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EFFECTS OF KAINIC AND DOMOIC ACIDS ON THE RELEASE OF GLUTAMATE AND ASPARTATE FROM RAT BRAIN SYNAPTOSOMES

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

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ABSTRACT

The neurotoxic effects of the excitatory amino acid (EAA), domoic acid (DOM), became apparent in 1987 when mussels contaminated with DOM caused 107 confirmed cases of human poisoning and three deaths in eastern Canada. Another EAA, kainic acid (KA), which has long been used in neuronal research for its selective neurotoxic effects, is structurally similar to DOM. It has been proposed that these compounds produce neurotoxicity by acting at EAA receptors, causing a prolonged depolarization which results in death of the postsynaptic neuron. This theory is known as the excitotoxic hypothesis. DOM has been shown to be 3 to 8 times more potent than KA at producing neurotoxicity. Kainic acid is known to increase the release of glutamate (GLU) and aspartate (ASP) from polarized presynaptic neurons. It has been suggested that this release of endogenous EAAs plays a role in the excitotoxic effects of KA. In this work, the effects of KA and DOM on the release of GLU and ASP from isolated presynaptic nerve terminals (synaptosomes) were compared. Amino acid determination was performed by reversed phase HPLC following derivatization with 9-fluorenylmethyl chloroformate (FMOC). Potassium chloride (40mM), a neuronal depolarizing agent, was used as a positive control. Responses to KA and DOM were similar. Concentrations of 0.5 and 1.0mM KA and DOM increased GLU release ($p \leq 0.01$), however only 1.0mM KA produced a significant increase in ASP release ($p \leq 0.05$). The calcium dependence of these effects was examined by comparing the effects of 1mM KA and DOM in the presence and absence of calcium. Calcium free media contained 1mM EGTA. It was found that amino acid release stimulated by KA and DOM was calcium independent. Potassium-stimulated GLU and ASP release were also calcium independent in this preparation. Results indicate that GLU and ASP release stimulated by KA and DOM originate from cytoplasmic stores, and may be released by reversal of the acidic amino acid uptake carrier. The high concentrations of agonist required to stimulate release, and similar potencies of KA and DOM suggest that this effect is not of primary importance to the neurotoxic mechanism of these compounds.

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USE OF ANIMALS

Research involving the use of animals was performed in accordance with the guidelines of the Canadian Council on Animal Care

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NOTATION

α	alpha
ASP	aspartic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoazolepropionate
ACPD	(1S,3R)-1-amino-cyclopentane-1,3-dicarboxylic acid
ATP	adenosine triphosphate
β	beta
$^{\circ}C$	degrees celsius
Ca^{2+}	calcium ion
CaCl	calcium chloride
CHCL	choline chloride
CH_3CN	acetonitrile
DOM	domoic acid
EAA	excitatory amino acid
EDTA	ethylenediamine tetraacetic acid
EM	electron microscope
GLU	glutamic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
H_2O	water
HPLC	high performance liquid chromatography
i.d.	internal diameter
KA	kainic acid
KCl	potassium chloride
L-AP4	L-2-amino-4-phosphonobutanoate
mOsm	milliosmoles
mg	milligram
$MgSO_4$	magnesium sulfate
min	minute
n mol	nanomoles
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	sodium phosphate
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline
NMDA	N-methyl-D-aspartic acid
%	percentage
±	plus or minus
QUIS	quisqualic acid
SD	standard deviation
Tris	tris(hydroxymethyl)aminomethane
μM	micromolar
X g	centrifugal force

1 GENERAL INTRODUCTION

1.1 Excitatory Amino Acids

Two excitatory amino acid (EAA) neurotransmitter candidates have been identified in the mammalian central nervous system, glutamate (GLU) and aspartate (ASP). Although these compounds do not fulfill all the criteria for identification of neurotransmitter substances, it is widely accepted that either glutamate alone, or both amino acids in conjunction, are the transmitters used at the majority of excitatory neurons in the mammalian central nervous system (CNS)(1-3).

Because of the ubiquitous nature of GLU and ASP, it has been difficult to differentiate neurotransmitter pools from other metabolic pools, or to locate specific glutamatergic or aspartatergic pathways. However, both compounds are released by electrical stimulation *in vitro* (4), elicit excitation when iontophoresed onto neurons *in vivo* (5, 6), and have selective binding/receptor sites on synaptic membranes (for review see 7). Specific reuptake carriers for these amino acids have been identified on presynaptic membranes and glia (8). Vesicular storage has been demonstrated for glutamate, with release being calcium dependent and stimulated by potassium (9), which is a general neuronal depolarizer.

The identification of EAA receptors has been a dynamic process. Initially, three subtypes of glutamate receptor were identified and characterized pharmacologically using selective agonists. These prototypical agonists were quisqualate (QUIS), kainate (KA) and N-methyl-D-aspartate (NMDA)(for review see 10). Due to the development and use of more selective agonists, additional receptors have been discovered, so that at present EAA receptor classification includes five receptor types (11, 12);

1) N-methyl-D-aspartatic acid (NMDA)
2) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)
3) Kainic acid (KA)
4) L-2-amino-4-phosphonobutanoic acid (L-AP4)
5) Metabotropic or (1S,3R)-1-amino-cyclopentane-1,3-dicarboxylic acid (ACPD).

Table I gives a brief overview of the location and function of each. Subtypes of the AMPA and KA receptors are now being identified using DNA cloning techniques (13, 14).

Table I, Excitatory Amino Acid Receptors: Localization and Function.

Receptor Type	Location/ Function
NMDA	Widely distributed in mammalian CNS. Usually recognized as the slow component in repetitive activity generated by non-NMDA receptors. Important in synaptic plasticity.
AMPA	Widespread in CNS, similar distribution to NMDA receptors. Involved in the generation of fast component of EPSPs. May be present in two forms, 'high' and 'low' affinity.
Kainate	Concentrated in stratum lucidum region of hippocampus. Difficult to distinguish from AMPA receptors because selective agonists are not available.
L-AP4	L-AP4 is thought to act as an agonist at presynaptic glutamate autoreceptors (decreasing transmitter release).
Metabotropic	Linked to IP_3 formation. May be involved in developmental plasticity.

Adapted from Watkins et al (25).

On the basis of the large number of EAA receptor types present and their variable expression in different areas of the CNS, it is apparent that EAA neurotransmission is complex. Several experimental models are commonly used to study these systems (see Ch.2), and consequently experiments investigating identical processes often yield different results. As well, receptor populations are dynamic, changing with age and producing different results at different ages (15-17).

For practical purposes, EAA receptors are often divided into two generic groups, NMDA and non-NMDA receptors. This is done because many specific competitive and non-competitive antagonists of the NMDA receptor have been found but selective non-NMDA antagonists have been discovered only recently. The most common of these antagonists are the quinoxalinediones, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) (18). These compounds have high binding affinity for KA and AMPA receptors, and have been found to antagonize the actions of KA and QUIS in neurochemical and electrophysiological studies, with only weak activity at NMDA receptors (for review see 10).

1.2 Domoic and Kainic Acids

Domoic acid (DOM) was originally isolated in 1958 by Takemoto, who extracted it from the macrophytic red algae, *Chondria armata* (19). The name, domoic acid, originated from the Japanese common name for *Chondria*, "domoi", meaning flowering willow. Takemoto was interested in isolating an insecticidal compound from *C. armata* because this plant was used traditionally in Japan as an insecticide and anthelminthic. DOM was subsequently found in another red algae, *Alsidium corralinum*, which grows in the Mediterranean (20). The insecticidal activity of DOM was investigated by Sakai in 1960 (21), and its action as a potent neuroexcitatory agent in insects, amphibians and mammals was investigated in the 1970's and 1980's (22-28).

In November 1987, DOM was identified as the causative agent in 107 cases of human poisoning and 3 deaths (29). The source of the toxin in this incident was cultured blue mussels (*Mytilus edulis*), grown in eastern Prince Edward Island, Canada. The toxin itself was produced by a marine diatom, *Nitzschia pungens*, which is consumed and concentrated by mussels (30-32). It took researchers several days to discover the origin of the toxin, as this was the first time that DOM was detected in unicellular algae (32). Toxic symptoms produced by

ingestion of contaminated mussels included vomiting, abdominal cramps, diarrhoea, headache, and loss of short-term memory. Twelve victims required intensive care because of seizures, coma, respiratory complications, or unstable blood pressure (29, 33). Post mortem investigations and subsequent trials in various species have revealed neuronal necrosis in several regions of the CNS, most notably the CA3 region of the hippocampus (34-37). This is likely the source of memory loss, as the hippocampus is generally believed to be involved in memory processing (38, 39).

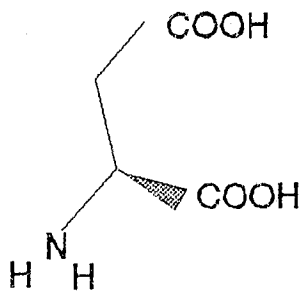
The physical constants and molecular structure of DOM are presented in Table II (19, 40). Domoic acid is very similar in structure to kainic acid (KA), another amino acid derived from marine algae, which has been extensively used in neurological research (41). Both DOM and KA are structurally related to the putative neurotransmitters, glutamic acid and aspartic acid (see Fig.1), with DOM and KA carrying a substituent on adjacent carbon atoms to the N and C3 atoms of the glutamate molecule, forming a pyrrolidine ring.

Both KA and DOM are thought to produce their neuroexcitatory and neurotoxic effects by interacting with EAA receptors on pre and postsynaptic membranes. Research into the relationship of the structure and activity of these compounds

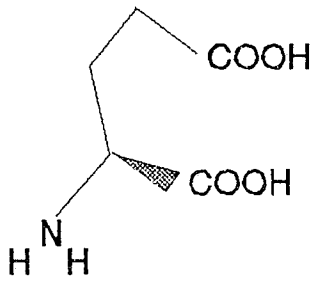
Table II, Physical Constants of Domoic Acid.

Chemical Composition:	$C_{15}H_{21}O_6N$
Molecular Weight:	311.3 daltons
Absorption Maximum:	244 nm
pKa Values:	2.10, 3.72, 4.93, 9.82
Protonation states:	
1) fully protonated cation	NH_2^+ COOH COOH COOH
2) neutral zwitter ion	NH_2^+ COO^- COOH COOH
3) singly charged anion	NH_2^+ COO^- COO^- COOH
4) doubly charged anion	NH_2^+ COO^- COO^- COO^-
5) triply charged anion	NH COO^- COO^- COO^-

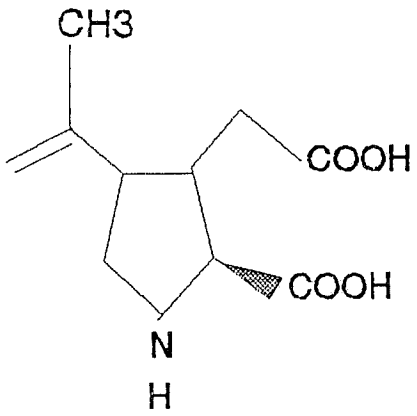
From Takemoto and Daigo (19), and Falk (40).



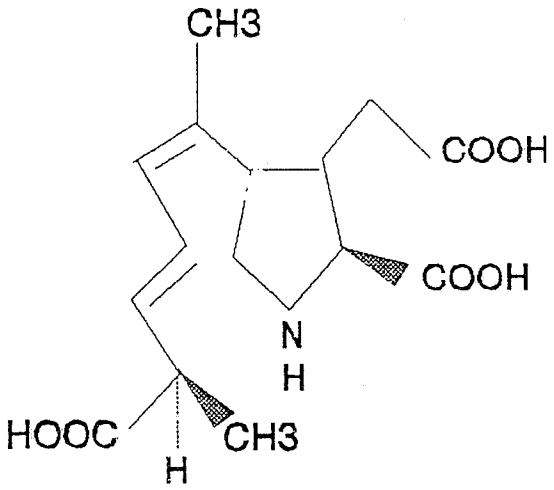
Aspartic Acid



Glutamic Acid



Kainic Acid



Domoic Acid

Figure 1. Structures of the Primary Isomers of Aspartic, Glutamic, Kainic and Domoic Acids.

indicates that the unsaturated side chains either bind to a lipophilic region of the receptor, or promote a conformation at the CH_2COO^- substituent of the pyrrolidine ring which is optimal for interaction with a cationic receptor site (11). It appears that KA and DOM have different affinities for two subtypes of KA receptor. Kainic acid is reported to have eightfold greater affinity for so-called KA-1 receptors (13), while DOM is about twofold more potent at [^{3}H]kainate-binding sites in rat cerebellar membranes (14).

Electrophysiological studies have shown DOM to be three times more potent than KA after iontophoretic administration (42), while studies using systemic administration have shown DOM to be eight times more potent than KA (43). Recent studies by Strain and Tasker (35) comparing the toxic effects of KA and DOM in mice, indicate that at equitoxic dosage, i.e. dosages producing the same levels of toxicity, DOM is more selective at producing damage in the CA3 region of the hippocampus. A possible explanation is that DOM is a more selective agonist at receptors mediating toxicity in this tissue. Administration of both compounds simultaneously has produced effects which are not completely additive indicating that, while most of their action is through a common mechanism, some of their effects are produced though different mechanisms (44, 45).

1.3 Excitatory Amino Acid Neurotransmission

Neuronal transmission occurs when an action potential is initiated and propagated down the axon, opening fast ion channels to allow Na^+ in and K^+ out. At the presynaptic bouton, the action potential causes voltage-gated calcium channels to open, allowing Ca^{2+} to enter and causing the release of neurotransmitter. It is thought that calcium acts presynaptically by complexing with calmodulin, activating a Ca^{2+}/calmodulin dependent kinase, which phosphorylates synapsin 1, a compound linking synaptic vesicles to actin filaments and microtubules. Phosphorylation of synapsin 1 causes vesicles to be released and allows their diffusion towards the presynaptic membrane (46). The precise mechanism causing exocytosis is uncertain, however, it is believed that the structural protein fodrin may be involved in this process (46).

The release of GLU following stimulation by 30mM KCl occurs in two stages. Approximately 20% is exocytosed within two seconds, and the remaining 80% is released slowly with a $T_{1/2}$ of 70 sec. (47). Released GLU diffuses across the synaptic cleft, inducing a conformational change in GLU receptors on the postsynaptic membrane which opens ion channels. The resulting influx of ions produces depolarization which, with sufficient stimulation, will reach the threshold necessary to

initiate an action potential. Specific amino acid carriers present on the presynaptic membrane and surrounding glia take up released transmitter, terminating its neuroexcitatory action (48).

Systemic GLU and ASP are effectively excluded from intracerebral fluid by the blood brain barrier, however, these amino acids exist in high concentration in the CNS (see Table III), where they are synthesized *de novo* (9). Several pathways for the formation of GLU and ASP exist and are outlined in Figure 2 (49). Most of the enzymes involved are ubiquitous, making localization of EAA neurons and pathways difficult.

Although CNS levels of these amino acids are high, extracellular levels are kept low by an acidic amino acid carrier. This carrier is selective for L-GLU, and D/L-ASP, and is localized on presynaptic and glial membranes (48). Transport across the membrane is achieved using the sodium ion gradient, as three sodium ions enter the cell with each molecule of GLU. By this mechanism, the extracellular glutamate concentration is maintained at around 1μM, while intracellular and vesicular glutamate concentrations are in the range of 10mM and 100mM, respectively (47).

Table III, Amino Acid Concentrations in Rat Brain.

Amino acid	Content (μmoles/g wet wt, ±sd)
Glutamate	13.6 ± 0.4
Taurine	4.8 ± 0.3
Glutamine	4.4 ± 0.2
Aspartate	3.7 ± 0.2
GABA	2.3 ± 0.1
Glycine	1.7 ± 0.1
Serine	1.4 ± 0.1
Alanine	1.1 ± 0.1
Lysine	0.4 ± 0.0

From McGeer et al (1).

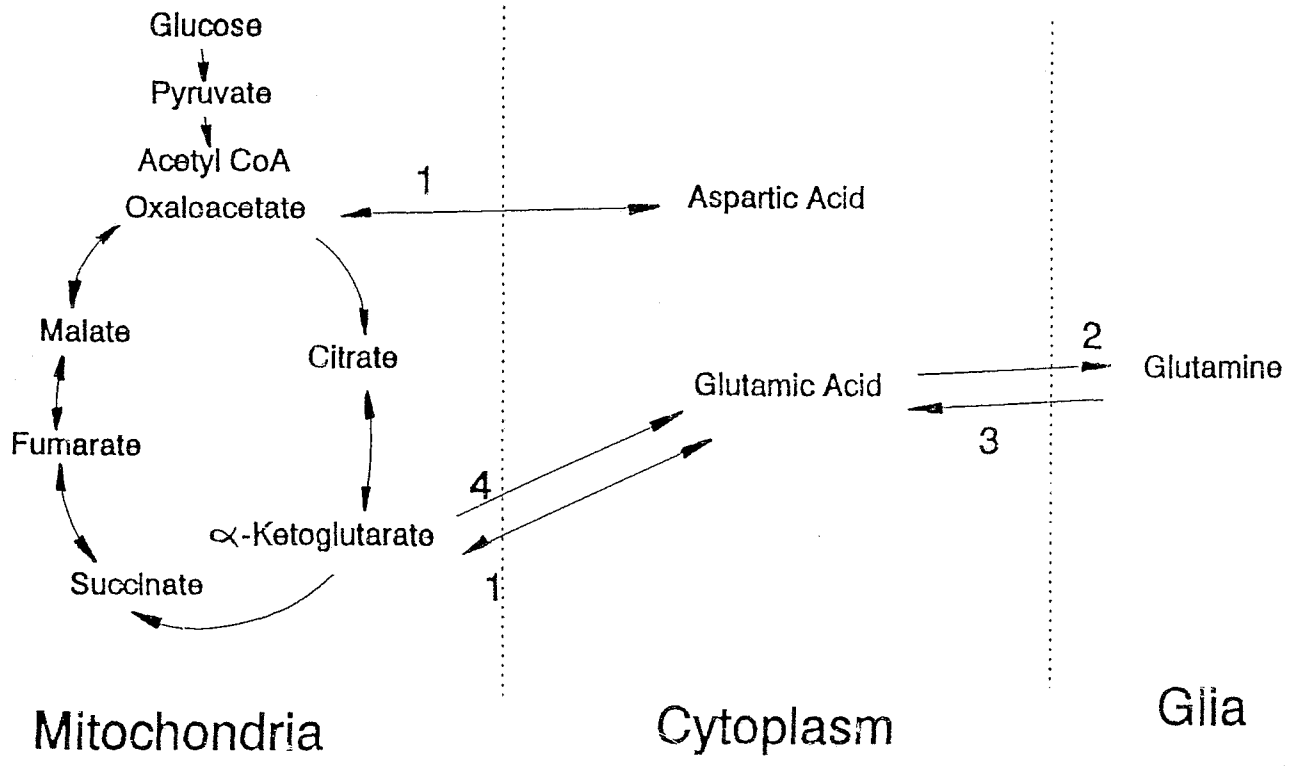


Figure 2. Major Pathways for the Formation of Glutamate and Aspartate. Enzymes involved are, 1: Aspartate transaminase, 2: Glutamine synthetase, 3: Glutaminase, 4: Glutamic acid dehydrogenase. Adapted from McGeer et al (1), and Erecinska and Silver (48).

