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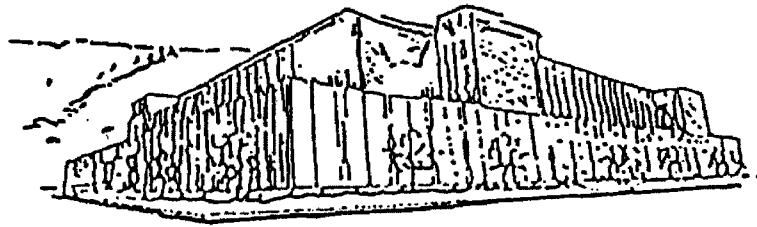
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AMINO ACID CARBAMATES AS INHIBITORS AND SUBSTRATES OF HIGH-AFFINITY GLUTAMATE TRANSPORT IN RAT BRAIN SYNAPTOSOMES

By

Colleen A. Baker

B.S., Chemistry, Montana College of Mineral Science and Technology

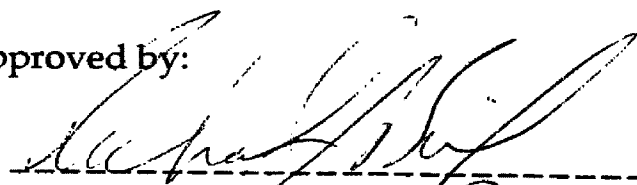
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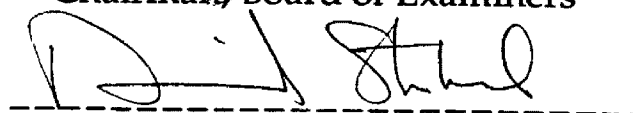
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Colleen A. Baker, M.S., 1995 Pharmacology

Amino Acid Carbamates As Inhibitors And Substrates Of High-Affinity Glutamate Transport In Rat Brain Synaptosomes (73 pp.)

Director: Richard J. Bridges, Ph.D. 

The process of excitotoxic-neuronal pathology is mediated through the excessive activation of excitatory amino acid (EAA) receptors by either endogenous transmitters (*e.g.*, glutamate, aspartate) or exogenous agonists (*e.g.*, NMDA, kainate). Recently a number of amino acids have been identified that appear to induce excitotoxic damage, yet are structurally dissimilar from glutamate (*e.g.*, β -N-methylamino-L-alanine, BMAA; α,β -diaminopropionate, DAP). The discovery that this toxicity was dependent upon the presence of bicarbonate led to the conclusion that the true agonist activity was attributable to a carbamate formed from the amino acid and carbon dioxide.

In the present study we have examined the ability of these amino acid carbamates to inhibit high affinity glutamate uptake. As this transport system is responsible for regulating extracellular glutamate levels and preventing excitotoxic damage, any loss in activity could potentiate EAA-mediated injury. We find the ability of DAP, but not BMAA, to inhibit the glutamate transporter in rat forebrain synaptosomes increased significantly in the presence of sodium bicarbonate. Inhibition of uptake by DAP/CO₂ was also stereoselective, with the L-enantiomer being the more potent blocker. While CO₂ may interact with either amino group on L-DAP, molecular modeling studies indicate that the β -carbamate is the active inhibitor. This work suggests that the neurotoxic action of DAP may include an action not only at the EAA receptors, but also at the EAA transporter.

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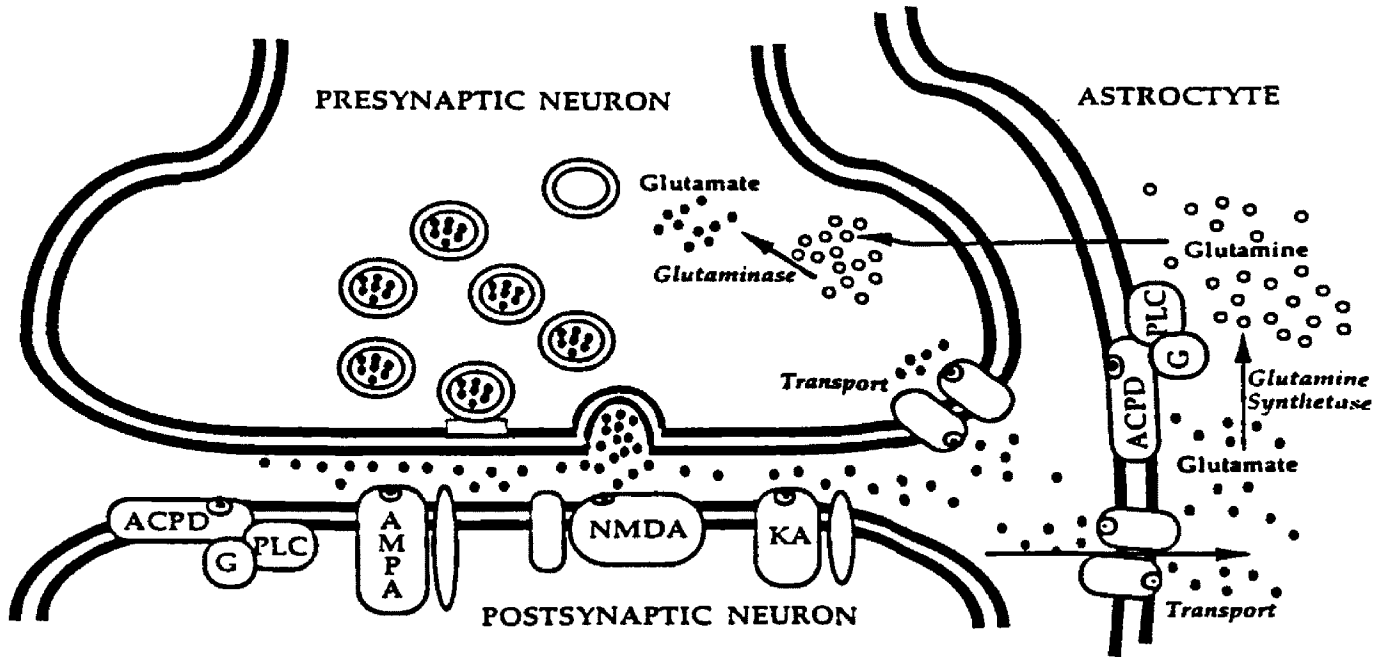
INTRODUCTION

I. GLUTAMATE

L-Glutamate was first reported to have an excitatory effect on spinal neurons by Curtis, Phillis, and Watkins in 1959 (Curtis, *et.al.*,1959). However; its distribution throughout the body and its involvement in intermediary metabolism set it apart from neurotransmitters such as γ -aminobutyric acid (GABA), dopamine, and acetylcholine, and make its postulated role in the CNS much more controversial. These metabolic roles include not only protein synthesis, but also a number of important biochemical pathways. As a substrate of aminotransaminases, glutamate is an important compound in ammonia and amino acid metabolism. It is also the direct precursor of α -ketoglutarate, a key tricarboxylic acid cycle intermediate. Glutamate can also be reduced to glutamic γ -semialdehyde, a precursor in the formation of proline (which eventually forms collagen) and ornithine (which leads to arginine and urea synthesis). When glutamate undergoes a pyridoxal phosphate-dependent decarboxylation by glutamic acid decarboxylase (GAD), it forms GABA, an important inhibitory neurotransmitter.

For these reasons, considerable effort over the past 30 years has focused on demonstrating that glutamate meets the criteria of a classic neurotransmitter. Studies have shown that glutamate is packaged in synaptic vesicles within presynaptic terminals, and released into the synaptic cleft in a Ca^{2+} -dependent manner (Figure 1) (Nicholls and Attwell, 1990). Exogenous

Figure 1. Excitatory Amino Acid Synapse.



applications of glutamate activate postsynaptic receptors and produce excitatory signals similar to those observed in the presence of endogenous neurotransmitter (Cotman, *et.al.*, 1987). Finally a high affinity glutamate transport system has been identified and postulated to play a role in signal termination (Balcar and Johnston, 1972; Rosenberg, *et.al.*, 1992). Taken together the results of these studies indicate that not only is glutamate a transmitter, but it is the principle excitatory neurotransmitter in the mammalian central nervous system (Cotman, *et.al.*, 1995; Monaghan, *et.al.*, 1989).

II. GLUTAMATE RECEPTORS

Given the critical role that the postsynaptic receptors play in neurotransmission, it is not surprising that considerable emphasis has been placed on defining the pharmacology of the excitatory amino acid (EAA) receptors. The receptors have been divided into two pharmacological categories: ionotropic (*i.e.*, those that are directly coupled to ion channels) and metabotropic (*i.e.*, those that are linked to second messenger systems) (Monaghan, *et.al.*, 1989; Cotman, *et.al.*, 1995). It is through the activation of ionotropic and metabotropic receptors (Figure 1), that glutamate mediates both fast excitatory transmission and complex signaling processes such as long term potentiation (LTP) and plasticity (Cotman, *et.al.*, 1989; Monaghan, *et.al.*, 1989; Collingridge and Wolf, 1991).

