EVIDENCE IN FAVOUR OF GLUTAMATE AS A MEDIATOR OF SYNAPTIC TRANSMISSION

by

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ABSTRACT

A microiontophoretic investigation of neurones in the spinal cord, cuneate nucleus, thalamus and cerebral cortex in the anaesthetized cat indicated that glutamic acid diethyl ester (GDEE), α-methyl-glutamate (αMG) and DL-methionine-DL-sulphoximine (MSO) could reversibly block, and glutamic acid dimethyl ester (GDME) and para-chloromercuriphenylsulphonate (pCMS) could enhance the excitation of neurones produced by glutamate and by orthodromic nerve stimulation. The action of GDEE was relatively specific in that it was possible to block glutamate-induced excitations without appreciably reducing the sensitivity of neurones to L-aspartate (Asp), L-cysteate (Cys), DL-homocysteate (DLH) and acetylcholine (ACh), whereas αMG and MSO showed no specificity of action and antagonized the excitatory effects of all the amino acids to the same extent. GDME and pCMS enhanced the action of glutamate and aspartate to a greater extent than that of DLH. The responses of neurones in the spinal cord and thalamus to electrical stimulation of branches of the sciatic nerve and of cuneate neurones to dorsal
column stimulation were blocked by GDEE and enhanced by GDME in the same manner as glutamate responses. GDEE blocked the responses of cortical neurones to glutamate and thalamic stimulation but the depressant effect was not as commonly observed as in other areas.

Studies on the uptake of labelled glutamate into crude synaptosomal preparations of rat cerebral cortex confirmed the existence of a high and low affinity uptake mechanism for glutamate and showed that GDME inhibits the high affinity uptake system. Glutamate and GDEE, but not αMG, enhanced the release of labelled glutamate from slices of rat cortex. No competition between glutamate and GDEE was observed in this system.

αMG was found to block the responses of the abdominal stretch receptor organ of the crayfish to applied glutamate. GDEE blocked the responses of the closer muscle in the claw to electrical stimulation of the excitatory nerve to this muscle.
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**LIST OF ABBREVIATIONS**

ACh  acetylcholine
Asp  L-aspartate
ATP  adenosine triphosphate
cm.  centimetre
C.N.S.  central nervous system
Cys  L-cysteate
DC  direct current
DFP  diisopropyl phosphorofluoridate
DLH  DL-homocysteate
e.j.p.  excitatory junctional potentials
EPSP  excitatory postsynaptic potentials
g.  gravities
GABA  γ-aminobutyric acid
GDEE  glutamic acid diethyl ester
GDME  glutamic acid dimethyl ester
Glut  L-glutamate
gm.  gram
HA 966  1-hydroxy-3-aminopyrrolidone-2
5-HT  5-hydroxy-tryptamine
I.V.  intravenous
kg.  kilogram
Km  Michaelis affinity constant
LSD  lysergic acid diethylamide
M  molar
mm.  millimetre
mM  millimolar
mCi.  millicurie
mg.  milligram
min.  minute
ml.  millilitre
msec.  millisecond
MSO  DL-methionine-DL-sulphoximine
MΩ  megohm
nA  nanoampere ($10^{-9}$ ampere)
NA  noradrenaline
p  probability
pCMS  para-chloromercuriphenylsulphonate
s  concentration
sec.  second
S.I.U.  stimulus isolation unit
v  velocity
V  Volt
VL  nucleus ventralis lateralis
VPL  nucleus ventralis posterolateralis
Vmax  maximum reaction rate
αMG  α-methyl-L-glutamate
γMG  γ-methyl-L-glutamate
μ  micron ($10^{-6}$ metre)
μA  microampere ($10^{-6}$ ampere)
μl.  microlitre ($10^{-6}$ litre)
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CHAPTER I

INTRODUCTION

The original suggestion that chemical agents might be involved in the transmission of nerve impulses came from T.R. Elliott in 1904, when he reported the conspicuous parallelism between stimulation of sympathetic nerves and the action of adrenalin upon the viscera, and postulated that adrenalin might be released by the terminals of nerve endings and be responsible for the sympathetic action. This hypothesis has been widely accepted and extended to include the parasympathetic nerves and transmission across neuromuscular junctions and synapses within the central nervous system. Since it was found that adrenalin was not the transmitter at all of these synapses the list of possible mediators of synaptic transmission has been expanded to include a wide variety of compounds. Latecomers to this list of putative transmitters have been the amino acids, of which L-glutamate, a dicarboxylic α-amino acid with a five carbon chain, has shown the most promise as a possible excitatory transmitter.

The history of glutamic acids dates back to 1866 when Ritthausen isolated an organic acid from wheat gluten hydrolysates. He observed that when wheat protein was hydrolyzed with sulphuric acid and the reagent removed with
calcium hydroxide, an amino acid sufficiently strong to decompose calcium carbonate was obtained. He named this substance glutamic acid, and was later able to isolate and crystallize it. The chemical synthesis of glutamic acid was carried out by Wolff in 1890, by means of a complex series of reactions, beginning with levulinic acid. There then followed investigations into the biological properties of this amino acid, beginning with the demonstration that glutamic acid was capable of reducing methylene blue in frog muscle preparations (Thunberg, 1920) and that it could be oxidised by minced peripheral nerve (Thunberg, 1923). Since then the metabolic role of glutamic acid, especially in nervous tissue because of its high concentration there (Krebs, Eagleston and Hems, 1949), and its probable importance in maintaining brain ammonium levels (Weil-Malherbe, 1950), has been studied in great detail. The principal metabolic pathways and enzymes considered to be of importance in the synthesis and breakdown of glutamic acid are shown in figure 1.

The observation of Krebs and Eagleston in 1949 that glutamate could reduce the loss of potassium ions from brain slices incubated in saline was the first indication that glutamate might have an influence on ionic fluxes in nervous tissue. Later Hayashi (1954) noted that application of sodium glutamate to the motor cortex caused clonic convulsions and suggested that glutamate accelerates the shift of sodium into nerve cells, causing excitation. However, it was not until Curtis et al., (1960) showed that iontophoretic
Figure 1. The principal enzymes and products involved in glutamate metabolism.
application of glutamate and other acidic amino acids to the immediate vicinity of individual neurones that the question of a possible synaptic role for glutamate was seriously raised.

Before glutamate can be considered as a mediator of synaptic transmission it must satisfy a number of criteria which have been established for all putative transmitters (McLennan, 1963; Werman, 1966). With the increased knowledge of the general mechanisms of synaptic transmission, the manner in which a criterion may be satisfied has changed, new criteria have been established while others have been subdivided. However, the six basic criteria remain as follows:

1. The putative transmitter must be present in the nerve endings where it is presumed to be active.
2. There must be a replacement mechanism for the transmitter which is released.
3. It must be demonstrated that the transmitter is released during synaptic activity.
4. An inactivation mechanism must be present to terminate the action of the transmitter.
5. The substance must mimic the action of the physiological transmitter in all its aspects.
6. The substance must react in a similar manner to the transmitter in the presence of pharmacological agents. The similar reaction of a pharmacological agent at a specific synapse and on the action of a given compound, is strong evidence to suggest that the transmitter at that synapse and the compound are identical. For this reason the last
criterion is gaining importance in determining which transmitters may be active in specific pathways in the brain.

The role of glutamate in the nervous system has been reviewed by a number of authors (Curtis, 1965; 1969; Kravitz et al., 1970; McLennan, 1970a; Johnson, 1972). Despite the numerous objections which have been raised, none of these articles seriously refutes the possibility that glutamate may act as a mediator of synaptic transmission. The reason for the difficulty in firmly establishing whether glutamate satisfies all of the criteria at any one synaptic junction has been that experiments have not been confined to one anatomical area of the nervous system and have been carried out on a wide variety of test animals. This discussion will be limited to the two general areas in which most of the research has been done, i.e. the mammalian central nervous system and the invertebrate neuromuscular junction.

THE MAMMALIAN C.N.S.

1. DISTRIBUTION OF L-GLUTAMATE.

The presence of L-glutamate in all areas of the nervous system has been established (Krebs et al., 1949; Tallan, 1962; Battiston et al., 1969; Duggan and Johnston, 1970a,b). In vertebrates, the concentration of free glutamic acid is higher in nervous tissue than in other tissues and in nervous tissue it is found in larger quantities than any other amino acid (Tallan, 1962). The concentration of glutamate is not
constant throughout the nervous system, but instead, has a characteristic distribution (Johnson and Aprison, 1971). It is at its lowest in sympathetic ganglia, increasing through the dorsal root ganglia, spinal cord grey matter to the thalamus and cerebral cortex to its highest concentration (10.9 - 12.4 μmoles/gm.) in the caudate nucleus, pyriform lobe and the sylvian gyrus.

As the glutamate content varies between higher and lower levels of the C.N.S., so it varies between areas at the same neural level. This latter variation is perhaps less likely to be linked to different metabolic needs of the tissues than the former. There is a higher level of glutamate in dorsal roots than in the peripheral nerves or ventral roots (Duggan and Johnston, 1970a,b), and glutamate content in lumbosacral grey matter is greater than in the white matter. Furthermore the sensory nuclei of the medulla have a significantly higher glutamate content than other medullary regions (Johnson and Aprison, 1970).

It appears that anatomical location is not the only factor which exerts an influence over the concentration of brain amino acids, since glutamate varies in the maturing mammal. Of all the tested amino acids, glutamate exhibited the greatest increase in the developing cortex of the kitten during maturation (Berl and Purpura, 1963). Since morphological studies show that the growth of cortical tissue and the development of neuronal connections in this animal follows the same time course as the increases in glutamate
concentration, (Noback and Purpura, 1961) it seems likely that these two processes are related. Experiments in young dogs (Dravid et al., 1965) showed that the changes in glutamate content up to maturity differed in the various regions of the brain and these variations may possibly be related to histological or functional differences.

Although studies on the anatomical localization of the total glutamate content of the brain are of interest, only a small portion of the total is likely to be important in synaptic transmission since the greatest part is involved in numerous metabolic processes. Presumably this portion, like other potential transmitters (Whittaker, 1965) should be located in the axonal terminals of neurones. Indirect calculations based on the amount of glutamate in grey and white matter have led Tower (1960) to suggest that most of the C.N.S. glutamate is associated with neurones rather than glia. However, subcellular fractionation studies (Mangan and Whittaker, 1966) have failed to show a uniquely synaptosomal localization for any amino acid. All amino acids examined had the same subcellular distribution as cytoplasmic markers, with only a small portion being retained in the particulate fraction. It is possible that this small amount is that portion of the total brain glutamate involved in synaptic transmission, since most of the particulate glutamate has been found to be associated with the synaptosomal fraction (Ryall, 1962). Further there appears to be sufficient glutamate in extracts of this fraction to elicit excitatory
effects when applied iontophoretically to single cells (Krnjević and Whittaker, 1965). In addition a synaptic role for glutamate is indicated by the observation that exogenously applied $^3$H-glutamate tended to localize in the synaptosomal fraction to a greater extent than other amino acids. The possibility that synaptosomal glutamate serves as a precursor for GABA as it does in other brain fractions (Berl, Nicklas and Clarke, 1970) seems unlikely since the synaptosomal fractions which accumulate glutamate differ from the corresponding fractions which accumulate GABA (Wofsey et al., 1971).

The suggestion has been made that functional compartmentation of glutamic acid in the nervous system (Berl and Purpura, 1966; Berl, Clarke and Nicklas, 1970; Berl et al., 1962; O'Neal and Koeppé, 1966) may be of significance in the determination of which portion of the total brain glutamate is important in synaptic transmission. This compartmentation may be unique to the brain since it is not evident in other tissues such as the liver (Berl et al., 1962). There are two distinguishable compartments or glutamate pools (Berl and Clarke, 1969); a small pool, associated with the so-called "synthetic tricarboxylic acid cycle" which appears to be the primary precursor of glutamine and has a rapid turnover; and a larger pool, associated with the "tricarboxylic acid energy cycle" which has a large quantity of transaminases, and is involved in energy metabolism of amino acids. It is interesting to note that in the immature animal a smaller percentage of the
total brain glutamate is involved in energy production than in the mature animal (van den Berg, 1970). Although there is no evidence as yet to connect either of these pools with a transmitter function, the probability of a dual system separating metabolic and transmitter pools of this amino acid has been suggested (Johnson, 1972).

Rizzoli (1968) presented further evidence in favour of glutamate having a transmitter function when he observed that glutamate concentrations in specific areas of the spinal cord decreased following spinal transection. This is unlikely to be due to metabolic changes since other amino acids (except glycine) remained at normal levels. He suggested that the degeneration of a descending pathway which utilizes a transmitter associated with glutamate caused this depletion.

2. REPLACEMENT OF THE TRANSMITTER.

Glutamate released from nerve endings must be replaced. Unlike transmitters such as ACh, numerous sources of glutamate exist which could fulfill this function, however the problem lies in determining which of these sources is drawn upon as the replacement mechanism.

An obvious consideration is that the "transmitter pool" of glutamate may have the ability to draw upon metabolic sources of glutamate precursors. Isolated nerve endings have the ability to metabolize glucose and form glutamate (Bradford and Thomas, 1969). Isolated synaptic vesicles from rat cortex have been shown to contain labelled glutamate following
an intraventricular injection of $^3$H-acetate (Farrow and O'Brien, 1971), thereby indicating an ability of the vesicles to pick up glutamate from the metabolic pool or to synthesize it directly from acetate. Dobkin (1970) found that there was a decrease in the glutamine content of the cerebral cortex but not in that of glutamate following afferent nerve stimulation and Johnson (1972) suggested that this might be due to a conversion of glutamine to glutamate in order to maintain the transmitter pool of glutamate.

The primary enzyme involved in glutamate synthesis is glutamate dehydrogenase and the question arises whether it is involved in synaptic transmission. When the spinal cord of the chick embryo was sectioned in such a manner that it developed in the absence of extrinsic neuronal connections, the glutamate dehydrogenase level in the spinal cord was found to be significantly lower than in control chicks (Burt and Narayanan, 1972). If the enzymatic deficit is associated directly with the operative deficit, these data would suggest that a certain amount of the over-all glutamate dehydrogenase activity is associated with the development of synaptic connections in the spinal cord. Topical application of diethyl-$\alpha$-fluoroglutarate, an inhibitor of glutamate dehydrogenase in the cerebral cortex, causes a decrease in the amplitude of the surface negative component of the response to thalamic stimuli (Cohen et al., 1972). However, depletion of numerous other metabolic intermediates and energy reserves also takes place. This latter observation makes it difficult
to conclude whether glutamate depletion is the sole cause of the reduction in electrical activity. It is interesting to note that a number of drugs such as chlorpromazine and phenothiazine which have definite behavioural effects are inhibitors of glutamate dehydrogenase (Skemisa and Fahien, 1971). Kinetic correlations between the inhibitory action on glutamate dehydrogenase and the antipsychotic activity of these drugs suggests that the two activities are related.

A second possible source is the axonal flow of glutamate from the cell body to the nerve endings. Such a flow has been observed for glutamate in the peripheral nerves of frogs and rats (Kerkut et al., 1967). It seems likely that a similar process for glutamate would exist in the C.N.S. since axoplasmic transport of other amino acids has been noted there (Foulkes and Robinson, 1970). This process is unlikely to be of prime importance in replacing transmitter glutamate, since the transport is in both directions (Kerkut et al., 1967). Further, the ease with which glutamate can be formed by synaptosomes from basic metabolites would make this mechanism superfluous.

It has been known for some time (Stern et al., 1949) that the brain has the ability to actively accumulate glutamate from its environment which is a further possible source of this amino acid. It appears that the blood-brain barrier in the adult allows fairly large amounts of glutamate to pass into the brain (Roberts et al., 1959). This is presumably balanced by an efflux of a similar amount since in
contrast to liver, muscle and kidney there is no net increase in the glutamate content of the brain following intravenous injection of large amounts of glutamate (Schwerin et al., 1950). This is not true in immature animals where the blood-brain barrier is not completely formed (Himwich et al., 1957). It is estimated that the contribution of plasma glutamate to the total glutamate pool of the brain is approximately 10% (Roberts et al., 1959), the remaining 90% originating primarily from glucose and other metabolites. Although glutamine can act as a precursor to glutamate, this interconversion is more likely to be of homeostatic significance than direct supply, since glutamine does not appear to cross the blood-brain barrier more rapidly than glutamate (O'Neal and Koepppe, 1966).

Finally, synaptosomal glutamate may be replaced by active accumulation from the extracellular spaces. With crude synaptosomal preparations from brain, glutamate (and aspartate) can be shown to be taken up by dual mechanisms, one of high affinity specific for the two and a second of lower affinity which is probably common to all amino acids (Logan and Snyder, 1972). Labelled glutamate taken up by brain slices can be released as glutamate following electrical stimulation (Arnfred and Hertz, 1971), suggesting that glutamate taken up by synaptosomes may be recycled in the transmitter pool.

3. **GLUTAMATE RELEASE DURING SYNAPTIC ACTIVITY.**

Although only a limited amount of research has been done
there are indications that glutamate is released under certain conditions following nerve stimulation. Jasper et al., (1965) showed that glutamate was released from the pial surface of the cerebral cortex of cats at a much higher rate during arousal states than when the EEG showed the constant spindle patterns characteristic of sleep. GABA showed the converse pattern of release. These data indicate a possible modulation of arousal and sleep by the two amino acids. Further studies indicated that a similar release of glutamate could be obtained following stimulation of the medial reticular formation (Jasper and Koyama, 1968, 1969). The arousal following stimulation of this area was accompanied by release of both glutamate and acetylcholine from the cortex. Following thalamic stimulation only acetylcholine could be collected, implying that the glutamate release following reticulo-cortical stimulation has some degree of specificity.