The role of neuroglia in EAA neurotransmission is uncertain. Originally these cells were thought to merely provide mechanical support for neurons, however it is now apparent that they also play a vital role in neuronal biochemistry. Glia are known to be involved in γ-aminobutyric acid (GABA) pathways, recycling released GABA by returning it to neurons in the form of glutamine (48). These cells also actively take up EAAs and so may be important in terminating the neurotransmitter action of these compounds (49). EAA receptors have also been identified on glia, however, the neurochemical role of these receptors has not been investigated. In conclusion, it is possible that glia play an important role in normal EAA neurotransmission, as well as in EAA toxicology and various pathological states. Investigation of these possibilities is ongoing and may produce some interesting results.

1.4 Neurotoxicity

1.4.1 Excitotoxic Hypothesis

Most EAA agonists, including glutamate and aspartate, are neurotoxic when administered in sufficient concentration. It was originally observed that the toxic potency of these compounds was generally proportional to their excitatory potency, indicating that both processes may be occurring via

the same mechanism, i.e. neuronal stimulation or excitation. This hypothesis, known as excitotoxicity, was first proposed by Olney *et al* in 1971 (49) and presently forms the basis for most theories on the neurotoxicity of EAA's.

Excitotoxicity is believed to occur when EAA agonists produce prolonged neuronal excitation. This excitation keeps post synaptic ion channels open, producing an ionic imbalance which results in the depletion of energy stores as the cell tries to maintain homeostasis. Increases in intracellular ion concentrations are followed by cellular swelling as water is drawn in to maintain osmolarity. Eventually, through ionic imbalance or energy depletion, cell death occurs. Observations of neuronal damage following EAA administration support this hypothesis. It has been found that neuronal damage is localized postsynaptically, is initially characterized by cell swelling (50), and that glucose and high-energy phosphate (ATP) levels are diminished (51).

Evidence is accumulating which indicates that spontaneously produced excitotoxicity is caused by excessive levels of endogenous glutamate, and may produce the neuronal damage associated with hypoxia, stroke, hypoglycemia, epilepsy, and neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's Chorea and motoneuron disease (50, 52, 53). This evidence includes; (a) elevated levels of endogenous GLU have

been reported in studies of neurotoxicity (9); (b) exogenously applied GLU and other EAAs produce histological changes similar to those seen in some of these conditions (9); (c) EAA receptor antagonists have demonstrated powerful anticonvulsant activity in animal models of epilepsy (54, 55); (d) GLU is extremely important as a neurotransmitter, as well as in several fundamental physiological pathways, indicating potential for a variety of problems (48).

1.4.2 Neurotoxic Mechanisms

Within the context of excitotoxic theory, it is possible to speculate on the specific actions of EAAs at presynaptic and postsynaptic receptors.

Histological examination of EAA induced neurotoxicity reveals swelling of dendrites and somata, clumping of nuclear chromatin and cell vacuolation (56, 57, 58). Post synaptic swelling occurs within an hour of exposure to EAAs (50), and is thought to be caused by prolonged depolarization, which draws chloride into neurons, producing an influx of cations to maintain electroneutrality. The hyperosmolar condition created by chloride and cation entry draws water into the cell (50). Neurodegeneration occurs more slowly and appears to be calcium dependent (52, 59). Biochemical work has shown rapid calcium influx in response to EAA's, with ions most likely

entering via receptor gated ion channels, especially NMDA-linked channels (50). Post-synaptically, increased levels of intracellular calcium may produce second messenger effects such as activation of lipases, proteases and kinases (50), and pre-synaptically, calcium influx may initiate neurotransmitter release from synaptic vesicles (60).

Kainic acid has been shown to increase extracellular GLU and ASP levels from presynaptic neurons, either as the result of increased release (61-65), or blocked uptake of these amino acids (66, 67). Increased release could originate from either vesicular or cytoplasmic stores of amino acid, which can be distinguished by eliminating extracellular calcium (47). It is believed that the vesicular release of neurotransmitter is calcium dependent, while cytoplasmic release is calcium independent (47).

The argument for blocked uptake is supported by the work of Pocock et al (66), who found that in synaptosomes prepared from cerebral cortex KA inhibited the uptake of radiolabelled D-aspartate in a concentration dependent manner, implying competition for the acidic amino acid carrier. Inhibition of the amino acid uptake carrier by KA was also demonstrated by Pastuszko et al (62) and Johnston et al (68). Pocock et al (66) concluded that the increase in extracellular GLU and ASP in response to KA was due to a combination of blocked uptake

and a persistent, slow leakage of these amino acids from cytoplasm, down their concentration gradient.

It should be noted that within this thesis the term 'release' is often used broadly to describe any increase in extracellular amino acid concentration, recognizing that it may not necessarily refer to active release but to a blockage of uptake.

There is some debate over whether the concentrations of KA required to affect neurotransmitter release correlate with those producing neurotoxic action. Several studies have noted that neurotoxic effects of KA occur at concentrations many times lower than those required to stimulate release (69, 70). These observations suggest that stimulation of neurotransmitter release from presynaptic neurons is not responsible for the neurotoxic action of KA. Pastuszko et al (62) noted that 0.5 mM KA was required to significantly increase efflux of endogenous glutamate and aspartate, with maximal release observed at 5 mM KA. Notman et al (69) obtained a similar result for radiolabelled aspartate release from rat striatal slices. Garthwaite and Garthwaite (70), however, found that neurotoxic damage is produced by concentrations as low as 5 μM KA, with acute toxicity being observed at 30 μM. Poli et al (65) have suggested that these discrepant results are due to the different sensitivities of

pre and postsynaptic neurons. In their view, production of toxicity at postsynaptic neurons may occur at a much lower threshold concentration than that required to stimulate amino acid release presynaptically.

Other workers contend that the increase in extracellular GLU and ASP is fundamental to the neurotoxic action of KA (62-64). In this model, increased levels of released EAAs have a direct effect on postsynaptic GLU receptors, while DOM and KA stimulate KA and possibly AMPA and/or metabotropic receptors. Novelli (60) has proposed that DOM's potent action at postsynaptic non-NMDA receptors opens fast ion channels, causing depolarization. This releases the Mg^{2+} blockade of NMDA receptors, allowing the excess synaptic GLU to activate these receptors. Under normal conditions NMDA receptors are only stimulated after repeated activation of non-NMDA receptors, while in the presence of DOM this activation is prolonged. The NMDA channels allow Ca^{2+} to enter the cell and initiate cellular changes that lead to local neuronal potentiation (12). This potentiation is thought to be the basis of neuronal plasticity leading to memory formation, and is known as long-term potentiation (LTP)(12, 71).

Research has shown that NMDA receptor activation and prolonged increases in intracellular Ca^{2+} are fundamental to GLU induced neurotoxicity (72, 73). Manev (72), working with cerebellar

granule cells in culture, found increased translocation of protein kinase C (PKC) from cytosol to membranes during GLU-induced toxicity. Addition of NMDA antagonists or blockers of PKC translocation produced decreases in both Ca^{2+} uptake and neuronal death.

1.5 Rationale and Research Objectives

In the neurotoxic mechanisms discussed above, an increase in the extracellular levels of neurotransmitter appears consistently as an important aspect of toxicity. KA has been shown to increase extracellular levels of both GLU and ASP, either by increasing release or blocking uptake (61-67). While it is believed that KA and DOM act by similar mechanisms, recent work indicates that DOM and KA have different affinities for KA receptor subtypes (13, 14). If this is the case, DOM may prove to be a valuable tool for investigating a specific receptor type. Further knowledge of the functions of different GLU receptor subtypes could assist in our understanding of memory formation, acute neuropathologies such as epilepsy, hypoxia, and hypoglycemia, and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's Chorea and motoneuron disease (50, 52, 53).

The theoretical objective of this research is to establish whether DOM affects the extracellular levels of GLU and ASP in rat brain synaptosome preparations, and if so, to determine if the origin of released amino acids is cytoplasmic or vesicular, based on calcium dependence. Because of the variability of results with KA in release experiments in the literature (see Section 1.4.2), effects of KA will also be investigated in this study. This will both confirm its effect in this preparation and allow for direct comparison with DOM. Results will allow comparison of the effects of KA and DOM on presynaptic EAA neurotransmitter release.

In addition to measuring extracellular amino acid levels, intracellular amino acid concentrations will be measured to determine what effects KA and DOM treatments have on intracellular pools of these amino acids. Previous studies (62) have shown that total concentrations of GLU and ASP increase over time in the presence of KA.

In order to meet the theoretical objectives, two technical objectives must be met. These are: 1) to develop techniques for synaptosome preparation, including demonstration of synaptosome purity by electron microscopy, and metabolic competence of synaptosomes by stimulating neurotransmitter release with potassium, and 2) to develop a technique for analysis of micromolar concentrations of amino acids using

high-performance liquid chromatography (HPLC). These aspects will be covered in chapters 2 and 3.

2 SYNAPTOSOMES AS A NEURONAL MODEL

2.1 Introduction

Synaptosomes are isolated presynaptic nerve endings produced by homogenizing and then separating nervous tissue by centrifugation. Gentle homogenization, using a Dounce-type homogenizer with uniform clearance, produces shearing forces which tear the club-like presynaptic boutons from their axons. The membranes of these presynaptic terminals reseal to form discrete particles, known as synaptosomes, which can be isolated from remaining tissue by conventional fractionation techniques. Extensive research using this preparation shows that these particles retain both the morphological and physiological properties of their intact counterparts (74, 75, 89).

This preparation was pioneered in 1962 by Gray and Whittaker (76) and De Robertis et al (77), and has been subsequently improved and characterized. Because they contain primarily presynaptic elements, synaptosomes are a valuable tool for the investigation of presynaptic neuronal biochemistry, especially the release of neurotransmitter substances.

Gray and Whittaker's procedure for synaptosome preparation involved homogenization of nervous tissue (10% w/v) in ice-cold isotonic sucrose medium, followed by centrifugation at 1000 X g. The resulting supernatant was centrifuged at 17000 X g to produce a crude synaptosomal pellet containing synaptosomes, small myelin and membrane fragments, and mitochondria (74). In the present study, the method of Booth and Clark (78) was employed in which the crude synaptosomal pellet was resuspended and subjected to density gradient centrifugation on a discontinuous Ficoll/sucrose gradient. This step eliminates most myelin fragments and free mitochondria, producing relatively pure, metabolically competent synaptosomes.

Two other *in vitro* models commonly used in this type of investigation are brain tissue slices and cell cultures. They have both advantages and disadvantages compared to the method chosen for this study. An advantage is that all the elements of a biological system remain relatively intact, producing results which are more representative of what occurs in living organisms. The fact that tissue slice and cell culture preparations contain most of the complex elements of intact tissue, including pre- and postsynaptic elements can also be viewed as a disadvantage, as these systems operate simultaneously and affect one another.

Another disadvantage of slice preparations is that they contain glial cells. These cells are believed to participate in removing released glutamate from the synaptic cleft, returning it in the form of glutamine (48)(see Fig.2, page 13), and so could affect extracellular glutamate levels. It appears that glia are essentially absent from synaptosomes prepared according to the method used in this work. Pastuszko et al (79), examining uptake of γ-aminobutyric acid (GABA) by synaptosomes, found that uptake into synaptosomes was not affected by β-alanine, a specific inhibitor of the glial GABA transporter. In addition, synaptosomes contain very little glutamine synthetase activity, the enzyme considered to be located specifically in glial cells (80).

By using cell cultures, researchers can avoid glial contamination by culturing a specific cell type or types. Cultured striatal neurons and cerebellar granule cells are two preparations which have been used in EAA-related work. Cell cultures are becoming increasingly popular as a research model for several reasons. First, their scientific merit i.e. cells are intact and alive in contrast to other preparations, second, the increasing concern over the use of animals in research, and third, the lower cost of maintaining cell cultures compared to live animals.

One disadvantage of the synaptosome preparation compared to brain slice or cell culture preparations is that the synaptosomes are more variable. The variability is caused by the lengthy purification technique, including variations in media, equipment and technique. To overcome the potential problem of variability, the preparation used in subsequent studies was examined with transmission electron microscopy to assess its purity. Also, all experiments included a positive control in the form of potassium stimulated amino acid release to verify metabolic competence.

Another neuronal model used in EAA research is the synaptoneurosome. These are essentially synaptosomes with a portion of the postsynaptic element attached. The postsynaptic dendrite or neurosome is similar to the synaptosome in having a resealed membrane and containing all postsynaptic cytoplasmic and membrane receptor elements. The neurosome is generally larger than the synaptosome, giving synaptoneurosomes the appearance of snowmen. This preparation is useful in examining receptor pharmacology (15), however, variability in this preparation is greater than that in synaptosome preparations and as well, glial contamination of this preparation appears significant (81).

Other models for examining the actions of excitatory amino acids *in vivo* or in isolated tissue preparations include

electrophoresis (4), microiontophoresis (42), microfluorimetry (82), patch clamping (47), dialysis-perfusion (83), and microdialysis (62, 84).

In conclusion, the major advantage of the synaptosome preparation is that it allows the investigation of presynaptic mechanisms in isolation, making it a useful tool for the examination of amino acid neurotransmitter release. However, it should be recognized that a compilation of experimental results from various *in vivo* and *in vitro* models will be needed to form a basis for understanding the complete mechanisms of neurotransmitter release and neurotoxicity of EAAs.

2.1.1 Rationale for Method Development

Method development involving synaptosomes progressed in three stages. First, the synaptosome preparation was examined with transmission electron microscopy to confirm the presence of intact synaptosomes and ascertain the purity of the preparation. Second, the release of glutamate and aspartate in response to elevated concentrations of potassium was demonstrated. This proved that the synaptosomes were intact and metabolically competent. The third stage of method development became necessary during the course of protocol

development, and involved an examination of the effect of osmolarity on GLU and ASP release.

Potassium Stimulated Release of Neurotransmitters: All known neurotransmitters are released from neurons following the addition of K^+, which is a nonspecific neuronal depolarizer. In fact, K^+-stimulated release is one of the major chemical criteria used for identification of neurotransmitter substances (1). Calcium-dependence of this effect is considered further evidence that a neurotransmitter is being released, as it is believed to indicate that the compound is being released from synaptic vesicles in cytoplasm (85).

Release from synaptic vesicles is believed to require an influx of extracellular Ca^{2+}. Calcium uptake is increased when the extracellular K^+ concentration is increased above 15-20 mM, with K^+ replacing Na^+ isosmotically (86). Maximum stimulation of Ca^{2+} uptake occurs at an extracellular K^+ concentration of 60 mM (86).

In previous studies, concentrations of KCl used to produce depolarization ranged from 30-80mM (15, 60, 61, 66), with NaCl concentrations in media being reduced accordingly to maintain isosmolarity. A 40mM solution of KCl was used in the current work as a positive control (i.e. to confirm that the

preparation was biologically active). Potassium at these concentrations produces depolarization by causing sodium channels to open, an effect also produced by the alkaloid, veratridine (15).

Osmolarity Trials: As the protocol for experiments developed, it became apparent that maintaining the same osmolarity in different treatment groups was a problem. If the primary objective was to maintain the same osmolarity between treatments, it would be necessary to commence incubation in medium containing reduced NaCl (100mM rather than 140mM). Addition of trial media would then include 40mM NaCl in control, DOM, and KA stimulated preparations, and 40mM KCl in potassium stimulated treatments. Initial experiments indicated a significant decrease in control levels of extracellular GLU and ASP during the course of the experiment. It was hypothesized that this could be a result of the increase in extracellular Na^+ stimulating the amino acid uptake carrier, as it is driven by the Na^+ gradient.

An alternative option (and the one which was followed), was to begin with 140mM NaCl in all tubes and allow the osmolarity of potassium treated tubes to increase by 80 mOsm with the addition of 40mM KCl, while all other tubes maintained their initial osmolarity. To assess this option properly, it was

necessary to determine the effect of osmolarity on GLU and ASP release.

2.2 Materials and Methods

2.2.1 Synaptosome Preparation

Synaptosomes were prepared following the method of Booth and Clarke (78). Male Sprague Dawley rats (Charles River Canada, St-Constant, Quebec), weighing between 250 and 300 g were group housed with food and water available *ad libitum*. Conscious rats were decapitated and their brains immediately excised and placed in ice-cold isolation medium (Appendix A). Blood and cellular debris were removed by adding more medium and decanting the supernatant. Whole brains were weighed and then cut into small pieces with a scalpel. This tissue was homogenized with 10 volumes of isolation medium in a Dounce-type glass homogenizer (clearance: 0.1mm, BDH Inc, Dartmouth, Nova Scotia), using 12 gentle up and down strokes. Homogenate was diluted to 24 ml with isolation medium and centrifuged at 1300 X g for 3 min in a Beckman model J2-21M/E centrifuge (Beckman Canada, Mississauga, Ontario).

Following the initial centrifugation, supernatant was carefully withdrawn and centrifuged at 17000g for 10 min. The resulting supernatant was removed and discarded, and the crude

microsomal/synaptosomal pellet was resuspended in 2ml isolation medium, diluted to 12ml with 12% Ficoll/sucrose medium (Appendix A), and gently homogenized. This suspension was placed in a centrifuge tube, layered with 3ml 7.5% Ficoll/sucrose medium (Appendix A), on top of which 2ml isolation medium was layered.

Discontinuous density gradient centrifugation was carried out in a Beckman model L8-70M ultracentrifuge (Beckman Canada, Missauga, Ontario) at 99 000 X g for 30 min. Using this procedure, myelin banded at the first interphase (between isolation medium and 7.5% Ficoll/sucrose layers), synaptosomes at the second (between 7.5% and 12% Ficoll/sucrose layers), and mitochondria pelleted at the bottom. The myelin layer was removed and discarded, and synaptosomes were then carefully pipetted off the interphase, diluted to 12 ml with isolation medium and gently homogenized. Synaptosomes were then diluted to 24 ml and centrifuged at 5500 X g for 10 min. Supernatant was decanted and the final synaptosomal pellet was made up in isolation medium to a final volume of 0.5ml, producing a protein concentration of 5.7 ± 1.2 mg/ml (sd, n=3).