The ionotropic receptors were named after the selective agonists that

were used to differentiate the various receptor subclasses: N-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA), and kainate (KA) (Monaghan, *et.al.*, 1989; Cotman, *et.al.*, 1995). Owing to the limited availability of selective antagonists, early pharmacological studies could readily distinguish AMPA and KA receptors from NMDA receptors, but not from each other (Monaghan, *et.al.*, 1989). For this reason they are often referred to collectively as “non-NMDA” receptors. Both KA and AMPA receptors are responsible for mediating the fast excitatory postsynaptic potentials at EAA synapses (Headley and Grillner, 1990). They are voltage independent and gate depolarizing currents mediated by the entry of sodium into the neuron (Monaghan, *et.al.*, 1989). It was not until both physiological and radioligand binding studies were carried out that distinctions between KA and AMPA receptors became more clear (Watkins and Evans, 1981; Fagg, 1985; Mayer and Westbrook, 1987). Studies demonstrate the receptors differ from each other with respect to agonist potency, anatomical location, and desensitization. KA receptors exhibit an agonist potency of: domoate>kainate>quisqualate>>L-glutamate (Davis, *et.al.*, 1979; Evans, *et.al.*, 1987), while AMPA receptors appear to have an agonist potency order of quisqualate \geq AMPA>L-glutamate>kainate (Monaghan, *et.al.*, 1989). These patterns are also observed in binding studies using ^3H -kainate (Simon, *et.al.*, 1976; London and Coyle, 1979; Slevin, *et.al.*, 1983) and ^3H -AMPA (Honore, *et.al.*, 1982; Murphy, *et.al.*, 1987; Olsen, *et.al.*, 1987). Studies using ^3H -kainate indicate that KA receptors are most

numerous in the hippocampal CA3 stratum lucidum, deep cerebral cortical layers, striatum, reticular nucleus of thalamus, and granule cell layer of the cerebellum (Monaghan and Cotman, 1982; Unnerstall and Wamsley, 1983). In contrast, AMPA receptors are most dense in the CA1 stratum radiatum, outer cortical layers, lateral septum, and molecular layer of the cerebellum (Monaghan, *et.al.*, 1984; Olsen, *et.al.*, 1987). KA receptors desensitize very slowly, whereas AMPA receptors do so rapidly (Mayer and Westbrook, 1987; Henley and Oswald, 1988; Mayer and Vlyklicki, 1989).

The recent identification of cDNAs has generated the most direct evidence of heterogeneity between the non-NMDA receptors (Egebjerg, *et.al.*, 1991; Bettler, *et.al.*, 1992; Herb, *et.al.*, 1992; Sommer, *et.al.*, 1992). Consistent with earlier findings, expressed clones produce responses separable into KA and AMPA sensitivities (Cotman, *et.al.*, 1995). Thus far, four AMPA subunits have been isolated (GluR1-GluR4, also referred to as GluRA-GluRD) and have been found to be active when expression is homomeric or heteromeric (Cotman, *et.al.*, 1995). RNA splicing generates alternate "flip" or "flop" variants in which the "flip" variants produce a larger sustained current than do the "flop" variants (Sommer, *et.al.*, 1990). Upon channel formation, these subunits are activated by AMPA and to some extent by KA (Cotman, *et.al.*, 1995). KA receptors have been divided into five subunits: GluR5-GluR7 and KA-1-KA-2, where GluR5 and GluR6 form active homomeric or heteromeric channels and GluR7, KA-1, and KA-2 form only active heteromeric channels (Cotman, *et.al.*, 1995). GluR5-GluR7 demonstrate low-affinity KA binding,

while GluR7, KA-1 and KA-2 have a high-affinity for KA binding (Cotman, *et.al.*, 1995).

Unlike non-NMDA receptors, NMDA receptors are voltage dependent and are permeable to Ca^{2+} , as well as Na^{+} and K^{+} . NMDA receptors appear to possess at least five discrete ligand binding domains: (i) a transmitter site to which an agonist such as L-glutamate binds), (ii) a regulatory or coactivator site to which glycine binds), (iii) a site inside the channel that binds phencyclidine and related compounds, (iv) a voltage-dependent site in the ion channel which binds Mg^{2+} , and (v) an inhibitory divalent cation Zn^{2+} binding site (Monaghan *et.al.*, 1989). The voltage dependency and Ca^{2+} permeability of the channel allow long term potentiation (LTP), long term depression (LTD), and developmental plasticity to occur (Muller, *et.al.*, 1988; Nicoll, *et.al.*, 1988). The cloning of the NMDA receptor has distinguished two families of subunits, NMDAR1 and NMDAR2. The NMDAR1 subunit has seven splice variants (*e.g.*, NMDAR1A-G) and can assemble homomeric or heteromeric channels (Moriyoshi, *et.al.*, 1991). The NMDAR2 subunit has four splice variants (*e.g.*, NMDAR2A-D) and is only capable of forming heteromeric channels (Ishii, *et.al.*, 1993).

In contrast to ionotropic receptors, metabotropic receptors are G protein coupled. These receptors have also been distinguished by the action of selective agonists such as 1-amino cyclopentane-*trans* - 1,3 -dicarboxylic acid (ACPD) and L-2-amino-4-phosphonobutanoic acid (L-AP4) (Monaghan, *et.al.*, 1989; Schoepp, *et.al.*, 1990; Schoepp, 1994; Cotman, *et.al.*, 1995). Widely

expressed throughout the brain, ACPD receptor subtypes have been shown to be linked to both phosphoinositide (PI) second messenger systems and cyclic adenosine monophosphate (cAMP) production (Berridge, 1987; Schoepp and Conn, 1993). L-AP4 receptors are thought to be present on presynaptic terminals and located in only a few brain areas. Electrophysiologically, activation of metabotropic receptors can produce either excitatory or inhibitory actions (Stratton, *et.al.*, 1990; Baskys and Malenka, 1991; Desai and Conn, 1991). ACPD can produce depolarization and reduction of the after-hyperpolarization, block cell firing, and reduce the postsynaptic potential. At least six subtypes of metabotropic receptors, mGluR1-mGluR6, have been cloned at this point (Nakanishi, 1992).