A similar release of glutamate has been observed following electrical stimulation of brain cortex slices (Arnfred and Hertz, 1971) which appears to be specific since the efflux of leucine by similar stimulation was not affected (Srinivasan et al., 1969). The observation that glutamate, lysine and leucine effluxes in the corpus striatum are all equally enhanced by electrical stimulation (Katz et al., 1969) may reflect some regional differences in function. It has been postulated that spreading depression which occurs in the cortex and retina is caused by a release of glutamate from neurones (van Harreveld, 1959). If glutamate was released
on stimulation of the cortex or the retina, it would cause a depolarization of the area with a further efflux of glutamate and K\(^+\) which in turn would depolarize adjacent neurones thus causing the spreading depression. A study of the excitability of cortical neurones during spreading depression (Phillis and Ochs, 1971) has confirmed that glutamate could be responsible for the excitatory and early depressive phases. Although attempts to detect a release of glutamate in the cortex during spreading depression have been largely unsuccessful (van Harreveld and Kooiman, 1965), similar attempts in the retina have had positive results (van Harreveld and Fifkova, 1970, 1972).

Another location where glutamate efflux has been observed following electrical stimulation is the peripheral nerves in frogs (Wheeler et al., 1966). Once again the release of glutamate appears to be highly specific as no efflux of aspartic acid, leucine, glycine or lysine occurred. It was considered to be energy dependent as it could be blocked by sodium azide. From experiments on desheathed nerves (De Feudis, 1971) it appears that only a portion of the total exchangeable glutamate can be released by electrical stimulation. The question whether all nerve fibres show an efflux of glutamate on stimulation is raised, however, the experiments by van Harreveld and Kooiman (1965) and Jasper and Koyama (1969) in the cortex, suggest that this might not be the case.
4. **INACTIVATION OF GLUTAMATE.**

There is a higher concentration of the enzymes involved in glutamate catabolism in non-cholinergic nerves than in cholinergic nerves. A study of these enzymes suggests that they are located intracellularly and tend to localize in specific fractions (Salganicoff and De Robertis, 1965). Most of the aminotransferases and other general metabolic enzymes can be found within the mitochondrial fraction, whereas glutamine synthetase is primarily located in the vacuolar system of the mitochondria and has a very low concentration in the synaptosomal fraction. On the other hand, glutamic acid decarboxylase is located primarily in the nerve endings and axoplasm of neurones, making it a more likely candidate for the catabolism of excess glutamate in this fraction. However, when labelled glutamate is taken up by isolated synaptosomes it is possible to extract labelled aspartate, glutamine, alanine and GABA (Bradford and Thomas, 1969) indicating that all of the enzymes necessary for glutamate catabolism are present to some extent in nerve endings.

Because of its location it is unlikely that glutamine synthetase has any importance in the inactivation process. The fact that methionine sulfoximine which inhibits glutamine synthetase causes a decrease, if anything in the excitability of a cell to glutamate (Curtis et al., 1972) supports this view. Glutamic acid decarboxylase on the other hand may be involved; since systemic administration of the inhibitor
thiosemicarbazide is followed by an increased neuronal excitability in subcortical structures (Preston, 1955), and the drug has also been shown to enhance the effect of iontophoretically applied glutamate to single cells (Steiner and Ruf, 1966, 1967). The latter phenomena may be explained in the following manner. Since glutamic acid decarboxylase is located intracellularly and re-uptake of released glutamate appears to be the primary inactivation process it is possible that a buildup of intracellular glutamate caused by the inactivation of the decarboxylase, might cause an increase in the concentration gradient, a decreased uptake, and therefore an enhanced action. The lack of extracellular catabolic mechanisms increases the probability that re-uptake is the preliminary step in inactivation.

The ability of isolated synaptosomes to accumulate glutamate is well documented. The demonstration in the cerebral cortex of a population of synaptosomes which selectively accumulates glutamic and aspartic acid has been taken to indicate that this mechanism may not be uniformly distributed about all cells (Wofsey et al., 1971). The presence of a high plus a low affinity uptake mechanism (Logan and Snyder, 1972) would make this system that much more efficient. The uptake appears to take place primarily into the synaptosomal fraction, rather than into the general cytoplasmic pool where the major part of endogenous glutamate is found (Kuhar and Snyder, 1970). The existence of several distinct types of transport systems for the uptake of amino acids in
isolated nerve endings has been postulated (Blasberg and Lajtha, 1966; Peterson et al., 1972). An amino acid may have an affinity to more than one transport system (Blasberg and Lajtha, 1966), so that in addition to a high affinity site specific for that amino acid it may have a lower affinity at another. This may explain the two-affinity system for glutamate and aspartate uptake described by Logan and Snyder (1972). Experiments by Peterson et al., (1972) on aspartate uptake, which, according to Blasberg and Lajtha (1966) utilizes the same mechanism as glutamate, suggest that the transport system is $\text{Na}^+$ dependent. It is possible that the transport of these amino acids may derive energy directly from a transmembrane $\text{Na}^+$ gradient and follow the downhill movement of $\text{Na}^+$ into the cell and the fact that the swelling observed when brain slices are incubated with glutamate is also $\text{Na}^+$ dependent (Lund-Andersen and Hertz, 1970), tends to confirm this possibility.

Organic mercurials have been shown to inhibit intestinal amino acid transport (Reiser and Christiansen, 1965) and the uptake of amino acids into brain slices (Curtis, Duggan and Johnston, 1970). As expected, iontophoretic application of these mercurials enhances glutamate action on spinal neurones, presumably by blocking the uptake and increasing the extracellular concentration (Curtis, Duggan and Johnston, 1970). Thiosemicarbazide, which enhances glutamate activity when applied systemically, has no effect when applied iontophoretically in amounts unlikely to affect intracellular
enzymes. This would be expected if uptake was the first step in the inactivation process.

5. COMPARISON OF ACTION OF GLUTAMATE AND THE PHYSIOLOGICAL TRANSMITTER.

Iontophoretic application of glutamate causes a powerful depolarization and excitation of C.N.S. neurones (Curtis et al., 1960) which appears rapidly with onset of the applied current and is terminated equally rapidly with its cessation. This is the first criterion of an excitatory transmitter candidate and this observation has led to most of the remaining experimentation and speculation.

Glutamate is able to excite neurones in most regions of the C.N.S. Examples of areas in which cells have been tested include the neocortex (Krnjević and Phillis, 1963a); paleocortex (Legge et al., 1966); thalamus (Curtis and Davis, 1962); caudate nucleus (McLennan and York, 1966); cerebellar cortex (Krnjević and Phillis, 1963a); amygdala (Straughan and Legge, 1965); corpus striatum (Fifkova and van Harreveld, 1970); hippocampus (Biscoe and Straughan, 1966); cuneate nucleus (Galindo et al., 1967); brain stem (Curtis and Koizumi, 1961); olfactory bulb (von Baumgarten et al., 1963) retina (Kishida and Naka, 1968); and spinal cord (Curtis et al., 1960). Glutamate also exerts a depolarizing action upon nerve terminals (Curtis and Ryall, 1966). One valid argument against glutamate as a transmitter is that it appears to have a widespread non-specific effect on all neuronal
membranes. However, not all cells are equally sensitive to glutamate or respond in the same way, thus Krnjević (1964) described some cortical cells which responded in a steady, easily graded discharge, while others reacted in short bursts in an "all-or-none" manner. The very high threshold of Betz cells to glutamate described by Krnjević (1964) as well as the observation that other cells such as motorneurones (Curtis et al., 1960), and Mauthner cells in fish (Diamond, 1963) are only depolarized subliminally by glutamate and very seldom fire an action potential, may be due to the large size of these neurones. However, the same argument cannot be used to explain the difference in the sensitivity of neurones in separate areas of the thalamus (McLennan et al., 1968) and between different neurones in the spinal cord (Duggan, 1971), to the excitatory amino acids. A similar difference in neuronal excitability to iontophoretically applied glutamate has been described in the olfactory bulb (von Baumgarten et al., 1963), however it seems probable that the lack of effect of glutamate on certain cells in this area is due to the action of smaller adjacent inhibitory neurones which block the excitation of the larger cells (McLennan, 1971). The fact that glutamate has no apparent effect in motor nerves or skeletal muscles (Takeuchi and Takeuchi, 1960) suggests that the glutamate "receptor" might not exist on all excitable membranes.

The ability of glutamate to excite neurones is not specific for this amino acid since any compound possessing
two acidic groups, one amino group and an optimal separation of 2 to 3 carbon atoms between the α-amino and ω-acidic groups, can excite the same neurones which are sensitive to glutamate (Curtis and Watkins, 1960, 1963). A number of naturally occurring amino acids such as aspartate and cysteate are as active as glutamate, while many non-natural compounds, especially those which have undergone N-alkylation (eg. N-methyaspartic acid) are considerably more potent than their natural counterparts. Glutamate is therefore neither the strongest nor the weakest of this group of excitants. Once again, however, there appears to be a regional difference in the sensitivity to these related compounds. Spinal neurones seem to be equally sensitive to the L- and D-enantiomorphs of glutamate (Curtis and Watkins, 1960), whereas cortical neurones are much more sensitive to the L form (Krnjević and Phillis, 1961). Similarly, spinal Renshaw cells appear to be relatively more sensitive to L-aspartate than to L-glutamate when compared with a population of dorsal spinal interneurones (Duggan, 1971). McLennan et al., (1968) found a difference in sensitivity of cells in the ventrolateral thalamus to DLH and glutamate. This led to the proposal that two types of receptor for the excitant amino acids may be present:— an unspecific receptor common to all cells, and a specific receptor activated only by glutamate.

The excitatory action of glutamate on the membrane "receptor" appears to be explicable in terms of changes in the ionic permeability. Glutamate has been shown to increase
the Na⁺ permeability fivefold relative to that of K⁺ in slices of cerebral cortex (Bradford and McIlwain, 1966) and these changes result in a net influx of Na⁺, which has been calculated to be sufficient to cause depolarization (Harvey and McIlwain, 1968). These changes in permeability are probably due to the excitation properties of glutamate since similar changes could be observed with other excitatory amino acids, but not with analogues which showed no excitatory action. A concomitant loss of energy-rich phosphate from the brain slices and the appearance of inorganic phosphate may be due to the acceleration of a Na⁺, K⁺-dependent ATPase by the additional Na⁺ entering the tissue. Although changes in membrane Ca²⁺ with an increased Ca²⁺ influx do occur following application of glutamate to the incubation medium (Ramsey and McIlwain, 1970), the chelating properties of glutamate do not appear to be responsible for its mechanism of action (Pull et al., 1970).

A loss of K⁺ and a gain of Na⁺, similar to that observed on incubating brain slices in solutions containing glutamate, has been shown following prolonged electrical stimulation of similar preparations (Keesey et al., 1965). Since then attempts have been made to determine whether these two means of stimulation have similar mechanisms of action. The effect of glutamate on Ca²⁺ fluxes differs from that of electrical stimulation, in that the former causes a net influx of Ca²⁺, whereas the latter causes an increased Ca²⁺ turnover but no net flux (Ramsey and McIlwain, 1970). These results
indicate a difference in mechanism, as do experiments with tetrodotoxin. Tetrodotoxin completely inhibits the electrically induced Na\(^+\) entry in neocortical tissue (McIlwain et al., 1969), but only partially inhibits the Na\(^+\) entry induced by glutamate (Pull et al., 1970). If one differentiates between the mechanism of slow Na\(^+\) influx during subthreshold depolarization and the mechanism of rapid Na\(^+\) influx characteristic of the actual spike potential it appears that tetrodotoxin blocks the spike generation caused by either electrical or glutamate stimulation, but has no effects on the conductance or depolarization caused by glutamate (Bernardi et al., 1972; Curtis et al., 1972; Pull and Zieglgänsberger, 1972).

A number of mechanisms have been proposed to explain the changes in membrane permeability caused by glutamate. One theory is that depolarization may be due to a redistribution of the pre-existing glutamyl groups of peptides linking a transmembrane pore and in this manner remove the obstruction to the flow of Na\(^+\) and K\(^+\). A second possibility is that glutamate may penetrate the membrane pore to its inner mouth, displacing Ca\(^{++}\), which acts as a counter ion to the acidic groups and limits the access of Na\(^+\) (Pull et al., 1970). Thirdly depolarization may be due to a carrier-type of process which transports the amino acid and Na\(^+\) into the cell, (Pull and Zieglgänsberger, 1972). None of these theories can be substantiated. However, the last suggestion seems unlikely since blocking of glutamate uptake enhances
rather than blocks the excitatory action of glutamate (Curtis et al., 1970).

It is essential that the mechanism of glutamate excitation should be shown to be identical to that of the synaptically evoked potential changes before glutamate can be accepted as a transmitter. However, attempts to compare the equilibrium potentials of glutamate and the evoked EPSP have been largely unsuccessful. The only serious attempt (Curtis, 1965) showed that the apparent equilibrium potential for the action of glutamate was at a higher level of polarization than that of the EPSP. Most reviewers of these results (McLennan, 1970; Curtis et al., 1972; Johnson, 1972) feel that this does not seriously weaken the likelihood of glutamate being a transmitter, since it is probable that the applied glutamate and the physiological transmitter act at different regions of the cell. For technical reasons the glutamate is applied near the recording electrode, which is located in the perikaryon of the nerve cell. Since most of the synapses involved in the monosynaptic reflex appear to be located in the dendritic regions of the cell (Rall et al., 1967), any estimate of the equilibrium potential of the EPSP based on an impalement of the cell body may be seriously in error. The observation that low doses of glutamate can produce a slow membrane depolarization without any obvious conductance change (Bernadi et al., 1972) also suggests a possible dendritic site of action of glutamate. The fact that alteration of intracellular chloride concentration is without effect on the
depolarization caused by glutamate application or the excitatory transmitter (Curtis et al., 1972), further suggests that these two processes are similar.

In 1957, Lucas and Newhouse reported an interesting finding which may or may not have any bearing upon the mechanisms of glutamate excitation. They showed striking degenerative changes in the infant mouse retina following subcutaneous treatment with monosodium glutamate. These findings were confirmed, and in addition discrete lesions of the arcuate nucleus have been found (Olney and Ho, 1970; Hanson, 1970; Olney, 1971). The lesions in the retina and arcuate nucleus are similar, being characterized by rapid swelling of neuronal dendrites and cell bodies, followed by nuclear pyknosis, and occurring within 6 to 8 hours after administration. The hypothalamic lesion is accompanied by obesity and neuro-endocrine disturbances (Olney, 1969; Redding et al., 1971), while the retinal lesion produces characteristic changes in the electroretinogram (Buckser, 1969).

The possibility that the neurotoxicity and the excitatory properties of glutamate may be related was raised by Olney et al., (1971) when they showed that identical lesions could be caused by aspartate, cysteate and DLH, whereas other non-excitatory amino acids were ineffective (Olney and Ho, 1970). Experiments by Perez and Olney (1972) showed that the arcuate nucleus but not the adjacent hypothalamic areas accumulate glutamate following subcutaneous injection in young mice. If glutamate is a transmitter, then the high affinity uptake
mechanism needed to inactivate it could perhaps accumulate sufficient glutamate to cause cellular edema. The other possibility is that glutamate induces depolarization, increases the permeability of the neural membranes to Na\(^+\) and in this manner causes swelling. This has been shown to occur in brain slices in vitro (Harvey and McIlwain, 1968).

6. **THE ACTION OF PHARMACOLOGICAL AGENTS ON GLUTAMATE AND SYNAPTICALLY EVOKED EXCITATIONS.**

Experiments in which the action of pharmacological agents on glutamate responses and on synaptically evoked responses have been compared are very few. One of the few observations which has been made is that thiosemicarbazide and the organic mercurials can enhance the effects of iontophoretically applied glutamate (Steiner and Ruf, 1966, 1967; Curtis et al., 1970). However, these studies did not include an investigation of the effects of these substances on evoked potentials. Apparently these drugs are of little use in the investigation of possible glutaminergic pathways since thiosemicarbazide is without effect on glutamate responses when applied iontophoretically to spinal neurones (Curtis et al., 1970), and there is great technical difficulty in using the organic mercurials.

Another investigation by Boakes et al., (1970) claimed that LSD could block the response of iontophoretically applied glutamate to brain stem neurones. However, this effect was only seen with cells which were excited by 5-hydroxytryptamine.
Again the action of LSD on evoked responses was not tested. Cells not excited by 5-HT, but still excited by glutamate, were reported to be unaffected by LSD. The lack of specificity of LSD effects, and lack of LSD effect on cells not excited by 5-HT makes it a poor tool for investigating glutaminergic synapses.

A more promising recent series of experiments in the search for glutamate antagonists were those performed by Curtis et al., (1972) using methionine sulfoximine and methoxy-aporphine. They were able to partially block the effects of iontophoretically applied DLH and glutamate without appreciably affecting the action of acetylcholine. They were not, however, able to demonstrate blocking of synaptic firing of neurones and they concluded that these compounds were unsuitable for the investigation of possible glutaminergic pathways. A similar depression of amino acid responses without appreciably affecting ACh excitation has been observed by Davies and Watkins (1972) using the compound 1-Hydroxy-3-aminopyrrolidone-2. Once again, however, no discrimination between glutamate and the other amino acid excitations was observed.

THE INVERTEBRATE NEURO-MUSCULAR JUNCTION

As early as 1952, Lewis reported the presence of high concentrations of glutamate and aspartate in leg nerves of several invertebrate species. With the observation of
Robbins (1959) that glutamate could enhance neurally evoked contractions in crustacean muscle at relatively low concentrations, and could induce contractions in the absence of nerve stimulation at higher concentrations, glutamate became a candidate for the transmitter substance at the neuromuscular junction in invertebrates.

The same criteria which must be met for transmitters in the mammalian C.N.S. must be satisfied in determining the validity of glutamate as the transmitter at the invertebrate neuromuscular junction. The advantage here, however, is the relative ease of access to this synapse, and the fact that apart from aspartate no other rival for the position of transmitter has been found.

1. DISTRIBUTION OF L-GLUTAMATE.

In 1941, Silber showed that leg nerves of lobsters had a very high concentration of free amino acids. Later studies (Lewis, 1952; Marks et al., 1970) have shown that most of these compounds were present in concentrations some 100-fold higher, on a wet weight basis in invertebrate nerves than in mammalian nerves. Although the concentration of glutamate was not found to be conspicuously higher than other amino acids, it was the only compound isolated from crab and lobster nerve by Kravitz et al., (1963), which showed excitatory activity. Kravitz et al., (1970) felt that glutamate could account for about half the excitatory activity present in isolated axons, however, when the axon extracts were treated
with the enzyme glutamic acid decarboxylase, essentially all of the excitatory activity was destroyed.