In the experiments described, tissues from two to four rats were pooled for synaptosome preparation. Total time of preparation was approximately 3 hours. Synaptosomes were

stored on ice, and experiments were completed within two hours after preparation to ensure viability.

2.2.2 Electron Microscopy

Synaptosome purity and integrity were examined by electron microscopy (EM). Two synaptosomal pellets from the final 5500 X g centrifugation step were fixed overnight in 2.5% glutaraldehyde in 0.1M Sorensen's phosphate buffer (pH 7.3)(87) and washed in the same buffer. Post fixing was done in 1% osmium tetroxide in the same buffer. Pellets were dehydrated in ethanol, cleared in propylene oxide (BDH Inc., Dartmouth, Nova Scotia), and embedded in Epon resin (BDH Inc.).

Sections of 70-90 nm thickness were cut on a Reichert-Jung ultracut E microtome (Leica, Halifax, Nova Scotia), mounted on grids, and stained with uranyl acetate and Satos lead stain. Examination was done on a Hitachi H-600 transmission electron microscope (Nissei Sangyo Canada, Toronto, Ontario).

2.2.3 Potassium Stimulated Release of Glutamate and Aspartate

Metabolic competence of the synaptosomal preparation was examined by exposure to 40mM K^+. Synaptosomes were prepared

from two rats as described in section 2.2.1. Tissues were pooled and made to 1ml with synaptosomal isolation medium. Four test tubes (13x100mm) were prepared, each containing 250μl of synaptosomal suspension. One ml Krebs-Henseleit HEPES medium (HEPES)(Appendix A) was added to each, and the tubes were incubated in a water bath at 30°C with gentle agitation. Most brain tissue incubation is carried out at 37°C, however, synaptosomes are more heat labile (78), so a lower incubation temperature is used to help maintain the viability of the preparation. A control sample (390μl) was taken at 5 min, then at 7 min, 30μl of 1M KCl in HEPES were added to two tubes, with the remaining two control tubes receiving 30μl HEPES only. This resulted in potassium stimulated preparations receiving additional ions (40mM KCl, or a total of 80 mOsm) to those already present in the HEPES medium. Additional samples of 390ul were taken at 10 and 15 min.

All samples were placed in microcentrifuge tubes and immediately centrifuged in a Beckman microcentrifuge (model 12, Beckman Canada, Mississauga, Ontario) at 1470 X g for 1 min. The resulting supernatants were removed to storage vials, acidified with 20μl 16% trichloroacetic acid (TCA) and frozen at -80°C for subsequent HPLC analysis (see Chapter 3). Pellets were resuspended in 380ul hypotonic medium (1.6% TCA) and centrifuged again at 1470 X g for 1 min. This supernatant, which represented the intracellular fraction, was

also placed in storage vials and frozen for later analysis. The remaining pellet was subjected to protein determination following the method of Lowry et al (88), as described in Appendix B.

2.2.4 Osmolarity Trial

The effect of changes in osmolarity on EAA release was examined for reasons discussed in Section 2.1.1. A treatment of 40mM choline chloride was used to elevate osmolarity, and tubes receiving control and 40mM KCl treatments were run in parallel. Choline is a major constituent of egg yolk and is commonly used for ionic substitutions in biological media as it is not known to produce any significant effect in biological preparations.

Synaptosomes were prepared from three rats as described in section 2.2.1. The final synaptosomal pellet was brought to 1.5ml with isolation medium (section 2.2.1). One trial was done using three treatments run in triplicate. To each of 9 13x100 mm test tubes, 650μl Krebs-Heseleit HEPES (Appendix A) and 150μl synaptosome suspension were added. Incubation in a 30°C water bath was commenced with gentle agitation. After 4 min incubation tubes were vortexed and 350 μl samples removed to microfuge tubes. At 10 min incubation time, 30 μl additions were made producing final solutions of control, 40mM

KCl and 40mM choline chloride treatment. A final sample of 350μl was taken at 18 min. All samples were centrifuged at 1470 X g for 1 min and the supernatants collected, acidified with 1.6% TCA and frozen as in potassium stimulated release trials (Section 2.2.3). Intracellular amino acids were not collected in this experiment. Pellets were frozen for later protein determination (Appendix B). GLU and ASP were quantified by HPLC as described in section 3.2.

2.3 Results

2.3.1 Electron Microscopy

An electron micrograph of the synaptosomal preparation is presented in Figure 3. The preparation contained putative synaptosomes in which synaptic vesicles and mitochondria were visible. Multilamellar bodies and free mitochondria, as well as other organelles, were also present. Electron micrographs produced were comparable to those reported in the literature (78, 89, 90).

2.3.2 Potassium Stimulated Release of Glutamate and Aspartate

Results of this preliminary trial are presented in Table IV. In control tubes, extracellular GLU and ASP concentrations decreased over time, declining to 30% of the initial

concentration after 15 min incubation. Whereas in K^+ stimulated tubes, extracellular GLU and ASP concentrations increased by 240 and 270%, respectively, after 15 min incubation. Extracellular glutamate levels ranged from 0.474 to 4.214 nmol/mg protein, while aspartate ranged from 0.256 to 3.107 nmol/mg protein.

Examination of intracellular amino acids (Table IV B) shows no significant difference between control and potassium stimulated samples. Intracellular glutamate levels ranged from 15.92 to 19.56 nmol/mg protein, and aspartate levels from 14.09 to 16.29 nmol/mg protein.

2.3.3 Osmolarity Trial

Results of the osmolarity trial are presented in Table V. Glutamate and aspartate levels in control and 40mM choline chloride-treated samples were comparable at both sampling times, while 40mM KCl stimulated the release of both amino acids. At 18 min incubation GLU levels were over 50 fold higher, and ASP levels approximately 20 fold higher, in potassium treated samples than in control and choline chloride samples.

These results indicate that the increase in EAA release produced by 40mM K^+ is not caused by the increase in

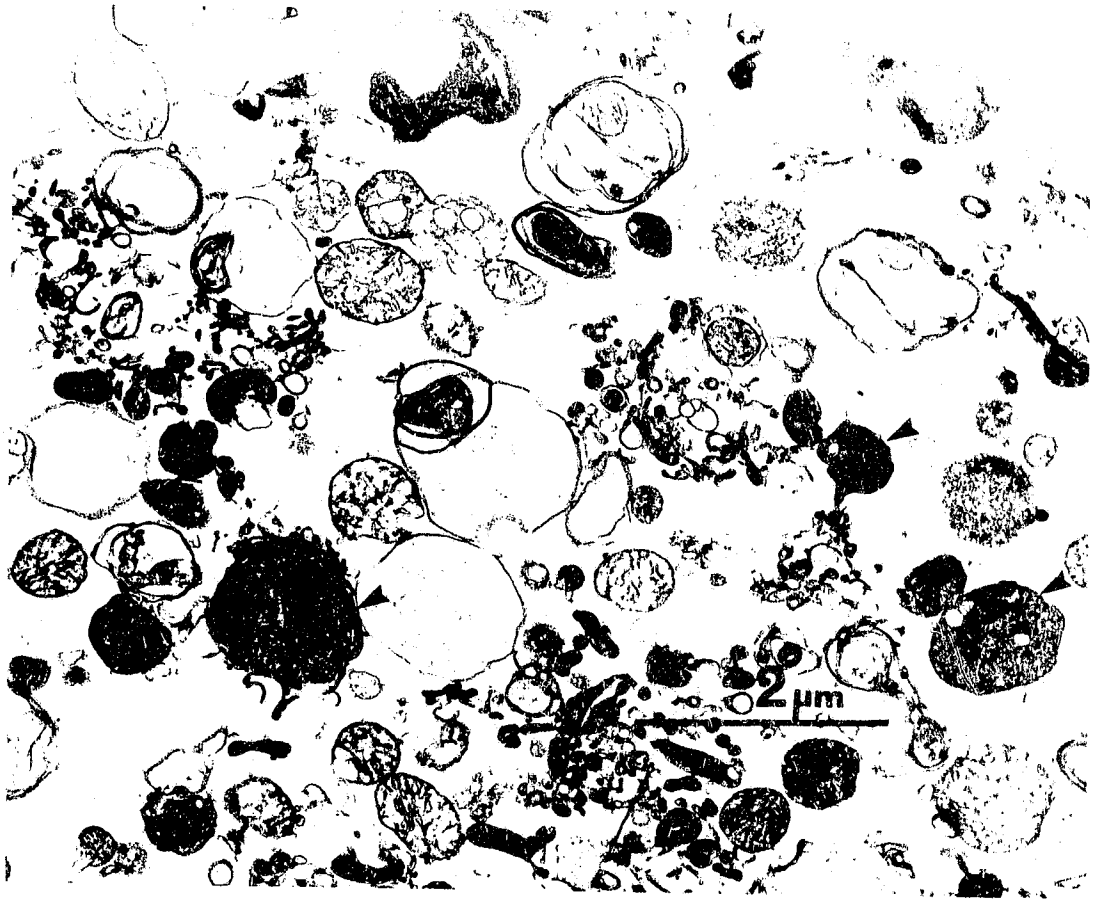


Figure 3. Electron Micrograph of Synaptosome Preparation. Arrowheads indicate putative synaptosomes.

Table IV, Effect of Potassium on the Release of Glutamate and Aspartate.

A) Extracellular Amino Acids

Time	Amino Acid	Control	40mM Potassium
5 min	GLU	1.51	1.77
	ASP	0.89	1.13
10 min	GLU	0.53	3.73
	ASP	0.28	2.73
15 min	GLU	0.47	4.21
	ASP	0.26	3.11

B) Intracellular Amino Acids

Time	Amino Acid	Control	40mM Potassium
5 min	GLU	19.56	19.42
	ASP	16.22	15.43
10 min	GLU	18.02	15.92
	ASP	14.93	14.09
15 min	GLU	18.33	17.29
	ASP	14.81	16.29

Data are in nmol/mg protein, means of duplicate samples, n=1.

Table V, Effect of Osmolarity on Glutamate and Aspartate Release.

Time	Amino Acid	Control	40mM KCl	40mM ChCl
4 min	GLU	0.293 ±.05	0.353 ±.03	0.411 ±.09
	ASP	0.462 ±.10	0.468 ±.07	0.475 ±.01
18 min	GLU	0.163 ±.10	9.378 ±.34	0.173 ±.03
	ASP	0.160 ±.09	4.275 ±.14	0.239 ±.13

Data are in nmol/mg protein, means of triplicate samples, n=1. 40mM KCl: control medium plus 40mM potassium chloride. 40mM ChCl: control medium plus 40mM choline chloride.

osmolarity, as responses to control and choline treatments are similar. Increases in response to 40mM K^+ must therefore be entirely due to a mechanism triggered by the potassium ion.

2.4 Discussion

Based on the available literature, synaptosomes are a useful, valid model for examining EAA release. Electron microscopic examination of the preparation described in Section 2.2.1 revealed synaptosomes with visible mitochondria and synaptic vesicles (see Fig 3). Subjective comparison of electron micrographs prepared in the present work with those in literature (78, 89, 90) showed similar composition.

Further morphological examination of the preparation would require analysis of purity based on percent composition of synaptosomes, free mitochondria, and other cellular debris as determined by image analysis of EM photographs (89, 91). Image analysis would be a valuable tool if one operator were to compare two preparations, but if, as in the present case, one preparation is to be compared with results in literature, the subjectivity of the process makes it of questionable value. In addition to morphological work, biochemical and biophysical indices are often used to assess this preparation. Hargittai et al performed comparative morphological (90), biochemical and biophysical studies (92) on three methods of

synaptosome preparation, including that used in the present work, and found no significant differences among preparations.

Stimulation of this preparation with 40mM K^+ (Table IV) produced an increase in extracellular GLU and ASP, while control samples showed a decrease in extracellular amino acids over time. At 15 min incubation, GLU and ASP concentrations in K^+ stimulated samples were 9 fold and 12 fold higher, respectively, than in control tubes. This response indicates that the synaptosome preparation is metabolically competent and responding in accordance with reports in the literature (66, 60, 61, 93, 94).

Comparison of the concentrations of amino acids in Table IV with values in the literature is difficult as results are expressed in many different ways. Results of Pocock et al (66) are comparable as they used synaptosomes and expressed concentrations in nmol/mg protein, however incubation media and protocol varied from those used in the current study. Control levels of GLU and ASP reported by Pocock et al (66) were 1.78 and 0.99 nmol/mg, respectively, which are very close to the control values of 1.51 nmol GLU/mg and 0.89 nmol ASP/mg reported here (Table IV). Intracellular amino acid levels reported by Pocock et al (66) were 24.72 nmol GLU/mg and 20.68 nmol ASP/mg, compared to 19.56 nmol GLU/mg and 16.22 nmol ASP/mg in this work (Table IV). Pastuszko et al (62), also

working with synaptosomes, measured control levels of intracellular GLU at 24.3 nmol/mg and ASP at 33.6 nmol/mg. Both of these papers (62, 66) measured amino acids by HPLC, using o-pthaldialdehyde derivatization and fluorescence detection.

Levi et al (67), examining potassium-stimulated release in cultured cerebellar granule cells, observed a rise in mean extracellular GLU from 0.96 to 10.06 nmol/mg, while ASP went from 0.55 to 1.95 nmol/mg. In the present work, extracellular GLU rose from 1.77 to 4.21 nmol/mg, and ASP, from 1.13 to 3.11 nmol/mg. The main difference in these results is that, while Levi et al (67) saw a preferential increase in GLU release (10.5 fold increase compared to 3.5 fold for ASP), levels of release of GLU and ASP were similar in this study (2.4 fold and 2.7 fold increases in GLU and ASP respectively).

Because the proposed experiments require changes in the ionic composition of the incubation medium (see Sect. 2.1.1), the potential impact of changes in osmolarity required study. Choline chloride (40mM) was used to elevate osmolarity, and this treatment was compared with 40mM KCl and control. Results demonstrated that the 80mOsm difference between control and ChCl samples had no apparent effect on extracellular GLU and ASP (Table V). These results also suggest that the

stimulating effect of 40mM KCl on EAA release is due to a specific action of the potassium ion.

In conclusion, several papers have suggested that synaptosomes are a valuable tool for the investigation of EAA neurotransmitter release (61, 64, 69). This is because presynaptic-postsynaptic interactions, and contributions from glial cell amino acid uptake or release which can complicate interpretation of results, are absent from this preparation. In developing a method for using synaptosomes, the ultrastructure and purity of the synaptosome preparation was examined by EM and found to be comparable to preparations published in literature. Next, the ability of the preparation to respond to stimulation was demonstrated by potassium-stimulated release of GLU and ASP. Finally, it was demonstrated that a 80 mOsm concentration difference in incubation medium does not affect the release of EAAs *per se*.

3. METHOD DEVELOPMENT OF AMINO ACID ANALYSIS BY HIGH PERFORMACE LIQUID CHROMATOGRAPHY

3.1 Introduction

Three general approaches to determining release of EAAs are described in the literature. These are:

a) incubation and measurement of isotopically labelled amino acids (65, 68, 93).

b) measurement of glutamate by a glutamate dehydrogenase based fluorometric assay (45, 65, 94, 95).

c) high performance liquid chromatography (HPLC) (60, 61, 63, 64, 65, 66, 96).

Each method has its advantages and disadvantages.

Use of isotopically labelled amino acids is a relatively simple technique, and has the advantage of allowing the detection of picomolar changes in concentration (97). However, there is controversy about how well these exogenouosly applied compounds are incorporated into various intracellular amino acid pools. It appears that labelled GLU and ASP are rapidly taken into the cytosol, however, incorporation into vesicular pools occurs more slowly, so that subsequent release of labelled amino acids may not be representative of endogenous EAA release (64).

The glutamate dehydrogenase method is also simple and sensitive. It involves the inclusion of glutamate dehydrogenase and nicotinamide adenine dinucleotide phosphate (oxidized form)(NADP+) in the synaptosomal incubation medium. Glutamate release is detected as an increase in fluoresence produced as GLU and NADP+ are converted to 2-oxoglutarate and fluorescent nicotinamide adenine dinucleotide phosphate (reduced form)(NADPH). Unfortunately the measurement of ASP release, which was one of the objectives of this study, is not possible with this method.

The fluorometric assay of derivatized amino acids following HPLC is more time consuming than the other techniques mentioned, but is the only method capable of measuring changes in the release of endogenous GLU and ASP, and was therefore selected for use in this study.

Chromatographic separation of amino acid mixtures is difficult because they are very similar in structure. In solution at neutral pH they are predominantly dipolar ions (zwitterions), with the amino group being protonated ($-NH_3^+$) and the carboxyl group dissociated ($-COO^-$). Glutamate and aspartate are acidic amino acids, having an extra carboxyl group which is predominantly dissociated at neutral pH, giving them a net charge of -1. Over the past twenty years techniques for their analysis have undergone tremendous improvement, resulting in

today's methods which use reversed phase HPLC and fluorescent detection to obtain high resolution of amino acid mixtures, and sensitivity in the picomolar range (97).

Chromatography using reversed phase HPLC allows the separation of compounds primarily on the basis of ionic and hydrophobic interactions. Figure 4 shows a schematic diagram of an HPLC system. The chromatographic column is packed with lipophilic material, usually long (up to 18 carbon), saturated hydrocarbon chains bonded to silica beads. Solutes injected onto the column are eluted by mobile phases, the pH and polarity of which are modified to obtain separation of the various components. In the case of glutamate and aspartate a low pH mobile phase (under pH 2.19 and 1.88 for GLU and ASP respectively) causes protonation of both carboxylic acid groups, rendering the molecules less polar and increasing their retention times. Reducing the polarity of the mobile phase, by adding acetonitrile to water for example, would reduce the retention time of lipophilic components.

Fluorescence detection is a convenient, sensitive and selective method of detection, however the solute must be present in a fluorogenic form. This can be achieved by derivatizing amino acids with a fluorescent compound, which is attached at the α-amino functional group. The derivatization process also facilitates separation by reducing charge on the

molecule (97). The most popular method of amino acid derivatization for fluorescent detection was developed by Hill et al (98), and uses o-phthaldialdehyde and ethanethiol to produce stable, fluorescent derivatives of primary amino acids.