III. GLUTAMATE TRANSPORTERS

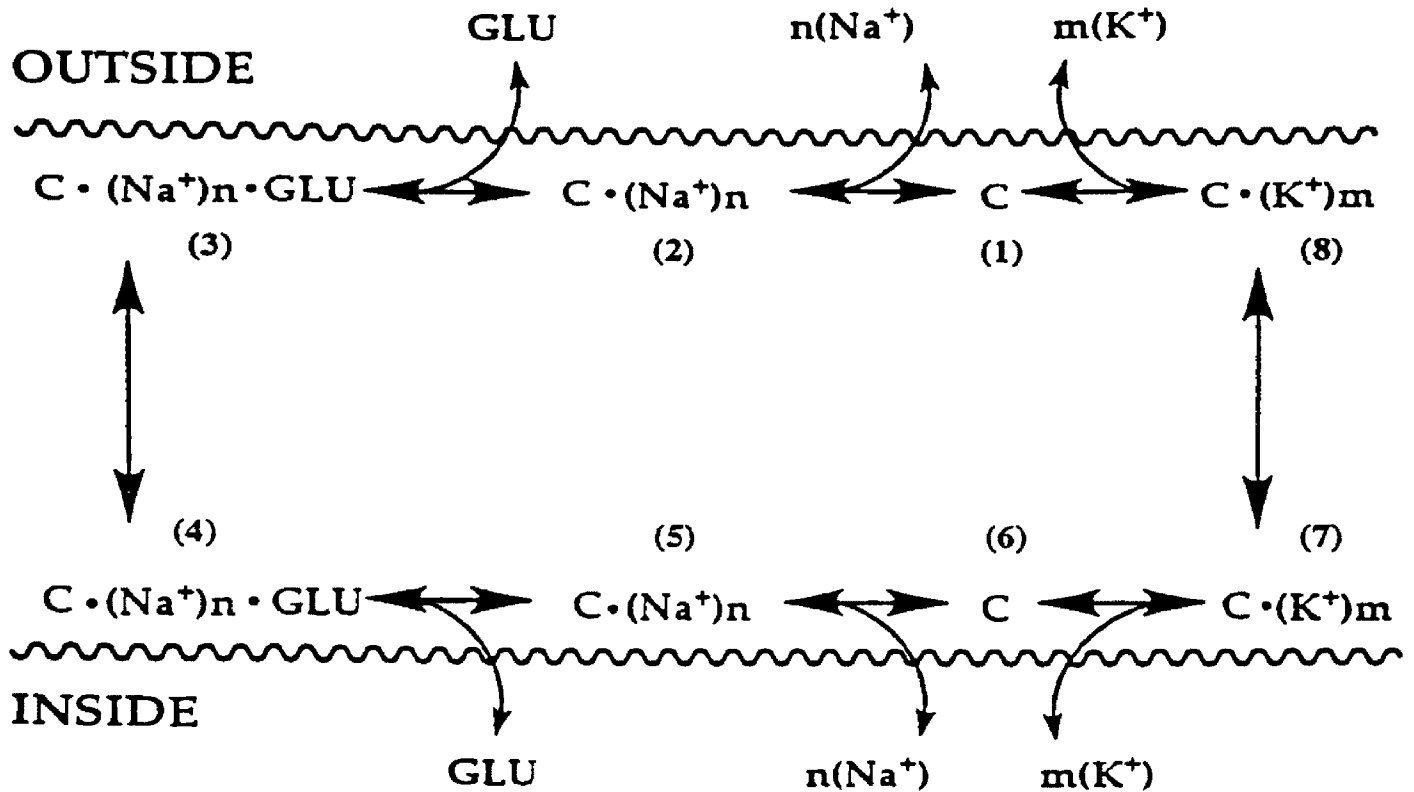
In addition to receptors, EAA synapses also contain transporter proteins that are responsible for the rapid clearance of glutamate from extracellular spaces (Nicholls and Attwell, 1990). It is the rapid removal of glutamate through these transporters that is thought to contribute to the termination of excitatory signals, the regulation of extracellular glutamate levels, and the recycling of glutamate through the glutamine cycle (Hamberger, *et.al.*, 1979; Shank, *et.al.*, 1981; Schousboe, *et.al.*, 1988; Rosenberg, *et.al.*, 1992). Thus, under normal circumstances the removal of synaptically released glutamate surrounding the receptors is thought to be dependent upon both diffusion and uptake (Nicholls and Attwell, 1990; Isaacson and

Nicoll, 1993).

Comparable to the heterogeneity observed with EAA receptors, evidence suggest subtypes of transporters can be differentiated on the basis of: ionic dependence (Na^+ vs. Cl^-), subcellular function (synaptosomal vs. synaptic vesicle), cellular type (neuronal and glial), and anatomical location (forebrain and cerebellar) (Balcar, *et.al.*, 1987; Anderson, *et.al.*, 1990; Kanai and Hediger, 1992; Chamberlin and Bridges, 1993; Robinson, *et.al.*, 1993). The Na^+ -dependent transporter, which is found on presynaptic terminals and glial membranes, is the most extensively characterized transporter with respect to mechanism, pharmacology, and distribution (Kanner and Schuldiner, 1987; Anderson, *et.al.*, 1990; Nicholls and Attwell, 1990; Arriza, *et.al.*, 1994).

An inwardly-directed electrochemical sodium gradient maintained by the action of Na^+/K^+ ATPase drives the uphill transport of the glutamate into neurons and glia (Figure 2) (Kanner and Schuldiner, 1987). The exact stoichiometry of the transport process remains unclear. Studies by Kanner and Sharon; Barbour, *et.al.*; and Pine and Kanner report that the translocation of glutamate into presynaptic terminals is accompanied by the cotransport of 3 Na^+ ions (or 2 Na^+ ions and 1 H^+ ion) into the cell and 1 K^+ ion out of the cell (Kanner and Sharon, 1978; Barbour, *et.al.*, 1988; Pine and Kanner, 1990). Evidence produced from kinetic and thermodynamic studies performed by Stallcup, *et.al.*; and Erecinska, *et.al.*, indicate a stoichiometry of 2 Na^+ : 1 glutamate is favored (Stallcup, *et.al.*, 1979; Erecinska, *et.al.*, 1983). If a OH^-

Figure 2. Model of High Affinity Na^+ -Dependent Glutamate Transport.



The above schematic represents the uptake of glutamate by the high-affinity Na^+ -dependent glutamate transporter (C) beginning with the binding of Na^+ (1-2), followed by the binding of glutamate (3), the internalization of the transporter (3-4), the release of glutamate into the extracellular environment (4-5), and the return of the transporter to the extracellular surface (5,6,7,8).

anion is countertransported out of the cell in addition to a K^+ cation, the stoichiometry is thought to be $2 Na^+ : 1 K^+ : 1$ glutamate (Bouvier, *et.al.*, 1992).

It should also be noted that while transport is generally thought of in terms of uptake, the action of the transporter protein is not unidirectional. Thus substrates can also be transported out of the cell depending upon ion and substrate gradients. Previous studies have demonstrated that the transporter can rapidly exchange an internal and external substrate without "net" transport taking place. If the two substrates are identical, this process is referred to as homoexchange; if not, this is referred to as heteroexchange (Christensen, 1975).

To characterize the specificity of the Na^+ -dependent transporter, large numbers of glutamate analogues have been screened for their ability to inhibit the uptake of radiolabeled substrates (*e.g.*, 3H -L-glutamate and 3H -D-aspartate) into synaptosomes, cultured astrocytes, and tissue slices (Robinson, *et.al.*, 1991; Chamberlin and Bridges, 1993). Generally, inhibitors possess the following characteristics: an α -amino acid with a second acidic group separated from the α -COOH by 2-4 methylene groups (Chamberlin and Bridges, 1993). Modification of the carbon backbone is tolerated to some degree as is substitution at the distal COOH group, which can be derivatized to a hydroxamate (*e.g.*, L-aspartate- β -hydroxamate) or replaced by a sulfonate group (*e.g.*, cysteic acid) (Curtis, *et.al.*, 1959). Studies have also found that this transporter is stereoselective, since D-glutamate is only a weak antagonist, while L- and D-aspartate are excellent substrates (Chamberlin and Bridges,

1993). The identification of transport inhibitors that are conformationally restricted analogues of L-glutamate (*e.g.*, L-trans-2,4-pyrrolidine dicarboxylate (L-trans-2,4-PDC), (2S,3R,4S)- α (carboxycyclopropyl) glycine (L-CCG-III) (Shinozaki, *et.al.*, 1989), and cis-1-aminocyclobutane-1,3-dicarboxylate (CACB) (Fletcher, *et.al.*, 1991)) has greatly added to the pharmacological characterization of this uptake system.

To date at least three glutamate transporters (GLAST (Stork, *et.al.*, 1992), GLT-1 (Pines, *et.al.*, 1992), and EAAC 1 (Kanai and Hediger, 1992)) have been cloned. Comparative analysis indicates that they: (i) are expressed in the brain; (ii) lack apparent signal sequences; (iii) contain carbohydrate moieties, and (iv) have molecular weights of about 57-64 kD. The first two are thought to be of glial origin, while the latter appears to be of neuronal origin (Kanai and Hediger, 1992). Recent studies have also identified a human counterpart for each of the clones (Arriza, *et.al.*, 1994).

While these transporters: (i) demonstrate a strong Na⁺-dependency; (ii) are enantioselective; (iii) are inhibited by uptake blockers (*i.e.*, dihydrokainate, β -threo-hydroxy-aspartate, and L-trans-2,4-PDC); and (iv) share about 50% homology among themselves; they exhibit little homology with other eukaryotic proteins (*i.e.*, the superfamily of GABA, noradrenaline, serotonin, dopamine, glycine, and choline transmitters) (Uhl, 1992). Studies indicate that these transporters are related to the proton-coupled glutamate transporters found in *E. coli* and other bacteria (glt-P (Tolner, *et.al.*, 1992)) and the dicarboxylic transporter (dct-A (Jiang, *et.al.*, 1989)) found in *Rhizobium*

meliloti.

While the exact role of transport in signal termination is debated (Nicholls and Attwell, 1990), it is generally accepted that transporters play a critical role in regulating the extracellular level of glutamate in the CNS (Nicoll, *et.al.*, 1988; Clements, *et.al.*, 1992; Isaacson and Nicoll, 1993). Failure to remove glutamate from the synaptic cleft leads to the accumulation of glutamate to toxic levels which cause overactivation of the EAA receptors (McBean and Roberts, 1985). Changes in transport activity may also be associated with neurodegenerative disease. Thus, while the affinity for glutamate appears to be unchanged in brain tissue isolated from amyotrophic lateral sclerosis (ALS) and Alzheimer's patients, studies suggest that the velocity of glutamate transport decreases in ALS patients (Rothstein, *et.al.*, 1992) and that the number of transport sites decreases in patients with Alzheimer's disease (Schwarcz and Meldrum, 1985; Rothstein, *et.al.*, 1992).