2. REPLACEMENT OF THE TRANSMITTER.

This criterion is perhaps the least difficult of all to fulfill for glutamate, since it is a normal component of general metabolism. Although no research has been done on peripheral nerves Bradford et al., (1969) carried out experiments on ganglia of the snail and locust and showed that glutamate, aspartate and a number of other amino acids could be formed from glucose by nervous tissue and that metabolic patterns were similar to those found in rat cortical tissue.

The large quantity of free amino acids in invertebrate nerves might be due to a breakdown of proteins and peptides in the axon. Marks et al., (1970) found large quantities of proteolytic enzymes in the peripheral nerves of crabs and lobsters and they suggest that these enzymes may contribute to a complex liberation of amino acids, from protein, during axoplasmic flow. It seems likely that free glutamate can also flow down the axon since in brain-nerve-muscle preparations of the snail, glutamate, injected into the brain, moved along the nerve and could be found in the muscle (Kerkut et al., 1967). Its movement was rapid and seemed to be specific, since similar movement was not observed for xylose.

Apart from anabolism in the nerve and flow from the cell body a third possible mechanism for the accumulation of
glutamate in the nerve terminal is uptake from the surrounding environment. Glutamate is present in the hemolymph of the lobster in a concentration of $7 \times 10^{-5}$M, which is too low to cause depolarization of muscle fibres (Kravitz et al., 1970). Presumably portions of this glutamate, plus any transmitter glutamate which may be released in the interstitial spaces, could be reabsorbed by an active process. A Na$^+$-dependent system capable of moving glutamate against a concentration gradient into a nerve-muscle preparation, has been demonstrated by Iversen and Kravitz (1968). The uptake mechanism seems to be specific for glutamate since close structural analogues had little or no effect on the process.

3. GLUTAMATE RELEASE DURING SYNAPTIC ACTIVITY.

A number of researchers have demonstrated the release of glutamate into a fluid perfusing neuromuscular junctions on stimulation of excitatory nerves (Kerkut et al., 1965; Usherwood, Machili and Leaf, 1968; Kravitz et al., 1970). Usherwood, Machili and Leaf (1968) also recovered significant amounts of alanine, glycine and aspartate during this procedure. An increase in the extracellular Ca$^{++}$ was found to increase glutamate and alanine release, but had no effect on the release of glycine or aspartate. Since Ca$^{++}$ potentiates nerve-muscle transmission, presumably by increasing the amount of transmitter released from the motor nerve terminals (Usherwood, 1963), the above phenomena would be expected to occur if glutamate (or alanine which has no excitatory activity)
was the transmitter. Also as expected there was a greater glutamate release with an increased rate of stimulation of excitatory nerve (Usherwood, Machili and Leaf, 1968), while no glutamate was released during stimulation of inhibitory nerves (Kravitz et al., 1970). Evidence to suggest that at least some of the glutamate released had been transported down the axon, came from Kerkut et al., (1967). He reported that labelled glutamate placed in the medium bathing the isolated brain of the snail, was not only transported to the nerve-muscle junction, but was released therefrom on stimulation of the brain. Once again the amount of radioactive glutamate recovered on nerve stimulation was found to be proportional to the number of stimuli given to the brain. It is interesting to note that under similar circumstances of excitatory nerve stimulation but in the presence of the metabolic inhibitor 2,4-dinitrophenol, there is a depletion of round synaptic vesicles from crayfish nerve terminals (Atwood et al., 1972). Since these synaptic vesicles are thought to contain the physiological transmitter (McLennan, 1970) it is not unlikely that the glutamate released on stimulation originated from these round vesicles.

4. INACTIVATION OF GLUTAMATE.

There are a number of enzymes in invertebrate nervous tissue capable of converting glutamate to inactive compounds. It is possible to recover GABA, other amino acids and citric acid cycle metabolites following the administration of
labelled glutamate to preparations of snail and locust ganglia (Bradford et al., 1969). As far as is known, however, these enzymes are all located intracellularly, require other substances and cofactors for their activity, and are probably not directly involved with the removal of extracellular glutamate.

It seems more likely that extracellularly released glutamate is inactivated through a transport system which removes it from the interstitial spaces. As mentioned earlier, a transport system which is activated by an increase in external Na\(^+\), and inhibited by an increase in external K\(^+\), has been described by Iversen and Kravitz (1968) in the lobster. Baker and Potashner (1971) describe two possible uptake systems; one of which can be competitively inhibited by other acidic amino acids but not by neutral compounds and a second which is Na\(^+\)-insensitive and more specific for glutamate. These data suggest that the glutamate uptake mechanism serves some special function as do the experiments of Faeder and Salpeter (1970) who showed that the uptake of glutamate in insect nerve-muscle preparations was enhanced by nerve stimulation whereas a similar uptake of leucine showed no increase and that uptake was greater at neuromuscular junctions than in other regions of the tissue. They are of the opinion that the sheath cells which surround the nerve terminals are probably very important in the inactivation process and could possibly act as a barrier to blood glutamate and control the supply of glutamate to the axon.
5. COMPARISON OF ACTION OF GLUTAMATE AND THE PHYSIOLOGICAL TRANSMITTER.

Robbins (1959) and van Harreveld and Mendelson (1959), showed that direct application of low concentration of glutamate could cause contractions in crustacean muscle, accompanied by a depolarization of the muscle. Iontophoretic application of glutamate to crayfish muscle by Takeuchi and Takeuchi (1964) demonstrated that the sensitivity of the membrane to glutamate was not uniform. L-glutamate sensitive spots on the muscle were very circumscribed; moving the tip of the glutamate pipette by less than 10 μ eliminated or greatly attenuated the response and the spots coincided with points of innervation characterized by extracellularly recorded excitatory junctional potentials. Intracellular injections of L-glutamate had no effect on the muscle potential. In the continued presence of L-glutamate there was an initial increase in sensitivity to both glutamate and the transmitter followed by tachyphylaxis to applied glutamate and a decline in the response to the excitatory synaptic transmitter (Robbins, 1959; Takeuchi and Takeuchi, 1964; Usherwood and Machili, 1968). This led Takeuchi and Takeuchi (1964) to conclude that the receptors that respond to L-glutamate were identical to those which respond to the physiological transmitter. Usherwood, Cochrane and Rees (1968) reduced the likelihood that the action of glutamate was to cause the release of the physiological transmitter from the nerve terminal, by carrying out similar
experiments on locust muscles denervated for a number of days. In these preparations the discrete glutamate-sensitive spots remained in the absence of motor innervation.

That the receptor is relatively specific for L-glutamate can be seen from the inability of other compounds to cause excitation of invertebrate muscle. Of the four amino acids glycine, alanine, aspartate and glutamate released on nerve stimulation, only glutamate and aspartate showed any excitatory activity (Usherwood and Machili, 1968). Crustacean muscle is very insensitive to D-glutamate even when applied directly to L-glutamate sensitive spots (Takeuchi and Takeuchi, 1964). The sensitivity to glutamine is 100 times less than to L-glutamate and glutamine in turn is 10 times more potent than L-aspartate. Further studies by McDonald and O'Brien (1972) showed that cysteic acid and DLH, as well as a number of like compounds have similar but generally a lesser effect than L-glutamate.

In an attempt to explain the mechanism of glutamate excitation Dudel and Kuffler (1960) proposed that a conductance increase to Na\(^+\), coupled with a decreased conductance to K\(^+\) or Cl\(^-\) or both, could account for the excitatory junctional potentials in crayfish muscle. The replacement of Na\(^+\) with Li\(^+\) or Tris reversibly abolishes the e.j.p. as well as the effect of iontophoretically applied glutamate suggesting that the physiological transmitter and glutamate use similar mechanisms of depolarization. Ozeki et al., (1966) showed that both glutamate excitation and the e.j.p.
are immune to tetrodotoxin and saxitoxin and concluded that both these processes differ from the mechanism of spike electrogensis. Baránek and Miller (1968) found identical reversal potentials for glutamate and the miniature excitatory potentials, at the glutamate sensitive areas on insect muscles. Using different techniques Taraskevich (1971) found a similar equality in reversal potentials at the neuromuscular junction of crayfish. These observations further suggest that at least at the latter two synapses, the permeability changes brought about by glutamate and the excitatory transmitter are similar.

6. **THE ACTION OF PHARMACOLOGICAL AGENTS ON GLUTAMATE AND SYNAPTICALLY EVOKED EXCITATIONS.**

To date this criterion has been impossible to fulfill, due to the lack of pharmacological agents which specifically affect glutamate excitation of cells. The only piece of evidence which may prove to be specific for glutamate, is the observation that 5'-ribonucleotides potentiate both the excitatory junctional potentials of crayfish muscles and the effects of iontophoretically applied glutamate (Ozeki and Sato, 1970). These authors are of the opinion that the potentiating effect of the nucleotides is due to a facilitation of the binding of the transmitter or glutamate with the receptor of the post-synaptic membrane.
THE PRESENT STUDY

It becomes obvious from the review of the literature that the weakest criterion for determining the importance of glutamate in synaptic function is the comparison of the action of pharmacological agents on excitations produced by glutamate and the physiological transmitter. Pharmacological agents which specifically interact with the postulated glutamnergic synapses would be of extreme importance in determining which pathways utilize glutamate as a transmitter. These agents would interact with the postsynaptic "receptor" and could be expected to give information regarding the form of the active centres and the specificity of the receptor.

The glutamate receptor apparently reacts with the three ionized sites on the glutamate molecule (Curtis and Watkins, 1960). By utilizing analogues which have a side chain attached to one or more of the probable active sites of the glutamate molecule, it may be possible to find a non-active compound which would combine competitively with the receptor and in this manner block or reduce glutamate activity. A number of these analogues have been used in the past as antimetabolites and have been shown to antagonize or inhibit enzymes involved in glutamate metabolism. α-Methyl glutamate and β-hydroxyglutamic acid for example, have been shown to inhibit competitively the utilization of glutamic acid by Lactobacillus arabinosis (Ayengar and Roberts, 1952).
α-Methyl glutamate is a competitive inhibitor of glutamic acid decarboxylase in acetone powders of rat brain (Roberts, 1952), and has the ability to inhibit partially glutamine synthetase (Weil-Malherbe, 1969) in guinea pig brain homogenates. Inhibition of growth and cell division of *Euglena gracilis* has been obtained with the diethyl and dimethyl esters of glutamic acid (Owens and Blum, 1969), presumably due to a competitive inhibition of the glutamic acid acyl t-RNA synthetase (Owens and Blum, 1966). If one assumes that the glutaminergic postsynaptic receptor is a protein with active sites not unlike those of the glutamate enzymes, it seems likely that compounds which compete with glutamate for these enzymes might very well compete in a similar manner for the receptor. It is interesting to note that certain glutamate analogues, when injected into test animals, cause profound neurological and behavioural reactions (Roberts, 1952; Desi et al., 1967). The observation by Marshall (1971) that α-methyl glutamate was able to block the effects of iontophoretically applied glutamate on thalamic cells, gave further promise to the investigation of these compounds.

Another compound which has inhibitory effects on glutamate enzymes, and which has been investigated by Curtis et al., (1972) for possible glutamate blocking action, is methionine sulfoximine. MSO is a powerful convulsant and has been shown to cause a non-competitive inhibition of the glutamine synthetase of brain (Sellinger and Weiler, 1963; Lamar and Sellinger, 1965). The period of inhibition
of glutamine synthetase however, does not correspond with the period of neurological symptoms (Lamar, 1968) and since none of the other enzymes tested has been shown to be inhibited by MSO (De Robertis et al., 1967) it is possible that the neurological seizures are due to interference with a different mechanism.

Two other compounds, cyclobenzaprine (N,N-Dimethyl-5H-dibenzocycloheptene-Δ5,γ-propylamine) and hydrastinine-HCL (l-Hydroxy-6,7-methylenedioxy-2-methyl-1,2,3,4-tetrahydroisoquinoline) were also tested for possible blocking action. Cyclobenzaprine is a tricyclic antidepressant related to imipramine (Grof and Vinar, 1965; Klein and Davis, 1969), while hydrastinine is related to narcotine which has central depressant properties (Stanek and Manske, 1954).

One difficulty encountered before commencing the experiments was to determine which areas in the C.N.S. one should use for the investigation. Glutamate is present throughout the nervous system and appears to excite almost all neurones. If there are non-specific as well as specific receptors for glutamate as suggested by McLennan et al., (1968), it is quite likely that specific and non-specific pharmacological agents could be found to interact with them. The specific receptors are more likely to exist in glutaminergic pathways, and certain areas of the C.N.S. appear to be likely candidates for such a pathway. The distribution of glutamate in anterior and posterior roots has led to the postulation that glutamate could be the excitatory transmitter.
at primary afferent terminals (Graham et al., 1967; Duggan and Johnston, 1970a,b). The thalamus seems to be the most likely candidate for the "specific" glutamate receptor since it was in this area that McLennan et al., (1968) determined the difference in cellular responses to iontophoretically applied glutamate. The release of glutamate from the cortex during EEG arousal (Jasper et al., 1965) and on reticular formation stimulation (Jasper and Koyama, 1968, 1969), suggested that glutamate may also be involved in this area. It was therefore decided to concentrate the investigation in these regions and to include the neuromuscular junction in the crayfish.
CHAPTER II

MATERIALS AND METHODS - CAT PREPARATIONS

Cats of either sex, weighing for the most part between 2.5 and 3.5 kg. were used. The cats were fasted for a minimum of sixteen hours prior to anaesthesia and were anaesthetised with sodium pentobarbitone (Nembutal, Abbott Lab.) 35 mg./kg. administered intraperitoneally. Anaesthesia was maintained by periodic additional doses of 10 mg. applied intravenously. A tracheotomy was performed and a glass Y-tube inserted into the trachea and secured. End-tidal CO$_2$ levels were monitored by connecting one arm of the Y-tube to a Beckman Medical Gas Analyzer or a Godart Capnograph. When the end-tidal CO$_2$ levels deviated significantly from the normal value of 4% the animals were paralyzed with gallamine triethiodide (Flaxedil, Poulenc Ltd.), 20 mg. intravenously and 20 mg. intramuscularly and the second arm of the Y-tube was attached to a Palmer artificial respiration pump. A similar procedure, together with a bilateral pneumothorax was carried out when it became necessary to reduce recording artifacts caused by excessive breathing movements.

A permanent femoral venous cannula was inserted for the administration of anaesthetic or other drugs. In cases of low blood pressure or following excessive surgical blood
loss, a bottle containing 5% Dextrose in saline was attached to the venous cannula and a continuous infusion of 0.2 ml./min. initiated. A femoral arterial cannula was attached to a pressure transducer and blood pressure monitored on one channel of a polygraph, while a second channel of the polygraph was connected to the CO₂ monitor. This enabled a permanent record of the blood pressure and respiration to be kept. In certain thalamic experiments a cardiac catheter for the intra-arterial application of drugs to the thalamus was inserted via the brachial artery. In this manner, it was possible to bring about higher concentrations of the drug in the brain than could be obtained intravenously. The depth of the cannula was estimated and no confirmation as to the location of the tip was made.

During the experiments on thalamic and cuneate neurones the rectal temperature of the animal was monitored by an EKEG temperature control unit. Body temperature was maintained at 37-38°C by a 6 volt battery-supplied heating pad placed under the animal.

Electrode placement.

Electrode placement was similar to that used by Marshall (1971). Recording from the thalamic, cortical and cuneate neurones required stabilizing of the head of the animal in a stereotaxic apparatus (Precision Cinematographique). This instrument uses the external auditory meatus and lower orbit for head fixation and for reference points. In the experi-
ments on the cerebral cortex steel stimulating electrodes were calibrated beforehand with reference to the inter-aural line at the midline position. The location and co-ordinates of the stimulation and recording sites were determined from the stereotaxic atlases of Snider and Niemer (1961) and Reinoso-Suárez (1961). The positions of the recording electrodes were confirmed by monitoring the spontaneous electrical activity and the responses to activation of the known neural pathways.

a) **Recording from thalamic neurones.**

Following placement of the arterial, venous and tracheal cannulae the cat's head was mounted in the stereotaxic frame. After a midline sagittal incision, the skull was exposed by scraping back the skin and temporal musculature. Using a trephine and bone forceps, the bone overlying the left side of the brain was removed, exposing the cortex from the inter-aural line anterior for about 20 mm., and from the midline lateral for about 15 mm. Bleeding from dura or brain was controlled by applying small pledgets of Gelfoam (Upjohn) while bleeding from bone was stopped with bone wax. The dura mater covering the exposed brain was removed and the surface vessels coagulated with a Birtcher coagulator. The cortical grey and white matter overlying the thalamus was removed by suction revealing the floor of the lateral ventricle. This procedure prevented breakage and tissue plugging of the electrode tips which often occurred if the
electrode was inserted through the cortical tissue overlying the thalamus. The resulting cavity was kept partly filled with warm Locke solution, except when the recording electrode position was being changed to a new track. In order to reach the VPL nucleus of the thalamus the electrode tip was placed 7 mm. lateral to the midline so that it passed just medial to the choroid plexus approximately 8 mm. anterior to the interaural line. The electrode track was vertical.