In the present work, the method of Pocklington et al (99) was adapted for quantitation of the amino acids, glutamate and aspartate. This method involves derivatization of amino acids using the reagent 9-fluorenylmethyl chloroformate (FMOC), followed by HPLC with fluorometric detection. The derivatizing reagent, FMOC, has the advantage of reacting rapidly under mild conditions with both primary and secondary (eg. proline and hydroxyproline) amino acids to yield stable derivatives (see Figure 5)(100). Derivatization is done prior to sample injection, with detection being carried out post-column.

Quantitation by post-column fluoresence detection is achieved by exciting the fluorescent components with light and measuring the subsequent photoemission, which is proportional to the amino acid concentration. A specific wavelength is required for excitation and is produced by focusing light from a Xenon lamp onto a concave diffraction grating. The desired wavelength is reflected through a slit and then focused by a spheroidal mirror onto a quartz flow cell. In the excited

state, an electron in the fluorescent molecule temporarily moves to a higher energy orbital, but soon returns and the corresponding energy is emitted, allowing detection. Excitation involves more energy than emission, and therefore excitation wavelengths are shorter. The energy difference between excitation and emission dissipates as heat. Detection of the emission wavelength is achieved by focusing the emitted light onto a photomultiplier which generates an amplified electrical signal for output (101). The two principal criteria for assessing the FMOC derivatization technique were: 1) that the technique be sensitive enough to quantitate GLU and ASP concentrations in the 0.25 to 10.0μM range, as this is the range of released GLU and ASP in literature (62, 66), and 2) that the technique be capable of resolving these amino acids from other constituents in synaptosomal extract. The objective of the present chapter is to describe the procedure used for quantitation of amino acids, examine its reliability in terms of recovery and variability, and discuss its strengths and weaknesses. In addition, the technique is used to compare the purity of domoic acid available commercially with that isolated in this laboratory, both of which were used at different stages of protocol development.

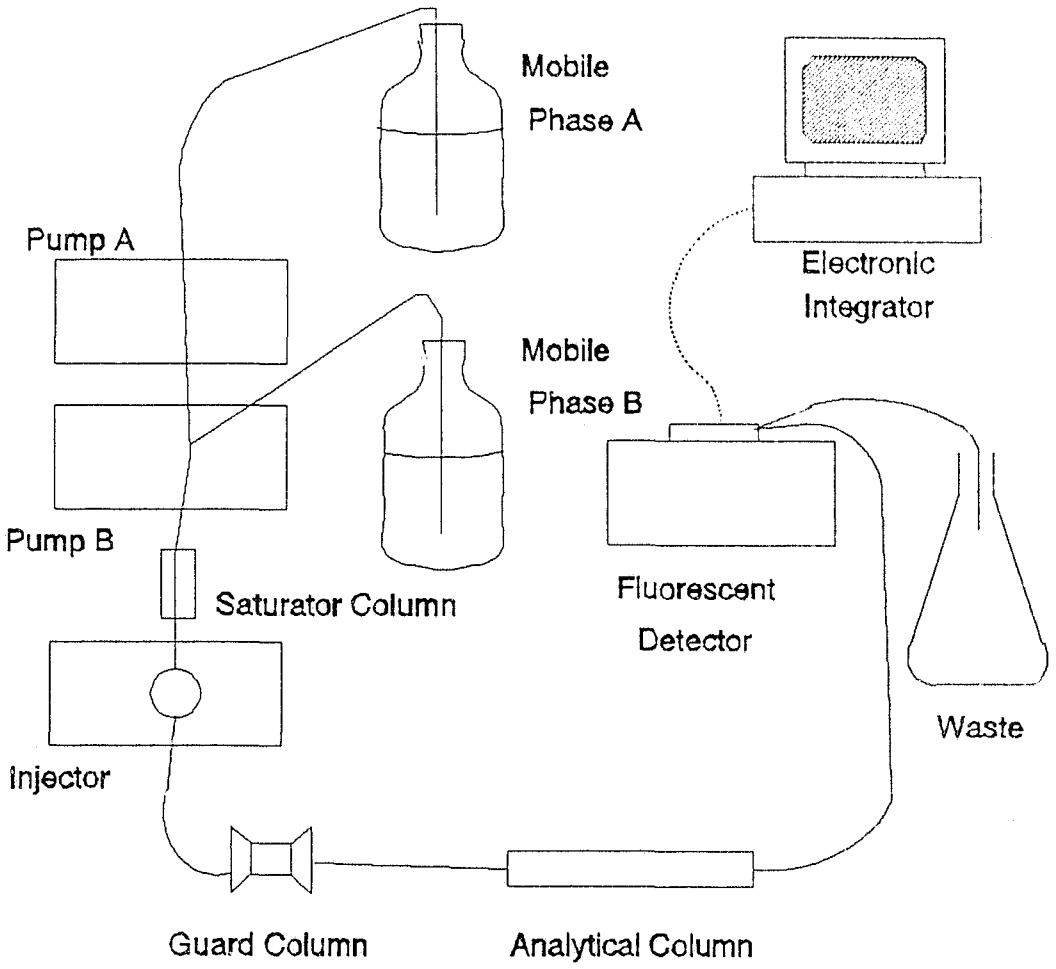


Figure 4. Schematic of High Performance Liquid Chromatograph.

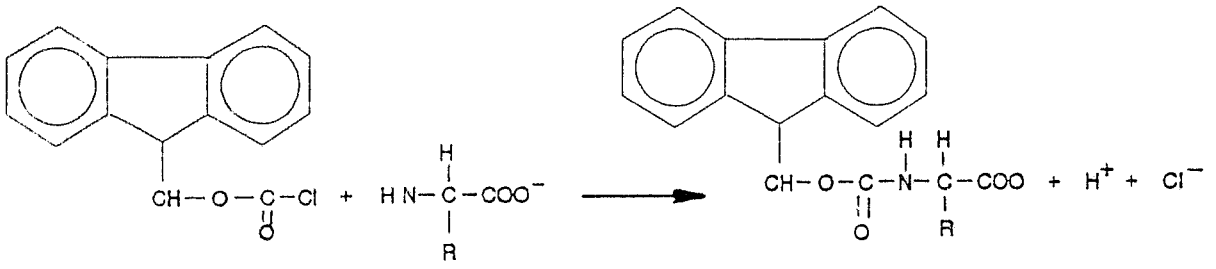


Figure 5. Derivatization of Amino Acids with 9-Fluorenylmethyl-Chloroformate.

3.2 Materials and Methods

3.2.1 Quantitation of Amino Acids

Glutamate and aspartate determinations were carried out, with minor modifications, according to the technique of Pocklington et al (99) that was developed for trace determination of domoic acid. Supernatants representing extracellular and intracellular synaptosomal fractions were obtained as described in section 2.2, and kept frozen at -80°C until analysis by HPLC.

Sample Preparation and Derivatization: Sample vials were removed from the -80°C freezer, thawed and adjusted to approximately neutral pH with 1N NaOH. Sample volumes of approximately 300μl required approximately 30μl NaOH. pH was verified using pH paper. For extracellular samples, 300μl sample plus 100μl double distilled H_2O (Milli-Q Water System, Millipore, Mississauga, Ont.) were placed in a test tube to make a total sample volume of 400μl. Intracellular samples were diluted 20 fold. Standards were prepared from a commercially produced amino acid solution (Standard H, Pierce, Rockford, Ill, USA) diluted to concentrations of 0.25, 1.0, 2.5 and 5.0μM GLU and ASP in 400μl.

To each sample, 100μl of 1M boric acid (Anachemia, Montreal) adjusted to pH 6.2 with 2N NaOH, and 40μl of 10μM dihydrokainic acid (DHKA)(Sigma Chemical Co., St Louis, MO) in acetonitrile (CH_3CN) and H_2O (10:90) for internal standard, were added. The mixture was vortex-mixed for 10 sec. Five hundred microlitres 9-fluorenylmethyl chloroformate (FMOC, 15mM in CH_3CN)(Aldrich Chemical Co., Milwaukee, Wis, USA) were added and mixed for exactly 45 sec, at which time 1.0ml ethyl acetate (HPLC grade, Fisher Scientific, Fair Lawn, NJ, USA) was added and mixing continued for an additional 10 sec. Ethyl acetate is not soluble in aqueous solution and stops the reaction by removing excess free FMOC. Test tubes were centrifuged for 2 min at 10 500 X g in a benchtop centrifuge (International Equipment Co., model CL, Needham Hts., Mass., USA) and the top (organic) layer was discarded. The remaining aqueous phase was washed with 1.0ml ethyl acetate and the final aqueous bottom layer was transferred to 2ml autosampler vials and sealed pending injection onto the HPLC.

High Performance Liquid Chromatography: Injections (20μl) were made on a Gilson HPLC system (Gilson Medical Electronics Inc., Middleton, WI, USA) consisting of a model 231 sample injector, 2 pumps (model 302), a System Interface Module (model 506B), and Gilson 714 HPLC System Controller Software. Mobile phases used were: A) 0.1% trifluoroacetic acid (Sigma Chemical Co, St Louis, MO, USA) in double distilled H_2O, and

B) trifluoroacetic acid in CH_3CN (HPLC grade, Fisher Scientific, Fair Lawn, NJ, USA)(pH 2.2). Mobile phase composition was modified according to a ramped gradient, starting with 30% B for the first five minutes, increasing gradually to 55% B at 22 min, rising to 85% B at 24 minutes and held there for 8 min before returning to initial conditions at 34 min, which were maintained until the end of the run at 35 min. Elution was carried out using a flow rate of 2ml/min and pressure of approximately 2Kpsi. A saturator guard column (3cm x 4.6mm, C_{18}, Phenomenex, Torrance, CA, USA) was inserted between the pumps and injection valve, and a Guard-Pak C_{18} guard column (Waters Chromatography Div., Milford, MA, USA) was placed between the sample injector and analytical column. Separation was accomplished using a 5μM C_{18} column (25cm x 4.6mm i.d.)(Phenomenex, Torrance, CA).

Detection was carried out using a Shimadzu fluoresence detector (Model RF-535, Shimadzu Corp., Kyoto, Japan), with excitation at 265nm and detection at 305nm.

Quantitation: To calculate amino acid concentrations, a standard curve was run for each group of derivatized samples. To produce the standard curve, standards of known GLU and ASP concentrations (0.25 to 5.0μM) were run in ascending order in duplicate and the corresponding peak area divided by internal standard peak area (GLU/DHKA and ASP/DHKA). A linear

regression analysis was then performed on these data. Sample concentrations were obtained by inserting GLU/DHKA and ASP/DHKA values in the line equations obtained by regression, and these values were subsequently normalized using the protein content of each sample. Protein determinations were carried out following the method of Lowry et al (88), as decribed in Appendix B. Sample calculations are given in Appendix C.

3.2.2 Recovery and Variability

Recovery: To determine percent recoveries one intracellular sample was selected and derivatized in triplicate as described above. Spiked samples at three different concentrations (0.25μM, 1.0μM and 2.5μM) were also derivatiized in triplicate. GLU and ASP concentrations were calculated as described in section 3.2.1, and recoveries for each concentration were determined.

Variability: Within-day and between-day variations in sample analysis were examined. To determine within-day variability, three standard solutions (containing 1.0, 2.5 and 5.0μM GLU and ASP), were run on the HPLC at three intervals over one day. The coefficient of variability was determined by dividing the standard deviation by the mean. Between-day variability was determined by analyzing the same standards for

four consecutive days. The standard deviation and mean were then used to calculate the coefficient of variability.

3.2.3 Analysis of Domoic and Kainic Acids

In the studies described in this thesis, commercially produced DOM (Diagnostic Chemicals Ltd., Charlottetown, PEI) and DOM purified in our laboratory from both toxic mussels and phytoplankton according to the method of Nijjar et al (102) were used at different stages of protocol development. To verify the equivalence of the different sources, two lots of commercially produced DOM (Diagnostic Chemicals Ltd, Charlottetown, PEI, Canada) and two lots produced in this lab were made into 100μM solutions, and derivatized and analyzed by HPLC as described in section 3.2.1.

A sample of kainic acid (Sigma Chemical Co., St Louis, MO, USA) was also derivatized and chromatographed to see that it did not interfere with GLU, ASP, or DHKA peaks.

3.3 Results

3.3.1 Quantitation of Amino Acids

Sample chromatograms for amino extracellular and intracellular synaptosomal samples are presented in Figure 6. ASP and GLU

eluted closely, and in extracellular samples these peaks were often on the shoulder of a very large glutamine peak. Peak areas for GLU were higher than for identical concentrations of ASP, indicating that the FMOC derivatization was more selective for GLU. Retention times for aspartate and glutamate were 13.2 and 13.5 minutes respectively, while the internal standard (DHKA) eluted at approximately 21 minutes. Standard curves showed linearity, with mean correlation coefficients (r) of 98.2% and 98.5% for GLU and ASP, respectively.

3.3.2 Recovery and Variability

Recovery: Recoveries of the different concentrations varied, ranging from over 100% recovery for the 0.25μM samples to approximately 80% recovery for the 2.5μM spikes (Table VI). This variability was probably due to slight variations in pH during derivatization, as it was found that pH levels greater than 7 increased the yield of derivatized GLU and ASP.

Variability: Results of within day and between day variability trials are presented in Table VII. For both of the amino acids measured, variability was lowest in the 5.0μM sample and highest in the 2.5 μM sample. Within- and between-day variability for GLU and ASP analysis ranged from 0.5 to 8.9%.

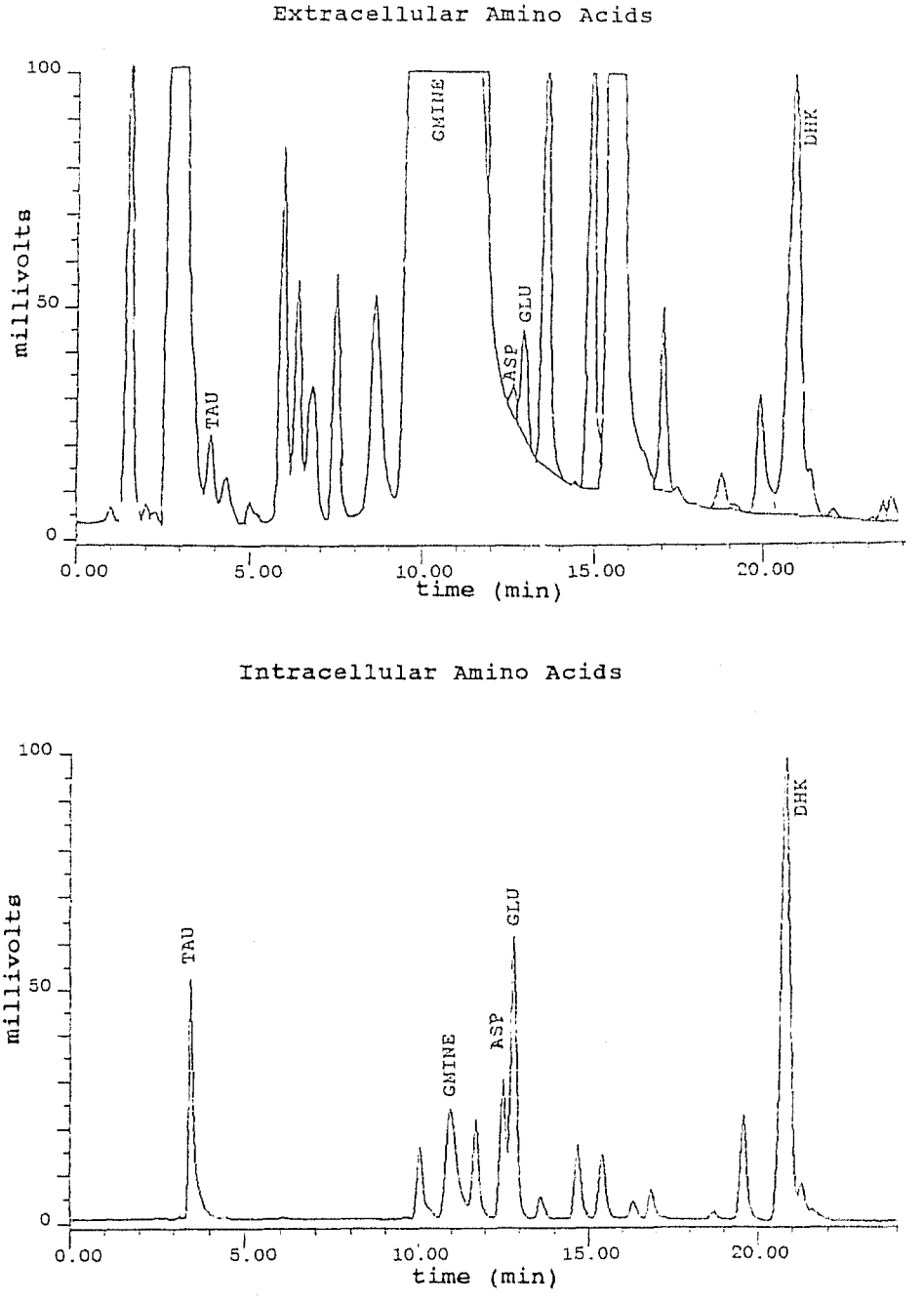


Figure 6. Chromatograms of Extracellular and Intracellular Amino Acids. Extracellular samples were diluted 1.33 fold, intracellular samples were diluted 20 fold. GLU: glutamate, ASP: aspartate, DHK: dihydrokainate (internal standard), TAU: taurine, GMINE: glutamine.

Table VI, Recoveries of Glutamate and Aspartate Following 9-Fluorenylmethyl Chloroformate Derivatization and High Performance Liquid Chromatography

Spike Level	% Recovery (±sd) Aspartate	Glutamate
0.25μM	121 (40)	111 (43)
1.0μM	95 (9)	89 (8)
5.0μM	81 (1)	78 (2)

Table VII, Coefficients of Within-Day and Between-Day Variability

A) WITHIN-DAY

Sample	n	mean	Standard Deviation	Coefficient of Variability
1.0μM ASP	3	0.94	0.022	0.023
1.0μM GLU	3	0.97	0.034	0.035
2.5μM ASP	3	2.50	0.221	0.089
2.5μM GLU	3	2.39	0.216	0.091
5.0μM ASP	3	4.98	0.045	0.009
5.0μM GLU	3	5.02	0.026	0.005

B) BETWEEN-DAY

Sample	n	Mean	Standard Deviation	Coefficient of Variability
1.0μM ASP	4	0.94	0.019	0.020
1.0μM GLU	4	0.98	0.053	0.054
2.5μM ASP	4	2.55	0.180	0.071
2.5μM GLU	4	2.50	0.176	0.070
5.0μM ASP	4	4.98	0.037	0.007
5.0μM GLU	4	4.99	0.054	0.011

3.3.3 Analysis of Domoic and Kainic Acids

The retention for DOM was approximately 19 minutes under the conditions described in Section 3.2. Comparison of HPLC profiles of FMOC-derivatized domoic acid produced in this lab with commercialy produced domoic acid indicated that: 1) DOM produced in this laboratory was purer, being less contaminated with other DOM isomers, and 2) DOM produced commercially varied in purity from lot to lot. Figure 7 shows HPLC chromatograms that support the first observation.

