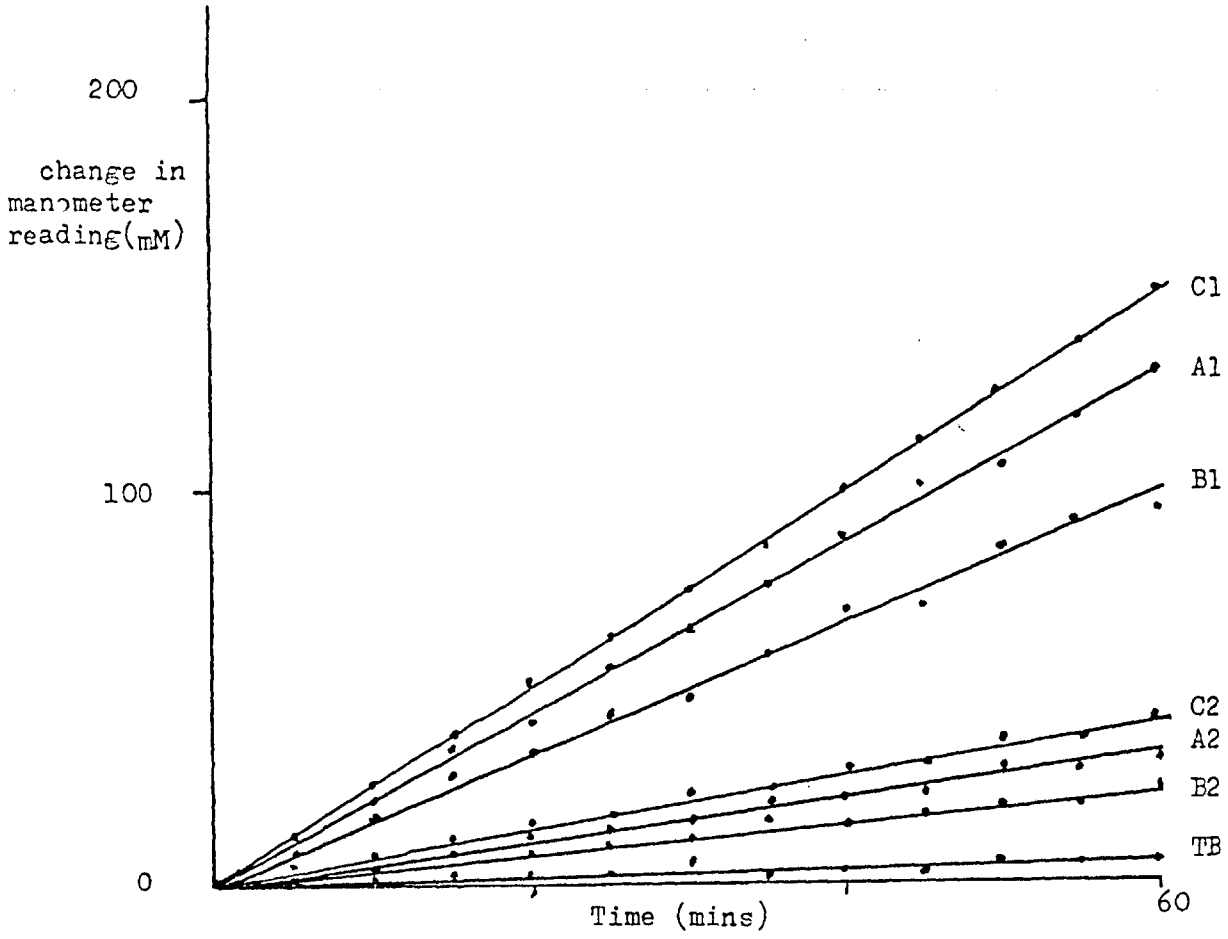


Figure 3.1 Respiration of glycerol protected ratcerebrocortical syraptosomes under different storage conditions.

A1 Fresh tissue +10mM glucose
A2 Fresh tissue -10mM glucose
B1 Tissue stored for 1 day, -10°C + 10mM glucose
B2 Tissue stored for 1 day, -10°C - 10mM glucose
C1 Tissue stored for 1 day, -70°C + 10mM glucose
C2 Tissue stored for 1 day, -70°C - 10mM glucose
TB Thermal barometer.

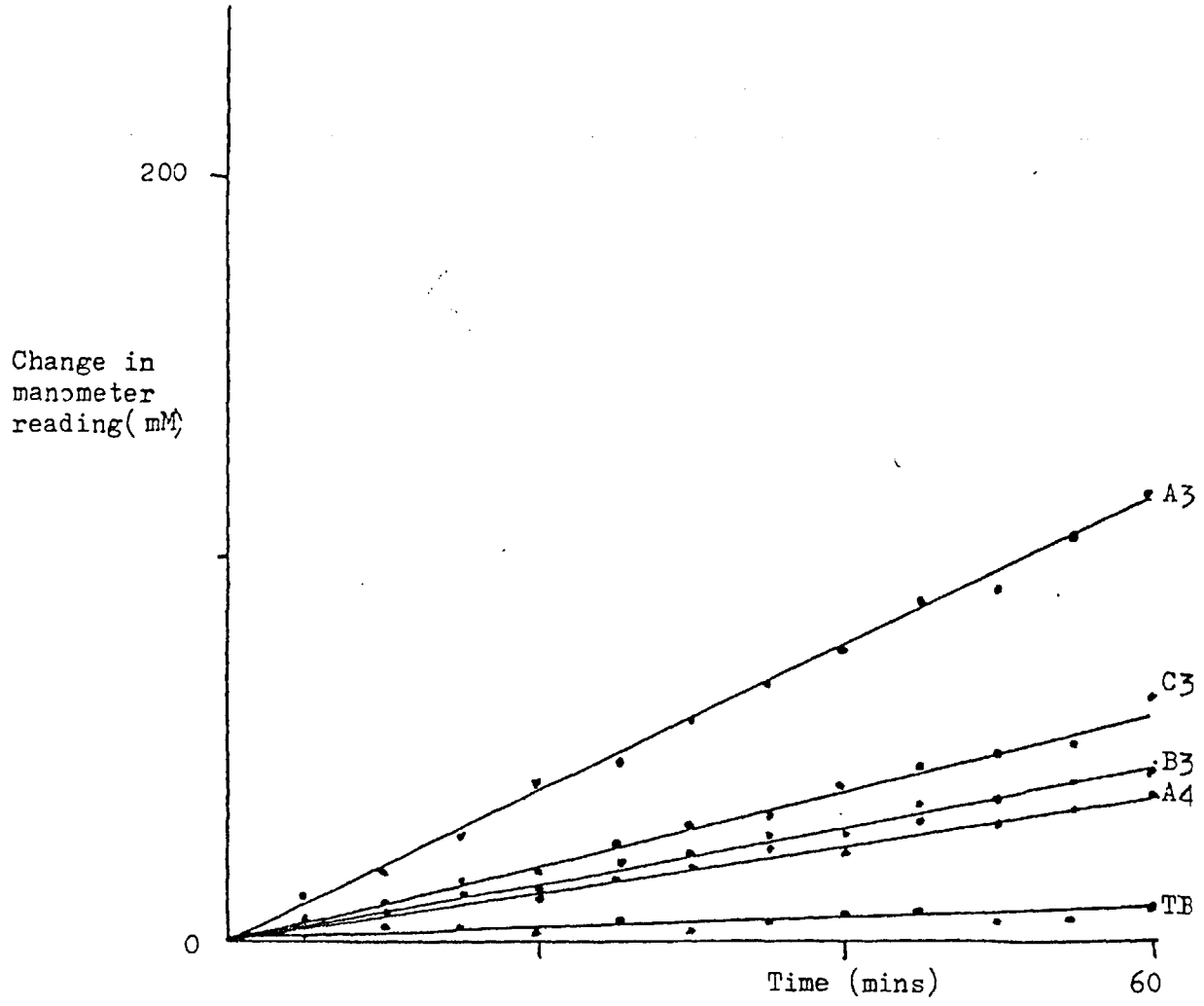


Figure 3.2 Respiration of rat cerebrocortical synaptosomes under different storage conditions without glycerol protection.

A3 Fresh tissue +10 mM glucose
A4 Fresh tissue -10 mM glucose
B3 Tissue stored for 1 day -10°C, +10mM glucose
C3 Tissue stored for 1 day -70°C, +10mM glucose

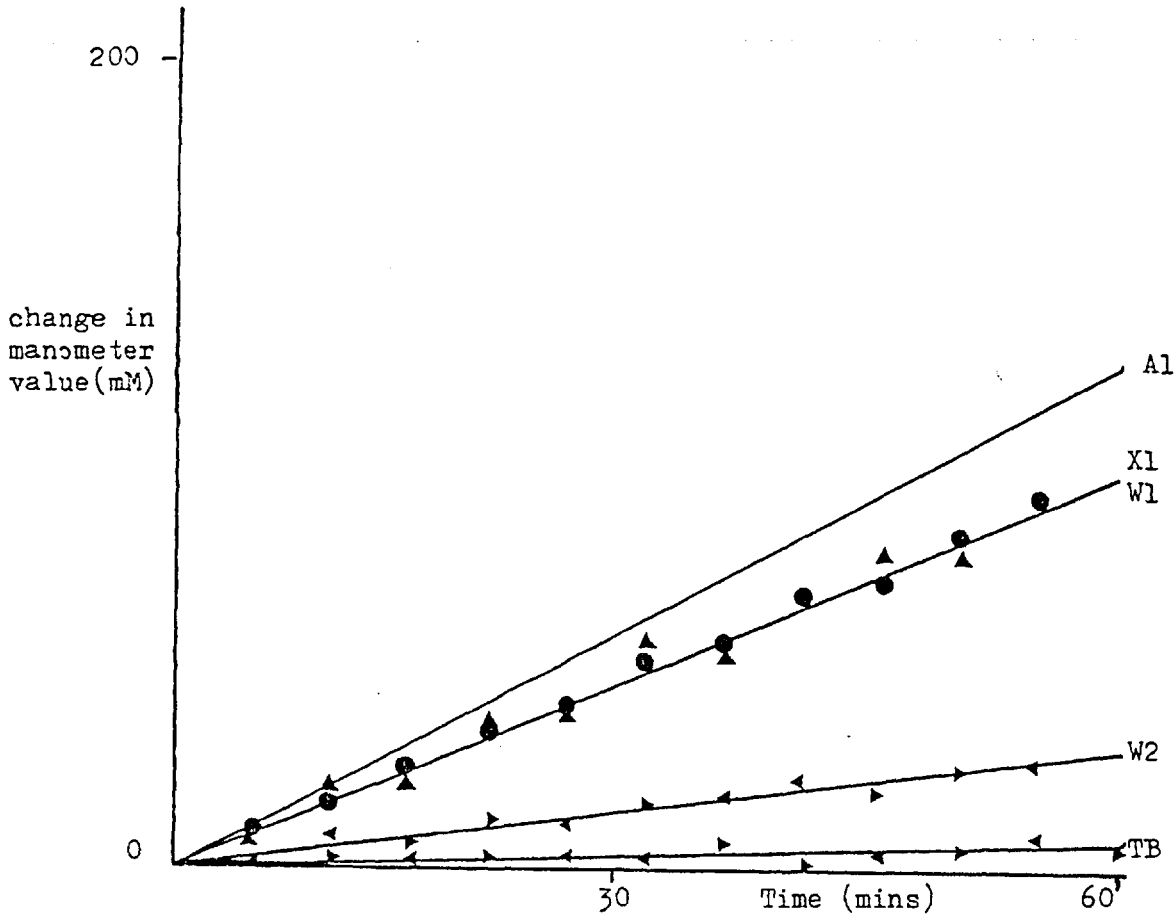


Figure 3.3 Respiration of glycerol protected rat Cerebrocortical Synaptosomes under different storage conditions.

W1 Tissue stored for 6 days, -10°C + 10mM glucose
W2 Tissue stored for 6 days, -10°C + 10mM glucose
X1 Tissue stored for 6 days, -70°C + 10mM glucose
A1 Fresh tissue
TB Thermal Barometer

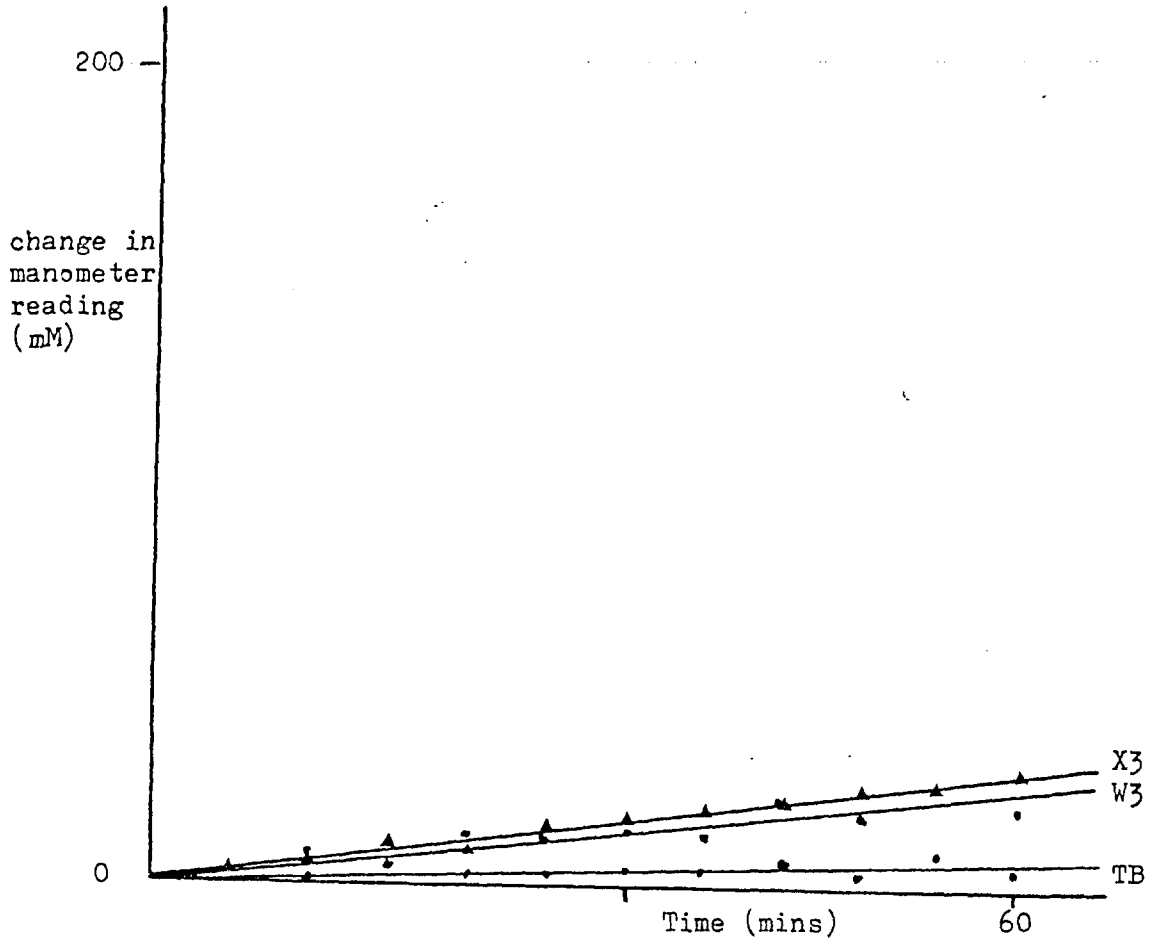


Figure 3.4 Respiration of rat cerebrocortical synaptosomes under different storage conditions, without glycerol protection.

W3 Tissue stored for 6 days, -10°C + 10mM glucose
W3 Tissue stored for 6 days, -70°C + 10mM glucose
TB Thermal barometer.

Time course measurements (Fig. 3.5) show that respiration was maximal for up to 6 days of storage (75% of controls); synaptosomes stored at $-70^{o}C$ performing slightly better than those stored at $-10^{o}C$.

(b) Potassium content

The potassium content of the incubated synaptosomal fractions was estimated for a variety of fresh and cryoprotected conditions. The results are shown in Table 3.1. The values obtained for nerve terminals frozen for 6 days at $-70^{o}C$ in a glycerol containing medium (16.3 μequiv. per 100mg protein) compare well with those obtained for freshly prepared synaptosomes (20.4 μequiv. per 100mg protein). Again it appears that the freezing temperature and duration of storage (up to 6 days) has a negligible effect (10-20% decrease) on the levels of potassium concentration obtained (Fig.3.6). The accumulation of this ion is evidence that the stored synaptosomes maintain a trans-membrane potential (Bradford et.al., 1973).

(c) Enzyme marker - LDH

As there is no known biochemical marker which is localized wholly within the presynaptic nerve terminal, the enrichment of viable stored synaptosome preparations had to be assessed from the abundance of substances which are significantly, but not exclusively, associated with nerve terminals. The previous results concerning K^{+} ion accumulation indicate viable intact tissue to a certain extent, so in addition to this lactate dehydrogenase activities were also measured. The results in Table 3.1 show that LDH activity was decreased by only 28% after 6 days in stored synaptosomes at -10^{o} or $-70^{o}C$

Legend to Table 3.1

Comparison of rates of oxygen uptake, potassium ion content and specific activity of LDH in fresh and cryoprotected rat cerebrocortical synaptosome preparations

Results are means $\pm$ S.E.M. , with the number of determinations in parentheses. Incubation was for a total of 60 minutes at 37^{o}C in Warburg respirometers; potassium ion levels and LDH activities were determined on tris buffer extracts (10mM, pH7.4) of the pellets as described in the Materials and Methods chapter. The freeze-thaw procedure for storage of the synaptosomes was as outlined in the legend to Figures 3.1-3.4.

Legend to Tables 3.2-3.5

Amino acid release from stored rat cerebrocortical synaptosomes

Values are means $\pm$ S.E.M. of the number of experiments shown. Incubations were for a total of 60 minutes in Krebs phosphate medium at 37^{o}C. Veratrine (75μM) and K^{+} ions (56mM) were added during the last 30 minutes where appropriate; TTX (1μM) was added 2 minutes before the addition of veratrine where appropriate.

Table 3.1

Comparison of rates of oxygen uptake, potassium ion content and LDH specific activity in fresh and glycerol cryroprotected rat cerebrocortical synaptosomes.

Storage conditions.	K^+ ion content (uequivalents per100mg protein)	LDH activity umol/min/ mg protein	O_2 uptake umoles O_2per 100mg protein per hour
Fresh	20.4±0.86(7)	2.64±0.05(8)	72±7(8)
-10° C 6 days	19.2±0.96(7)	1.89±0.08(3)	53±(6)
-70° C 6 days	16.3±0.72(4)	1.90±0.03(3)	56±3(4)
Lysed fresh synaptosomes	11.5±1.4(18)	0.63±0.05(18)	20±4(3)

* values are means ± SEM

Table 3.2 Amino acid release from freshly prepared rat cerebrocortical synaptosomes, (amino acid levels given in units of nmoles/100mgs protein).

	Control (5)	Potassium (5)	Veratrine (5)	Veratrine + TTX (4)
Aspartate	226±30	567±57	609±31	300±9
Threonine	328±23	444±39	426±24	370±30
Serine	445±41	590±55	605±27	520±16
Glutamate	541±72	1449±148	2226±72	697±33
Glycine	302±26	430±42	469±11	356±23
Methionine	254±22	367±50	328±23	298±24
Valine	145±14	197±28	186±19	180±25
Leucine	373±37	503±67	471±37	438±41
Phenylalanine	131±9	189±22	193±18	166±14
Tyrosine	178±19	238±35	227±19	209±29
Gaba	61±9	450±71	840±16	97±17

Amino acid	Control	Veratrine
Aspartate	296±34	518±90
Threonine	342±18	245±12
Serine	660±39	498±32
Glutamate	342±15	1395±257
Glycine	394±17	355±29
Alanine	741±97	578±71
Methionine	186±17	148±9
Valine	125±26	114±14
Leucine	242±23	186±14
Phenylalanine	100±7	86 ±5
Tyrosine	112±5	86±5
GABA	74±13	393±79
	n = 5	n = 4

Table 3.3 Amino acid release from freshly prepared rat cerebrocortical synaptosomes suspended in Krebs-phosphate glycerol medium (amino acid levels are given in units of nmoles/100mgs protein).

Amino acid	Control		Veratrine Stimulation	
	-10°C (6)	-70°C (12)	-10°C(6)	-70°C (5)
Aspartate	3508±287	3650±185	4076±134	3710±190
Threonine	3119±234	2002±85	2919±36	1925±189
Serine	2089±87	2807±302	2199±41	3433±227
Glutamate	1598±182	1080±77	1583±59	1281±308
Glycine	930±113	1243±139	836±45	1365±234
Methionine	434±77	1051±89	440±82	1080±140
Valine	357±14	591±49	372±21	474±78
Leucine	716±85	681±46	581±14	612±77
Phenylalanine	310±68	569±51	210±14	416±60
Tyrosine	288±28	-	258±5	-
GABA	429±27	345±30	516±53	316±64

Table 3.4 Amino acid release from stored rat cerebrocortical synaptosomes (6 days; in glycerol Krebs-phosphate-glucose medium), (amino acid levels expressed as nmoles/100mgs protein).

Amino acid	Fresh (5)	Fresh+Glycerol (5)	-10°C (6)	-70°C (12)
Aspartate	226±30	296±38	3508±287	3650±185
Threonine	328±23	342±18	3119±234	2002±85
Serine	445±41	660±39	2089±87	2807±302
Glutamate	541±72	342±15	1598±182	1080±77
Glycine	302±26	394±17	930±113	1243±139
Methionine	254±22	186±17	434±77	1051±89
Valine	145±14	125±26	357±14	591±49
Leucine	373±37	242±23	716±85	681±46
Phenylalanine	131±9	100±7	310±68	569±51
Tyrosine	178±19	112±5	288±28	-
GABA	61±9	74±13	429±27	345±30

Table 3.5 Amino acid resting release from rat cerebrocortical synaptosomes stored for 6 days in a Krebs-phosphate-glucose-glycerol medium (amino acid levels expressed as nmoles/100mgs protein).

Figure 3.5 Time course for Oxygen uptake into synaptosomes when stored in a glycerol containing medium at temperatures of -10° and -70°C

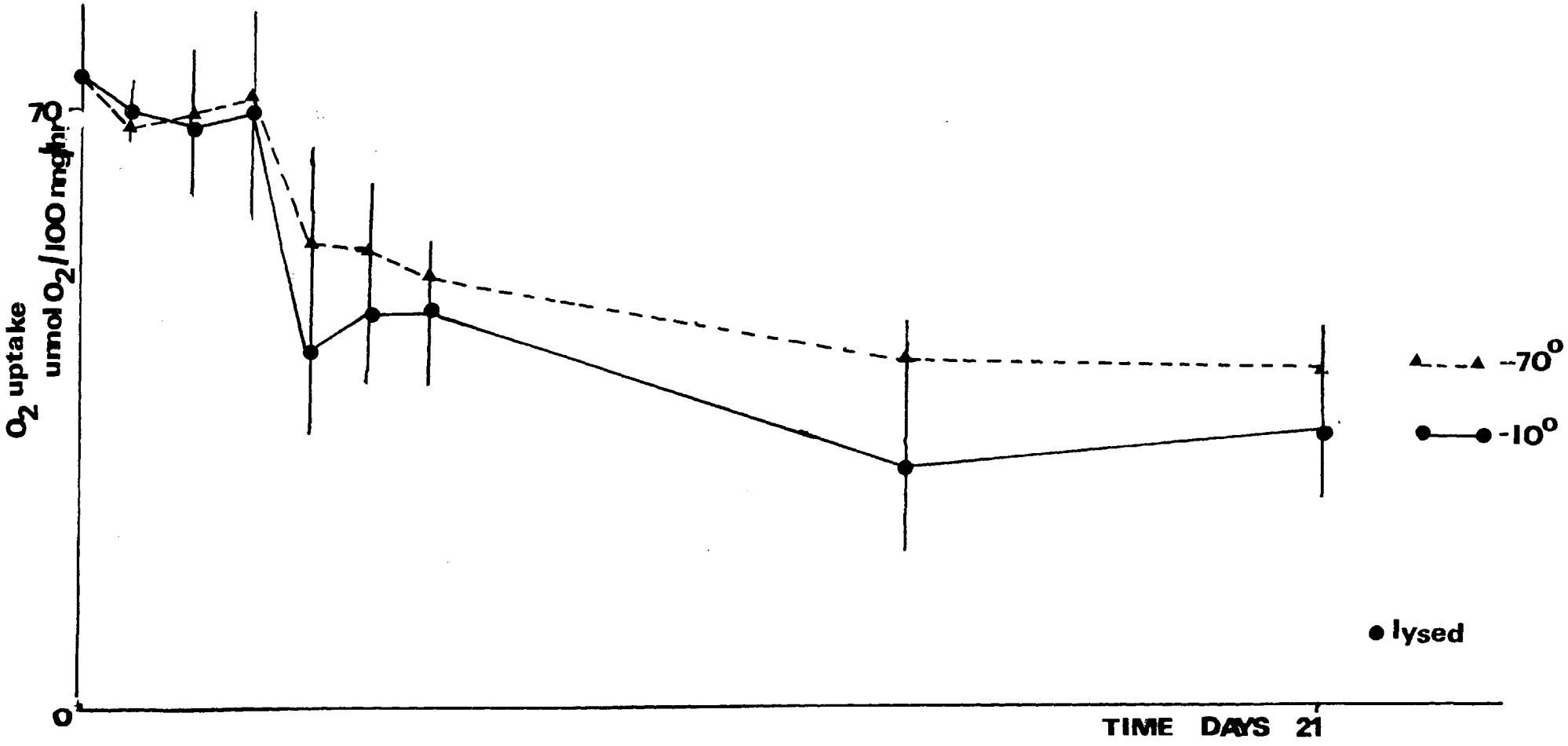


Figure 3.6 Time course for K^+ ion content of synaptosomes, when stored in a glycerol medium at temperatures of -10° and -70°C.

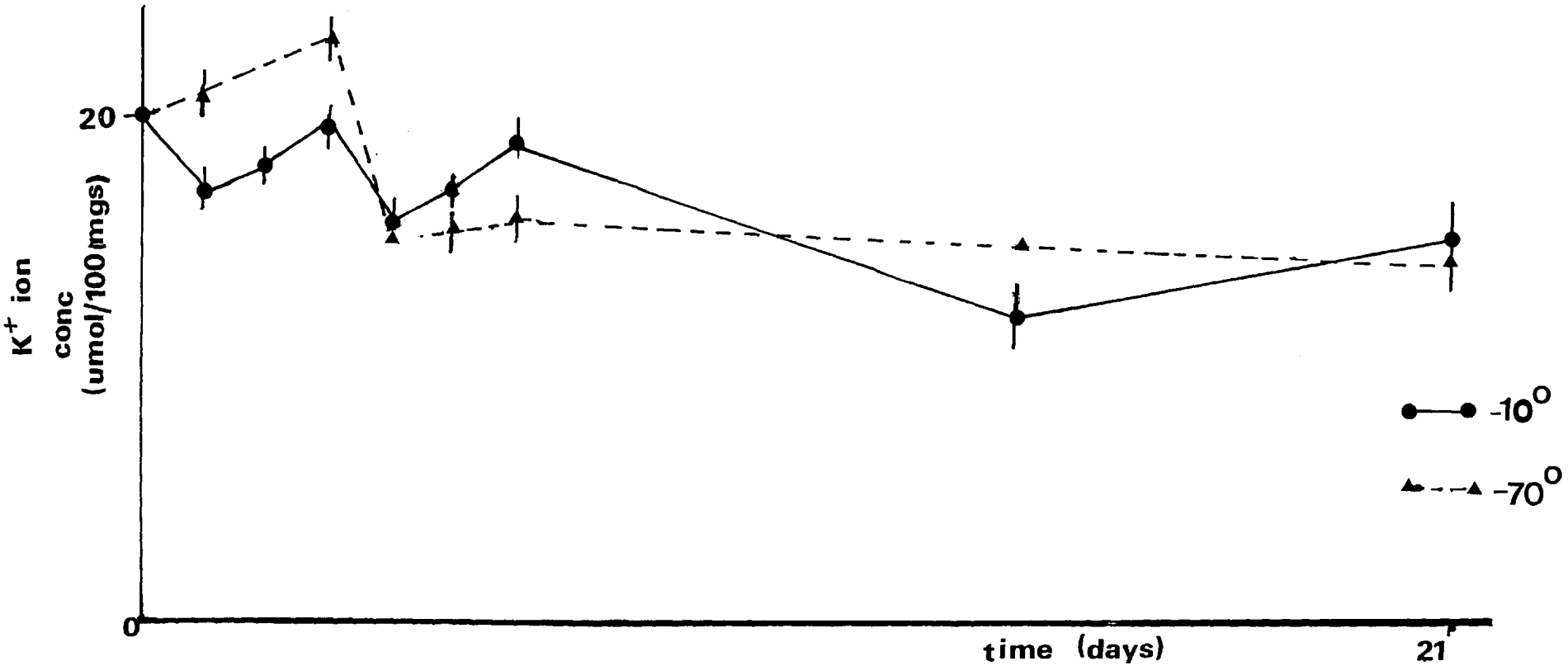
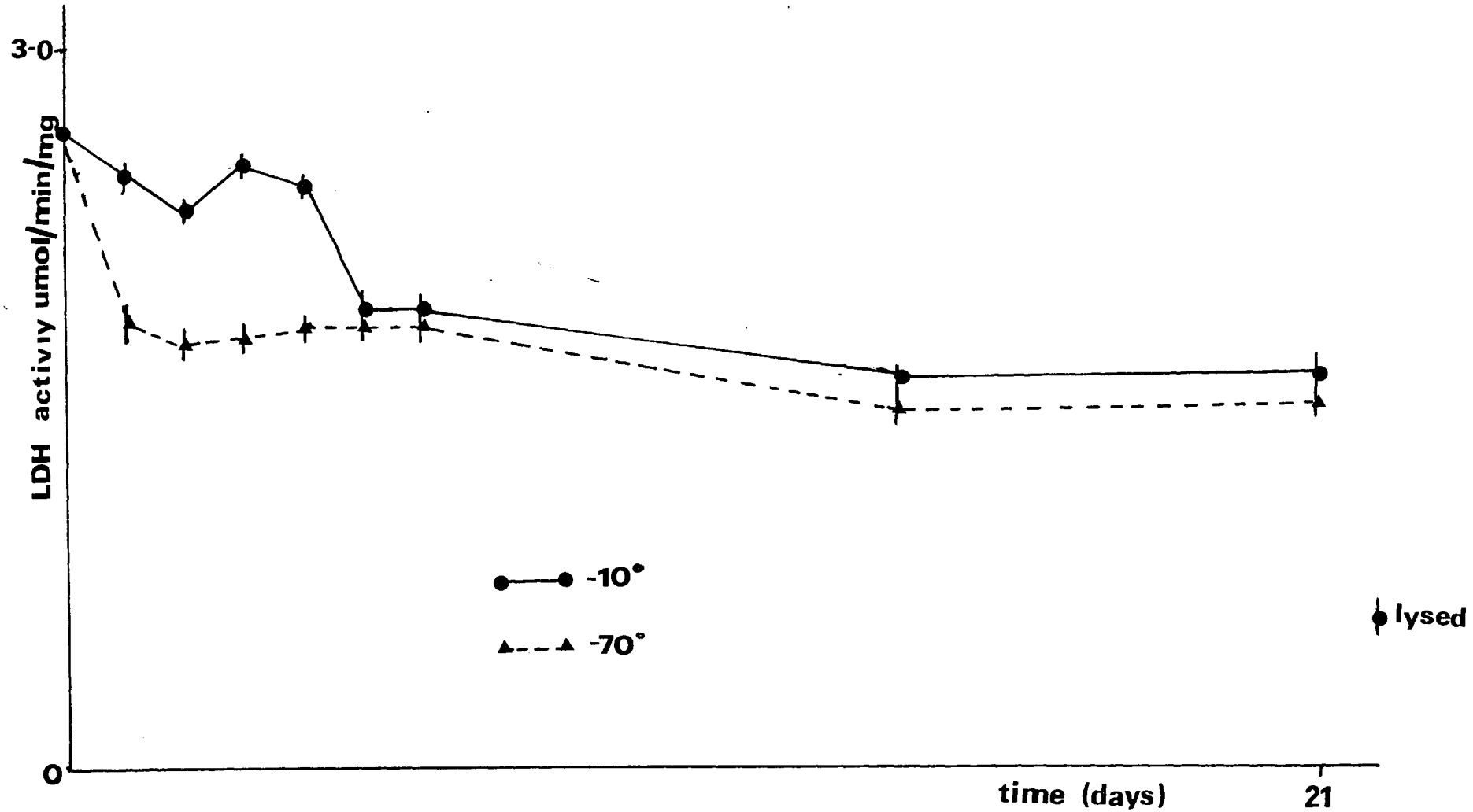


Figure 3.7 Time course for LDH activity of synaptosomes when stored in a glycerol medium at temperatures of -10°C and - 70°C.



Legend to Table 3.6

The effect of different storage conditions on the exogenous amino acid uptake into rat cerebrocortical synaptosomes

The values shown represent means $\pm$ S.E.M. of 6 separate preparations for the incorporation of ^{14}C isotope (0.5μCi, 1μM) into 1-3mgs of tissue. Incubations were carried out in Krebs phosphate medium for a total of 35mins at 37°C, the radioisotope being added for only the last five minutes. The experiment was terminated at room temperature by bench centrifugation (15,000 rpm, 2mins) and the synaptosome pellets were washed with fresh medium (3 x 1ml). Tris buffer extracts (10mM, pH4.7) of the pellets were then prepared for scintillation counting as described in the Materials and Methods chapter.

Legend to Figures 3.8 and 3.9

Morphology of stored synaptosome suspensions after incubation

Synaptosomes were pelletted from the medium by centrifugation at room temperature and fixed, stained and studied by electron microscopy as described in the Materials and Methods chapter. Magnification was x32,000 in all cases. Incubation was for 60 minutes in Krebs phosphate medium at 37°C.

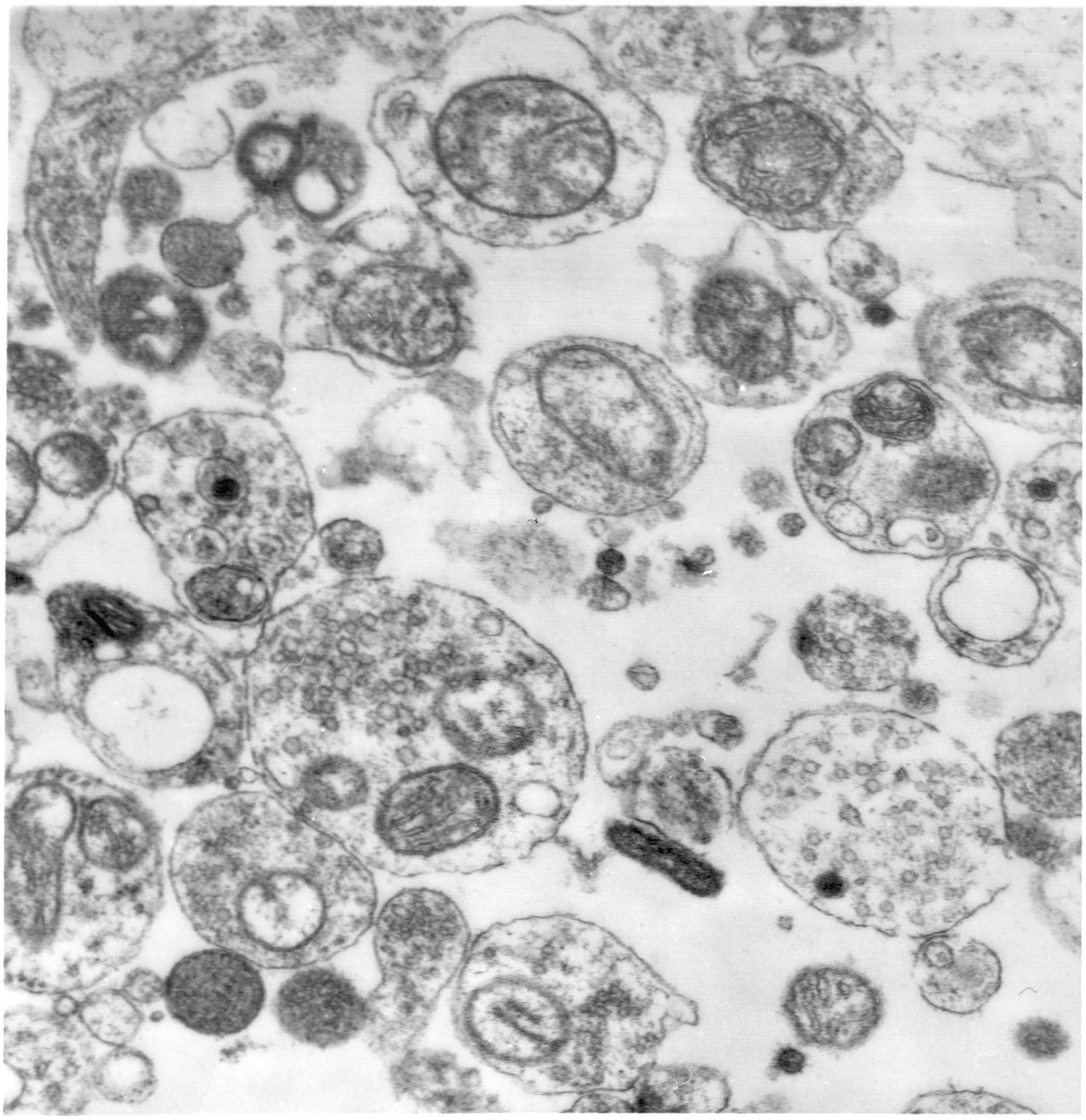
Figure 3.8 electron micrograph of fresh synaptosomes

Figure 3.9 electron micrograph of synaptosomes stored for 6 days at -70 °C.

Amino acids	Fresh (6)	Fresh+Glycerol (6)	-10°C (6)	-70°C (6)
Glutamate	63±1.9	59.4±3.3	6.1±0.3	5.8±0.2
GABA	69±3.0	70.9±4.7	7.1±0.4	11.1±1.1
Leucine	14.2±0.9	12.9±0.4	5.2±0.1	5.3±0.5
Tyrosine	23.4±1.3	18.5±0.5	6.1±0.1	6.1±0.6
Glutamine	56.6±2.8	54.3±2.4	6.3±0.3	5.5±0.5

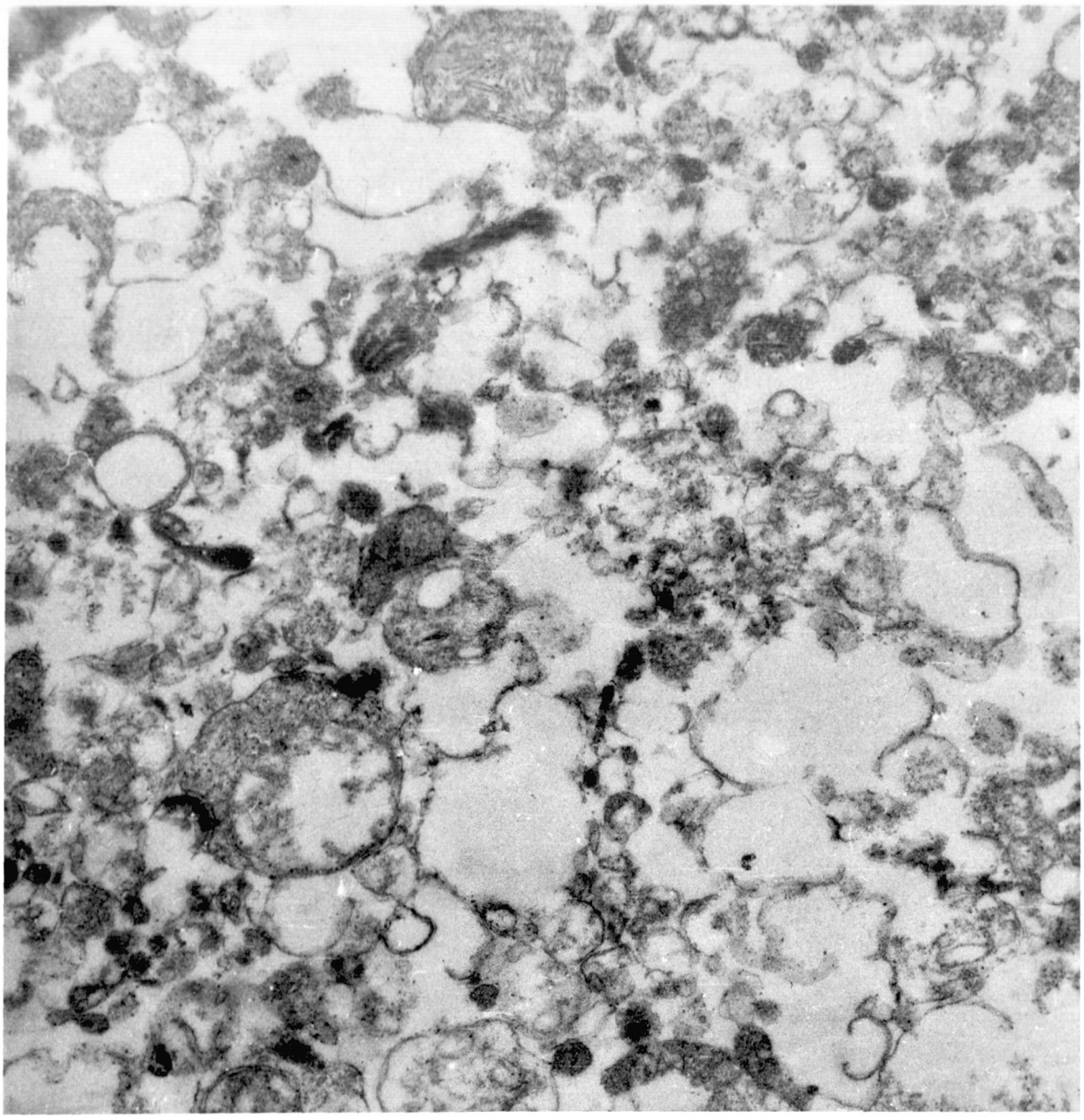
Table 3.6 Exogenous ^{14}C amino acid uptake into rat cerebrocortical synaptosomes stored for 6 days in glycerol medium, (values are expressed in units of dpm/100mg protein 10^{-6}).

Figure 3.8 (opposite) Electron micrograph of freshly prepared rat cerebrocortical synaptosomes.



1 um

Figure 3.9 (opposite) Electron micrograph of rat cerebrocortical synaptosomes stored for 6 days at -70°C.


1 um

and that this value was apparently constant for up to 21 days storage (Fig. 3.7). Hence as with the K^+ assay, the enzyme marker assay would indicate an enrichment of synaptosomes with an intact plasma membrane.

(d) Release and uptake of transmitter-candidate amino acids

The encouraging results of oxygen uptake, K^+ ion accumulation and LDH activity in our stored synaptosomal preparations, led quickly to the next investigation that was carried out, i.e. an analysis of putative amino acid release and reuptake. Obviously, any evidence that the stored synaptosomes could transport the physiologically active agents glutamate, GABA, and aspartate would imply that complex neurochemical functions are being exhibited in these preparations. Reuptake of neurotransmitter substances into presynaptic nerve endings or glia is known to constitute an efficient mechanism be which post-synaptic action can be determined.

Initially, freshly prepared synaptosomes were analysed for their evoked release of glutamate, aspartate, and GABA by potassium ion (56mM) and veratrine induced stimulation (75μM). It was found that for these three amino acids, the concentrations in the medium increased substantially (3-10 fold)when the synaptosomes were depolarized, whereas the levels of the non-candidate amino acids remained unchanged or increased only slightly (Table 3.2). The evoked release of glutamate, GABA and aspartate was abolished when the sodium ion channel associated with neurotransmitter release was blocked with tetrodotoxin (1μM). Unfortunately, when synaptosomes which had been stored frozen either at -10°C or

at -70°C, this pattern of evoked release for the candidate transmitters was not repeated. Veratrine induced depolarization did not significantly enhance the level of glutamate, GABA or aspartate above that of controls (Table 3.4). This lack of enhanced release from the stored preparations is probably not due to the inclusion of glycerol in the medium since freshly prepared synaptosomes in a Krebs phosphate-glucose-glycerol medium did exhibit veratrine induced depolarization successfully (Table 3.3) with enhanced release of the putative transmitter amino acids. Also, another striking feature concerning the stored preparations were the high levels (2-10 fold) of amino acid resting release (Table 3.5) compared to that in freshly prepared synaptosomes. This effect was not limited to only glutamate, GABA, and aspartate, but was true for every amino acid concentration measured.

The lack of detection of stored synaptosomes to show stimulated release of transmitter led to an investigation in the reuptake of amino acids from the extracellular medium (Table 3.6). Using exogenous ^{14}C isotopes, the effect of freezing the synaptosomes was to reduce their uptake capability by approximately ten-fold. This was true for not only GABA and glutamate, but also for the non-transmitter candidates leucine, tyrosine and glutamine. The inclusion of glycerol in the medium with fresh synaptosomes had no apparent significant effect on the uptake of the amino acids measured.

(e) Morphology of stored synaptosomes

Synaptosomes which have been incubated in a physiological medium are known to show improved staining properties and clearer morphological features when compared with nerve endings

taken directly from the sucrose gradient and fixed for electron microscopy (Jones, 1975). For this reason, only incubated synaptosome preparations were compared in the present study. Freshly prepared synaptosomes (Figure 3.8) revealed large numbers of presynaptic nerve terminals characterised by an intact plasma membrane enclosing vesicles and mitochondria, and with postsynaptic thickenings where the plane of sectioning was appropriate. By comparison, synaptosomes which had been stored frozen at -70^{o}C for 6 days in a glycerol medium (10%v/v), showed primarily membranes and synaptosomal ghosts indicating substantial disruption of nerve endings (Figure 3.9).

3.3 Discussion

Although the literature would suggest that some complex neurochemical functions can be protected from freezing damage by agents such as dimethyl sulphoxide (DMSO) and glycerol (Pascoe, 1957; Tower and Young, 1973; Suda et.al., 1974; Houle and Das, 1980), the problem of finding a suitable cryoprotective process is complex since damage to synaptosomal tissue may occur at several stages. The conditions which could be manipulated include: the holding temperature and the storage time at that temperature; the rate of cooling and rewarming of the frozen tissue; and also the concentration of cryoprotective agent. Because of this complexity, the selection of freezing conditions used was arbitrary, although based on the experience of other workers in the field (Bird and Iversen, 1974; Enna et.al., 1976; Farrant, 1980).

Preservation of the synaptosomes seems to be relatively insensitive to the holding temperature because only marginally different results of O_2 uptake/K^+ retention and LDH activity

were obtained using holding temperatures between -10°C and -70°C, for storage times of 1 and 6 days. However, the fact that these frozen preparations did not subsequently exhibit amino acid transmitter release and uptake is a disappointment regarding their potential use in the future as a neurochemical tool for post-mortem brain work. Along with the discouraging morphological analysis, it would appear from the results presented here that the neurosecretory processes in nerve endings are much more sensitive to the trauma of freezing and thawing than the synaptosomal elements concerned with metabolic activities.

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IN VITRO approaches to Amino Acid Neurotransmitter Transport

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CHAPTER 4

IN VITRO Approaches to Amino Acid Transport and Release: Synaptosome suspensions

4.1 Introduction

In recent years, synaptosomes have been utilized in a large number of studies to analyse some of the properties of the pre-synaptic nerve terminal - eg. uptake, storage, release of putative neurotransmitters. The results obtained have provided an alternative method of study to that of the electrophysiological approach (see chapter 6). The primary purpose of the work presented here, was to use various amino acid analogues as biochemical tools to investigate neurotransmitter uptake and release coupling mechanisms. In part A of this chapter, uptake and 'resting' release of the candidate amino acid neurotransmitters was investigated using conventional flask incubations of synaptosomes. Another type of mechanism for uptake and release, called 'Homoexchange' was investigated in part B of this work, using a synaptosome superfusion system.

In the last section in this chapter (part C), the possible interaction between amino acid release and prostaglandins was investigated in conventional synaptosomal incubations.

Part A

4.2 Introduction

It is well known that Na^+-dependent amino acid transmitter re-uptake in the pre-synaptic terminal is responsible for its own inactivation at the synapse (Logan and Snyder, 1971), and so any transmitter present in the medium after stimulation represents a net enhancement of the outward flux over the inward flux for the molecules. However, although this has been extensively investigated for the depolarised situation, very

little is known about synaptosomal amino acid 'resting release'.

It is well known from our own laboratory and other workers (Benjamin and Quastel, 1977; Bradford et.al., 1975), that amino acids,both candidate and non-candidate transmitters,are detected in a physiological medium just from incubating neural tissue and the question investigated in this chapter is - how and why are they there? Are they there because

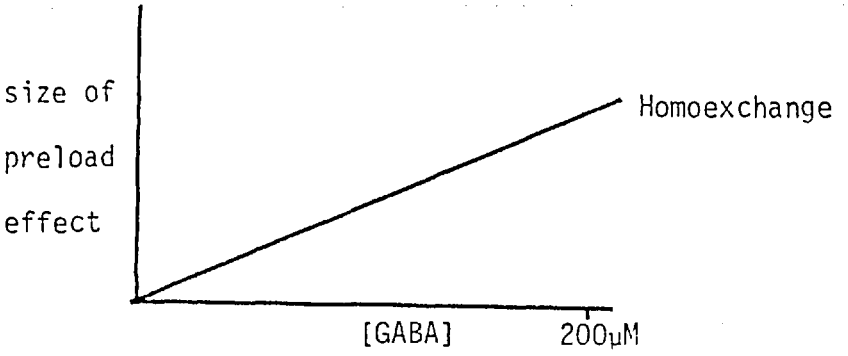
(1) of synaptosomal leakage/tissue breakdown of amino acids down concentration gradients, or

(2) do they represent a mepp mechanism analogous to ACh in NMJ systems (Fatt and Katz, 1952), ie an organised continuous resting release effect that is ion gated.