The right rear hindlimb was clamped in an extended position. The skin incision was made along the groove formed between the semitendinosus and the semimembranosus muscles on the medial side, and the biceps femoris on the lateral side. These muscles were then separated and the fascia lata and popliteal fat pad removed after carefully tying off the blood supply to the adipose tissue. This procedure uncovered the floor of the popliteal fossa and revealed the sciatic nerve with its numerous branches. Bipolar silver electrodes were attached to the two main branches of the sciatic nerve i.e. the anterior tibial and peroneal nerves and these nerves were cut distal to the electrodes. A reservoir was formed by tying the skin surrounding the area to supporting rods and the entire fossa was filled with warm paraffin oil, which covered the nerves and electrodes.

b) **Recording from cuneate neurones.**

Following placement of the cannulae and mounting of the cat's head in the stereotaxic frame, a midline skin incision
was made from the top of the skull posteriorly to the area of the fourth cervical vertebra. The suboccipital and upper cervical musculature were separated from the occiput, posterior arch of atlas and the spinous process of axis. Where necessary the Birtcher coagulator-desiccator was used to cut the muscles and to prevent bleeding. The posterior aspect of the occiput, the posterior arch of atlas and the spinous and lamina of axis were removed using bone forceps. The dura mater was cut away and the caudal aspect of the cerebellum removed by suction. This allowed easy access to the cuneate nucleus and the posterior columns. The recording electrode was placed visually in the cuneate tubercle and bipolar silver stimulating electrodes were placed so that they just touched the dorsal columns on the same side. A reservoir was made around the area and filled with warm paraffin oil.

c) **Recording from spinal cord neurones.**

Following placement of the cannula a midline skin incision was made from the first sacral to the second lumbar spinous processes. The erector spinae muscles were dissected from both sides of the spine and the spinous processes and laminae of the third to the seventh lumbar vertebrae were removed with bone forceps. The dura mater was then cut away making the spinal cord easily accessible. Extreme care was taken not to touch the spinal cord and the area was kept moist with wads of cotton wool dipped in warm Locke
solution. The animal was suspended by means of clamps tightly secured to the L2 and S1 spinous processes. The anterior root of the L7 spinal nerve was severed and suspended over bipolar silver stimulating electrodes. The recording electrode track was either vertical just medial to, or at an angle of 18° just lateral to the entrance of the L7 dorsal root into the spinal cord. Again a reservoir was made and the area covered with paraffin oil.

Stimulating electrodes were placed on branches of the sciatic nerve in a manner similar to that used when recording from the thalamus. In this case, however, electrodes were placed on the following nerves; tibial, plantaris, gastrocnemius, sural and peroneal.

d) Recording from the cerebral cortex.

The cannulae were fixed in position, the head mounted in the stereotaxic frame and the skull exposed. In order to avoid touching the brain, a hand drill fitted with a small round dental burr was used to cut a window the same size as that used in the thalamic experiments, but extending anteriorly as far as the frontal pole. The dura was removed and a small tear made in the pia in the area of the precruciate gyrus. This allowed easy penetration of the cortex by the recording electrode.

A small slot was made in the contralateral side of the skull in an area opposite the thalamus. A parallel pair of steel electrodes with 2 mm. separation was then lowered
to co-ordinates previously calculated as being at the level of the VPL nucleus of the ipsilateral thalamus. These electrodes were constructed from 25 gauge needle tubing and insulated to within 0.5 - 1 mm. of the tip with Insl-X. A trephine hole in the skull was made posterior to the window and a concentric bipolar electrode lowered to the co-ordinates of the ipsilateral pyramids. These electrodes were constructed by threading fine teflon-coated stainless steel wire through 23 gauge stainless steel tubing until the wire extended $\frac{1}{2} - 1$ mm. beyond the tip of the tubing. The other end of the wire was led through an opening in the side of the tubing and the outside of the electrode was coated with Insl-X. The teflon insulation of the inner electrode and the Insl-X coating of the outer electrode were removed from the tip.

The position of the thalamic electrodes was confirmed histologically. The histological technique used was a modification of that described by Marshall (1971) and Green (1958). A positive current was passed (20 µA for 30 sec.) through the electrode while it was in position, in order to deposit iron from the uninsulated tip. The electrodes were removed, the dorsolateral surfaces of the brain exposed, and a 1 cm. section of brain was cut parallel to the electrode tracks and fixed in 10% formalin for 2 - 3 days. The blocks were then dehydrated, cleared and embedded in paraffin wax. Sections were cut 25 µ thick, mounted on gelatinized slides, placed in formalin vapour, dried and
brought to water. The sections were immersed in a 1% H$_2$O$_2$ solution to prevent reduction of the iron to the ferrous form by formaldehyde and then placed for 5 min. in a 5 - 10% solution of potassium ferrocyanide. 10% HCL (half the volume of the potassium ferrocyanide) was then added and the sections left for 20 - 30 min. during which time the Prussian Blue reaction took place and a blue spot in the area where the iron was deposited became obvious. The slides were washed in distilled water, counterstained in 1% safranin, dehydrated, cleared and mounted. Paired sections were stained with cresyl violet to facilitate identification of topographical structures in the neighbourhood of the electrode tip.

**Recording electrodes.**

The centre barrel of a 7-barrelled glass electrode assembly was used for the extracellular recording while the outer barrels were used for iontophoretic application of chemical compounds. The glass electrode blanks were supplied by Vancouver Scientific Glassblowing as an array of seven fused glass capillaries, drawn to an overall diameter of 2.5 - 3.5 mm. These blanks were heated and pulled in a vertical Canberra-type micro-electrode puller and the resulting tips were broken back, under microscopic observation, to a total diameter of 5 - 10 μ. The electrodes were then filled by boiling in distilled water for 20 min. After cooling, the distilled water was removed from the wide part of the shaft with a syringe and a 30 gauge needle, and
replaced by an electrolyte. 4M NaCl was used for the recording barrel while all the drugs used in the outer barrels are listed in table I. These drugs were dissolved in distilled water or 0.15M NaCl, the pH was adjusted as indicated to bring it well away from the iso-electric point (Krnjević and Phillis, 1963) and the solutions were filtered through a millipore filter. The solutions diffused to the tip within 24 - 48 hours. A platinum wire extending into the NaCl of the centre barrel was connected to the amplifier probe while silver wires extending into the remaining barrels connected the solutions in these barrels to the iontophoretic panel. Before using the glass electrodes, the DC resistance of each barrel was measured. The usual ranges were 5 - 12 MΩ for the recording barrel and 70 - 100 MΩ for drug containing barrels.

In initial experiments a large Narashige micromanipulator mounted on a bar attached to the stereotaxic frame was used for holding and moving the glass electrode; later an AB Transvertex micromanipulator with an electronically controlled stepping motor was used.

To prevent the unwanted diffusion of active ions, retaining currents were applied to each of the drug-containing barrels. Currents used to retain or pass drugs iontophoretically were measured on a Cambridge galvanometer and were passed to the barrels of the electrode through 1000 MΩ series resistors. Relatively small variations in resistance at the electrode tips therefore did not appreciably affect
<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc.</th>
<th>Solvent</th>
<th>pH</th>
<th>Active ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  Acetylcholine Bromide</td>
<td>1.00M</td>
<td>water</td>
<td>4.0</td>
<td>cation</td>
</tr>
<tr>
<td>2  Na-L-Glutamate</td>
<td>0.50M</td>
<td>water</td>
<td>8.0</td>
<td>anion</td>
</tr>
<tr>
<td>3  Na-DL-Homocysteate</td>
<td>0.20M</td>
<td>water</td>
<td>8.0</td>
<td>anion</td>
</tr>
<tr>
<td>4  Na-D-Glutamate</td>
<td>0.50M</td>
<td>water</td>
<td>8.0</td>
<td>anion</td>
</tr>
<tr>
<td>5  Na-DL-N-methylglutamate</td>
<td>0.50M</td>
<td>water</td>
<td>8.0</td>
<td>anion</td>
</tr>
<tr>
<td>6  Na-p-aminobenzoyl-L-glutamate</td>
<td>0.50M</td>
<td>water</td>
<td>8.0</td>
<td>anion</td>
</tr>
<tr>
<td>7  Na-p-nitrobenzoyl-L-glutamate</td>
<td>0.50M</td>
<td>water</td>
<td>8.0</td>
<td>anion</td>
</tr>
<tr>
<td>8  Na-L-glutamate-α-methylester</td>
<td>0.50M</td>
<td>water</td>
<td>8.0</td>
<td>anion</td>
</tr>
<tr>
<td>9  L-glutamic acid-dimethylester.HCl</td>
<td>0.50M</td>
<td>water</td>
<td>4.0</td>
<td>cation</td>
</tr>
<tr>
<td>10 L-glutamic acid-diethylester-HCl</td>
<td>0.50M</td>
<td>water</td>
<td>4.0</td>
<td>cation</td>
</tr>
<tr>
<td>11 Na-4-fluoroglutarate</td>
<td>0.50M</td>
<td>water</td>
<td>8.0</td>
<td>anion</td>
</tr>
<tr>
<td>12 Na-L-aspartate</td>
<td>0.50M</td>
<td>water</td>
<td>8.0</td>
<td>anion</td>
</tr>
<tr>
<td>13 Na-L-cysteate</td>
<td>0.50M</td>
<td>water</td>
<td>8.0</td>
<td>anion</td>
</tr>
<tr>
<td>14 DL-methionine-DL-sulphoximine</td>
<td>0.50M</td>
<td>water</td>
<td>3.5</td>
<td>cation</td>
</tr>
<tr>
<td>15 Hydrastinine HCl</td>
<td>0.01M</td>
<td>0.15M</td>
<td>3.0</td>
<td>cation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NaCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 Cyclobenzaprine HCl</td>
<td>0.50M</td>
<td>water</td>
<td>4.0</td>
<td>cation</td>
</tr>
<tr>
<td>17 p-chloro-mercuri-phenyl-sulphonic acid</td>
<td>0.02M</td>
<td>0.15M</td>
<td>8.0</td>
<td>anion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NaCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 Atropine sulphate</td>
<td>0.05M</td>
<td>0.15M</td>
<td>7.0</td>
<td>cation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NaCl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
the current flow.

Occasionally depressant effects similar to those observed by Curtis and Koizumi (1961) and Krnjević and Phillis (1963a) on application of cation-ejecting currents to the drug containing barrels were seen. In order to separate the drug effects from those due to the current flow the depressant effects of the current was counteracted with a current of equal strength but opposite polarity, passing from another barrel containing an inactive ion (usually Na⁺) (Salmoiraghi and Steiner, 1963). The current effects were distinguished by their instantaneous onset and termination, followed by rebound excitation (Krnjević and Phillis, 1963a).

**Electrical equipment.**

The stimulating electrodes were connected through a selection panel and stimulus isolation units to a Grass S8 stimulator. The signal from the recording electrode was fed through a preamplifier or impedance converter and displayed on a dual beam oscilloscope which was triggered from the stimulator. A second parallel oscilloscope which was not triggered from the stimulator monitored the continuous electrical activity. The amplified signal from the oscilloscope was fed to a loudspeaker and an EKEG ratemeter, the integrated ratemeter output being recorded on an Esterline-Angus Rectilinear recorder. The triggering level was determined by feeding the ratemeter output into the second channel of the oscilloscope and matching the electrical
potentials with the triggering potentials. In this way the activity of a single cell could be represented in the rate-meter output signal.

The output from the ratemeter was also fed into a PDP-8/L Digital computer programmed to display post stimulus time and latency histograms. The computer was triggered from the stimulator so that it counted potentials following each stimulus and added those following successive stimuli which had the same poststimulus latency. The number of sweeps to be summed as well as the bin width and total number of bins could be varied, depending on the type of response being recorded. The histogram was either displayed on an oscilloscope and photographed or plotted on an X/Y recorder. Figure 2 represents a block diagram of the electrical equipment and connections used.
Figure 2. Block diagram of the electrical equipment used in the cat experiments.
CHAPTER III

RESULTS - CAT PREPARATIONS

The cats under barbiturate anaesthesia breathed spontaneously with an end tidal CO\textsubscript{2} of 3.5 - 4.5\%. As the animal started recovering from the anaesthetic, the respiratory rate increased and the end-tidal CO\textsubscript{2} decreased. Maintenance doses of 10 mg. Na-pentobarbitone caused, in many cases, a momentary hesitation in the respiratory movements, followed by a slowing of respiration and an increase in the end-tidal CO\textsubscript{2}. With injection of the anaesthesia, the blood pressure rose during the apnoeic period following which the blood pressure was depressed for a considerable time. Figure 3 shows an example of these changes. The cats on gallamine triethiodide and respired artificially, maintained an end-tidal CO\textsubscript{2} of 4\%. All experiments were carried out after the end-tidal CO\textsubscript{2} and blood pressure levels had settled to constant values.

a) Neuronal firing patterns and evoked responses.

Neurones in VPL showed spontaneous bursts of action potentials separated by periods of inactivity characteristic of this area (Andersen and Curtis, 1964; Andersen et al., 1967; Marshall, 1971). Stimulation of branches of the sciatic nerve evoked bursts of 1 - 4 spikes at latencies
Figure 3. The effects of an intravenous injection of 10 mg. Na-pentobarbitol in 1 ml. saline on tidal CO₂ and blood pressure. Injection at arrow.
between 8 and 20 msec. to the first action potential.
Spinal interneurones showed no consistent pattern of spontane­
aneous activity and the evoked response obtained by stimu­
lating branches of the sciatic nerve consisted of bursts of
1 - 10 spikes with minimum latencies usually between 6 and
10 msec. The cuneate neurones showed the typical spon­
taneous activity described by Schwartz et al. (1964) and
Galindo et al. (1968), i.e. double spikes occurring at a
frequency of 5 - 10 per min. The response of these cells
to dorsal column stimulation was 1 - 3 spikes at latencies
between 1.5 and 8 msec. Under pentobarbitone anaesthesia
the cortical neurones showed little or no spontaneous
activity and were often completely silent until excited by
one of the amino acids. Evoked potentials in the cortex
elicited by stimulation of VPL consisted for the most part
of a single spike with a latency between 1.5 and 4 msec.,
and on only two occasions were multiple evoked spikes seen.
The frequency of firing and the number of action potentials
in the evoked responses in all areas was dependent on the
strength of the stimulus, which could be decreased to a
point at which the cell fired with one or two spikes follow­
ing every second or third stimulus. The attempts at
blocking the response were done at this threshold stimulus
strength.

Cells in the four areas tested were readily excited
by the acidic amino acids. The time-lag between commence­
ment of the iontophoretic current and excitation of the cell
by the drug varied from cell to cell, and from one excitant to another. Often the effect was immediate (figure 9), at other times there was a period of 1 min. duration or longer before the cell was excited (figure 12). This was probably due to the variable distance between the tip of the electrode and the stimulated cell. The response always stopped immediately after cessation of the current. The ejecting current needed to produce excitation with DLH was considerably lower than that needed for Glut. Glut was generally equal to, or a more effective excitant than Asp, whereas Cys was the least active of the four amino acids tested. Dose-responses of a single cell for the four amino acids were determined in the cuneate nucleus, and indicated that the sulphur-containing amino acids were active at lower ejecting currents and were able to induce higher maximum firing rates than did the two dicarboxylic amino acids (figure 4).

The order of potency of the amino acids to excite the cells in the spinal cord was not as regular as that observed in other areas. DLH was invariably the greater excitant, however some cells showed a far greater sensitivity to Glut than to Asp, while others showed the reverse.

The effects of nine Glut analogues on the spontaneous activity and excitation of thalamic neurones by Glut are listed in table II. Only two, aMG and GDEE showed any ability to antagonize the excitation of neurones induced by Glut, whereas GDME caused a definite potentiating effect. Na-4-fluoroglutamate had a greater excitatory action than
Figure 4. The response of a neurone in the cuneate nucleus to increasing iontophoretic current strengths of DLH, Glut, Asp and Cys.
### TABLE II
THE ACTION OF DERIVATIVES OF GLUTAMATE ON THALAMIC (VPL) NEURONES

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Upon spontaneous activity</th>
<th>Upon L-glutamate-induced activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na-D-glutamate</td>
<td>Excitation</td>
<td>None</td>
</tr>
<tr>
<td>Na-DL-α-methylglutamate</td>
<td>None</td>
<td>Blockade</td>
</tr>
<tr>
<td>Na-DL-N-methylglutamate</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Na-p-aminobenzoyl-L-glutamate</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Na-p-nitrobenzoyl-L-glutamate</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Na-L-glutamate-γ-methylester</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>L-glutamic acid-dimethylester-HCl</td>
<td>None</td>
<td>Enhancement</td>
</tr>
<tr>
<td>L-glutamic acid-diethylester-HCl</td>
<td>None</td>
<td>Blockade</td>
</tr>
<tr>
<td>Na-4-fluroglutamate</td>
<td>Excitation</td>
<td>(Not tested)</td>
</tr>
</tbody>
</table>
L-Glut and could therefore not be tested for possible blocking action. αMG, γMG, GDEE and GDME occasionally caused an increase in the background firing rate of cells, but only when very high ejection currents were used for prolonged periods of time.

b) Glutamic acid diethyl'ester.

Table III gives the results of the action of GDEE on the induced firing of neurones in the spinal cord, cuneate nucleus, VPL and cerebral cortex, tabulated in each case as a ratio of the total number of cells examined. It should be noted that this table gives no indication of the degree of blockade produced. It is evident that in the spinal cord, cuneate nucleus and VPL GDEE more frequently antagonized excitations induced by Glut than those induced by the other amino acids or ACh. Two examples of the results from which this table was compiled are illustrated in figure 5 (in the thalamus using DLH, Glut and ACh) and figure 6 (in the cuneate using DLH, Glut and Cys). The time taken for full recovery of the Glut response varied after cessation of the GDEE ejection current, but usually complete recovery occurred within 5 – 10 min., as can be seen from figure 5. When the action of DLH was reduced by GDEE it was always less affected than the response produced by Glut, and full recovery of the DLH-induced response was also more rapid. In the thalamus GDEE had no appreciable effect on neuronal excitations caused by ACh even when the response to DLH was
TABLE III

THE EFFECT OF GDEE ON SINGLE CELL EXCITATIONS. EACH SET OF FIGURES INDICATES THE RATIO OF THE NUMBER OF CELLS IN WHICH ANY REVERSIBLE ACTION ON INDUCED FIRING WAS OBSERVED, TO THE TOTAL NUMBER EXAMINED.