Kainic acid eluted at approximately 20 min, indicating it would not interfere with GLU and ASP analysis. However KA did elute very close to the internal standard, DHKA, a fact which made it necessary to limit KA concentrations to 1mM and lower (see Section 3.4).

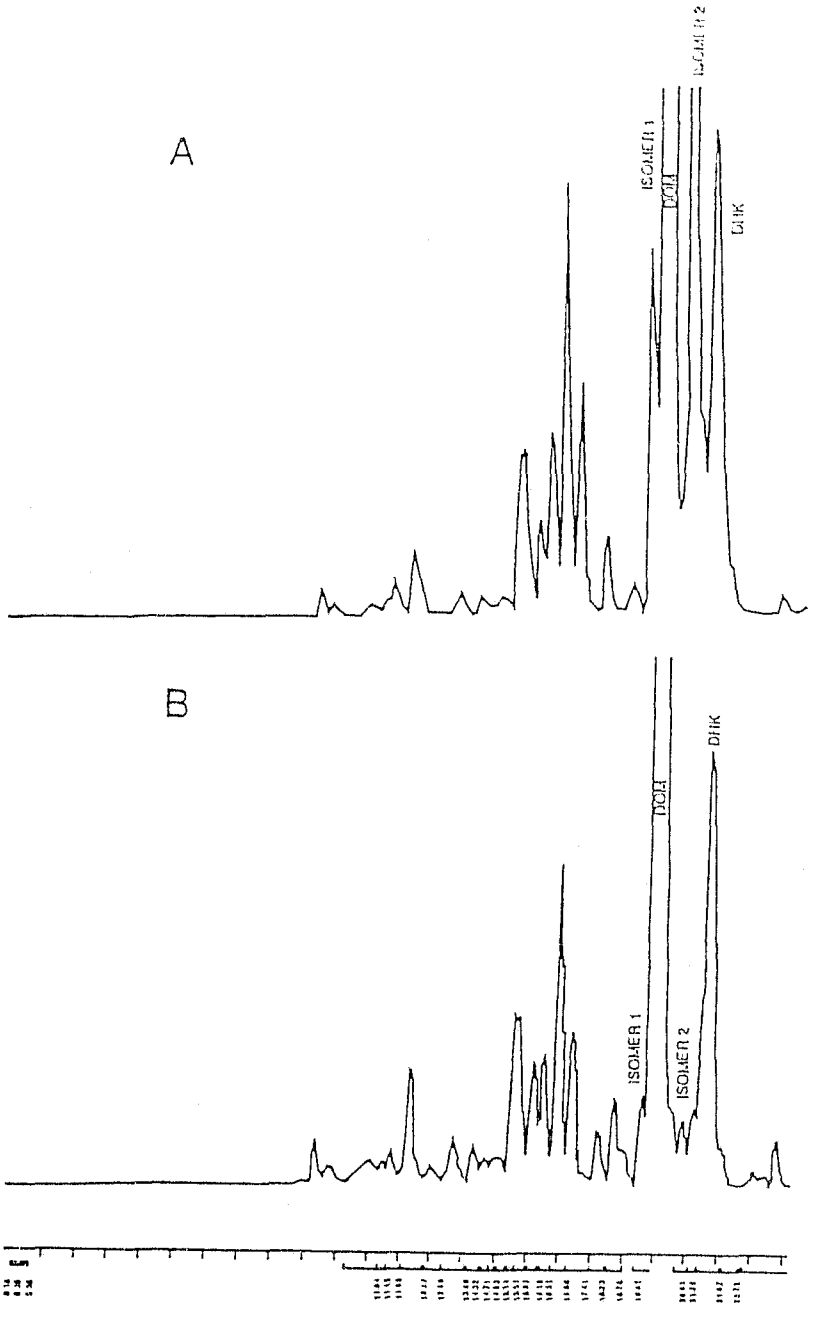


Figure 7. Chromatograms of Domoic Acid Produced Commercially and In House. Commercially produced DOM (A) shows greater contamination with isomers than that produced in house (B). DOM: domoic acid, DHK: dihydrokainic acid (internal standard).

3.4 Discussion

Although HPLC quantitation of amino acids by fluorometric detection of FMOC derivatives is a very sensitive technique for amino acid determination, many difficulties were encountered in its use in this application. As stated, GLU and ASP eluted very closely and made integration of their peak areas difficult, particularly in extracellular samples where high concentrations of glutamine produced a large peak which eluted shortly before the amino acids of interest (Fig 6). Resolution of ASP from the glutamine peak in these samples was the greatest problem. The large amount of time required for manual derivatization of samples was another significant drawback of this technique.

It was determined that the optimal pH of the buffered sample prior to addition of the FMOC reagent recommended by Pocklington et al (99) for DOM determination (7.5 ±0.2) was not optimal for derivatization of GLU and ASP. A higher pH (8.0 - 8.5) produced a higher yield of derivatized GLU and ASP, while reducing the yield of FMOC-glutamine. No difference in the yield of derivatized internal standard was noted.

It was also found that multiple washing of samples with ethyl acetate to remove excess FMOC-OH product was not necessary.

This step was introduced by Pocklington et al (99) to eliminate FMOC-OH, as the retention time for this compound is close to that of DOM. Elimination of this step would have facilitated the derivatization process (which was very time consuming) by making it possible for the entire derivatization process to be programmed on the Gilson model 401 dilutor, as described for o-pthaldialdehyde derivatization (97, 98).

Another option that would have facilitated amino acid analysis is the automated Amino System 1 (Gilson Medical Electronics Inc., Middleton, WI), although this would have necessitated the purchase of additional equipment. Dedicated amino acid analyzers are also commercially available. These systems use HPLC with a temperature gradient and multiple mobile phases to achieve selective elution of individual amino acids, derivatization is carried out with ninhydrin, and detection is by uv spectrophotometry.

The close elution of KA with DHKA proved to be another difficulty. In studies described in Chapter 4, it essentially limited the maximum dose of KA which could be administered to 1.0mM, because higher levels of KA produced peak areas so large that they interfered with the DHKA peak. Fortunately this concentration was adequate (see Chapter 4), although 5.0mM is the concentration which has been found to produce the

maximal response in some studies on the release of EAAs (60, 61).

In retrospect, it appears that o-phthaldialdehyde derivatization would have been a more suitable technique for quantifying GLU and ASP. Other researchers investigating release of endogenous EAA's have reported success using this technique (60, 61, 63-66, 95). GLU and ASP elute much earlier in this technique, both appearing before 5 min, and are well separated. Glutamine elutes at approximately 10 min, so there is no problem of interference. As well, automated derivatization techniques using the Gilson model 401 dilutor and sample controller are available, and in fact have been developed in this lab for taurine determination since the conclusion of this work. The automated technique has good repeatability, so an internal standard is not necessary.

Nevertheless, the recovery of GLU and ASP and variability reported in section 3.3.2 were satisfactory, and FMOC-derivatization with HPLC and fluorometric detection was shown to be capable of providing the data necessary to answer the questions addressed in this work. However, it is evident that additional time spent in method development would have resulted either in improvements to this technique, which would have reduced processing time and could have given more

consistent results, or in the use of an alternate technique such as o-pthaldialdehyde derivatization.

The variablity observed between DOM produced commercially and that produced in our laboratory has previously been reported by Nijjar et al (102) using HPLC with ultraviolet detection. Due to the observed differences, one lot of commercially produced DOM was selected for use in experiments described in Chapter 4.

4 EFFECTS OF KAINIC AND DOMOIC ACIDS ON THE RELEASE OF EXCITATORY AMINO ACIDS

4.1 Introduction

Kainic acid has been reported to stimulate the release of excitatory amino acid neurotransmitters (glutamate and/or aspartate) from numerous preparations (see Section 1.4). Several authors have proposed that this is an important aspect of the neurotoxic mechanism of KA (45, 50, 59, 61, 63, 103). The neurotoxic properties of another EAA, DOM, have recently been discovered. On the basis of similarities in their molecular structure, neuroexcitatory properties, and in their behavioral (43) and histological manifestations of toxicity (35, 104, 105), it has been proposed that KA and DOM produce their neurotoxic actions via similar mechanisms. We compared the effects of KA and DOM on the release of GLU and ASP from isolated presynaptic nerve endings. Results were then examined in the context of EAA neurotoxicity.

In this work the effects of KA and DOM on basal EAA release, i.e. from resting (polarized) synaptosomes are studied. This is the approach traditionally presented in the literature. However, more recently researchers have examined the effects of these compounds on preparations which have been stimulated by depolarizing agents (45, 64, 93, 106). This is achieved by

administering the compound of interest in conjunction with an agonist known to cause depolarization and EAA release, such as high concentrations of potassium. Each method has its own merit, as the examination of basal efflux provides information on how transmitter release is affected in resting cells, while examination of the stimulated system indicates how transmitter release may be modified during neurotransmission.

Initial requirements for the study of the effects of KA and DOM on EAA release were; 1) the development of a synaptosomal preparation and demonstration of its metabolic competence, as reflected in the potassium stimulated release of EAA neurotransmitters (described in Chapter 2); and 2) an analytical system capable of accurately measuring GLU and ASP concentrations in synaptosomal extracts in the 0.25 to 10.0μM range (Chapter 3). This Chapter discusses the final stages of method development, including time and dosage trials and experiments with amino acid uptake-blockers, followed by release experiments and calcium dependence trials. Rationales for these experiments are discussed below.

4.1.1 Determination of Sampling Time and Agonist Dosages

Sampling Times: Sampling times were selected on the basis of findings reported in current literature, and on the basis of results of preliminary experiments which were conducted in our laboratory.

Pocock et al (65) studied the effects of KA on the release of GLU and ASP from synaptosomes prepared from guinea pig cerebral cortex. In this work the release of endogenous GLU was monitored continuously for 12 minutes following the addition of agonist using the glutamate dehydrogenase fluorometric assay. The release of endogenous and exogenous (radiolabelled) GLU and ASP were compared using measurements taken 30 minutes after addition of agonist. The glutamate dehydrogenase assay was also used by McMahon et al (106) to study the kinetics of GLU release from guinea pig cerebral cortical synaptosomes. In the present study, GLU release was monitored for 8 minutes following the addition of agonist. Poli et al (64) used synaptosomes prepared from rat brain hippocampus in a superfusion apparatus, collecting effluent fractions every 4 min for a total of 12 min. Increases in extracellular amino acids were measured in fractions obtained at 4 and 8 min after addition of KA.

In the only work of this kind involving DOM, Terrian et al (45) examined the release of GLU from hippocampal mossy fibre synaptosomes using the superfusion technique. Fractions were collected every 5 min, with stimulation of GLU release being measured in a fraction obtained between 3 and 8 min after addition of the agonist.

In this thesis, sampling time was examined in two preliminary trials. In each trial, one sample was taken before and two samples were taken after the addition of experimental media. Samples for Trial I were taken at 5 and 10 min, and for Trial II at 2 and 8 min after the addition of appropriate media. Sampling times were changed from one trial to the next because the low yield of synaptosomes and experimental time constraints made it difficult to examine more than three sampling times in one experimental trial. Consequently, sampling times were staggered from one trial to the next.

Agonist Dosages: Dosages of KA and DOM were selected on the basis of relevant work in the literature, what was found to be effective in preliminary trials, and on limitations imposed by the analysis technique used. In the literature, KA concentrations ranging from 10μM (64) to 10mM (68) have been used to examine EAA release. Maximum dosages were in the range of 1 to 10mM (45, 60, 61, 64, 65, 68, 93). In these studies, KA consistently increased extracellular EAA levels in

a dose dependent manner, with significant increases reported at dosages between 100μM and 1mM. Histological evidence of KA neurotoxicity is apparent at KA levels as low as 5μM (69). The discrepancy between this dosage and that required to stimulate release have placed the physiological and neurotoxic implications of these results in dispute (68, 69).

Terrian et al (45), examining the effects of DOM on EAA release, found a dose dependent increase in potassium-stimulated GLU release using DOM concentrations between 30 and 300μM.

Preliminary trials were conducted with several concentrations of each agonist, ranging from 33μM to 1mM. The maximum dosages of KA and DOM were limited by interference of these peaks with the internal standard, DHKA. DOM elutes at approximately 19 min, closely followed by KA at 20 min and DHKA at 21 min (see Section 3.3). It was found that KA concentrations above 1mM and DOM concentrations above 1.5mM produced broad peaks which interfered with DHKA peak integration.

4.1.2 Use of a Glutamate and Aspartate Uptake Blocker in Release Studies

Preliminary trials demonstrated a decline in control levels of extracellular GLU and ASP over time. It was hypothesized that this change was due to uptake through the acidic amino acid uptake carrier during the course of incubation. This carrier actively transports L-glutamate and D- and L-aspartate into neurons via a mechanism which is driven by the Na^+ gradient. We speculated that the addition of an appropriate concentration of uptake carrier blocker might stabilize extracellular amino acid concentrations in control samples. As well, if KA or DOM stimulated the release of EAAs by acting at an EAA receptor(s), an uptake blocker would theoretically increase the level of release seen with these agonists.

The difficulty in finding an effective uptake blocker is that most compounds which block GLU and ASP uptake are also active at EAA receptors. Dihydrokainic acid (DHKA) has a low affinity for EAA receptors, however it is also relatively weak as an uptake inhibitor (107, 108), and could not be used in this work as it was also the internal standard used in HPLC analysis. The blocker used in these trials was DL-threo-β-hydroxyaspartic acid (HAA), a highly effective uptake inhibitor (109, 110) which also has significant affinity

(D > L) for NMDA receptors (111). Balcar and Johnston (112) found that 4μM HAA reduced the uptake of GLU and ASP in rat brain slices by 50%, while Johnston (110) found that 10μM produced a 28% inhibition of ASP uptake. On the basis of these findings, a concentration of 20μM HAA was employed in trials examining EAA release in the presence of an uptake blocker.

4.1.3 Calcium Dependence of Excitatory Amino Acid Release Stimulated by Kainate and Domoate

As stated earlier (Section 1.4.2) the term 'release' is used broadly in this work to refer to an increase in the extracellular concentration of GLU or ASP. In fact, these amino acids are maintained in an equilibrium which, for GLU, consists of a 10,000 fold higher concentration intracellularly than extracellularly. Due to this high concentration gradient, GLU and ASP are constantly leaking from cytoplasm, probably through large membrane channels, and are efficiently returned to the cell via the acidic amino acid carrier, which is driven by the Na^+ gradient. Consequently, increases in extracellular GLU and ASP can occur as a result of several factors, including:

1) The exocytosis of synaptic vesicles containing EAA's.
2) Blockage of the acidic amino acid carrier.
3) Reversal of the acidic amino acid carrier.

4) Increased cytoplasmic leakage.

Of these factors, exocytosis is the only process which is Ca^{2+}-dependent.

In this work, the Ca^{2+}-dependence of GLU and ASP release stimulated by KA and DOM was examined in an effort to determine whether the origin of these EAAs was cytoplasmic or vesicular. Previous studies have shown that high concentrations of potassium produce neurotransmitter release which is largely Ca^{2+}-dependent (60, 64, 66, 68, 93, 95), whereas reports of this effect for KA-stimulated EAA release vary widely (60, 61, 63-66, 68, 95, 106).

Many researchers have also examined changes in intracellular and extracellular Ca^{2+} concentrations in response to EAA agonists. The rationale for these studies is similar to that for Ca^{2+}-dependence, i.e. to determine whether Ca^{2+} is involved in the mechanism of action of these compounds through stimulation of vesicular release, or possibly via other second messenger effects of Ca^{2+} (114). Results of studies examining changes in intracellular and extracellular Ca^{2+} concentrations in response to KA have varied widely (45, 61, 82, 83), as have Ca^{2+}-dependence trials (see above), indicating that there may be a specific reason underlying these discrepant results, most likely due to the predominance of different mechanisms in the different preparations used.

4.2 Materials and Methods

4.2.1 Experiments Examining Sampling Time

Two trials were conducted to examine changes in the release of GLU and ASP over time. In Trial I, two male Sprague-Dawley rats were used, while in Trial II three rats were used. Synaptosomes were prepared as described in Section 2.2.1. Aliquots of 250μl each of the resulting synaptosomal suspension were placed in 13 x 100mm test tubes (4 tubes in Trial I and 6 in Trial II). One ml of Krebs Heseleit HEPES medium (HEPES, Appendix A) was added to each tube, the mixture was vortex-mixed and placed in a water bath at 30°C with gentle agitation.

After 5 min of incubation, control samples of 390μl were removed to microcentrifuge tubes. At 10 min, 30μl of appropriate media were added to each tube and mixed to produce the following solutions. 1) Trial I: Control, 40mM KCl, 33μM KA and 170μM KA. 2) Trial II: Control, 40mM KCl, 330μM KA, 170μM DOM, 330μM DOM and 1mM DOM. Test media were prepared by dissolving appropriate concentrations of agonist in HEPES medium.

Two additional samples of 390μl were collected, at 3 and 8 min after addition of media in Trial I, and at 5 and 10 min after

addition of media in Trial II. It will be noted that sampling times varied in several experiments described in method development (Sections 4.2.1-4.2.3). This was done in order to observe changes in amino acid levels over time. Due to the low yield of synaptosomes, and experimental time constraints, it was difficult to examine more than three sampling times in one experimental trial. Consequently, in this and other experiments sampling times were staggered from one trial to the next.

Samples were immediately centrifuged and treated as described in Section 4.2.4 to produce extracellular, intracellular and pellet fractions. Extracellular fractions were frozen at -80°C for subsequent amino acid as described in Section 3.2.1. Pellets were frozen at -80°C and later analyzed for protein content as outlined in Appendix B. Intracellular fractions were discarded.

4.2.2 Experiments Examining Agonist Dosage

Data from Time Trials I and II were used as a basis for dosage determination with one additional trial being run (Trial III). The protocol for Trial III was the same as that for Trial II (see Section 4.2.1), except that the treatments examined were control, 40mM KCl, 0.5mM KA, 1mM KA, 0.5mM DOM and 1mM DOM, and only two samples were taken, one before the addition of

trial media (0 min) and one at 10 min after the addition of test media.