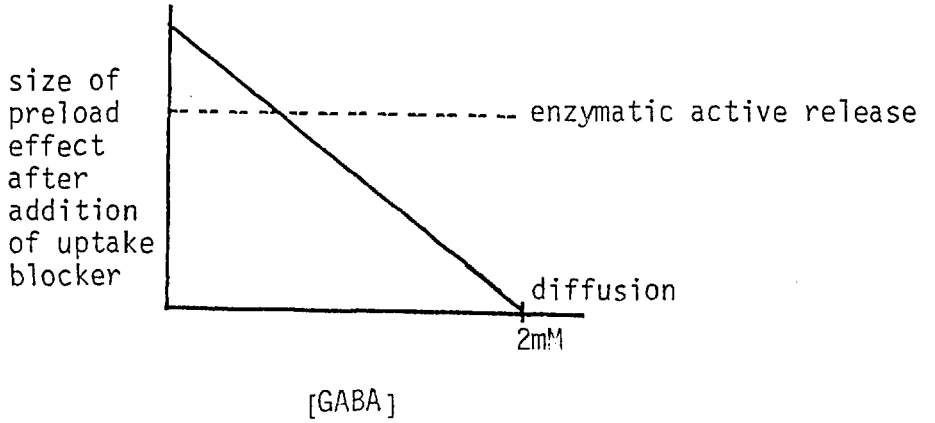
Using various amino acid uptake blockers evidence is presented to favour the idea that synaptosomes exhibit a continuous resting release for the candidate neurotransmitters GABA, glutamate, but not for leucine. This continuous release process, if closely associated with Na^+-dependent re-uptake channels has been termed a 'transport shuttle' by Cox and Bradford (1978): a process through which if organised and ion gated could account for many amino acid neurotransmitter uptake/release phenomena eg. homoexchange, depolarized release, and resting release. Calcium dependence and Tetrodotoxin (TTX) sensitivity for the resting release of preloaded transmitter in nerve terminals was investigated.

It was also determined whether the release and uptake processes were just simple manifestations of homoexchange. Adding external GABA at concentrations approaching the external synaptosome levels and measuring the subsequent GABA 'preload effect', might illuminate the different processes taking place

by the shape of the graph, eg:



It was also determined whether the so-called resting release was, in fact, diffusion of amino acids down concentration gradients, which is seen predominantly for the candidate amino acid neurotransmitters anyway since they have a high concentration intracellularly. This was studied by firstly adding an uptake blocker to the incubating pre-labelled synaptosomes, and then adding external GABA to the medium (0-2mM). Curves reflecting the different processes occurring might be:



4.3 Results

(a) The effect of various amino acid analogues on candidate amino acid transport in rat cortical synaptosomes

Modulation of neurotransmitter amino acid transport in rat cortical synaptosomes was studied using the following compounds:

(1) parachloromercuriphenyl sulphuric acid (PCMPS, 1mM).

(2) Nipecotic acid (1mM) and isonipecotic acid (1mM).

(3) Gamma-vinyl-gaba (GVG, 1mM).

(4) 2(Alpha)-amino-4-phosphonobutyrate (APBA, 1mM).

(5) Kainic acid (KA, 1mM) and dihydrokainate (DHK, 1mM).

(6) Threo-hydroxy-aspartate (THA, 1mM).

(1) Action of p-chloromercuriphenyl sulphonic acid (Figures 4.1a, 4.1b)

PCMPS at a concentration of 1mM was found to significantly elevate counts in the medium for gaba and glutamate but not for leucine, whether or not the amino acid was preloaded or not preloaded. This picture was also mirrored by the tissue levels in that labelled GABA and glutamate were decreased by 64% and 96% respectively. Exogenous leucine levels remained unchanged.

(2) The Effect of Nipecotic acid (1mM) and Isonipecotic acid (1mM)

This experiment illustrated the isomeric conformation of the GABA (transport molecule (receptors)) with regard to exhibiting the preload effect. Figures 4.2a and 4.2b show that nipecotic acid increased and decreased exogenous gaba in the medium and tissue respectively whereas isonipecotic acid had no effect.

(3) The action of γ-vinyl-gaba (1mM) (Figure 4.3)

GVG (1mM), when incubated with rat cerebrocortical synaptosomes, was found to significantly increase exogenous gaba in the medium and conversely decrease it in the tissue, (typically 170% increase in the medium and a 55% decrease in the tissue, when ^{14}C GABA was preloaded). Whether this represents GVG exhibiting a preload effect or whether it is a manifestation of homoexchange in static incubations is discussed later.

Legend to Figures 4.1a and 4.1b

The effect of parachloromercuriphenyl sulphonic acid (PCMPS, 1mM) on exogenous ^{14}C glutamate (2μM), ^{14}C GABA (2μM), ^{14}C leucine (1mM) transport in rat cortical synaptosomes

Synaptosomes were preincubated in Krebs phosphate medium and treated as outlined in the Methods and Materials section, for an uptake/release experiment. Values are means $\pm$ S.E.M. for 4-6 determinations.

(1) Preloaded, control.

(2) Preloaded (+) PCMBS (1mM).

(3) Not preloaded, control.

(4) Not preloaded (+) PCMBS (1mM) in the medium.

* $p < 0.001$

** $p < 0.01$

Fig. 4.1a reflects medium cpm/mg protein expressed as % control.

Fig. 4.1b reflects tissue cpm/mg protein expressed as % control.

Figure 4.1a The action of PCMPS on synaptosomal medium amino acid transport.

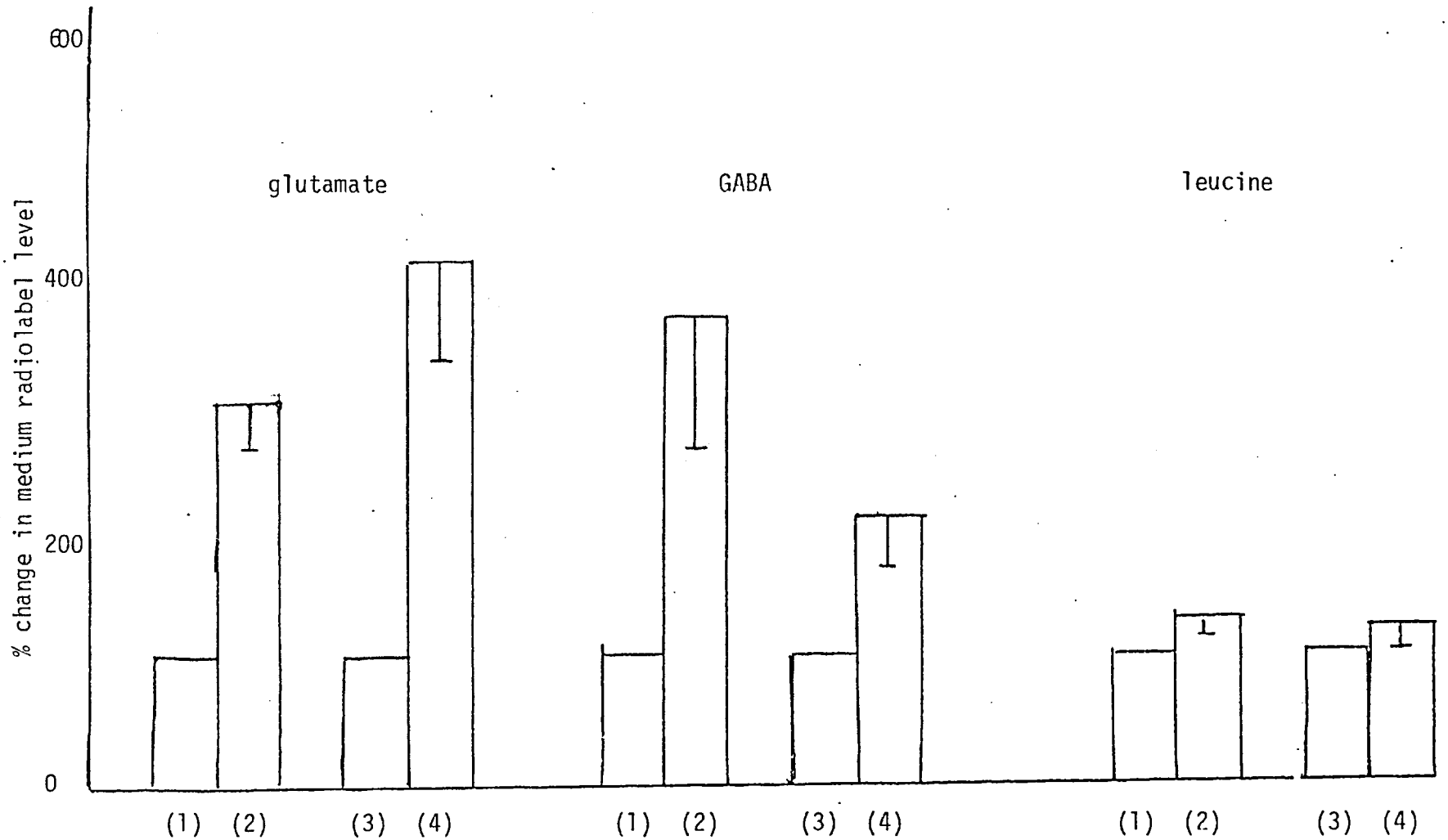
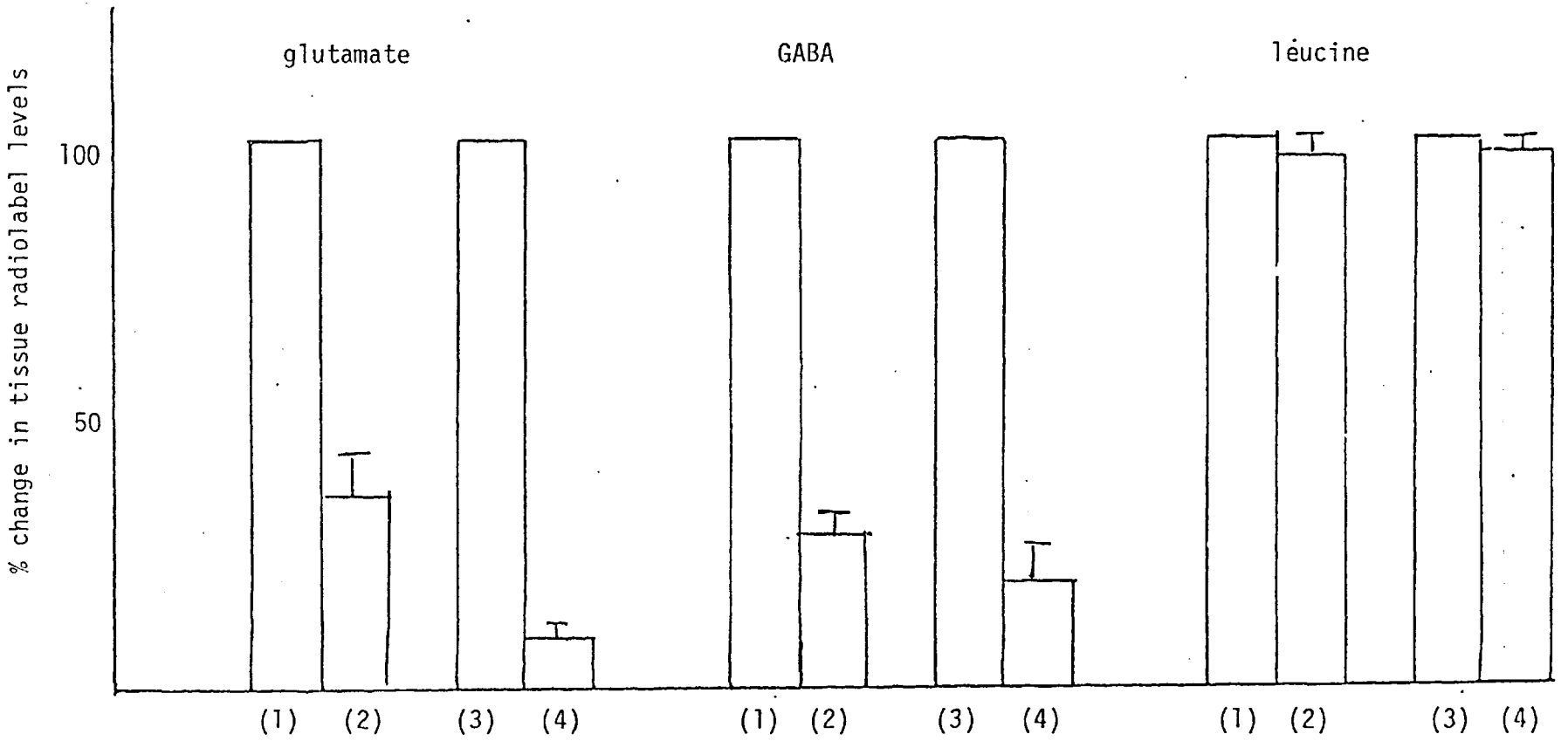


Figure 4.1b The action of PCMPS on tissue synaptosomal amino acid levels.



Legend to Figures 4.2a and 4.2b

The effect of nipecotic acid (1mM) and isonipecotic acid (1mM) on on the transport of exogenous ^{14}C GABA (2μM) in rat cerebrocortical synaptosomes

Synaptosomes were incubated in Krebs phosphate medium and preloaded or not preloaded as indicated with ^{14}C GABA. Nipecotic acid and isonipecotic acid were added as for an uptake/release experiment (see Materials and Methods section). Values are means $\pm$ S.E.M. for 4-6 determinations. cpm /mg protein expressed as % of control. Change in release or uptake due to presence of nipecotic acid, $p < 0.001$ *.

(1) Preloaded control with ^{14}C GABA (2μM).

(2) Preloaded (+) nipecotic acid or isonipecotic acid.

(3) Not preloaded control.

(4) Not preloaded (+) nipecotic acid or isonipecotic acid followed by addition of 2μM ^{14}C GABA.

Legend to Figure 4.3

The effect of γ-vinyl gaba (GVG, 1mM) on exogenous ^{14}C GABA uptake/release from rat cerebrocortical synaptosomes

Synaptosomes were preincubated in Krebs phosphate medium and treated with GVG (1mM) as outlined before with these uptake/release experiments. Values are means $\pm$ S.E.M. for 2-3 determinations. cpm/mg protein expressed as % of controls.

(1) Preloaded control, 2μM ^{14}C GABA.

(2) Preloaded; GVG (1mM) added.

(3) Not preloaded control.

(4) Not preloaded; GVG (1mM) added followed by ^{14}C GABA (2μM).

Figure 4.2a The effect of nipecotic and isonipecotic acid on medium amino acid levels.

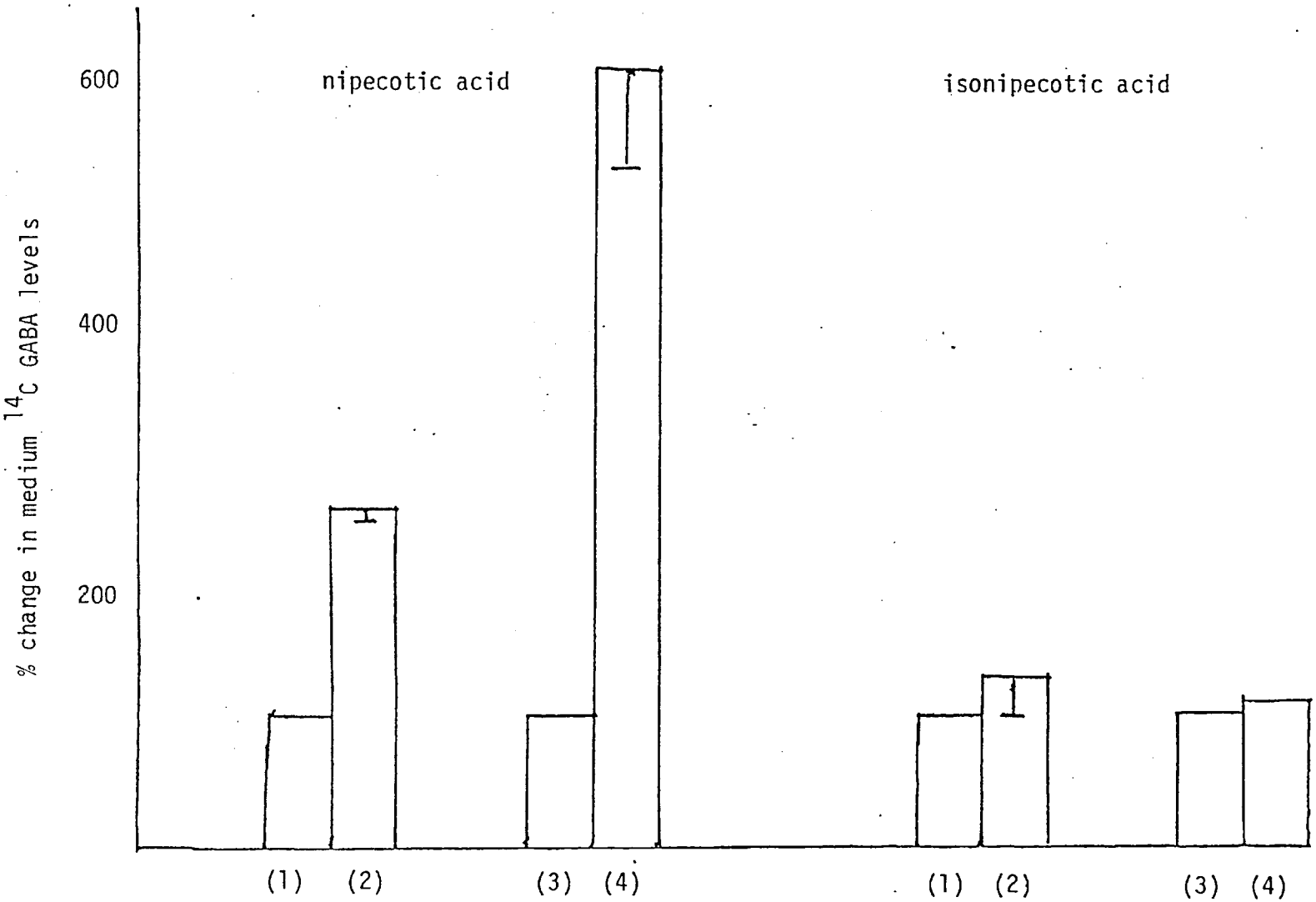


Figure 4.2b The effect of nipecotic acid and isonipecotic acid on ^{14}C GABA tissue levels.

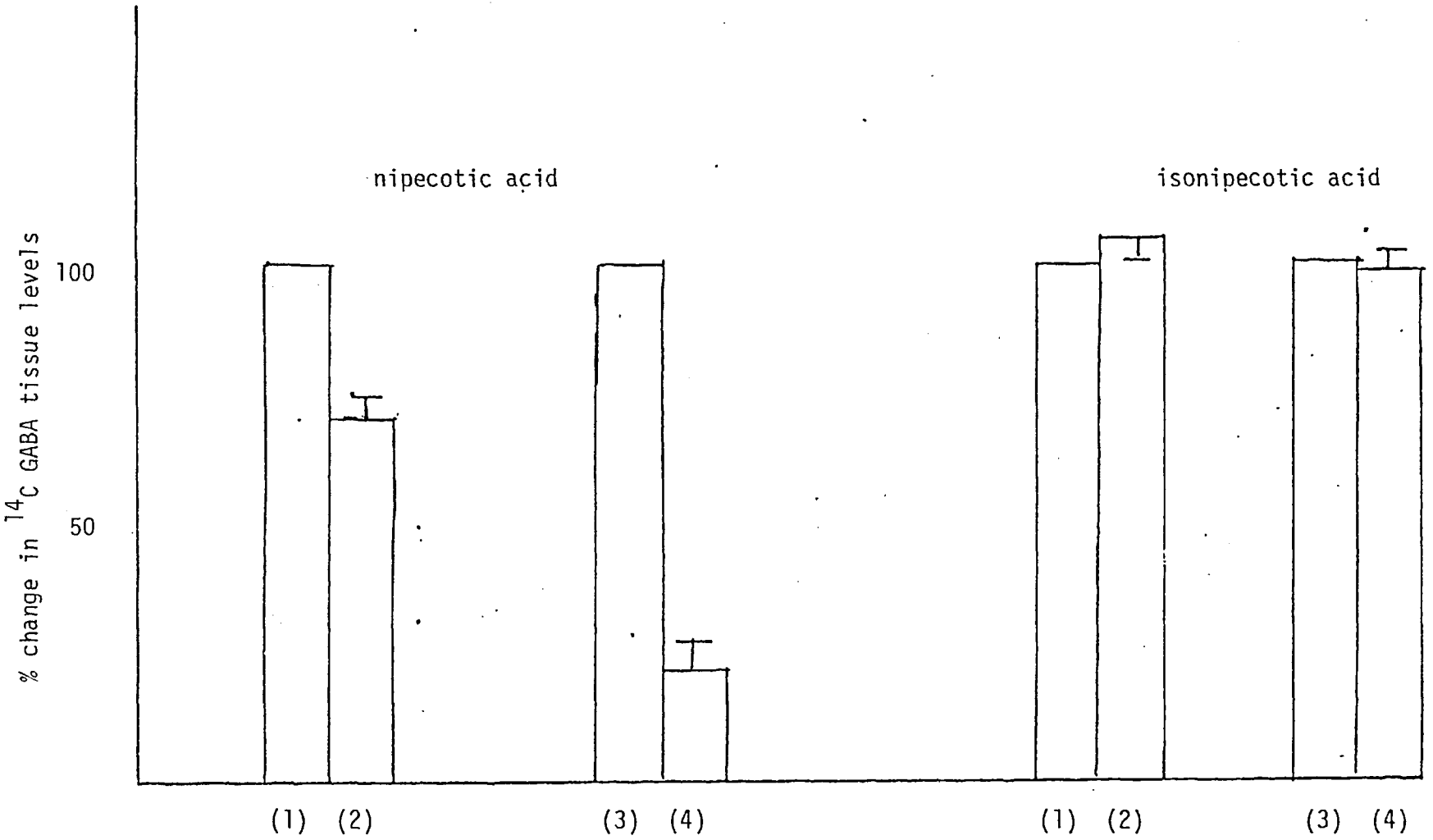
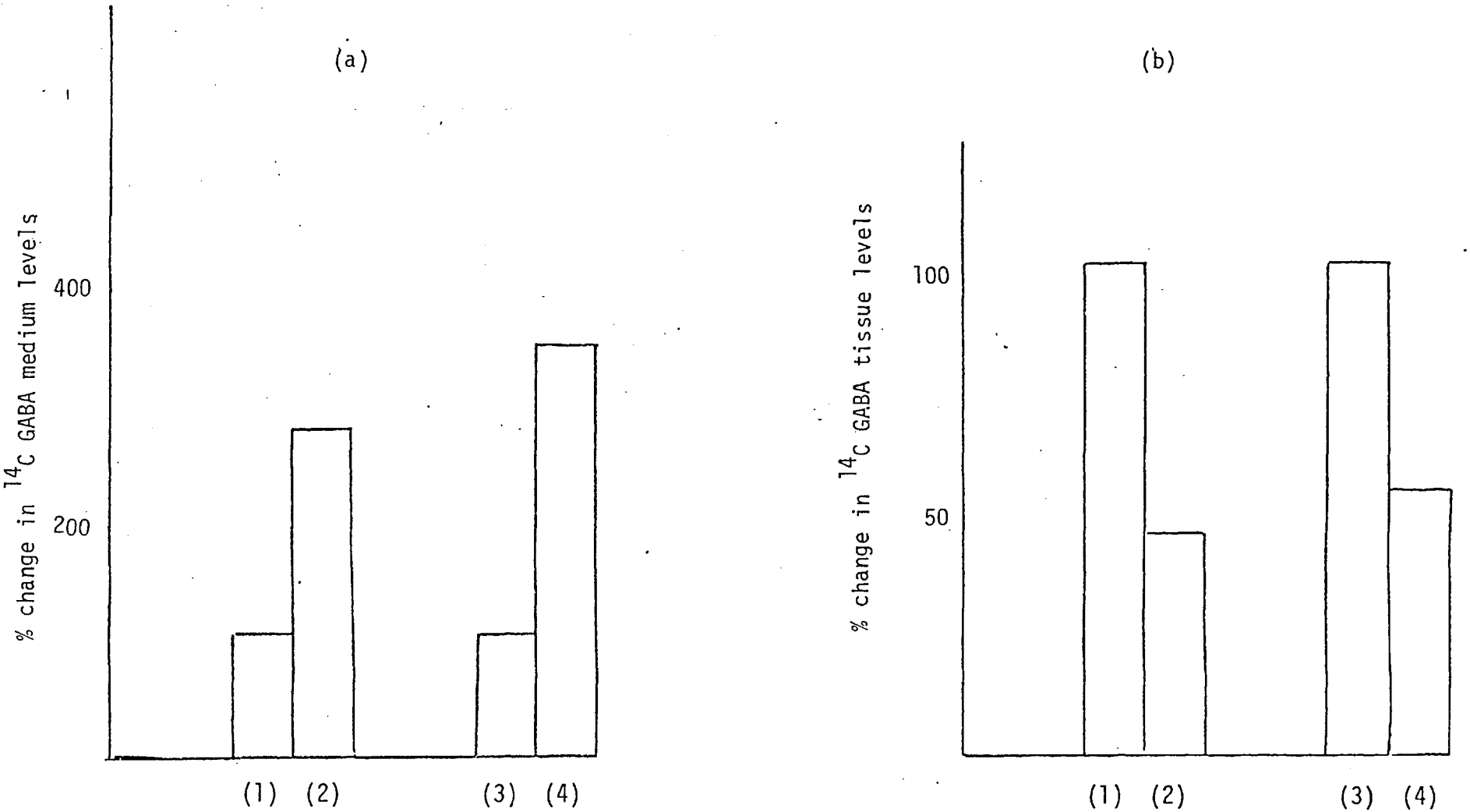


Figure 4.3 The action of γ-vinyl GABA on synaptosomal amino acid levels.



(4) The effect of 2-amino-4-phosphonobutyrate (APBA, 1mM) (Figure 4.4)

APBA, a structural analogue of glutamate, was found not to exhibit a preload effect with exogenous ^{14}C glutamate. Figure 4.4 shows no significant difference in levels of radioactivity between controls and test suspensions, irrespective of whether the synaptosomes were exogenously preloaded or unlabelled.

(5) The action of kainic acid (1mM) and dihydrokainate (DHK, 1mM) (Figure 4.5)

In this experiment, isomeric cyclic compounds with the same carbon chain length as glutamate were used to probe the receptor activities of the synaptosomes. Kainate was found to exhibit the preload effect, elevating medium exogenous pre-labelled glutamate by approximately five-fold. Tissue ^{14}C glutamate levels were decreased by 80% when preloaded and nonpreloaded respectively.

DHK at the same concentration, however, was found to be inactive at showing a preload effect either in tissue or medium levels of exogenous glutamate. The effect of KA could be explained by action as a necrotizing agent, or as a reuptake blocker (Olney et.al., 1974; McGeer et.al., 1976, Krogsgaard-Larsen et.al., 1979).

(6) The effect of Threo-hydroxy aspartate (THA, 1mM)

THA (Figure 4.6) was found to exhibit a preload effect with exogenous ^{14}C glutamate. Medium levels were found to increase by 390% and 300% when preloaded and non preloaded amino acid was used respectively. Conversely, tissue levels were decreased with preloaded ^{14}C glutamate by 65% and by

Legend to Figure 4.4

The effect of α-amino-4-phosphonobutyrate (APBA, 1mM) on ^{14}C glutamate uptake/release from rat cerebrocortical synaptosomes

Synaptosomes were preincubated in Krebs phosphate medium and preloaded with ^{14}C glutamate (2μM) as indicated, and the action of APBA was followed. Values are means for 2-3 determinations.

(1) Preloaded control, ^{14}C glutamate (2μM).

(2) Preloaded with ^{14}C glutamate, followed by addition of ^{14}C glutamate.

(3) Not preloaded control, addition of ^{14}C glutamate (2μM).

(4) Not preloaded, addition of APBA (1mM); addition of ^{14}C glutamate (2μM).

Legend to Figure 4.5

The effect of kainic acid (1mM) and dihydrokainate (DHK, 1mM) on the release/uptake of exogenous ^{14}C glutamate (2μM) in rat cerebrocortical synaptosomes

Synaptosomes were preincubated in Krebs phosphate medium and were preloaded or not preloaded with ^{14}C glutamate. Kainate (1mM) or dihydrokainate (1mM) were added as indicated in the Materials and Methods section for these uptake/release experiments. Values are means for 2-3 determinations.

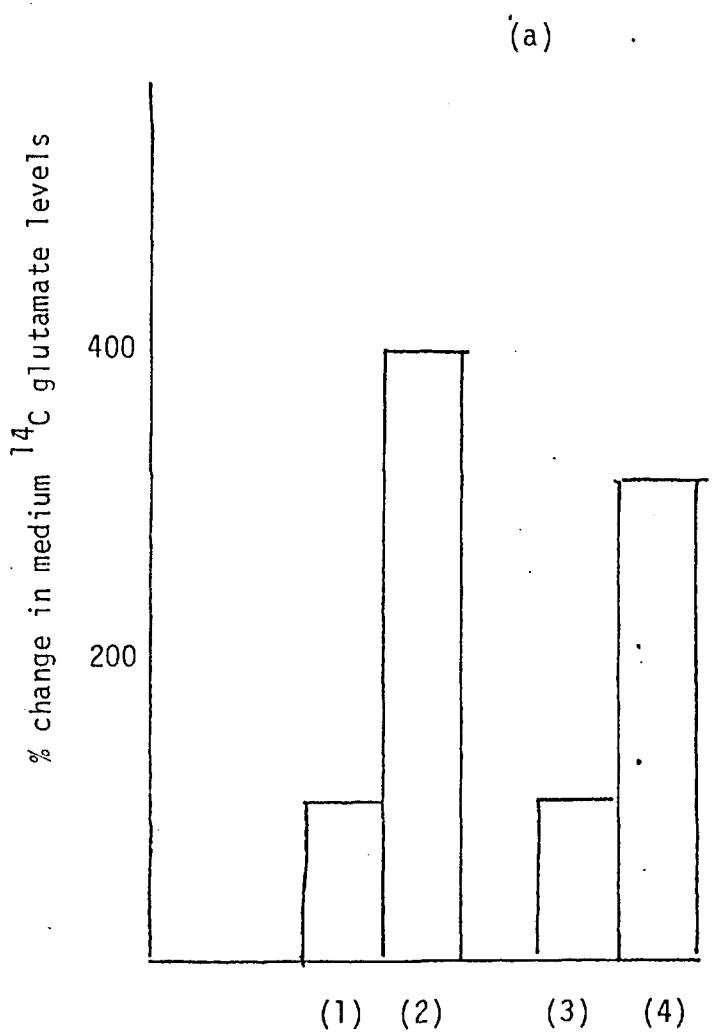
(1) Preloaded control with ^{14}C glutamate (2μM).

(2) Preloaded (+) kainate (1mM) or dihydrokainate (1mM).

(3) Not preloaded control.

(4) Not preloaded, (+) kainate or DHK followed by addition of ^{14}C glutamate (2μM).

Figure 4.4 The effect of α-amino-4-phosphono butyrate on ^{14}C glutamate levels.



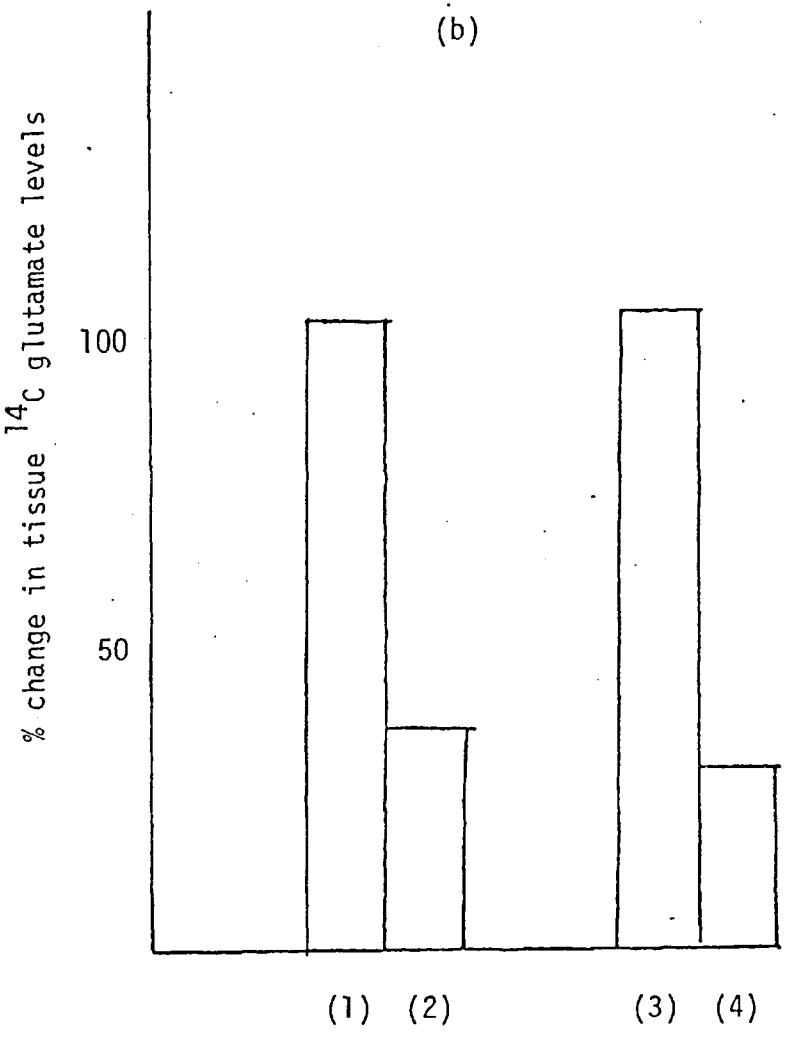
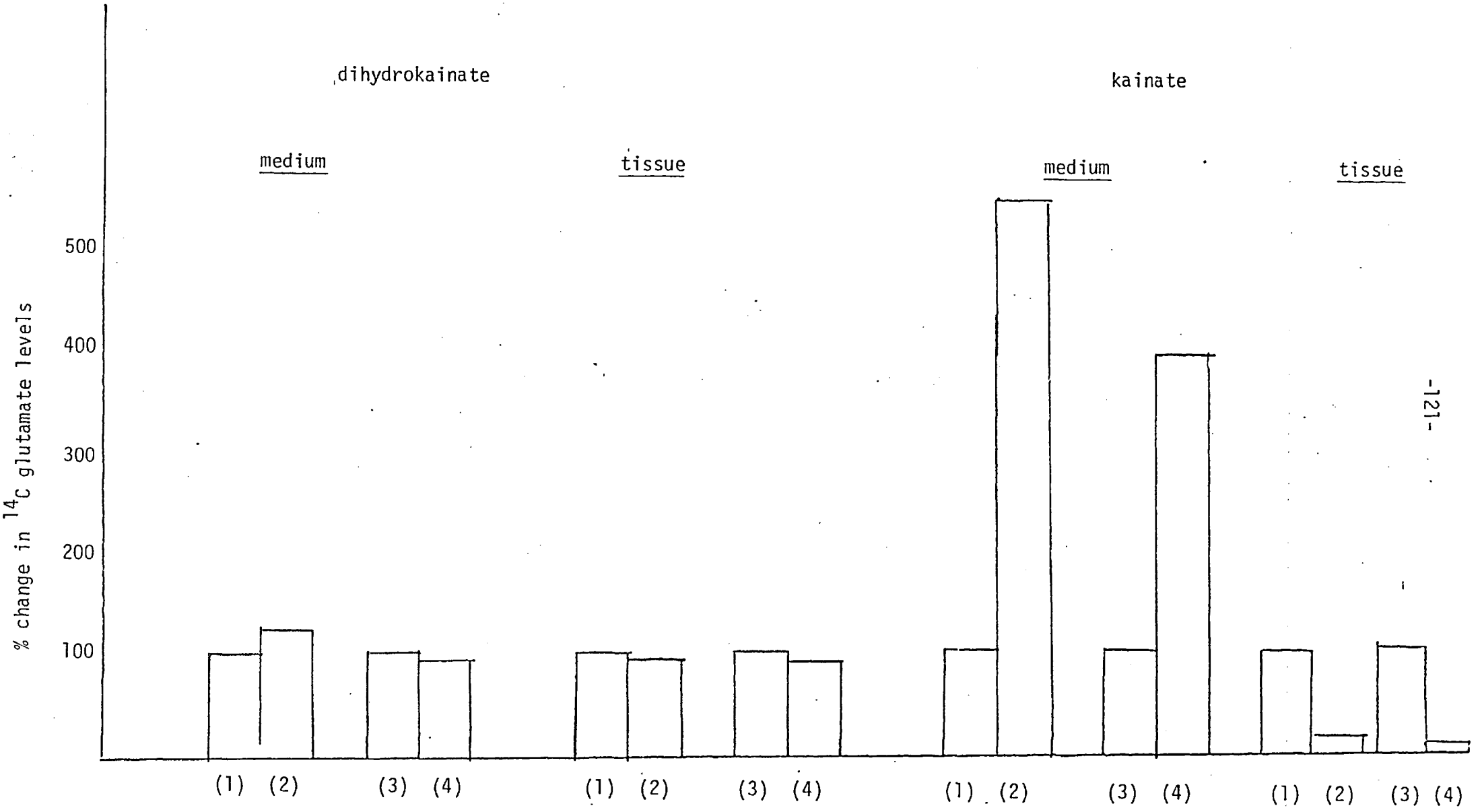


Figure 4.5 The action of kainic acid and dihydrokainate on ^{14}C glutamate levels.



Legend to Figure 4.6

The effect of threo-hydroxy aspartate (THA, 1mM) on exogenous ^{14}C glutamate (2μM) transport in rat cerebrocortical synaptosomes

Synaptosomes were incubated and treated as outlined in the Materials and Methods section for an uptake/release experiment. In the suspensions indicated, the exogenous label was ^{14}C glutamate (2μM). Values are means for three determinations.

(1) Preloaded control.

(2) Preloaded (+) THA (1mM).

(3) Not preloaded control.

(4) Not preloaded, addition of 2μM ^{14}C glutamate followed by addition of THA (1mM).

Legend to Figure 4.7

Time course for the action of PCM^{PS} (1mM) on the exogenous ^{14}C GABA and ^{14}C leucine release from rat cerebrocortical synaptosomes

Synaptosomes (P_2B) were preincubated at 37°C for 30 mins. and then preloaded with ^{14}C GABA (2μM) or ^{14}C leucine (1mM). Following the addition of PCM^{PS} (1mM) to the incubate, the radioactivity released into the medium over a period of 5 mins was determined by liquid scintillation counting. Points represent the means ± S.E.M. for 4-6 values.

Figure 4.6 The action of threo-hydroxy aspartate on ^{14}C glutamate levels.

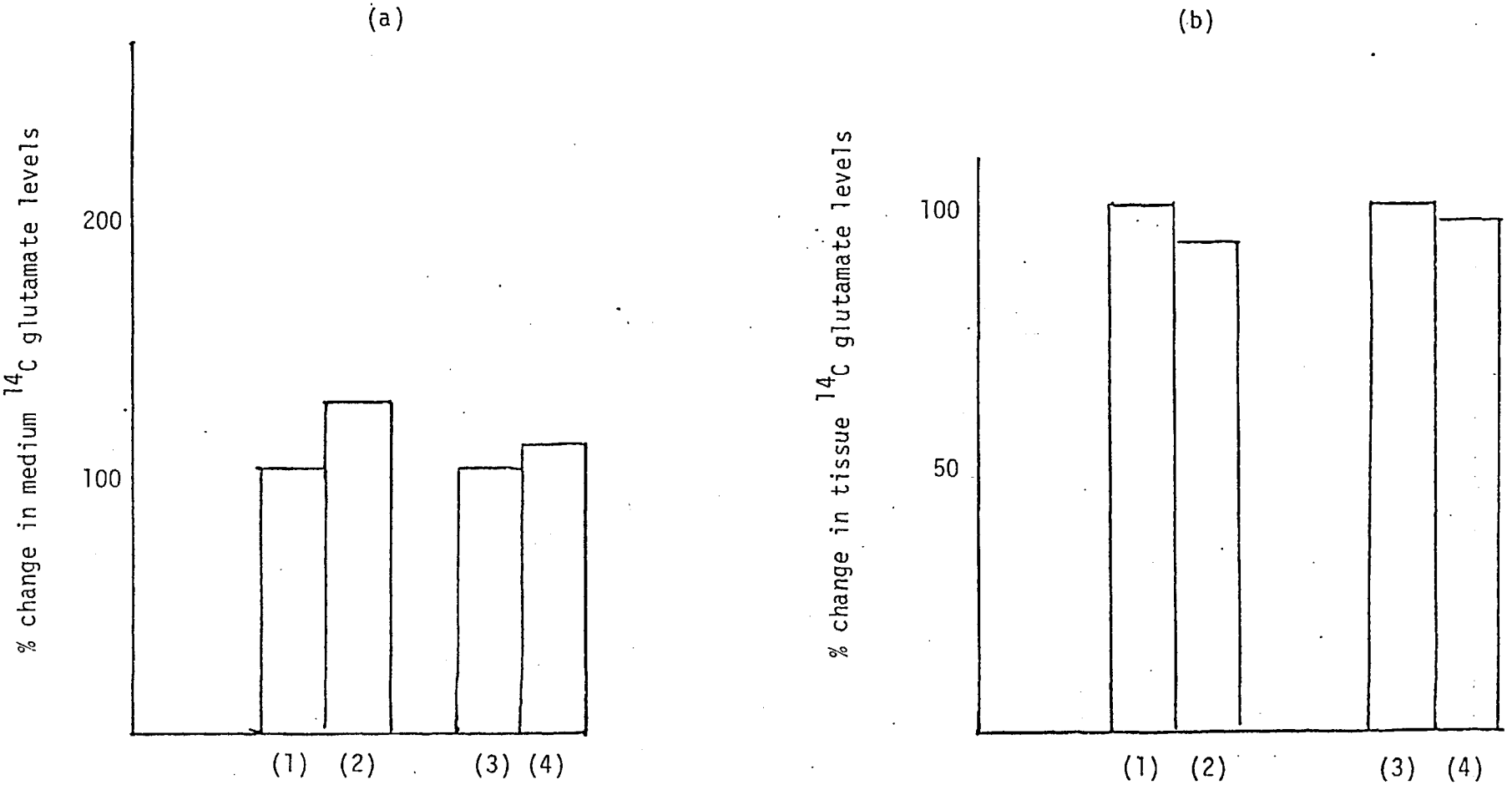
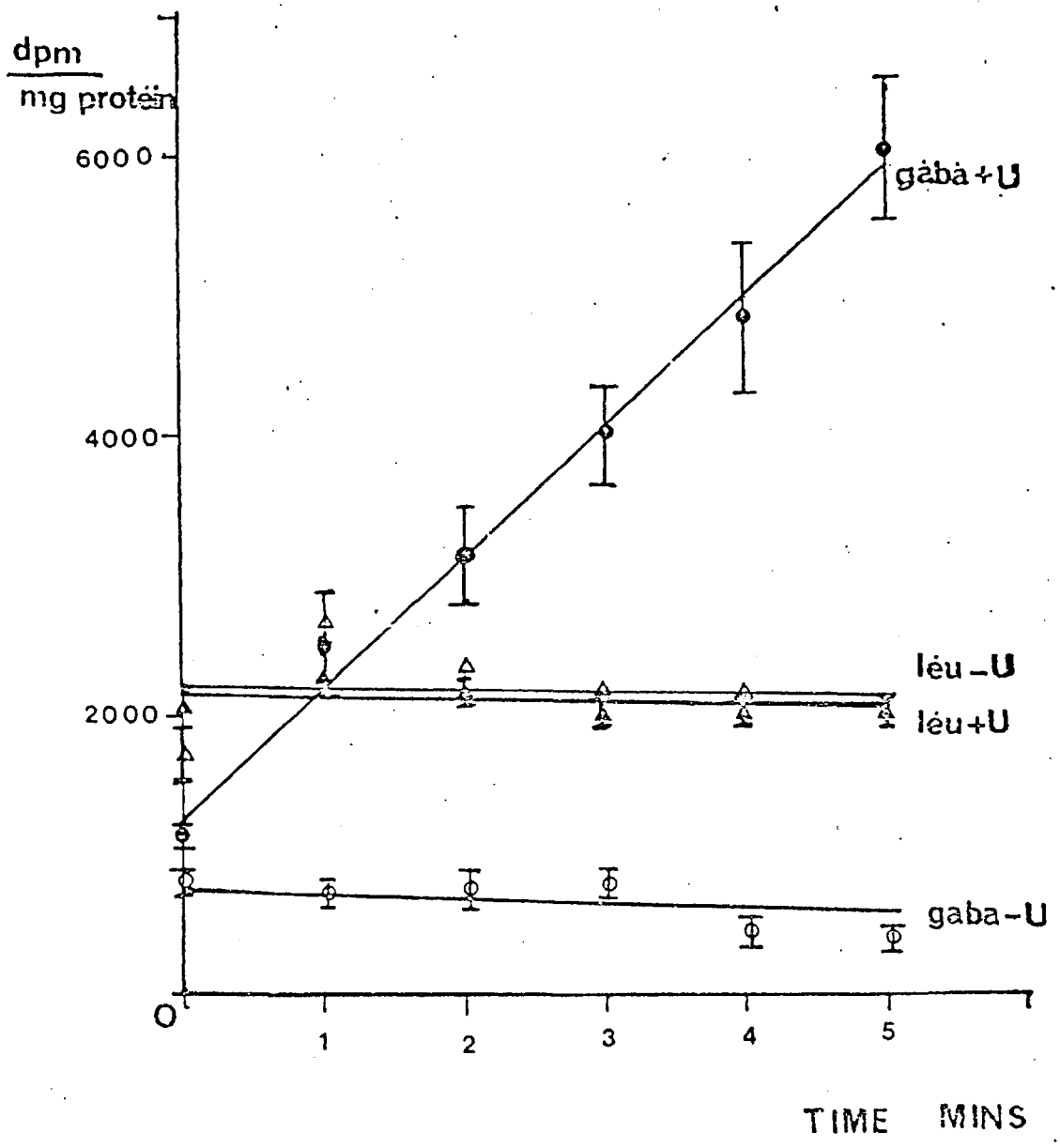


Figure 4.7

Graph to show rates of release of prelabelled amino acids in the prescence and abscence of an uptake blocker (pcmbs) 1mM.



70% with non preloaded ^{14}C glutamate. The action of THA as a glutamate and an aspartate reuptake blocker had been described by Balcarand Johneston (1972).

(b) Effect of various amino acid analogues on Potassium retention by rat cerebrocortical syaptosomes (Table 4.1)

Some aspects of the preload effects described earlier could be explained by the fact that the analogues were inducing synaptic plasma membrane depolarization rather than any manifestation of a transport shuttle. If this was the case, then it would be expected that following incubation with the test compound, synaptosomal potassium retention would be reduced. Table 4.1 shows the results from this type of experiment. Control levels of K^+ were found to be 23 µequivalents/100 mgs protein, which after treatment with veratrine fell by 52% to 11 µequivalents/100 mgs. This loss of K^+ taken to be a result of chemical induced depolarization (see General Introduction) was found to be completely blocked by TTX (1µM).

PCMPS (1mM), γ-vinyl-gaba (1mM), nipecotic acid (1mM), isonipecotic acid (1mM), 2-amino-4-phosphono-butyric acid (1mM), threo-hydroxy-aspartate (1mM) were found not to induce a significant loss of K^+ retention. Of the analogues tested only kainate induced a loss of potassium from the nerve terminals (43%). This was interpreted as a necrotizing action of kainate rather than any physiologically related depolarization.

(c) Time course for the action of PCMPS (1mM) on the exogenous ^{14}C GABA and ^{14}C leucine release from rat cerebrocortical synaptosomes

Legend to Table 4.1

The effect of various compounds on potassium retention of synaptosomes.

Rat cerebrocortical synaptosomes were prepared and pre-equilibrated (37°C, 30mins) as described in the Methods section. Following incubation for 10 mins with the test compound at the concentration indicated, the synaptosomes were pelleted by bench centrifugation, the supernatants being discarded. The pellets were lysed with TCA (1ml, 10%) by leaving on ice for 30 minutes with frequent vortexing. The protein was then pelleted at room temperature again using the bench centrifuge, before aliquots of the supernatants were stored for K^+ determination the following day. K^+ levels were analysed by comparison with standards using an EEL flame emission spectrophotometer.

Table 4.1 Potassium ion content of rat cerebrocortical synaptosomes following incubation with various analogues.

Test compound	Concentration	K^+ level of synaptoplasm after incubation (μequivalents/100ngs protein)
Control		23.2 ± 1.6
PCMPS	1mM	21.4 ± 1.8
γ-vinyl GABA	1mM	22.1 ± 1.7
Nipecotic acid	1mM	23.1 ± 1.5
Isonipecotic acid	1mM	22.5 ± 1.5
α-amino-phosphono-butyric acid	1mM	21.9 ± 1.4
Threo-hydroxy-aspartate	1mM	21.8 ± 1.7
Kainate	1mM	10.1 ± 0.8
Veratrine	75μM	11.2 ± 0.9
Veratrine + TTX (1μM)	75μM	22.3 ± 1.5

Values represent the means ± S.E. of 5-6 determinations.

In this experiment a resting release rate value for GABA was attempted using PCMPS (1mM). Figure 4.7 shows the time course of exogenous ^{14}C preloaded GABA and leucine detected in the medium over the five minute period. As expected for leucine, there was no net release when PCMPS was added to the medium. For gaba, however, a release rate of approximately 700dpm/min/mg protein is detected in the presence of the uptake blocker. Figure 4.8a shows how the tissue activity of exogenous GABA increases in time from 6×10^{-3}moles/dpm at t=0 to 9.6×10^{-3}moles/dpm at t=5 mins. With these experimentally-determined tissue-specific activities of GABA and by assuming a similarity of hot/cold ratios in the medium to that in the tissue, a theoretical time course for the resting release of GABA can be plotted, expressed in absolute amounts. Figure 4.8b shows a resting release rate for gaba of 1nmole/min/mg protein approximately.

(d) Tetrodotoxin (TTX)sensitivity and calcium-independence of exogenous U^{14}C gaba resting release from rat cerebrocortical synaptosomes

In this experiment, active Na^+ channel dependence of resting release was tested by including TTX (1μM) in the incubation medium. TTX was found to reduce the rate of resting release of ^{14}C GABA by 65% from approximately 700 to 240 dpm/min/mg. No significant difference was observed for leucine, however (Fig. 4.9).

In a similar type of experiment designed to test calcium-dependence, nerve terminals were incubated in a calcium-free Krebs-phosphate medium containing EGTA (0.5mM), and with or without the presence of PCMPS (1mM). Figure 4.10 indicates

Legend to Figure 4.8a

Measurement of tissue GABA cold/hot ratios after addition of PCMPS (1mM) in rat cerebrocortical synaptosomes

Synaptosomes (P_2B) were preloaded with exogenous ^{14}C GABA (2μM) in Krebs phosphate medium. PCMPS (1mM) was then added for a period of up to 5 minutes before the medium was separated from the protein by centrifugation (see Materials and Methods section). Radioactivity in the medium was determined by scintillation counting, whereas non-isotopic levels of GABA were measured by automatic ion exchange chromatography. Values are means for three determinations.