IV. EXCITOTOXICITY

In parallel with the characterization of glutamate as a neurotransmitter, studies have also demonstrated that ionotropic EAA agonists, are potent neurotoxins (Choi, 1992; Rothstein, *et.al.*, 1992). Accumulating evidence suggests this toxicity is mediated through overactivation of EAA receptors and is referred to as excitotoxicity (Choi, 1992; Rothstein, *et.al.*, 1992). Areas of the brain containing high levels of glutamate receptors, such as the hippocampus, are most sensitive to excitotoxic damage

(Cotman, *et.al.*, 1987; Meldrum and Garthwaite, 1990; Lodge and Collingridge, 1991). Furthermore, *in vitro* and *in vivo* studies have demonstrated that excitotoxic neural damage can be reduced by EAA receptor antagonists (Schwarcz and Meldrum, 1985; Willis, *et.al.*, 1993). Similarities between EAA mediated neuronal injury and the pathology associated with a wide range of neurological disorders, such as ischemia, amyotrophic lateral sclerosis, Huntington's disease, and Alzheimer's disease, suggest that excitotoxicity is a significant pathological mechanism in the CNS (Olney, *et.al.*, 1976; Nunn, *et.al.*, 1987; Rothman and Olney, 1987; Spencer, *et.al.*, 1987; Choi, 1988; Meldrum and Garthwaite, 1990).

In the neurological disorders discussed above, the excitotoxic pathology is attributed to the action of endogenous glutamate. However, a number of amino acids found in the environment have also been found to produce excitotoxic damage when consumed by humans (Nunn, *et.al.*, 1987; Stewart, *et.al.*, 1990). For example β -N-oxalyl-L- α , β -diaminopropionic acid (β -ODAP) which is present in the chickling pea, *Lathyrus sativus*, produces a permanent spastic paralysis of the legs referred to as neurolathyrism (Spencer and Schaumburg, 1983; Nunn, *et.al.*, 1987; Spencer, *et.al.* 1987; Bridges, *et.al.*, 1989; Weiss, *et.al.*, 1989b). Domoic acid, which originates in the plankton that blue mussels feed upon, produces varying degrees of temporal lobe damage with amnesia when ingested by humans (Stewart, *et.al.*, 1990; Sutherland, *et.al.*, 1990). These compounds, having similar structures to glutamate (two carboxyl groups and an amino group separated by 2-3 methylene groups),

have been shown electrophysiologically to act as agonists and biochemically to bind to the EAA receptors (Chamberlin and Bridges, 1993). Studies with neuronal cultures have shown these exogenous EAAs to be potent neurotoxins. Importantly, this toxicity can be blocked by EAA antagonists (Stewart, *et.al.*, 1990; Willis, *et.al.*, 1993). It has been further suggested that a contributing factor to excitotoxic properties may reside in the fact that these agonists are not substrates of the glutamate transporter, consequently making it difficult to remove these agents from the extracellular environment around the receptors. Not surprisingly these compounds have become useful tools for studying the EAA transmitter system, the mechanisms of neurotoxicity, and several neurodegenerative diseases.

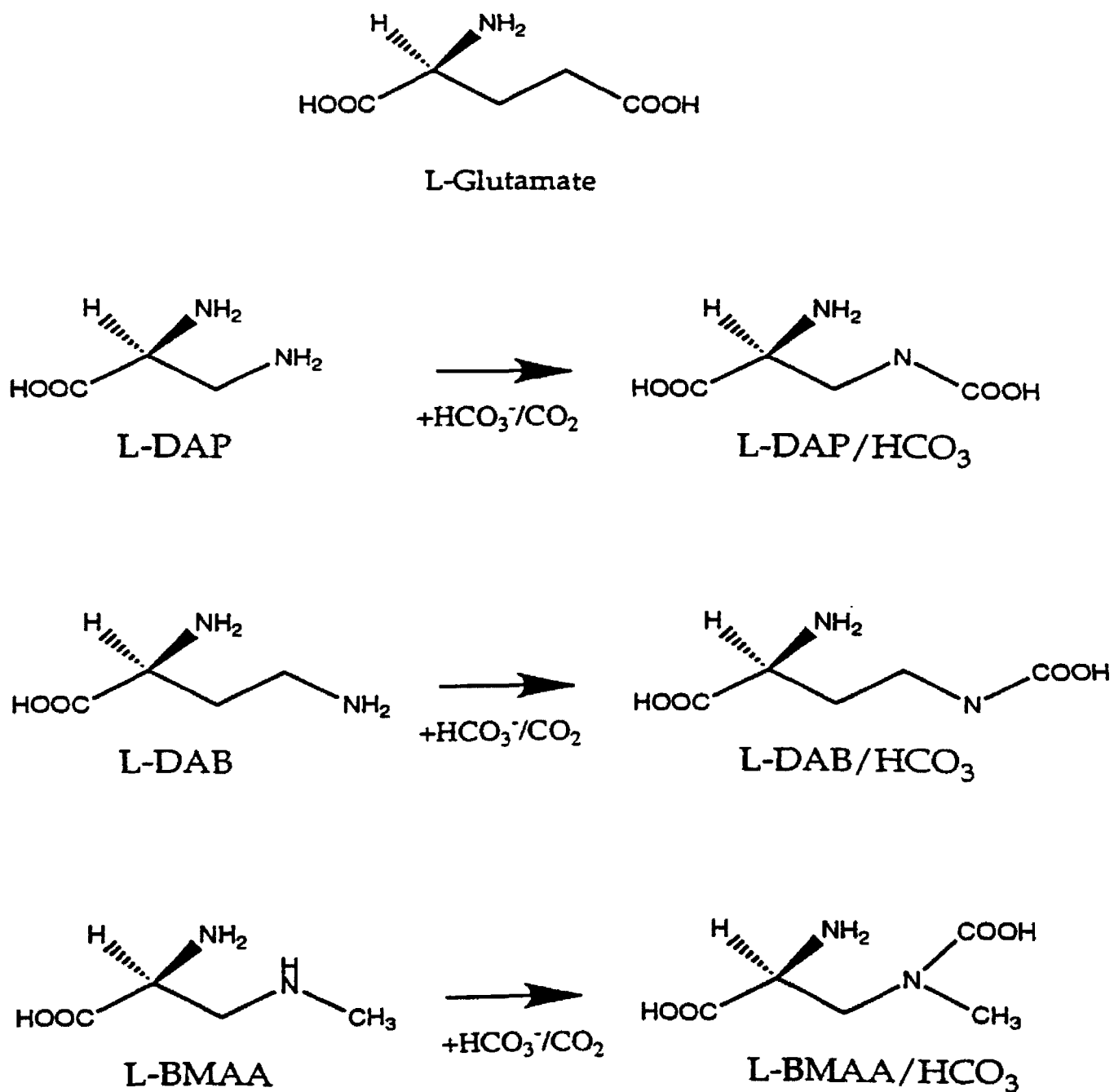
V. CARBAMATES

Interestingly, a number of compounds (*e.g.*, β -N-methylamino-L-alanine (BMAA) and L-cysteine) have recently been identified that produce excitotoxic damage, yet are structurally dissimilar to glutamate (*i.e.*, they possess only 1 carboxyl group) (Weiss and Choi, 1988; Weiss, *et.al.*, 1989a, 1989b; Olney, *et.al.*, 1990; Copani, *et.al.*, 1991; Kisby, *et.al.*, 1992; Lehmann, *et.al.*, 1993). Progress in this apparent inconsistency came from the observation that the neurotoxic action of these compounds was dependent on the presence of bicarbonate (Weiss and Choi, 1988; Davis, *et.al.*, 1991). Thus, while the amino acids alone produced little excitotoxic damage, they became toxic when combined with bicarbonate (Weiss and Choi, 1988; Duncan, *et.al.*,

1990). Consistent with the action at EAA receptors, this toxicity can be inhibited by glutamate antagonists. While it has been suggested that bicarbonate may modify the glutamate receptors, studies demonstrate that this event is unlikely since the single-channel conductance activated by BMAA with bicarbonate is the same as that activated by NMDA or glutamate (Weiss and Choi, 1988; Weiss, *et.al.*, 1989). In light of these observations, it was hypothesized that when carbon dioxide adds to a free amine group on a fraction of the amino acids it forms a new compound, a carbamate, which more closely resembles classic EAA agonists (Figure 3). Thus, it is thought to be the carbamate which is the true EAA agonist and the mediator of the neuronal injury induced by these unusual amino acids (Weiss and Choi, 1988; Davis, *et.al.*, 1991).