4.2.3 Use of a Glutamate and Aspartate Uptake Blocker in Release Experiments

Two experiments were performed using the uptake blocker, DL-threo-β-hydroxyaspartic acid (HAA). The first compared the effects of 40mM K^+ and 600μM KA in the presence and absence of the blocker, while the second examined the effects of 40mM K^+ and 1mM KA in the presence of blocker.

For the first experiment, synaptosomes were prepared from three rats as described in section 2.2.1, and brought to 1.5 ml with isolation medium (Appendix A). In each of 6 test tubes, 1ml Krebs-Henseleit HEPES (Appendix A) and 250μl synaptosome suspension were added. Incubation in a water bath at 30°C with gentle incubation was commenced immediately. At 5 min incubation time, tubes were vortex-mixed and an aliquot of 390μl removed as a control sample. At 10 min, 30μl additions were made to each tube, producing two each of control, 40mM potassium and 600μM KA solutions, one of each having 20μM uptake-blocker (DL-threo-β-hydroxyaspartic acid, Sigma Chemical Co., St. Louis, MO, USA) added. Additional samples of 390μl were taken 8 and 13 min after addition of agonists to determine amino acid levels at these times.

All samples were placed in microcentrifuge tubes, centrifuged and intracellular and extracellular supernatants collected as in Release Experiments (Section 4.2.4). Pellets were frozen for later protein determination (Appendix B). GLU and ASP were quantified by HPLC as described in section 3.2. This experiment was repeated once.

In the second experiment involving the use of an uptake blocker, a similar protocol was followed except that blocker was added to all samples, along with the experimental media. The experiment was divided into two trials with three test tubes per trial. Each trial examined the effects of control, 40mM K^+, and 1mM KA, however sampling times for the two trials were different. In the first trial, the initial control sample was taken 1 min after incubation commenced, experimental media were added at 6 min incubation and susequent samples were taken at 5 and 10 min after the addition of media. In the second trial, the initial sample was taken after 8 min incubation, media were added at 10 min and subsequent samples were taken 8 and 15 min after addition of media.

Intracellular and extracellular samples were collected as described in Section 4.2.4. Final pellets were frozen for subsequent protein determination (Appendix B). Intracellular fractions from this experiment were discarded.

4.2.4 Changes in Intracellular and Extracellular Glutamate and Aspartate in Response to Kainate and Domoate

Four rats were killed and synaptosomes prepared as described in Section 2.2.1. The final synaptosomal pellet was made up to 1.9 ml with isolation medium (Appendix A) and stored on ice. Aliquots (150μl) of synaptosomal suspension were placed in each of 12 test tubes (13 x 100mm), and 650μl of HEPES medium was added. The mixture was vortex-mixed and tubes were placed in a 30°C water bath with gentle agitation. After 5 min incubation, tubes were mixed again and aliquots of 350μl were removed and placed in microcentrifuge tubes.

At 10 min incubation, 30μl of experimental media were added to each tube to produce duplicate samples of control, 40mM KCl, 0.5mM KA, 1mM KA, 0.5mM DOM and 1mM DOM. Experimental media were produced by dissolving appropriate concentrations of agonist in HEPES medium. Eight minutes after the addition of media (18 min incubation time), tubes were vortexed and samples of 350μl were removed to microcentrifuge tubes as before.

All samples were immediately centrifuged in a Beckman ultracentrifuge (model 12, Beckman Canada, Dartmouth, NS) at

1470 x g for 1 min. The resulting supernatants were removed to storage vials, acidified with 20µl 16% TCA and frozen at -80°C for subsequent HPLC analysis as described in Section 3.2.1. Pellets were resuspended in 380µl hypotonic medium (1.6% TCA) and centrifuged again at 1470 X g for 1 min. This supernatant, which represented the intracellular fraction, was placed in storage vials and frozen for later analysis. The remaining pellet was subjected to protein determination as described in Appendix B.

This experiment was repeated four times with all measurements in duplicate. Results for each sampling time were examined separately by analysis of variance (ANOVA) using the general linear model (GLM). Treatment groups were then compared by the Student Newman-Keuls (SNK) method. Statistical calculations are presented in Appendix D.

4.2.5 Calcium Dependence of Stimulated Excitatory Amino Acid Release

Synaptosomes were prepared from the whole brains of four rats as described in Section 2.2.1, brought to 1.3ml with isolation medium and stored on ice. Aliquots of synaptosome suspension (150µl) were placed in each of 8-13 x 100mm test tubes. 650µl of Krebs-Henseleit HEPES medium (Appendix A) were added to four of the tubes, while the remaining tubes received a

modified Krebs-Henseleit HEPES medium without Ca^{2+} and with 1mM ethyleneglycol-tetra-acetic acid (EGTA, Sigma Chemical Co., St Louis, MO, USA), a selective Ca^{2+} chelator, added.

Incubation and sample collection were carried out as outlined in Section 4.2.4. Experimental media were added to produce control, 40mM KCl, 1mM KA, and 1mM DOM solutions, with one of each in the regular HEPES medium (with Ca^{2+}) and one in the modified HEPES medium (without Ca^{2+}, plus 1mM EGTA). HPLC analyses of known concentrations of GLU and ASP in both types of media were carried out as a control (see Section 3.2.4).

This experiment was repeated three times, and results were compared by ANOVA (GLM) as in Section 4.2.4.

4.3 Results

4.3.1 Determination of Sampling Time

Extracellular levels of glutamate and aspartate measured in time trials are presented in Table VIII (Trials I and II). Responses of GLU and ASP were similar in all treatments, although GLU concentrations were consistently higher. Extracellular GLU and ASP in control samples decreased over time in both trials. 40mM potassium produced the greatest response in GLU and ASP release, however the profile of this

response varied in the two trials. In Trial I, potassium stimulated a rapid increase in GLU and ASP release at 3 min, which leveled off at 8 min, while Trial II showed a more linear increase. These variations in response and in the absolute levels of release are probably the result of variations in the synaptosomal preparations. For example, differences in protein contamination from preparation to preparation will affect estimation of amino acids, as they are expressed on a per mg protein basis.

Both trials demonstrated an increase in the difference between control and agonist treated amino acid release over time. In Trial I, GLU and ASP release in response to 170μM KA increased rapidly at the three minute sample, and appeared to have levelled off by 8 minutes (Table VIII). Based on these results and the literature discussed in Section 4.1.1, a final sampling time of 8 min was selected for the release experiments.

4.3.2 Determination of Agonist Dosages

Glutamate and aspartate release results from three trials are presented in Tables VIII and IX. The range of amino acid release varied greatly between preparations, and may have been due to differences in protein contamination between synaptosome preparations, as discussed previously. Responses

were consistently dose-dependent and similar in pattern, i.e. K^+ always stimulated release although the magnitude of the effect varied. On the basis of these results, on data reported in the literature, and limitations of the analytical method discussed in Section 4.1.1, concentrations of 0.5 and 1mM KA and DOM were selected for the final release studies.

4.3.3 Effects of an Uptake Blocker on Glutamate and Aspartate Release

In experiments conducted in the absence of HAA, extracellular levels of GLU and ASP in control samples decreased over time. These results imply active uptake of EAAs by synaptosomes and are consistent with the observations of other investigators (106, 113). The primary justification for using an uptake blocker was to stabilize extracellular amino acid levels by reducing reuptake. Results for GLU and ASP release in the two uptake blocker experiments are presented in Table X. It appears that HAA was effective at reducing GLU and ASP uptake, as 20μM HAA stabilized extracellular GLU and ASP in control tubes. In the first experiment, control GLU levels in the absence of HAA declined from 1.541 at the first sample, to 0.579 nmol/mg protein at 8 minutes and remained low thereafter. GLU levels in the presence of HAA were more stable, with an initial value of 1.362 nmol/mg protein, and

Table VIII, Effects of High Potassium and Kainic and Domoic Acids on Glutamate and Aspartate Release Over Time.

A) Trial I, Effect of 33μM and 170μM Kainic Acid on Glutamate Release

Treatment	Time 1 (0 min)	Time 2 (3 min)	Time 3 (8 min)
Control	0.59	0.43	0.42
Potassium (40mM)	0.67	3.61	4.18
Kainic Acid (33μM)	0.63	0.70	0.65
Kainic Acid (170μM)	0.56	1.29	1.31

B) Trial II, Effect of 330μM Kainic Acid and 170μM, 330μM, and 1000μM Domoic Acid on Glutamate and Aspartate Release

Treatment	Amino Acid	Time 1 (0 min)	Time 2 (5 min)	Time 3 (10 min)
Control	Asp	2.96	1.58	1.22
	Glu	5.42	2.80	2.27
Potassium (40mM)	Asp	2.15	3.60	5.99
	Glu	3.77	6.92	11.40
Kainic Acid (330μM)	Asp	1.78	2.02	2.80
	Glu	2.94	3.69	5.28
Domoic Acid (170μM)	Asp	2.39	2.22	1.93
	Glu	4.00	2.91	3.21
Domoic Acid (330μM)	Asp	3.38	4.04	*
	Glu	5.79	4.02	*
Domoic Acid (1mM)	Glu	5.02	3.67	6.42

Data in nmol amino acid/ mg protein. For each trial, n=1 with samples in duplicate. * data incomplete.

Table IX, Effects of High Potassium and 0.5 and 1mM Kainic and Domoic Acids on Glutamate and Aspartate Release.

Treatment	Amino Acid	Time 1 (0 min)	Time 2 (10 min)
Control	Asp	1.569	0.581
	Glu	1.633	0.585
Potassium (40mM)	Asp	1.548	12.623
	Glu	1.695	18.446
Kainic Acid (0.5mM)	Asp	1.069	1.020
	Glu	1.260	1.384
Kainic Acid (1mM)	Asp	1.606	1.145
	Glu	1.641	1.611
Domoic Acid (0.5mM)	Asp	0.973	0.745
	Glu	1.185	0.849
Domoic Acid (1mM)	Asp	1.261	0.928
	Glu	1.448	0.926

Data are in nmol/mg protein, n=1.

Table X, Effect of an Uptake Carrier Blocker on Extracellular Glutamate and Aspartate.

Time	Amino Acid	Treatment: Control	Control + Block	Potassium (40mM)	Potassium + Block	Kainic Acid (0.6mM)	Kainic Acid + Block
0 Min	Asp	1.31	1.17	1.18	1.09	1.05	1.01
	Glu	1.54	1.36	1.44	1.31	1.31	1.30
8 Min*	Asp	0.43	0.95	1.17	1.47	0.55	0.58
	Glu	0.58	1.33	1.74	2.01	0.84	0.84
13 Min	Asp	0.49	0.72	1.61	1.61	0.63	0.59
	Glu	0.67	1.01	2.15	2.19	1.07	0.87

Data are in nmol/mg protein, n=2. * n=1 for observations at 8 minutes. Block: 20μM DL-threo-β-hydroxyaspartic acid added.

subsequent values of 1.329 and 1.013 nmol/mg protein. In the second experiment, control levels of extracellular GLU and ASP increased in the presence of HAA, indicating that GLU and ASP uptake were effectively blocked.

Results obtained in experiments using HAA were variable, and while control levels of EAA release were more stable, responses to high potassium, KA and DOM were weak. It is possible that KA and DOM act primarily as amino acid uptake blockers in this preparation, as KA has been shown to be an effective uptake blocker (61, 65, 110). This mechanism would explain the weak response to KA and DOM, and is further discussed in Section 4.4.2. Because of the variable results in experiments using HAA, and concern that HAA might interfere with the actions of KA and DOM, further use of HAA was abandoned.

4.3.4 Changes in Intracellular and Extracellular Glutamate and Aspartate in Response to Kainate and Domoate

Extracellular GLU and ASP levels recorded in release experiments are presented in Table XI. Data from each sampling time were analysed separately. There was no significant difference in GLU or ASP release measured in samples collected before addition of experimental media (0 min, Table XI). In

samples collected 8 min after addition of media, GLU release was significantly greater than control in all treatment groups ($p \leq 0.01$)(Fig 8). Dose dependence was demonstrated for DOM, as GLU release stimulated by 1mM DOM was significantly greater than that produced by 0.5mM ($p \leq 0.05$). Mean levels of ASP release were also increased in all treatment groups, however this effect was significant only for 1mM KA ($p \leq 0.05$) and 40mM K^+ ($p \leq 0.01$)(Figure 8).

It should be noted that statistical analysis was done in the absence of potassium stimulated data. This was done because the mechanism of potassium stimulated release is different from that of KA or DOM stimulated release, with 40mM KCl producing on average an approximately 10 fold greater response, with 10 times greater variability. Including this variability in SNK analysis masked differences among the other treatments examined.

Intracellular Glutamate and Aspartate: Table XII contains GLU and ASP data from intracellular samples. There were no significant differences in intracellular GLU and ASP levels among the treatment groups.

4.3.5 Calcium Dependence of Glutamate and Aspartate Release

Extracellular Glutamate and Aspartate: Data comparing the release of GLU and ASP stimulated by 40mM potassium, 1mM KA, and 1mM DOM in normal and calcium-free medium containing EGTA are presented in Table XIII. As in the previous experiment, there were no significant differences among treatments in samples collected 5 min before addition of experimental media when analyzed by ANOVA (Table XIII, A). Analysis of data collected after 8 min incubation in the presence of experimental media indicate that the GLU and ASP release stimulated by KA and DOM are not Ca^{2+}-dependent (Fig 9). In fact, mean amino acid release stimulated by these agonists is approximately 50% greater in the absence of Ca^{2+}. However, this difference was not found to be significant when analyzed by ANOVA or Student's T-test.

Potassium stimulated release also showed Ca^{2+}-independence, as levels of GLU and ASP in the presence and absence of Ca^{2+} were similar. However, if amino acid release is calculated on the basis of % total, i.e. (extracellular/ intracellular + extracellular) X 100, Ca^{2+}-dependence on the order of 20% for GLU and 17% for ASP is evident. This result is unexpected, as much higher levels of Ca^{2+}-dependence, especially for K^{+}-stimulated release of GLU, are reported in the literature (60,

64, 66, 68, 93, 95). These results are discussed further in Section 4.4.2.

Intracellular Glutamate and Aspartate: There were no significant differences in intracellular amino acid levels among the treatment groups when analyzed by ANOVA (Table XIV). It was noted that intracellular levels of these amino acids increased over time in samples containing Ca^{2+}-free medium. It may be that these conditions stimulated intracellular amino acid biosynthesis (61).

Table XI, Effects of High Potassium and Kainic and Domoic Acids on Glutamate and Aspartate Release.

Time	Amino Acid	Treatment: Control	Potassium (40mM)	Kainic Acid (0.5mM)	Kainic Acid (1.0mM)	Domoic Acid (0.5mM)	Domoic Acid (1.0mM)
0 Min	Asp	0.48 ±0.4	0.36 ±0.2	0.32 ±0.1	0.40 ±0.2	0.43 ±0.3	0.41 ±0.3
	Glu	0.41 ±0.2	0.40 ±0.2	0.59 ±0.5	0.58 ±0.2	0.62 ±0.4	0.69 ±0.4
8 Min	Asp	0.24 ±0.1	6.35 ±5.4[1]	0.35 ±0.3	0.75 ±0.6[2]	0.43 ±0.3	0.53 ±0.3
	Glu	0.28 ±0.2	7.28 ±3.4[1]	0.76 ±0.3[1]	1.00 ±0.6[1]	0.83 ±0.3[1]	1.40 ±0.3[1]

Data are in nmol/mg protein, ±sd, n=4. 1 $p \leq 0.01$, 2 $p \leq 0.05$.

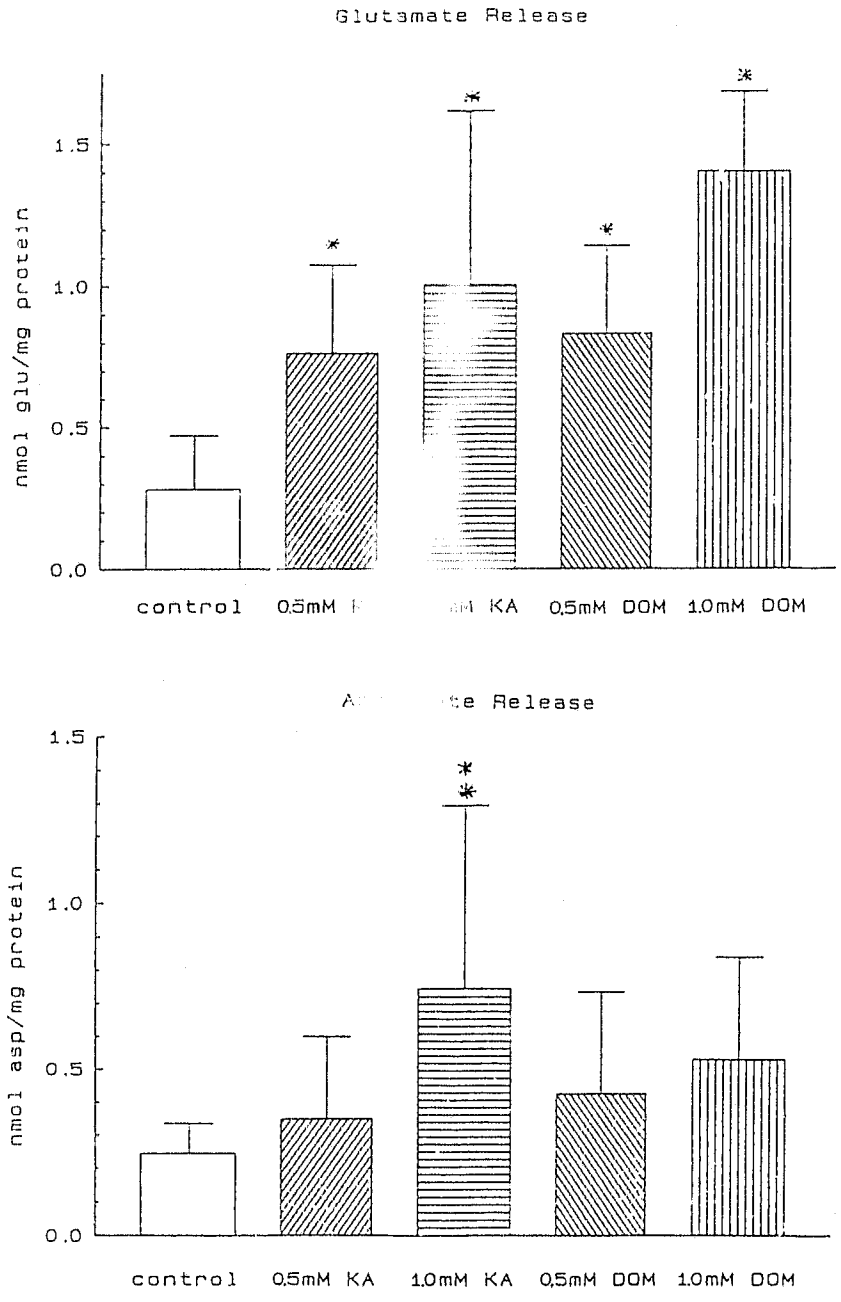


Figure 8. Release of Glutamate and Aspartate After Eight Minutes Incubation with Kainic and Domoic Acids. Error bars indicate ±sd, n+4. * Significantly different from control, $p \leq 0.01$. ** Significantly different from control, $p \leq 0.05$.