Legend to Figure 4.8b

Using experimentally determined tissue cold/hot ratios, a theoretical data based graph of GABA release was plotted. The difference between the two gradients yields an expected net rate of release of GABA for the active process of 1.3 nmoles/mg/min.

Legend to Figure 4.9

Tetrodotoxin sensitivity of resting release of exogenous ^{14}C GABA from rat cerebrocortical synaptosomes

Synaptosomes were preloaded with exogenous ^{14}C gaba (2μM). TTX (1μM) was added to the Krebs phosphate medium one minute before the addition of PCMPS (1mM) or saline for the controls. The radioactivity in the medium occurring over a five minute period was determined by liquid scintillation counting. The values represent the mean of 4-6 determinations, and straight line graphs were plotted by the method of linear regression. Standard errors are omitted for reasons of clarity.

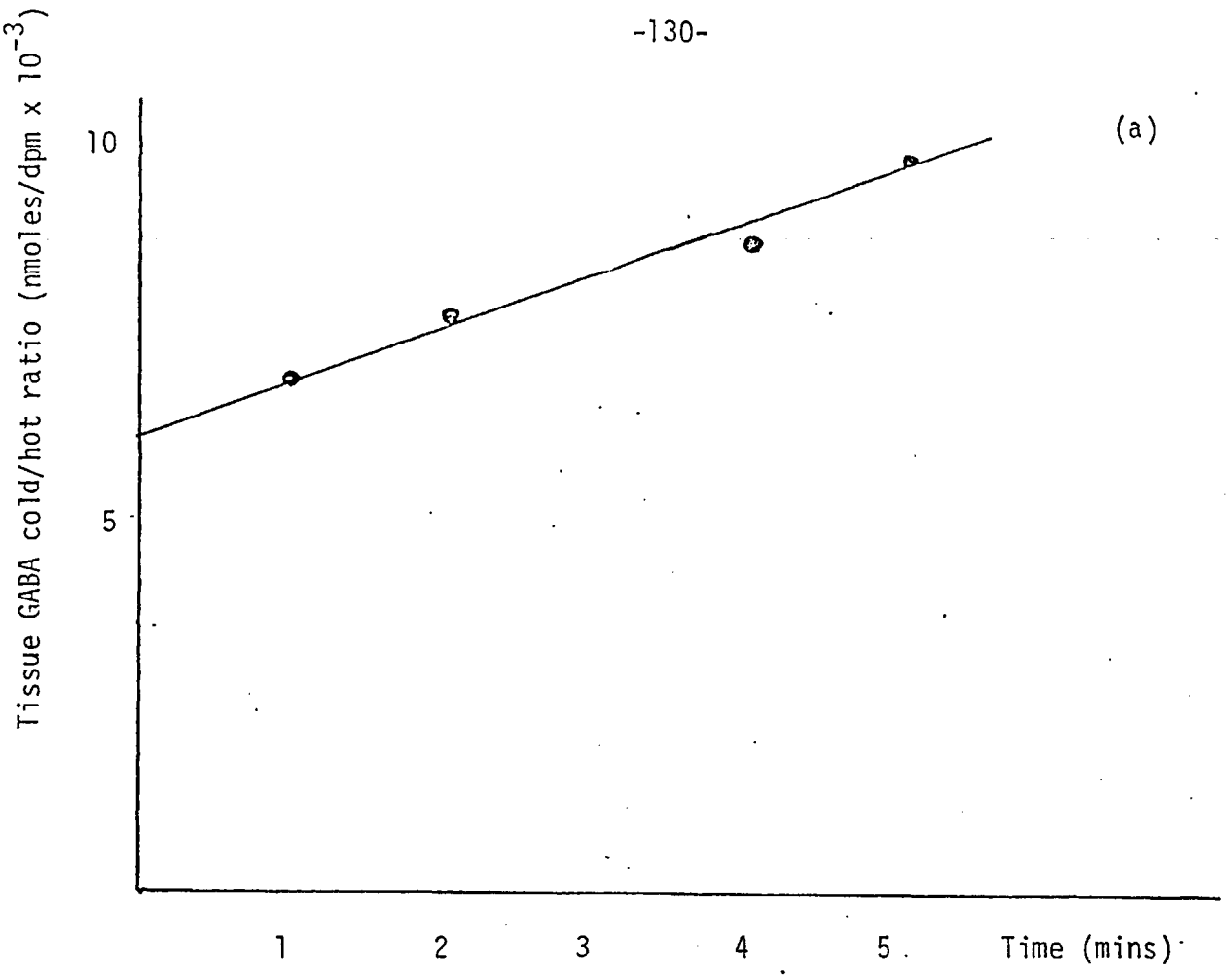


Figure 4.8 Changes in tissue specific activities of GABA in synaptosomes.

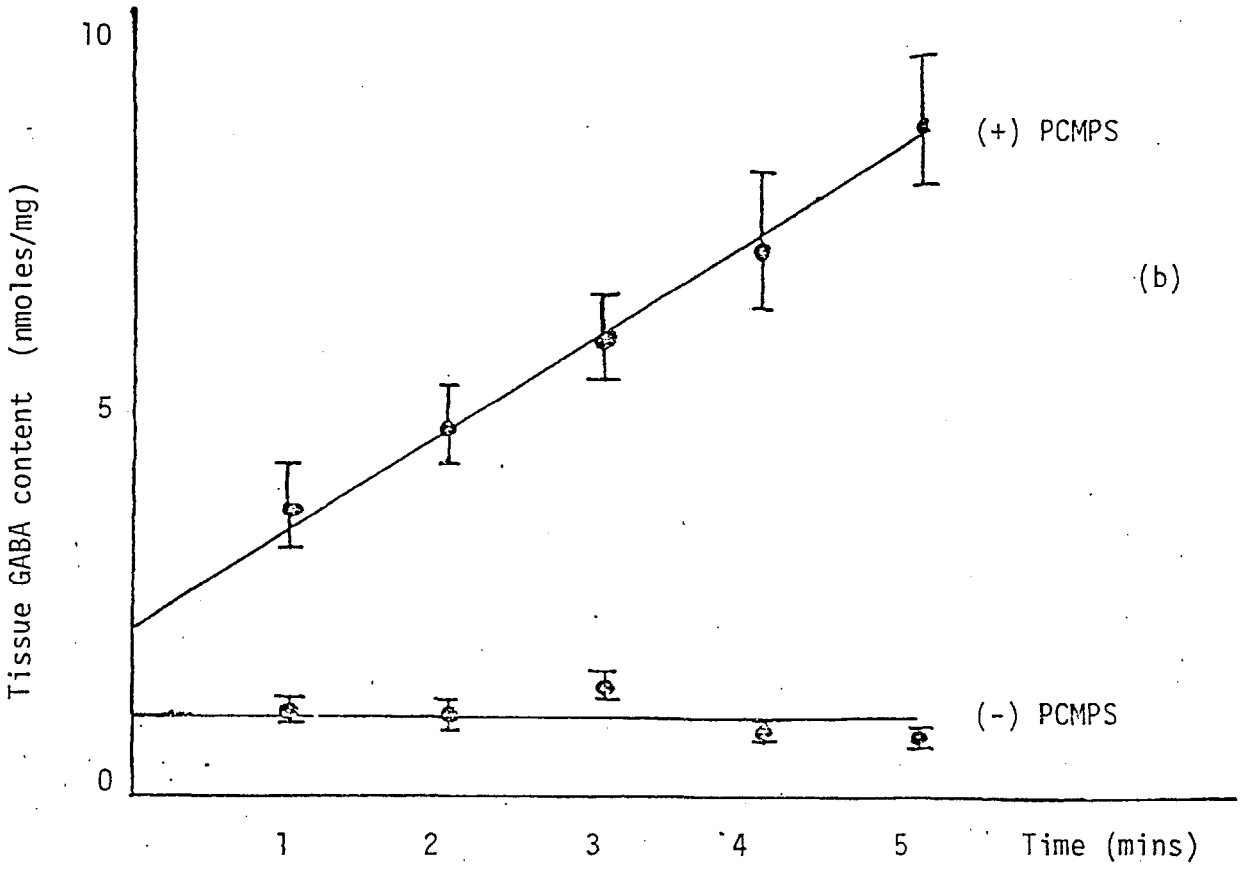
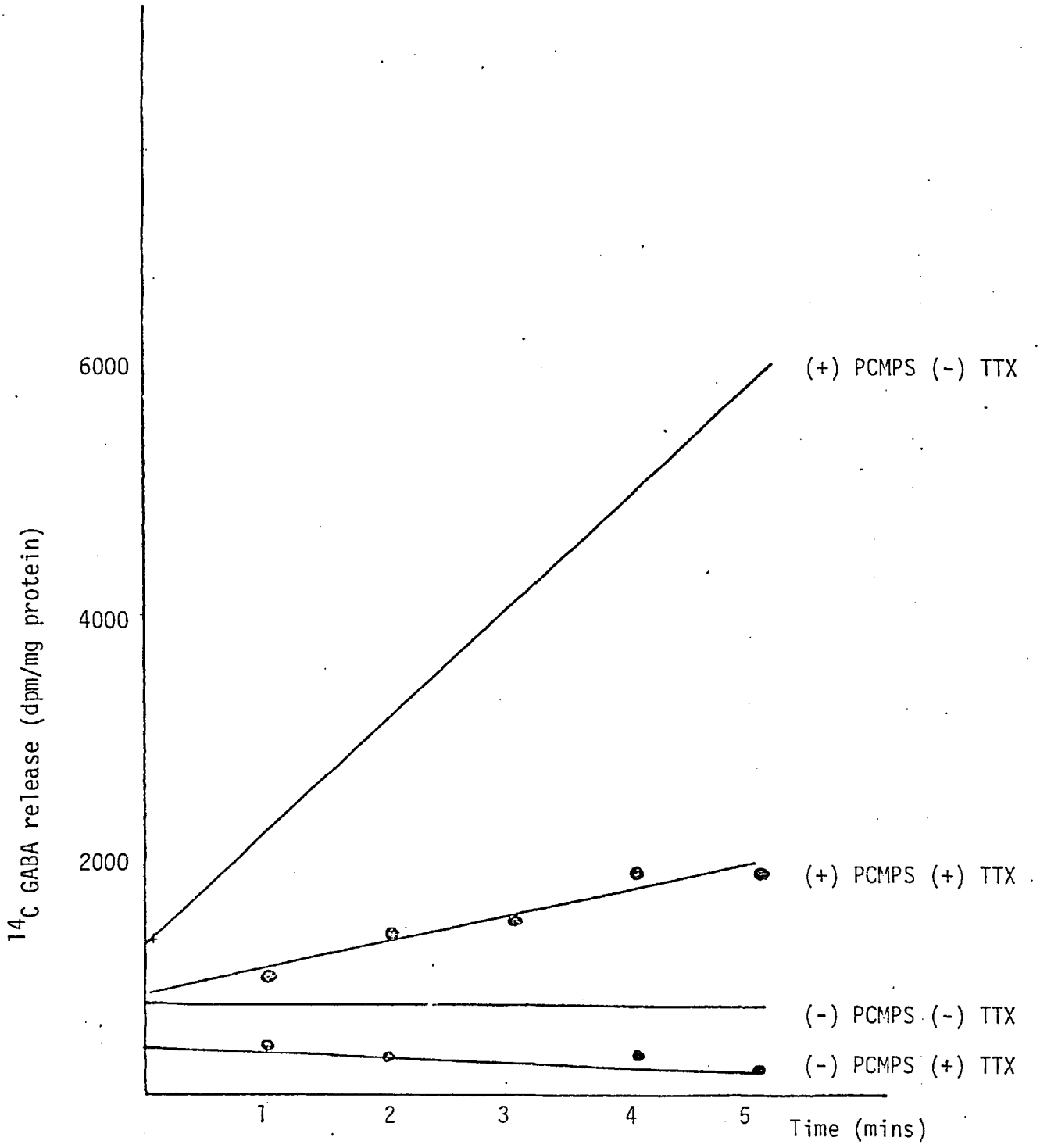


Figure 4.9 TTX sensitivity of [U-^{14}C]-GABA release from nerve endings.



Legend to Figure 4.10

Calcium independence of exogenous ^{14}C GABA release from rat cortical synaptosomes

Nerve terminals from the rat cerebrocortex were prepared and prelabelled with U^{14}C GABA (2μM) as described previously, except that calcium was omitted from the medium, and EGTA (0.5mM) was added in an attempt to chelate any endogenous calcium. Control incubations were carried out in a normal Krebs phosphate medium. PCMPS (1mM) was then added to the incubates as indicated and the radioactivity occurring in the medium over a five minute period was determined. Points represent the mean of 4-6 determinations, the graphs being plotted by linear regression methods. S.E.'s are omitted for reasons of clarity.

† no significance

Legend to Figure 4.11

The effect of extracellular ^{12}C GABA (0-200μM) on the exogenous resting release of ^{14}C GABA from rat cerebrocortical synaptosomes

Synaptosomes were prepared and preloaded with ^{14}C GABA (2μM) in Krebs phosphate medium as described before. After washing (3 x 1ml) away excess radioactivity, the nerve terminals were then subjected to incubations for five minutes at 37^{o}C with ^{12}C GABA in the medium (0-200μM). After the five minute incubation period, the suspensions were rapidly centrifuged and the radioactivity in the medium was determined by liquid scintillation counting. Curve A shows the experimental results, whilst curve B shows the adjusted radioactivities from a zero baseline. Values represent the means of 2-3 determinations.

Figure 4.10 Calcium independence of [U-^{14}C]-GABA release from synaptosomes.

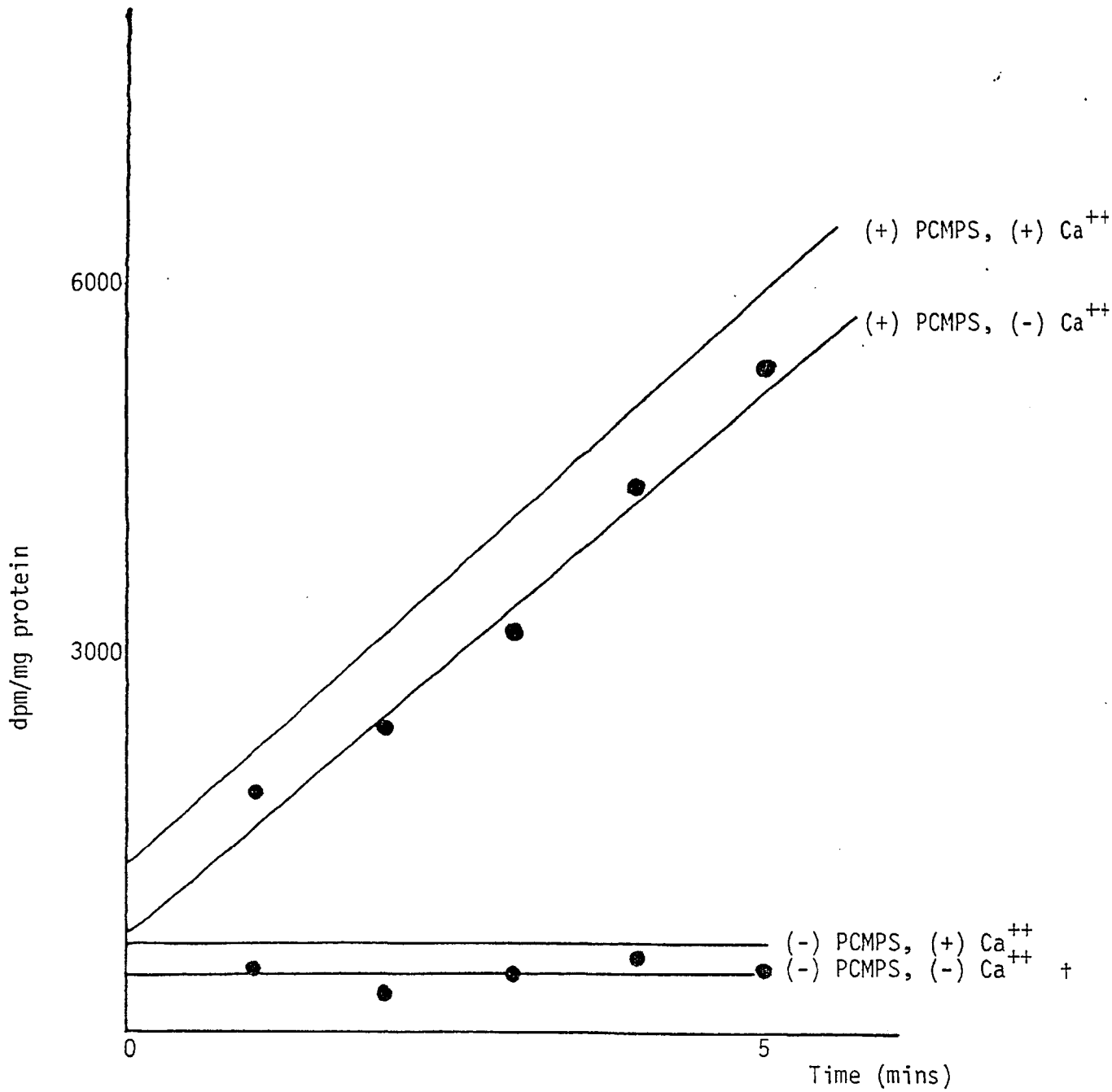
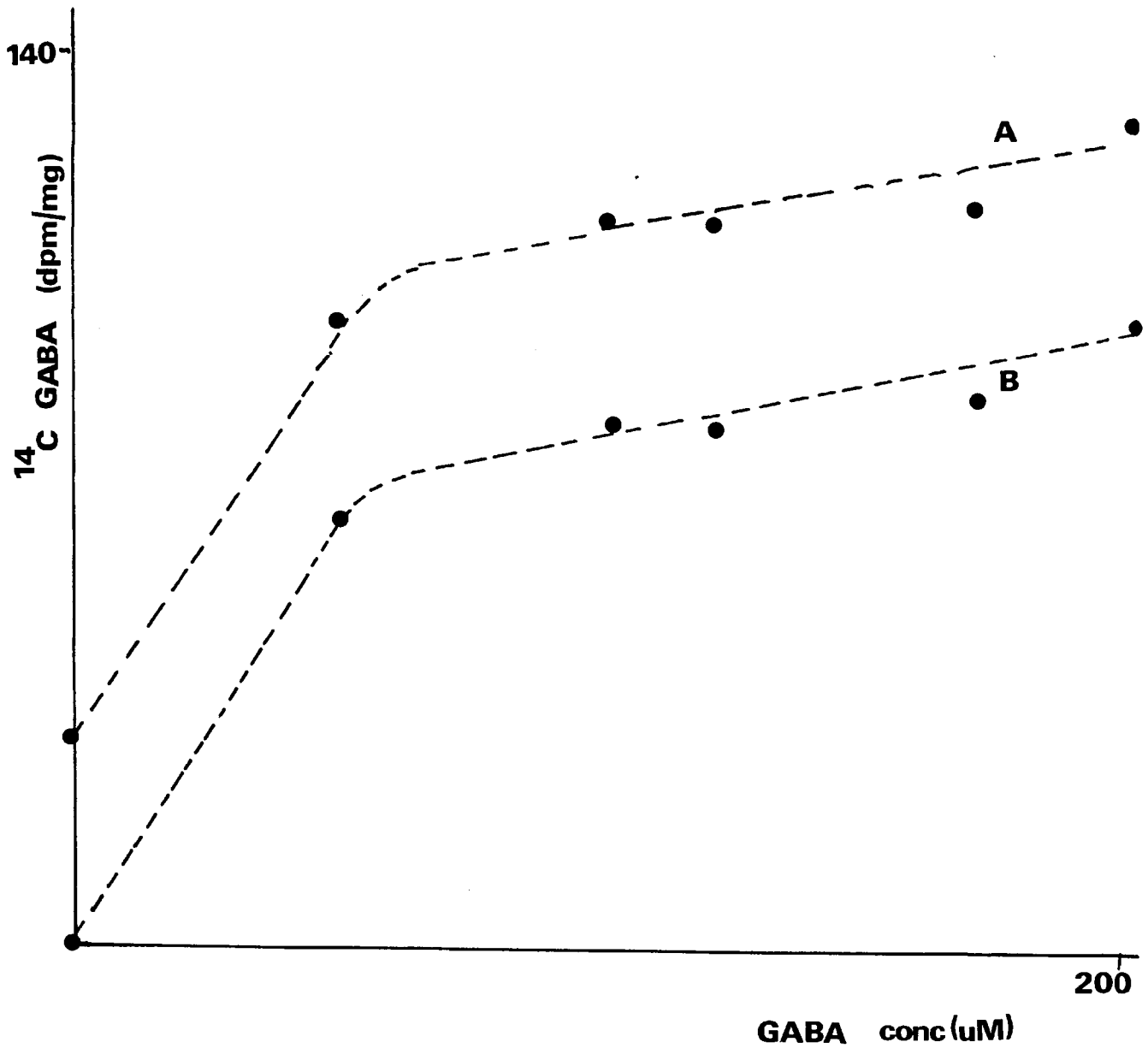


Figure 4.11 Graph to show how exogenous GABA detectable in the incubation medium varies as extracellular ^{12}C GABA concentration is increased.



no decrease in the resting release rate for exogenous ^{14}C GABA, in this calcium-free medium.

(e) The behaviour of the 'preload effect' of exogenous ^{14}C GABA from rat cerebrocortical synaptosomes in the presence of extracellular ^{12}C GABA

These series of experiments were attempts to distinguish the GABA uptake/release couple from other processes such as homoexchange and diffusion. Figure 4.11 depicts the levels of exogenous ^{14}C GABA detected in the medium as the extracellular ^{12}C GABA concentration is raised from 0-200μM. The saturating plot obtained is not explained by a homoexchange mechanism (Raiteri et.al., 1975), nor can it be due to an isotope dilution effect since Figure 4.12 shows that radiolabelled GABA accumulation into the tissue is linear over the 0-200μM concentration range.

Figure 4.13 illustrates an experiment to determine the extent of a diffusion component to the preload effect of GABA With PCMPS (1mM) in the medium, the extracellular ^{12}C GABA concentration was raised from 0-200mM. The graphs show a constant level of radiolabel in the medium over the concentration range tested, suggesting an active release process taking place rather than passive leakage of amino acid along a concentration gradient.

(f) Resting release of Dopamine from rat cortex and sheep corpus striatal synaptosomes

The graphs in Figure 4.14 show that no dopaminergic preload effect was detected using PCMPS (1mM) or the more specific dopamine uptake blocker nomifensen (Raiteri et.al., 1979), despite the fact that the striatum has a large number of

Legend to Figure 4.12

^{14}C GABA (0-200μM) accumulation into rat cerebrocortical synaptosomes

Synaptosomes were prepared and preincubated at 37°C as described in the methods chapter. After this period, the medium concentration of ^{14}C GABA was raised from 0-200μM for a further five minute period, before the tissue was separated from the medium by bench centrifugation. The tissue was then washed (3 x 1ml fresh medium) and then lysed by 1mg/ml protein of TCA (10%). Tissue GABA levels were then determined by liquid scintillation counting of TCA aliquots, using a toluene-based scintillant.

Legend to Figure 4.13

The extent of a diffusion component to the 'preload effect' of ^{14}C GABA resting release from rat cerebrocortical synaptosomes

Synaptosomes were prepared and prelabelled with exogenous ^{14}C GABA (2μM) in the usual manner. After 3 x 1ml washes with fresh Krebs phosphate medium, they were then pre-treated with PCMPS (1.0mM) to inhibit reuptake of neurotransmitter, before being subjected to extracellular concentrations of ^{12}C GABA (0-200mM) for a five minute period. Radiolabel levels in the medium were then determined by liquid scintillation counting. Values represent the means of 2-3 determinations.

Figure 4.12 GABA accumulation into synaptosomes.

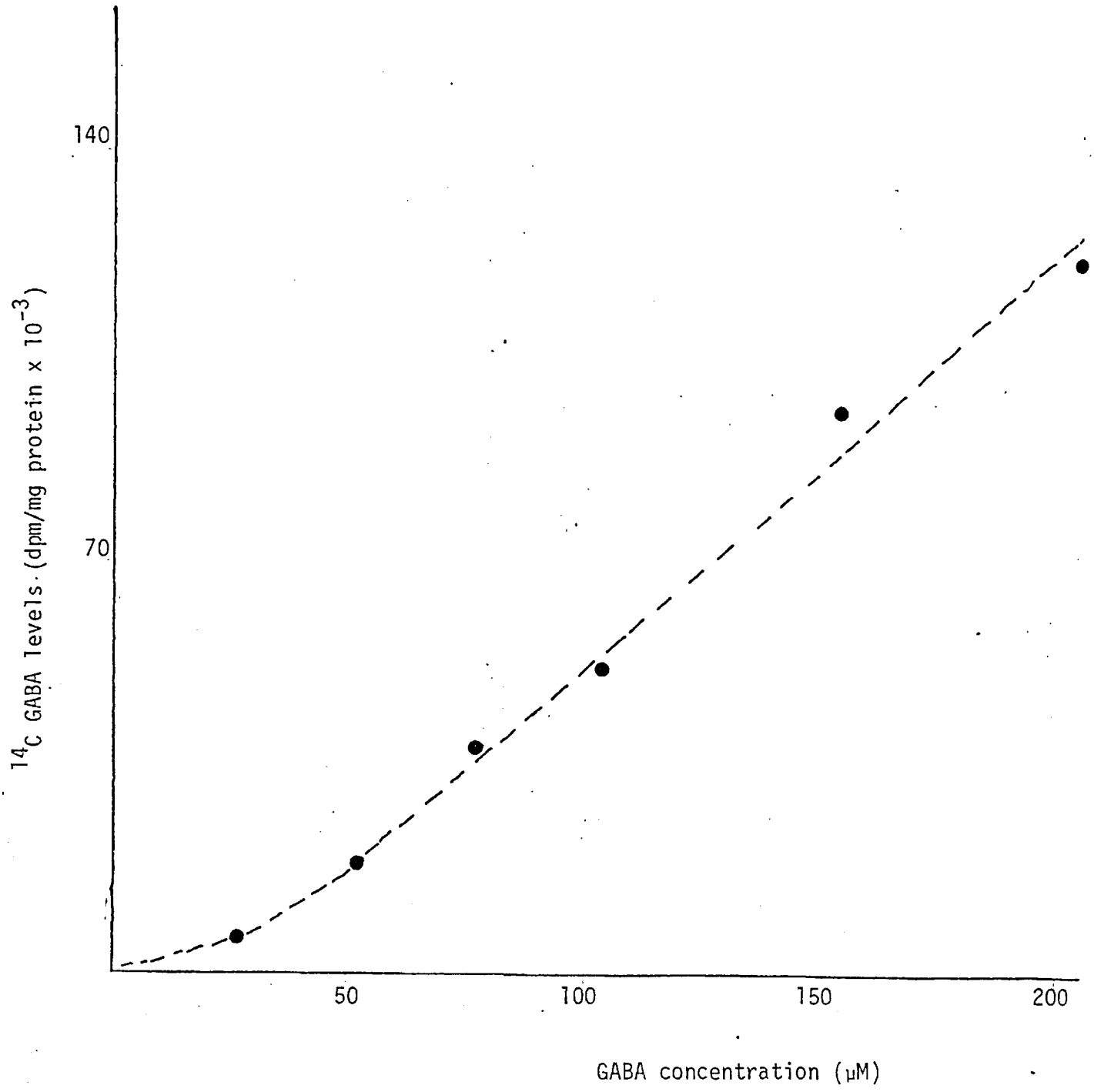
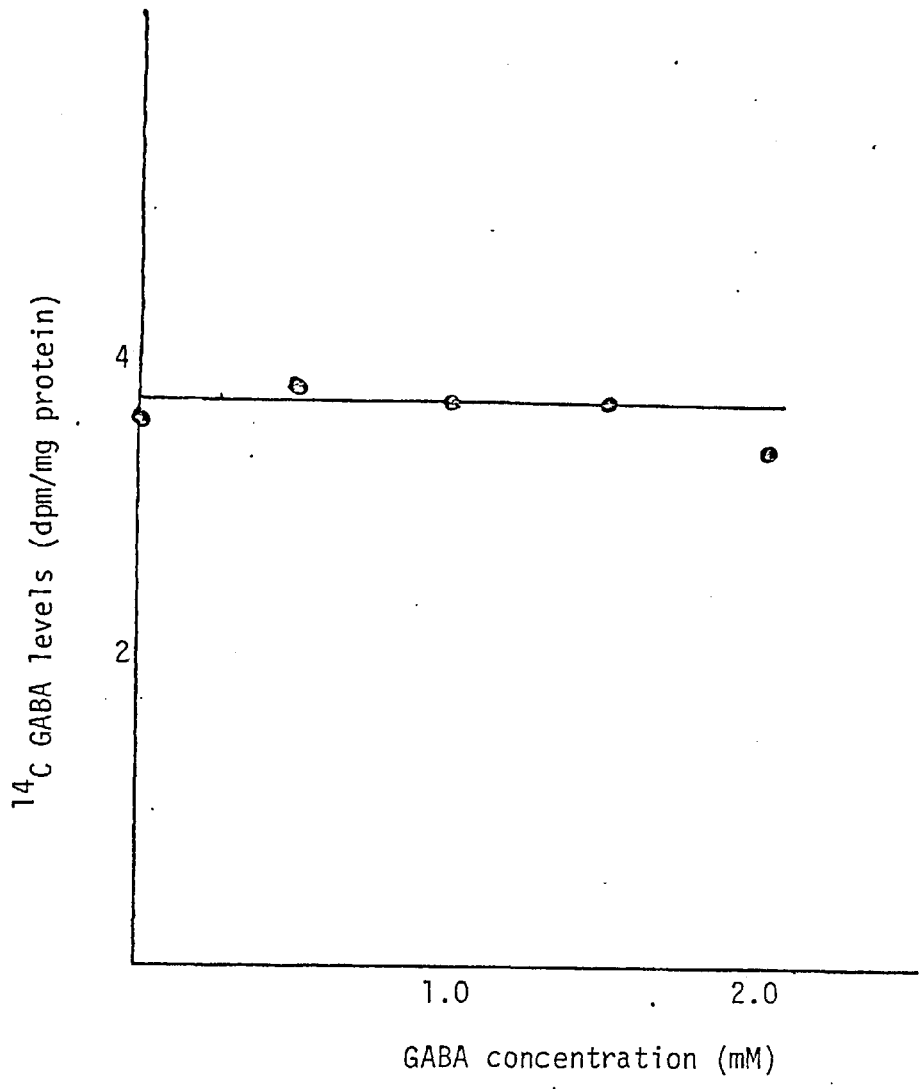


Figure 4.13 Graph to show the extent of a diffusion component to the GABA "preload effect"

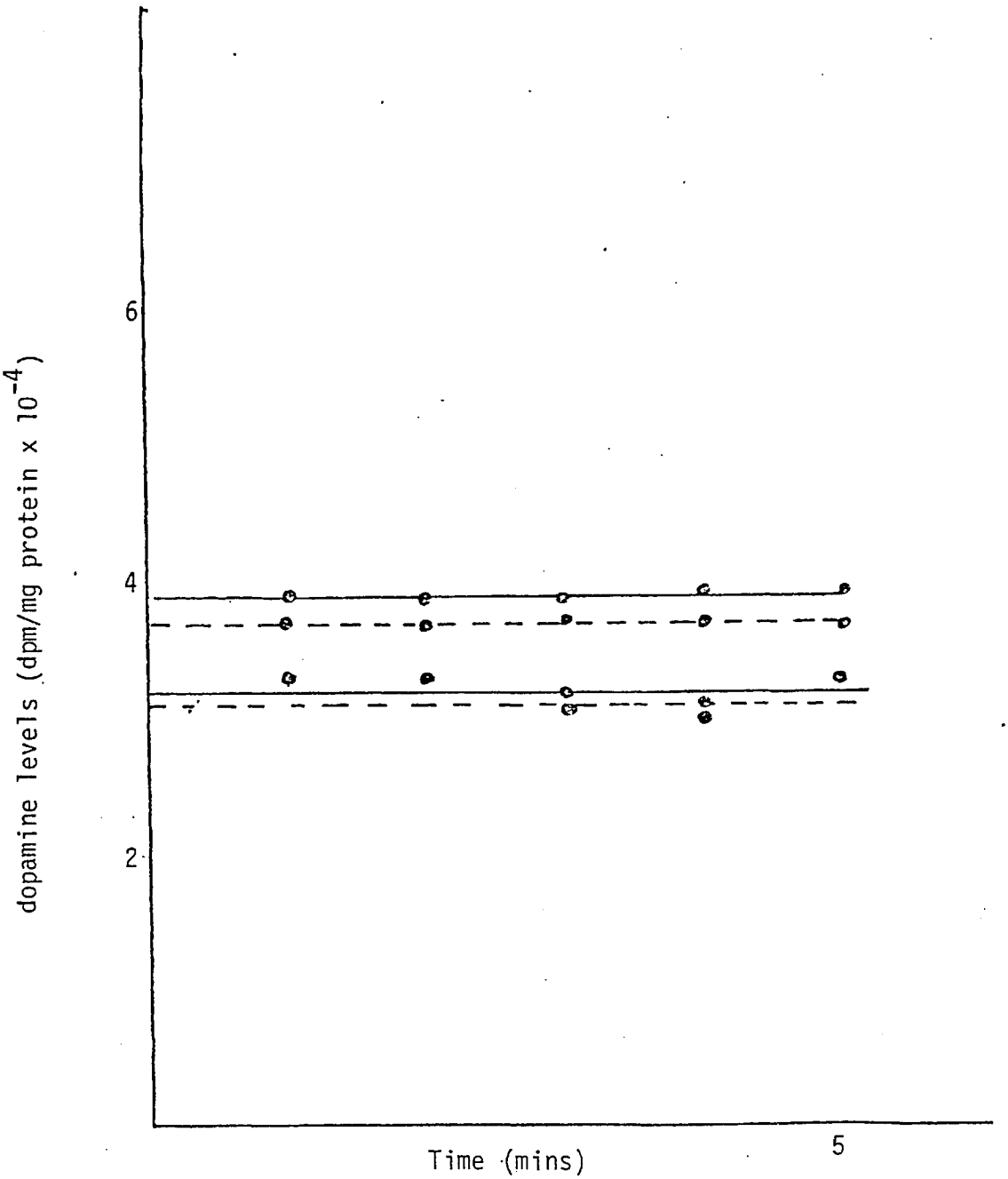


Legend to Figure 4.14

Experiment to detect a 'preload effect' for Dopamine from rat cortex and sheep corpus striatum synaptosomes

Synaptosomes (P_2B) from the rat cortex and sheep striatum were prepared as outlined in the methods section. After preincubation (37^oC, 30mins) and prelabelling with exogenous ^{14}C dopamine (1μM) in Krebs phosphate medium containing ascorbate (1mg/ml), the washed nerve terminals were incubated with PCMPS (1mM) or nomifensen ($10^{-5}M$) as indicated, over a five minute period. The tissue was then separated from the medium by bench centrifugation,and the radiolabel in the medium was determined by liquid scintillation counting. The graph shows mean values for 2-3 determinations.

Figure 4.14 Resting release of Dopamine from rat cortex and sheep striatal synaptosomes.



dopaminergic neurons (Fuxe and Anden, 1966). It is tempting to interpret these results and those of GABA resting release as a consequence of the site of origin of release of neurotransmitter, ie. GABA release thought to be from cytoplasmic pods (de Belleroche and Bradford, 1977), whereas dopamine release is thought to be of predominantly vesicular origin (de Belleroche et.al., 1976). This most interesting question relates, of course,to other factors such as site of origin of endogenous and exogenous neurotransmitter release and is discussed more fully in the next section of this chapter, (4.4d).

4.4 Discussion

(a) The primary aim of the work presented in this chapter was to determine the existence and characteristics of uptake/release couples in the presynaptic membrane for the candidate amino acid neurotransmitters. Amino acid analogues and associated compounds were used as biochemical tools to probe these transport couples that might be expected to exist in mammalian synaptosomal preparations.

(1) Action of PCMBS

PCMPS has been shown to inhibit GABA uptake by as much as 90% (Iversen et.al., 1971), and also to inhibit glutamate uptake (Curtis et.al., 1970; Roberts and Watkins, 1975) in rat brain slices. The results presented in Figure 4.1 indicate that,with prelabelled rat cortical synaptosomes, PCMPS induces a net release enhancement of GABA, glutamate, and a corresponding decrease in the tissue content. The fact that this was not shown for leucine (even though a prelabelling concentration of 1mM was used) reflects either a non-specificity of PCMPS for leucine uptake channels, or more probably, a rather slow

rate of 'release' (leakage of leucine itself). Of course the idea that faster rates of release exist for GABA, glutamate, and other neurotransmitters may in itself reflect 'functional specialization'.

(2) The effect of Nipecotic and Isonipecotic acid

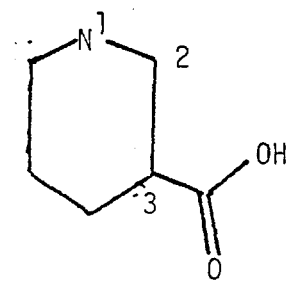
Johnston et.al. (1975) has shown nipecotic acid (1mM) to be a specific uptake receptor blocker of GABA into rat brain slices. Figure 4.2ashows that nipecotic acid (1mM) added to the ^{14}C prelabelled synaptosomes gives an increase in detected radioactivity of 260% in the medium with a decrease in the tissues of 30%. No difference in exogenous release or reuptake patterns are revealed with the isomer isonipecotic acid (Fig. 4.2b). This isomer, with a movement of the carboxyl group from position 3 in nipecotic acid to position 4 in isonipecotic acid,probably reflects the different structural activities of gaba transport molecules (receptors)(Fig. 4.15). Krogsgaard-Larsen et.al. (1979a) have reported that isonipecotic acid is a selective GABA agonist at rat brain membranes and at cat spinal interneurons (Beart et.al., 1972).

This evidence together with the above would suggest that transport enzyme receptors (presynaptic) are different,either in structure or in cofactors required, to postsynaptic membrane receptors. Apart from Na^+ dependence associated with reuptake of putative amino acid neurotransmitters, little biophysical information about uptake receptor structures themselves is available at the moment. Johnston 's theory (Johnston et.al., 1975) is that conformational changes of GABA are responsible for different interactions at the pre and post synaptic sites.

Figure 4.15 Chemical structures of GABA and glutamic acid analogues.

glutamic acid	$HOOCCH_2CH_2CH(NH_2)COOH$
$\tilde{\gamma}$-amino butyric acid	$H_2NCH_2CH_2CH_2COOH$
aspartic acid	$HOOCCH_2CH(NH_2)COOH$
homocysteic acid	$HOOCCH(NH_2)CH_2SO_3$
β-alanine	$H_2NCH_2CH_2COOH$
adipiate	$HOOCCH_2CH_2CH_2CH_2COOH$
aspartic acid	$HOOCCH_2CH(NH_2)COOH$

nipecotic acid



isonipecotic acid

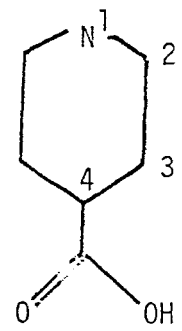
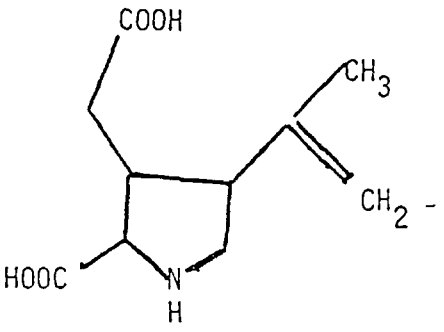
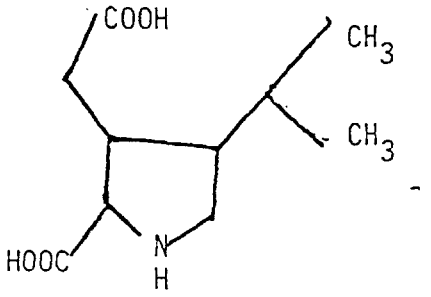


Figure 4.15 (continued)

kainic acid



dihydrokainic acid



(3) The action of γ-vinyl-GABA

The results in Figure 4.3 show that GVG appears to be acting as a reuptake blocker towards exogenous ^{14}C GABA revealing net enhancement of molecules in the medium, and a net loss from the tissue. However, if this compound is a reuptake blocker by virtue of its ability as a competitive inhibitor to the transport receptor site, then the results could easily be explained by an isotope dilution effect with GVG acting as a GABA mimicker creating a homoexchange situation; ie. GVG could be displacing GABA from the synaptosome. This would seem to be a more tangible explanation of the events, than those put forward by Adul-Ghani et.al. (1980), who invoke an apparent releasing effect of GABA in vivo and in vitro by partial activation of Na^+ active channels. They also report, as do Jung et.al. (1977), that GVG can selectively and irreversibly inhibit GABA transaminase.

(4) The effect of α-amino-4-phosphono butyric acid

Figure 4.4 shows no action of APBA of being an uptake blocker of exogenous ^{14}C-glutamate in the experimental conditions used. There is no indication of relevant net enhancement or net loss of molecules to the medium or from the tissue respectively. This would seem to be in agreement with the work of Cull-Candy et.al. (1976), Watkins (1978), and White et.al. (1977), who classify APBA as a glutamate and aspartate antagonist electrophysiologically. This mode of action has also been used to explain the APBA suppression of epileptic manifestations seen in cobalt-induced convulsions in the rat. Taken together, this evidence would imply that postsynaptic membrane receptor activation is different in some way as yet

unclear, to that of presynaptic reuptake receptors, despite the fact that glutamate as the neurotransmitter must intervene physiologically with both types.

(5) The action of kainate and dihydrokainate

Kainic acid has recently been the subject of much investigation because of its potency as a glutamate receptor probe. Lakshmenan et.al. (1974), McGeer et.al. (1975), have reported that KA reduces the uptake of glutamate with rat striatal crude mitochondrial fractions and whole brain P_2B extracts by 60-70%. Roberts and Watkins (1975), however, report a smaller inhibition of uptake. The results presented in Figure 4.5 probably reflect the fact that kainate at this concentration has a toxicological effect. These neurotoxic effects have been well documented by Biscoe et.al. (1975), Olney et.al. (1974), McGeer and McGeer (1976).

DHK appears to be a much weaker receptor probe than kainate in that its potency is some 200 times smaller (Johnston et.al., 1979). Certainly in our experiments no uptake blocking action towards exogenous radiolabelled glutamate was observed.

(6) The effect of threo-hydroxy aspartate

Balcar and Johnston (1972) have reported this compound to be a very specific uptake blocker of both aspartate and glutamate in rat brain slices. Our results show a transport shuttle effect towards glutamate (see Figure 4.6) demonstrating continuous release and uptake for the exogenous radiolabel.

(b) K^+ retention after addition of amino acid analogues

The question arises as to whether the preload effects are manifestations of depolarized release of candidate transmitter, rather than manifestations of continuous uptake and release. To answer this question, potassium retention was used as a criterion

to indicate whether depolarized induced release had occurred rather than resting release (see Chapter 1). Table 4.1 shows that, of the analogues tested, only kainate was found to lower potassium retention in synaptosomes. Thus it was concluded that the preload effects seen with PCMPS, nipecotic acid, γ-vinyl-GABA, cannot be easily explained by membrane depolarization. The fact that kainate induced a 43% loss of potassium (from 23.2 to 10.1 μequivalents/100mgs protein) probably relates to its necrotizing action discussed earlier.

(c) Estimation of GABA resting release rate

When using exogenous radiolabel as a tracer in neurochemical studies one has to assume that the ^{14}C GABA for example, measured in the medium, originates as part of the organized total release from inside the synaptosome, rather than the binding to surface binding sites. These alternatives were investigated by examining expected changing tissue-specific activities that occur during the time course experiment. Figure 4.7 shows that the tissue activity increases with time consistent with the idea that as label is lost from the releasable pool, it is replenished by newly synthesized transmitter from a metabolic pool (de Belleroche and Bradford, 1977). The experimentally-determined data in Figure 4.8awas used to plot a theoretical time course for gaba resting release. Figure 4.8b shows that this rate was found to be approximately 1nmole/min/mg protein. This determination would seem to be unreasonably high when compared to the total GABA content of 83nmoles/mg (Bradford et.al., 1975). Thus if the release rate was physiological, the releasable pool would be empty in about eighty three minutes. This discrepancy is probably explained by the fact that our estimation of tissue activities

relates to total GABA activity rather than releasable pool GABA activity, ie. our experimental hot/cold ratios are too small because in vivo the releasable pool of GABA is only a fraction of the total synaptosomal GABA. This underestimation in the calculation would hence yield a higher than expected determination of cold GABA. The means to overcome this problem could be solved if metabolic pools and releasable pools could be characterized independently or if cold GABA in the medium was measured directly eg. by fluorescence chromatography (de Belleroche et.al., 1976) or by microenzymatic methods (Graham and Aprison, 1966).

(d) Modulation of resting release by ion channels

If the resting release is an active process rather than diffusion down concentration gradients, it would seem likely to be ion-gated. In the experiment depicted in Figure 4.10, no calcium sensitivity was observed towards resting release of exogenous GABA once reuptake had been blocked with PCMPS. This result conflicted with an original idea that the resting release channels might be the same as those used for stimulated release of transmitter. Calcium-dependence of potassium-stimulated release of amino acid transmitter has been shown by Olsen et.al. (1977) and also of electrical-stimulated release by Szerb (1979). Calcium-dependence of mepps and stimulated release of acetylcholine has been well documented by Katz et.al. (1971) and Miledi (1973). In the past, calcium-dependence has been associated with quantized release of transmitter, ie. of discrete integral numbers of molecules probably being released from vesicles (Katz et.al., 1971). Thus when considering the site of origin of amino acid neurotransmitter release in the above experiment, this would imply a non-vesicular

involvement, or at least a non-detectable involvement, in our system of calcium-mediated release, assuming of course that the EGTA employed is an efficient endogenous calcium chelating agent. The fact that de Belleroche et.al. (1977) have assigned a cytoplasmic site of origin for GABA, glutamate, and aspartate, would correlate well with the fact that the release and uptake channels are situated in the presynaptic membrane.

Apart from calcium channels, Na^+ active channels seemed a likely candidate for resting release modulation, because of their association with stimulated release. Na^+ active channels were blocked by the inclusion of TTX in the incubation medium (Nakamura, 1965) followed by the addition of the uptake blocker PCMPS. The results in Figure 4.9 show that TTX reduces the rate of release of exogenous ^{14}C GABA by 70%. This together with the lack of calcium dependence would imply that the resting release is mostly a non-quantized Na^+ active channel dependent process, a proposal that would correlate well with acetylcholine studies of Vizi et.al. (1979), Katz and Miledi (1977). Working with mouse diaphragm and giant squid axon preparations, they have tried to quantitate the ion dependencies of vesicular and cytoplasmic release to <u>total</u> acetylcholine release. Their results can be summarized as follows:

	Resting Release	Stimulated Release
Vesicular release component mepp characteristics - intracellular Ca^{++}-dependent	3%	50%
Cytoplasmic component non-quantized Ca^{++}-independent Na^{+} active channel-dependent	97%	50%
Total ACh released	100%	100%

(e) Modulation of resting release through homoexchange channels

Although active Na^{+} channel dependence has been detected for the preload effect, the possibility still exists that resting release for GABA takes place through other known mechanisms. Homoexchange would fall into this category. Homoexchange is defined as the one to one exchange of a neurotransmitter molecule between the intracellular space and extracellular medium (Raiteri et.al., 1975). Debate frequently occurs in the literature as to whether this process is a manifestation of high affinity uptake or not (Iversen, 1975; Benjamin and Quastel, 1976). The experiment performed in Figure 4.11 shows a saturation type graph as the external concentration is raised to 200μM. The most likely explanation of this graph would be that reuptake sites are being saturated by cold GABA competitively inhibiting ^{14}C GABA reuptake, and so an almost constant resting releasing of exogenous neurotransmitter is revealed. A homoexchange mechanism is not explained by this plot, since it is non-linear.

(f) Is diffusion a component to the preload effects?

The resting release effects described earlier could be

explained by diffusion, in part anyway, as TTX sensitivity is only observed for 65% of the effect. Diffusion of amino acid across a concentration gradient might explain the other 35%. In order to test this, the experiment depicted in Figure 4.13 was carried out. When reuptake of exogenous GABA is inhibited with PCMPS (10mM) and the external GABA concentration is elevated to 2mM, a graph reflecting an enzymatic process is found. If diffusion was taking place from inside the synaptosome preparation, a straight line graph of negative slope would have been expected.

(g) Resting release of dopamine

When considering the interpretation of release data, especially with exogenous radiolabel, it is important to consider the physiological storage sites of the neurotransmitter compared to the site of origin of release of prelabelled transmitters. Physiologically, it is considered that dopamine in the nerve terminal is stored and released from vesicle pools (De Belleroche et.al., 1976a). The question then arises as to whether the preloading methods of the experiment in Figure 4.14 actually labels these pools rather than non-neural pools. If this was so, then there is no reason to assume that a resting release effect would not be detected. Of course, dopamine is at a lower concentration than the other amino acid neurotransmitters (pmoles/100mg rather than nmoles/100mg protein), but this should not be too much of a problem for liquid scintillation counting techniques (De Belleroche et. al., 1976b).

Resting release predominantly occurring from vesicles rather than cytoplasm might have different characteristics, notably

calcium dependence and fast-acting Na^+ channel dependence. The release would also be expected to be quantized with mepp-like characteristics as described by Katz (1966).

Part B IN VITRO Approaches to Amino Acid Transport: Superfusion

4.5 Introduction

Although nerve terminals are useful tools for studying the biochemistry of the nervous system, their use in classical *in vitro* studies is limited since phenomena such as uptake and release of neurotransmitters to and from synaptosomes can never be examined in isolation from each other. Thus in a study to determine the mechanism of action for candidate anticonvulsants for example, the observed action of the test compound could be inferred either to modulate the release of a neutotransmitter or to affect its reuptake. Hence, using conventional synaptosome flask incubations, the assignment of biological observations to neurochemical mechanisms remains uncertain. A superfusion system in which released neurotransmitter reuptake is inhibited would obviously alleviate this problem.

The limitations of flask incubations were realized more than ten years ago and various superfusion systems of nerve tissue have been described in the literature, e.g. Baldessarini and Koplin (1967), Srinivassen et.al. (1969), Hopkin and Neal (1970), McIlwain and Snyder (1970). All of these workers used slices of nervous tissue sandwiched between quick-transfer holders. De Belleroche and Bradford (1972a,b,c) modified this system somewhat to sandwich synaptosomes, to form a 'bed' between nylon gauze. However, it was found that even in superfusion of these systems, reuptake from the bed was not prevented.