<table>
<thead>
<tr>
<th></th>
<th>VPL</th>
<th>SPINAL CORD</th>
<th>CUNEATE NUCLEUS</th>
<th>CORTEX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>response</td>
<td>response</td>
<td>response</td>
<td>response</td>
</tr>
<tr>
<td>Glut</td>
<td>41/49</td>
<td>1/49</td>
<td>12/18</td>
<td>0/18</td>
</tr>
<tr>
<td>DLH</td>
<td>20/40</td>
<td>5/40</td>
<td>5/16</td>
<td>1/16</td>
</tr>
<tr>
<td>Asp</td>
<td>5/11</td>
<td>2/11</td>
<td>5/14</td>
<td>1/14</td>
</tr>
<tr>
<td>Cys</td>
<td>3/4</td>
<td>1/4</td>
<td></td>
<td></td>
</tr>
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<td>ACH</td>
<td>9/19</td>
<td>1/19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evoked</td>
<td>15/18</td>
<td>0/18</td>
<td>11/13</td>
<td>0/13</td>
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</table>
Figure 5. The effects of GDEE on DLH, Glut and ACh excitation of a VPL neurone. Ratemeter records of the excitations elicited by homocysteate (DLH), 25 nA; glutamate (GL), 80 nA; and acetylcholine (ACh), 84 nA. A: before, B: during GDEE application for 4 min. with a current of 80 nA; C: 1 min. after cessation of the GDEE current, D: 7 min. later.
Figure 6. The effect of GDEE on the DLH, Glut and Cys excitation of a neurone of the cuneate nucleus. Ratemeter records are of excitations caused by DLH (160 nA), Glut (200 nA) and Cys (240 nA). A: control, B: following application of GDEE (80 nA) for 1 min., C: 1 min. following cessation of the GDEE application.
depressed.

The greater effectiveness of GDEE as a Glut antagonist was confirmed when quantitative comparisons of the extent of blockade were examined. The sustained frequency of firing of neurones in the presence and absence of GDEE was regulated by adjusting the ejection currents for the amino acids so that the control firing frequencies were roughly similar. In the thalamus 34 direct comparisons were conducted between Glut and DLH, 11 between Glut, DLH and Asp, and 4 with all four amino acids. The results are given in table IV, and indicate that the effect of GDEE on excitation elicited by Glut was significantly greater (p<0.02 in every case using the paired T-test) than for any of the other three amino acids. On the basis of these experiments there was no significant difference between the action of GDEE on DLH, Asp or Cys, although Asp does appear to be slightly more affected than the sulphonate compounds (in the last 4 cells in table IV the difference between the effects of GDEE on Glut and Asp was not significant). The mean maximal sustained frequencies of firing were estimated by eye (figure 7).

Occasionally after prolonged application at high iontophoretic currents GDEE had a slight excitatory action and increased the background firing rate of the neurone under investigation.

Although it is difficult to draw conclusions (Curtis et al., 1971), the antagonism between Glut and its derivative appears to be competitive. When the responses to increasing
### Table IV

**Blocking Action of GDEE on the Firing of Thalamic Neurones.** The figures indicate the average ratios of the firing frequencies attained in the presence and absence of GDEE, ± S.E.

<table>
<thead>
<tr>
<th>Glut</th>
<th>DLH</th>
<th>Asp</th>
<th>Cys</th>
<th>n</th>
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</thead>
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<tr>
<td>0.41 ± 0.049</td>
<td>0.82 ± 0.034</td>
<td></td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>0.42 ± 0.102</td>
<td>0.95 ± 0.045</td>
<td>0.79 ± 0.094</td>
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<td>11</td>
</tr>
<tr>
<td>0.31 ± 0.106</td>
<td>1.00 ± 0.000</td>
<td>0.59 ± 0.227</td>
<td>0.85 ± 0.069</td>
<td>4</td>
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</tbody>
</table>
Figure 7. The effect of GDEE on the response of a VPL neurone to the excitant amino acids. Ratemeter records of the excitation of a thalamic neurone induced by the electrophoretic application during the periods indicated of DLH (60 nA), Glut (120 nA), Asp (200 nA) and Cys (200 nA). A: control, B: following the application of glutamate diethylester (GDEE) for 4 min. from another barrel of the electrode assembly, C: 5 min. after cessation of the GDEE-ejecting current. The horizontal lines indicate the manner in which the mean maximal sustained firing frequencies were estimated by eye.
intensities of the Glut-ejecting current, in the absence and presence of the antagonists were compared, the dose-response curve was shifted parallel to the control, indicating that a higher Glut current was required to elicit a similar degree of neuronal excitation in the presence of the inhibitor. The maximum sustained firing rate was the same in the inhibited as in the control situation (figure 8). Increased "doses" of Glut in both circumstances led to diminished rates of firing through excess depolarization and inactivation of the cell membrane.

Intra-arterial administration of GDEE through a cardiac cannula inserted via the brachial artery caused a similar decrease in Glut response in the thalamus. A control injection of 0.5 ml. Locke solution had no effect on the excitation of a single cell caused by the iontophoretic application of the amino acids (figure 9A,B). A single injection of 30 mg. GDEE (0.25 ml. of a 0.5 N solution) followed by 0.25 ml. Locke solution to clear the cannula caused a substantial decrease in the Glut excitation, without appreciably affecting the excitations produced by DLH, Asp and Cys (figure 9C,D). The effect could be seen within 2 - 3 min. after the injection, and recovery of the response was complete within 8 - 12 min. Larger amounts of GDEE resulted in a reduction of the excitations caused by all four amino acids, although Glut was always reduced to the greatest extent and took longest to recover.

Table III also indicates that iontophoretic application
Figure 8. Dose-response curves for Glut excitations on a VPL neurone. Ordinate: maximum sustained frequency of cell firing; abscissa: intensity of Glut-ejecting current. 0—0 before; X—X during the application of GDEE (160 nA): •—• recovery.
Figure 9. The effect of an intra-arterial injection of GDEE on the responses of a VPL neurone to the excitant amino acids. Ratemeter records of the excitations elicited by DLH (120 nA), Glut (160 nA), Asp (160 nA), Cys (160 nA). A: control, B: 5 min. following the injection of 0.5 ml. Locke solution, C: 3 min. following the injection of 30 mg. GDEE, D: 8 min. following the injection of GDEE.
of GDEE caused a reversible reduction in the responses evoked by electrical stimulation of afferent nerves in a significant number of cells in all areas tested. In those cells which showed blockade of the evoked responses, excitations by Glut were also blocked concurrently and recovery occurred pari passu with restitution of the Glut sensitivity (figures 10,11,12,13). This blockade of synaptic activation was not observed in every cell in which the response to Glut had been abolished; and when an inhibition was observed, the reduction in the evoked response was very often not as great as the reduction in Glut excitation. In no case however, was a reduction in the evoked response found without a concomitant decrease in the effect of applied Glut. In the thalamus similar reductions in the evoked response could be obtained with the intra-arterial injection of GDEE (figure 14).

There appeared to be some variation in the effects on GDEE in the different areas of the C.N.S. The reduction in the evoked responses in the cuneate were generally less than those observed in other areas. In the spinal cord, those cells which showed a greater sensitivity to Asp than to Glut reacted to the application of GDEE with a equal depression of both Asp and Glut responses while the DLH response remained unchanged. Although it was possible to reduce Glut relatively specifically (figure 13), the results in the cortex were not as consistent as those observed in other areas. GDEE had an effect on a smaller percentage of cells in the cortex than in the other areas, and it was also more difficult to
Figure 10. The effect of GDEE on amino acid and synaptically evoked excitations in VPL. A neurone was excited by the electrophoretic application of DLH (20 nA) and Glut (100 nA) and by electrical stimulation of the contralateral tibial nerve (0.1 msec., 3V). The figure shows ratemeter records of the amino acid induced firings on the left, and on the right computed histograms showing the summed evoked responses to 50 stimuli delivered at a frequency of 1/4 sec., which in this case usually consisted of two spikes. A: before, B: during application of GDEE (160 nA) for 6 min., C: 5-6 min. after cessation of the GDEE current. In this figure and all subsequent figures the stimulus artifacts are indicated by arrows.
Figure 11. The effect of GDEE on amino acid and synaptically evoked excitations of a spinal interneurone. Ratemeter records are of excitations produced by DLH (80 nA), Glut (80 nA) and Asp (80 nA). The histograms show the summed evoked responses to 40 stimuli (1/2 sec., 0.1 msec., 7V) applied to the ipsilateral sural nerve. A: control, B: following application of GDEE (80 nA) for 1-4 min., C: 3-4 min. following cessation of the GDEE ejecting current.
Figure 12. The effect of GDEE on the amino acid and synaptically induced excitations of a neurone in the cuneate nucleus. Ratemeter records are of excitations produced by DLH (120 nA) and Glut (160 nA). Histograms show the summed responses to 50 stimuli (1/2 sec., 0.1 msec., 3V) applied to the ipsilateral dorsal column. A: control, B: following application of GDEE (160 nA) for 12-13 min., C: 3-4 min. following cessation of the GDEE application.
Figure 13. The effect of GDEE on amino acid and synaptically evoked excitations of a neurone in the cerebral cortex. Ratemeter records are of excitations produced by DLH (50 nA), Glut (140 nA) and Asp (120 nA). Histograms show the summed responses to 40 stimuli (1/2 sec., 0.1 msec., 4.5V) applied to VPL. A: control, B: following application of GDEE (160 nA) for 2-3 min., C: 13-16 min. following cessation of the GDEE ejecting current.
Figure 14. The effects of an intra-arterial injection of GDEE on amino acid and synaptically evoked excitations in VPL. A neurone was excited by the electrophoretic application of DLH (160 nA) and Glut (120 nA) and by electrical stimulation of the contralateral tibial nerve (0.1 msec., 4V). Ratemeter records of the amino acid induced firing is shown on the left and on the right the computed histograms of the summed evoked responses to 50 stimuli delivered at a frequency of 1/2 sec. The stimulus artifacts are indicated by arrows. A: before, B: 2-3 min. following a 50 mg. injection of GDEE, C: 7-8 min. following the injection of GDEE.
demonstrate a specific action towards Glut. However, as can be seen from the two examples given (figures 13, 24), Glut responses could be blocked to a greater extent than those produced by DLH and Asp. In the cortex the evoked potentials were occasionally blocked completely and thereafter took a long time for recovery which was often incomplete.

The blocking effects of GDEE were dependent on the strength of the ejecting current. At high iontophoretic currents (above 160 nA) GDEE very often caused a reduction in the sensitivity of a cell to all the excitatory amino acids, whereas at low currents there was no significant blocking of the excitations produced by any of the amino acids. As the strength of the GDEE ejecting current required to block Glut responses specifically varied from one cell to another, it was often necessary to try a number of ejecting current strengths. In the example in figure 15 there was no significant blocking effect at 80 nA, while at 125 nA GDEE blocked the Glut and evoked responses without reducing the sensitivity of the cell to Asp.

c) Glutamic acid dimethyl ester.

The action of GDME on amino acid induced and evoked excitation of cells in VPL varied slightly from that observed in the spinal cord, cuneate nucleus and cerebral cortex (table V). The effects of GDME on VPL neurones depended to a large extent on the iontophoretic current used. At currents of 160 nA or larger, there was usually a blockade
Figure 15. The effect of different current strengths of GDEE on the amino acid and evoked responses of a spinal interneurone. The ratemeter records are of excitations produced by Glut (20 nA) and ASP (100 nA). The histograms show the summed evoked responses to 40 stimuli (1/2 sec., 0.1 msec., 12V) applied to the ipsilateral sural nerve. A: control, B: following application of 80 nA GDEE, C: following application of 125 nA GDEE, D: recovery.
TABLE V

THE EFFECT OF GDME ON SINGLE CELL EXCITATIONS. EACH SET OF FIGURES INDICATES THE RATIO OF THE NUMBER OF CELLS IN WHICH ANY REVERSIBLE ACTION ON INDUCED FIRING WAS OBSERVED, TO THE TOTAL NUMBER EXAMINED.

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<td>response</td>
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<td>response</td>
<td>response</td>
</tr>
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<td>1/4</td>
<td>3/4</td>
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<td>1/3</td>
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<td></td>
</tr>
<tr>
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<td>3/5</td>
<td>0/3</td>
<td>2/3</td>
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</tr>
</tbody>
</table>

76
of the Glut response (figure 16). The blocking effect was not as specific as that obtained with GDEE since a decrease in the responses to Asp and Cys were usually observed. On cessation of the iontophoretic current the responses of the cell to the excitatory amino acids invariably increased to values higher than the controls, before finally returning to the initial levels after 5 - 15 min. As can be seen in figure 16 the potentiation of the Glut and Asp responses was greater than the DLH and Cys responses. At iontophoretic currents below 100 nA GDME caused an increase in the Glut-induced responses of 7 out of 8 cells tested in the thalamus. In 4 of these cells where only Glut and DLH were tested, Glut responses increased to an average of twice the control value, while DLH responses showed no significant increase.

The results in the spinal cord, cuneate and cortex appeared to be more consistent, with GDME causing an increase in the responses to Glut and Asp at all current strengths (figures 17,20,21).

Glut-induced excitations in all areas usually showed a greater potentiation than Asp responses, which were in turn increased to a greater extent than the DLH responses (figure 17) and the onset of the excitation induced by Glut and Asp was often more rapid in the presence of GDME. The changes could usually be observed within 5 min. after commencement of the application of GDME, and the responses of the cells invariably returned to control values within 10 min. after
Figure 16. The effects of GDME on amino acid excitations of a VPL neurone. Ratemeter records of the excitations produced by DLH (160 nA), Glut (120 nA), Asp (200 nA) and Cys (200 nA) are shown. A: before, B: after application of GDME for 1 min., C: 1 min. following cessation of the GDME-ejecting current, D: 5 min. following cessation of the GDME-ejecting current.
Figure 17. The effect of GDME on amino acid excitation of a neurone in the cerebral cortex. Ratemeter records are of excitations produced by DLH (40 nA), Glut (80 nA) and Asp (80 nA). A: control, B: following application of GDME (60 nA) for 6 min., C: 5 min, following cessation of the GDME ejecting current.
cessation. The recovery of the amino acid responses in the thalamus tended to be slower than in the other areas and very often it was not complete even after 15 min.

The effects of GDEE and GDME could be demonstrated at the same neurone. Figure 18 is an example of a cell in the cuneate in which GDEE blocked the excitation of Glut and Asp and, after recovery, GDME caused a reversible enhancement of the responses of the cell to these amino acids, while the response to DLH was largely unaffected by both esters.

The effect of GDME on the evoked responses in the thalamus, cuneate and spinal cord was similar to the effect on Glut responses. In those cells in the thalamus in which GDME reduced Glut excitation, the evoked response showed a concomitant decrease which returned with the recovery of the Glut response, whereas those cells in which a potentiation of the amino acid responses was observed showed an increase in the evoked response (figures 19, 20, 21). This increase could only be observed if the stimulus was set near threshold, such that the cell fired with every second or third volley. Under these circumstances the cell would fire more consistently on application of GDME. The increase in evoked response with GDME was never as great as the increase in Glut response, however, recovery to control values followed the same time course as the Glut response. In the two cuneate cells which showed an altered evoked response the number of spikes following each stimulus was increased rather than the probability of firing at short latencies. The number
Figure 18. A comparison of the effects of GDEE and GDME on the amino acid induced excitations of a neurone of the cuneate nucleus. Ratemeter records are of the excitations produced by DLH (60 nA), Glut (120 nA) and Asp (140 nA). A: control, B: following application of GDEE (200 nA) for 2 min., C: 5 min. following cessation of GDEE application, D: following application of GDME (200 nA) for 2 min., E: 5 min. following cessation of GDME application.
Figure 19. The effects of GDME on amino acid and synaptic excitation of a VPL neurone. Stimuli were applied to the contralateral tibial nerve (2.8V, 0.1 msec.) and the histogram shows the summed evoked responses to 40 stimuli (1/2 sec.). The histogram and ratemeter records of excitations produced by DLH (40 nA), Glut (120 nA) and Asp (120 nA) are shown A: before, B: following application of GDME (50 nA) for 5-6 min., C: 10-16 min. following cessation of the GDME ejecting current.
Figure 20. The effect of GDME on the amino acid and synaptically evoked excitations of spinal interneurons. The ratemeter records are of excitations produced by DLH (100 nA), Glut (120 nA) and Asp (120 nA). The histograms show the summed evoked responses to 40 stimuli (1/2 sec., 0.1 msec., 10V) applied to the ipsilateral sural nerve. A: control, B: following application of GDME (80 nA) for 3-5 min., C: 4-5 min. following cessation of the GDME ejecting current.
Figure 21. The effect of GDME on the amino acid and synaptically evoked excitations of a neurone of the cuneate nucleus. Ratemeter records are of excitations produced by DLH (80 nA), Glut (160 NA) and Asp (140 nA). Histograms show the summed responses to 50 stimuli (1/2 sec., 0.1 msec., 2-5V) applied to the ipsilateral dorsal columns. A: control, B: following application of GDME (200 nA) for 4-8 min., C: 1-5 min. following cessation of the GDME ejecting current.
of tests conducted in this area was small, since GDME itself usually caused a marked increase in the "spontaneous" firing of the cuneate neurones. The action of GDME on evoked responses in the cortex was not examined.

d) Alpha-Methylglutamate.

From table VI it is evident that αMG blocked Glut excitation in approximately 40% of the cells tested in the thalamus, but had no effect on the responses of cuneate cells to any of the excitants. Inhibition occurred only when high iontophoretic currents (above 200 nA) were used and in most of the cells where Glut excitations were blocked there was a similar inhibition of the responses to the other excitatory amino acids and ACh; however, the reduction of the DLH response was always less than that of the Glut excitations. In 4 out of 10 cells tested in the thalamus, αMG caused a reduction in the evoked response; however in all of these cases there was a concommitant decrease in the amino acid and ACh responses. No reductions in the evoked responses were observed in the cuneate.

αMG often caused an increase in the background rate of firing of neurones, for the most part following prolonged application at high current strengths, and indeed in certain cells, especially in the cuneate, αMG appeared to be as effective an excitatory agent as Glut itself. In a number of cells αMG caused a potentiation of the responses to the excitatory amino acids (figure 22). The effect was
TABLE VI

THE EFFECTS OF αMG ON SINGLE CELL EXCITATIONS. EACH SET OF FIGURES INDICATES THE RATIO OF THE NUMBER OF CELLS IN WHICH ANY REVERSIBLE ACTION ON INDUCED FIRING WAS OBSERVED, TO THE TOTAL NUMBER EXAMINED.