Carbamate formation has been studied using NMR and reverse liquid phase chromatography (RPLC). In NMR studies on L-BMAA, DL- α,β -diaminopropionic acid (DL-DAP), and DL- α,γ -diaminobutyric acid (DL-DAB) in the presence and absence of hydrogencarbonate, carbamate peaks appeared between 50 and 60 ppm in all of the spectra (Davis, *et.al.*, 1991). Although carbamates can be envisioned to form at either of the amine groups on DAP and DAB, the greatest structural similarity to glutamate is found when a carbamate is formed on the side chain amine group of these compounds (see Figure 3) (Weiss, *et.al.*, 1989a; Davis, *et.al.*, 1991). Related studies using RPLC have been carried out to determine carbamate formation constants and mole fractions of carbamates formed from 11 L- α -amino acids and peptides (Chen,

Figure 3. Amino Acids (L-DAP, L-DAB, and BMAA) and Their Carbamate Forms Compared to the Structure of Glutamate.



et.al, 1993). Even though no direct relationship between the formation constant and the amine pKa has been found, the authors observed a correlation between the amine pKa and the percentage of carbamate formed at a given pH (Nunn and O'Brien, 1989; Chen, *et.al*, 1993). In using the equilibrium constant extrapolated to a physiological value for the bicarbonate concentration (\cong 25 mM), it is suggested that 26% carbamate formation is seen at pH=7.4 for the compound BMAA (Nunn and O'Brien, 1989). In similar studies, it has been suggested that significant amounts of carbamates do not form on α -amines of amino acids with pKa values greater than 9.5 in physiological conditions (Chen, *et.al*, 1993). Taken together, these data suggest that carbamates can form on distal amine groups of selected amino acids at physiologically significant levels and support their potential contribution to receptor activation and excitotoxicity (Nunn and O'Brien, 1989; Nunn, *et.al.*, 1993).

While studies of carbamates have focused primarily on their toxic effects or their actions as EAA agonists, little is known about their potential action at the glutamate transporters. Such an activity is particularly important because glutamate uptake plays an important role in regulating EAA levels and preventing excitotoxic damage (Rosenberg and Aizenman, 1989; Robinson, *et.al.*, 1991; Rosenberg, *et.al*, 1992; Rothstein, *et.al.*, 1992). If carbamates inhibit the transporter, they could potentially lead to the extracellular accumulation of excitotoxic levels of glutamate. Beyond their potential action as glutamate uptake inhibitors, it is also important to

determine if carbamates are substrates of the transporter. Specifically, this will answer the question as to whether or not carbamates can be removed from the extracellular space by uptake and influence the amount of time to which receptors are exposed to these excitotoxins. Thus, a better understanding of the interactions between carbamates and the glutamate transport system will provide insight into the toxic mechanisms of carbamate forming amino acids as well as into transporter pharmacology.

VI. SPECIFIC OBJECTIVES

The overall goal of this project was to investigate the ability of amino acid carbamates to inhibit and serve as substrates of the high affinity, Na⁺-dependent glutamate uptake system found on synaptosomes prepared from rat forebrain. This was carried out by the following objectives:

- A. To determine if *in vitro* conditions favorable to carbamate formation are also compatible with transporter function.
- B. To assay amino acids of interest in the presence and absence of sodium bicarbonate for their ability to inhibit uptake of ³H-D-aspartate into rat brain synaptosomes.
- C. To kinetically characterize those compounds that form amino acid carbamates capable of inhibiting the synaptosome transport system.
- D. To use the process of heteroexchange to determine if those amino acid carbamates that inhibit uptake can also act as substrates for the transporter.

MATERIALS AND METHODS

Male Sprague-Dawley rats (160-200 gms) were obtained from Simonsen Labs, Inc. (California) and were tested according to AALAL approved methods. $^3\text{H-D-Aspartate}$ (28 Ci/mmol) was obtained from New England Nuclear (Boston, MA). All chemicals were obtained from Sigma (St. Louis, MO), except for D-DAP, L-DAP, D-DAB, and L-DAB which were given to the lab by Professor Peter Nunn (King's College). BMAA was purchased from Cambridge Research Biochemicals, Ltd. (England). Liquiscint was obtained from National Diagnostics (Atlanta, Georgia). BCA Protein Assay Reagents were obtained from Pierce (Rockford, Illinois). Ficoll was used from Lot 34H7100. Whatman GF/F filters were used.

Synaptosomal Preparation. Synaptosomes were prepared as described by Booth and Clark using a Ficoll/sucrose gradient configuration (Booth and Clark, 1978). Two male Sprague-Dawley rats (160-200 gms) were decapitated, their forebrains were rapidly removed, and the brain tissue was placed in approximately 20 mls of homogenization buffer (0.32 mM sucrose and 10 mM Tris-acetate, pH 7.4). The buffer was decanted and 15 mls of fresh homogenization buffer were added. The brain tissue was then cut into small pieces with scissors, homogenized in a 15 ml Wheaton Glass/Glass Homogenizer (12 strokes), and suspended in approximately 40 mls of homogenization buffer. After being centrifuged for 3 minutes at 3,700 RPM

(1,636 x g), the supernatant was collected and re-centrifuged for 10 minutes at 13,700 RPM (22,433 x g). The supernatant was then decanted and the remaining membrane material suspended in 10 mls of 12% Ficoll. This mixture was homogenized in a 10 ml B. Braun Melsungen AG Homogenizer and used to form the bottom layer of a discontinuous gradient. 10 mls of 7.5% Ficoll was then gently layered onto the homogenate. Finally, a third layer consisting of 10 mls of homogenization buffer was added. The gradient was centrifuged for 30 minutes at 27,800 RPM (138,957 x g). Using a Pasteur pipette, the synaptosome layer, which had formed between the 12% and 7.5% Ficoll layers, was removed, homogenized in 10 mls of homogenization buffer, and resuspended to approximately 25 mls. Following centrifugation for 20 minutes at 19,000 RPM (43,146 x g), the supernatant was decanted, the tube was dried with a Kimwipe, and the remaining pellet weighed. The wet weight of the pellet (mg) was divided by 5 and multiplied by 1 ml/mg to give a resuspension volume for uptake assays while the mg pellet weight was divided by 27 and multiplied by 1 ml/mg to give a resuspension volume for exchange assays. The pellet was then homogenized in a 15 ml Wheaton Homogenizer and suspended in the appropriate volume of HEPES/Control Buffer (10 mM D-glucose, 128.5 mM NaCl, 5 mM KCl, 1.5 mM NaH₂PO₄, 1 mM MgSO₄, 10 mM Tris, 1 mM CaCl₂, 50 mM HEPES), pH of 7.4. At this point in both uptake and exchange assays, an aliquot (\cong 1 ml) of the suspension was removed for subsequent protein analysis.

Synaptosomal Screening and Kinetics. The uptake of $^3\text{H-D}$ -aspartate was quantified using the method of Kuhar and Zarbin (Kuhar and Zarbin, 1987) as described by Bridges (Bridges, *et.al.*, 1991). Synaptosomes were prepared, suspended in HEPES/Control Buffer, and stored on ice as previously described. All buffers were bubbled under N_2 gas for 15 minutes prior to being used in assays. Aliquots (100 μL) of the suspended synaptosomes were pipetted into ice cold test tubes (16 x 125 mm). Following a 5 minute temperature preincubation at 25°C , the assay was initiated by adding a 100 μL aliquot containing $^3\text{H-D}$ -aspartate (2.5 μM , \cong 125,000 cpm) with potential inhibitors in HEPES/Control Buffer, also preincubated to 25°C . The reaction was allowed to proceed for 2 minutes, after which 5 mls of ice cold HEPES/Control Buffer were added to the test tubes to stop the reaction. The solutions were rapidly filtered (Whatman GF/F filters 2.5 cm) and rinsed with an additional 5 mls of HEPES/Control Buffer. The filters were dried for 2 minutes, placed into scintillation vials, and submerged in 4 mls of scintillation fluid (Liquiscint, National Diagnostics). Radioactivity retained on the filter was quantified in a Beckman LS6500. All values reported have been corrected for background and non-specific uptake, as determined by following uptake at 4°C . Lineweaver-Burk plots and associated kinetic analysis of the transport inhibitors were carried out using k•cat kinetic program (Biometallics Inc.) with weighting based on constant relative error.

K_T values were estimated on the basis of a replot of K_m , app values.