In 1974, however, Raiteri et.al. put forward a different technique for superfusion of synaptosomes. In this method, synaptosomes were sedimented onto a millipore filter situated at the bottom of a thermostatted chamber. Physiological saline was then pumped over and through the filter at a rate of 0.5mls/min.

In this way, reuptake of recently released transmitter is claimed to be inhibited. The other advantages claimed for this system are: (a) experiments require very little protein, typically 0.1 - 0.5mg protein per filtre; (b) parallel experiments can be performed easily and quickly with minimum variability of results between experiments even on different days; (c) the method provides a complete pattern of release compared to static incubations, where the sampling is discontinuous.

A similar superfusion system to that described above was set up in this laboratory to examine the following:

(1) K^+-stimulated release of ^{14}C GABA, ^{14}C glutamate

(2) Unlabelled GABA, glutamate-stimulated release of ^{14}C GABA, ^{14}C glutamate respectively

(3) Compartamentation of candidate amino acid neurotransmitters with regard to metabolic and releasable pools.

(4) Release of ^{14}C GABA and ^{14}C glutamate using amino acid analogues.

4.6 Results and Discussion

(a) Potassium stimulation and Homoexchange of ^{14}C glutamate and ^{14}C GABA from superfused synaptosomes

Figure 4.16a shows that the peak GABA efflux for K^+ (56mM) stimulation and homoexchange (GABA 100μM) are 4% and 5% respectively, the results expressed as per cent of radioactivity per fraction. Raiteri and Levy (1974) in their system obtained corresponding values of 6% for K^+ stimulation and 7.5% for homoexchange. The evidence that the radiolabel represents release from an intrasynaptosomal site of origin, rather than exchange at non-specific membrane receptor sites is given by:

(a) After the nerve terminals have been loaded onto the filtre,

Incubation procedures for Figures 4.16-4.22

This was essentially as described by Raiteri et.al. (1974). After 30 minutes incubation and preloading (2μM, 0.5μCi/ml of radiolabel)in Krebs phosphate medium, synaptosomes were deposited by suction onto Millipore filtres of 0.8μ pore size (see Materials and Methods, Chapter 2). Fresh medium at 37°C was then added and pulled through the synaptosome layer by a peristaltic pump at a rate of 0.5ml/minute.

Legend to Figures 4.16a and 4.16b

Potassium stimulation and homoexchange of ^{14}C GABA and ^{14}C glutamate from superfused synaptosomes

Using ^{14}C glutamate or ^{14}C GABA radiolabelled synaptosomes as indicated, following a control period of four minutes, medium containing 100μM glutamate or 56mM K^+ ions was introduced into the superfusion chamber (indicated by the arrow in the figure). Samples (0.5mls) from synaptosomes subjected to these media were then taken for a further six minutes. The fractions (0.5mls) were then subjected to liquid scintillation counting.

Values represent the mean of 2-3 determinations.

The results are expressed as % of total radioactivity and represent the mean of 2-3 determinations.

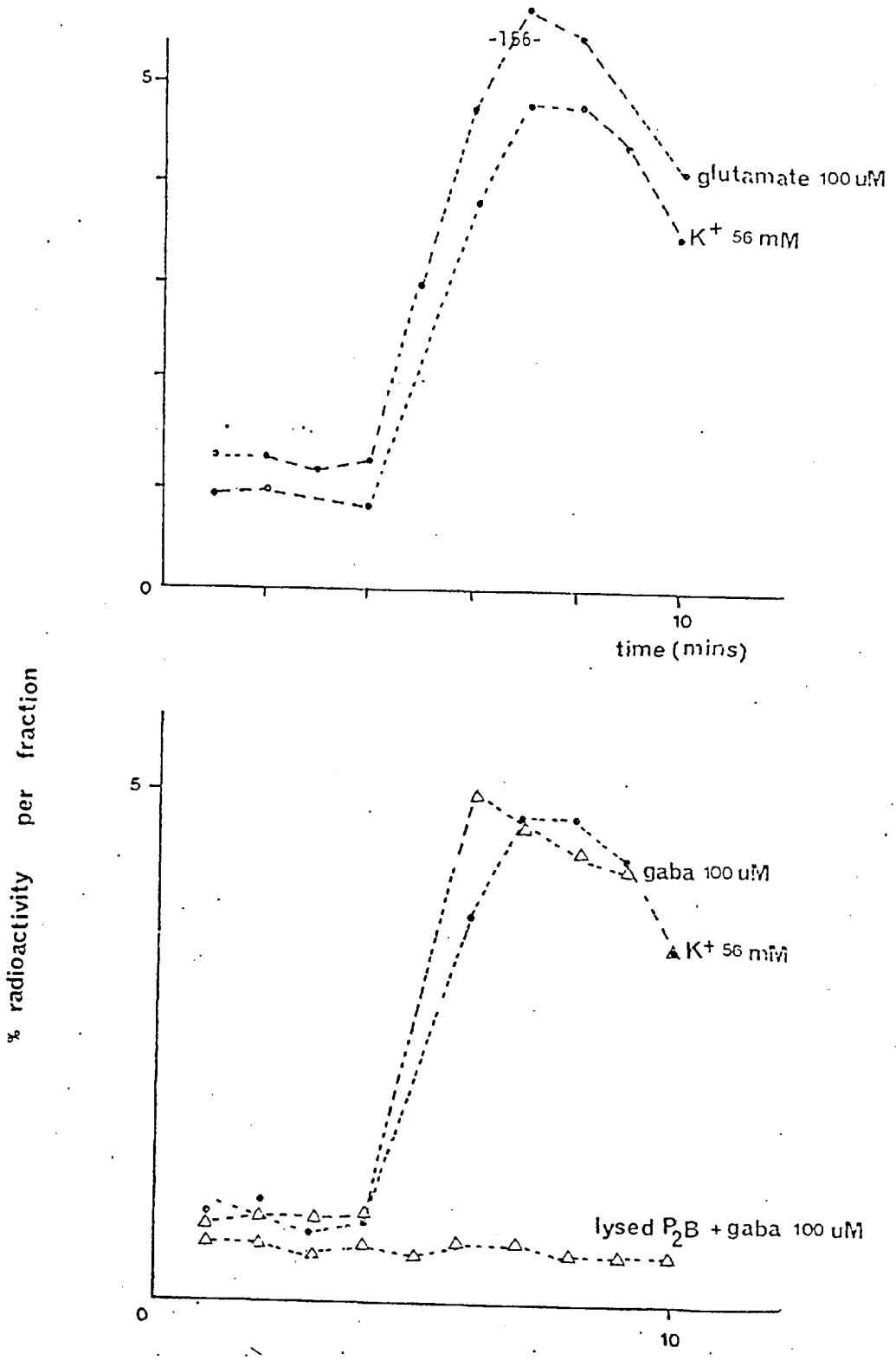


Figure 4.16 Homoexchange and K^+ stimulation of ^{14}C glutamate and ^{14}C GABA.

as a matter of procedure the protein was washed twice with fresh medium,

(b) At the end of an experiment there is typically

(i) in a control experiment, 95% of total radioactivity associated with the pellet (Raiteri obtains 95%)

(ii) in a homoexchange experiment (GABA 100μM), 71% of total radiolabel is associated with the pellet (Raiteri obtains 75%).

(c) With synaptosomes that have been preloaded and then lysed, no homoexchange is seen.

The peak efflux for potassium-stimulated release and homoexchange of glutamate in our system was found to be 4.8% and 5.7% respectively (see Figure 4.16b).

Standard homoexchange signals have also been documented for glycine (Levi et.al., 1974), dopamine and glutamate (Raiteri et.al., 1975), serotonin (Raiteri et.al., 1978), and noradrenaline(Levi et.al., 1976).

(b) Release of endogenous amino acids from superfused synaptosomes

In this experiment, an attempt was made to deplete synaptosomes of their transmitter pools of amino acids by subjecting them to two ten minute stimuli with high K^+ medium (56mM). Synaptosomes clearly responded to the first period of potassium-induced depolarization by selective increased release of glutamate, aspartate and GABA but not valine (Figures 4.17a,b,c,d). They responded weakly or not at all to the second stimulus. At this point there were considerable amounts of amino acids left in the pellet (Table 4.2) which, it is inferred, represents the minimum metabolic pool levels for the amino acids. Thus using a prolonged supramaximal stimulus, 80% of the physiologically active amino acids

Table 4.2 Amino acid release and content of synaptosomes when superfused in a Raiteri and Levi superfusion system.

	Total released*	Tissue content**
Asp	2484 ± 219	551 ± 78
Glu	3375 ± 355	707 ± 135
Gly	3361 ± 336	2142 ± 492
Ala	2362 ± 424	926 ± 176
Val	434 ± 82	252 ± 37
GABA	1562 ± 161	223 ± 57
	n = 6	n = 6

* - Total amount released into medium in 40 minutes of superfusion, including two 10 minute pulses of 56mM K^+ medium.

**- Tissue content at end of superfusion.

Values are nmol/100mg protein, means ± SEM. Conditions of incubation are the same as described for Figures 7 - 10.

Legend to Figures 4.17a, 4.17b.,4.17c.,4.17d

Release of endogenous amino acids from superfused synaptosomes

Freshly prepared synaptosomes from the rat cerebral cortex were initially incubated in Krebs phosphate medium for a period of 30 minutes. At the end of this period, 0.5mg of protein was transferred to the superfusion chamber. Fresh medium at $37^{o}C$ was then added and perfused using the peristaltic pump. Twenty two minute samples, as well as the synaptosome layer remaining at the end of the incubation, were taken for amino acid analysis.

Treatment of results

Valine release is expressed as nanomoles per ml of superfusing medium $\pm$ S.E.M. (n=4). The values for other amino acids are expressed as the ratio of the amount released to that of valine.

The bar indicates 10 minute periods when high K^{+} medium replaced the normal medium in the superfusion chamber.

Legend to Figure 4.18

The effect of veratrine-induced depolarization preceding homoexchange of ^{14}C glutamate from perfused rat cerebrocortical synaptosomes

Synaptosomes were incubated and labelled with ^{14}C glutamate (final concentration 2μM, activity concentration 0.5μCi/ml); before 0.5 mg portions were transferred to the superfusion chambers. Perfusion was carried out for a period of 5 minutes.

Test media at the concentrations shown were superfused for the time indicated.

Figure 4.17a

Valine Release from Superfused Synaptosomes

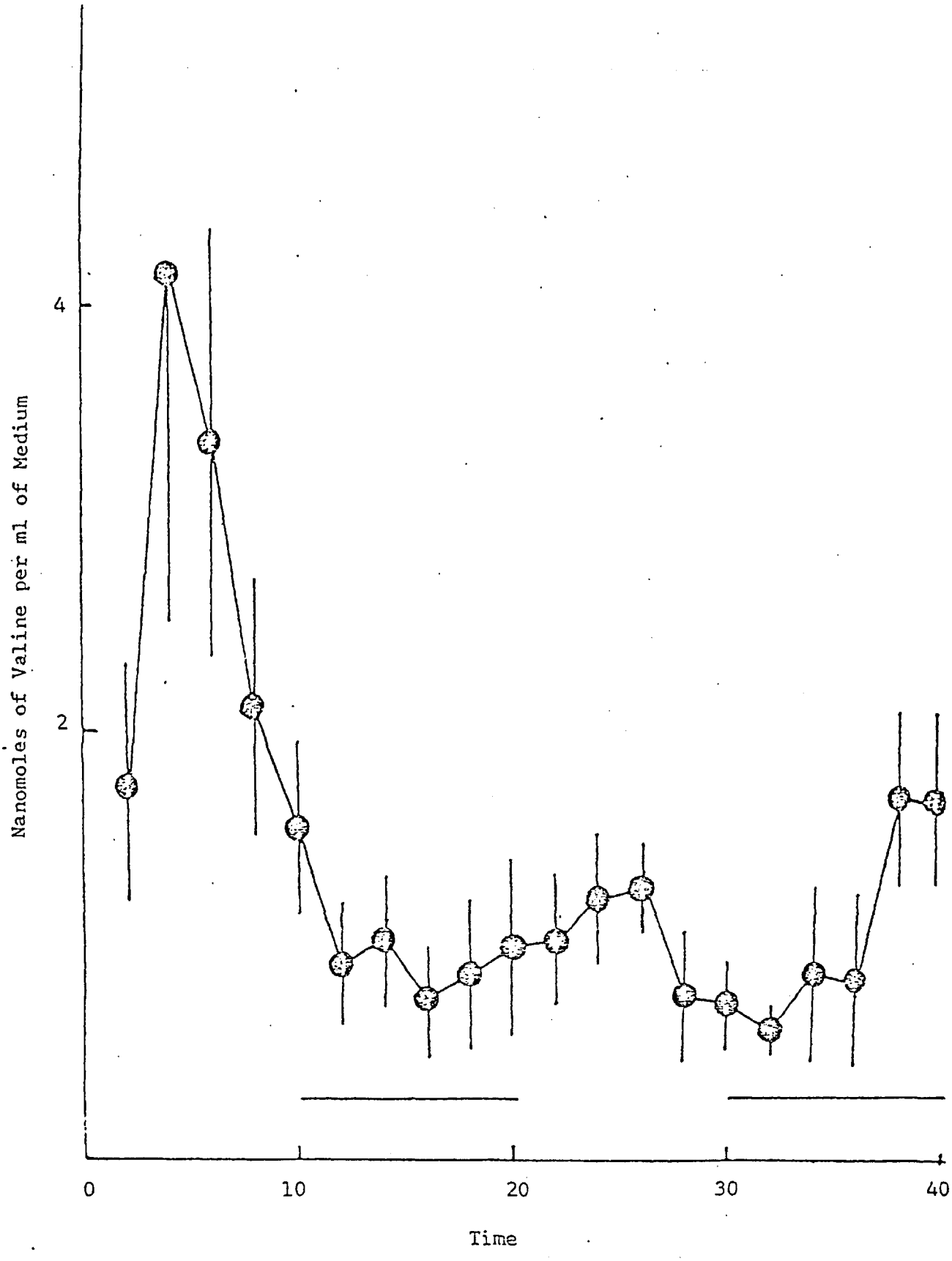


Figure 4.17b Glutamate : Valine Ratios in Medium from Synaptosome Superfusion Experiments

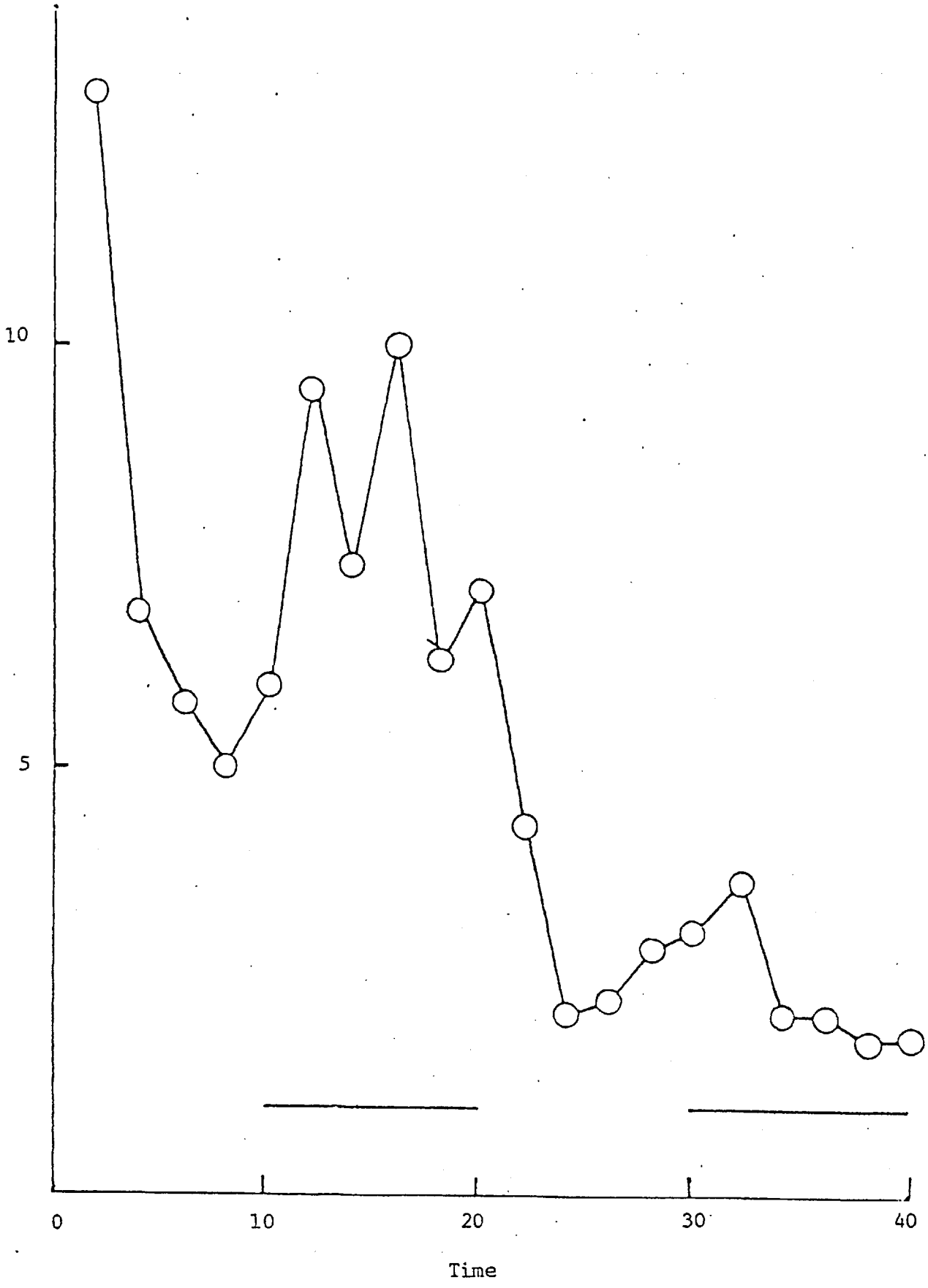


Figure 4.17c Aspartate : Valine and GABA : Valine Ratios in Medium from Synaptosome Superfusion

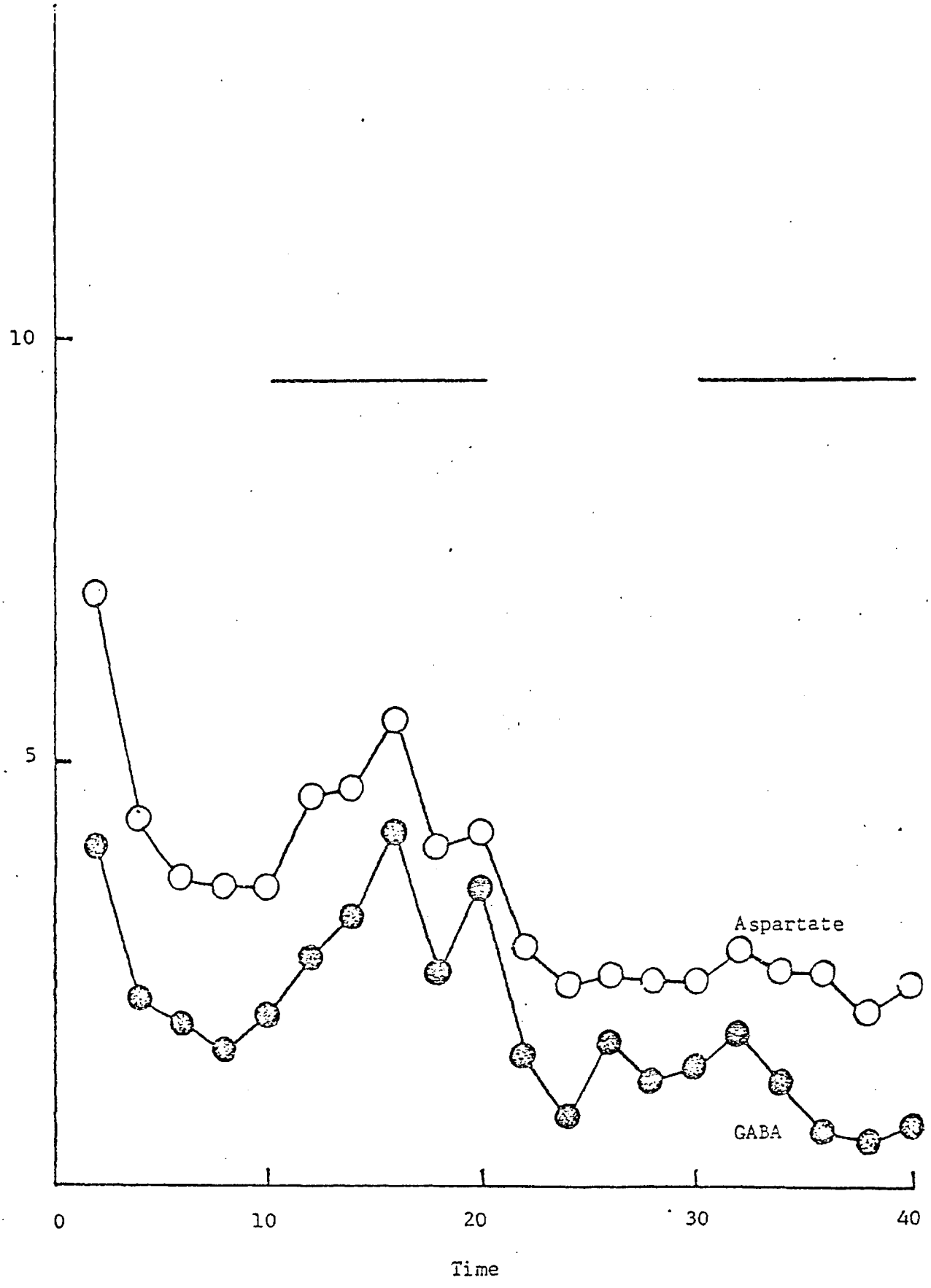


Figure 4.17d Glycine : Valine and Alanine : Valine Ratios in Medium from Synaptosome Superfusion

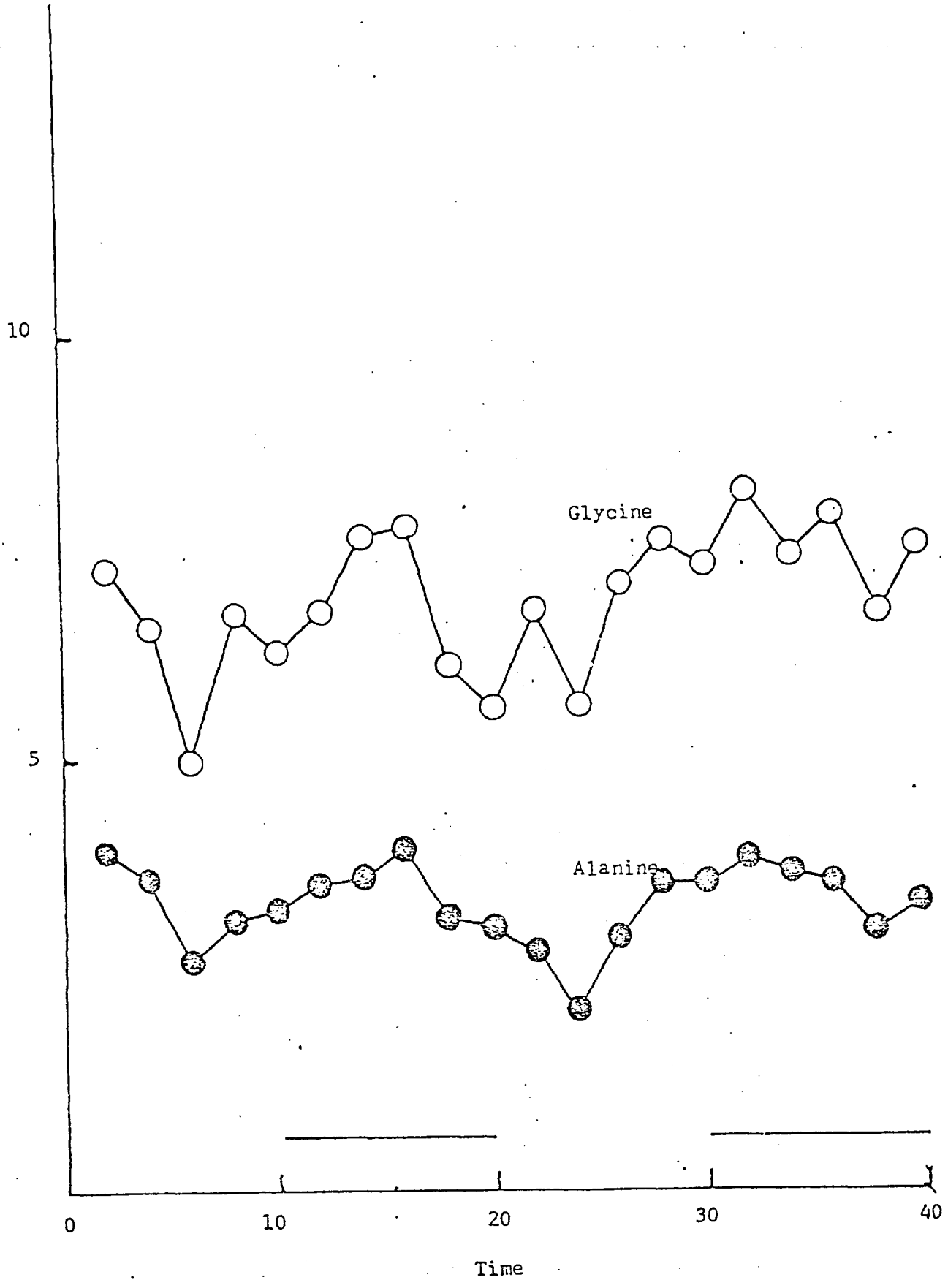
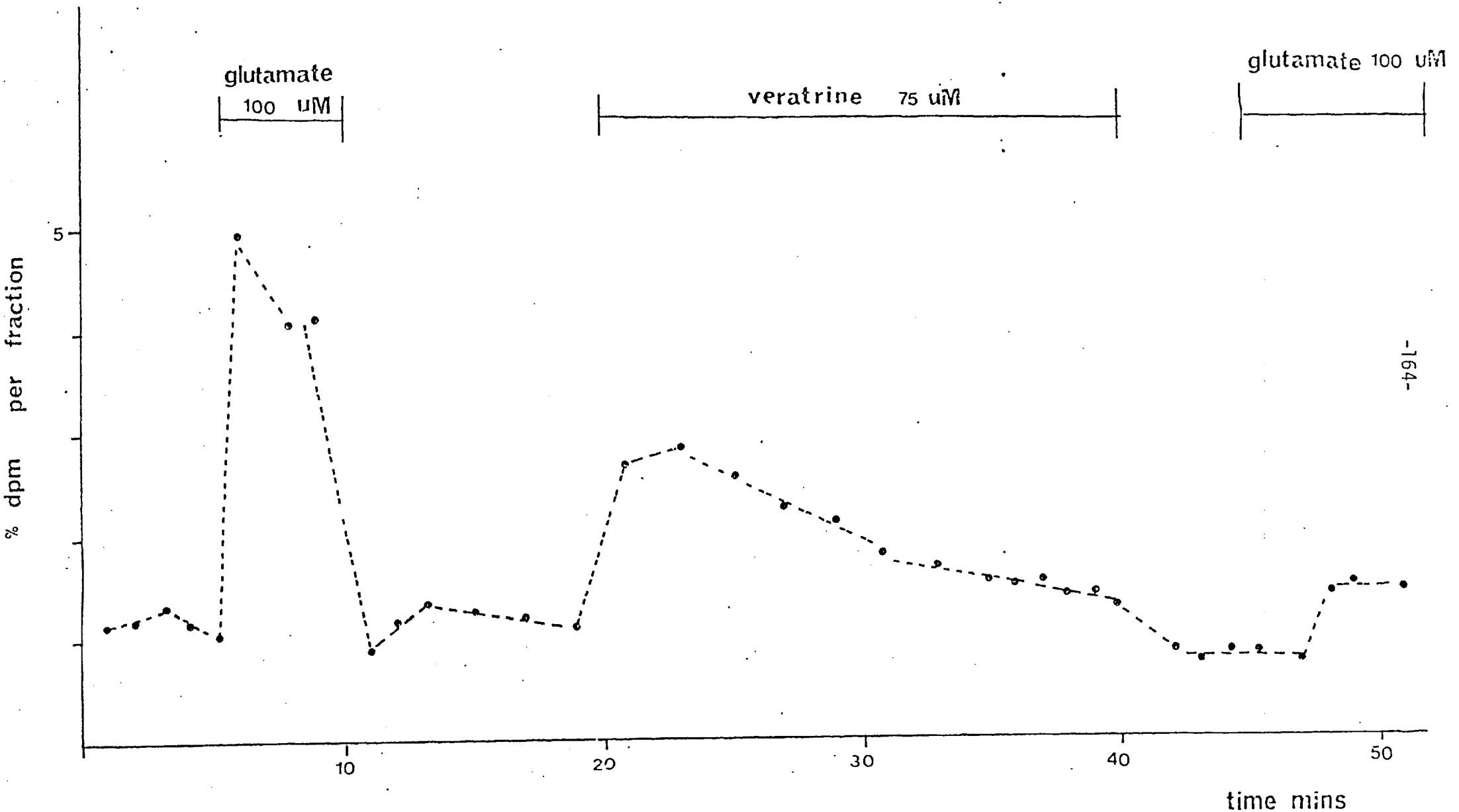


Figure 4.18 The effect of veratrine-induced depolarization preceeding homoexchange of ^{14}C glutamate.



aspartate, glutamate and GABA, can be released. In vivo of course the percentage actually released would be very much less since physiological stimulation is pulsed stimulation, whereas in our in vitro experiments using chemical stimulation the ion channels are open continuously and so transmitter release is exaggerated.

(c) The effect of veratrine-induced depolarization preceding homoexchange of ^{14}C glutamate from perfused synaptosomes

Figure 4.18 shows that despite homoexchange of ^{14}C glutamate being detected initially, following veratrine-induced depolarization, negligible homoexchange is observed, even though a recovery period of ten minutes was allowed and there was 34% of the total radiolabel associated with the pellet at the end of the experiment.

Specific activities were measured at points A and B to ensure that hot and cold glutamate were depleted to the same extent by homoexchange and depolarization:

Specific activity at point A - 58.9 picoCi/nmole/mg protein

Specific activity at point B - 59.3 picoCi/nmole/mg protein

Due to the similarity of these activities, together with the evidence above, it is implied that neurotransmitter homoexchange and depolarization occur from the same pool(s) via the same or similar mechanism(s). It also appears that before homoexchange of glutamate can occur, a full homeostatic complement of transmitter is necessary. If this is the case, the question then arises as to the physiological role of homoexchange. Certainly, during stimulated release of neurotransmitter, homoexchange would not occur since the releasable pool would be depleted. Raiteri et.al. (1974) consider that

Legend to Figures 4.19a, 4.19b, 4.19c, 4.19d

Ion channel sensitivities of ^{14}C GABA homoexchange and basal efflux from perfused nerve endings

Rat cerebrocortical synaptosomes were incubated and prelabelled with ^{14}C GABA (2μM final concentration 224mCi/mmole) as described previously. The calcium free medium contained EGTA (0.5mM) or verapamil (100μM), from zero time. Similarly the medium designed to test active sodium ion sensitivity contained TTX (1μM) from zero time.

Legend to Figures 4.20a and 4.20b

The effects of anticonvulsants on the IN VITRO release of ^{14}C GABA from perfused synaptosomes

Following a 30 minute preincubation at 37°C, synaptosomes were preloaded with 14C GABA as described in the Materials and Methods section. Aliquots of the incubates were then superfused for 8 minutes with oxygenated Krebs phosphate medium. After this control period, the test medium was added to give the final concentrations as indicated in the figure. Except for phenytoin and carbamazepine, all compounds were prepared with oxygenated Krebs phosphate medium, pH7.4. Phenytoin was dissolved in a small volume of medium, pH > 11.7, followed by medium dilution to pH 7.4. Carbamazepine was dissolved in a few drops of absolute alcohol followed by medium dilution to pH 7.4. GAG, γ-acetylinic GABA; GVG, γ-vinyl GABA; DPA, di-n-propyl acetate; Phenob, phenobarbitone; Phenyt, sodium phenytoin; Ethos, ethosuccimide; Carb, carbamazepine; Hist, histidine; Leu, leucine; Tau, taurine.

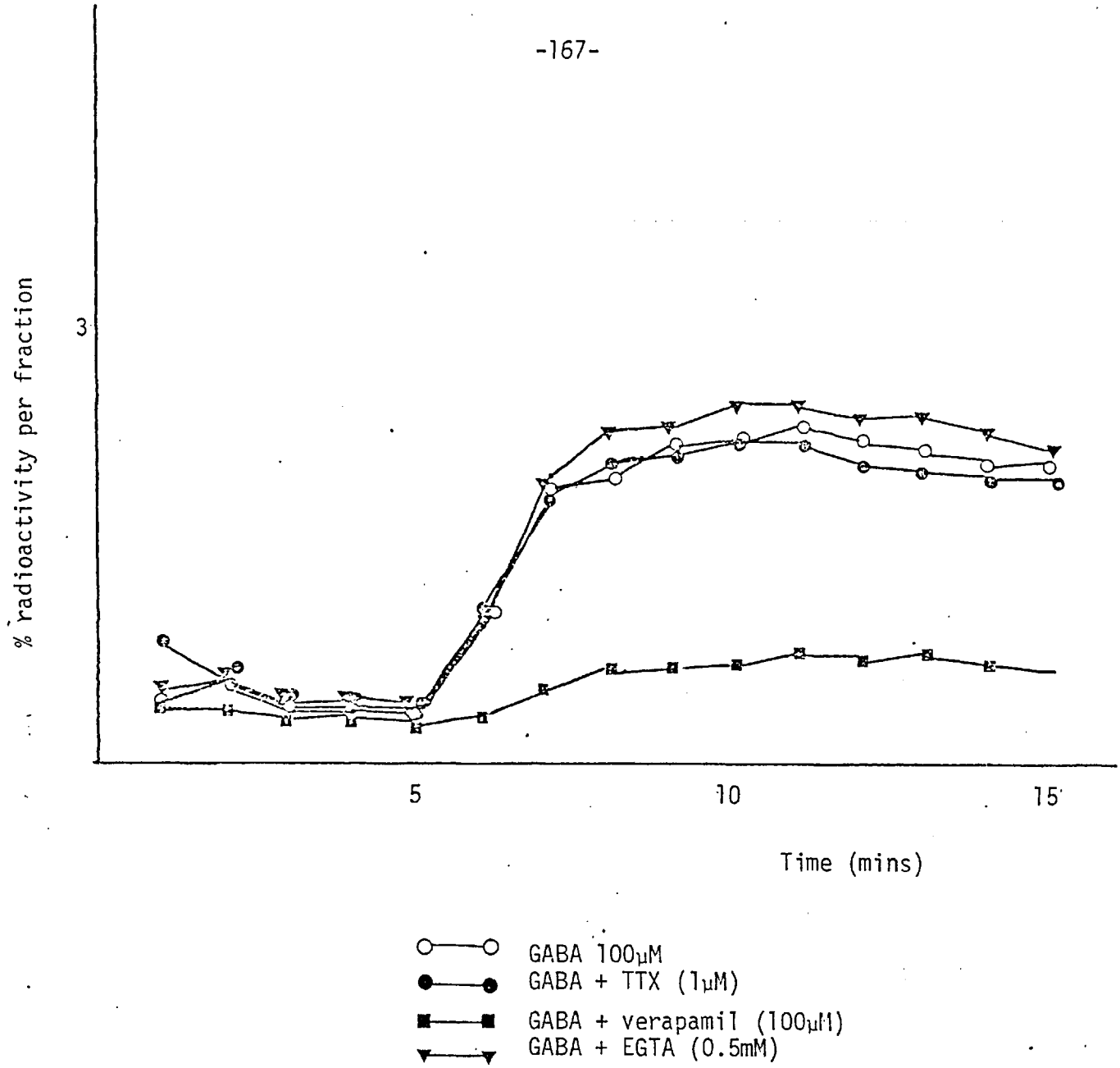
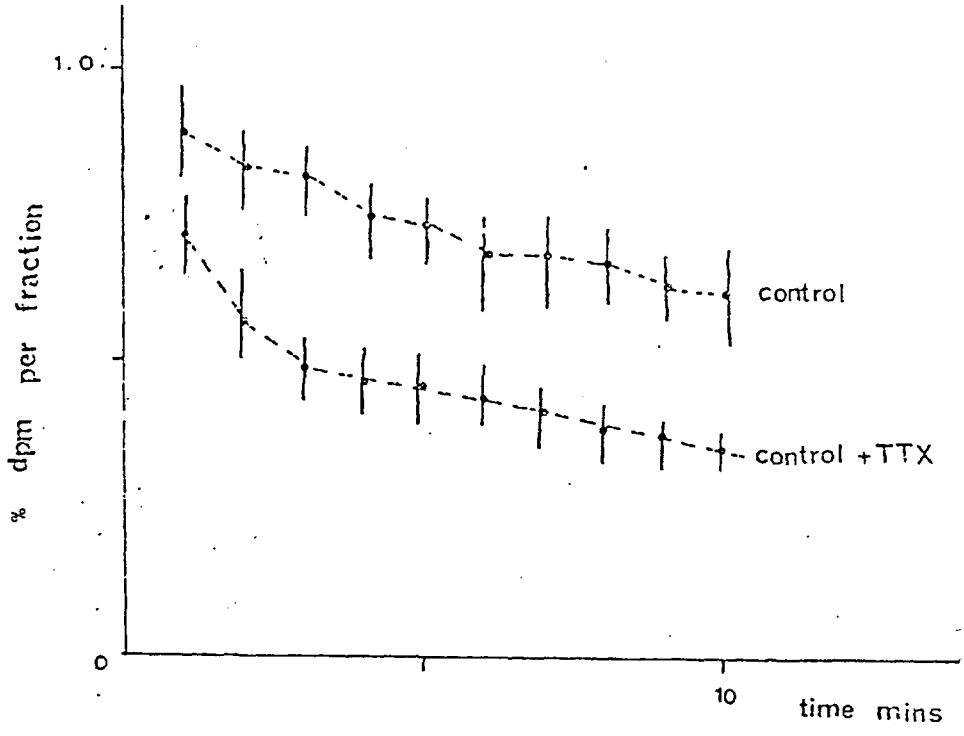


Figure 4.19a TTX sensitivity and calcium dependence of ^{14}C GABA homoexhange.

Figure 4.19b TTX sensitivity of basal ^{14}C GABA efflux from perfused synaptosomes.



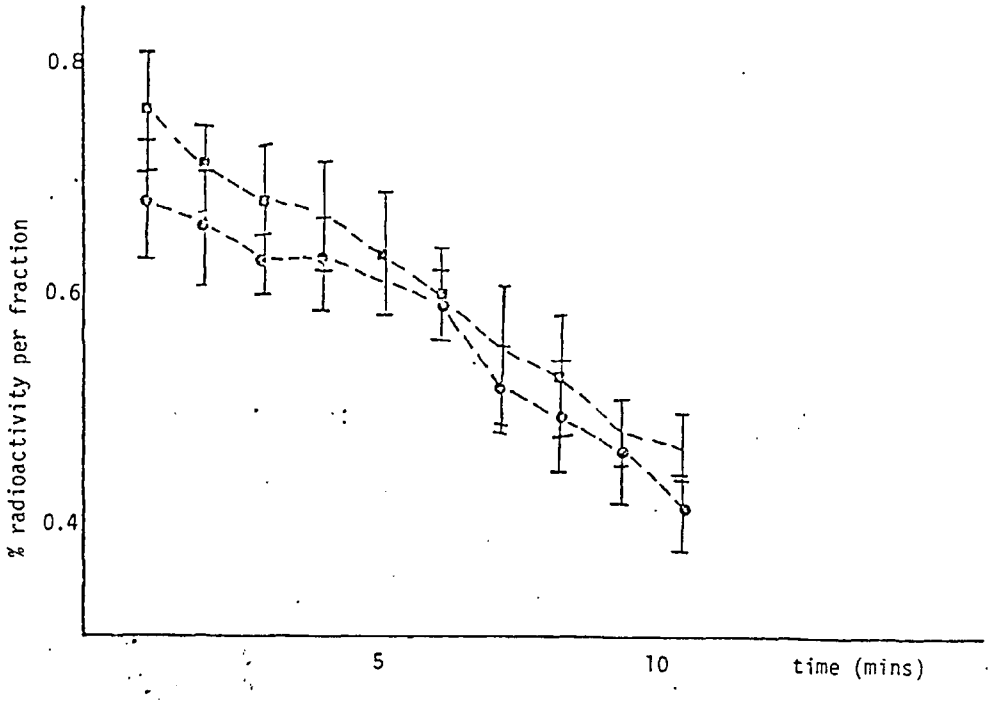
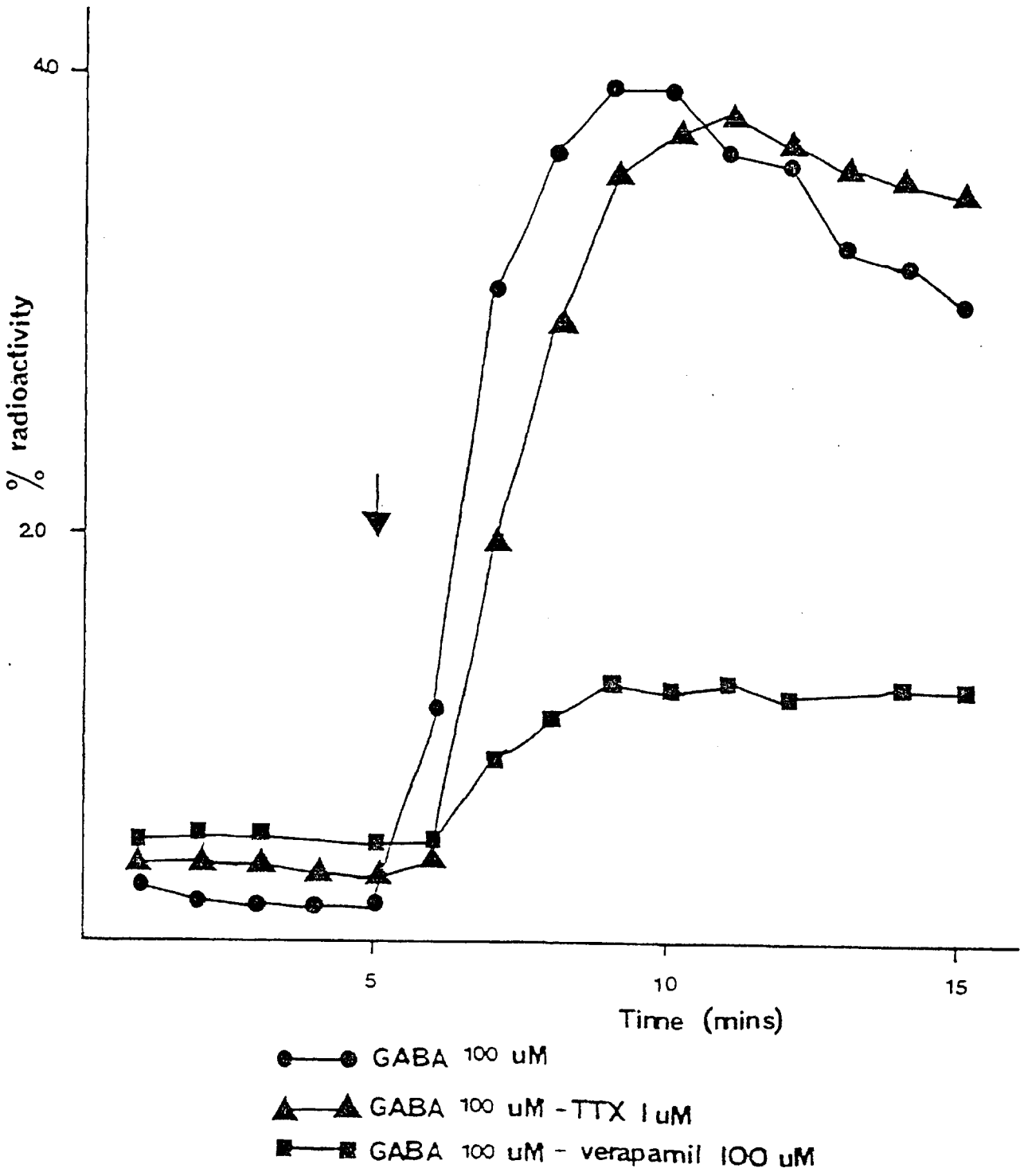


Figure 4.19c Ca^{2+} independence of ^{14}C GABA efflux from perfused synaptosomes.

Figure 4.19d TTX and verapamil sensitivity of GABA-induced homoexchange in synaptosomes.



The effects of anticonvulsants on the in vitro release of ^{14}C GABA from perfused synaptosomes.

Fig. 4.20a

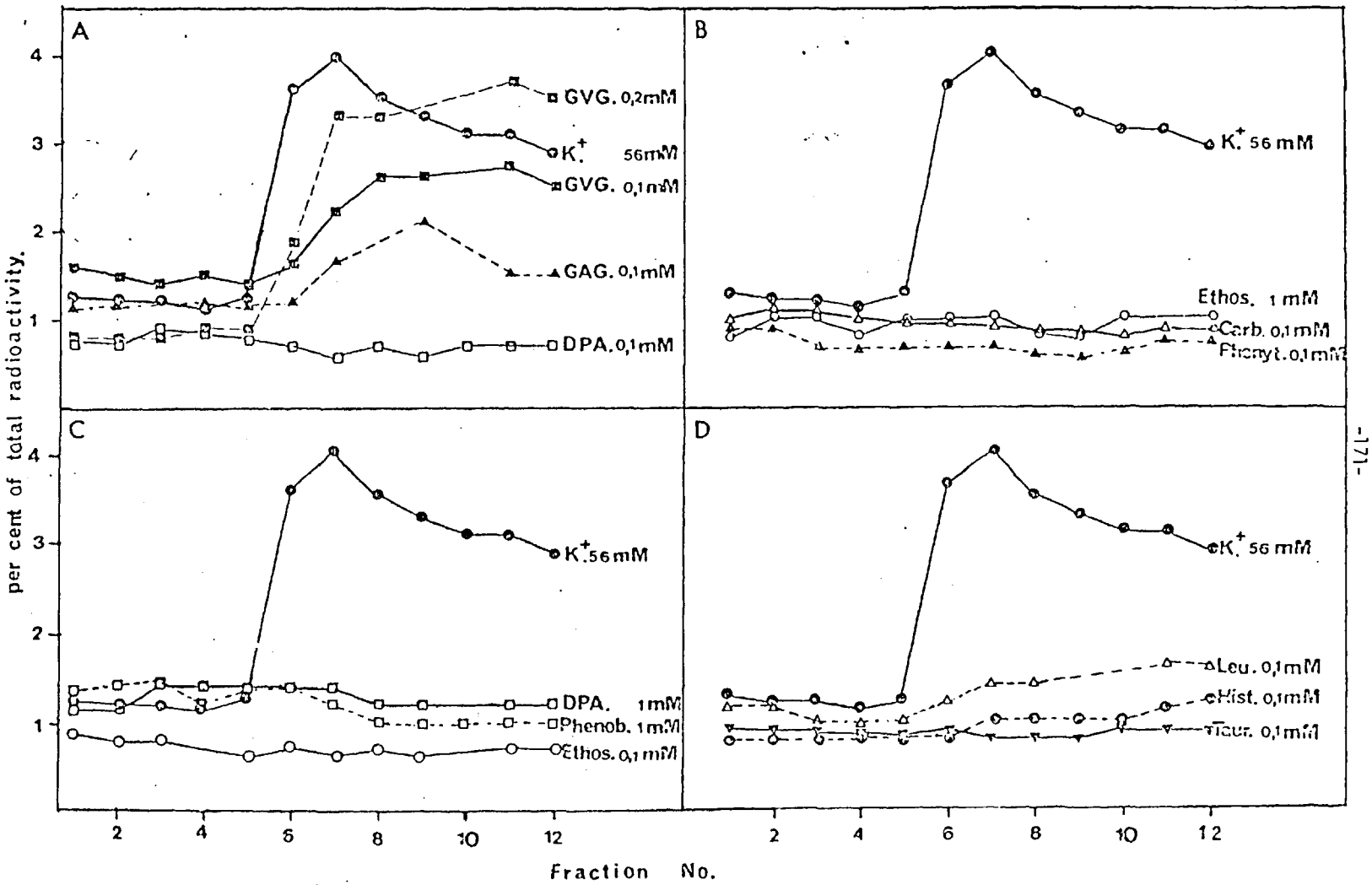
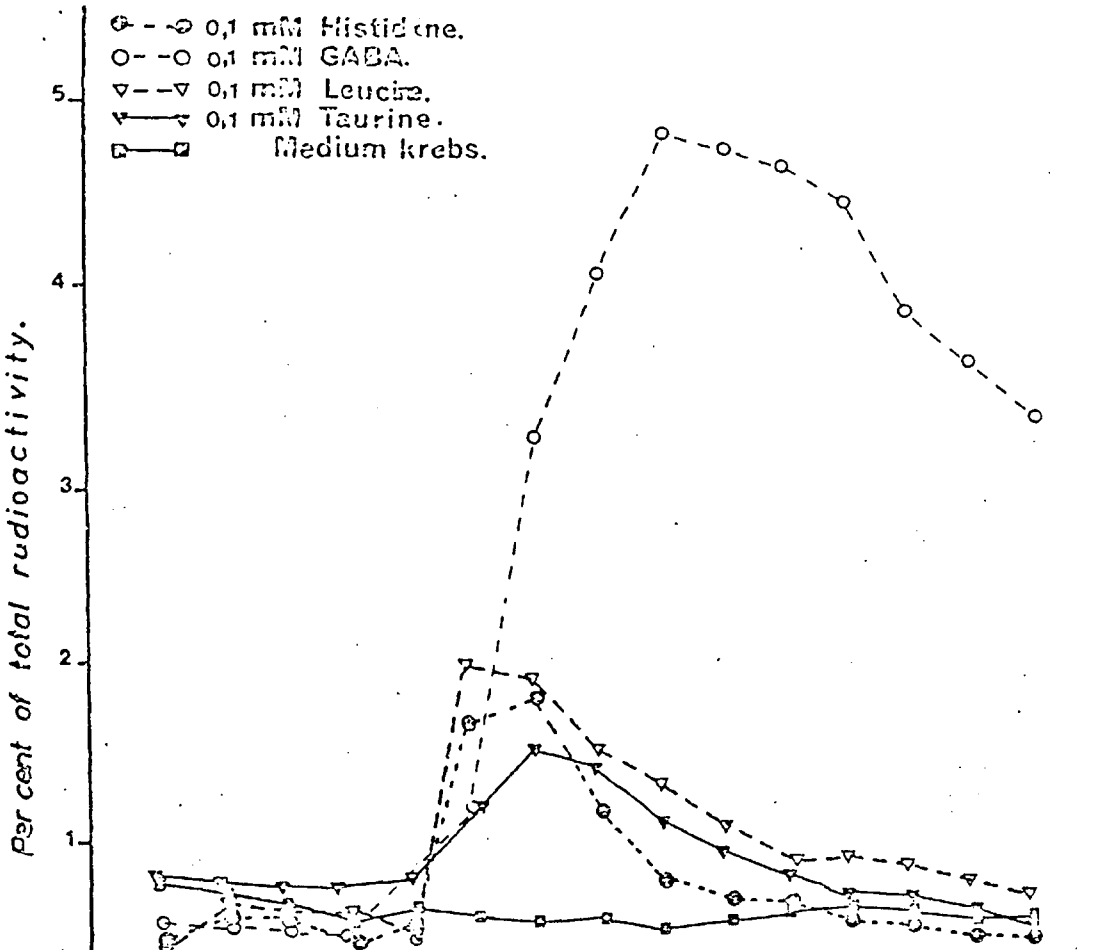


Figure 4.20b Exogenous ^{14}C GABA release from perfused crude synaptosomal fractions.



at this point, as the Na^+-K^+ ATPase activity is modulated, the stoichiometry of homoexchange would change, such that uptake would exceed the release component. However, invoking this theory implies changing the definition of homoexchange itself.