<table>
<thead>
<tr>
<th></th>
<th>VPL</th>
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<td>Glut</td>
<td>9/24</td>
<td>4/24</td>
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<td>0/5</td>
</tr>
<tr>
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<td>0/13</td>
<td>1/5</td>
<td>1/5</td>
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<td>Asp</td>
<td>1/4</td>
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<td>0/5</td>
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<td>Evoked</td>
<td>4/10</td>
<td>1/10</td>
<td>0/2</td>
<td>0/2</td>
</tr>
</tbody>
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Figure 22. The potentiating effects of αMG on amino acid excitation of a VPL neurone. Ratemeter records are of the excitations elicited by DLH (80 nA), Glut (160 nA) and Asp (120 nA). A: before, B: following application of αMG (240 nA) for 7 min., C: 8 min. after cessation of the αMG application.
non-specific, affecting DLH, Glut and Asp in the same manner.

e) DL-Methionine-DL-Sulphoximine.

As can be seen from table VII, MSO had no marked or consistent effects on the responses of VPL cells to Glut, Asp, DLH, ACh or on the evoked response. Iontophoretic currents of 40 - 160 nA were used to apply the drug for periods up to 15 min. An intra-arterial injection of 18 mg. MSO did not change the responses of cells in the thalamus. On two occasions when currents of 160 nA were passed, MSO increased the background "spontaneous" rate of firing, which returned to control levels after the MSO-ejecting current was terminated. In these cells also there was no obvious effect on the amino acid or ACh responses.

The effects of MSO on spinal interneurones and cortical neurones were more dramatic than its effects on thalamic neurones. At iontophoretic current strengths of 60 - 80 nA, MSO caused a decrease in the responses of the cell to all amino acids tested, although the DLH excitations were often reduced to a lesser extent than were those elicited by Glut and Asp. Figure 23 illustrates the responses of the only cell in the spinal cord in which MSO had a reversible effect on evoked potentials and in which Glut responses were decreased to the greatest extent. In the other spinal interneurones in which a reduction was observed, the Glut responses were reduced by no more than 30%, whereas in the cortex the amino acid responses were often reduced to 70%
TABLE VII

THE EFFECT OF MSO ON SINGLE CELL EXCITATIONS. EACH SET OF FIGURES INDICATES THE RATIO OF THE NUMBER OF CELLS IN WHICH ANY REVERSIBLE ACTION ON INDUCED FIRING WAS OBSERVED, TO THE TOTAL NUMBER EXAMINED.

<table>
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<tr>
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<th>SPINAL CORD</th>
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<td>1/9</td>
<td></td>
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<td>2/9</td>
<td></td>
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</tr>
<tr>
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<tr>
<td>ACh</td>
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<td></td>
<td>1/5</td>
<td>0/5</td>
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</table>
Figure 23. The effect of MSO on the amino acid and synaptically evoked excitation of a spinal interneurone. Ratemeter records are of excitations produced by DLH (80 nA) and Glut (80 nA). The histograms show the summed evoked responses to 40 stimuli (1/2 sec., 0.1 msec., 7V) applied to the ipsilateral sural nerve. A: control, B: following application of MSO (80 nA) for 1-6 min., C: 2-5 min. following cessation of the MSO ejecting current.
of control values.

Figure 24 compares the effects of equal ejecting currents of GDEE and MSO on the same cortical neurone. Although both GDEE and MSO reduced all the amino acid responses, GDEE reduced Glut to a far greater extent than DLH and Asp responses, whereas MSO affected all three responses to the same extent.

f) Other miscellaneous drugs.

Cyclobenzaprine was tested in the thalamus and spinal cord where it caused an equal reduction in the sensitivity of neurones to application of Glut, Asp and DLH in an appreciable percentage of the cells tested (table VIII). Reduction of amino acid sensitivity was usually accompanied by a reduction in the evoked responses. At iontophoretic current strengths of 80 - 100 nA the inhibition of the evoked responses was seldom more than 40% of the control levels and was always less than the reduction in the amino acid responses (figure 25). An intra-arterial injection of 40 mg. of CB caused an immediate cessation of respiration in the two cats tested. This was not investigated further.

Only three cells in the thalamus were tested with hydrastinine. In one of these there was a significant increase in the spontaneous firing rate with 80 nA of the drug, however, none of the cells showed any change in their responses to Glut or ACh, nor was there any change in the evoked response.

pCMS caused an enhancement of the sensitivity to the
Figure 24. A comparison of the effects of GDEE and MSO on the amino acid excitations of a neurone in the cerebral cortex. Ratemeter records are of excitations produced by DLH (60 nA), Glut (60 nA) and Asp (80 nA). A: control, B: following application of GDEE (60 nA) for 1 min., C: 1 min. following cessation of GDEE application, D: following application of MSO (60 nA) for 1 min., E: 1 min. following cessation of MSO application.
### TABLE VIII

The effect of pCMS and cyclobenzaprine on single cell excitations. Each set of figures indicates the ratio of the number of cells in which any reversible action on induced firing was observed, to the total number examined.

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</tr>
<tr>
<td></td>
<td>response response</td>
<td>response response</td>
<td>response response</td>
</tr>
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</tr>
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<td>2/9 3/9</td>
</tr>
<tr>
<td>Asp</td>
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</tbody>
</table>
Figure 25. The effect of cyclobenzaprine on the amino acid and synaptically evoked excitations of a VPL neurone. Ratemeter records are of excitations produced by DLH (40 nA), Glut (80 nA) and Asp (80 nA). The histograms show the summed evoked responses to 50 stimuli (1/2 sec., 0.1 msec., 3V) applied to the contralateral tibial nerve. A: before, B: following application of CB (80 nA) for 1-4 min., C: 15-16 min. following cessation of the CB ejecting current.
excitatory amino acids of a high percentage of the VPL neurones tested (table VIII, figure 26). The excitatory activities of Glut and Asp were enhanced to the same extent, and the effect of pCMS on these excitations was much greater than on that produced by DLH. The potentiation of amino acid responses was often accompanied by an increase in the response evoked by a perithreshold stimulus (figure 27). In some of these experiments Glut and Asp caused a burst of spikes at high frequencies followed by depolarization of the cell during pCMS ejection and for some time after its cessation. This effect would last up to 20 min. following a 10 min. application of pCMS, after which time normal amino acid responses would return. During this period the evoked response was usually depressed. No increase in background "spontaneous" firing of the cells comparable to that described in the spinal cord by Curtis et al. (1970) was observed when the pCMS ejecting current was terminated.
Figure 26. The effect of pCMS on amino acid excitations of a VPL cell. Ratemeter records of the excitation elicited by DLH (30 nA), Glut (80 nA) and Asp (80 nA). A: before, B: following the iontophoretic application of pCMS (50 nA) for 7 min., C: 11 min. after cessation of the pCMS ejecting current.
Figure 27. The effects of pCMS on amino acid and synaptically evoked excitations of a VPL neurone. Ratemeter records of excitations produced by DLH (40 nA) and Asp (160 nA) are shown. The histograms show the summed evoked responses to 50 stimuli (1/2 sec., 0.1 msec., 8V) to the contralateral tibial nerve. A: before, B: 8-9 min. after the cessation of the pCMS ejecting current which was applied for 10 min., C: 19-22 min. following cessation of the pCMS ejecting current.
CHAPTER IV

MATERIALS AND METHODS - RAT CORTEX PREPARATIONS

Adult male rats of the Long-Evans strain were used. The animals were decapitated and the entire brain removed and placed in cold Locke solution. Four slices weighing between 25 and 75 mg. were cut from the dorsal and lateral aspect of the cerebral cortex with the aid of an instrument similar to that used by Stadie and Riggs (1944).

a) Release of glutamate from brain slices.

A technique similar to that described by Arnfred and Hertz (1971) was used to study the effects of glutamate and its analogues on Glut release from brain cortex slices. After weighing, the cortical slices were placed in transfer holders and incubated at 37°C in test tubes containing 4 ml. of the basic medium. The holders (figure 28) were similar to those used by Arnfred et al. (1970). They consisted of a glass tube with a nylon mesh bottom, and a rubber stopper through which passed a short glass tube as well as a long glass tube which reached almost to the nylon mesh. Gas containing 95% O₂ and 5% CO₂ was continuously passed through the longer tube. When the transfer apparatus was placed in the test tube containing the basic medium, bubbling produced by the air kept the brain slices mobile in the
Figure 28. A diagram of the transfer holder used to transfer brain slices from one test tube of medium to another.
solution. The holder made it possible to transfer the brain slice from one solution to another quickly and easily.

The basic medium contained the following salts,

- KCl 5.0mM
- NaCl 120.0mM
- NaHCO\textsubscript{3} 15.0mM
- MgCl\textsubscript{2} 1.0mM
- CaCl\textsubscript{2} 1.5mM
- Glucose 1.0mM

In the Glut-containing medium 10mM NaCl was replaced by 10mM Glut. In the media containing GDEE and aMG, 30mM NaCl was replaced by 30mM of the respective Glut analogue. In the combined media, 40mM NaCl was replaced by 10mM Glut and 30mM of the analogue. Glut and the analogues were neutralized with NaOH before addition to the media. (U-\textsuperscript{14}C)-L-glutamic acid (206 mCi/mM) was obtained from New England Nuclear Corporation. The \textsuperscript{14}C-Glut medium was mixed by adding 100 µl. \textsuperscript{14}C-Glut to 20 ml. of basic medium.

The brain slices were incubated in the basic medium containing the \textsuperscript{14}C-Glut for a period of 40 min. After incubation the slices were washed for approximately 5 sec. in unlabelled medium and then incubated for 10 min. periods in each of a series of twelve test tubes with non-radioactive medium. The first four test tubes contained basic medium, the second four contained the Glut and/or its analogues and the third set of four test tubes contained basic medium. The slices were then drained, weighed and dissolved in
5 ml. of 1M-NaOH in a boiling water bath and the solution was diluted with distilled water to 25 ml. The $^{14}\text{C}$ released from the slices into each test tube was determined by counting 1.0 ml. of the medium from each tube in a liquid scintillation counter. Each sample was dissolved in 12 ml. of a mixture of 588 ml. ethanol and 412 ml. toluene in which was dissolved 4 gm. of Omnifluor (NEN cat #NEF-906A) and counted for a minimum of 10 min. (i.e. an efficiency of 1.0 - 5.0% compared to an internal standard). One ml. of the solution containing the dissolved brain slice was acidified with 0.1 ml. formic acid before dissolving it in the counting solution.

The amount of radioactivity found in the series of test tubes after the washout, was added to that which was left in the tissue, giving the total amount in the slice at the start of the washout. No correction was made for $^{14}\text{C}$ lost from the system as $^{14}\text{CO}_2$. The amount left at the beginning of each 10 min. period of the washout was calculated by subtracting the amount of radioactivity already washed out in previous test tubes from the total. The percentage loss of tracer into each test tube (rate coefficient, Shanes and Bianchi, 1959) was calculated as

\[
\frac{\text{c.p.m. in the test tube}}{\text{c.p.m. in the slice when transferred to this test tube}} \times 100
\]

b) **Glutamate uptake into synaptosomes.**

A technique similar to that used by Logan and Snyder
(1971) to measure Glut uptake into crude synaptosomal preparations of the cerebral cortex of rats was employed.

The cortical slices were homogenized in 20 volumes of cold 0.32M sucrose solution using a Potter-Elvehjem glass homogenizer fitted with a "Teflon" pestle. The homogenate was centrifuged for 10 min. at 1000 g. and 0.2 ml. aliquots of the supernatant were added to 3.8 ml. of Krebs-Henseleit solution containing:

\[
\begin{align*}
\text{NaCl} & : 118.0\text{mM} \\
\text{KCl} & : 4.7\text{mM} \\
\text{CaCl}_2 & : 2.5\text{mM} \\
\text{NaHCO}_3 & : 25.0\text{mM} \\
\text{KH}_2\text{PO}_4 & : 1.2\text{mM} \\
\text{MgSO}_4 & : 1.2\text{mM} \\
\text{Glucose} & : 11.1\text{mM}
\end{align*}
\]

A series of 8 test tubes containing various concentrations of \((U^{-14}\text{C})-\text{L-Glut}\) between 0.1 and 1mM were used in each experiment. The mixtures were equilibrated with 95% \(\text{CO}_2\) and 5% \(\text{O}_2\) and incubated for 4 min. at 37°C. The solutions were then immediately centrifuged at 27,000 g. for 15 min. at 0°C and the supernatants were poured off. The radioactivity in 50 μl. aliquots was assayed in a liquid scintillation spectrometer. The pellets were washed in 1 ml. of cold 0.15M NaCl and recentrifuged at 27,000 g. for 10 min.; the radioactivity in the pellet was extracted into 0.5 ml. of 1M hyamine hydroxide in methanol, dissolved
in 12 ml. toluene and Omnifluor and counted. Identical measurements were made following incubation in media containing 0.25 or 1mM GDME or 1mM GDEE in addition to the labelled Glut. From these measurements the amount of Glut taken up by the synaptosomal preparations could be calculated and plotted against concentration in the incubating medium.
a) **Release of glutamate from brain slices.**

The loss of $^{14}\text{C}$ from brain slices loaded with $^{14}\text{C}$-Glut and washed out in solution was increased by incubating the slices in a medium containing 55mM $K^+$ or 10mM Glut. The increased efflux of label when Glut was added to the incubation medium was greatest in the first test tube and then gradually decreased until it was just above control values by the fourth test tube. With further incubation of the brain slice in Glut-free solution the efflux of label usually continued at the same rate as the control. These results are similar to those observed by Arnfred and Hertz (1971).

Four brain slices were incubated simultaneously, each with a different combination of Glut and/or its analogues added to the solution. Figure 29 illustrates one example of a series in which i) Glut and analogue-free solution, ii) 10mM Glut, iii) 30mM GDEE, iv) 30mM GDEE and 10mM Glut was added to the solution of the 5th through the 8th test tubes. The GDEE caused an increased efflux of Glut similar but smaller than that brought about by Glut. The effects of Glut and GDEE were not additive, the net increase in
Figure 29. Plots of the effects of Glut and GDEE on the rate coefficient of Glut efflux from slices of cerebral cortex. All points marked with open markers and with crosses were obtained with washout in the basic medium. The points marked with solid markers were obtained when a) 10mM Glut, b) 30mM GDEE, c) 10mM Glut and 30mM GDEE was added to the basic medium between 40 and 80 min. from the beginning of the washout.
efflux with both of these compounds in the medium was equal to that caused by Glut alone.

A similar series of experiments using αMG instead of GDEE was carried out (figure 30). The addition of 30mM αMG to the incubation solution had very little, if any, effect on the Glut efflux and the addition of 10mM Glut and 30mM αMG did not change the efflux induced by Glut alone.

b) Glutamate uptake into synaptosomes.

The rate of accumulation of Glut by the homogenates incubated in solutions containing different concentrations of Glut, in the absence or presence of GDEE or GDME, were drawn on double reciprocal plots as functions of the Glut concentration (figure 31). The points are mean values obtained from 8 control experiments, 5 experiments with 1mM GDME, 4 with 0.25mM GDME, and 4 with 1mM GDEE. The regression lines were calculated by a least squares fit. Regression lines were also calculated for the Glut data with appropriate weighting to allow for the increasing variance of the $\frac{1}{V}$ values as $\frac{1}{S}$ increased, by the method of constrained regression. Since these procedures resulted in negligible changes, simple regression equations were used for all experimental data. It was impossible to describe the experimental points by a single line for the control and GDEE situations (cf. Logan and Snyder, 1971, 1972); all of the points however fell close to a single line when GDME was present in the incubation solution.
Figure 30. Plots of the effects of Glut and αMG on the rate coefficient of Glut efflux from slices of cerebral cortex. All points marked with open markers and with crosses were obtained with washout in the basic medium. The points marked with solid markers were obtained when a) 10mM Glut, b) 30mM αMG, c) 10mM Glut and 30mM αMG was added to the basic medium between 40 and 80 min. from the beginning of the washout.
Figure 31. Double reciprocal plots of the velocity (v: μmoles/gm. fresh tissue/min.) of the uptake of L-Glut by homogenates of rat cerebral cortex, as a function of the concentration (s: mM) of Glut in the incubation medium. In the three graphs where GDME and GDEE were added, the dashed lines indicate the control values. The regression lines have been calculated as described in the text.
From these plots, values of the Michaelis affinity constant ($K_m$) for a high and a low affinity uptake were calculated by determining the reciprocal of the intersection of the $\frac{1}{S}$ axis. The probability that the values differed from the control was computed from the residual variances about the regression lines (table IX). The data indicate that neither ester exerted a significant effect upon the low affinity uptake of Glut, but that 0.25mM GDME partially and 1mM GDME completely inhibited the high affinity system. GDEE also appeared to reduce the high affinity uptake of Glut, but the difference from the control situation was not significant.
TABLE IX
KINETICS OF GLUTAMATE UPTAKE INTO HOMOGENATES OF RAT BRAIN.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$K_m$ values ($\mu$M)</th>
<th>High affinity</th>
<th>Low affinity</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td></td>
<td>22</td>
<td>98</td>
<td>-</td>
</tr>
<tr>
<td>+0.25mM GDME</td>
<td>64</td>
<td>^0.025</td>
<td>64</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>+1.00mM GDME</td>
<td>98</td>
<td>0.005</td>
<td>98</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>+1.00mM GDEE</td>
<td>31</td>
<td>^0.100</td>
<td>109</td>
<td>&gt;0.1</td>
</tr>
</tbody>
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CHAPTER VI

MATERIALS AND METHODS - CRAYFISH PREPARATIONS

Experiments were carried out on adult Columbia River crayfish of the species *Pacifastacus leniusculus*. Van Harreveld's (1936) salt solution consisting of,

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<tbody>
<tr>
<td>NaCl</td>
<td>200.50mM</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>5.37mM</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.25mM</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.70mM</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10.00mM</td>
<td></td>
</tr>
</tbody>
</table>

was used as the bathing medium. All test solutions were made up in this medium by replacing equimolar amounts of NaCl with the sodium salts of the drugs used and adjusting the pH to 7.8 using NaOH.

a) **The abdominal stretch receptor.**

Methods similar to those used by Wiersma et al. (1953) and Florey (1957) were employed for dissecting and recording from the abdominal stretch receptors. The tergal parts of the abdominal segments were dissected free from the rest of the abdomen in the form of a single strip containing all of the extensor musculature. The proximal end of the first tergite was placed in a clamp with the ventral surface up.
The ventral and superficial extensor musculature with the exception of the musculus superficialis lateralis was removed. This muscle serves to protect the receptors from being stretched by pull on the nerve. A thread was fixed on the last segment by means of a hook, making it possible to flex the abdominal strip. The dorsal half-shell of the "tail" of the crayfish in this way served as a receptacle for the test solutions. Removal of the tergal strip necessarily severs the nerve trunk which supplies the stretch receptors, but a sufficient length of nerve remains to allow for the placement of electrodes. Solutions to be tested were applied topically to the stretch receptor organ, and after 10 sec. exposure, the excess fluid was sucked away, and the effect upon the discharge of the slowly adapting receptor noted.