Synaptosomal Exchange. Synaptosomes were loaded with $^3\text{H-D}$ -aspartate essentially as described above, (V_{TOT} 10 ml, 1 mg/ml, 15 minutes at 25°C). After reisolating the synaptosomes by centrifugation, the pellet was rinsed and resuspended in HEPES/Control Buffer (e.g., 2 mg wet pellet wt./ml) at 4°C . At this point in time and throughout the assay, aliquots were removed to quantify the synaptosomal content of $^3\text{H-D}$ -aspartate. The exchange process was initiated by adding 100 μl of this suspension to 2.9 mls of HEPES/Control Buffer containing variable amounts of amino acids in the absence and presence of added bicarbonate. The samples were allowed to incubate for 2 minutes at 37°C , after which they were rapidly filtered (Whatman GF/F 2.5 cm), and then rinsed twice with 4 ml aliquots of cold HEPES/Control Buffer. The radioactivity remaining in the synaptosomes retained on the filter was quantified by liquid scintillation counting as previously described.

Bicinchoninic Acid (BCA) Protein Assay. The Pierce BCA Protein Assay was used to determine the protein contents in all experiments. This assay was chosen because of its tolerance for high lipid concentrations. Standard curves were prepared with BSA (Sigma) and assays carried out as described by Smith (Smith, *et.al.*, 1985). Spectrophotometric measurements

were made on a Beckman DU 650. Standards ranged from concentrations of 0-5.5 mg/ml (0-15 mg/ml*), contained 100 μ l of HEPES/Control Buffer (50 μ l*), and were spended to 200 μ l volumes with nanopure water. (*These values were used when synaptosomal samples prepared for exchange assays were being measured.)

Statistical Analysis. Statistical analysis was carred out with the InStat Software (Graph Pad Software). Groups were compared and p values (two tailed) calculated using an alternate Welch t test with unpaired values. The Alternate Welch t test is comparable to a student t test in which the Gaussian population of the two groups have unequal S.D.

RESULTS

Effects of sodium bicarbonate on the pH of control buffer and HEPES control buffer.

The overall goal of this project was to investigate the ability of carbamates to inhibit and serve as substrates of the glutamate uptake system. Prior to assessing the activity of carbamates to block uptake, conditions conducive to both carbamate formation and transport ability were identified. The standard control buffer used for transport assays had a pH of 7.4 and contained D-glucose, sodium chloride, potassium chloride, sodium phosphate (monobasic), magnesium sulfate, tris, and calcium chloride (anhydrous) as described in the materials and methods. However; to examine the activity of carbamates as potential inhibitors of the transport system, the assays needed to be carried out in the presence of added NaHCO_3 . Table 1 shows the effects of added NaHCO_3 (10, 25, and 50 mM) on the pH of control buffer. Increases in pH were observed at each NaHCO_3 concentration, particularly at the physiological relevant level of 25 mM. To avoid the interpretational complications associated with such pH changes, 50 mM HEPES was included in the control buffer to increase buffer capacity. As shown in Table 1, the pH of HEPES/Control Buffer was maintained close to 7.4 for NaHCO_3 concentrations of up to 50 mM.

Uptake of $^3\text{H-D-Aspartate}$ into rat forebrain synaptosomes in HEPES/Control Buffer and N_2 saturated HEPES control buffer.

In addition to maintaining pH, it was also important to ensure that the 50 mM HEPES/Control Buffer was as free as possible of carbon dioxide prior to the preparation of drug solutions. Thus, nitrogen gas was bubbled through the HEPES/Control Buffer to provide a carbonate free solution. Upon doing so, it also was necessary to ensure that the HEPES/Control Buffer, by itself, or with N_2 saturation did not affect synaptosomal transporter activity. Thus, uptake assays were carried out with each of the buffer conditions. The results, which are depicted in Table 2, verify that neither the HEPES nor N_2 saturation significantly ($P>0.05$) altered the uptake rates of $^3\text{H-D-Aspartate}$ (5 μM) into rat forebrain synaptosomes. In view of these results, it was decided that N_2 saturated HEPES/Control Buffer was to be used in all subsequent experiments.

Effects of bicarbonate concentrations on uptake of $^3\text{H-D-aspartate}$ into rat forebrain synaptosomes.

Prior to assessing the action of carbamate forming amino acids on the activity of the glutamate transporter, experiments were first carried out to evaluate the potential effect of NaHCO_3 itself. Table 3 shows that about 10% inhibition was observed at the high concentrations of NaHCO_3 (20 and 25 mM). As 20

mM approximates the physiological concentration of NaHCO_3 , it was chosen for the subsequent experiments.

Uptake of $^3\text{H-D-aspartate}$ by synaptosomes in the solutions containing various amino acids in the presence and absence of sodium bicarbonate (20 mM).

A series of amino acids were chosen on the basis of previously reported excitotoxic activity and structural similarities of their corresponding carbamates to glutamate. These compounds were initially tested for their ability to inhibit the uptake of $^3\text{H-D-aspartate}$ ($5\ \mu\text{M}$) at a concentration of 1 mM in the presence and absence of NaHCO_3 (20 mM). Those compounds which produced a significant level of inhibition were also evaluated at 100 μM . The results of these experiments are shown in Table 4. In the absence of added NaHCO_3 , L-BMAA produced only about 10% reduction in the uptake of $^3\text{H-D-aspartate}$. When NaHCO_3 was added with BMAA, the rate of transport was reduced by another 10%, comparable to what is produced by the addition of NaHCO_3 alone. These results indicate that BMAA is a very weak inhibitor of the high affinity Na^+ -dependent uptake system and that its corresponding carbamate is inactive. In contrast, L-DAP inhibited transport activity in both the absence and presence of added NaHCO_3 . Indeed, $^3\text{H-D-aspartate}$ uptake was totally blocked by 1000 μM L-DAP/+ NaHCO_3 and inhibited 83% at 100 μM L-DAP/+ NaHCO_3 . In this regard, L-DAP carbamate

appeared to be one of the most potent inhibitors identified in these assays. Studies with D-DAP allowed the stereoselectivity of this activity to be evaluated. While at comparable doses, both in the absence and presence of NaHCO_3 , the D-enantiomer was considerably less potent than L-DAP; some inhibition was observed. Thus, the increased inhibition produced by the inclusion of NaHCO_3 with D-DAP (89 to 38%), suggested that the D-enantiomer of the corresponding carbamate can be accommodated in the transporter binding site. This is not a surprise, as D-aspartate is known to be an excellent substrate.

L- and D-DAB are the one carbon homologues of L and D-DAP. When added alone, L- and D-DAB did not significantly ($P > 0.05$) inhibit the uptake system at 1 mM. However; in the presence of NaHCO_3 , a small degree of inhibition was observed with L and D-DAB, 18% and 15% respectively, although the values were not statistically significant ($P > 0.05$). This level of inhibition was difficult to distinguish from the inhibition produced by NaHCO_3 alone. The loss of activity observed with these increases in carbon backbone length is consistent with the loss of activity observed in experiments comparing glutamate to α -amino adipic acid, which is inactive as an inhibitor.

The amino acid L-cysteine was also included in these tests, as it has exhibited CO_2 -dependent excitotoxicity (Olney, *et.al.*, 1990). While the inhibition produced by cysteine was not that large (*i.e.*, 18% at 1000 μM), it did

appear to increase upon the addition of NaHCO_3 (*i.e.*, 42% at 1000 μM). Once again this 10% difference is most likely attributable to NaHCO_3 . In evaluating the actions of cysteine, it is important to take into consideration its possible oxidation to cystine, a dicarboxylic compound that more closely resembles the structure of glutamate. Uptake experiments examining the effect of 100 μM L-cystine (data not included in Table 4) demonstrated that it inhibits transport of ^3H -D-aspartate (5 μM) in rat brain synaptosomes by about 49%. In light of this finding, it is difficult to resolve any inhibition observed with L-cysteine (in the presence or absence of NaHCO_3) from that which might be attributable to L-cystine. Replacing the SH group of cysteine with an OH moiety yields the amino acid serine. As shown in Table 4, L-serine was also a very weak inhibitor in either the presence or absence of added NaHCO_3 .

Kinetic analysis of compounds demonstrating inhibition.

The amino acids and their respective carbamates that were identified as inhibitors in the initial assays were kinetically analyzed in greater detail. Lineweaver-Burk plots are shown for the inhibition produced by D-DAP/+ NaHCO_3 , L-DAP, and L-DAP/+ NaHCO_3 (Figure 4, 5, and 6). All three of these compounds produced patterns consistent with competitive inhibitors (*i.e.*, change in $K_{m,app}$ with no change in V_{max}). K_I values were estimated by replotting $K_{m,app}$ values determined in the Lineweaver-Burk plots vs. inhibitor concentrations. The replots are shown as insets within Figures 4-6.