(d) Ion channel dependencies of ^{14}C GABA homoexchange and basal efflux from perfused nerve endings

When nerve terminals are perfused with calcium-free medium, containing EGTA (0.5mM), no difference in the basal efflux of ^{14}C GABA is detected from controls (Figure 4.19a). Here of course we are assuming that the EGTA is efficient at chelating the endogenous calcium ions available. Figure 4.19b, however, shows that the basal efflux is to some extent active sodium channel dependent since, with TTX included in the superfusion medium, spontaneous release is significantly reduced. This would imply that the efflux has an active release component. Plotting a cumulative graph of the data in Figure 4.19b , background leakage of the radiolabel in this system would appear to account for only 50% of the basal efflux.

The experiment depicted in Figure 4.19d reveals no TTX (1μM) sensitivity for gaba (200μM) homoexchange itself. However, when verapamil (100μM) is included in the medium from zero time, then the gaba homoexchange process is abolished.

Similar results were obtained when the GABA analogue γ-vinyl-GABA was used as the exchange compound. Thus at the concentrations shown in Figure 4.19d, GVG-stimulated efflux was found to be TTX insensitive and Ca^{++}-independent under our test conditions (Ca^{++}-free medium, EGTA 0.5mM). However, verapamil (100μM), a supposed Ca^{++} antagonist, was found to

ihibit this 'heteroexchange'. GVG(100μM) was only 37% as effective as cold GABA at the same concentration (100μM) in evoking ^{14}C GABA release.

The work of Simon et.al. (1974) is now widely accepted as evidence of Na^+ ion dependence for GABA homoexchange. These authors claim to be detecting ion dependence of the efflux component, after firstly having depleted the synaptosomes of intracellular sodium. However, this result could easily be explained by the results of Levi et.al. (1974), that the uptake component of homoexchange (i.e. the Na^+-dependent high affinity enzyme) is being inhibited.

The literature evidence for Ca^{++} dependence of homoexchange is also somewhat conflicting. Olsen et.al. (1977), for example, showed no calcium dependence for gaba homoexchange when using, as we did, a calcium-free medium and EGTA. However, Levi et.al. (1976) reported increasing GABA homoexchange with increasing concentrations of the ionophore A23187 and a calcium-containing medium. The discrepancy between these results has yet to be explained.

Further remarks on the use of verapamil

Recently Nachstenand Blaustein (1979) have studied the usefulness of verapamil as a calcium 'antagonist' in synaptosomes. They conclude that although verapamil and D-600 appear to be potent antagonists of Ca^{++} currents in heart and smooth muscle, in nerve terminals Ca^{++} currents may be insensitive towards them. Also, these and other workers (Baker et.al., 1973; Van der Kloot and Kita, 1975) suggest that these compounds are potent antagonists of Na^+ channels as well.

(e) The effects of anticonvulsants on the release of exogenous ^{14}C GABA from perfused synaptosomes

Agents which raise brain GABA content are of special interest as potential anticonvulsants because of the potent inhibitory action of this compound, and also because of the observed aberrations in the storage and metabolism of GABA in focal epileptic tissue obtained from experimental animal models, and in cases of human epilepsy (Bradford, 1976). The question therefore arises as to whether commonly employed clinically effective anticonvulsants, and potential candidates for this role, produce their effects partially, or entirely, by raising brain GABA content, or by causing a rise in the concentration of this inhibitory agent in the extracellular fluid of the brain. This possibility was tested by following the release of exogenous GABA from perfused rat brain synaptosome preparations. As shown previously, high concentrations of K^+ ions (56mM) caused a 3.5 fold increase of pre-loaded [U^{14}C]-GABA (Figure 4.20a). The two alkyl GABA derivatives at 100μM also evoked substantial [U^{14}C]-GABA release (1.5-2.2 fold). At 200μM γ-vinyl-GABA, this rate could be increased by 6-fold. None of the other anti-convulsants (sodium di-n propyl acetate, carbamazepine, phenobarbitone, ethosuccimide, phenytoin) tested had any detectable influence on the rate of release of preloaded GABA to the superfusion fluid, even at concentrations up to 1mM. These results suggest that neither the benzodiazepines (carbamazepine), hydantoins (phenytoin) nor the barbiturates (phenobarbitone) exert their anticonvulsant actions by immediately releasing GABA from nerve terminals. However, the potential anticonvulsant candidates γ-vinyl-GABA and

γ-acetylinic GABA where shown, show an apparent release of exogenous GABA in the perfusion system. This correlates well with the results of Bergamini et.al. (1974) who have demonstrated stimulated endogenous GABA release and an ability to raise GABA levels in the CSF and blood (Ferkany et.al., 1979; Loscher, 1979) using these compounds.

Taurine at 100μM doubled the rate of [U^{14}C]-GABA from crude synaptosome fractions over a 5-minute period (Fig. 4.20b) but this was not specific to taurine, since histidine and leucine at 100μM were equally effective. Unlabelled GABA itself at this concentration was 2-3 times as effective as the other amino acids in releasing [U^{14}C]-GABA. However, pure synaptosomal preparations did not show this problem of taurine-induced gaba release, or any changes with histidine or leucine.

Taurine has been found to exhibit anticonvulsant properties in a wide range of test systems (Wheeler et.al., 1974; Van Gelder et.al., 1972; Deraeux et.al., 1973; Izumi et.al., 1973), and also to be clinically effective in containing fits in human epilepsy (Barbeau and Donaldson, 1974). The evidence presented here shows that taurine has a non-specific effect in releasing [U^{14}C]-GABA from crude synaptosomal fractions, nor was it a potent one when compared to gaba itself. Also the effect was not a property of purified synaptosomal preparations. Pasantes-Morales (1980) has reported a similar action. Thus, it is proposed from those in vitro results that the primary anticonvulsant action of taurine is not through enhancing gaba release in vivo.

(f) Exogenous GABA release from perfused crude synaptosomal fractions

Since many workers (Levi et.al., 1974; Brennan et.al., 1979) have used perfused crude synaptosomal preparations (P_2) rather than purified preparations (P_2B) to study release and exchange mechanisms, an experiment was performed to see if the presence of whole brain mitochondria and glial cells affect homoexchange from neurons.

Crude synaptosomal fractions were prepared, preloaded with [U^{14}C]-GABA, and perfused in the manner described in the Materials and Methods section.

Figure 4.21a shows that following a control period of 8 minutes, the Krebs phosphate medium was substituted for a medium containing veratrine (50μM), veratrine + TTX (1μM) or control medium, as indicated. Veratrine was found to elicit a four-fold increase in release of exogenous GABA, which was completely inhibited by TTX. Because of this TTX sensitivity, the site of origin of release of exogenous ^{14}C gaba is implied as neuronal rather than glial. This would be so despite the fact that glial cells possess high affinity reuptake systems (Iversen et.al., 1975). Of course this event does not rule out whole brain mitochondria regulating the transmitter release, by their ability to transport Ca^{++} ions as suggested by Sandoval (1980). Whether it is specifically synaptosomal mitochondria that are important in this process is as yet unknown.

Figure 4.21b demonstrates homoexchange from P_2 with various concentrations of GABA (10, 50, 100μM). The cold GABA elicited a peak efflux of [U^{14}C]-GABA of 5-5.5, 2.5-3, 1-1.5 fold increases over controls respectively. Hence the evidence presented here suggests that perfusion of prelabelled crude synaptosomal fractions show similar patterns of release

Legend to Figures 4.21a and 4.21b

The release of ^{14}C GABA from crude mitochondrial fractions

Experimental conditions are as described previously in Materials and Methods, chapter 2. After 8 minutes of superfusion with standard medium, the test medium was added, to give the indicated final concentrations. TTX where indicated was included in the medium (1μM) from zero time. All reagents were prepared in oxygenated Krebs phosphate medium, pH 7.4.

Legend to Figures 4.22a, 4.22b, 4.22c, 4.22d

The action of various glutamate analogues on perfused ^{14}C labelled nerve endings

Experimental conditions for incubation, radiolabelling, and superfusion were as described previously. All the reagents were freshly prepared in oxygenated Krebs phosphate medium, pH 7.4, at the concentrations indicated.

Figure 4.21a

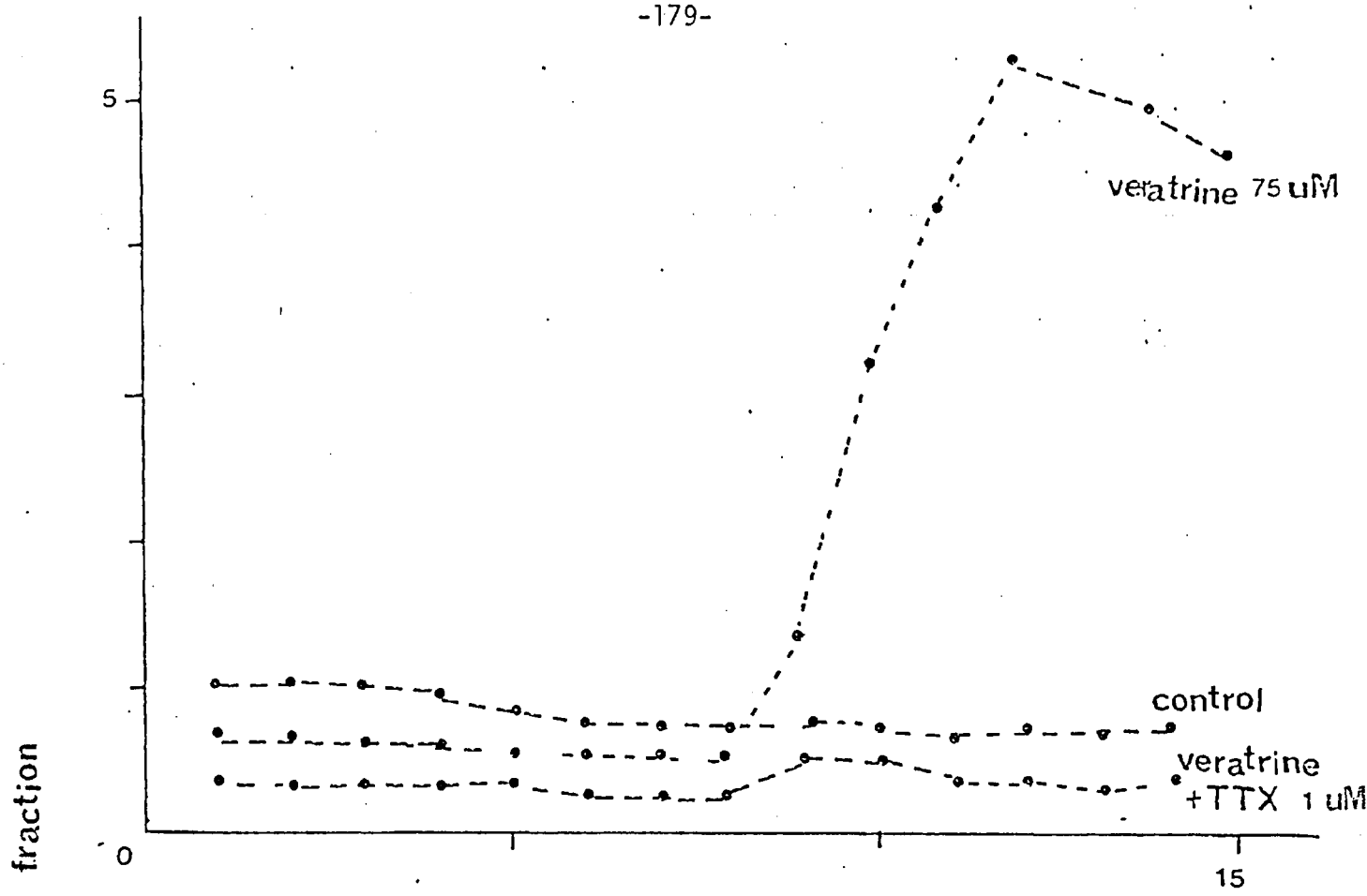
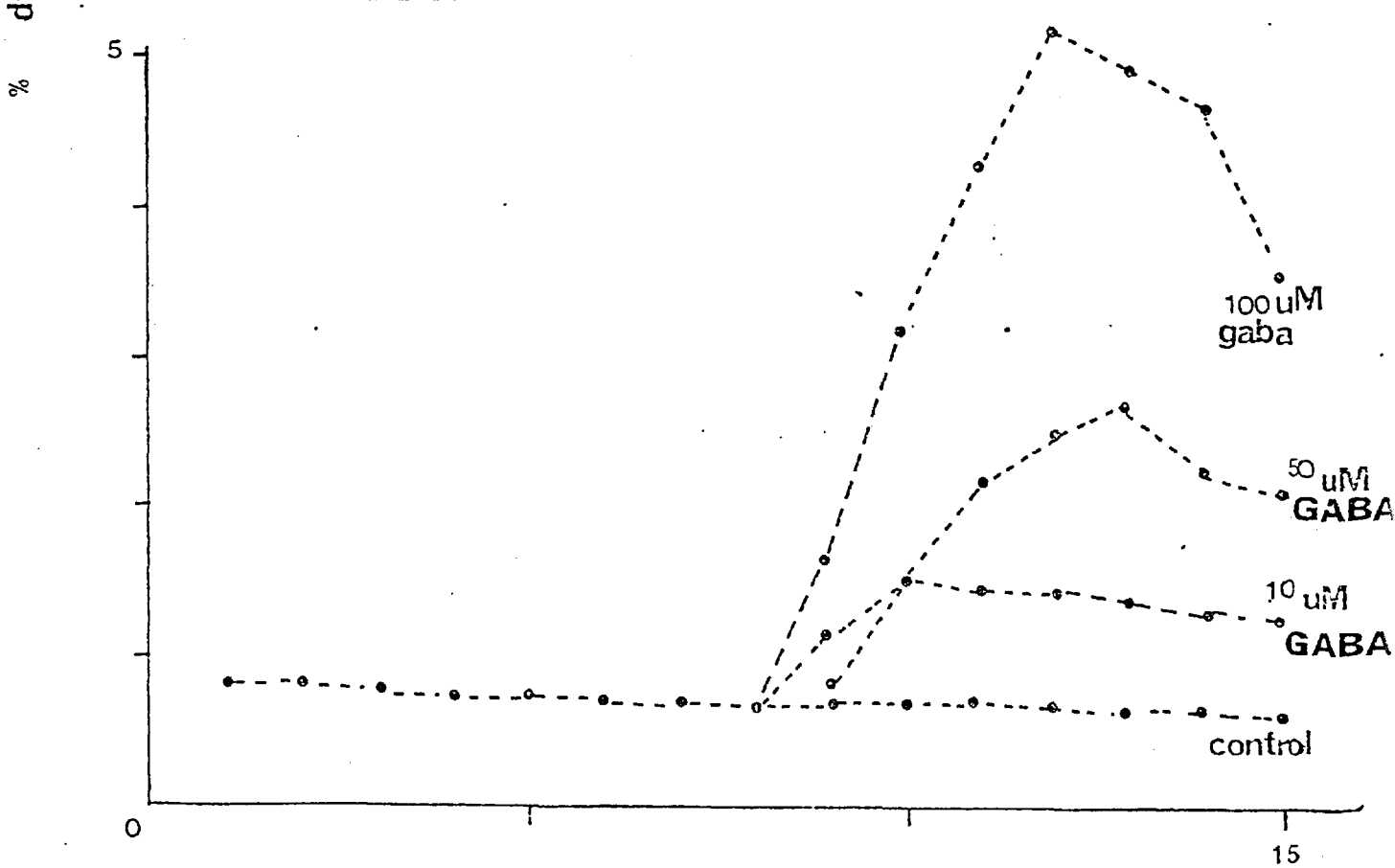


Figure 4.21b



% dpm per fraction

time mins

Figure 4.21 The release of ^{14}C GABA from crude mitochondrial fractions.

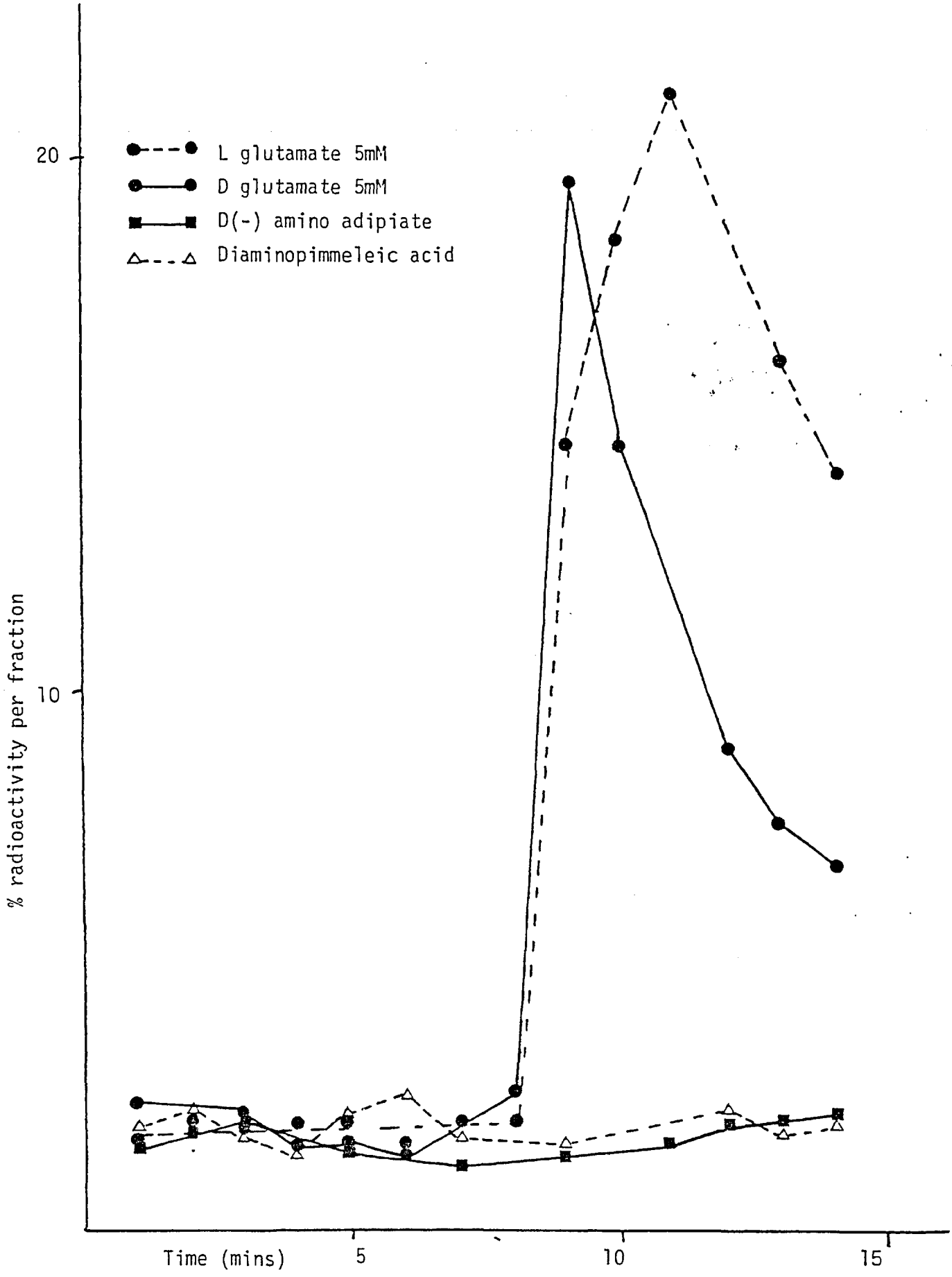


Figure 4.22a The action of D and L glutamate, D(-) amino adipiate, and diaminopimmeleic acid on ^{14}C glutamate release from perfused synaptosomes.

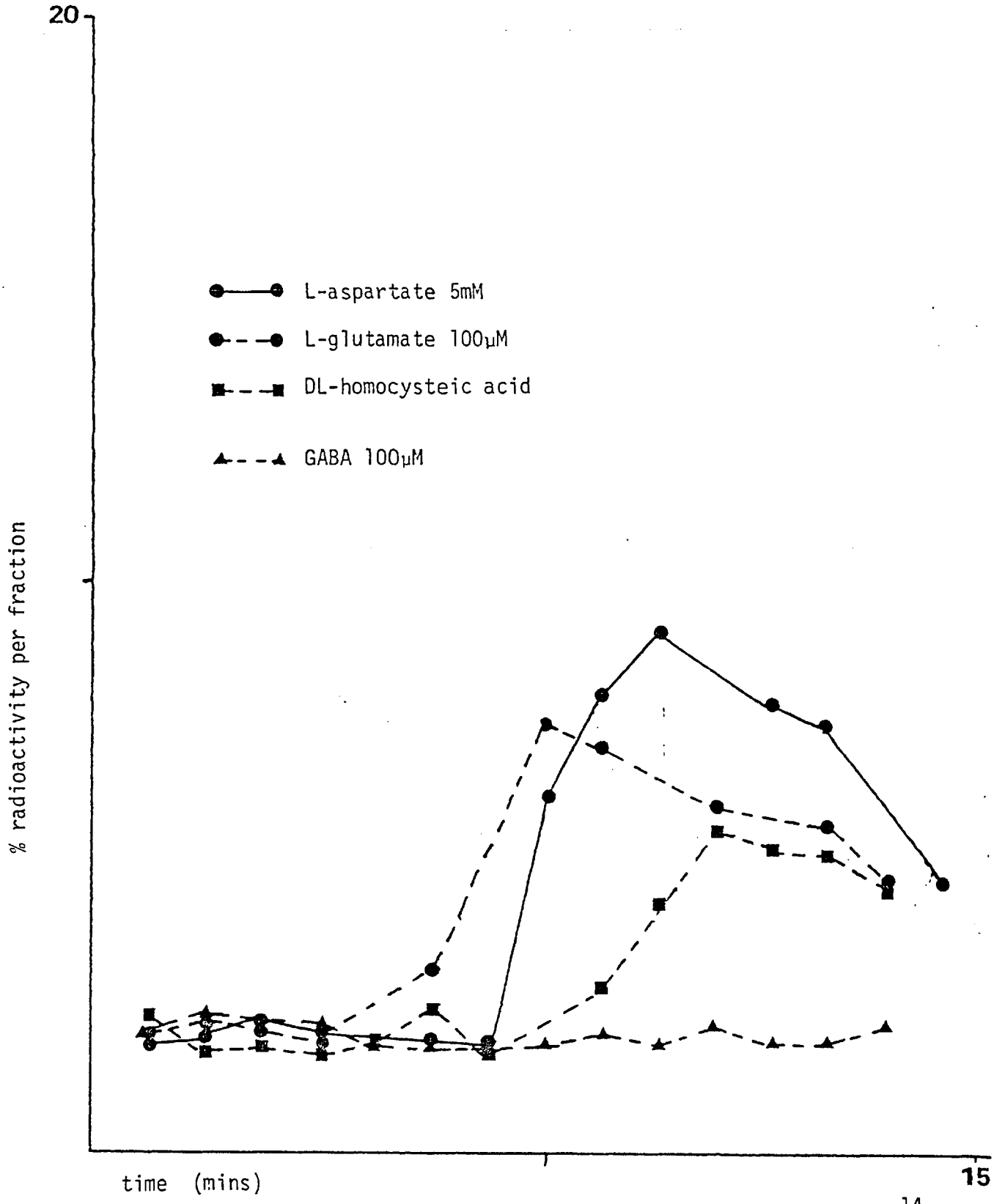


Figure 4.22b The effect of L-aspartate, DL-homocysteic acid on ^{14}C glutamate release from perfused synaptosomes.

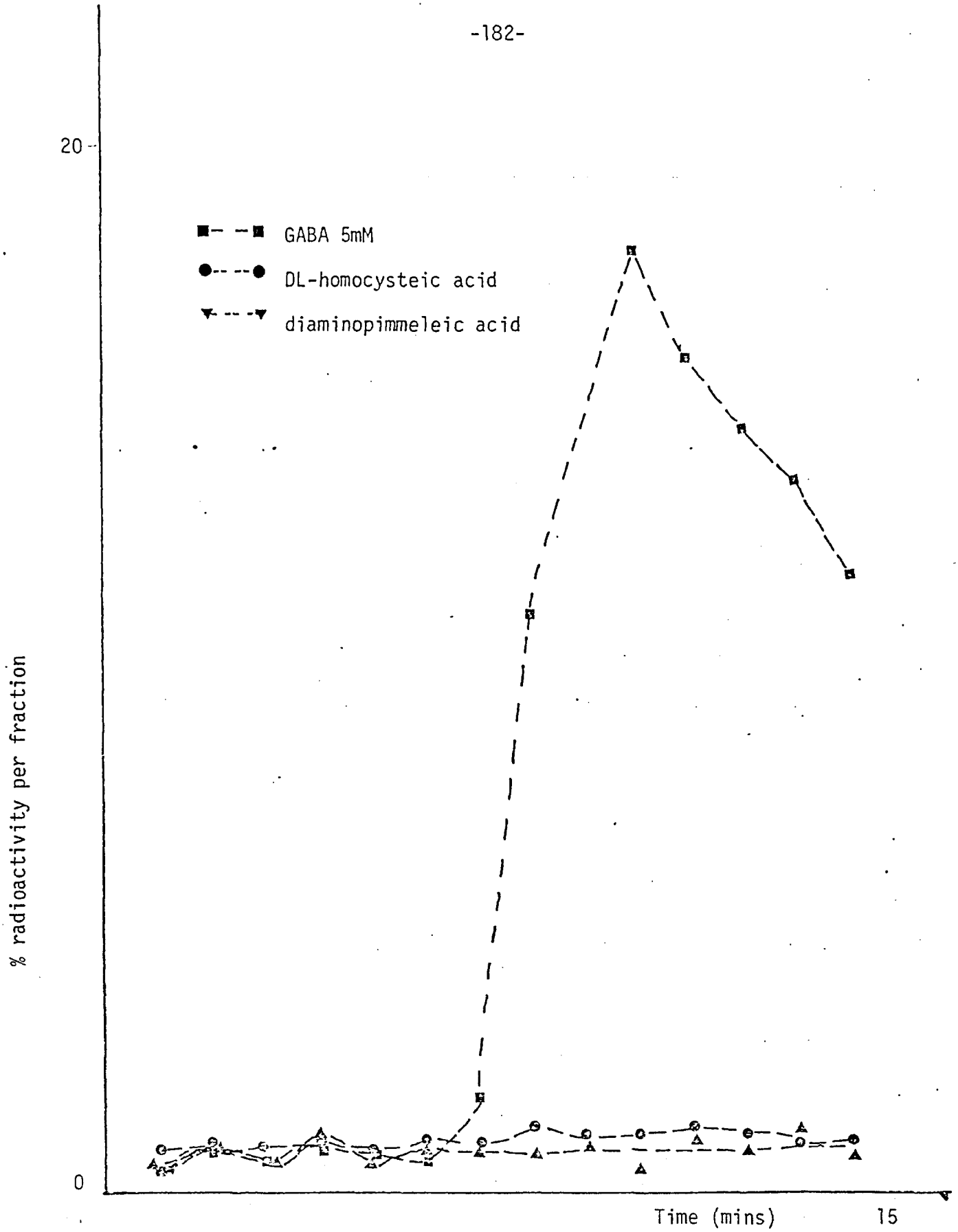


Figure 4.22c The action of GABA, diaminopimmeleic acid, and homocysteic acid on ^{14}C GABA release from perfused synaptosomes.

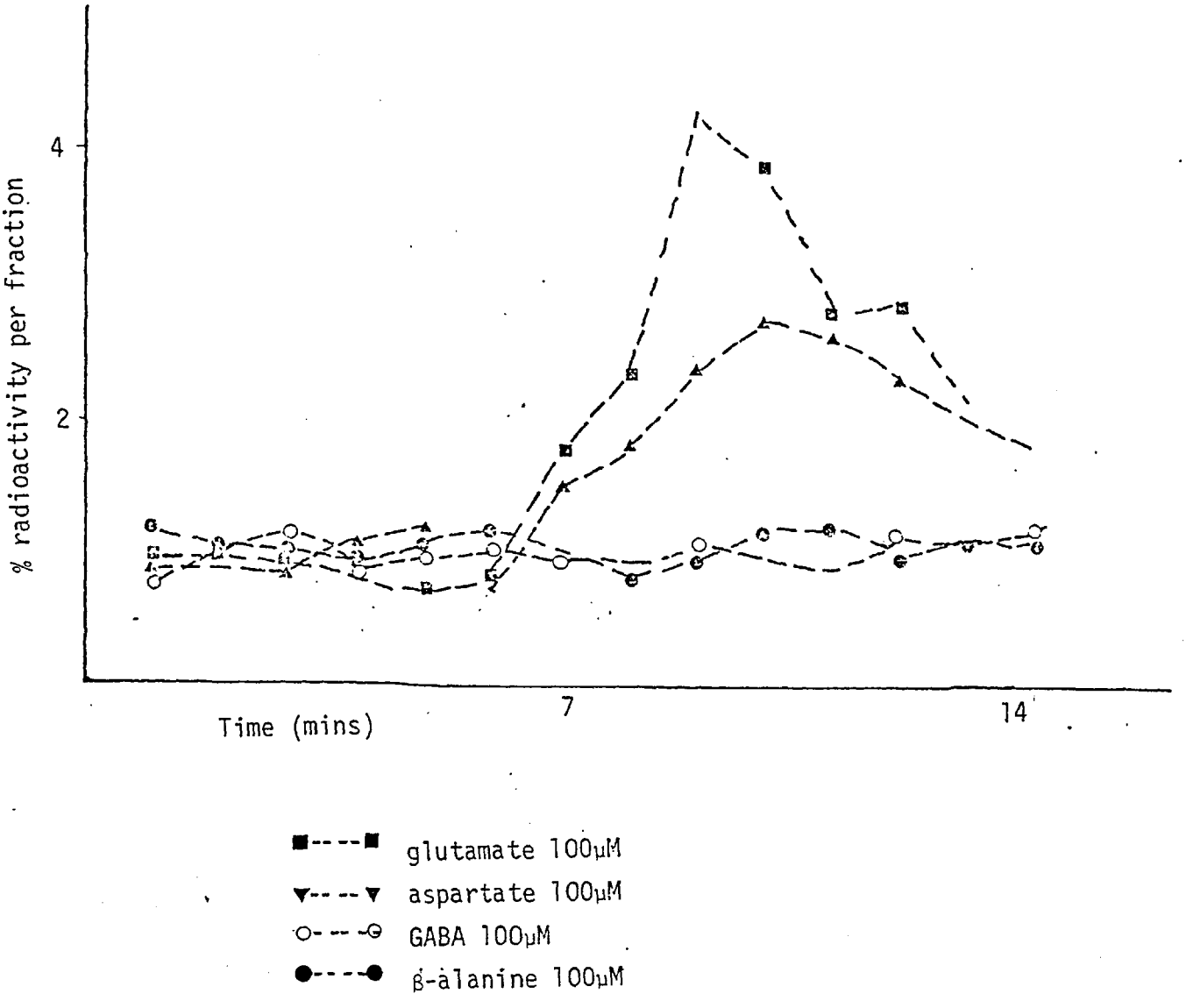


Figure 4.22d The action of GABA and β-alanine on ^{14}C-glutamate release from perfused nerve endings.

to perfusion of purified synaptosomal fractions.

(g) The action of various glutamate analogues on perfused [U^{14}C]-glutamate prelabelled synaptosomes

Structural activity of various glutamate analogues towards glutamate homoexchange was examined in purified synaptosomes. In particular, attempts were made to correlate homoexchange activity as a function of the bond distance between the two terminal acidic groups on the glutamate molecule, by employing suitable compounds that selectively varied the number of CH_2 groups in their 'glutamate-like' chain. Thus in Figure 4.22a, although no stereospecificity was detected between D and L glutamate (5mM) evoked release of ^{14}C glutamate (10-11 fold), no efflux was observed when either D(-) aminoadipiate or diaminopimeleic acid was introduced into the superfusion medium at the same concentration; nor when included at the lower concentration of 100μM (results not shown). D(-)-AP and DAP have 5 and 4 CH_2 groups respectively between their two terminal carboxy groups, compared to glutamate which has three (see Table 4.3). However, L-aspartate (5mM), and DL-homocysteic acid (5mM), were found to evoke [U^{14}C]-glutamate release by 6.5 and 3.9 fold respectively (Figure 4.22b). These relatively short chain glutamate analogues were specific in their homoexchange activity since none of the compounds evoked [U^{14}C]-GABA release from perfused synaptosomes (Figure 4.22c). Also, cold GABA and β-alanine did not evoke any [U^{14}C]-glutamate release in our experimental conditions (Figure 4.22d), presumably because they contain only one terminal acid group in their structure.

Table 4.3 Chemical structures of GABA and glutamic acid analogues

Compound	Structure	number of CH_2 groups in the molecule between terminal acid groups
glutamic acid	$HOOCCH_2CH_2CH(NH_2)COOH$	3
homocysteic acid	$HOOCCH(NH_2)CH_2SO_3$	2
aspartic acid	$HOOCCH_2CH(NH_2)COOH$	2
adipic acid	$HOOCCH_2CH_2CH_2CH_2COOH$	4
diaminopimmeleic acid	$HOOCCH_2CH_2CH_2CH_2CH_2COOH$	5
β-alanine	$H_2NCH_2CH_2COOH$	-
γ-aminobutyric acid	$H_2NCH_2CH_2CH_2COOH$	-

Part C Investigating Regulation of Amino Acid Release by Prostaglandins, using Synaptosome Suspensions

4.7 Introduction

Prostaglandins (PG's) are synthesized by homogenates or slices of mammalian brain (Abdel-Halim and Anggard, 1979). The role of these compounds in the central nervous system remains to be elucidated, although prostaglandin E_2 inhibits noradrenalin release from rat cortical slices (Aillier and Templeton, 1980). Prostaglandins of the E series also inhibit noradrenergic transmission in the peripheral nervous system (Hedqvist, 1977). Further investigation of a possible synaptic function of prostaglandins is complicated by contamination of homogenates or slices of cerebral tissue by non-neuronal tissue, that synthesises and is sensitive to prostaglandins. These problems were avoided in studies (Blair et.al., 1981; Blair et.al., 1980) of a cloned somatic cell hybrid that was derived from neuronal cells. Prostacyclin (PGI_2) and other prostaglandins activate adenylate cyclase in these cells. The process is receptor mediated, and binding of ^{3}H-prostacyclin is regulated by divalent cations. The interaction between the prostaglandin receptor and the adenylate cyclase molecule is dependent on the presence of guanosine-5'-triphosphate, suggesting that the receptor-enzyme coupling is similar to that observed in other hormonal (Lad et.al., 1977) and neurotransmitter (Sabol and Nirenberg, 1979) systems. In order to extend our studies, rat cortical synaptosomes have been examined. Their ability to synthesize prostaglandins has been investigated. The effect of depolarization on prostaglandin release, and the effect of prostaglandins on amino acid release has been determined. Finally, radioligand binding

techniques have been employed to resolve whether synaptosomes possess a prostacyclin receptor.

4.8 Results

Suspensions of freshly prepared synaptosomes in Krebs phosphate medium (pH 7.4) were allowed to equilibrate for 30 mins prior to stimulation. Control samples were incubated for a further 10 mins, whilst parallel incubations were treated for the same period with selected reagents. The incubations were terminated by centrifugation at 10,000g and the supernatants analysed for prostaglandin content by GC-MS. Under the control incubation conditions, rat cortex synaptosomes synthesized prostaglandins. PGD_2 (10.9ng/mg protein) was found in the highest concentration (Table 4.3), while 6-oxo-$PGF_{1\alpha}$ was found in concentrations close to the limits of detection. TXB_2 was not detected. In parallel incubations, in which the synaptosomes were depolarized either with potassium (55mM KCl) or Veratrine (75μM), there was no measurable increase in the prostaglandin content of the media, whilst differential release of neurotransmitter amino acids did occur, showing the responsiveness of these synaptosomes to the stimulating reagents.

The binding of 11β-^{3}H-PGI_2 to synaptosomes was studied by employing a modification of previously described methods (Blair and MacDermot, 1981). Experiments were carried out in triplicate and binding of radiolabel was allowed to proceed for 15 mins. Specific binding to the synaptosomes represented only 7% of the total binding. A positive control employing NCB-20 neuronal cells in the assay resulted in about 60% specific binding.

The synaptosomes used in these experiments were tested for their ability to release physiologically important amino acids both from endogenous and exogenous stores. Incubations were

Table 4.3 Synthesis of prostaglandins by rat cortical synaptosomes

Conditions	$PGF_{2\alpha}$	6-oxo-$PGF_{1\alpha}$	D_2	E_2
Control	5.3 ± 1.6	0.8 ± 0.2	10.9 ± 0.8	6.8 ± 3.3
High K^+	5.2 ± 0.9	0.7 ± 0.2	7.6 ± 1.3	7.6 ± 1.1
Veratrine	4.4 ± 0.3	0.3 ± 0.2	6.8 ± 0.9	4.5 ± 0.5

Results expressed in ng/mg^{-1} protein represent determinations from incubations of synaptosomes ± S.E.M. (n=3).

Table 4.4 Endogenous release of amino acids by rat cortex synaptosomes

	Asp	Glu	GABA	Gly
Control	118 ± 24	168 ± 23	84 ± 19	371 ± 55
PGD_2 (1 μM)	168 ± 69	238 ± 27	83 ± 10	405 ± 66
PGE_2 (1 μM)	198 ± 71	216 ± 14	96 ± 21	507 ± 21
K^+ (55 mM)	344 ± 38	814 ± 123	466 ± 53	617 ± 84
PGD_2 + K^+ (55 mM)	359 ± 46	991 ± 141	438 ± 59	657 ± 111
PGE_2 + K^+ (55 mM)	357 ± 36	848 ± 102	512 ± 89	711 ± 116

Results expressed in nmoles/100mg protein ± S.E.M. (n=5).

Table 4.5 Release of exogenous GABA by rat cortex synaptosomes

Sample	5 mM K^+	55 mM K^+
Control	1.63 ± 0.22	4.03 ± 0.79
Control + PGD_2	1.77 ± 0.21	4.43 ± 0.90
Control + PGE_2	1.60 ± 0.25	4.37 ± 0.86

Results expressed as dpm/100 mg protein ± S.E.M. (n=8-11).

carried out under the same conditions as described for the prostaglandin synthesis. Potassium stimulation evoked release of endogenous aspartate, glutamate and GABA, with only a modest release of other amino acids (Table 4.4). No measurable release of amino acids occurred following addition of 1μM PGD_2 or 1μM PGE_2. These prostaglandins also had no effect on the potassium-dependent release.

When synaptosomes were prelabelled with exogenous ^{14}C-GABA, potassium added to 56mM caused release of the label (Table 4.5). No release was evoked with 1μM PGD_2 or 1μM PGE_2. Similarly, these prostaglandins had no inhibitory effect on the potassium-dependent release of ^{14}C-GABA.

4.9 Discussion

Synaptosomes derived from rat cerebral cortex are able to function as viable biochemical units under appropriate incubation conditions. They function in a similar way to rat cortex slices but are relatively free of non-neuronal prostaglandin sensitive tissue. Synaptosomes are well organized cytoplasmic units which maintain the complexity of the original synapse, and have been used widely as models of central synapses (Bradford, 1975). It has been possible to use this system to detect the release of putative neurotransmitters, and also to investigate the factors that regulate their release.

Analysis of the supernatants from synaptosomal incubations in the present experiments has revealed that cortical synaptosomes synthesize prostaglandins. By employing a stable isotope dilution analysis based on GC-MS, PGD_2, PGE_2, $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$ were identified in the supernatants. Quantification by means of the appropriate heavy isotope internal standard showed that PGD_2 was the most abundant prostaglandin formed, with 6-oxo-$PGF_{1\alpha}$ being

formed only in amounts close to the limit of detection. These findings agree with earlier studies (Abdel-Halim et.al., 1977) which demonstrated that PGD_2 is the most abundant prostaglandin formed from homogenates of rat cortex.

Stimulation of rat synaptosomes with either potassium or veratrine caused no detectable further release of prostaglandins beyond control levels. Thus, depolarization in this system is not likely to be a functionally important mediator of prostaglandin release.

Previous studies have shown that prostaglandins mediate activation of adenylate cyclase in neuronal cells (Blair et.al., 1980). This biological activity was shown to be dependent on the occupation of a specific prostacyclin receptor which was characterised by radioligand binding techniques. The present study employing the rat cortical synaptosomes cannot be totally excluded, but they would have to be present at low concentrations.

Although depolarisation of synaptosomes with high potassium concentrations caused release of aspartate, glycine and GABA, with a more modest release of other amino acids, PGD_2 and PGE_2 caused no increased release. Prostaglandins D_2 and E_2 also produced no inhibition of the potassium evoked amino acid release. Experiments in which exogenous radiolabelled GABA was taken up into the synaptosomes demonstrated that although potassium partially released preloaded ^{14}C-GABA, neither PGE_2 nor PGD_2 was able to. These results contrast with the demonstration that PGE_2 is a potent inhibitor of potassium-evoked noradrenaline release from slices of rat cerebral cortex (Hillier and Templeton, 1980).

In conclusion, cerebral cortical synaptosomes synthesise prostaglandins. They release putative amino acid transmitters

on depolarization but not when treated with PGE_2 or PGD_2. These prostaglandins have no inhibitory effect on potassium-evoked amino acid release. Finally, prostaglandins are not released by depolarization with potassium or veratrine.

Chapter 5

Studies on Kinetics of ^{3}H -Glutamic Acid Binding

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CHAPTER 5

Studies on Kinetics of ^{3}H -Glutamic Acid Binding

5.1 Introduction

Although glutamic acid was first shown to have a neuro-excitatory effect at the invertebrate neuromuscular junction, much evidence has been accumulated in recent years to suggest that it is a major excitatory neurotransmitter in the central nervous system (CNS). Curtis and Whatkins (1960) showed that glutamate has an excitatory effect on mammalian CNS neurons, and Krnjevic and Phillis (1963) showed that application of glutamate by iontophoresis to the cerebral cortex results in firing of cells. This had a short latency of onset and offset which is typical of a neurotransmitter. Another factor in favour of glutamate as a neurotransmitter is its release from brain slices (Snyder, 1973) and synaptosomes (Bradford, 1970) upon depolarization. A high affinity uptake system has also been shown for glutamate (and other putative amino acid transmitters) (Divac, 1972; Snyder, 1971). However, there are still reservations in regarding glutamate as a transmitter, such as the widespread occurrence of 'glutamatergic neurons' in the CNS and the problem of finding potent and specific agonists/ antagonists. In connection with this last point, it was considered that a more precise index for the transmitter role of glutamate might be obtained from receptor binding studies.

Evidence that there must be a membrane receptor for glutamate on such neurons comes from the finding that glutamate will only produce an excitatory effect if it is applied at the nerve surface and not intracellularly (Coombs et.al., 1955). Putative glutamate receptors have recently been identified

and partially purified by Michaelis (1975).

The criteria for identification of a receptor have been detailed by Cuatrecasas et.al. (1976). Binding must be of a high affinity (usually in the nanomolar range for a neurotransmitter), be saturable (ie. to a finite number of sites) and be specific. Binding should also be shown to occur only when receptors are known or suspected to occur, and these tissues should be biologically responsive to the ligand. Since neurotransmitters must operate under physiological conditions (pH 7.4, temperature 37^{o}C), binding must be observed at these conditions. A further important requirement is that agonists and antagonists of the ligand should compete with the ligand for the binding site at comparable potencies. Thus binding studies in the test tube can be investigated using the appropriate radioligand, which is usually in the tritiated form, since high specific activities and stable compounds can be obtained. The bound ligand is then separated from the free usually by filtration or centrifugation. Receptor binding follows kinetics similar to classical enzyme substrate interactions, hence:

$$R + L \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} RL$$

where (R) is the concentration of unoccupied receptors, (L) the concentration of free ligand, and (RL) the concentration of receptor-ligand complex; and

$$K_d = \frac{K_{-1}}{K_1} = \frac{[R]\,[L]}{[RL]}$$

where(K_d)is the dissociation constant, (K_{-1}) the dissociation rate constant, and (K_1) the association rate constant. Also

since

$$[RL] + [R] = B_{max}$$

where (B_{max}) is the maximum number of binding sites, the Satchard equation is derived:

$$\frac{B}{F} = \frac{B_{max} - B}{K_d}$$

where (B) is bound ligand and (F) is free ligand. Thus the dissociation constant and B_{max} of the binding site can be calculated.

The aims of the experiments presented in this chapter were:

To establish a glutamate binding assay using the displacement of L-(^{3}H)-glutamate by unlabelled L-glutamate, to obtain a Scatchard plot and hence the K_d and B_{max} of the binding site(s).

To compare these values with those obtained by the direct (saturation) methods.

The study the distribution of glutamate-binding in different brain areas.

To show the inhibition of L-(^{3}H)-glutamate binding to this site(s) by various analogues.

To study the effect of induced depolarizing conditions on specific binding of L-(^{3}H)-glutamate.

Specific binding was defined as the difference between that observed in the presence of radioligand only (total binding), and the binding observed in the presence of excess (2mM) unlabelled ligand (non-specific binding).

5.2 Results

(a) Kinetics of binding of L-(^{3}H)-glutamic acid by the displacement method

Since it is necessary to have a rough estimation of the dissociation constant in order to know what concentration of radioactive ligands to use in binding assays, extensive experiments were performed to calculate the dissociation constants for different brain regions.

Using the displacement method, the counts obtained ranged from approximately 800 to 2000 cpm (counts per minute). The binding of L-(^{3}H)-glutamate to the filtre paper was measured and found to be 342 cpm in the absence of membranes at this concentration of radioligand (28.5 nM). Picomoles of L-(^{3}H)-glutamate bound per mg of protein were calculated. The concentration of free L-(^{3}H)-glutamate was calculated by subtraction from the total isotopic glutamate added in the experiment. It was assumed that the ratio of bound:total radiolabelled ligand was the same as that for the unlabelled ligand; thus since the total amount of L-glutamate present was known, the quantities of bound and free nonisotopic glutamate could be calculated. Therefore to calculate the bound glutamate per mg protein and the bound/free ratio for the Scatchard plots, values for hot and cold glutamate levels were added together. The slope of a Scatchard plot is $-\frac{1}{K_d}$ and the intercept on the x-axis is equivalent to B_{max}.

(1) Binding to cerebral cortex membranes

An experiment performed in the manner previously described, using concentrations of unlabelled L-glutamate in the range of 0 - 2 x 10^{-5}M, yielded displacement curves

Legend to Figure 5.1

Inhibition of specific binding of L-(^{3}H)-glutamate by nonisotopic glutamate in rat cerebro-cortical membranes.

Crude synaptosomal membranes prepared from the rat cerebral cortex were incubated with L-(^{3}H)-glutamate (29 nM) and with various nonisotopic glutamate concentrations in the range of 0 to 10^{-5}M. The data obtained by following the experimental procedures for glutamate binding assays (see section 2.13, chapter 2) was used to plot the displacement curve in Figure 5.1. Each point is the mean of three experiments. Standard errors (less than 15%) are not shown for reasons of clarity.

Legend to Figure 5.2

Structural plots of total glutamate binding to cerebral cortex membranes.