The action potentials were recorded from the axon by a silver wire electrode and the clamp holding the preparation served as the indifferent electrode. The action potentials generated by the slowly adapting stretch receptor, which were the only ones occurring spontaneously in this preparation, were amplified and displayed on an oscilloscope. The frequency of firing was determined with a ratemeter and recorded on paper chart. Figure 32 gives a block diagram of the preparation and equipment.

b) The closer muscle of the claw.

The cheliped of the crayfish was removed at the ischiopodite. The medial half of the exoskeleton of the
Figure 32. A block diagram of the equipment used to record from the abdominal receptor in crayfish.
meropodite was removed and the nerves exposed. The point of the fixed finger of the propodite was cut and a cannula inserted. Fluid running through this cannula circulated through the claw and leaked out at the meropodite. The entire claw was pinned firmly to a wax plate. The nerve was dissected free and divided. Each division was stimulated using bipolar silver electrodes until the excitatory nerve to the closer muscle was found. In order to record the contraction of the closer muscle, the point of the dactylopodite of the claw was connected to a force transducer which in turn was connected to one channel of a polygraph. Figure 33 illustrates the preparation. A mariotte bottle containing van Harreveld's salt solution was elevated above the claw and attached to the cannula in the propodite. The continuing circulation of fluid kept the muscles and nerves moist. The drugs were applied through the same cannula and washed out again with van Harreveld's solution.
Figure 33. A block diagram of the equipment used to record closer muscle contraction in the claw of the crayfish.
CHAPTER VII

RESULTS - CRAYFISH PREPARATIONS

a) The abdominal stretch receptor.

By flexing the dorsum of the tail of the crayfish it was possible to make the stretch receptor fire at a constant rate. This rate of firing could be varied from zero to approximately 25 spikes/sec., by increasing the tension on the line flexing the abdominal segments. The stretch receptor was sensitive to temperature, increasing its rate of discharge when flushed with a cold solution, while a decrease was observed when warm solution was applied. A number of Glut analogues were tested on this preparation and the ratemeter recording of the pattern of firing was used to determine whether these analogues had any excitatory or inhibitory action on the stretch receptor. Table X gives the list of substances used in order of excitatory potency. N-methyl-dl-glutamic acid had by far the greatest excitatory effect on the stretch receptor and at high concentrations (10 mg./ml.) it would cause a burst of potentials which increased in frequency and decreased in amplitude until the receptor was depolarized and stopped firing.

Glut was a good excitant, although repetitive applications caused successively decreasing responses. As could
TABLE X

THE EFFECTS OF A NUMBER OF GLUTAMATE ANALOGUES ON THE FIRING RATE OF THE STRETCH RECEPTOR IN THE CRAYFISH. THE COMPOUNDS ARE LISTED IN ORDER OF THEIR EXCITATORY POTENCY.

<table>
<thead>
<tr>
<th>Glut analogue</th>
<th>Order of potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Methyl-DL-Glutamic acid</td>
<td>Very strong</td>
</tr>
<tr>
<td>4-Fluoroglutamic acid</td>
<td></td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>Strong</td>
</tr>
<tr>
<td>L-Glutamic acid diethyl ester</td>
<td></td>
</tr>
<tr>
<td>para-Nitrobenzoyl-L-Glutamic acid</td>
<td></td>
</tr>
<tr>
<td>DL-Homocysteic acid</td>
<td></td>
</tr>
<tr>
<td>L-Glutamic acid γ-Methyl ester</td>
<td></td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>Weak</td>
</tr>
<tr>
<td>N-Methyl-DL-Aspartic acid</td>
<td></td>
</tr>
<tr>
<td>α-Methyl-L-Glutamic acid</td>
<td></td>
</tr>
<tr>
<td>D-Glutamic acid</td>
<td></td>
</tr>
<tr>
<td>α-Amino-L-Adipic acid</td>
<td></td>
</tr>
<tr>
<td>Acetyl-L-Glutamic acid</td>
<td></td>
</tr>
<tr>
<td>para-Aminobenzoyl-L-Glutamic acid</td>
<td></td>
</tr>
</tbody>
</table>
be expected the greatest effects of Glut on the receptor were observed when the firing rate was initially low, whereas at high firing rates a much smaller response was observed. αMG had little or no excitatory effects on the firing rate of the stretch receptor. In those preparations where high concentration (10 mg./ml.) of αMG did not affect the firing rate, application of αMG prior to Glut would reduce or eliminate the response of the receptor to Glut (figure 34). The sensitivity of the receptor to Glut returned following thorough washing with fresh van Harreveld's solution. The effects of αMG did not appear to be specific for Glut since a similar depression of the effects of low concentrations of N-methyl-Glut could also be obtained. GDEE was as effective an excitant as Glut and it was therefore impossible to determine any blocking action by this compound.

b) The closer muscle of the claw.

Stimulation of the nerve in the meropodite with repetitive stimuli at a frequency of 50/sec. caused a contraction of the closer muscle and exerted tension up to 20 gm. on the transducer. The only drug tested in this preparation was GDEE (figure 35). After a constant level of contraction with successive stimuli was obtained, 0.2 mg. of GDEE in van Harreveld's solution was perfused through the claw. Stimuli following application of GDEE at first caused erratic contractions followed by successive reductions in the force of the contraction. After the claw was perfused
Figure 34. The effect of αMG on the Glut excitations of the abdominal stretch receptor in the crayfish. Ratemeter records show the effect of Glut (10 mg./ml.) alone, Glut following application of αMG (10 mg./ml.) and finally after rinsing with van Harreveld's solution.
Figure 35. The effect of GDEE on synaptically induced contractions of the closer muscle of the claw of the crayfish. Records show the tension produced by the closer muscle on application of a stimulus (50/sec., 0.2 msec., 50V) to the excitatory nerve of this muscle. 0.75 ml. of a lmM solution of GDEE was applied through a cannula in the claw. Rinsing was accomplished with the use of van Harreveld's solution.
with fresh van Harreveld's solution the response to stimuli slowly recovered until sustained contractions were again maintained.
CHAPTER VIII

DISCUSSION

The results obtained in this investigation demonstrate that it is possible to block reversibly or to enhance the excitatory action of glutamate on single neurones of the central nervous system by the local application of suitable pharmacological agents. The action of these agents appeared to be relatively specific for glutamate, in that the activation of the neurones by the exogenous application of other excitatory agents was often not affected. The synaptic activation of neurones in a number of sensory pathways was affected by the pharmacological agents in a similar manner to that of the glutamate excitation, which suggests that glutamate may be the physiological transmitter of excitation at these synapses.

Of the compounds tested GDEE showed the greatest blocking action on glutamate responses. Blockade was most noticeable in the spinal cord, cuneate and thalamus and was observed following either iontophoretic or systemic administration of GDEE. That the glutamate excitation was not depressed in every cell may be attributed to the limitations of the iontophoretic technique which makes it impossible to determine the exact concentration of the
compound in the region of the receptor (Curtis and Crawford, 1969).

GDEE showed a large degree of specificity in as much as it antagonized the glutamate-induced neuronal excitations to a greater extent than those produced by the other excitatory amino acids and ACh. The degree of specificity, however, was dependent on the amount of GDEE applied, thus with large iontophoretic currents, GDEE would usually block the action of all of the amino acid excitations, but even under these circumstances neuronal excitation by ACh could be spared. With suitable adjustment of the GDEE current it was usually possible to block the glutamate effect specifically without affecting the response of the cell to aspartate, cysteate and DLH, and therefore in the present investigation the sensitivity of the cell to DLH was used as a control. The fact that ACh responses were very little affected may explain why Curtis et al. (1972) were unable to observe the same degree of specificity towards the amino acids, since they used the excitations produced by ACh as controls.

The question whether DLH is an appropriate compound to compare with glutamate however, requires cautious consideration. The fact that in every case the DLH-ejecting current used was lower than the glutamate current required to produce an equivalent excitation (McLennan, 1970b), indicates that the net effectiveness of DLH upon the cell membrane is greater. The relative failure to antagonize
the action of DLH might therefore merely reflect a quantitative rather than a qualitative difference between the two amino acids; however, the same consideration would not apply to the specific effect of GDEE on glutamate relative to ACh, aspartate and cysteate (figures 5,7). The specificity of action also indicates that GDEE does not exert its effect by depressing the overall excitability of the cell.

Another consideration is that since glutamate and aspartate are known to be transported into nerve endings by a high affinity system (Logan and Snyder, 1971; Wofsey et al., 1971), whereas other amino acids (including presumably DLH and cysteate) are not, the non-natural sulphonic acids ejected into the perineuronal extracellular space might diffuse to affect more distant receptors than could be influenced by glutamate or aspartate (Curtis, Duggan and Johnston, 1970a,b; Curtis et al., 1972). Such distant receptors might therefore be too far removed from the blocking action of GDEE to be affected by the drug, and thus the antagonist would appear less effective against DLH and cysteate. On the other hand, the finding that aspartate was antagonized to a lesser degree than glutamate (figures 7,9,11) would argue against this view and in favour of the existence of neuronal sites specifically or at least preferentially sensitive to glutamate.

This concept of the division of receptors into a group which is unspecific and reacts with all the excitatory
amino acids and one which is more specific for glutamate was first proposed by McLennan et al. (1968) when they showed that the sensitivity of thalamic neurones to DLH and glutamate did not invariably run parallel. McLennan (1970b) also found that excitations of cortical neurones induced by iontophoretically applied glutamate were depressed for a longer time by stimuli to neighbouring cortex than were comparable excitations produced by DLH. The experiments of Boakes et al. (1970) indicated that in certain brain stem neurones it was possible to block glutamate but not DLH responses with LSD, and they also suggested that glutamate and DLH react with different receptors.

The observation that different populations of spinal neurones show a greater relative sensitivity to aspartate than to glutamate (Duggan, 1971) indicates that in addition to a non-specific receptor site for the acidic amino acids and one which is more sensitive to glutamate there is also likely to be a receptor which has a greater sensitivity to aspartate than to the other excitants. The existence of neurones with different relative sensitivities to glutamate and aspartate in the spinal cord was confirmed in the present investigation, and it is interesting to note that in those neurones which tended to be more sensitive to aspartate, GDEE blocked the effects of glutamate and aspartate to the same extent.

The receptor which reacts with ACh can be readily distinguished from the receptors sensitive to the acidic
amino acids. Drugs such as atropine or dihydro-β-erythroidine which block ACh excitation have very little if any effect on amino acid excitation (Krnjević, 1964; McLennan, 1970a), while MSO (present investigation; Curtis et al., 1972), 2-methoxy-aporphine (Curtis et al., 1972) and the compound L-hydroxy-3-aminopyrrolidone-2 (HA-966) (Davis and Watkins, 1972) on the other hand appear to block amino acid-induced excitations without appreciably affecting those produced by ACh. The ability of MSO to block the amino acid responses in the present investigation was not as great as that observed by Curtis et al. (1972). This is probably due to the fact that Curtis et al. used the L-isomer of MSO, whereas in the present investigation the DL-isomer was used, especially since the convulsant activity and the ability to inhibit glutamate synthetase depends on the isomeric configuration (Rowe and Meister, 1970). In neither case, however, was any significant degree of specificity towards glutamate excitations shown. The difference in the effects of MSO and GDEE becomes obvious when they are compared on the same cell (figure 24).

αMG was the only other compound tested which showed any blocking action. In contrast to the results reported by Marshall (1971) its effect was found to be rather weak and inconsistent. More commonly, effects similar to those described by Curtis et al. (1972) were observed, where αMG had a tendency to potentiate the effects of all other excitatory compounds (figure 22). The latter effect could well
be due to a subthreshold depolarization of the neurone, thus increasing its excitability, since αMG tended to increase the spontaneous firing rate of a number of cells when applied at high iontophoretic current strengths.

A potentiating effect on the excitations produced by the acidic amino acids (especially glutamate and aspartate) was observed following application of GDME in all areas of the nervous system tested. These effects do not appear to be due to a subthreshold depolarization since the effects of DLH were not increased to the same extent (figures 17, 19, 20). However, GDME may cause some degree of depolarization since an increase in the background firing rate was occasionally observed. The occasional blocking action observed with GDME (figure 16) may be due to its structural similarity to GDEE; however, even in these cases there was a potentiating effect on cessation of the GDME-ejecting current. The fact that the potentiating effect of GDME and the blocking effect of GDEE can be demonstrated on the same cell (figure 18) where the pH concentration and ejecting currents of the two esters are the same, indicates that the "receptors" which are responsible for the action of these drugs have a high degree of specificity. GDME occasionally caused blockade of the responses of neurones to glutamate (figure 16) and GDEE showed some inhibition of glutamate uptake into synaptosomes (figure 31). This suggests that glutamate excitation and glutamate uptake are inhibited by both esters, GDEE having its predominant effect
on glutamate excitation whereas GDME had the greatest effect on glutamate uptake.

The potentiating effects of GDME can be explained in terms of inhibition of the uptake system for glutamate and aspartate. The experiments on glutamate uptake confirmed those of Logan and Snyder (1971, 1972), which indicated that high affinity as well as low affinity mechanisms exist for the uptake of glutamate into crude synaptosomal preparations. The inhibition produced by GDME is limited to the high affinity system (figure 31). The marked increase in $K_m$, the Michaelis affinity constant, suggests a competitive inhibition; however, concomitantly there is an apparent increase in $V_{\text{max}}$ and the data therefore do not fit any of the classical models of inhibition for a single enzyme system. Preliminary experiments on leucine uptake suggest that the inhibitory action of the GDME is not specific for glutamate uptake. The value determined for $K_m$ for the high affinity uptake of glutamate is comparable to that reported by Logan and Snyder; however, that for the non-specific low affinity system is only about one-tenth of the minimum value reported by those authors. No evident explanation for the difference presents itself.

The data support the suggestion that transport into cellular elements may well be the mechanism for the inactivation of the excitatory amino acids (Curtis, Duggan and Johnston, 1970). It may be argued (McLennan, 1970a) that a prolonged excitatory action on cessation of the
glutamate-ejecting current as well as a potentiation of the excitation could be expected on inhibition of the uptake mechanism. However, this would not be the case if the inhibition was only partial, or as in this case, only one of the two uptake systems was affected. Assuming that under normal circumstances the uptake is not close to saturation the residual uptake mechanism after inhibition by GDME could well be sufficient to inactivate the glutamate on cessation of the ejecting current with no noticeable increase in the inactivation time; however, the inhibition of the high affinity uptake mechanism would allow a greater concentration of glutamate to accumulate extracellularly and thus cause a greater excitation. Similar potentiating effects without prolonged excitation observed following systemic administration of thiosemicarbazide (Steiner and RUF, 1966) could be explained in terms of inhibition of glutamate decarboxylase which is located intracellularly (Salganicoff and De Robertis, 1965) thus increasing the intracellular content of glutamate. This would have the effect of increasing the concentration gradient across the cell membrane and thus inhibit the uptake mechanism.

Both pCMS (which also inhibits amino acid uptake into slices of cerebral cortex (Curtis et al., 1970)) and GDME have a potentiating effect on evoked responses of cells in the thalamus as well as enhancing the excitations produced by glutamate and aspartate (figures 19, 27) and GDME has a similar effect in the spinal cord and cuneate nucleus.
(figures 20,21). This would suggest that the transmitter released at these synapses is inactivated by a mechanism which is inhibited by these agents. However, the possibility that this effect is the result of a subthreshold depolarization cannot be excluded.

The primary sensory pathway.

The simultaneous blocking of the glutamate responses and the synaptically evoked potentials in spinal interneurones and in cuneate neurones by GDEE (figures 11,12,15) is strong evidence to suggest that glutamate may be the mediator of synaptic transmission at primary afferent terminals. The preliminary observation by Davies and Watkins (1972) that HA-966, which has a depressant effect on the glutamate excitations of cuneate cells blocks the response of these cells to cutaneous stimulation further supports this possibility.

The suggestion that glutamate may be the transmitter at the primary sensory terminals was made by Aprison et al. in 1965 when they demonstrated a higher concentration of glutamate in dorsal roots and spinal cord grey matter than in the ventral roots or spinal white matter. Further studies (Duggan and Johnston, 1970a,b; Johnson and Aprison, 1970, 1971) have shown that the centrally directed portion of nerve roots from spinal ganglia have a higher glutamate content than the peripherally directed nerves and that concentrations in the dorsal regions of the spinal cord are
higher than those of the ventral. Similarly glutamate concentrations have been shown to be present in significantly higher concentrations in the cuneate and gracile nuclei than in more ventral regions of the brain stem. These data are consistent with the suggestion that glutamate is a transmitter in these regions if one accepts the idea that glutamate is compartmentalized into metabolic and transmitter "pools" in the C.N.S. Under these circumstances one would expect those areas which utilize glutamate as a transmitter to show a higher level of this amino acid by an amount equal to the transmitter pool. The fact that none of the other amino acids showed similar differences in concentration further suggests that glutamate serves some specialized function in these regions.