The K_I value shown in the Figures are representative of that individual experiment, while a summary of all of the kinetic data is reported in Table 5. L-DAP yields a $K_{I,app}$ of 171 μM in the absence of added NaHCO_3 . The $K_{I,app}$ decreased by 50-fold (*e.g.*, $3.4 \pm 1 \mu\text{M}$) when NaHCO_3 was included in the assay. This value is quite similar to the K_I value determined for the endogenous substrate L-glutamate (4.9 ± 2.6), identifying the carbamate as a potent inhibitor. Although D-DAP was much less potent than L-DAP, a dramatic decrease in the apparent K_I as also observed in the presence of added NaHCO_3 (*e.g.*, 1000 μM to 268 μM). It should also be kept in mind that the $K_{I,app}$ values reported in Table 5 for the carbamates are overestimations, as they are based on the total amino acid concentration and not that fraction of the amino acid that is in carbamate form. Thus, in the instance of BMAA, NMR studies suggest a more appropriate value would be 26% of the value reported in Table 5 (Nunn and O'Brien, 1989). In light of these corrections, L-DAP/+ NaHCO_3 appears to be a very potent inhibitor.

Exodus of ^3H -D-aspartate from synaptosomes in the presence of amino acids and carbamates.

In addition to determining if carbamates could inhibit the high-affinity Na^+ -dependent transporter, we also wanted to assess whether or not carbamates could also serve as substrates. While this could be accomplished with radiolabeled compounds, expense and availability made this approach

prohibitive. As an alternative, we have found that heteroexchange can be used to identify potential substrates. Previous studies with synaptosomes have demonstrated that the addition of an external substrate can stimulate the rate of exodus of another substrate (radiolabeled) previously loaded into the synaptosomes. In order for a compound to stimulate the rate of exodus, it must be able to exchange with an internal compound and therefore must also be a substrate. Thus, a series of amino acid and amino acid carbamates were tested to determine if they alter the rate of exodus of $^3\text{H-D-aspartate}$ previously loaded into synaptosomes.

Technically these experiments are more difficult as they are comprised of two phases. First synaptosomes need to be filled with $^3\text{H-D-aspartate}$ (2.5 μM , 15 minutes, 25° C), reisolated, and then assayed for rates of exodus. To assure that appropriate comparisons are made, the synaptosomes must be filled with similar amounts of $^3\text{H-D-aspartate}$ as internal levels could be easily envisioned to effect rates of exodus. In the preceding experiments the level of $^3\text{H-D-aspartate}$ in the loaded synaptosomes was 1485 ± 44 (mean \pm SEM, $n=19$) pmol/mg protein. Experiments that fell well outside of a set range (1100-1750 pmol/mg protein) were not included. The exodus of $^3\text{H-D-aspartate}$ was followed at 37° C. Importantly, little or no exodus was observed during the course of individual assays if the synaptosomes were maintained at 4° C. Control values reported in Table 6 represent the exodus of $^3\text{H-D-aspartate}$ from the synaptosomes at 37° C in the absence of external amino acids. Data

in Figures 7 and 8 illustrates the time course of the exodus of $^3\text{H-D-aspartate}$ from the synaptosomes. Values are reported as % of the initial $^3\text{H-D-aspartate}$ content of the synaptosomes. In the absence of added compound, (Control curves, Figures 7 and 8) only about 10% of the $^3\text{H-D-aspartate}$ in the synaptosomes was released into the media during the time course of the experiment. However; when $10\ \mu\text{M}$ L-glutamate was present in the assay buffer, the level of exodus increased to about 50% of the loaded $^3\text{H-D-aspartate}$ remaining in the synaptosomes. This time course demonstrates the process of heteroexchange, as glutamate acts as a substrate of the transporter and is exchanged for the $^3\text{H-D-aspartate}$ in the synaptosomes. A different result is found when DHK, a nontransportable inhibitor, is included in the assay buffer. In contrast to glutamate, the rate of exodus does not change from control values. Furthermore, when DHK was included in the assay with glutamate, the rate of exodus was not stimulated beyond control values, consistent with DHK binding to the transporter and preventing glutamate exchange.

Figure 8 shows the results of heteroexchange assays with L-DAP and L-DAP/+NaHCO₃. The synaptosomal content of $^3\text{H-D-aspartate}$ was determined 1, 2, 4, and 6 minute after the addition of the potential substrates ($10\ \mu\text{M}$ L-DAP, $10\ \mu\text{M}$ L-DAP/+NaHCO₃, and $10\ \mu\text{M}$ L-DAP/+NaHCO₃/DHK). L-DAP/+NaHCO₃ displayed a similar pattern to that of glutamate, suggesting

that the carbamate is also a substrate. When the NaHCO_3 was not included in the assay, the exodus of $^3\text{H-D-aspartate}$ returned to control levels. Similar to its effect on glutamate, DHK inhibited the DAP/+ NaHCO_3 stimulation of $^3\text{H-D-aspartate}$ exodus. It should also be noted that solutions of NaHCO_3 alone did not stimulate exchange and produced data resembling control values.

Table 6 summarizes the ability of a number of amino acids to stimulate the efflux of $^3\text{H-D-aspartate}$ from the synaptosomes. The data are reported as % control \pm SEM as determined at 2 minutes. L-Glutamate and L-DAP/+ NaHCO_3 were the only compounds tested under these conditions that significantly ($P < 0.01$) increased the exodus of $^3\text{H-D-aspartate}$ from synaptosomes. As was observed in the time course experiment, including DHK with the compounds significantly ($P < 0.01$) reduced the level of exchange. It is important to note that direct comparisons of potential substrates at similar concentrations, (Table 6) fail to take into account variations in binding affinities. For example, a compound may be a substrate, but would not exchange because its concentration is insufficient to produce the necessary level of binding. Thus, to study the compounds on a more equal basis, each was added to assays at substrate concentrations equal to about 3X its K_I value (Table 7). Studied in this manner, the addition of 500 μM L-DAP stimulated the exodus of $^3\text{H-D-aspartate}$ similar to that produced by 10 μM L-DAP in the presence of NaHCO_3 . Studies with increased amounts of D-

DAP/+NaHCO₃ (800 μM) also demonstrated that it could participate in heteroexchange. Taken together these results include that both the L and D carbamates of DAP are substrates of the high-affinity Na⁺-dependent glutamate transporter.

Table 1. Effect of Added NaHCO₃ on the pH of Control Buffer and HEPES/Control Buffer.

NaHCO ₃ Concentration (mM)	Vol. (mls)	Temperature (°C)	Control Buffer pH	50 mM HEPES Control Buffer pH
0	50	20±1	7.37±0.06 (4)	7.36±0.08 (4)
10	50	20±1	7.51±0.06 (4)	7.38±0.08 (4)
25	50	20±1	7.64±0.07 (4)	7.41±0.07 (4)
50	50	20±1	7.77±0.09 (4)	7.45±0.05 (4)

The concentrations of sodium bicarbonate listed were evaluated for the effects of pH on control buffer with and without 50 mM HEPES. Values reported represent the mean±S.D. (n=number of times experiment was performed).

Table 2. Effect of HEPES/Control Buffer and N₂ on the Rate of Synaptosomal Uptake of ³H-D-Aspartate.

Control Buffer	Uptake Rate of ³ H-D-Aspartate (5μM) % of Control
Control Buffer alone	100±7 (4)
HEPES (50 mM)	111±8 (3)
N ₂ saturated HEPES (50 mM)	108±14 (4)

The buffers were screened for effects on the uptake of ³H-D-aspartate into rat forebrain synaptosomes. The control rate of synaptosomal uptake was 1.37±0.12 nmol/min/mg. Values were corrected for background and leakage. Data is presented as means±S.D. (n=number of duplicate assays).

Table 3. Effect of NaHCO₃ on Synaptosomal Uptake of ³H-D-Aspartate .

NaHCO ₃ Concentration (mM)	Uptake Rate of ³ H-D-Aspartate (5 μM) % of Control
0	100±8 (9)
10	95±10 (6)
15	91±8 (6)
20	*92±6 (6)
25	*89±8 (6)

Varying concentrations of compounds were analyzed for effects on ³H-D-aspartate uptake into forebrain synaptosomes. The control rate of uptake was 1.58±0.40 nmol/min/mg. Results are shown as means±S.D. (n=number of duplicate assays). Statistical comparisons were made as described in Methods (*P<0.05).