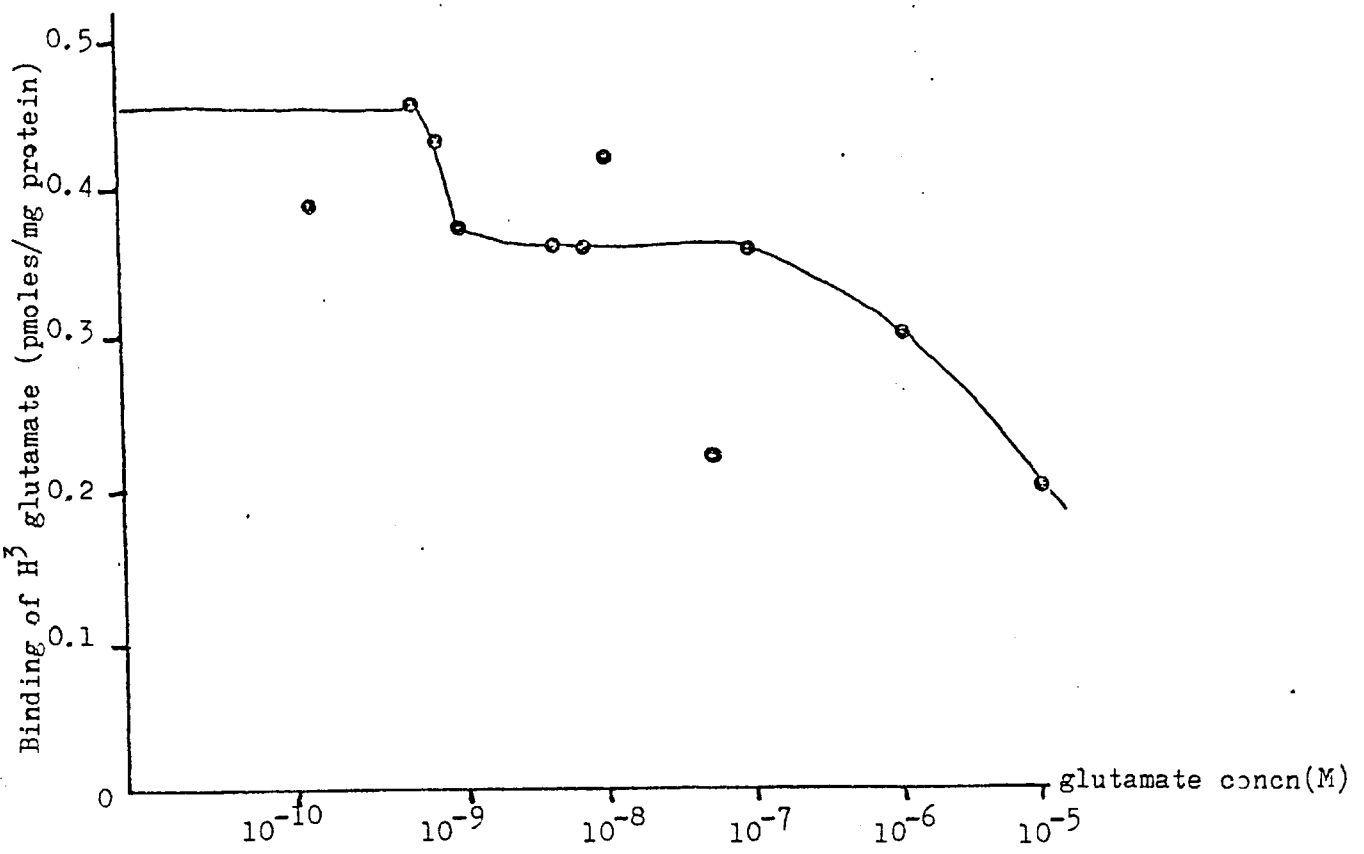
The data displayed in Figure 5.1 was subjected to Scatchard plot analysis. Since

$$\frac{\text{Bound glutamate}^{hot}}{\text{Total glutamate}^{hot}_{\text{used in the expt.}}} = \frac{\text{Bound glutamate}^{cold}}{\text{Total glutamate}^{cold}}$$

then Bound glutamate^{cold} can be calculated. Hence free glutamate^{cold} can be calculated.

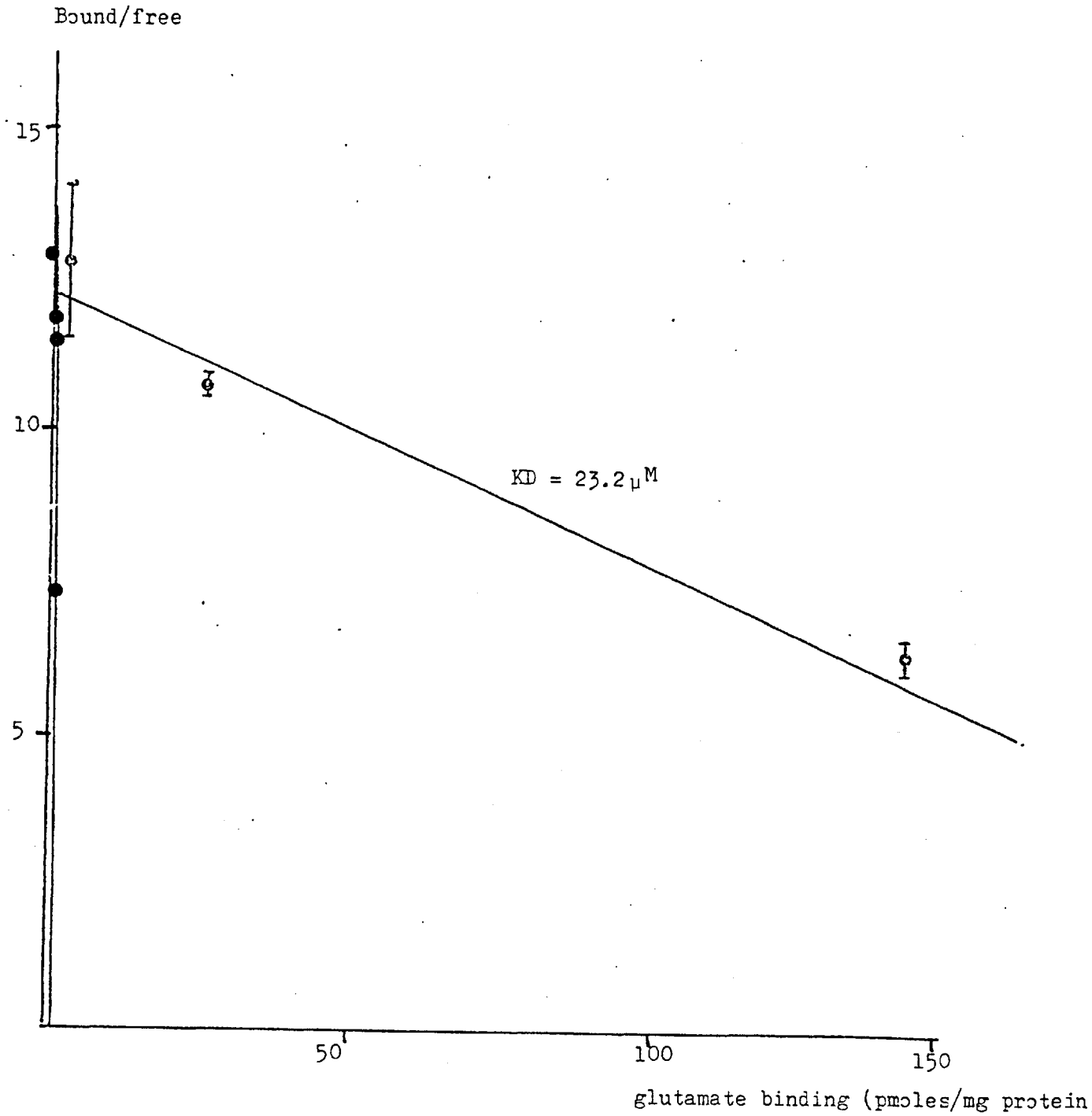
The graph in Figure 5.2 of $\frac{\text{Bound}^{(hot + cold)}}{\text{Free}^{(hot + cold)}}$ vs Bound$^{(hot + cold)}$glutamate was then constructed. Each point is the mean of three experimental determinations carried out in triplicate (n=3), $\pm$ SEM. The lines of best fit were drawn by the method of linear regression.

Figure 5.1



Inhibition of specific binding of ^3H glutamate binding by non-isotopic glutamate, in rat cerebral cortex membranes.

Figure 5.2



Scatchard plots of total glutamate binding to cerebral cortex membranes.

Legend to Figure 5.3

Enlarged Scatchard plot of total glutamate binding to rat cerebral cortex membranes.

The x-axis of Figure 5.2 has been enlarged in this graph to show the high affinity site. Each point represents a mean value $\pm$ SEM (n=3).

Legend to Figure 5.4

Hill plot of total glutamate binding to cerebral cortex membranes.

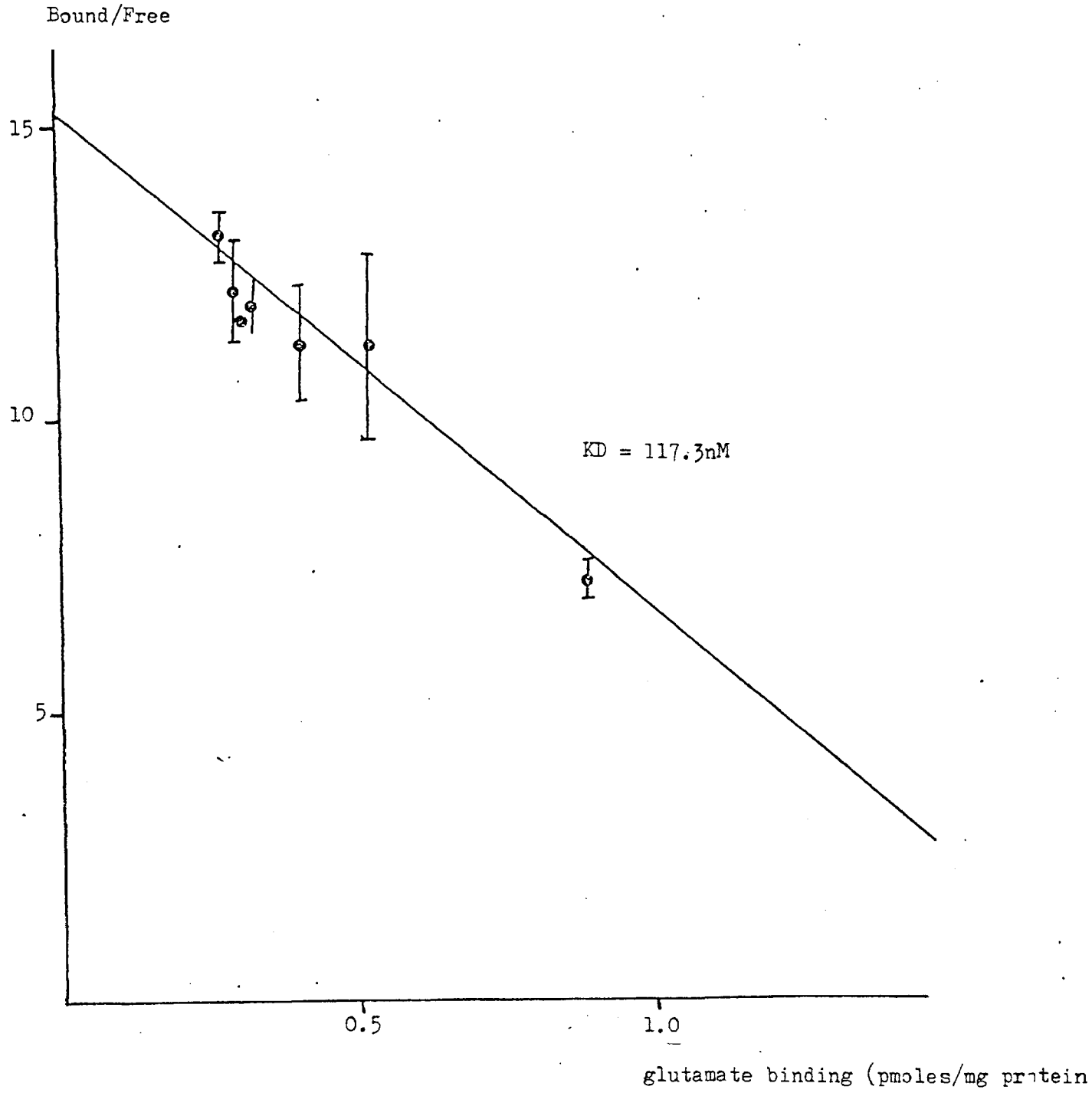
The data for Figures 5.2, 5.3 was subjected to Hill plot analysis. Points forming a line with K_d of 117.3nM were plotted with a B_{max} of 1.76 pmoles/mg protein, and the rest were plotted using a B_{max} of 280 pmoles/mg protein.

Legend to Figure 5.5

Inhibition of specific binding of ^{3}H-glutamate by nonisotopic glutamate to rat cerebellum membranes.

Crude synaptosomal membranes prepared from the cerebellar cortex were incubated with L-(^{3}H)-glutamic acid (29nM) and with varied concentrations (0-10^{-3}M) of nonisotopic glutamate. The binding assay performed according to section 2.13, chapter 2, produced a displacement curve as shown in Figure 5.5. Each point is the mean of two experiments (n=2), carried out in triplicate. Standard errors (not shown) were 10% or less than the means.

Figure 5.3



Enlarged Scatchard plot of total glutamate binding to cerebral cortex membranes.

Figure 5.4 Hill plot of total glutamate binding to cerebral cortex membranes.

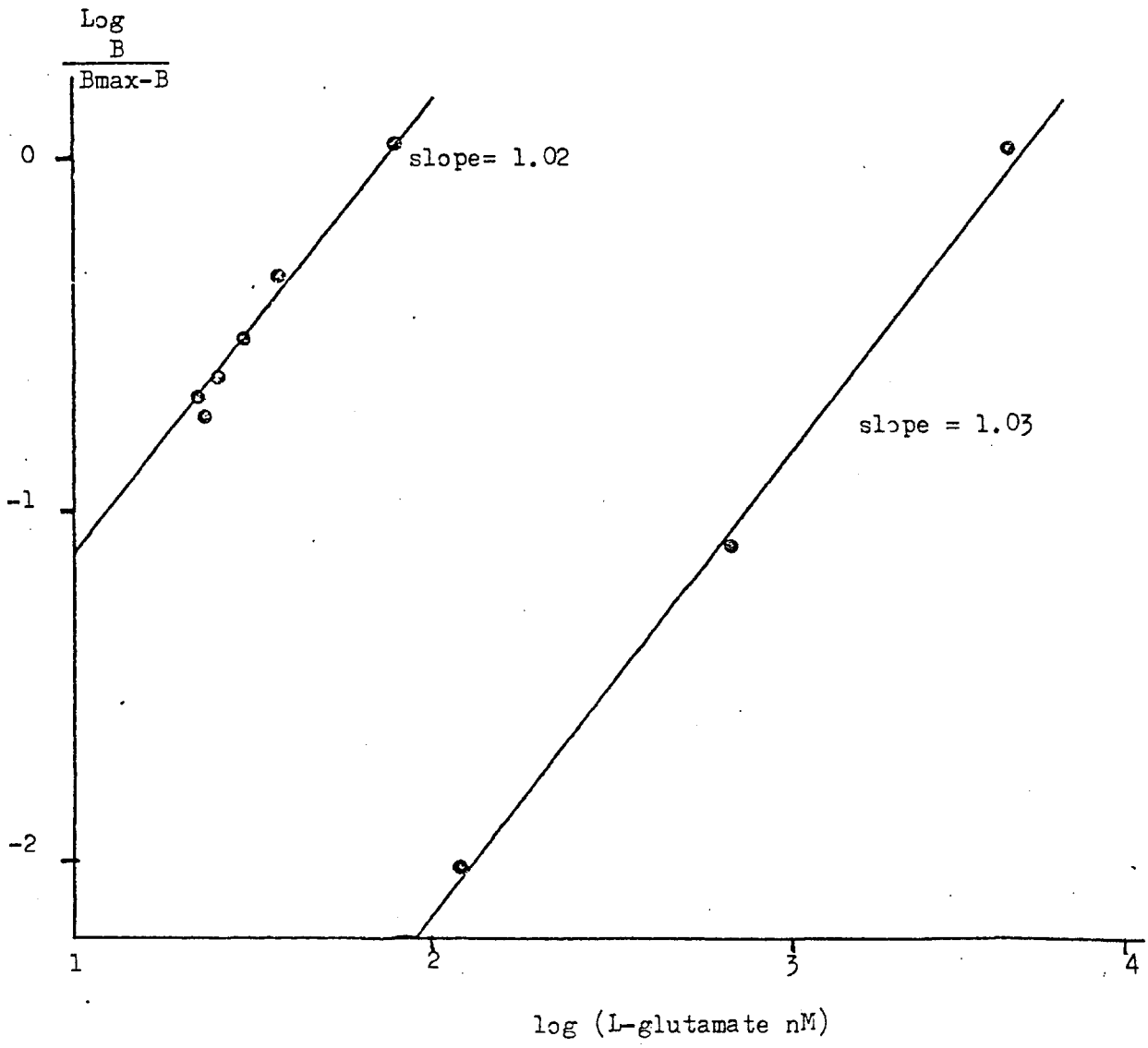
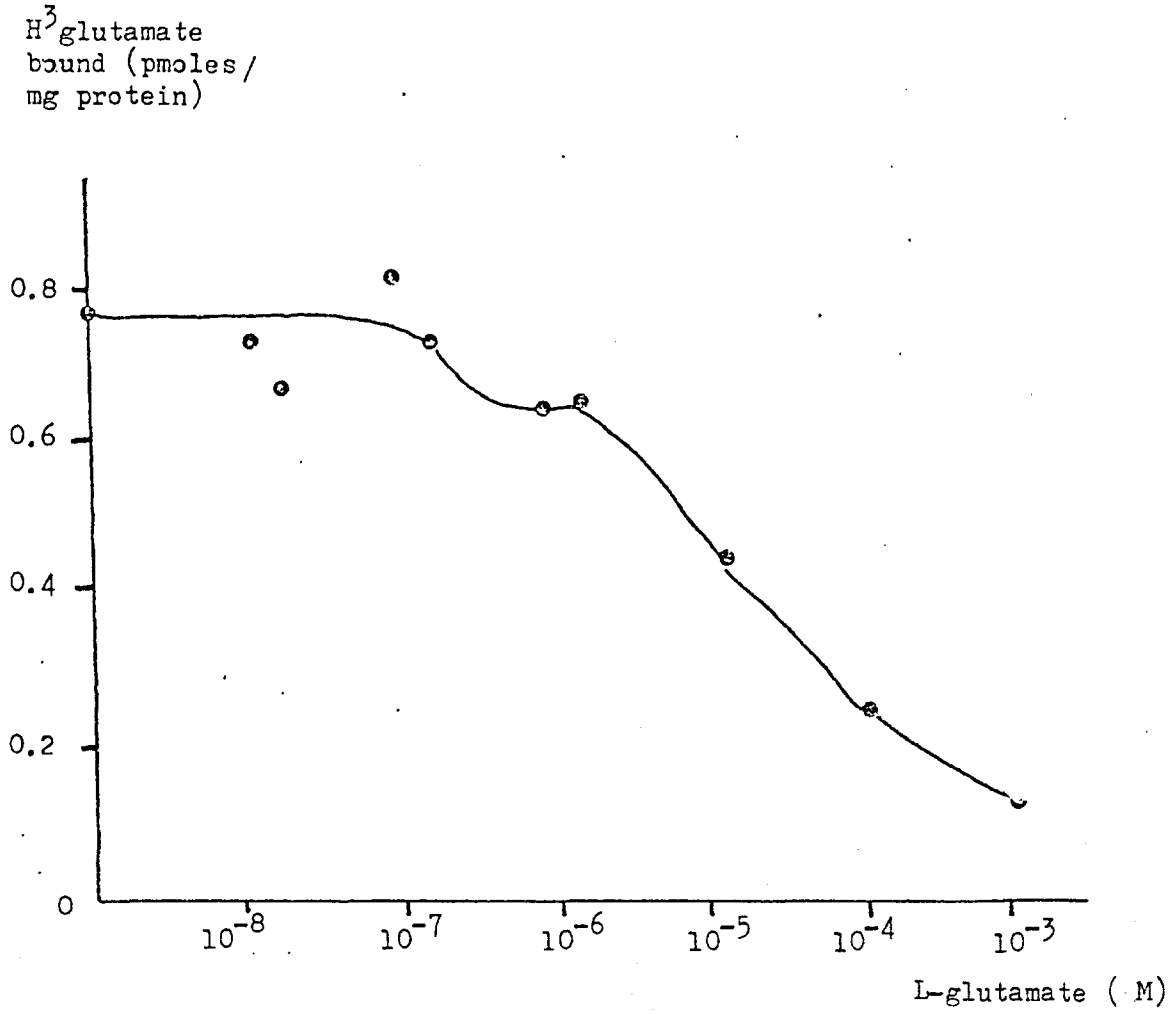


Figure 5.5



Inhibition of specific binding of (^{3}H) glutamate by nonisotopic glutamate in rat cerebellum membranes.

similar to that shown in Figure 5.1. The presence of specific binding (a consequence of displacement) and of two binding sites, one in the nanomolar and one in the micromolar range, is indicated.

When the results were subjected to Scatchard analysis, two separate binding plots were observed (Fig. 5.2). The high affinity plot can be seen more clearly when the scale is enlarged (Fig. 5.3) and has a K_d of 117.3 nM and a B_{max} of 1.76 pmoles/mg. The low affinity site has a K_d of 23.2μM and a B_{max} of 280 pmoles/mg protein.

Since the Scatchard plot (Fig. 5.2) is not of the usual L-shaped curve, it would seem as if the two sites were totally unconnected. This is confirmed by Hill plots (Fig. 5.4) which show both sites to possess slopes not significantly different to 1.0, indicating the absence of cooperativity.

(2) Binding to cerebellum membranes

Binding studies to the cerebellum revealed similar results to those of cortex. A typical displacement curve is shown in Figure 5.5. This again shows the presence of two binding sites, one in the nanomolar range and one in the micromolar range.

When subjected to Scatchard analysis, two binding sites were obtained as before (Fig. 5.6). The high affinity site had a K_d of 188 nM and a B_{max} of 5.22 pmoles/mg. The low affinity site had a K_d of 6μM and a B_{max} of 1.61 pmoles/mg protein. A Hill plot shows again that these binding sites were uncooperative (Fig. 5.7).

Legend to Figure 5.6

Scatchard plot of total glutamate binding to cerebellar membranes.

The data displayed in Figure 5.5 was subjected to Scatchard plot analysis as described in the legend to Figure 5.2. Lines of best fit were drawn by the method of linear regression.

Legend to Figure 5.7

Hill plot analysis of total glutamate binding to rat cerebellum membranes.

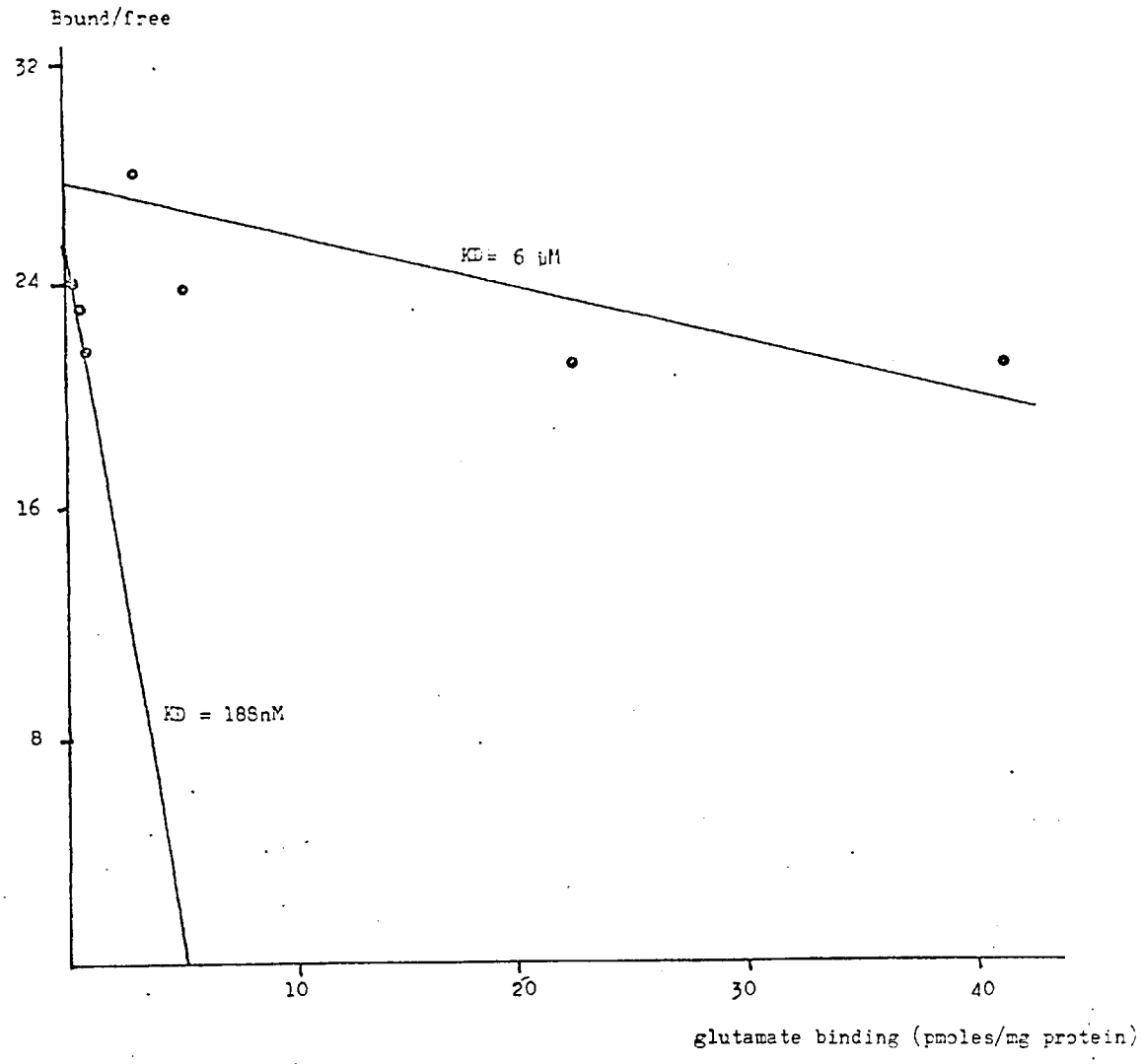
The values obtained in Figure 5.6 were subjected to Hill plot analysis. Points forming a straight line with a K_d of 1.88nM were plotted with a B_{max} of 5.22 pmoles/mg protein, whilst the others were plotted using a B_{max} of 162 pmoles/mg protein.

Legend to Figure 5.8

Inhibition of specific binding of ^{3}H-glutamate by non-isotopic glutamate to rat striatal membranes.

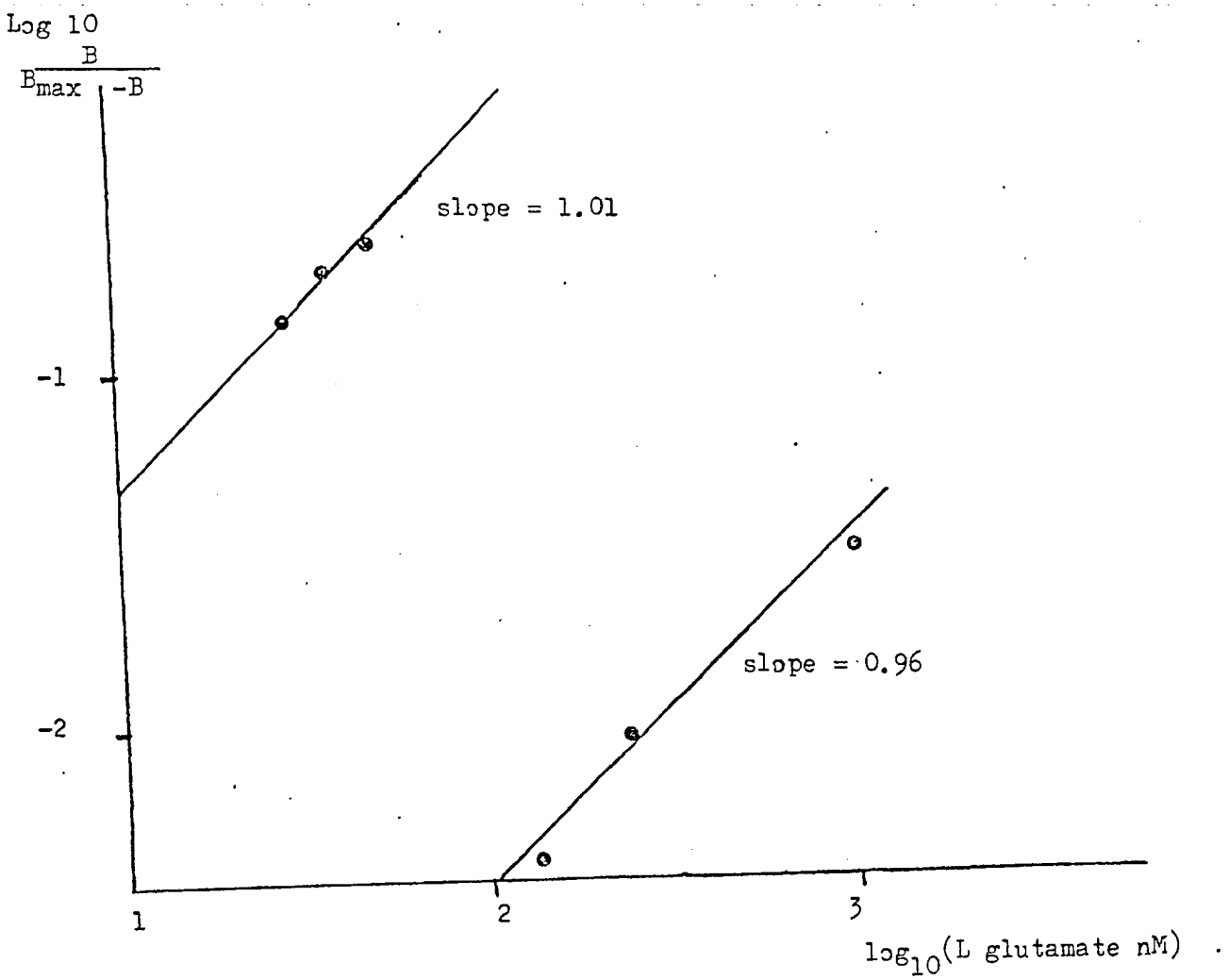
Crude synaptosomal membranes prepared from rat corpus striatal membranes were incubated with isotopic glutamate (29nM) and nonisotopic glutamate (0 - 10^{-4}M) as described previously. The data obtained was used to plot the displacement curve shown. Each point is the mean of three experiments. Standard errors have been omitted for reasons of clarity.

Figure 5.6



Scatchard plot of total glutamate to cerebellum membranes.

Figure 5.7



Hill plot of total glutamate binding to cerebellum membranes.

Figure 5.8 Inhibition of specific binding of ^{3}H-glutamate by nonisotopic glutamate in striatal membranes.

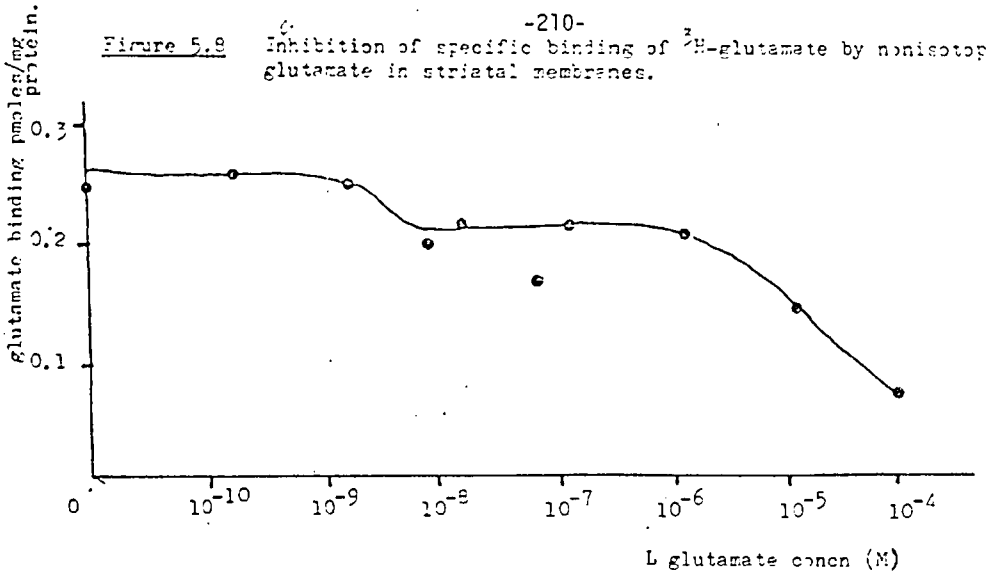
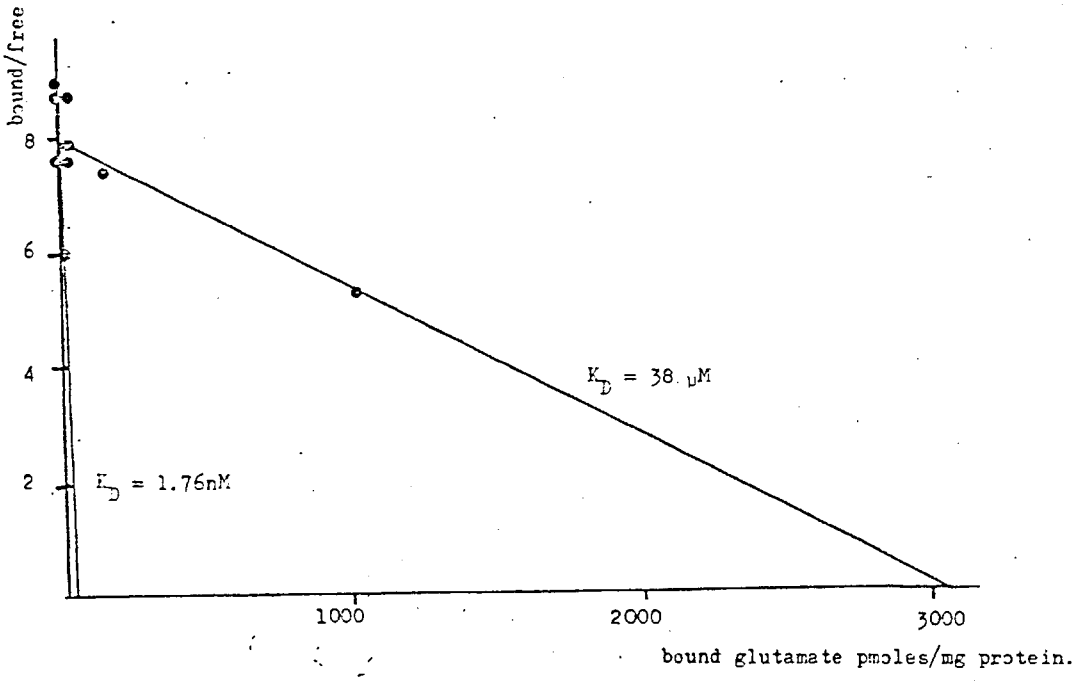


Figure 5.9 Scatchard plot of total glutamate binding to striatal membranes.



(3) Binding to neostriatum membranes

The displacement curve obtained for binding to the corpus striatum does not conclusively reveal two binding sites (Fig. 5.8). However, Scatchard analysis (Fig. 5.9) reveals two separate binding plots, one a high affinity one (enlarged in Fig. 5.10) with a K_d of 176 nM and a B_{max} of 1.75 pmoles and the other a low affinity site with a K_d of 38.3μM and a B_{max} of 297 pmoles/mg. Hill plots (Fig. 5.11) again reveal lines with slopes not significantly different from 1.0.

(4) Cerebral cortex binding in Krebs phosphate medium

A few experiments were carried out in Krebs Ringer phosphate buffer since it is a more physiological medium than Tris HCl. Unfortunately, since it contains sodium it is likely that at least some of the binding observed would be to the high affinity reuptake sites. However, it was decided to include one such result to show what effect the presence of sodium does have in this assay system.

A displacement curve (Fig. 5.12) and a Scatchard plot (Fig. 5.13) were produced which show the presence of two binding sites. It is interesting to note that the shape of the Scatchard plot is of the usual L curve shape, not V-shaped as in the studies in Tris buffer. The high affinity site has a K_d of 15.1μM and a B_{max} of 358 pmoles/mg protein, whilst the low affinity site has a K_d of 1.88mM and a B_{max} of 20,000 pmoles/mg protein. These are within the range of values for high affinity reuptake sites.

Legend to Figures 5.9, 5.10

Scatchard plot of total glutamate binding to rat striatal membranes.

The data displayed in Figure 5.8 was subjected to Scatchard plot analysis as described in the legend to Figure 5.2. In Figure 5.10, the x-axis has been enlarged to indicate a high affinity binding site.

Legend to Figure 5.11

Hill plot analysis of total glutamate binding to rat striatal membranes.

The results obtained in Figures 5.9 and 5.10 were subjected to Hill plot analysis. The data is graphically represented in Figure 5.11.

Legend to Figure 5.12

Inhibition of specific binding of ^{3}H-glutamate by nonisotopic glutamate to cerebral cortex membranes in Krebs phosphate buffer.

Inhibition of ^{3}H-glutamate (29nM) by nonisotopic glutamate (0 - 10^{-3}M) was examined in this experiment. However, the binding assay was carried out in Krebs phosphate medium rather than in Tris HCl, but the experimental procedure for the assay was as described in section 2.13, chapter 2 (Materials and Methods). The displacement curve is plotted in Figure 5.12. Each point is the mean of three experiments. Standard errors were less than 10%.

Figure 5.10 Enlarged scatchard plot of total glutamate binding to striatal membranes.

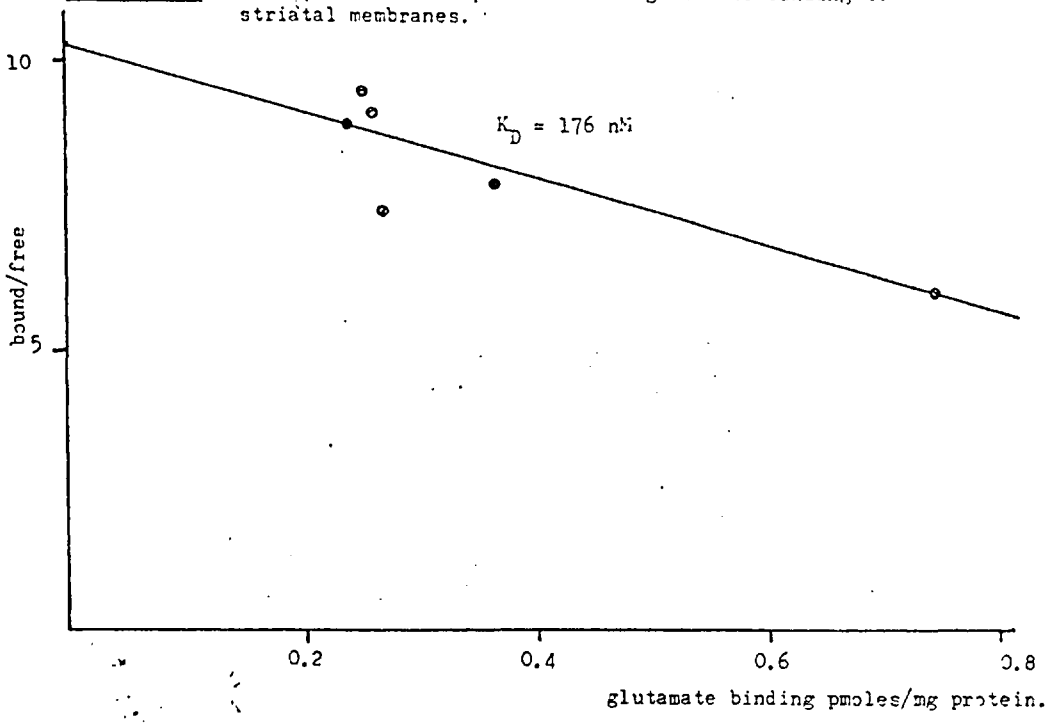


Figure 5.11 Hill plot of total glutamate binding to striatal membranes.

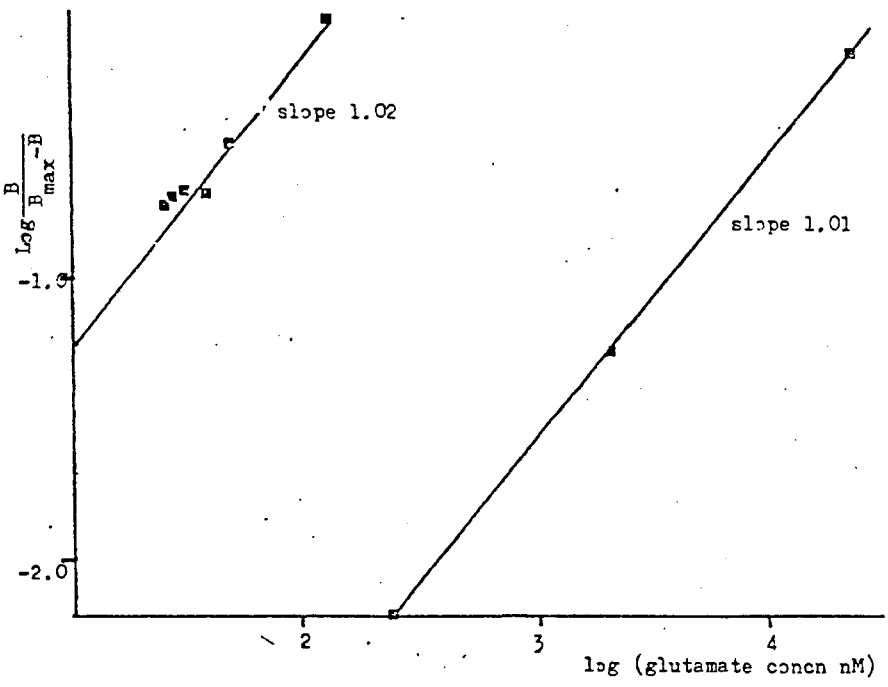
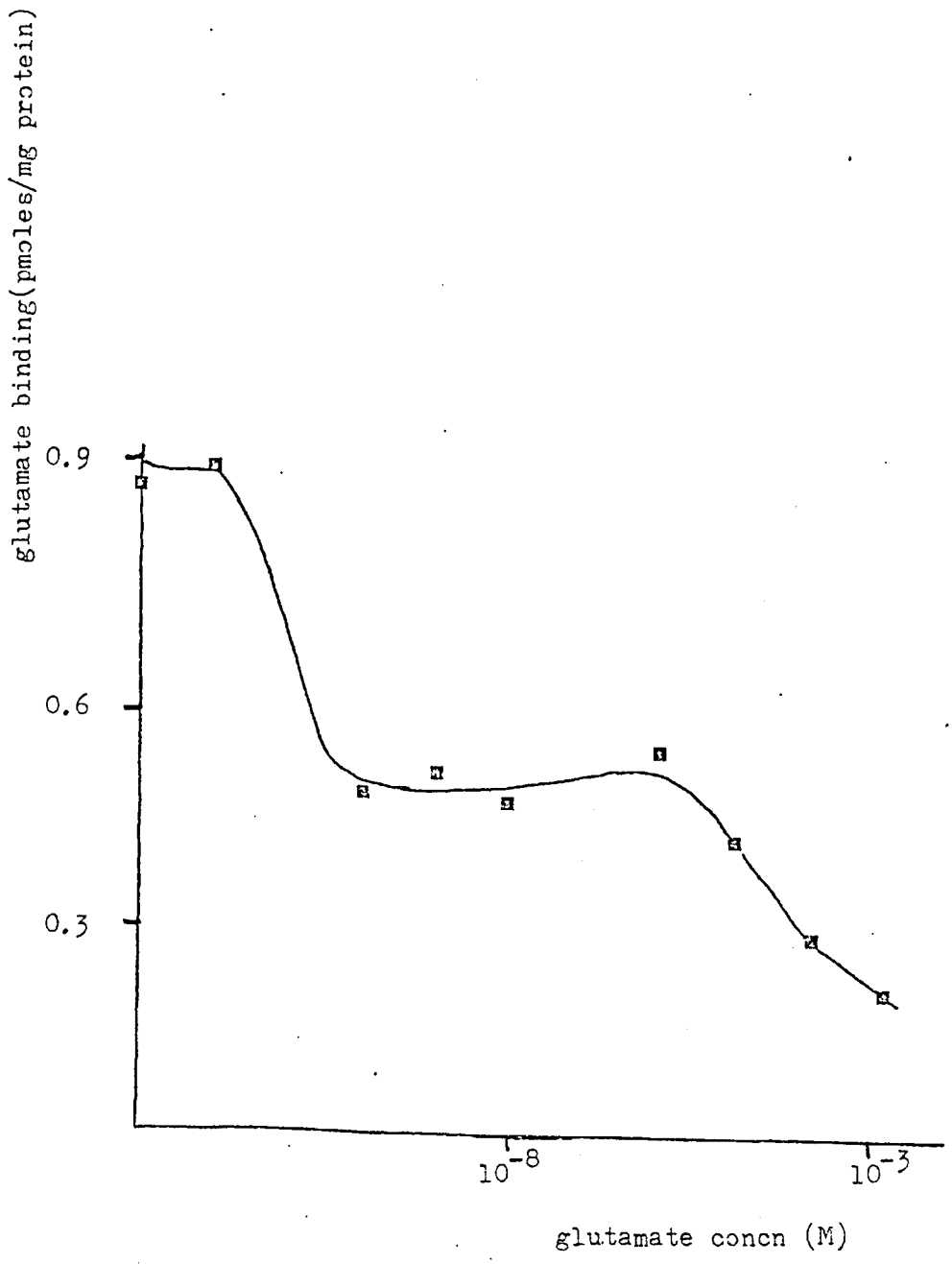


Figure 5.12 Inhibition of specific binding of ^{3}H-glutamate by nonisotopic glutamate to cortical membranes in Krebs-phosphate medium.



Legend to Figure 5.13

Scatchard plot of total glutamate binding to cerebral cortex membranes in Krebs phosphate buffer.

The results shown in Figure 5.12 were subjected to Scatchard plot analysis as described in the legend to Figure 5.2.

Legend to Figure 5.14

Specific binding of ^{3}H-glutamate to rat cerebro-cortical membranes.

Binding studies of L-(^{3}H)-glutamate (0.001 - 10μM) to rat cortical membranes were carried out in the absence/presence of nonisotopic glutamate (2mM) so that non-specific as well as total binding in Tris HCl (10mM, pH 7.4) could be determined. The experimental method was as described in Materials and Methods, chapter 2.

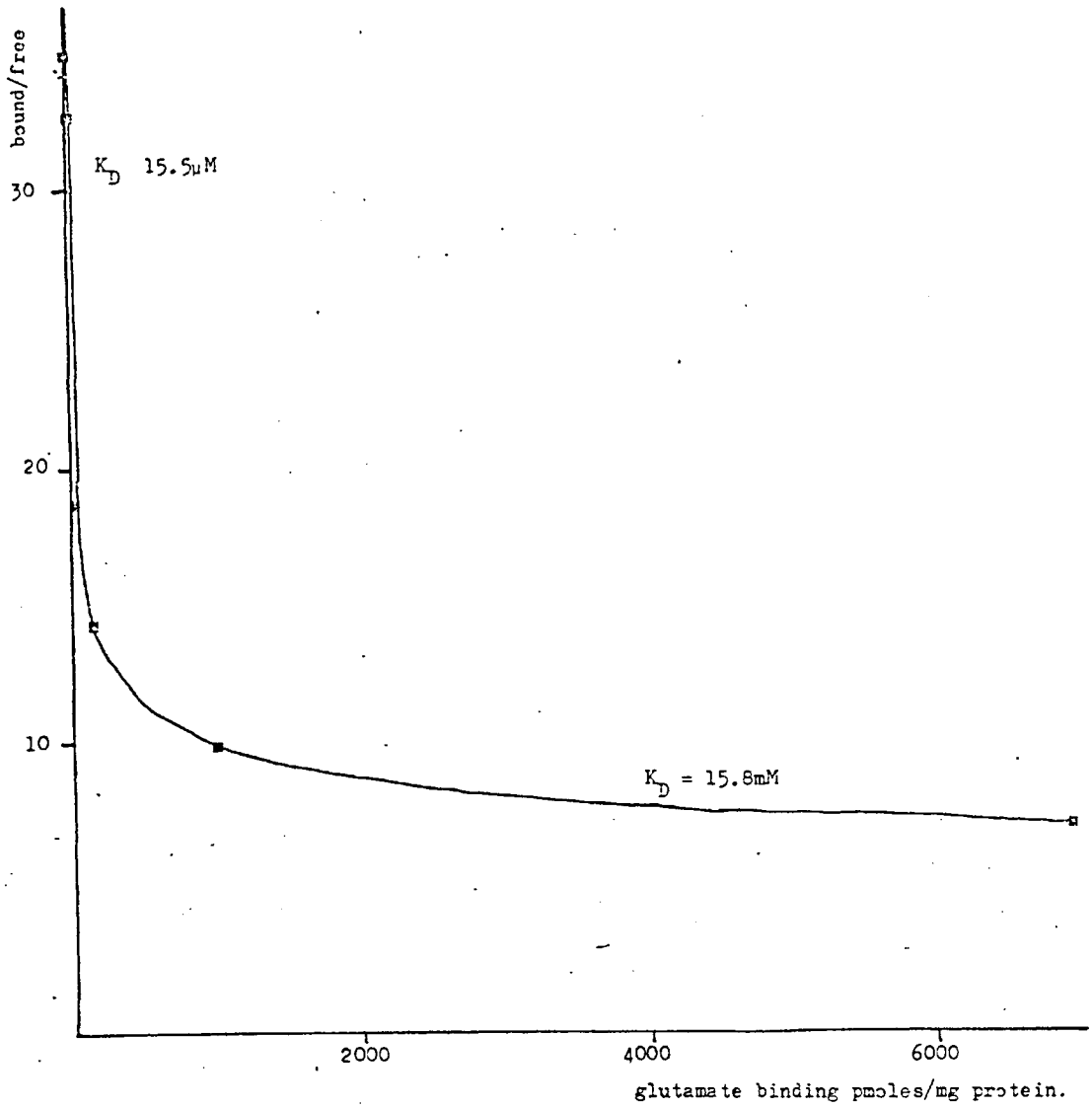
In the graph, each point represents the mean of three experiments. Standard errors (not shown) were less than 10%.

Legend to Figure 5.15

Scatchard plot analysis of specific L-(^{3}H)-glutamic acid binding to cerebral cortex membranes.

The results obtained in Figure 5.14 were subjected to Scatchard plot analysis. Each point is the mean of three experiments. K_ds and B_{max}s were calculated by linear regression.

Figure 5.13



Scatchard plot of total glutamate binding to cerebral cortex membranes in Krebs-phosphate medium.

Figure 5.14 Specific binding of ^{3}H glutamate to cerebral cortex membranes.