A number of other compounds have been suggested as mediators of synaptic transmission at primary afferent neurones. Amongst these are ACh (Büllbring and Burn, 1941), ATP (Holton and Holton, 1954), substance P (Lembeck, 1953) and histamine (Holton and Perry, 1951). The evidence suggests that ACh is not the transmitter at primary afferent terminals since dorsal roots and the medullary sensory nuclei contain very little ACh or choline-acetyltransferase (McIntosh, 1941; Feldberg and Vogt, 1948). Further evidence against ACh serving as a transmitter at primary afferent terminals is the inability (Curtis et al., 1961; Steiner and Meyer, 1966; Galindo et al., 1967) or inconsistent ability (Weight and Salmoiraghi, 1966) of ACh to
excite motorneurones, interneurones and cuneate neurones when applied iontophoretically, and the lack of effect of ACh blocking agents on the response of spinal neurones to sensory stimulation (Curtis et al., 1961).

Substance P, histamine and ATP have been proposed as possible mediators of primary afferent synapses on the grounds that they are present in distal portions of cutaneous nerves and are able to cause vasodilation in the rabbit ear (McLennan, 1970a). This reasoning stems from the Dale hypothesis which states that a neurone releases the same compound at all of its terminal branches and since antidromic stimulation of peripheral sensory nerves can cause vasodilation it is likely that the transmitter in these nerves is responsible for this action. It seems unlikely however, that either substance P or histamine are involved in central transmission at primary afferent synapses since neither of them have any excitatory ability when applied directly to cuneate cells (Galindo et al., 1967). It is possible that the presence of histamine in distal portions of cutaneous neurones may be the result of trauma (McLennan, 1970a) and that substance P may in some manner bind with the transmitter and influence its activity (Umrath and Grallert, 1967).

ATP on the other hand, does show powerful excitatory action when applied to cuneate and gracile neurones (Galindo et al., 1967). This action has been attributed to the calcium chelating properties of ATP, since other chelating agents show similar excitatory activity (Curtis, Perrin and
Watkins, 1960; Galindo et al., 1967). However, the observation that spinal (Curtis et al., 1961) and cortical (Krnjević and Phillis, 1963a) neurones are unaffected by ATP infers that cuneate neurones may be particularly sensitive, and this together with the observation that liberation of catecholamine from the adrenal medulla is accompanied by ATP release (Douglas and Poisner, 1966) has led to the suggestion that ATP liberated together with the transmitter may act to modify the postsynaptic response (McLennan, 1970a).

The suggestion that aspartate may act as an excitatory transmitter of spinal interneurones was made by Davidoff et al. (1967) when they observed that there was good correlation between the loss of spinal interneurones and the decrease in aspartate concentration following spinal cord ischaemia. This possibility is further enhanced by the observation that Renshaw cells appear to be relatively more sensitive to aspartate than to glutamate, in comparison with a population of dorsal spinal interneurones (Duggan, 1971) and the current observation that those neurones which tended to be more sensitive to aspartate did not show the same degree of specificity to the glutamate blocker, GDEE. It is interesting to note that glutamate increases the excitability of primary afferent terminals in the cuneate nucleus whereas aspartate has no such effect (Davidson and Southwick, 1971).
The secondary sensory pathway.

Neurones in the VPL nucleus of the thalamus showed the same response as spinal interneurones to the application of GDEE and GDME (figures 10, 14, 19), suggesting that glutamate may be the mediator of synaptic transmission at the secondary sensory synapses as well as at the primary afferent terminals. The fact that ventrobasal neurones in the thalamus are excited by the acidic amino acids has been known for some time (Curtis and Watkins, 1963) and as mentioned in the introduction, these neurones are relatively more sensitive to glutamate than are more superficial thalamic neurones (McLennan et al., 1968). The thalamus contains fairly large amounts of free glutamic acid (Johnson and Aprison, 1971). On autopsy the concentration of glutamate in the thalamus of humans has been shown to be higher than in the substantia nigra and red nucleus, but not as high as in the basal ganglia and cortex (Perry et al., 1971). This fairly specific localization might well be due to changes which occur following death, however, the lack of correlation between glutamate concentrations and those of GABA, glutamine and aspartate suggest that it may be involved in specific functions unrelated to metabolism in those regions where it is present in high concentrations.

Additional evidence in favour of glutamate as a transmitter at sensory synapses in the thalamus is the fact that none of the other putative transmitters appear to be involved. There are only moderate amounts of acetylcholine, acetyl-
cholinesterase and choline acetyltransferase in the mammalian thalamus in comparison to basal ganglia and ventral roots (McIntosh, 1941; Feldberg and Vogt, 1948; Oliver et al., 1970) which suggests that the major afferent pathways to the thalamus are not cholinergic. There do appear to be some regional differences in cholinesterase staining within the thalamus (Oliver et al., 1970), and it seems likely that inputs other than the primary sensory ones may utilize a cholinergic mechanism (McCance et al., 1968; Marshall, 1971). Further evidence to suggest that any cholinergic input to ventrobasal neurones is unrelated to the primary afferent pathway is the fact that whereas most of these neurones are readily excited by acetylcholine it is possible to block this excitation with atropine or dihydro-β-erythroidine without affecting the response to cutaneous nerve stimulation (Andersen and Curtis, 1964).

The catecholamines and 5-HT are present in the thalamus (McGeer et al., 1963), and NA and 5-HT have been shown to excite a few cells there (Phillis and Tebécis, 1976; but see Frederickson et al., 1971 and Jordon et al., 1972). However, there is no evidence to suggest that these compounds are responsible for any excitatory synaptic mechanisms. On the contrary, they have been proposed as inhibitory transmitters since their prime effect on neurones is one of depression. The observation that 5-HT inhibits the response of lateral geniculate neurones to optic nerve stimulation, but does not affect the responses of ventrobasal thalamic
neurones to impulses in cutaneous nerves, has led to the suggestion that the spinothalamic and opticogeniculate pathways utilize different transmitter mechanisms (Curtis, 1966).

The thalamocortical pathway.

GDEE blocked the response of cortical neurones to thalamic stimulation and glutamate application (figure 13); however, the action of GDEE in this region was much less consistent than in the spinal cord, cuneate nucleus and thalamus. This inconsistency could be due to the extreme neuronal complexity of this structure and the likelihood that a variety of pathways with different synaptic transmitters are involved in its function.

The observation that GDEE was able to block the excitatory effects of glutamate fairly specifically at certain cells, whereas at others GDEE and MSO had either no effect or a non-specific effect on the sensitivity of the neurones to the excitatory amino acids, might be due to differences in sensitivity of different cells to glutamate. Crawford (1970) was unable to show any difference in the ratio of the sensitivity of any population of cortical cells to DLH, aspartate and D-glutamate when compared to that of L-glutamate; however, the wide variation in the responses of neurones to DLH and L-glutamate which he attributed to limitations in the technique of iontophoresis, might well have been due to real differences in the sensi-
tivity of the neurones to the two amino acids. McLennan (1970b) on the other hand, found that excitations of cortical neurones induced by iontophoretically applied L-glutamate were depressed for a longer time by stimuli to neighbouring cortex than were equivalent excitations produced by DLH. The differences seen were not the same at all cortical depths, but were more pronounced in layers II and V, suggesting that the pyramidal cells giving rise to cortical efferents which lie in those zones may be somehow specialized in this regard.

Additional evidence in favour of glutamate playing some synaptic role in the cortex is that glutamate is present in high concentration in cortical tissue (Johnson and Aprison, 1971; Perry et al., 1971) and a portion of it is located in the synaptosomal fraction (Kuher and Snyder, 1969). Increased amounts of glutamate and ACh can be collected from fluids bathing the cerebral cortex during arousal states and following reticulocortical stimulation (Jasper and Koyama, 1968, 1969), whereas on thalamic stimulation only ACh could be collected. This might be interpreted as suggesting that the thalamocortical pathway utilizes a cholinergic transmission mechanism, and there is a fair amount of additional evidence to suggest that ACh is involved in a thalamocortical pathway. ACh and its synthetising enzymes are present in high concentrations in the cortex (Feldberg and Vogt, 1948) and are decreased in chronically undercut cortical slabs (Collier and Mitchell, 1967), and cells in the deeper cortical layers are strongly excited.
by acetylcholine (Krnjević and Phillis, 1963b; Crawford, 1970).

ACh does not appear to be the transmitter in the main sensory pathway, since atropine has been found to abolish the cholinceptive responses without any effect upon the discharge evoked by sensory stimulation (Krnjević and Phillis, 1963b). However, the responses of cells in the cortex to thalamic stimulation can sometimes be blocked by the iontophoretic application of atropine (Stone, 1972). In these experiments the latter part of the burst response elicited in the cortex by stimulation of VPL was blocked by atropine while the first few spikes were unaffected, and this suggests that two mechanisms may be involved in the thalamocortical pathway. Similar conclusions can be drawn from the observation that inhibition of cholinesterase in the cortex by DFP increases the amplitude of the late response to stimulation of the forepaws in rats without affecting the primary complex of the evoked potentials (Bhargava, 1972).

The presence of a "cholinergic" as well as a possible "glutaminergic" pathway between the thalamus and the cortex could well explain the present inconsistent results. It is possible that those cells which have a cholinergic input have only the non-specific type of receptor and respond to GDEE with a decrease in all the amino acid responses, while those which have a glutaminergic input respond to GDEE with a specific decrease in the glutamate and evoked responses. It is possible that glutamate is responsible for the direct
pathway from somatic afferents and ACh for a second pathway of unknown function.

The anaesthetic and muscle relaxant.

Barbiturate anaesthesia has been shown to depress the mean spontaneous firing rate of cortical neurones (Curtis and Crawford, 1970) and is responsible for the spontaneous spindle activity in the thalamus (Andersen et al., 1967; Baker, 1971). In the spinal cord it reduces the number of spikes in the response evoked by peripheral nerve stimulation (Wall, 1967), but does not affect the initial part of the response. Similarly, the short latency responses of ventrobasal thalamic nuclei to stimulation of cutaneous nerves (Baker, 1971) appear to be unaffected by barbiturates. The effects of barbiturates on iontophoretically applied excitants seems to vary, depending on the area tested, however, in all cases it has either little effect on the sensitivity of neurones to glutamate and DLH (Krnjević and Phillis, 1963a; Bloom et al., 1965; McCance et al., 1968) or depresses the sensitivity of the neurone to all forms of excitation (Crawford and Curtis, 1966).

The possibility that gallamine triethiodide, which was used to paralyze the animals, might have some effect on the results should also be considered. This compound applied systemically has been shown to augment the after discharge of neurones in the intact and isolated cortex of unanaesthetised cats (Halpern and Black, 1967), to increase the mean
spontaneous firing rate of neurones in the feline cuneate nucleus (Galindo et al., 1968) and can modify transmission through this nucleus. These observations suggest that gallamine, which has an excitatory effect on neurones when applied iontophoretically (Crawford and Curtis, 1966; Galindo et al., 1968), penetrates the blood-brain barrier. However, it is possible that the effects which follow systemic application may reflect alterations in the activity of peripheral sensory receptors (Curtis and Crawford, 1969).

The release of glutamate from brain slices.

In the experiments on slices of rat cortex the release of labelled glutamate obtained by adding glutamate or K\(^+\) to the incubation medium is thought to be due to a depolarization of the cells in the slice (Arnfred and Hertz, 1971). If this is true, then the present results suggest that GDEE has a similar depolarizing effect on the cells and aMG is unable to block the depolarization caused by glutamate. The total amount of label released, in excess of the basal release seen in the control experiments (figure 29; Arnfred and Hertz, 1971), appeared to be constant since the presence of glutamate and GDEE in the bathing medium did not increase the glutamate release over that released by each of these compounds by itself, and the increased release over control values was initially very marked but returned to control levels by the end of 40 min. even though the concentration of glutamate and/or GDEE in the bathing medium
remained constant. This suggests that the extra labelled glutamate released by these compounds, and that released continuously into the solution might possibly originate from different "pools". Similar conclusions were drawn by De Feudis (1971), who showed that only a portion of the total exchangeable glutamate absorbed into desheathed nerves could be released on electrical stimulation.

The crayfish neuromuscular junction.

As can be seen from table X the relative sensitivity of the abdominal stretch receptor in the crayfish to the excitatory amino acids differs from that observed in the mammalian C.N.S. For example, the stretch receptor is more sensitive to glutamate than to DLH, whereas the opposite is true in the mammalian C.N.S. The stretch receptor is also very sensitive to N-methyl-DL-glutamic acid and insensitive to the corresponding aspartate compounds, whereas in the spinal cord, neurones show the opposite relative sensitivity (Curtis and Watkins, 1960, 1963). It is possible that these compounds act on the stretch receptor neurones as does ACh (McLennan and York, 1966) rather than on the muscle directly, since no excitatory innervation of the muscle has been found (Jansen et al., 1971). However, the fact that nerve-muscle preparations in the grasshopper are also more sensitive to glutamate than to DLH (McDonald and O'Brien, 1972) suggests that the receptors for glutamate
in invertebrates do differ from those in the mammalian C.N.S.

The blocking of the response of the stretch receptor to glutamate by αMG (figure 34) and of the contraction of the closer muscle by GDEE (figure 35) require cautious interpretation. It is possible that these compounds competitively depress the sensitivity of the membrane both to glutamate and to the physiological transmitter. However, both of the compounds cause some degree of excitation of the stretch receptor and it is thus possible that they block the responses by causing a depolarization of the muscle, although if this was the case one would expect an initial contraction on application of the drug.

The ability of GDEE to block glutamate responses specifically, without affecting the sensitivity of cells to DLH, aspartate or cysteate, together with the other observations on differences in the relative sensitivity of neurones to the acidic amino acids, suggest that the active sites on membranes for glutamate differ from those for the other amino acids. Curtis and Watkins (1960) have shown that the size of the amino acid and the number of ionized groups it contains determine to a large extent its activity. In an attempt to make the ionized groups on three excitatory amino acids conform to the classical "3-point" receptor site proposed by Curtis and Watkins (1960), von Gelder (1971) had to propose that the amino acids adopt a folded molecular arrangement. If, however, one assumes that there are
different receptor sites on the membrane which react with amino acids with different numbers of carbon atoms between the acidic groups and with different steric configurations, then such complex proposals become unnecessary. The hypothetical existence of a receptor site for a five carbon dicarboxylic amino acid leads to the speculation of the existence of a receptor site which is preferentially sensitive to the four carbon atom equivalent. If this is the case, it should be possible to find a compound which selectively depresses the sensitivity of cells to aspartate.

The series of experiments in this thesis was undertaken in an attempt to satisfy one of the criteria for glutamate as a transmitter i.e. that the physiological transmitter at specific synapses must react in the same manner in the presence of pharmacological agents as does glutamate. That the evoked response at primary and secondary sensory afferent terminals responded to the presence of a variety of agents in the same manner as glutamate-induced excitations, decreasing when glutamate responses decreased and increasing when they increased, suggests that the attempt has been successful. It is hoped that these results may be useful in future experiments to satisfy more completely other criteria for amino acids as transmitter agents at specific pathways. The use of GDME to block the inactivation of glutamate should permit better recovery of this compound from the region of a nucleus if it is released from terminals which end there. The ability of GDEE to block glutamate
excitations specifically and MSO to block all of the amino acid excitations should prove valuable in intracellular investigations in further pursuit of the criterion of "identity of action". The substantiation of the proposed mode of action of the various blocking and potentiating agents will depend to a large extent on an intracellular investigation; and the possibility of specific "receptors" for aspartate and that this compound too may act as a transmitter will require a great deal of further research.
SUMMARY AND CONCLUSIONS

1. Following an examination of the literature on Glut as a potential mediator of synaptic transmission, it became apparent that the criterion which states that the physiological transmitter must respond to the actions of pharmacological agents in the same manner as Glut had not been fulfilled.

2. The iontophoretic or intravenous application of GDEE was found to block the responses of neurones in the thalamus to Glut without appreciably affecting the responses to DLH, Asp, Cys and ACh. This specific action suggested that receptors exist which are preferentially sensitive to Glut. The action of Glut and GDEE appeared to be competitive since the dose-response curve of a neurone to Glut shifted to the right in the presence of GDEE.

3. The concomitant blocking of Glut-induced excitations and the responses in the thalamus and spinal cord evoked by electrical stimulation of peripheral nerves, and those in the cuneate nucleus evoked by dorsal column stimulation, were interpreted as suggesting that Glut could well be the physiological transmitter at primary and secondary afferent terminals.

4. The response of cortical neurones to application of
Glut and thalamic stimulation could be blocked by GDEE, but the depression was less consistent than that observed in other areas. This could be explained by the presence of two pathways between the thalamus and cortex of which only one utilized Glut as a transmitter.

5. αMG and MSO also showed some ability to block the responses of cells to Glut; however, the inhibition produced by these compounds was never as great as that produced by GDEE, and did not show the same specificity of action. MSO appeared to be more active in the cerebral cortex and spinal cord than in the thalamus and αMG often increased the spontaneous rate of firing and enhanced the activity of the excitatory amino acids.

6. The application of GDME potentiated the responses of neurones to Glut and Asp without appreciably affecting the responses to DLH. The simultaneous potentiation of the evoked responses in the spinal cord, cuneate nucleus and thalamus further increased the possibility that Glut may act as a transmitter in these regions.

7. The potentiating effects of GDME appeared to be due to an inhibition of Glut uptake mechanisms, since the high affinity uptake mechanism for Glut in crude synaptosomal preparations could be inhibited by adding GDME to the incubation medium. A much smaller inhibition was obtained using GDEE.

8. pCMS also caused a potentiation of the responses of thalamic neurones produced by Glut and Asp or by
stimulation of peripheral nerves.

9. The addition of GDEE or Glut to the incubation medium of slices of rat cortex caused a significant increase in the release of labelled Glut. The amount of Glut which could be released over control levels from the brain slice by these compounds appeared to be constant and may well have originated from a specific "pool" of Glut.

10. The application of various Glut analogues to the abdominal stretch receptor in the crayfish indicated that this preparation was more sensitive to Glut and N-methyl-glutamate than to DLH or N-methyl-aspartate. Since the opposite is true in mammalian neurones it is possible that the receptors for the amino acids differ in these two tissues.

11. αMG blocked the response of the abdominal stretch receptor to Glut, and GDEE blocked the response of the closer muscle in the crayfish to electrical stimulation of the motor nerve, suggesting that these compounds may have similar properties at the crayfish neuromuscular junction and in the mammalian C.N.S.
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