Table XII, Effects of High Potassium and Kainic and Domoic Acids on Intracellular Excitatory Amino Acids.

		Treatment					
Time	Amino Acid	Control	Potassium (40mM)	Kainic Acid (0.5mM)	Kainic Acid (1.0mM)	Domoic Acid (0.5mM)	Domoic Acid (1.0mM)
0 Min	Asp	34.6 ±20	42.7 ±38	42.3 ±22	37.8 ±23	42.7 ±24	31.9 ±12
	Glu	60.1 ±33	66.9 ±57	71.0 ±39	64.2 ±40	72.9 ±40	57.0 ±24
8 Min	Asp	43.4 ±21	36.0 ±13	44.0 ±23	41.2 ±19	40.7 ±22	41.7 ±20
	Glu	68.9 ±27	52.2 ±15	72.8 ±37	67.6 ±28	70.2 ±32	74.6 ±29

Data are in nmol/mg protein, ±sd, n=5.

Table XIII, Effect of Calcium on Excitatory Amino Acid Release Stimulated by High Potassium and Kainic and Domoic Acids.

A) Extracellular Glutamate and Aspartate, T= 0 min.

Treatment	Amino acid	$+Ca^{2+*}$	$-Ca^{2+**}$
control	Glu	0.23 ±0.1	0.28 ±0.0
	Asp	0.15 ±0.1	0.16 ±0.1
potassium (40mM)	Glu	0.19 ±0.1	0.20 ±0.0
	Asp	0.09 ±0.1	0.09 ±0.0
kainate (1mM)	Glu	0.13 ±0.1	0.22 ±0.1
	Asp	0.06 ±0.1	0.09 ±0.1
domoate (1mM)	Glu	0.13 ±0.1	0.20 ±0.1
	Asp	0.18 ±0.2	0.13 ±0.0

B) Extracellular Glutamate and Aspartate, T= 8 min.

Treatment	Amino acid	$+Ca^{2+*}$	$-Ca^{2+**}$
control	Glu	0.21 ±0.0	0.23 ±0.0
	Asp	0.22 ±0.2	0.18 ±0.1
potassium (40mM)	Glu	3.78 ±0.4	3.77 ±1.6
	Asp	2.57 ±0.3	2.36 ±1.1
kainate (1mM)	Glu	0.71 ±0.8	1.14 ±0.7
	Asp	0.52 ±0.4	0.85 ±0.3
domoate (1mM)	Glu	0.69 ±0.5	1.01 ±0.4
	Asp	0.50 ±0.3	0.80 ±0.3

Data are in nmol/mg protein, ±sd, n=3.
* Normal Krebs Henseleit HEPES medium (1.3mM CaCl).
** Krebs Henseleit HEPES without CaCl, with 1mM EGTA added.

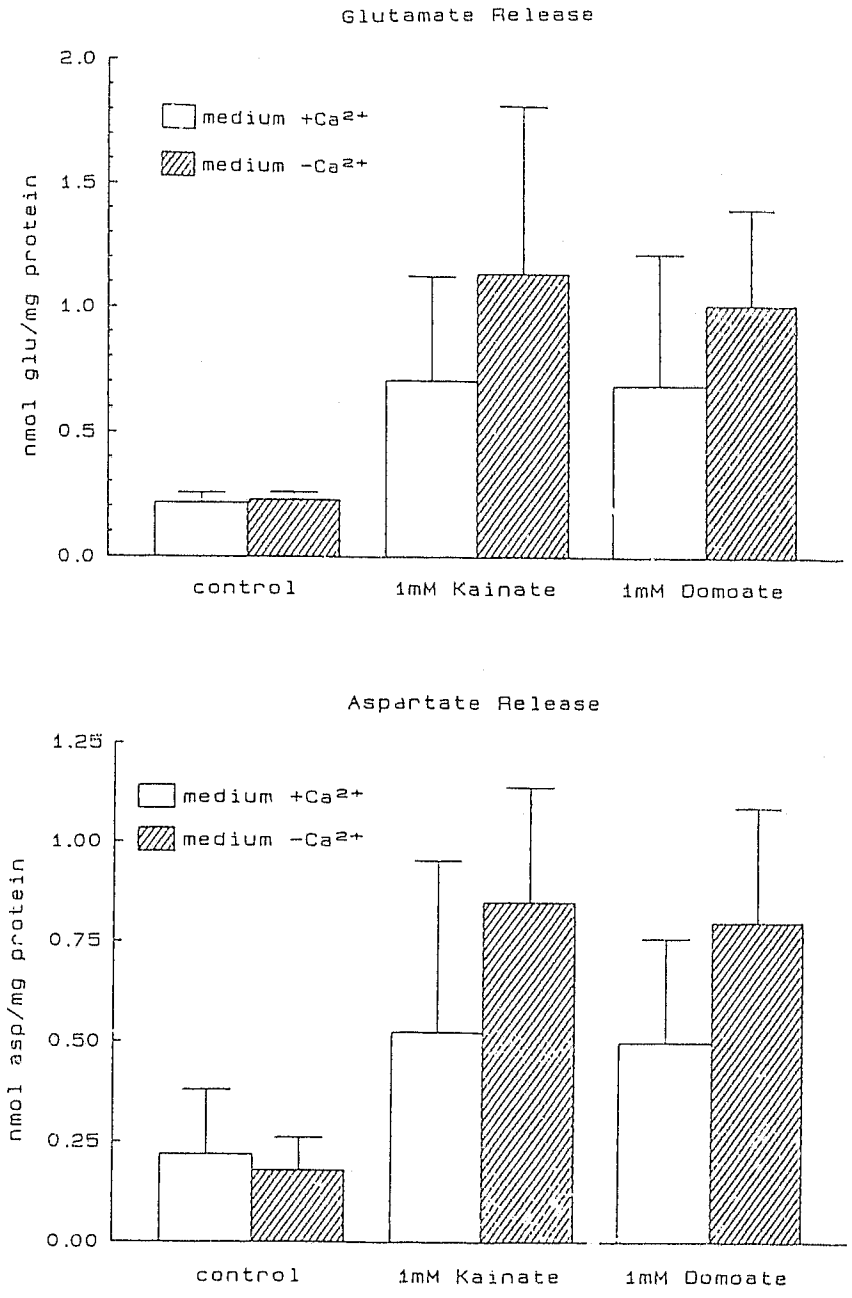


Figure 9. Calcium Dependence of Glutamate and Aspartate Release Stimulated by Kainic and Domoic Acids after Eight Minutes Incubation. Error bars indicate ±sd, n=3.

Table XIV, Effect of Calcium on Intracellular Excitatory Amino Acids in the Presence of High Potassium and Kainic and Domoic Acids.

A) Intracellular Glutamate and Aspartate, T= 0 min.

Treatment	Amino acid	$+Ca^{2+*}$	$-Ca^{2+**}$
control	Glu	90.6 ±23	91.1 ±6
	Asp	70.7 ±22	70.7 ±5
potassium (40mM)	Glu	93.0 ±29	86.4 ±8
	Asp	76.4 ±27	65.4 ±8
kainate (1mM)	Glu	101.9 ±0.1	101.0 ±33
	Asp	76.4 ±26	76.3 ±24
domoate (1mM)	Glu	117.4 ±47	100.8 ±29
	Asp	85.2 ±35	80.8 ±27

B) Intracellular Glutamate and Aspartate, T= 8 min.

Treatment	Amino acid	$+Ca^{2+*}$	$-Ca^{2+**}$
control	Glu	87.9 ±24	104.6 ±23
	Asp	68.5 ±17	82.8 ±20
potassium (40mM)	Glu	93.8 ±24	117.7 ±32
	Asp	74.7 ±9	82.8 ±22
kainate (1mM)	Glu	92.6 ±34	102.4 ±28
	Asp	70.7 ±24	76.8 ±26
domoate (1mM)	Glu	109.8 ±50	113.9 ±35
	Asp	82.9 ±42	88.7 ±33

Data are in nmol/mg protein, ±sd, n=3.
* Normal Krebs Henseleit HEPES medium (1.3mM CaCl).
** Krebs Henseleit HEPES without CaCl, with 1mM EGTA added.

4.4 Discussion

4.4.1 Stimulation of Excitatory Amino Acid Release

Results from release experiments indicate that, at the doses used in this experiment, both KA and DOM increase the basal release of GLU from synaptosomes, while KA increases the release of ASP (Table XI). Results from release experiments and Ca^{2+}-dependence experiments showed overall potencies of KA and DOM were similar, however variability was significant. Differences between synaptosome preparations, particularly the amount of extrasynaptosomal protein contamination (see Section 2.1), and difficulties with the HPLC quantitation (Section 3.4), are likely responsible for much of this variability.

Variability is clearly demonstrated by comparing results from release trials (Section 4.3.4), with results from the control group of Ca^{2+}-dependence trials (Section 4.3.5), which were carried out under identical conditions. In the release trial, 1mM KA produced increased mean extracellular GLU concentrations by 259% and ASP by 205% over control, and 1mM DOM produced increases of 401 and 117%, respectively (Table XI), whereas in the control group of Ca^{2+}-dependence trials (samples incubated in plain HEPES medium), 1mM KA produced increases in GLU and ASP of 230 and 138%, and 1mM DOM, 222 and 127% (Table XIII).

While the increase in basal EAA release observed with KA is supported by a large body of literature (59, 60-66, 103), the release stimulated by DOM is in conflict with the only published work on the topic (45). Terrian et al (45), using 300μM DOM, found no increase in the basal efflux of GLU. However, this discrepancy is not surprising as there are several important differences in the preparations and procedures used in these experiments. First, the 300μM dosage used by Terrian may have been too low, or too high, to produce a significant increase. In our work, 500μM DOM did produce a significant increase in extracellular GLU, but based on the apparent dose dependence of this effect, it is doubtful that a significant result would have been observed with 300μM.

As well, Terrian et al (45) used synaptosomes isolated from hippocampal mossy fibres which were incubated in a superfusion bath, whereas in our work whole brain synaptosomes were used with stationary incubation. This mossy fibre preparation may contain different proportions of receptors than whole brain preparations, or glial fragments may be present in one or the other preparation. Also, we speculate that release into superfusion media is diffuse, while release under stationary incubation conditions is more cumulative. While this gives the superfusion technique the advantage of presenting a more accurate profile of release over time, it also makes it more difficult to detect small changes in levels of EAA release.

One final and important difference between the procedures employed by Terrian et al (45) and this work is that they preincubated synaptosomes in a medium containing 50μM D-aspartate, in order to, "reduce the level of cytosolic glutamate and thereby enhance the calcium dependence of measured glutamate release". This treatment stimulates the exchange of cytoplasmic GLU and extracellular D-ASP across the acidic amino acid carrier, reducing cytoplasmic GLU concentrations (115). If the basal release of GLU stimulated by DOM originates from cytosolic rather than vesicular stores, the release will be masked by preincubation with D-ASP.

Research comparing the neuroexcitatory and neurotoxic effects of KA and DOM have consistently found DOM to be more potent. Domoic acid is reported to be 3 times more potent at producing neuroexcitation when applied directly to the hippocampus (42), and at enhancing potassium-stimulated GLU release from hippocampal mossy fiber synaptosomes (45), and 8 times more potent at producing behavioral symptoms of neurotoxicity when administered systemically (43, 116). If the basal release of EAAs is a primary aspect of the neurotoxic mechanism of these compounds, we would expect DOM to stimulate proportionally higher levels of GLU or ASP release. Alternatively, if GLU and ASP act synergistically as co-transmitters to produce neuroexcitation, we would expect KA and DOM to release different ratios of GLU/ASP, which would produce different

levels of activity at post-synaptic receptors. As previously indicated, the potencies of KA and DOM in stimulating the basal release of GLU and ASP in this preparation were very similar and therefore support the conclusion that this effect may not constitute a major part of the neurotoxic mechanism of these compounds. The high concentrations of KA and DOM (0.5 to 1mM) required to produce a significant increase in EAA release also support this conclusion (68, 69)(see Section 4.1.1).

There is preliminary evidence which indicates that modification of stimulated release may be an important aspect of neurotoxicity. In experiments comparing the effects of KA and DOM on K^+ stimulated release, Terrian et al (45) found DOM to be approximately 3 times more potent than KA at stimulating release of GLU from synaptosomes, with 1mM KA being equipotent with 0.3mM DOM. As well, a concentration of 0.1mM DOM produced a significant increase in this response, making this result more comparable with observations of neurotoxic potency.

4.4.2 Calcium Dependence of Stimulated Release

Calcium dependence trials were performed in an attempt to determine whether the source of released neurotransmitter was cytoplasmic or vesicular. Ca^{2+}-independent release of GLU and

ASP results from the reversal or blockage of t) uptake carrier (113) and/or a slow cytoplasmic leak (65), whereas Ca^{2+}-dependent release relies on an influx of extracellular Ca^{2+} to produce presynaptic depolarization and exocytosis of vesicularly-stored amino acids (64, 66).

Calcium Dependence of Glutamate and Aspartate Release Stimulated by Potassium: It is widely recognized that both Ca^{2+}-dependent and independent mechanisms contribute to the release of GLU from neuronal preparations (64, 66, 68, 93, 94, 95, 106, 103). Results presented in Table XIII demonstrate Ca^{2+}-independence for K^{+}-stimulated GLU and ASP release. However, if amino acid release is expressed on the basis of percent of total GLU or ASP (as described in Section 4.3.5), Ca^{2+}-dependence of 20% for GLU and 17% for ASP is evident. This low level of Ca^{2+}-dependence for K^{+}-stimulated GLU release is in conflict with results presented in the literature. Reports of this effect in the literature range from 53% to 75% (60, 93), while reports of ASP release range from no dependence to 42% dependence (3, 64).

There appear to be three possible explanations for the low level of Ca^{2+}-dependence for K^{+}-stimulated GLU release observed in this work. First, our time of sampling after addition of agonists was 8 minutes, which may have been too late to observe Ca^{2+}-dependence. Nicholls and Sirah (94) examined the

kinetics of K^+-stimulated release from synaptosomes using the glutamate dehydrogenase-based spectrophotometric assay. They found that in the presence of Ca^{2+} (1.3mM), 30mM K^+ produced a rapid initial increase in extracellular GLU, which leveled off after approximately 4 minutes, while in Ca^{2+}-free medium to which 0.5mM EGTA was added, K^+ produced a gradual, linear increase in extracellular GLU which had not leveled off by the end of 4 minutes. In a subsequent work, McMahon et al (106) continued these observations for a total of 6.5 minutes using the same system and found the linear increase in the absence of Ca^{2+} still had not leveled off. It would appear that due to the kinetics of K^+ stimulated release, the difference between Ca^{2+}-dependent and independent GLU release is most notable between 1 and 4 minutes after addition of the agonist i.e. during the vesicular release phase, and is much reduced by 8 minutes.

Second, it may be that Ca^{2+}-independent release was more significant in our preparation because the synaptosome preparation was very fresh and preincubation times were kept to a minimum (5 min). Nicholls et al (113) observed that the Ca^{2+}-independent release of GLU, which occurs by reversal of the uptake carrier, is fastest from freshly suspended synaptosomes, and that preincubation causes a time dependent decrease in the activity of the uptake carrier. Other workers

who found Ca^{2+}-dependence used extensive preincubation periods ranging from 15 to 40min (64, 113).

A third possible explanation for the low level of Ca^{2+}-dependence seen with K^+-stimulated GLU release is that GLU uptake was decreased in the absence of Ca^{2+}, thereby increasing the apparent level of release. Balcar (117), examining GLU uptake in cultures of glial and GABAergic neurons, found that GLU uptake was reduced by 30-40% in the absence of Ca^{2+}. This effect was independent of the K^+ ion concentration.

Another factor which could account, at least in part, for the low level of Ca^{2+}-dependence for GLU release stimulated by 40mM K^+ is that synaptosomes were not washed repeatedly, or preincubated with EGTA, and therefore may have contained enough endogenous Ca^{2+} to saturate the EGTA in Ca^{2+}-free media. The remaining Ca^{2+} may have been sufficient to produce a balance of intracellular to extracellular Ca^{2+} which allowed normal mechanisms of Ca^{2+}-dependent release to function. Most of the published works which demonstrate Ca^{2+}-dependent K^+ stimulated EAA release, have Ca^{2+} levels in preparations reduced by repeated washing of tissues (3, 93). As well, Holopainen and Kontro (93) found that reducing Ca^{2+} in tissues by preincubating in a Ca^{2+} free medium containing 1mM EDTA resulted in a more pronounced Ca^{2+}-dependence for K^+ stimulated release.

Calcium Dependence of Glutamate and Aspartate Release Stimulated by Kainate and Domoate: Unlike the Ca^{2+}-dependence of EAA release stimulated by high K^+, which has been consistently demonstrated in the literature, reports of the Ca^{2+}-dependence of KA stimulated release vary widely. Several *in vitro* experiments have shown that KA enhances the release of endogenous GLU or radiolabelled ASP in a largely Ca^{2+}-dependent manner (60, 61, 63, 64, 66, 95). These works support the hypothesis that KA acts at presynaptic receptors, allowing Ca^{2+} to enter through receptor operated channels and causing the vesicular release of amino acid. The inhibition of the amino acid uptake carrier by KA is recognized in some of these works for contributing to the Ca^{2+}-independent portion of released amino acid. In contrast with these results, other *in vitro* studies have found that KA increases extracellular GLU and ASP concentrations in a Ca^{2+}-independent manner. Suggested mechanisms have included non-neuronal elements (most likely glia)(84, 118), and the inhibition of the amino acid carrier (neuronal and glial)(68, 106) coupled with slow leakage from the cytpolasm (65). Results of complementary *in vivo* experiments using microdialysis have also been contradictory (62, 84).