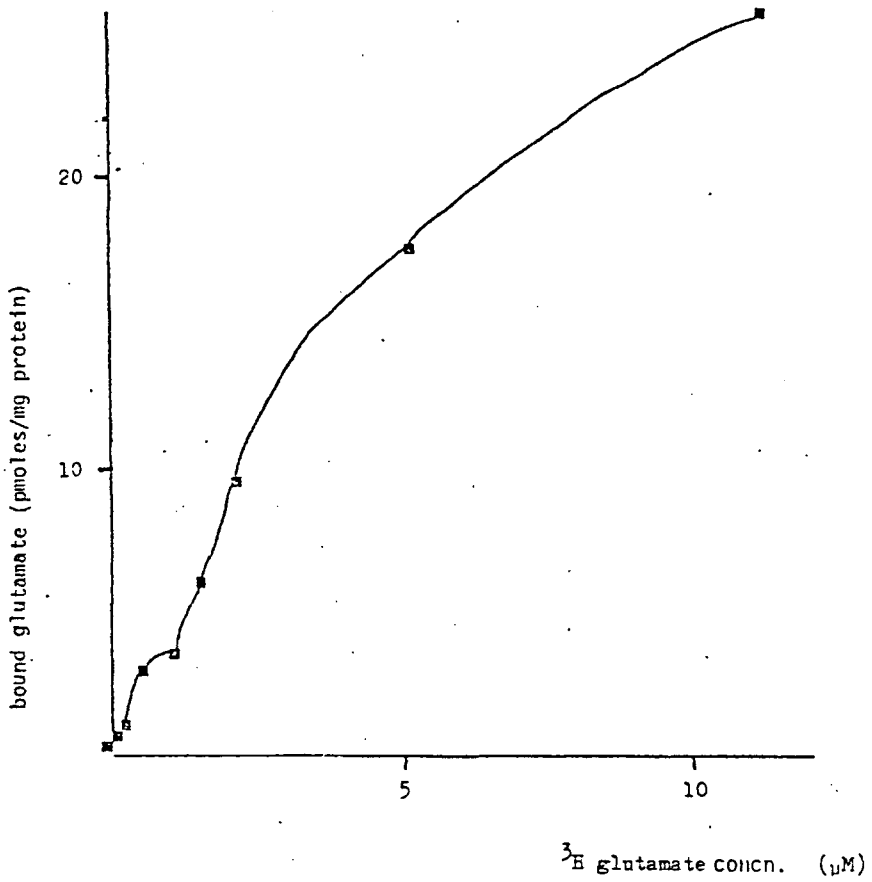
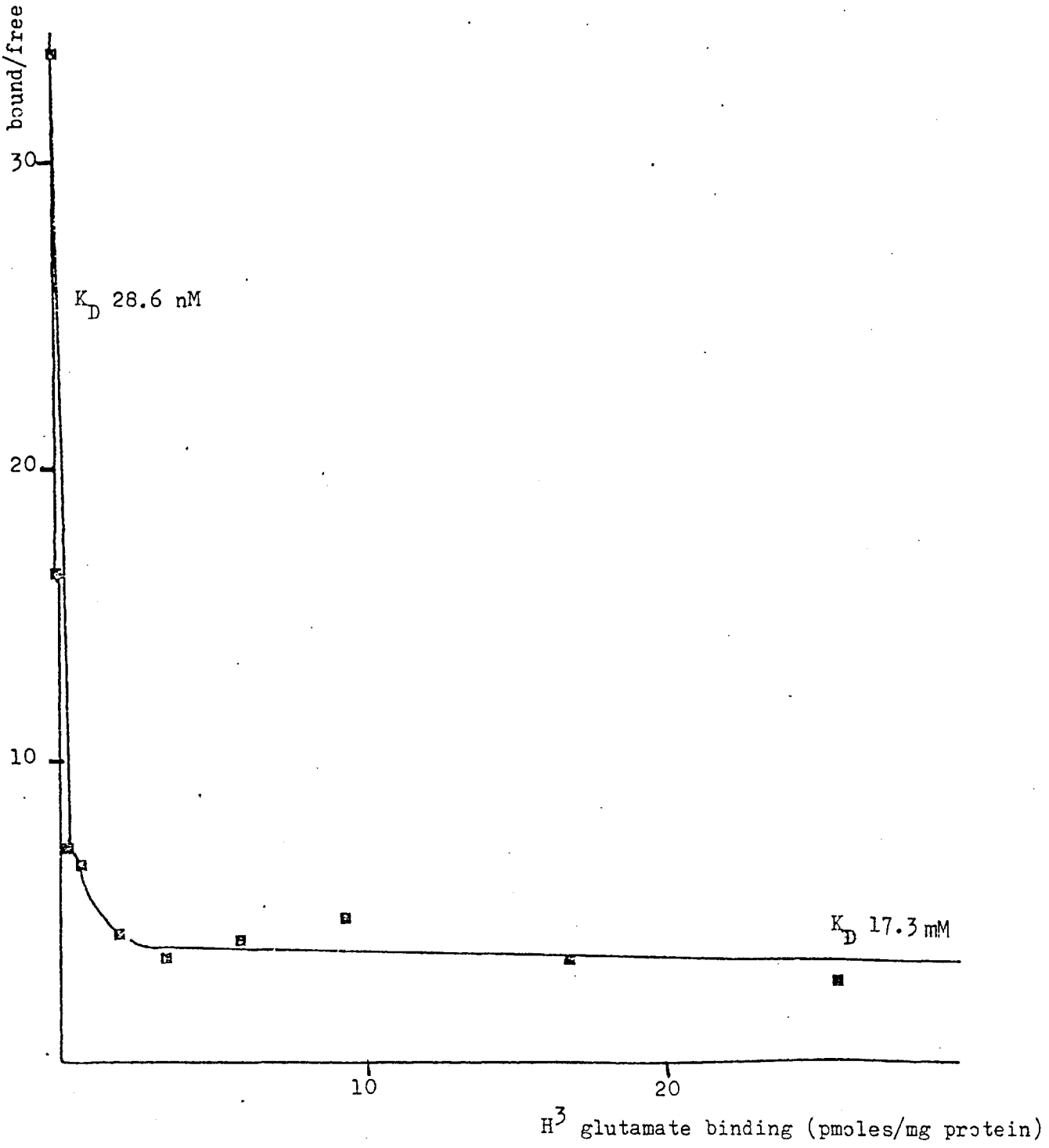


Figure 5.15 Scatchard plot of specific ^{3}H glutamate binding to cerebral cortex membranes.



(b) Kinetics of binding of L-(^{3}H)-glutamate by the direct method

An experiment was performed using various concentrations of L-(^{3}H)-glutamate (0.001 - 10μM) in order to compare results obtained by this and the previous displacement method. The specific activity of L-(^{3}H)-glutamate was diluted to 70μCi/100mmoles and non-specific as well as total binding was measured to cerebral cortex membranes.

The results showed that non-specific binding was linear but high, between 20 and 60% of the total and, no doubt, much of this was to the filtre paper. The specific binding was saturable as shown in Figure 5.14. The deviation in the curve at low concentrations indicates the presence of two binding sites.

When the data was subjected to Scatchard analysis (Fig. 5.15), two binding sites were revealed, one a high affinity site with a K_d of 28.6 nM and a B_{max} of 1.1pmoles/mg protein, and the other a low affinity site with a K_d of 17.3μM and a B_{max} of 75pmoles/mg protein. A Hill plot of the data (Fig. 5.16) shows that there is no cooperativity between the sites.

(c) Demonstration of specific binding to various brain regions

Since the studies on L-(^{3}H)-glutamate binding by the displacement method measure only total binding, it was necessary to perform further experiments to demonstrate that this binding was specific. Since the dissociation constants obtained by displacement were all between 100 and 200 nM and the concentration of radioligand present in binding studies should be one fourth or less than that of

Legend to Figure 5.16

Hill plot analysis of specific L-(^{3}H)-glutamate binding to rat cerebral cortex membranes.

The results obtained in Figure 5.15 were subjected to Hill plot analysis. Points forming a straight line with a K_d of 28.6 nM were plotted using the B_{max} of 1.1 pmoles/mg protein, whilst those forming a straight line with a K_d of 17.3μM were plotted using a B_{max} of 75 pmoles/mg protein.

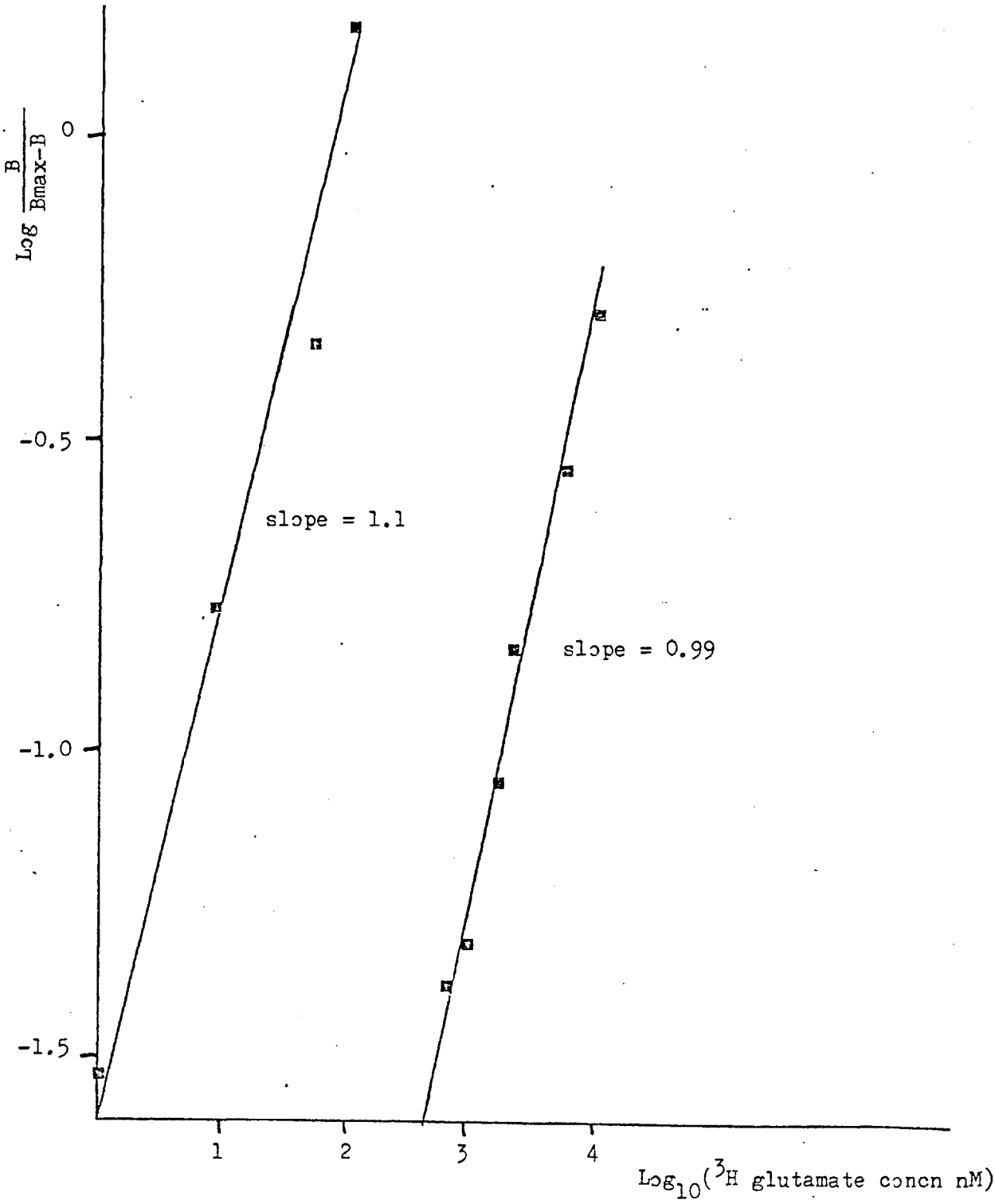
Legend to Figure 5.17

Chromatograms showing endogenous glutamate present in membrane samples.

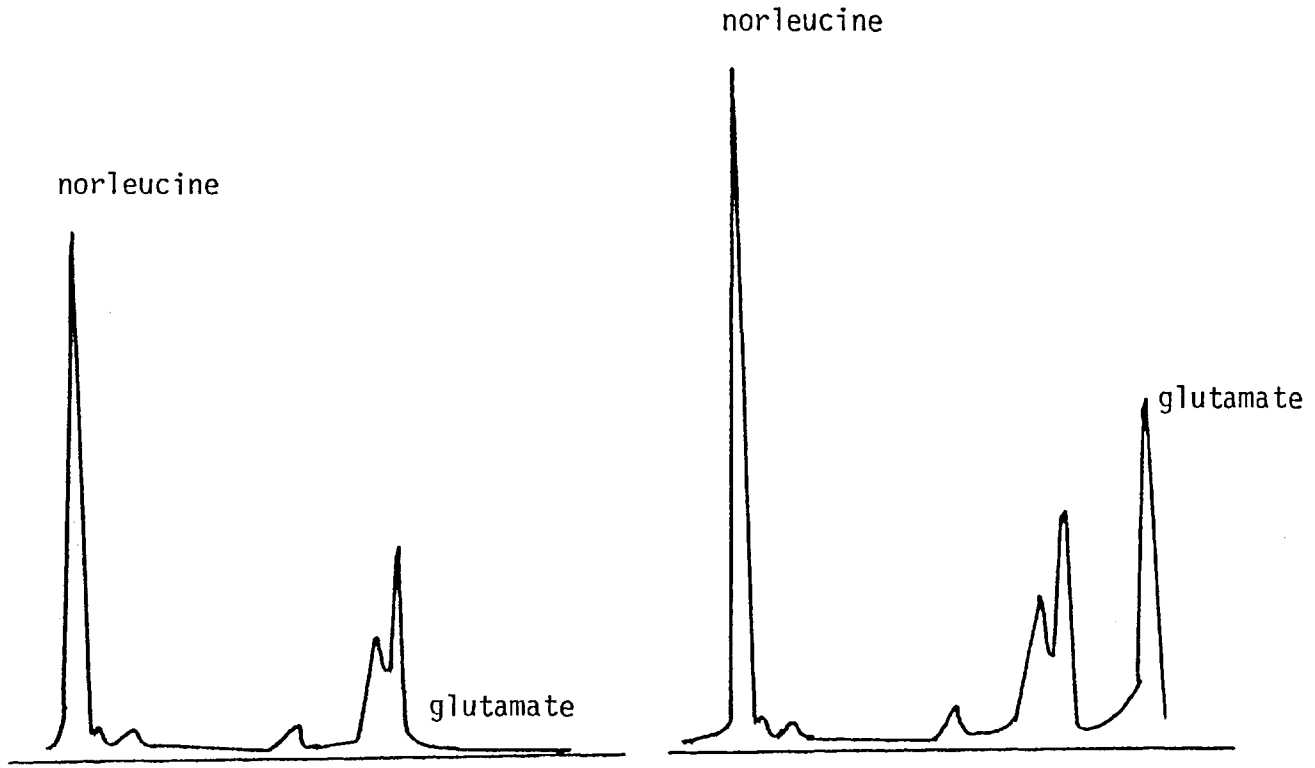
Samples of rat cerebro-cortical membrane preparations were incubated in the absence and presence of veratrine (75μM) for 15 minutes at 37^{o}C. Endogenous glutamate was then extracted from the samples by the following procedure. The membranes were pelleted by centrifugation (50,000g, 5 minutes) and the supernatants taken to dryness by vacuum dessication. The dried extracts were then dissolved in methanol and left on ice for 30 minutes. The methanol contained an internal standard of Norleucine (500 pmoles/ml). After precipitation of the soluble proteins, the methanol extracts were decanted off, before being taken to dryness once again. These dried extracts were then prepared for amino acid autoanalysis by dissolution in 1 ml of 25mM hydrochloric acid.

The figure shows the chromatograms obtained from membranes incubated with and without the depolarizing alkaloid veratrine.

Figure 5.16



Hill plot analysis of specific ^{3}H glutamate binding to rat cerebral cortex membranes.



(a) Control

(b) Synaptic membranes incubated with veratrine.

Figure 5.17 Chromatograms showing the endogenous glutamate present in membrane samples used for binding assays.

the K_d (Burt et.al., 1978), it was decided to use 28.5 nM L-(^{3}H)-glutamate in this and further experiments. Non-isotopic glutamate was used at a final concentration of 2 mM.

The results in Table 5.1 show that although binding to the striatum and cerebral cortex is about the same, that to the cerebellum is considerable less (28%).

Table 5.1 Specific binding of L-(^{3}H)-glutamic acid to various brain regions and to the liver.

Tissue	Specific binding of L-(^{3}H)-glutamate pmoles/mg protein
Cerebral Cortex	0.215
Cerebellum	0.157
Striatum	0.226
Liver	$\simeq$ 0 ie not significant

The results obtained above were the average of experiments done in triplicate. Repetition of the experiments produced similar results.

Liver glutamate binding was found to be zero as detected in our assay system.

(d) Inhibition of Specific Binding of L-(^{3}H)-glutamate by analogues

In order to obtain more information on the type of receptor involved in glutamate binding, the inhibition of specific binding to cerebral cortex membranes of L-(^{3}H)-glutamate by various agonists and antagonists was measured at concentrations of 1mM. The results are shown in Table 5.2.

Legend to Table 5.1

Specific binding of L-(^{3}H)-glutamic acid to membranes from various rat brain regions and from the liver.

Specific binding to the membrane preparations was determined by calculating the difference between the total binding of ^{3}H-glutamate (29nM) and the non-specific binding of nonisotopic glutamate (2mM).

The results were the average of two experiments performed in triplicate.

Legend to Table 5.2

Inhibition of specific binding of L-(^{3}H)-glutamate by amino acid analogues.

Specific binding to rat cerebrocortical membranes by isotopic glutamate was determined in the presence of various competitive ligands (1mM) by the method described in the legend to Table 5.1. The results expressed as % inhibition are the mean from three experiments performed in triplicate. Standard errors (not shown) were less than 10%.

Legend to Table 5.3

Effects of various depolarizing agents on the specific binding of L-(^{3}H)-glutamate to rat cerebro-cortical membranes.

Crude synaptosomes (0.5 ml, 1mg/ml) were incubated for 10 minutes at 37^{o}C with the depolarizing agents veratrine (75μM), veratrine and TTX (75μM and 1μM respectively), and potassium (56mM). Alternatively the synaptosomes were stimulated electrically for 10 minutes with a.c. stimulus of 0.5 volts, 0.7 amps, 50 Hz (square wave).

Membranes from the crude synaptosomes were then prepared (see Materials and Methods, chapter 2) for specific glutamate binding studies, described in the legend to Table 5.1.

The results expressed as % decrease in specific binding are the mean of 2 - 5 determinations $\pm$ SEM, each experiment being a triplicate assay. Values for controls were in the range of 0.2 - 0.5 pmoles ^{3}H-glutamate bound/mg protein.

Legend to Table 5.4

The effect of adding L-glutamic acid to crude synaptosomes on specific L-(^{3}H)-glutamate binding.

L-glutamate (50 nmoles) was added to crude rat cerebro-cortical synaptosomes at the same time as Tris buffer (50mM, 5ml) was added to lyse them. The membranes were then analysed for specific glutamic acid binding as described in the legend to Table 5.1.

The results expressed as a % decrease from controls in specific binding are the mean from three experiments $\pm$ SEM.

Table 5.2 Inhibition of specific ^{3}H glutamate by glutamic acid analogues.

Competative ligand 1mM	% Inhibition
DL homocysteic acid	99.4
L aspartate	98.1
kainic acid	52
dihydrokainate	52
L glutamate diethylester	58
diamino pimmeleic acid	50

Table 5.3 Effects of various depolarizing agents on the specific binding of ^{3}H-glutamate.

Depolarizing agents	% decrease of control
veratrine	30.0±0.76
veratrine + TTX	9.5±0.44
K^{+} ions	51.1±1.33

Table 5.4 Effect of addition of L-glutamate to crude synaptosomes on binding.

nmoles of glutamate added	% decrease in specific binding
50	29.4 ± 3.46

Homocysteic acid and aspartate were the most potent inhibitors producing almost 100% inhibition at 1mM. Kainate and dihydrokainate, however, only produced 50% inhibition at the same concentration. The antagonists GDEE and diaminopimmelate produced just over 50% inhibition as well.

(e) Effects of depolarizing agents on specific binding of L-(^{3}H)-glutamate

It is known that exposure of receptors to their ligands can result in a reduction in the number of binding sites. This constitutes a possible homeostatic mechanism in interneuron communication by desensitizing the target neuron. Therefore, it was decided to investigate the effects of veratrine, potassium and electrical stimulation on the specific binding of L-(^{3}H)-glutamate to cerebrocortical membranes.

The procedure was as described in the methods chapter with veratrine (final concentration 75μM), veratrine and tetrodotoxin (final concentrations of 75μM and 1μM respectively) and potassium (final concentration 56mM) being added to the incubating crude synaptosomes for 10 minutes at 37^{o}C. Alternatively, the synaptosomes were stimulated electrically for 10 minutes with an a.c. stimulus of 0.5 volts, 0.7 amps at 50 Hz.

The percentage decreases with respect to the control are shown in Table 5.3. The results obtained with veratrine and potassium indicate a receptor loss of 30% and 50% respectively. Since the effect of veratrine is blocked by tetrodotoxin,it seems likely at first that the observed

losses caused by veratrine were a consequence of depolarization, and not an effect of veratrine as a chemical.

Since depolarizing agents increase the release of neurotransmitter amino acids and also increase metabolism of proteins etc, it seemed plausible that veratrine and potassium may be causing an increase in endogenous glutamate levels in the assay and thus block the receptors from the radioligand. This was tested in two ways.

Firstly, since veratrine is known to release 2,000 to 3,000 nmoles of glutamate/100 mgs of protein, 50 nmoles of glutamate were added to tubes containing approximately 2-5mg of protein at the same time as Tris buffer was added to lyse them. As can be seen from Table 5.4, this produced an observed decrease in the number of binding sites of 29%, which is of the same order as that produced by veratrine.

Secondly, the endogenous glutamate levels in the membrane suspensions used for assay were measured by amino acid analysis in samples incubated with and without veratrine. The results shown in Figure 5.17 indicate that addition of veratrine to the incubating membranes results in increased levels of glutamate (final concentration of 520 nM) in the membranes for assay.

Thus it seems likely that the apparent effect of veratrine in inducing receptor loss is,at least in part, due to a resultant increased level of endogenous glutamate in the assay.

5.3 Discussion

(a) Choice of Method

Since binding to different brain regions was to be studied and compared, it was essential that the same membrane fraction should be used in each case. Thus, since preparation of synaptosomes from the cerebellum has proved difficult by the procedure used, it was decided to use crude synaptosomal membranes (P2) throughout. Although this fraction contains mitochondria, it is unlikely that these bind glutamate, particulartly since no specific binding was observed to liver mitochondrial membranes we prepared.

Incubation of the membranes was carried out at 37^{o}C since this is the most physiological temperature. The decision to terminate the reaction afer 10 minutes was based upon the work of Roberts, 1974, Foster and Roberts, 1978, Head et.al., 1980, who have studied the time course of binding, and have found that maximum specific binding occurs within 10 minutes. Filtration was chosen instead of centrifugation as a rapid and efficient method to terminate the assay. However, it is interesting to note that although most workers in the field terminate the reaction by centrifugation, Baudry and Lynch (1979a) obtain very similar results by filtration.

During the first few experiments (the results of which are not shown) the methods were modified slightly to improve the number of cpms obtained. Since it is common practice to dissolve filtres before radiolabel counting, sodium hydroxide (3M) at 60 - 80 ^{o}C was added.

However, this proved to be unsuccessful and although some counts dissolved out into solution most remained trapped in the now pulp-like filtre paper.

Sodium dodecyl sulphate (10%) was also used to dissolve the membrane and protein on the filtre paper. However, althouth this was an improvement, the counts were still low (350 - 450 cpms)and it seemed the SDS was causing quenching. Thus SDS was omitted and, instead, scintillant was added directly to the filtre papers. This was a considerable improvement (600 - 5000 cpms) and the method remained as such throughout the experiments.

Up to this stage the assay had been terminated be the addition of 5 mls of ice cold Tris buffer to the test tube, followed by filtration, washing, etc. Since the membranes were however in contact with the 5 mls of buffer for several seconds, it seemed possible that dissociation of glutamate from the receptor could be occurring. Hence the membranes were taken up by pipette and then placed on the filtre paper before being washed under mild suction.

(b) Kinetics of binding of L-(^{3}H)-glutamic acid by the displacement method

The studies presented here would indicate that L-glutamate binds to two sites on crude synaptic membranes in the cerebral cortex, cerebellum and striatum. One of these is of high affinity with a K_d of 117 - 188 nM and a B_{max} of 1 - 4 pmoles/mg protein, whilst the other site is of low affinity with a K_d of 6 - 38μM and a B_{max} of 161 - 28 pmoles/mg protein (Table 5.5). These

results are well in keeping with those of other workers (Biziere and Coyle, 1980; Roberts, 1974,1978; Head and Tunnicliff, 1980; and Baudry and Lynch, 1979). Whether or not a low affinity site is detected seems to depend upon the ligand concentration used. If the range is broad enough, such a site is usually detected (Michaelis et.al., 1974; Head et.al., 1980).

Table 5.5 A comparison of data obtained for glutamate binding to the cerebral cortex, cerebellum and striatum.

Brain Region	High Affinity Site		Low Affinity Site	
	K_d	B_{max}	K_d	B_{max}
Cerebral Cortex	117.3nM	1.76pmoles/mg	23.2μM	280pmoles/mg
Cerebellum	118 nM	5.22pmoles/mg	6 μ M	161pmoles/mg
Striatum	176.5nM	1.75pmoles/mg	38.3μM	297pmoles/mg

The Scatchard plots from which these values were obtained appear somewhat strange in that they are a V shape rather than L-shaped. The question arises as to whether the high affinity site was in fact an artifact or not, seen at low concentrations; however, since the displacement curves indicate the presence of two sites,it would seem that both sites are in fact real (Dickerson, 1981, and Hughes, 1981, have both agreed with this interpretation). It appears in fact as if they are two separate plots superimposed on the same graph and, since Hill plots apparently reveal two independent uncooperative sites, this could be possible.

There are several explanations which could account for these separate sites: 1) one site could be on glial cells, not neurons; 2) the low affinity site may be of high affinity uptake; 3) there may be two (or more) subpopulations of receptors for glutamate on neurons.

It is unlikely that glutamate is binding to glial cells since it was seen in the depolarization experiment that tetrodotoxin blocked the effect of veratrine. The action of tetrodotoxin is specific to neurons. That the low affinity site is the high affinity glutamate reuptake site is more plausible since the K_d is in the same range (Logan and Snyder, 1971). However, the reuptake process has an absolute requirement for the presence of sodium ions, and sodium was absent in these experiments. An experiment which would confirm whether the low affinity binding site was the high affinity glutamate reuptake channel or not would be to perform the binding studies in the presence of a glutamate uptake blocker. If the micromolar K_d then vanished, it could be concluded that the site is one for reuptake. However, although this should produce a clear-cut result in theory, in practice it would be difficult due to the non-specificity of uptake blockers.

(c) Effect of sodium ions upon binding

When the assay was performed in Krebs buffer (which contains sodium) binding sites with different characteristics were observed. The high affinity site had a K_d of only 15.5μM and the low affinity site a K_d of 1.88mM. These values correspond poorly to those of glutamate binding to the

receptor, but excellently to those for high affinity uptake, whose K_ds are 20μM and 1mM respectively (Snyder et.al., 1973).

Since lysed membranes are used in the assay, it seems strange that reuptake can be observed, since binding to the actual transport process is expected to be only transient. However, it must be considered that only 60 to 80% of synaptosomes are actually lysed in Tris buffer and, as Krebs buffer encourages even more to reseal, reuptake itself could be occurring . It seems, therefore, that in Krebs-Ringer phosphate buffer, sodium-dependent high affinity uptake occurs, competing with the receptor sites for glutamate. (Note - it must, of course, be remembered that Krebs buffer contains other additional ions compared to Tris buffer, not only sodium).

(d) Kinetics of binding of L-(^{3}H)-glutamate by the direct method

Experiments performed by the direct method (saturation) revealed binding to be saturable and to involve two sites. One was of a high affinity with a K_d of 20nM (B_{max} 1.1 pmoles/mg) and the other of low affinity with a K_d of 17.3μM (B_{max} 75 pmoles/mg).

Since this method measures specific binding only, whilst the displacement method measures total binding, it seems reasonable to expect and find binding sites with a higher affinity and smaller B_{max}. However, as the data obtained for the two sites by both methods are in the same range it seems valid to use either.

(e) Specific binding to various brain regions

It was found that although specific binding to the cerebral cortex and striatum was about the same (0.22 pmoles/mg), specific binding to the cerebellum was 28% less (0.15 pmoles/mg).

These results are comparable to those of Sharif and Roberts (1980) and Biziere et.al.(1980), although they found only half as much specific binding to the cerebellum as that to the cortex and striatum . These results are in keeping with the fact that all three areas are suspected to contain glutamatergic neurons.

(f) Inhibition of specific L-(^{3}H)-glutamate binding by analogues

The results show (Table 5.2) that DL-homocysteic acid and L-aspartic acid are strong inhibitors of specific binding of glutamate. Kainate and dihydrokainate were found to be weaker in their action, inhibiting only 50% of binding at a concentration of 1mM, as were the antagonists glutamate diethyl-ester (GDEE) and diaminopimmelate. These results are in excellent agreement with those of Baudry and Lynch (1979a), Biziere et.al. (1980), Head et.al. (1980), and Foster and Roberts (1978), with the exception that Foster and Roberts did not find aspartate to be a strong inhibitor.

It seems, therefore, that the extended, short-chain analogues (DL-homocysteate and L-aspartate) are more potent receptor blockers than the folded, long-chain analogues kainate and GDEE. The fact that kainate does not produce a large inhibition of binding adds further evidence towards the growing body of data which suggests that it acts either indirectly with the glutamate receptor or binds to a specific site (de Feudis, 1981). Since diaminopimmelate is only a weak antagonist, strong inhibition of binding was not expected; and the effect of GDEE is in keeping with the results of Clarke and Straugham (1977) who challenge its potency and

selectivity as a glutamate antagonist.

The finding that DL-homocysteate was a potent inhibitor of binding in this assay whilst it has no inhibitory effect on sodium-dependent high affinity uptake (Baudry and Lynch, 1979a) is further evidence that the specific glutamate binding observed in these studies is not to the high affinity re-uptake process.

(g) Effects of depolarising agents on L-(^{3}H)-glutamate specific binding

The results in Table 5.3 indicate that depolarisation results in reduction of the number of glutamate receptor sites. This is in good keeping with the findings of Luqmani et.al. (1979) in that depolarisation produces a decrease in muscarinic acetylcholine receptors.

Further investigation, however, revealed that this decrease was due (at least for veratrine depolarisation) to the increased levels of free endogenous glutamate in the assay system, caused by synaptosomal release and increased protein metabolism. This extra endogenous glutamate escaped complete removal during the washing procedure and bound to the receptors thus blocking the binding sites.

However potassium, a weaker depolarising agent than veratrine, produced a decrease in the number of receptors of 50%. This cannot all be accounted for by the release of endogenous glutamate (veratrine produced a 30% reduction). It seems likely, therefore, that potassium is having some effect on the receptor as an ion. Studies by Baudry and Lynch (1979b) have shown that several monovalent cations (including potassium) do seem to decrease the number of glutamate receptors

although they do not affect the K_d. It seems likely, therefore, that the effect of potassium upon receptor numbers is due to its properties as a cation rather than as a depolarising agent.

In conclusion, the studies presented here have indicated that glutamate binds to specific high affinity sites on membranes which are probably those of neurons . These sites are considered to be different to those for glutamate high affinity reuptake, which have an absolute sodium ion requirement. As soon as potent, specific antagonists of this binding are identified, more will be learned about the physiological role of such putative receptors and of glutamatergic function in nervous conditions such as Huntingdon's chorea and epilepsy.

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CHAPTER 6

The action of various glutamic acid analogues at the locust neuromuscular junction

6.1 Introduction

Electrophysiology provides an alternative approach to the study of neurochemistry to that of *in vitro* nerve terminal preparations or tissue nerve cell culture. In practice the main advantages of this technique are that animal preparations can remain relatively intact with all neuronal and non-neuronal cells retaining their interconnectivity. Thus a higher level of organization can be studied than with respect to synaptosomes, for example.

Many vertebrate and invertebrate preparations have been used for electrophysiological analysis - the frog neuromuscular junction (Fatt and Katz, 1952) and the rat phrenic nerve preparation, where the transmitter is ACh, are some examples. Other ACh preparations that have been useful are the giant South American cockroach (Hoyle, 1955a, b) and the giant squid axon (Hodgkin and Katz, 1949). The crayfish and the cat spinal Renshaw interneuron (Krogsgaard-Larsen et.al., 1979) have also been useful since the principal neurotransmitter effects have been cited as inhibitory, being the result of GABA as the putative neurotransmitter.

The locust neuromuscular junction has been well characterised by Usherwood and his colleagues (1968), where the excitatory amino acid glutamate has been determined as

* A post-synaptic effect was assumed on the basis that the glutamate analogue behaves as a false transmitter. Where plots of mepp frequency change only at one or two threshold levels, this represents a change in mepp amplitude. Where all plots at all thresholds change in the same way, this represents a change in mepp frequency.

the primary neurotransmitter. In this work, the spontaneous miniature end-plate potentials, detected by a microelectrode in the extensor tibialis methothoracic leg muscle of the locust schistoceria gregaria, were studied. Miniature end-plate potentials are of interest since they are thought to represent spontaneous quantized release of neurotransmitter (Katz, 1966), which in the locust preparation is thought to be glutamate, as already mentioned. The extensor tibialis muscle has been shown to be particularly rich in glutamate receptors of which there are thought to be two types, termed junctional and extra-junctional (Cull-Candy et.al., 1973; Usherwood et.al., 1974). The extra-junctional receptors are of two types themselves termed D and H receptors (Cull-Candy 1975, 1976) which account for the biphasic response, seen in localized areas, of depolarization preceeding hyper-polarization; observed when glutamate is applied ionotophoretically to these receptor areas.

A similar locust neuromuscular junction preparation was used for mepp analysis in our laboratory. In our system, the mepps were quantified by using a six channel pulse analyser which monitored mepp frequency at six threshold voltages (50-250mV) as outlined in the materials and methods section. Thus a change in mepp frequency would represent changes at the presynaptic level and a change in mepp amplitude would represent a change at the post-synaptic level. The work presented here shows the effect of various glutamate analogues which were studied in this system with the purpose of examining their properties as glutamate receptor antagonists/agonists and their action on the synaptic release

of glutamate.

6.2 Results

(a) 2-Amino-4-Phosphonobutyrate (APB)

Application of 2 x 10μl(-)APB (10mM barrell concentration; Table 1) from an additional pipette situated just above and in front of the recording electrode, produced a large decrease in frequency (95%) and virtual disappearance of mepps in all of 5 muscle fibres which were examined. A typical response is illustrated in Fig.1. The maximum inhibitory action was usually reached after about 9 mins. and was found to be completely reversible as the drug diffused away, assisted by changing the medium in the bath. This action of (-)APB was induced several times on each fibre by further additions after recovery and responses of equivalent size were seen each time. This decrease in mepp frequency was not accompanied by depolarization of the muscle membrane potential.

Additions of (-)APB were alternated with additions of equivalent amounts of (+)APB. This enantiomer had no detectable influence on either mepp frequency or muscle membrane potential even when multiple additions were made to the fibres studied (Fig.6.1).

Additions (10μl) of KCl (200 mM) from the additional pipette caused both depolarization of the muscle membrane and a large increase in mepp frequency (Fig.6.2). Calculation from the Hodgkin-Huxley equation showed that a dilution factor of 20 must be applied to the

Legend to Figure 6.1

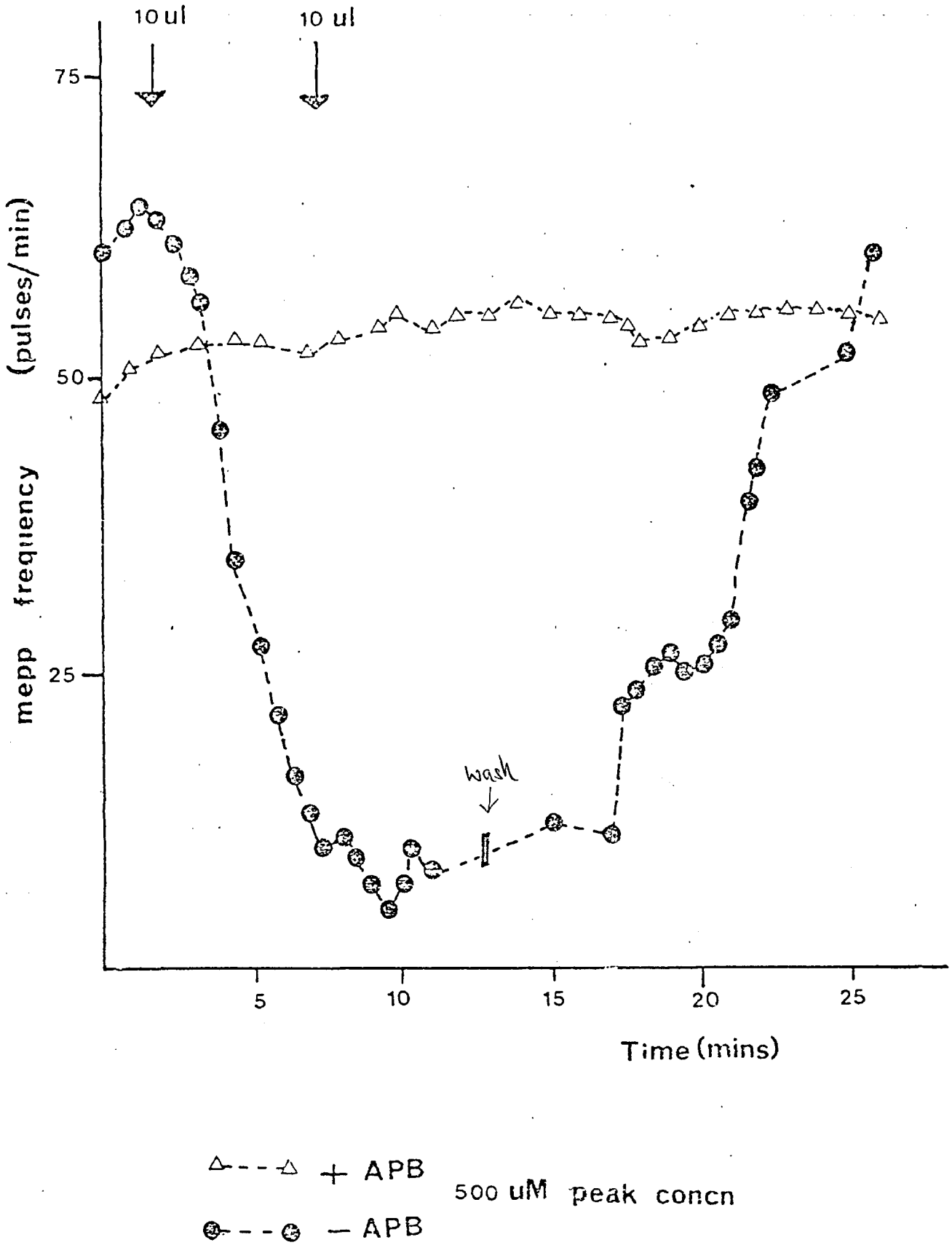
Effect of (+) and (-) amino-phosphono-butyric acid on spontaneous miniature end-plate potentials at the locust neuromuscular junction.

Recordings of 100 μV amplitude mepps were made from a muscle fibre using a glass filled microelectrode. After a brief control period, 10 μl additions (as indicated by the arrows) of either (+) or (-) APB (10 mM barrel concentration, dissolved in locust saline pH6.5) were made from a micro-addition pipette employing a Hamilton syringe.

The graphs show a typical result from three experiments (i.e. 3 fibres from 3 different locust legs). In each experiment, additions of either enantiomer were made on the same fibre with the microaddition pipette and recording electrode in the same position relative to each other. Bath washing with fresh locust saline was carried out between each addition.

No changes in resting membrane potentials were detected (Not shown for reasons of clarity).

Figure 6.1 Effect of (+)and (-) amino phosphono butyric acid on spontaneous miniature end plate potentials at the Locust NMJ.



Legend to Figure 6.2

The effect of Potassium ions on miniature end-plate discharges.

Muscle fibres were impaled with a microelectrode, special note of the membrane potential being taken. After a short equilibration period, 10µl of a concentrated K^+-ion solution (in locust ringer) was added from a micropipette situated above the fibre. The change in mepp frequency was recorded using a pulse analyser, and the change in membrane potential was noted from the oscilloscope display. Figure 6.2 shows a typical increase in 100µV threshold mepps after the addition of 10µl of KCl solution (10 mM). The response is almost immediate from 31 to 672 pulses/min, and lasts some 15 minutes before the rate of mepps returns to a constant frequency. The figure also shows the corresponding peak membrane potential change. The graph shows a typical result from 6 experiments (i.e. multiple additions on 6 fibres each from different insects).

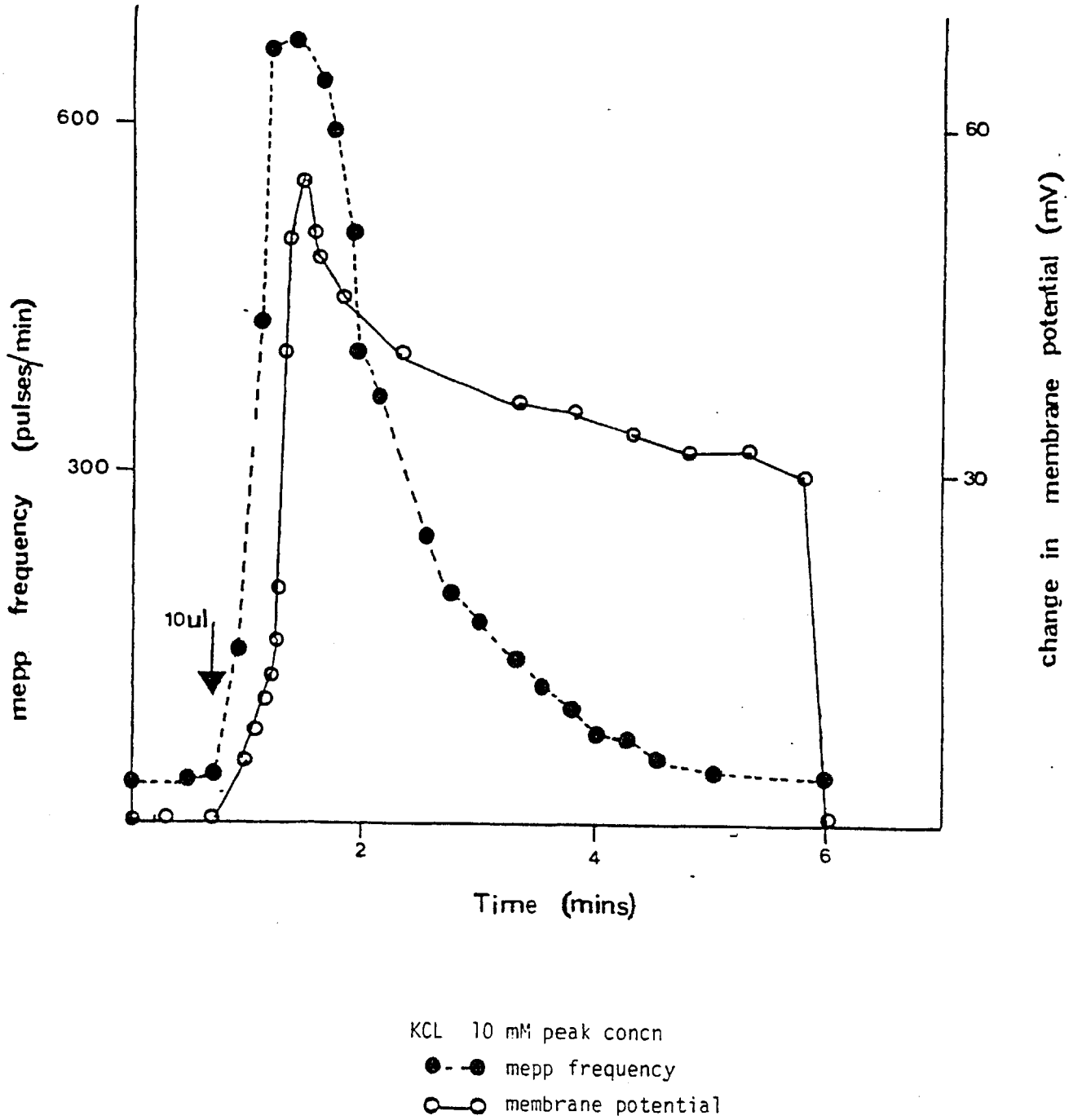
Legend to Figure 6.3

The action of 2-amino-5-phosphono-valeric acid on spontaneous miniature end-plate potentials.

The resting end-plate potential and miniature end-plate potential of 100µV amplitude were recorded at the locust neuromuscular junction. After a brief control period, 10µl of APV, 10mM, dissolved in locust saline pH6.5, were topically applied from a microaddition pipette, and the recording continued.

The plots show changes in membrane potential and mepp frequency occurring simultaneously from a typical experiment. The experiment was perfomed on two separate locust leg fibres.

Figure 6.2 The effect of potassium ions on miniature end plate potentials.



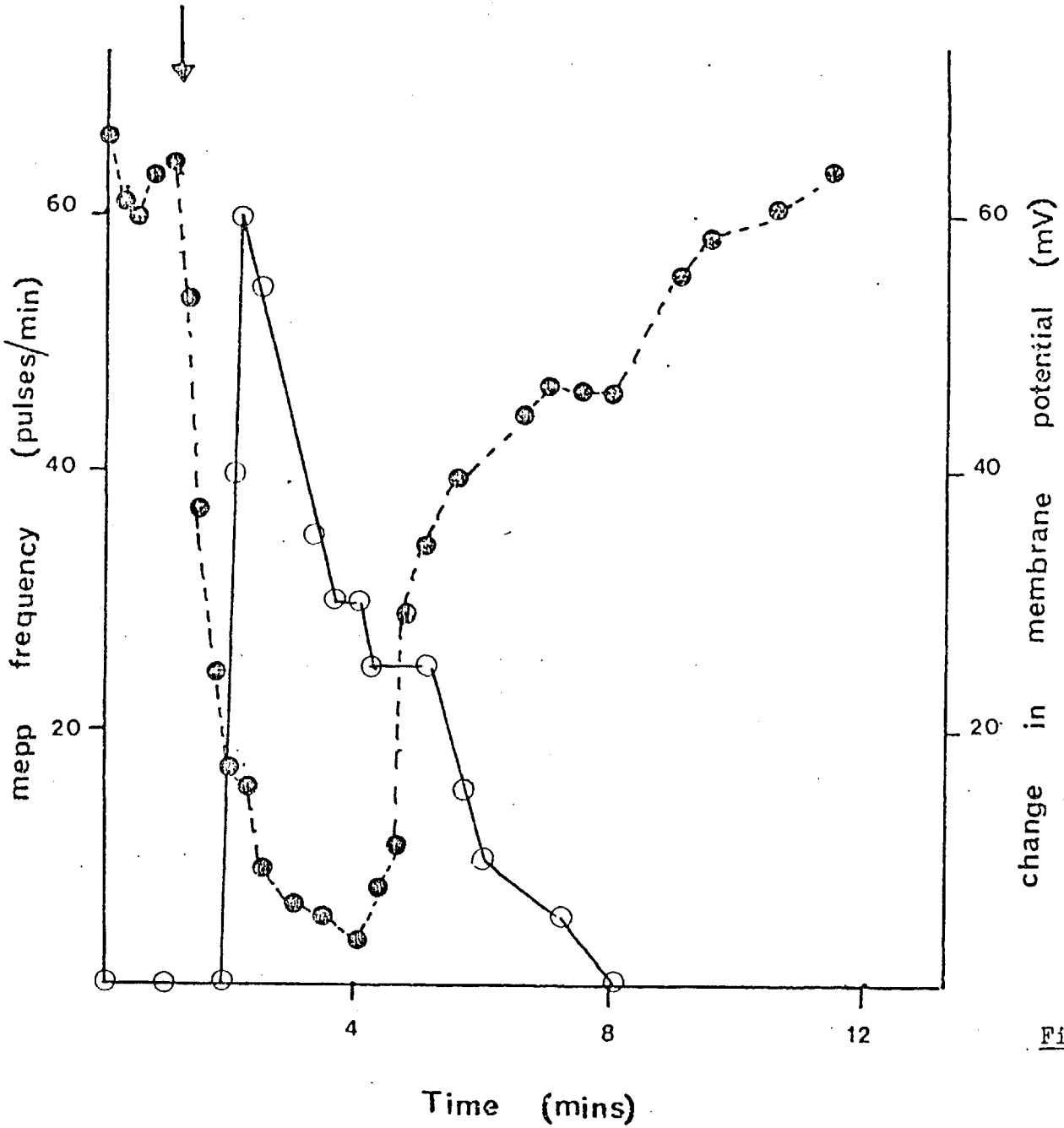
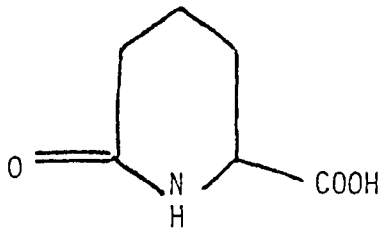


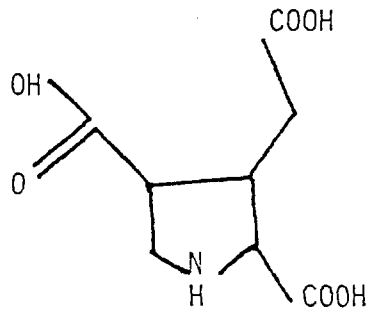
Figure 6.3 The action of 2 amino phosphono-valeric acid on spontaneous miniature end plate discharges.

Table 6.1 Chemical structures of glutamic acid analogues.

glutamic acid	$HOOCCH_2CH_2CH(NH_2)COOH$
2-amino-4-phosphono butyric acid	$H_2O_3PCH_2CH_2CH(NH_2)COOH$
2-amino-5-phosphono valeric acid	$H_2O_3PCH_2CH_2CH_2CH(NH_2)COOH$



pyroglutamate



kainic acid

KCl in the barrel to obtain the concentration at the surface of the muscle fibre from which the recording was made. This factor when applied to APB gave a maximum concentration in the range 500μM at the muscle surface which would greatly diminish with time due to diffusion.

(b) 2-Amino-5-Phosphono Valerate (APV)

This compound is an analogue of APB with an extra methylene group (Table 6.1). In contrast to the lack of influence of (+ or -) APB on the muscle resting membrane potential, this compound caused a rapid depolarization by some 60 mV following addition of 10μl(10 mM). This would have given a maximum concentration of 500μM at the fibre surface. The response was rapid, being maximal by about 30 sec. It was fully reversible following washing effected by changing the bath saline (Fig.6.3). At the same time, this agent caused a large (95%) decrease in mepp frequency which was reversible and followed a similar time course to the depolarization (Fig.6.3).

(c) DL-Pyroglutamate (DL-PG)

This cyclic form of glutamate was found to behave similarly to (-)APB. Additions of 10μl (40 mM) induced an immediate decrease in frequency and subsequent disappearance of mepps. The maximum concentration at the muscle surface was estimated at 2mM. Maximum mepp inhibition was reached about 6 mins. after addition but no change in membrane potential was observed during this period (Fig.6.4). The effect was reversible by applying saline washes.

Legend to Figure 6.4

Effect of DL-pyroglutamate on miniature end-plate potential discharge.

100 μV threshold potentials were recorded for a control period whereupon an addition of DL-pyroglutamate (10 μl, 40mM in locust saline) was made from a microaddition pipette, and recording continued.

The figure shows the results of a typical experiment from 6 additions conducted on 3 different fibres each one from a separate locust.

No change in E_m was observed (Not shown for reasons of clarity).

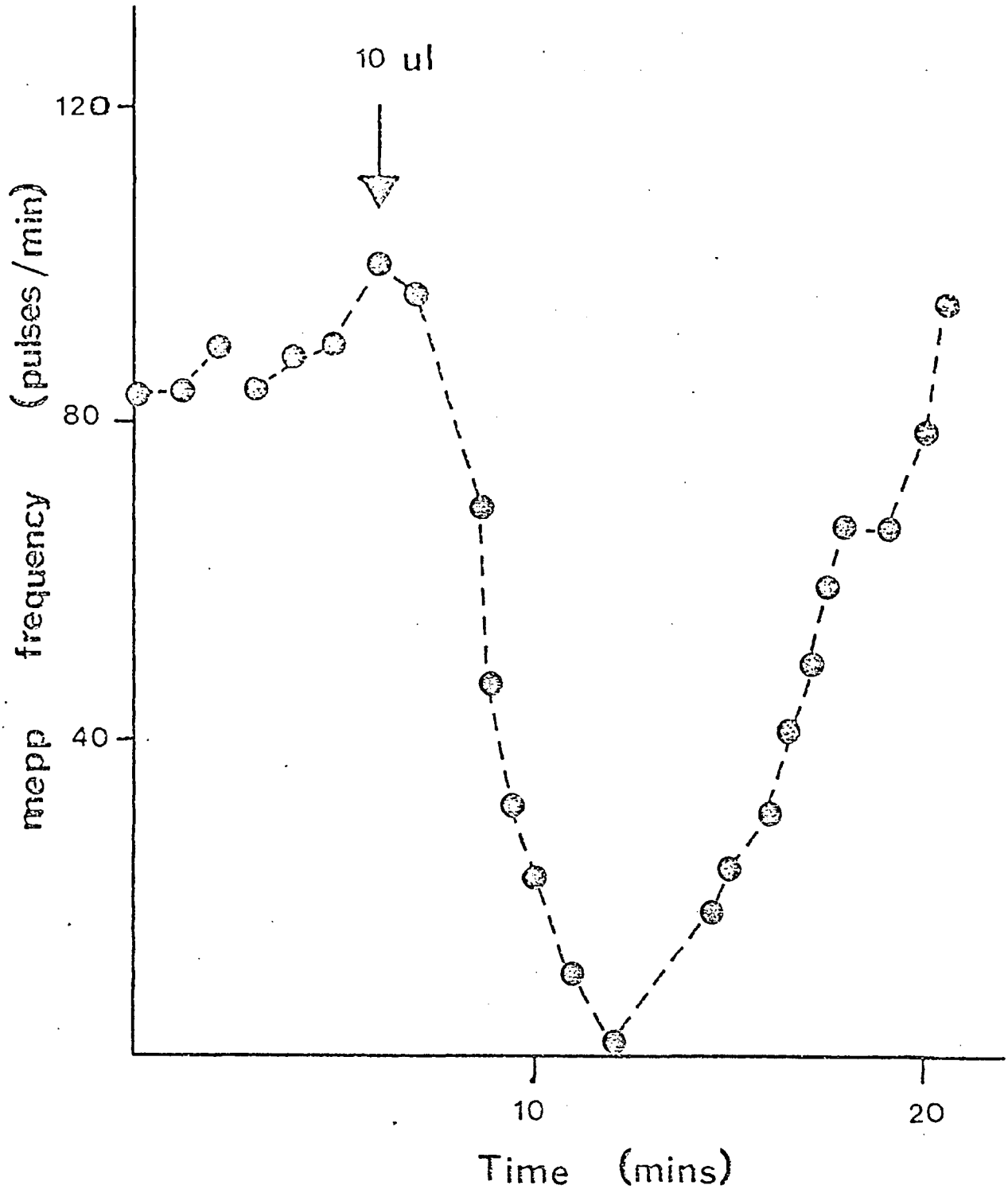
Legend to Figure 6.5

Effect of glutamic acid and kainic acid on spontaneous miniature end-plate potentials.

The resting membrane potential and miniature end-plate potentials of 150 μV amplitude were recorded and monitored intracellularly at the locust neuromuscular junction. After a brief control period, 10 μl additions of either glutamate or kainate (50mM, dissolved in locust saline pH6.5) was applied, as indicated by the arrow in the figure, and recording was continued.

The figure shows a typical result from 3 experiments.

Figure 6.4 Effect of DL-pyroglutamate on minature end plate potential discharges.

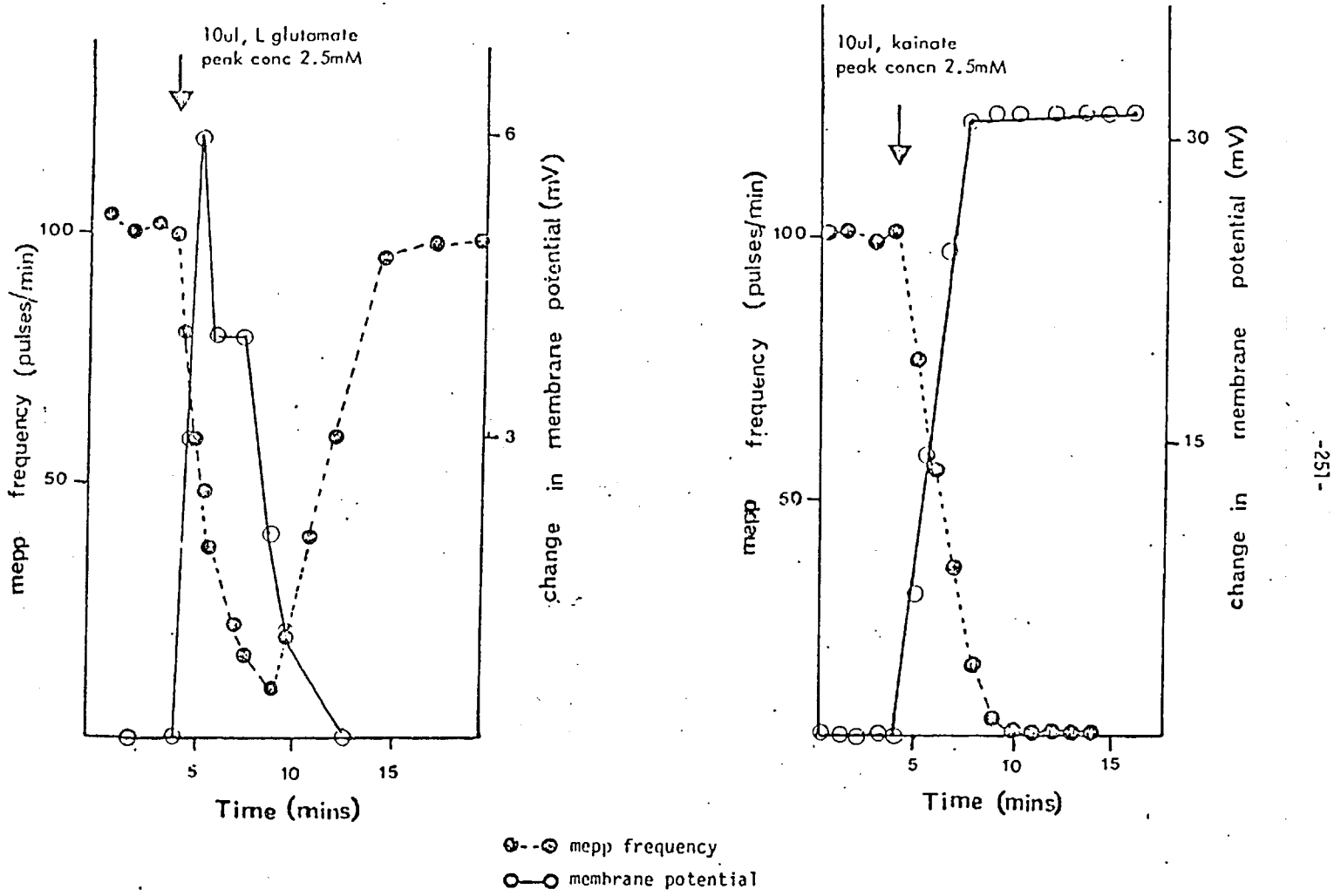


DL pyroglutamate, 2 mM peak concn

mepp frequency

membrane potential

Figure 6.5 Effect of glutamic acid and kainic acid on spontaneous miniature end plate potentials.



When 2-pyrrolidone (cyclic GABA; Table 6.1) was applied in similar or even larger (20 to 100 mM) concentrations, no effect on either mepp frequency or muscle membrane potential was seen.

(d) L-Glutamate and Kainic Acid

Each of these compounds (50 mM) when added from the addition pipette (10μl) produced very similar effects on the pattern of mepps. Thus, glutamate caused substantial reduction of theirfrequency whilst producing depolarization of the muscle membrane by 7-8 mV. This effect was reversible. Kainate showed a more powerful action, causing a rapid decrease in frequency and then a disappearance of mepps. The effect was not reversible by washing even over 30 min. periods (Fig.6.5). The depolarization induced by Kainate was total, the membrane potential falling to zero. Even with extensive washing with saline, no recovery was observed to occur over 30-60 mins. The mepp frequency showed a similar pattern with an irreversible decrease to zero following closely on addition (Fig.6.5).

6.3 Discussion

This simple neuromuscular preparation from locust allows the study of agents which are proposed agonists or antagonists acting at glutamate or aspartate receptors since glutamate is thought to be the excitatory neurotransmitter at this junction (Usherwood, 1968).

(a) Effects of 2-Amino-4-Phosphonobutyrate enantiomers

The present studies show that only the (-) enantiomer of APB interacts with glutamate receptors, thereby reducing

mepp frequency, though dilution by washing with saline reduced the response to zero showing the reversible binding of this agonist. The (+) form of APB was quite without effect. Since neither enantiomer affected the level of membrane potential, a direct interaction of (-)APB with glutamate receptors is indicated rather than an indirect depolarizing action. Others have recently demonstrated the effectiveness of (-) and inactivity of (+)APB in various systems including inhibition of radiolabelled glutamate binding to mammalian brain membranes (Roberts et.al., 1981) and effectiveness in blocking glutamate and quisqualate-induced excitation in spinal cord (Watkins et.al., 1981).

(b) Effects of 2-Amino-5-Phosphono-Valerate

APV has been characterized as an antagonist of aspartate-type receptors, activated by N-methyl-D-Aspartate and related compounds (Watkins et.al., 1980). In the present system, DL-APV caused a large and reversible reduction in mepp frequency as did (-)APB, but this was accompanied by an almost total depolarization of the muscle fibre, indicating a complex action. This decrease in mepp frequency induced by APV contrasts with the increase in mepp frequency which occurs during muscle-membrane depolarization caused by high-K^+ levels. In the latter case it is assumed that the presynaptic nerve terminal was also depolarized and this led to Ca^{2+}-influx and hence augmented quantised transmitter release detected as an increased mepp frequency. With APV it seems likely that both nerve-end

terminal and muscle fibre are depolarized but APV effectively blocks glutamate receptors preventing the increased quantised glutamate release from being detected. Why APV and not (-)APB should cause depolarization is not clear. DL-APB has been shown to block all on-channel responses in mud puppy retina induced by light-stimulation whilst leaving off-channels intact, probably by mimicking the photoreceptor transmitter (Miller and Slaughter, 1981). APB and APV also reduced the light-evoked release of acetylcholine from cholinergic amacrine cells of retina with an ED_{50} of 10μM (Collins , 1981).

(c) Effects of DL-Pyroglutamate

This compound appears to be an effective glutamate antagonist which acts directly on the receptors without causing depolarization, as did (-)APB. The ineffectiveness of 2-pyrrolidone (cyclic form of GABA) indicates the requirement of the carboxyl group at position 5 on the ring for effective interaction with the glutamate receptor.

(d) Effects of L-glutamate and Kainate

The effect of applied L-glutamate in reducing mepp frequency was expected, since it presumably prevents detection of the quantised release of endogenous glutamate. The depolarizing action is most likely due to inactivation of the excitatory ionphores(Na^+-gates) associated with the glutamate receptors.

The similar but irreversible action of Kainate reflects its excitotoxic action (McGeer et.al., 1978) and appears to involve a permanent and irreversible interaction between Kainate and glutamate receptors.

CHAPTER 7

IN VIVO Central Manipulations: Measurement of Amino Acid Storage and Release

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CHAPTER 7

IN VIVO Central Manipulations; measurement of amino acid storage and release

7.1 Introduction

The majority of the work presented in previous chapters of this thesis represents an investigation into a number of possible synaptic events occurring at the nerve terminal *in vitro*. The primary technique used to study such events was the isolated nerve terminal preparation. In this chapter, neurotransmitter releasing properties of synaptosomes were investigated after the two types of central manipulation had been carried out *in vivo*. The types of pre-treatment instigated were (1) the systemic i.p. injection, into adult rats, of chemical convulsant drugs, and (2) the placing of a stereotaxic lesion, again in the rat, to ablate the cortico-striatal pathway. It was considered that synaptosomal data from such pre-treatments could provide useful information on (a) possible neurochemical correlates of induced seizures and (b) the identity of the neurotransmitter(s) at cortico-striatal projections.

(a) Experimental induced convulsions

Currently, a great deal of interest is centred on the possible involvement of certain amino acid neurotransmitters in the mechanism(s) of convulsions. This stems from the early observation by Tower et.al., (1960), that glutamate and GABA are diminished in concentration in human epileptogenic cortex. However, since the availability of human tissue for routine biochemical analysis is restricted,

it was considered that an understanding of the dynamics of brain metabolism in seizure could be obtained from suitable animal models. How closely such models represent the human condition is of course critical, and their validity must be judged upon whether the experimental approach produces aberrations consistently found in human epilepsy. Studies on excised human epileptogenic cortex have so far generally confirmed the work of Tower, revealing diminished levels of glutamate and GABA (Berl et.al., 1959; Van Gelder and Cartois, 1972; Van Gelder et.al., 1972; Bradford, 1976). These results could suggest a reduction in the performance of the GABA inhibitory transmitter system (Meldrum, 1974); thus drugs which raise brain GABA levels tend to show anti-convulsant properties, and those lowering the content are often convulsants (Bradford, 1976).

Another proposal might be that the excitatory neurotransmitter glutamate is involved in neuronal hyperactivity. When administered systemically, it often causes convulsions (Bagara et.al., 1971; Johnston, 1972; Bradford and Dodd, 1975), and its diminished levels seen in experimental focal tissue correlate frequently with its appearance in fluids superfusing the tissue when in situ (Dodd and Bradford, 1976; Koyama and Jasper, 1977; Koyama, 1978; Dodd et.al., 1980). However, such simultaneity does not necessarily prove any causal relationship between glutamate release and seizure activity. In an attempt to obtain indications of such a causal relationship, a study of chemical induced convulsions, which were found to be

antagonized by glutamate diethyl ester (GDEE) and glutamate dimethyl ester (GDME), were achieved in the rat.

(b) Lesioning of the cortico-striatal pathway

The mammalian cerebral cortex sends many excitatory projections to the neostriatum and thalamus (Anderson et.al., 1964; Kitai et.al., 1976; Feger et.al., 1979). Biochemical studies of the cortico-striatal pathways following stereotactically-placed lesions have been limited to demonstrating a lower rate of glutamate accumulation, by crude synaptosomal fractions, relative to other amino acids (McGeer et.al., 1977; Divac et.al., 1977). A more frequently used method for assessing cortical projection has been ablation of varying amounts of cortical tissue, and measuring the biochemical consequence of this more severe lesion. A number of cortical ablation studies have shown decreased amino acid content and uptake selectively for glutamate in both striatum and thalamus (Kim et.al., 1977; Young et.al., 1981; Mclennan, 1980; Rowlands and Roberts, 1980; Fonnum et.al., 1981), and often these are of greater magnitude than following the more limited lesions described above. However, it may be argued that the removal of so much tissue is more likely to produce no specific alterations, which may overestimate the amount of striatal glutamate associated with the cortical projection.

In the study presented here, we have used the cortico-striatal lesion described by McGeer et.al., (1977), in association with three different measures of glutamate activity in the striatum. We have measured tissue content

of a number of amino acids and, since glutamate is present in high concentration in both non-neuronal and neuronal cells, we also looked at K^+-stimulated release of glutamate from synaptosomes as a more specific indicator of neuronal glutamate. Finally, to see is there was any change in postsynaptic glutamate receptors, as a result of decreased glutamate input, the specific glutamate binding to crude synaptosomal membranes was measured. It was expected that such studies would provide further evidence that the cortico-striatal pathway is glutamatergic and, in addition, a decorticated animal would provide a complimentary model for the study of Huntington's chorea to that of the kainic acid model (McGeer and McGeer, 1976).

7.2 Results

(a) The effect of glutamate antagonists in delaying the onset of chemically-induced convulsions (Table 7.1)

Injections i.p. of strychnine (5mg/kg) and pentylene-tetrazol (70mg/kg) were found to induce convulsions in adult rats, on average after 4.16 and 7.87 minutes respectively. When glutamate diethyl ester (GDEE, 200 ,/kg) or glutamate dimethyl ester (GDME, 200mg /kg) were injected 2 minutes prior to the injections of the convulsant drugs, the time to onset of seizures was significantly delayed (Table 7.1). Thus injections of GDEE and GDME prior to the injection of strychnine delayed the time to onset of convulsions by 335% and 221% respectively. Similarly, injections of GDEE and GDME delayed the time to onset of pentylenetetrazol (PTZ)-

Legend to Table 7.1

Effect of glutamate antagonists in delaying the onset of chemically-induced convulsions.

Rats were injected i.p. with the convulsants strychnine and pentylenetetrazol (PTZ) and the time taken to the onset of convulsions was measured. The effect in delaying the onset of seizures by injecting the glutamate antagonists glutamate diethyl ester (GDEE) and glutamate dimethyl ester (GDME) one minute prior to the administration of the convulsants is also indicated. Values represent means $\pm$ SEM.

Legend to Table 7.2

The concentration of amino acids in whole cerebral cortex at the time of onset of chemically-induced convulsions.

Animals were treated with the convulsants strychnine and PTZ and, where indicated, pretreated with the glutamate antagonists GDEE and GDME according to the legend to Table 7.1. When animals were treated with the convulsants only, animals were sacrificed at the onset of seizure, the brain removed and the cerebral cortices placed in ice-cold methanol (5mls) in order to extract the endogenous amino acids. When animals were pretreated with glutamate antagonists, animals were sacrificed four minutes after the injection of strychnine and eight minutes after the injection of PTZ. Again the brains were removed and the cortices placed in ice cold methanol, ready for amino acid analysis (see Materials and Methods, chapter 2).

The values shown represent means $\pm$ SEM for the amino acid and treatment indicated.

Table 7.1 Effect of glutamate antagonists in delaying the onset of chemically-induced Convulsions.

glutamate antagonist	convulsant	dose(mg/kg)	No.of animals	onset of convulsions (mins.)
-	strychnine	5	10	$4.16^{+}_{-}0.75$
GDEE (200mg/kg)	strychnine	5	5	$13.96^{+}_{-}1.10$
GDME (200mg/kg)	strychnine	5	13	$9.21^{+}_{-}0.74$
-	PTZ	70	21	$7.87^{+}_{-}0.64$
GDEE (200mg/kg)	PTZ	70	16	$37.48^{+}_{-}4.25$
GDME (200mg/kg)	PTZ	70	5	$20.60^{+}_{-}4.36$

6 animals did not convulse at all.

Table 7.2 Amino Acid content of rat brains following systemic injection with chemical convulsants. Effect of pre-treatment with glutamate antagonist.

	Aspartate	Threonine	Serine	Glutamate	Glutamine	Glycine	Alanine	GABA
Control	3.43±0.19(2)	0.79±0.05(7)	1.15±0.07	11.40±0.27	4.82±0.27	0.81±0.02	0.98±0.08	2.34±0.14
Strychnine 5mg/kg	3.30±0.32(3)	0.95±0.15(3)	1.11±0.09(3)	11.29±0.34(3)	3.89±0.29(2)	0.89±0.14(3)	1.13±0.11(3)	2.63±0.05(3)
GDME 200mg/kg +strychnine (no convulsions)	2.84±0.10(4)	0.62±0.04(10)	0.86±0.02(4)	10.84±0.23(4)	4.15±0.08(14)	0.78±0.03(14)	0.99±0.07	2.38±0.11(14)
GDEE+strychnine (no convulsions)	2.45±0.14(3)	0.86±0.07(3)	1.17±0.08(3)	10.40±0.70(3)	5.15±0.04(3)	0.94±0.05(3)	3.21±0.23	2.43±0.14(3)
PTZ 70mg/kg	2.16±0.17(10)	0.65±0.09(10)	1.00±0.01(10)	9.55±0.48(10)	4.24±0.35(10)	0.89±0.07(10)	1.97±0.38(10)	2.49±0.06(10)
GDME+PTZ	2.56±0.14(7)	0.55±0.07(7)	0.91±0.05(7)	10.56±0.27(7)	3.90±0.13(7)	0.71±0.05(7)	1.11±0.06(7)	2.39±0.11(7)
GDEE+PTZ	2.40±0.17(11)	0.60±0.06(11)	1.03±0.09(11)	9.19±0.44(11)	4.23±0.30(11)	0.90±0.06(11)	1.95±0.25(11)	2.51±0.10(11)

induced convulsions by 476% and 262% respectively. Hence, of the glutamate antagonists tested for their anticonvulsant action, GDEE was found to be more potent than GDME.

The observation that GDEE and GDME provided a degree of protection towards chemically-induced convulsions prompted a study for a neurochemical correlate of the convulsant action and for the protective treatment of the glutamate antagonists. The analysis was centred upon the existence, if any, of differential amino acid transmitter content and/or release in the mammalian brain.

(b) Cerebral cortex amino acid levels at the time of onset of chemically-induced convulsions

At the time of onset of PTZ (70mg/kg) and strychnine (200mg/kg) induced convulsions,the animals were sacrificed and the brains removed by exsanguination. The freshly dissected cerebral cortices were then analyzed for their endogenous amino acid content, the results of which are displayed in Table 7.2. In addition, animals were injected i.p. with GDEE or GDME at the dose indicated 2 minutes prior to injections of the chemical convulsants. Animals were then sacrificed 4 minutes after the i.p. injection of strychnine and 10 minutes after the administration of PTZ. As before, the endogenous amino acid levels were determined in the cerebral cortex.

The results show that neither strychnine nor PTZ induced any significant detectable changes in either the concentration of the putative amino acid neurotransmitters

glutamate, aspartate, GABA, glycine, or the physiologically inactive amino acids threonine, serine, glutamine, alanine, from the controls. The glutamate antagonists GDME and GDEE also produced no corresponding changes in amino acid levels when administered two minutes prior to strychnine or PTZ injections.

(c) The effect of PTZ-induced convulsions on amino acid levels in discrete areas of the cerebral cortex

Since no change in amino acid content was detected in whole cerebral cortex after administration of the convulsant drugs, an experiment was performed in which discrete areas of the brain, i.e. the occipital cortex, frontal cortex, and the sensorimotor cortex (SMC), were studied in order to determine the existence or not of an amino acid neurochemical correlate. Thus adult rats were injected i.p. with PTZ (70mg/kg) and the brains removed at the onset of convulsion. The freshly dissected occipital cortex, frontal cortex and SMC were then analysed for their endogenous amino acid content. Rats were also injected with GDEE (200mg/kg) two minutes prior to PTZ administration, and sacrificed after six minutes. Amino acid content of the discrete cortical areas was then determined.

The results displayed in Table 7.3 show that PTZ at the dose indicated did not induce any significant changes in the amino acid concentrations of the frontal cortex, SMC, or the occipital cortex. Furthermore, the glutamate antagonist produced no corresponding detectable change in amino acid levels, consistent with its anticonvulsant

Legend to Table 7.3

The concentration of amino acids in subcortical areas of rat brain, at the time of onset of PTZ convulsions.

Animals were treated with PTZ but, where indicated, pre-treated with the glutamate antagonist GDEE. This was achieved by i.p. injection one minute prior to the administration of PTZ. The values shown (mean $\pm$ SEM) represent the amino acid concentration in either the frontal cortex, the sensory motor cortex, or the occipital cortex, at the onset of convulsions. However, when rats were pretreated with GDEE, the amino acid levels were determined eight minutes after the injection of PTZ as described in the legend to Table 7.2.

Legend to Table 7.4

The performance of amino acid release and storage in cerebro-cortical synaptosomes prepared from rats which had been treated with glutamate diethyl ester and PTZ.

Rats were injected i.p. with PTZ or GDEE and PTZ as described in the legend to Table 7.1. When animals were injected i.p. with PTZ only, at the onset of seizures, the rats were sacrificed and synaptosomes were prepared from the cerebral cortex. When animals were injected with GDEE one minute prior to the injection of the convulsant, the rats were sacrificed and the brains removed eight minutes after the administration of the PTZ. Synaptosomes were then prepared. Amino acid release in response to high levels of K^+ ions was then determined as well as the ability of nerve endings to store amino acids. The values shown represent means $\pm$ SEM (n=4).

Table 7.3 The effect ofPTZ and GDEE induced convulsions on amino acid content in various regions of the cerebral cortex.

	Frontal cortex	SMC	Occiptal Cortex
Protection with GDEE			
Asp	6.14±1.72	5.28±1.20	3.94±1.24
thre	0.56±0.02	0.43±0.02	0.63±0.19
ser	1.03±0.09	0.87±0.06	0.86±0.06
glu	7.96±0.20	8.14±0.28	8.17±0.71
glu	3.12±0.12	2.95±0.12	3.14±0.41
gly	0.45±0.03	0.34±0.03	0.47±0.08
ala	0.87±0.10	0.73±0.12	0.70±0.19
gaba	1.59±0.25	1.65±0.11	1.42±0.17
PTZ convulsion			
asp	4.02±0.16	3.58±0.22	4.18±0.56
thre	0.73±0.07	0.69±0.02	0.78±0.13
ser	1.37±0.10	1.26±0.13	1.54±0.12
glu	15.76±0.98	12.69±0.66	17.07±2.61
glu	5.23±0.46	4.99±0.70	5.79±0.52
gly	0.70±0.09	0.54±0.10	0.65±0.13
ala	1.61±0.08	1.05±0.29	1.39±0.44
gaba	1.90±0.04	1.86±0.08	2.05±0.15
Control			
asp	4.07±0.52	4.12±0.44	4.00±0.40
thre	0.61±0.08	0.57±0.06	0.63±0.10
ser	1.39±0.18	1.19±0.07	1.36±0.07
glu	12.66±1.11	12.09±1.36	13.96±1.40
glu	4.21±0.91	4.20±0.52	5.01±0.78
gly	0.56±0.08	0.59±0.02	0.81±0.05
ala	1.00±0.04	1.01±0.12	1.00±0.20
gaba	1.51±0.09	1.71±0.08	1.92±0.11

n = 3

Table 7.4 The action of PTZ and GDEE on nerve terminal amino acid release and storage *in vivo*.

	In vivo treatment	Pellet amino acid content nmoles/100mgs protein.	
	saline	PTZ	GDEE + PTZ
C asp	3353±1027	3399±568	2590±507
O ser	436±64	389±42	374±134
N glu	9356±584	9161±1661	7410±703
T gly	308±9	344±30	353±51
R ala	727±123	733±103	784±185
O gaba	1882±151	1763±276	2038±379
L			
		Medium amino acid level	nmoles/100mgs protein.
C asp	44±3	44±7	57±12
O thre	163±40	132±49	94±23
N ser	176±31	160±43	193±46
T glu	151±50	91±30	117±25
R gly	123±20	107±39	117±26
O ala	260±74	203±65	255±72
L GABA	19±4	21±8	18±4
asp	141±8	112±12	180±43
K^+ thre	126±29	122±28	156±49
S ser	158±53	169±31	252±87
T glu	394±55	270±55	427±115
I gly	99±14	106±20	156±58
M ala	220±35	229±50	280±84
GABA	98±20	85±18	121±39

action.

(d) The action of PTZ at nerve terminals IN VIVO and IN VITRO

The fact that no biochemical correlate of chemically-induced convulsions could be detected in whole cortical tissue was not attributed, at this stage, to an inactivity towards involvement in amino acid metabolism, but rather a masking effect by glial cells of events occurring at the synapse. Thus, the presence of amino acids in high concentration in glial cells and their high rates of metabolic activity might have precluded the changes in neurotransmitter storage and release mechanisms that do take place from being detected. Hence in this experiment, the convulsive effect of PTZ *in vivo* and *in vitro* at isolated nerve endings on amino acid content and release was investigated.

The results from an *in vivo* experiment, depicted in Table 7.4, show an apparent inactivity of i.p. injected PTZ towards amino acid metabolism in the nerve terminal, as evident by the fact that no significant changes in synaptosomal content was detected from controls. Correspondingly, the anticonvulsant action of GDEE, injected i.p. two minutes prior to PTZ administration, was found not to induce any change in amino acid concentration. Complimenting these results of synaptosomal content is the data concerning release of amino acids (Table 7.4) in response to depolarizing levels of K^+ ions (55mM). The *in vivo* administration of PTZ did not appear to significantly affect the ability of synaptosomes to release

putative amino acid neurotransmitters. Thus when cortical synaptosomes were prepared from animals with PTZ-induced convulsions, neither the resting release not the stimulated release of aspartate, glutamate and GABA were found to be different from the control i.p. injection of saline (NaCl, 0.95%). The concentrations of the other amino acids serine, glycine and alanine were also unaltered. In addition, the results did not provide an explanation, in terms of synaptosomal amino acid neurotransmitter release, of the protective action of GDEE towards PTZ-induced convulsions, since no changes in the release of any of the amino acids measured were detected when animals were pretreated with GDEE.

It was considered that the lack of detection of a neurochemical correlation to the convulsant actions of PTZ and strychnine might be due to the nature of the in vivo experiment. Any temporary changes in synaptic release and metabolism that do take place as a result of pretreatment of the animal with various agents might not be detected after synaptosomes had been prepared, five hours later. For this reason, an in vitro experiment was performed in which the responses of synaptosomes to additions of PTZ and GDEE (final concentration 1 mM) were determined in flask incubations. Thus, as shown by the results in Table 7.5, PTZ was found not to induce any significant change in synaptosomal content or in release of the amino acids studied. This was taken as evidence that the convulsive action of PTZ could not be interpreted as a depolarizing action by this compound.

Legend to Table 7.5

The effect of PTZ and GDEE on synaptosomal amino acid levels.

Synaptosomes were prepared from the cerebral cortex of untreated rats. Following resuspension and preincubation in Krebs phosphate medium, additions of GDEE (1mM), PTZ (1mM), GDEE plus PTZ (one minute later) were made. After eight minutes of incubation with the reagents, the synaptosome suspensions were pelleted by bench centrifugation (10,000g, 2 mins). Amino acid levels in the pellet and the medium were then determined as described in the Materials and Methods, chapter 2. The values shown represent means $\pm$ SEM (n=4).

Table 7.5 The effect of PTZ and GDEE on synaptosomal amino acid levels in vitro.

	CONTROL	GDEE(1mM)	PTZ(1mM)	GDEE(1mM)+ PTZ(1mM)
Asp	80±10	114±6	61±10	76±10
thre	110±3	126±10	107±8	122±13
ser	235±25	296±32	212±12	244±38
glu	141±10	2028±415	94±11	1980±128
gly	160±16	266±34	161±11	236±27
ala	193±11	294±22	194±12	265±22
GABA	<1	<1	<1	<1

Values are expressed as nmoles/100mgs protein ± SEM (n=4)

The addition of GDEE (1mM) to synaptosome suspensions did not appear to change the concentration of aspartate, threonine, serine, glycine, alanine, and GABA either in the incubation medium or in the synaptoplasm. However, GDEE was found to increase glutamate levels in the incubation medium, but did not appear to change the glutamate concentration inside synaptosomes. Hence this elevation of glutamate was attributed to the hydrolysis of GDEE over the time course of the experiment (10 mins) rather than any direct stimulatory influence initiated by the drug itself.

(e) Protein yield changes following cortical ablation

An initial indication that severe neuronal degeneration and gliaiocis had taken place, following denervation of the cortico-striatal tract, was obtained from protein yield measurements of the P_2 pellet. Table 7.6 shows that a 47% decrease in protein yield was found to occur in crude synaptosomes, prepared from the lesioned side of the neostriatum In contrast to this, however, the tissue wet weights remained unchanged. This result was interpreted by assuming firstly that an increase in the number of glial cells occurs after decortication, and secondly that these glial cells were inefficiently sedimented by centrifugation in our preparative method.

Since protein recovery in the P_2 pellet seemed to be variable, tissue wet weights were used for amino acid concentration indexing; as explained in the discussion, this is in common with most other authors working in this field (Kim et.al., 1977; Young et.al., 1981; and Rowlands

Table 7.6

Comparison of corpus striatum tissue weights with crude synaptosomal protein yield following leisoning of the cortico-strital tract.

	Tissue wet weight(mgm)	Protein yield(mgm)
Lesion	37 ± 3.4 (n=9)	*0.18 ± 0.04 (n=10)
Control	40 ± 3.1 (n=9)	0.35 ± 0.03 (n=10)

*$p<0.01$ 47% decrease from control.

and Roberts (1980).

(f) Differential amino acid release and storage

Figure 7.1 shows that when crude synaptosomes, prepared from the lesioned side (RHS) and the control side (LHS) of the animals, were stimulated with high K^+ levels (56mM), a significant difference in glutamate and aspartate release was obtained (approximately 25% decrease from controls). However, no differences were detected in stimulated GABA release, or in the release of the amino acids serine, glycine, alanine, valine, leucine (Table 7.7). The decrease in stimulated aspartate and glutamate release was not explained by a change in resting release levels (see legend to Figure 7.1), but was interpreted rather, by the fact that lesioning initiates a degeneration of the cortical projection to the neostriatum, which is revealed in this study by differential evoked aspartate and glutamate release.

At the completion of incubations, amino acid levels were analyzed in the crude synaptosomal tissue itself. Figure 7.2 shows that for crude synaptosomes prepared from lesioned neostriata, a significant decrease was observed in glutamate content, when compared to controls (from 301 nmoles/gm tissue to 213 nmoles/gm tissue). No significant differences were detected for the non- neurotransmitter amino acids measured: serine, glycine, alanine (Table 7.7).

(g) Specific binding of ^{3}H-glutamate

Table 7.8 illustrates the results of specific glutamate

Legend to Figure 7.1

The K^+-stimulated release of transmitter amino acids from striatal synaptosomes two weeks after lesioning of the cortico-striatal pathway.

Control release is measured in synaptosomal fractions prepared from striates of the unlesioned sides of the same rat brains. Release is expressed as the mean $\pm$ SEM % of resting release, which was 15.3 $\pm$ 2.3 and 11.0 $\pm$ 1.2 nmoles aspartate/gm tissue, 68.7 $\pm$ 9.3 and 52.1 $\pm$ 4.2 nmoles glutamate/gm tissue, and 12.2 $\pm$ 1.8 and 9.9 $\pm$ 1.3 nmoles GABA/gm tissue for lesioned and control synaptosomes respectively. Significant differences between lesioned and control release were found at the p-level indicated using Student's *t*-test (n=9).

Legend to Figure 7.2

Transmitter amino acid content of crude synaptosomal fractions of striates from cortico-striatal lesioned and unlesioned control sides of rat brain.

Significant differences between lesioned and control mean $\pm$ SEM (n=5) values were found at the p-level indicated using Student's *t*-test.

Figure 7.1 The K^+ stimulated release of transmitter amino acids from striatal synaptosomes, two weeks after lesioning of the cortico-striatal pathway.

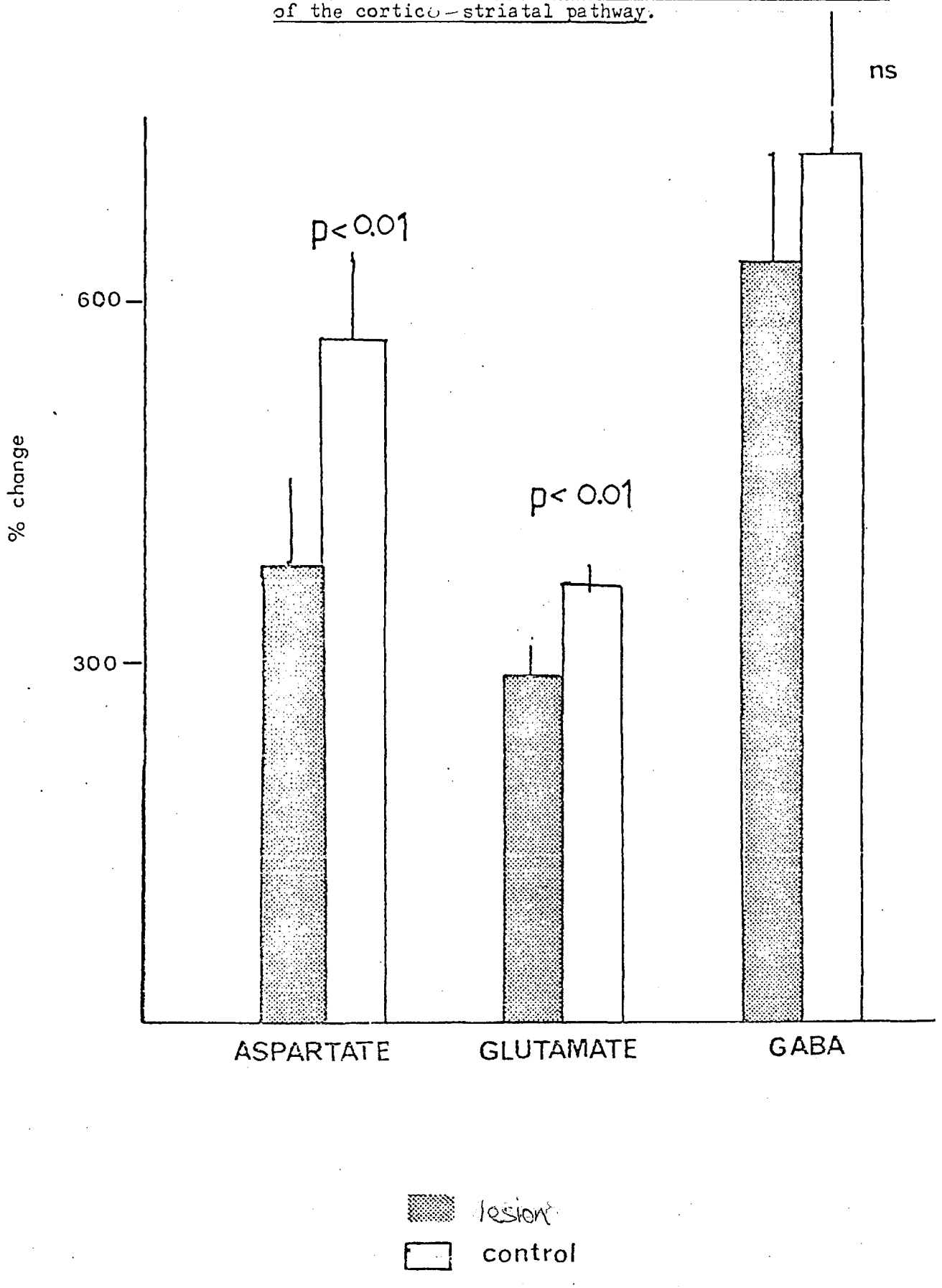

Figure 7.2 Transmitter amino acids content of crude synaptosomal fractions of striates from cortical-striatal lesioned and unlesioned control sides of rat brains.

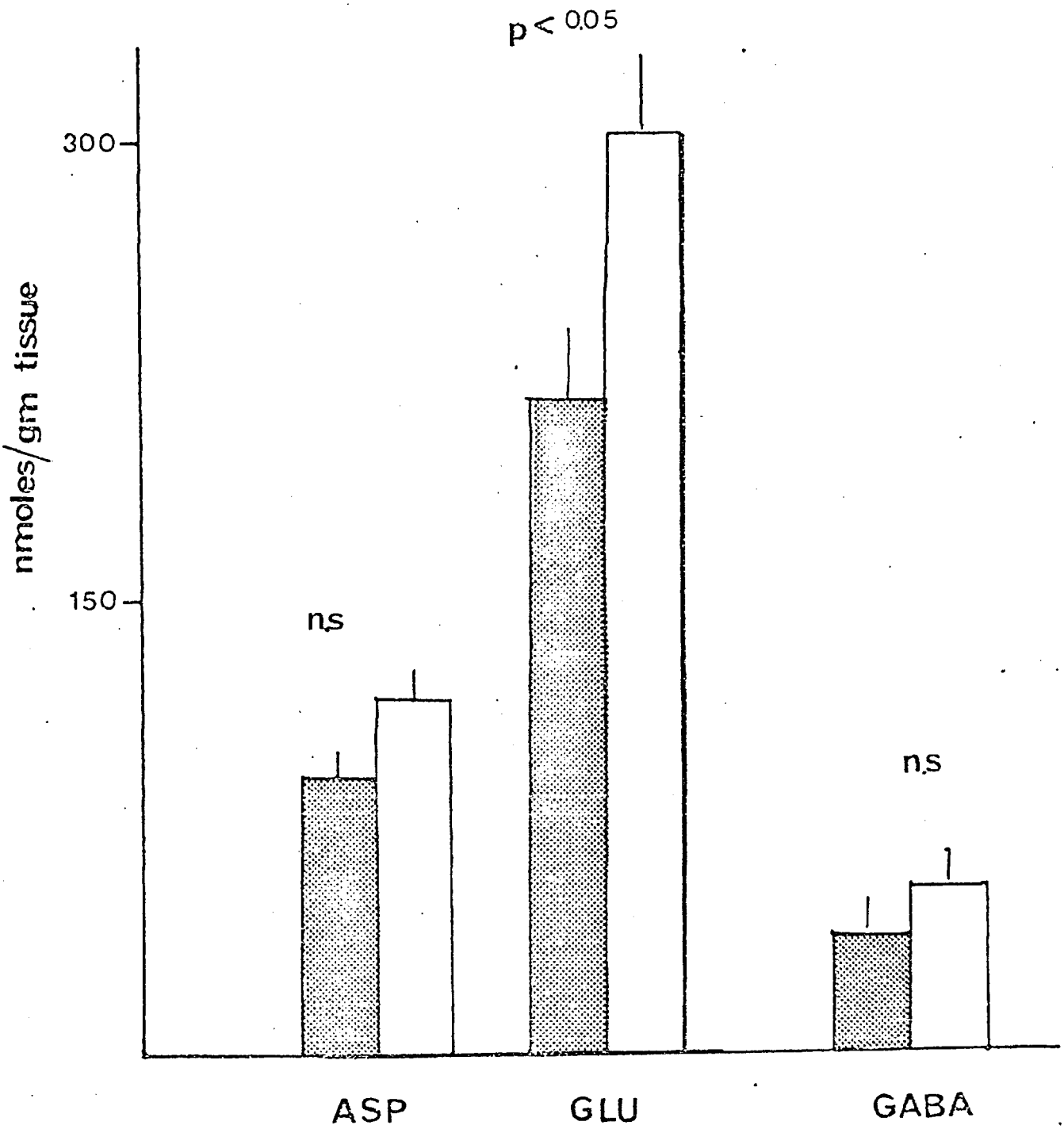

Table 7.7 Non-transmitter amino acids K^+ stimulated release, and crude synatosomal content from rats with unilateral cortical-striatal lesions.

	K+ stimulated release(a)		Synatosomal content(b)	
Amino acid	Lesioned	Control	Lesioned	Control
Serine	116.1±7.9	116.9±7.5	181.0±19.2	163.0±17.5
Glycine	123.4±13.8	121.2±16.3	108.6±12.3	102.3±10.1
Alanine	121.8±13.5	122.8±12.4	111.5±19.0	102.1±10.0
Valine	109.9±19.6	100.0±7.9	ND[c]	ND
Luecine	114.5±21.3	96.9±5.3	ND	ND

(a) Mean ± S.E.M. % of resting release (n=9)
(b) Mean ± S.E.M. nmoles/gm tissue (n=9)
(c) Not detectable.

Table 7.8 Specific Binding of H^3 - Glutamate.

	Lesioned side (RHS)	Control side (LHS)
specific binding expressed as a % of control	99.1 ± 18.9 (n=10)	100

Specific binding of H^3 glutamate to crude synaptosomal membranes of the rat corpus striatum following lesioning of the cortico-striatal tract.

Data is mean ± SEM

binding to crude neostriata membranes. No differences in specific binding were detected between the membranes prepared from the lesioned side and the yoked control side of the neostriata.

7.3 Discussion

(a) Central manipulations by systemic i.p. injections

There are few conditions within the realm of brain metabolism that have been so intensively studied as convulsive disorders. These studies are aimed at the discovery of the biochemical "characteristics" of convulsions, the assumption being that the participation of neurotransmitters in the control of the level of neuronal firing is somehow upset and a chain of events is initiated which culminates in seizure activity. In addition to such studies casting light on the mechanisms that precipitate seizures, they could also yield information on how convulsions could by terminated with the use of therapeutic agents.

Almost without exception, data on in vivo biochemical correlates of seizures has been obtained on experimental animals in which convulsions have been induced either by electroshock, or by administration of convulsive drugs. Some of these models (Purpura et.al., 1972) may be only remotely related to the human condition but, nevertheless, give detailed information on events occurring during the seizure discharge. Unfortunately, the results concerning induced seizure activity from other workers is both confusing and conflicting. Thus, whilst the results of Killam and Bain (1957) indicate no change in whole brain

GABA, glutamate, taurine , and aspartate; de Feudis and Elliot (1968) detected a 17% increase in GABA concentration during PTZ-induced seizures; however, De Ropp and Snedeker (1961) obtained a 13% decrease in glutamate, but a 13% increase in glutamine concentration in similar experiments with rats. Strychnine-induced seizures in rats have been reported to elevate GABA levels by 15% (De Ropp and Snedeker, 1961) as well as to elevate glutamate (18%) and GABA (21%) in the rabbit whole brain (Saito and Tokunaga, 1967). With cobalt-induced experimental epilepsy, experimentors have generally found decreases in GABA levels (18 - 50%), decreased glutamate content (12 - 50%), and decreased aspartate levels (20 - 60%) (Van Gelder, 1972; Koyama, 1972; Emson and Joseph, 1974).

However, since many of these amino acids are important intermediates in mainstream carbohydrate and energy metabolism, it is possible that the displaced amino acid levels detected on seizure initiation or propagation are aberrations in metabolic processes, rather than modifications of neurotransmission processes. For this reason, we chose to examine K^+-stimulated release of amino acids in addition to the synaptosomal content, as a more specific marker for the involvement of amino acid neurotransmitter systems.

From the results presented earlier, central manipulations mediated by PTZ and strychnine were found not to affect the amino acid content of the cerebral cortex in seizure induced rats. Cortical synaptosome studies from PTZ-convulsed animals showed no changes from controls in their amino acid content, nor did they exhibit any apparent

differential amino acid release, in response to high levels of potassium. Hence, in our experimental conditions, it would appear that the chemical convulsants tested are inactive towards amino acid transmitter storage and release in the cerebral cortex. It is possible that their convulsive action could be attributed to involvement at another area of the nervous system not yet tested, e.g. the spinal cord. Alternatively, the site of action may not be at the pre-synapse as examined here, but rather at the postsynaptic membrane. Thus if PTZ was found, for example, to elevate the number or affinity of glutamate receptors, synaptic excitation would be explicable as would the anticonvulsant action of the glutamate antagonist GDEE. If this was found experimentally, a search for a similar receptor aberration in human epileptogenic brain tissue would be necessary.

(b) Central manipulation by cortical ablation

Since the existence of the cortico-striatal pathway has been defined, much work has been carried out to identify the transmitter of these projections. Initially Divac et.al. (1977) showed that a lesion in the frontal cortex was accompanied by a large reduction in the high affinity uptake of L-glutamate in the anterior part of the neostriatum. This was confirmed by McGeer et.al. (1977) in P_2 preparations of the corpus striatum. Other workers later showed that a frontal cortical ablation was followed by a larger reduction in the content of L-glutamate than that of any other amino acid examined. Kim et.al.(1977) reported this using homogenates of rat neostriata, whereas Young et.al. (1981) reports a reduction in glutamate content only in

homogenates of the cat ventrolateral thalamus, and not in the corpus striatum. Such data highlights the difficulties of interpretation when using the relatively non-specific ablation technique. We have sought more specific methods to acquire data on the nature of the cortico-striatal pathway. Our results show a specific decrease in stimulated glutamate and aspartate release from P_2 preparations prepared from rat neostriatum. This correlates well with the work of Rowlands and Roberts (1980) who also showed a decrease in stimulated glutamate release after cortical ablation. Differential release of a candidate neurotransmitter is thought to be a more specific index to the synaptic nature of the projection concerned, than differential amino acid content. Also the specificity of a glutamatergic/aspartic component to the nerve tract is further enhanced by the fact that when the parasaggital lesions were made more medially than outlined, reduced differential glutamate release and content was not observed (unpublished observations).

The fact that protein yield was decreased following lesioning of the pathway is an indication of severe degeneration of neurones and glialosis taking place in the neostriatum. Fractionation of the brain homogenates results in almost a 50% decrease in yield of P_2, probably because of the relative inability of glial cells to sediment (compared to nerve terminals) using the centrifugation spin speeds in our preparation. Hence in our calculations we have used tissue wet weights as concentration parameters for our amino acid levels. This is in common with most other authors

(Kim et.al., 1977; Young et.al., 1981; Rowlands and Roberts, 1980).

The fact that no differences were detected in specific binding of glutamate to membranes after lesioning implies no changes in receptor affinity or number. Mclennan (1980) has also found no evidence of supersensitivity either with L-glutamate or the more potent ligand - kainic acid. However, glutamate receptor levels could follow a time course after lesioning/ablation, such that maximum changes occur after one month. Therefore we cannot rule out that receptor analysis may provide useful additional information as to the identity of the transmitter at these projections.

This analysis of differential transmitter release and content provides not only information confirming the role of glutamate as a neurotransmitter, but it may also provide complimentary animal models to many disorders of the human nervous system, e.g. Huntington's chorea and tardive dyskinesia (McGeer and McGeer, 1976; McGeer et.al., 1978; Coyle and Schwarcz, 1976) currently of unknown aetiology.

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