Our results show KA and DOM acting in a Ca^{2+}-independent manner. Since it has been shown that glia are not present to any extent in this preparation (see Section 2.1), we conclude

this release to be the result of blockage or reversal of the uptake carrier (113), and/or cytoplasmic leakage (65). The argument that KA and DOM may be blocking the amino acid carrier through a non-specific action would explain much of the results obtained. As well, this hypothesis is supported by the results of Pocock et al (66) and McMahon et al (106), who used cerebrocortical synaptosomes from guinea pig in a static incubation system very similar to that used in this study.

The non-specific blockage of the amino acid carrier would explain why high concentrations of KA and DOM are required to increase extracellular EAAs, and why potencies of KA and DOM are the same. It would also account for why the increase in extracellular GLU and ASP accumulated slowly, and then plateaued, as this would be the response as a new intracellular/extracellular equilibrium of these amino acids was established. As well, this model would explain why results in this work are in conflict with those of Terrian et al (45), who found no increase in extracellular GLU in response to DOM using mossy fibre synaptosomes in superfusion. In the superfusion technique, released amino acids are constantly removed, so that blockage of reuptake is not evident.

If results for GLU and ASP release are expressed as percent of total GLU or ASP ((extracellular amino acid/ intracellular + extracellular)x 100) and compared, we find that levels of GLU and ASP released by KA and DOM are almost identical. This suggests that GLU and ASP are being released from a common pool, probably cytoplasmic, and is consistent with the model proposed.

Further experiments designed to test this hypothetical mechanism of action for KA and DOM would be useful. To determine whether KA and DOM block the uptake of GLU and ASP, the uptake of isotopically labelled GLU and ASP into synaptosomes in the presence or absence of varying dosages of KA and DOM could be measured. As well, the actions of these compounds could be compared with those of known uptake blockers, such as DHKA, HAA, or trans-PCD. Modification of the effect should be possible through an increase in extracellular GLU, ASP, and Na^{+}, all of which should counteract the action of a blocker by influencing the equilibrium towards increased uptake.

Not only did KA and DOM demonstrate Ca^{2+} independence, it was found that EAA release stimulated in the absence of Ca^{2+} was approximately 50% greater. Although this trend was not statistically significant I have speculated on three possible explanations for this result. First, the profiles of GLU and

ASP release over time may be similar to those described for K^+-stimulated release, i.e. a rapid increase and subsequent leveling off or decline in the presence of Ca^{2+}, and a more gradual increase in the absence of Ca^{2+}, so that at the 8 min sampling time amino acid levels are lower in the presence of Ca^{2+}. The second hypothesis is that EAA uptake is reduced in the absence of Ca^{2+}, resulting in an increase in extracellular amino acid levels in Ca^{2+}-free media compared to Ca^{2+}-containing media. This hypothesis is supported by the work of Balcar (117), who found that GLU uptake was decreased by 30-40% in the absence of Ca^{2+}. A third possible explanation is that in Ca^{2+}-free media to which EGTA is added, the electrochemical gradient is reduced by chelation of extracellular positive ions and reversal of the uptake carrier is increased. This theory is supported by observations that reduction of the Na^+ gradient accelerates reversal of the uptake carrier (113, 119).

Experiments conducted by Kauppinen et al (119) indicate that Ca^{2+}-independent GLU release may be a major factor in the neurotoxic mechanisms of hypoglycaemia and anoxia. If this is the case, KA and DOM may be acting in our model to mimic some of the neuronal mechanisms leading to hypoglycaemic or anoxic brain damage.

In conclusion, KA and DOM produced similar responses in the present study, suggesting a common mechanism of action. Percentages of total GLU and ASP released were the same, suggesting that released amino acids probably originated from a common pool, probably cytoplasmic. Our results show that KA and DOM increase the release of both GLU and ASP in what appears to be a Ca^{2+}-independent manner, suggesting that under the experimental conditions used, KA and DOM act to release GLU and ASP from cytosol, probably through blockage or reversal of the uptake carrier, and possibly combined with cytoplasmic leakage. While this result for KA (Ca^{2+}-independent stimulation of EAA release) is supported by several reports (65, 68, 106), it is also refuted by numerous others who have identified receptor-mediated stimulation of Ca^{2+}-dependent EAA release in response to KA (60, 61, 63, 64, 66, 95). It appears that at least two mechanisms are present, one Ca^{2+}-dependent, one independent, and that the predominant mechanism varies according to the preparation used.

The Ca^{2+}-independence demonstrated for K^{+}-stimulated EAA release is inconsistent with the findings of other investigators. This discrepancy may be due to differences in experimental conditions, and makes the conclusions regarding the mechanism of DOM and KA action on GLU and ASP release tentative. Further investigations into the role of Ca^{2+} in KA

and DOM-stimulated release of EAAs from brain tissue are necessary.

Based on the similar potencies of KA and DOM in stimulating basal GLU and ASP release, and on the high dosages required to produce a significant effect it does not appear that the increase in extracellular EAAs observed in this work is a major aspect of the neurotoxic mechanism of action of these compounds. However, the increase in extracellular EAAs may play a role in exacerbating increases in stimulated EAA release.

5 SUMMARY AND CONCLUSIONS

5.1 Summary

Domoic acid (DOM) is an amino acid which produces neuronal excitation when applied at low concentrations (26, 42), and neurotoxicity at higher concentrations (35, 60). In 1987, DOM was found to be the cause of numerous poisonings and three fatalities which resulted from consumption of contaminated mussels (*Mytilus edulis*)(29). The toxin in mussels originated from a marine diatom, *Nitszchia pungens* (32). Histological examination of fatally affected individuals revealed extensive neuronal damage, particularly in the hippocampus and amygdaloid nucleus, and severely affected individuals acquired a lasting short-term memory deficit (29).

Kainic acid is another excitatory amino acid (EAA) produced by marine algae, and has been used extensively in research for its selective neurotoxic actions (41). Kainic acid (KA) is a prototypical agonist at a major class of glutamate receptors in the mammalian central nervous system, known as KA receptors (10). It is believed that KA produces its neurotoxic effects through a mechanism similar to that which produces excitation; this effect is known as excitotoxicity (49, 50).

KA and DOM are both structural analogs of the endogenous EAAs, glutamate (GLU) and aspartate (ASP), with their amino groups restricted by an imino ring structure. Domoic acid also has an elaborate five carbon side chain with an additional carboxyl group (19). It has been proposed that KA and DOM produce their toxicity through the same mechanism, and that this mechanism involves the stimulation of pre- and/or post-synaptic KA receptors, resulting in increased release of GLU, and possibly ASP, pre-synaptically (60-66, 68), and prolonged neuroexcitation post-synaptically (42, 50, 59). According to excitotoxic theory, the post-synaptic excitation produced by both exogenous and endogenous agonists result in ionic imbalances, energy depletion, neuronal swelling and eventually cell necrosis (50, 52, 120). Another factor contributing to elevated levels of GLU and ASP in the synaptic cleft may be the blocking (61, 65, 110), and perhaps even reversal (113), of the acidic amino acid carrier by KA or DOM, which slows the removal of EAAs by neurons and glia.

The neurotoxic potency of DOM is approximately 3 to 8 times that of KA (42, 43, 45). Recent research indicates this difference may be due to different binding affinities for KA and DOM at kainate receptor subtypes. Werner *et al* (13) demonstrated that KA has a higher affinity for the so called high affinity kainate binding site (KA-1), while Nielsen et al (121) have shown that DOM has higher affinity for the low

affinity kainate binding site, and that antagonism of this site selectively reduces DOM toxicity. If this is the case, the neurotoxic effects of these EAAs could be the result of their action at the low affinity site.

In this work, a method for the preparation of synaptosomes from rat brain was assessed, and a technique for the analysis of intracellular and extracellular amino acids in this preparation, using reversed-phase HPLC and fluorometric detection of FMOC derivatized amino acids was developed. Using these techniques, the effects of KA and DOM on the basal release of GLU and ASP were compared, and the Ca^{2+}-dependence of these effects was examined. High levels of potassium, a general neuronal depolarizer, were used as a positive control.

Concentrations of 0.5 and 1.0 mM KA and DOM both produced significant increases in GLU release ($p \leq 0.01$). Dose dependence was demonstrated for DOM, as GLU release stimulated by 1.0 mM DOM was significantly greater than that produced by 0.5 mM ($p \leq 0.05$). Mean levels of ASP release were also increased, but this effect was significant for 1.0 mM KA only ($p \leq 0.05$). High potassium (40mM) produced significant increases in both GLU and ASP release ($p \leq 0.001$).

The calcium dependence of EAA release in response to KA and DOM was studied to determine whether released EAAs originated

from vesicular or cytoplasmic stores. Release of GLU and ASP stimulated by KA and DOM demonstrated calcium-independence, as did release stimulated by high potassium. This result for potassium is inconsistent with the findings of other investigators (see Section 4.4.2), and makes conclusions regarding the mechanism of KA and DOM's action on GLU and ASP release tentative. Despite this fact, the data for KA and DOM are consistent with the hypothesis that these agonists increase extracellular GLU and ASP concentrations by blocking the acidic amino acid uptake carrier.

Results were examined to determine the relevance of these observations to the neurotoxicity of KA and DOM. Determining the neurotoxic mechanism of DOM will have two major implications; first, it will greatly assist in the development of an antagonist for domoic acid intoxication, so that no further losses will be suffered due to consumption of contaminated shellfish; and second, in the field of neuroscience, DOM may prove to be a selective agonist at one KA receptor sub-type (13, 121), thereby providing a tool for obtaining information on several aspects of neuronal function, ranging from memory formation to chronic and acute neuronal diseases, such as Alzheimer's disease, Huntington's chorea, epilepsy, and hypoxia (52, 120).

5.2 Conclusions

Conclusions and recommendations based on this work can be summarized as follows:

1) KA and DOM both increase the basal release of endogenous GLU from rat brain synaptosomes, while only KA raised extracellular ASP in a statistically significant manner. Glutamate and aspartate were released in what appears to be a Ca^{2+}-independent manner. These results suggest that EAAs are being released from cytoplasmic rather than vesicular stores, possibly by blockage or reversal of the acidic amino acid carrier (113), and/or by increased diffusion from the cytoplasm through other channels (65). The lack of calcium-dependence observed with potassium-stimulated release makes these conclusions tentative. Further examination of the role of Ca^{2+} in the mechanism of GLU and ASP release would be useful. This work would involve the investigation of the time course of EAA release in the presence and absence of Ca^{2+}. As well, selective non-NMDA receptor blockers such as CNQX or NBQX (123) could be used to determine if KA receptor activation is involved in the mechanism of EAA release. The hypothesis that KA and DOM are acting as EAA uptake blockers should also be examined. The use of isotopically labelled GLU and ASP on synaptosomes is recommended for this work.

2) Two aspects of the results presented in this thesis suggest that the increase in basal EAA release observed in response to KA and DOM are not directly responsible for the toxicity of these compounds. First, equal concentrations of KA and DOM produced comparable increases in basal EAA release. This result is in contrast with the 3 to 8 fold difference in toxic potencies reported for these compounds (42, 43, 45). Second, the fact that high concentrations of KA and DOM (0.5-1mM) were required to stimulate this release, while neurotoxic concentrations are in the 5-100μM range (68, 69), suggests that these responses are not related. However, while increased basal release may not be a primary mechanism of the neurotoxicity of these EAAs, it could contribute to neurotoxicity by maintaining high extracellular concentrations of GLU and ASP produced via other mechanisms, such as increased EAA release during neuronal stimulation (see Section 4.4.1).

3) The estimation of amino acids by derivatization with FMOC, followed by HPLC with fluorometric detection was very time consuming. Continued research using this technique would require development of the technique to reduce processing time and improve separation of GLU and ASP from glutamine. It would also be beneficial to find an internal standard which would elute at a time well resolved from that of KA and DOM. Alternatively, o-phthaldialdehyde derivatization followed by

reversed phase HPLC could be an improvement for determination of GLU and ASP, or glutamate-dehydrogenase spectrophotometric assay for determination of GLU alone.

4) Further studies examining effects of KA and DOM in neuronal preparations may provide answers regarding the mechanism(s) of their neurotoxic action. Research examining EAA release using synaptosomes from different regions of the brain, e.g. cerebral cortex, striatum, or hippocampus, would yield results specific to these regions of the brain. As well, continued investigation of the effects of DOM and KA on potassium-stimulated release of EAAs may be useful.

APPENDIX A, Biological Media

Synaptosomal Preparation Media:

Synaptosomes were prepared from whole rat brain following the method of Booth and Clarke (78).

1) Isolation Medium

Conc.	Compound	Source
320 mM	sucrose	Sigma (1)
1 mM	potassium EDTA*	Sigma (1)
10 mM	Tris	Sigma (1)

* ethylenediamine tetraacetic acid

2) Ficoll Sucrose Media

Media were prepared as described above with the addition of 12% or 7.5% Ficoll (Type 400, Sigma (1)).

Incubation Medium:

Krebs Henseleit HEPES*

Synaptosomes were suspended in Krebs Henseleit HEPES medium for incubation as described in Section 4.2.

Conc.	Compound	Source
140 mM	NaCl	BDH (2)
5 mM	KCl	BDH (2)
1.3 mM	$MgSO_4$	Fisher (3)
5 mM	$NaHCO_3$	Sigma (1)
1.3 mM	$CaCl_2$	Fisher (3)
1 mM	Tris	Sigma (1)
10 mM	D-glucose	Sigma (1)
10 mM	HEPES*	Sigma (1)
1 mM	NaH_2PO_4	Sigma (1)

* hydroxyethylpiperizine ethanesulfonic acid

(1) Sigma Chemical Co., St Louis, MO.
(2) BDH Chemicals, Toronto, Ont.
(3) Fisher Scientific Co., Fair Lawn, NJ.

APPENDIX B, Sample Calculations of Amino Acid Concentration

Glutamate and aspartate concentrations were calculated similarly using Minitab software (Minitab Inc., State College, PA). Sample calculations presented here are for the glutamate concentration in the control sample taken at 5 min incubation in the experiment described in Section 2.2.3. The glutamate concentration for this sample was 1.506 nmol/mg protein (see Table IV). This value was obtained from duplicate samples using the following calculations;

1) Peak areas for glutamate (GLU) were:
   Sample 1- 109020
   Sample 2- 119000

2) Peak areas for the internal standard (DHKA) were:

   Sample 1- 208732
   Sample 2- 194048

3) The linear equation for GLU concentrations in standards run on the same day was:

$$\mu M\ GLU = 0.219 + 6.33\ GLU/DHKA$$

   - Substituting values for GLU and DHKA into this equation gives the following GLU concentrations:

   Sample 1- 3.525 μM
   Sample 2- 4.100 μM

4) Protein determination (described in Appendix C) gave protein quantities of:
   Sample 1- 1.34 mg
   Sample 2- 1.36 mg

5) Final GLU concentrations were calculated using the equation:

$$X = \frac{Y \times 400/300 \times 0.4\ ml}{Z}$$

Where; X = n mol GLU/mg protein
Y = μM GLU, converted to n mol/ml (i.e. X 1)
Z = mg protein in sample
400/300 = dilution factor, and
0.4 ml = total sample volume

For Sample 1: $X = \frac{3.525\ \mu M \times 400/300 \times 0.4\ ml}{1.34\ mg}$

$X = 1.403$ n mol/ mg protein

For Sample 2: $X = 1.608$ n mol/ mg protein

6) When these values are averaged, the result of 1.506 n mol/ mg protein is obtained.

APPENDIX C, Protein Determination

The method of Lowry et al (88) was used for protein determinations. In this technique, the phenolic hydroxyl groups of amino acid side chains are estimated by the Folin colour reaction.

Procedure

1) Pellets were thawed and suspended in 2.5 ml distilled, deionized water by triturating with a pasteur pipette. In 13 x 100 mm test tubes, 50 μl aliquots of sample were diluted with 150 μl water. Standards containing 5, 10, 15, 25, and 50 μg protein from bovine serum albumin and a reagent blank (200 μl H_2O) were also prepared. All samples were run in duplicate. 200 μl of 1 N NaOH were added to each tube.

2) Two ml of copper carbonate solution were added to each tube, samples mixed and let stand for 10 minutes. Copper carbonate solution was prepared fresh daily by mixing 50 ml 2% Na_2CO_3 with 1 ml copper tartrate solution. Copper tartrate solution was prepared by mixing 50 ml 2% Na tartrate, 50 ml 1% $CuSO_4$, 2.5 ml 1N-NaOH. Sodium hydroxide and tartrate were mixed before adding copper sulfate. Copper tartrate solution was made up and stored for up to 8 weeks at room temperature.

3) To each tube, 200 μl diluted Folin Reagent (1:1) (Sigma Chemical Co., St Louis, MO) were added and immediately mixed. Samples were allowed to stand for 30 minutes before being analyzed.

4) Absorbance was read on a Hewlett Packard 8452A diode array spectrophotometer (Hewlett Packard Ltd., Mississauga, Ont.) at 750 μm. Absorbance of standards was plotted against concentration and unknowns were quantified using Hewlett Packard UV/VIS quantitation software.

5) Protein quantities given in μg were converted to total mg protein in the sample using the following equation:

$$X = \frac{Y/50\mu l \times 2500\mu l}{1000}$$

Where; X = mg protein in sample
Y = μg protein
50μl = aliquot taken in 1.
2500μl = total sample volume, and
1000 = conversion to mg

APPENDIX D, Statistical Analysis of Glutamate and Aspartate Release

After glutamate and aspartate concentrations were calculated as described in Appendix B. Data were then analyzed using a two-way factorial design (analysis of variance, general linear model), on Minitab software (Minitab Inc., State College, PA). The extracellular amino acid concentrations after 8 min incubation in the presence of agonists (see Table XI), from experiments described in Section 4.2.4 will be used as an example.

Glutamate: There were 5 treatment levels (control, 0.5 and 1.0mM kainic acid, and 0.5 and 1.0mM domoic acid), and 4 experiments. This gave 4 treatment degrees of freedom (df), 3 experiment df, 12 interaction df, and 20 error df. Treatment, experiment, and treatment x experiment effects were all significant ($F_{4,20}= 23.90$, $p\leq0.001$, $F_{3,20}= 7.99$, $p\leq0.001$, and $F_{12,20}= 3.40$, $p\leq0.01$, respectively). The mean square error term used in subsequent tests of comparison was 0.05546.

Aspartate: As for glutamate, there were 5 treatments and 4 experiments. The error df were reduced to 15 due to incomplete data. Treatment, experiment and interaction effects were all significant ($F_{4,15}= 6.33$, $p\leq0.01$, $F_{3,15}= 8.28$, $p\leq0.01$, and $F_{12,15}=2.64$, $p\leq0.05$, respectively). The mean square error term was 0.04669.

Multiple comparisons were performed by hand using the Student Newman-Keuls method decribed by Ferguson (124).

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