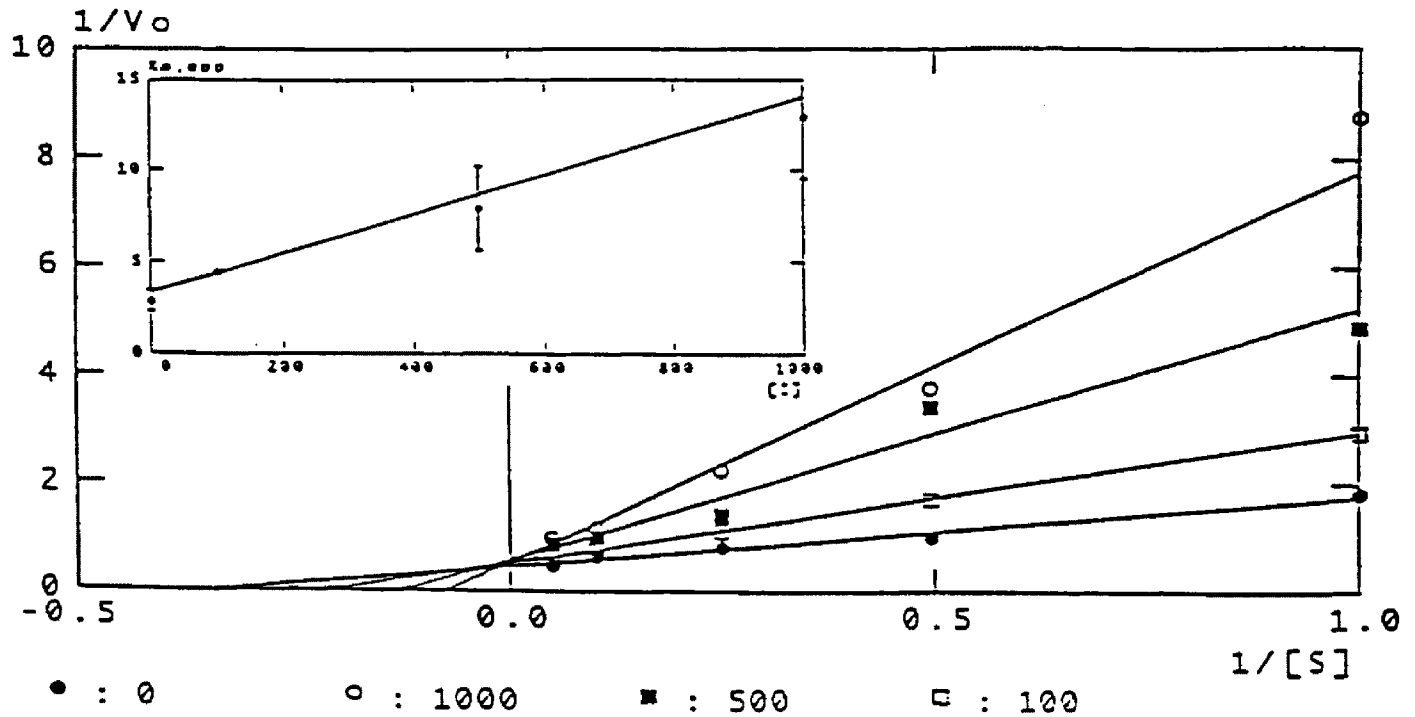
Table 4. Inhibition of $^3\text{H-D-Aspartate}$ Uptake: Structure Activity Study with Carbamate-Forming Amino Acids.

Compound (μM)	Uptake Rate of $^3\text{H-D-Aspartate}$ (5 μM) % of Control	
	Absence of NaHCO_3 (20mM)	Presence of NaHCO_3 (20mM)
Control	100 \pm 2 (40)	** 90 \pm 2 (11)
L-BMAA (1000 μM)	*** 89 \pm 1 (5)	** 79 \pm 2 (3)
(100 μM)	93 \pm 6 (4)	81 \pm 8 (4)
L-DAP (1000 μM)	*** 38 \pm 4 (3)	*** 0 \pm 0 (3)
(100 μM)	76 \pm 9 (4)	*** 17 \pm 2 (4)
D-DAP (1000 μM)	*** 79 \pm 2 (4)	*** 38 \pm 2 (6)
(100 μM)	*** 82 \pm 2 (5)	* 78 \pm 4 (9)
L-Cysteine (1000 μM)	* 72 \pm 7 (5)	** 58 \pm 8 (11)
(100 μM)	90 \pm 8 (4)	84 \pm 4 (10)
D-Cysteine (1000 μM)	96 \pm 9 (3)	86 \pm 2 (3)
L-DAB (1000 μM)	90 \pm 5 (3)	82 \pm 3 (3)
D-DAB (1000 μM)	96 \pm 7 (3)	85 \pm 6 (3)
L-Serine (1000 μM)	87 \pm 4 (3)	75 \pm 7 (3)
D-Serine (1000 μM)	98 \pm 6 (3)	80 \pm 5 (3)

The above compounds are amino acids which can potentially form carbamates in the presence of bicarbonate. These amino acids were tested for their ability to inhibit $^3\text{H-D-aspartate}$ uptake into forebrain synaptosomes in the presence and absence of added 20 mM sodium bicarbonate. The average control rate of uptake was 1.38 \pm 0 nmol/min/mg. The data are reported as %

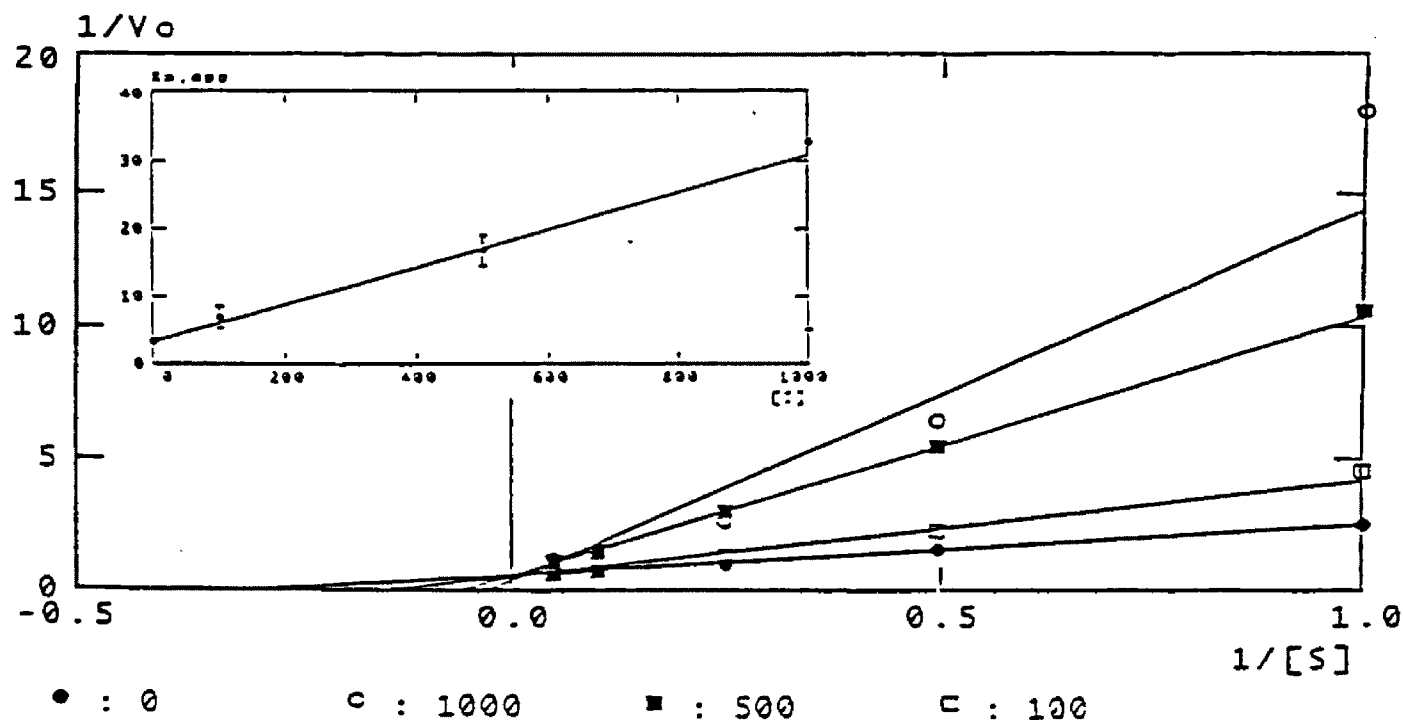
of the control \pm SEM (n=number of duplicate assays). Comparisons were made between experimental points and the appropriate control values, *i.e.*, in the absence (*) and presence (†) of added NaHCO₃. Levels of significance are denoted by *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Lineweaver-Burk Plot of the Inhibition of ^3H -D-Aspartate Uptake by D-DAP/+NaHCO₃ (20 mM).



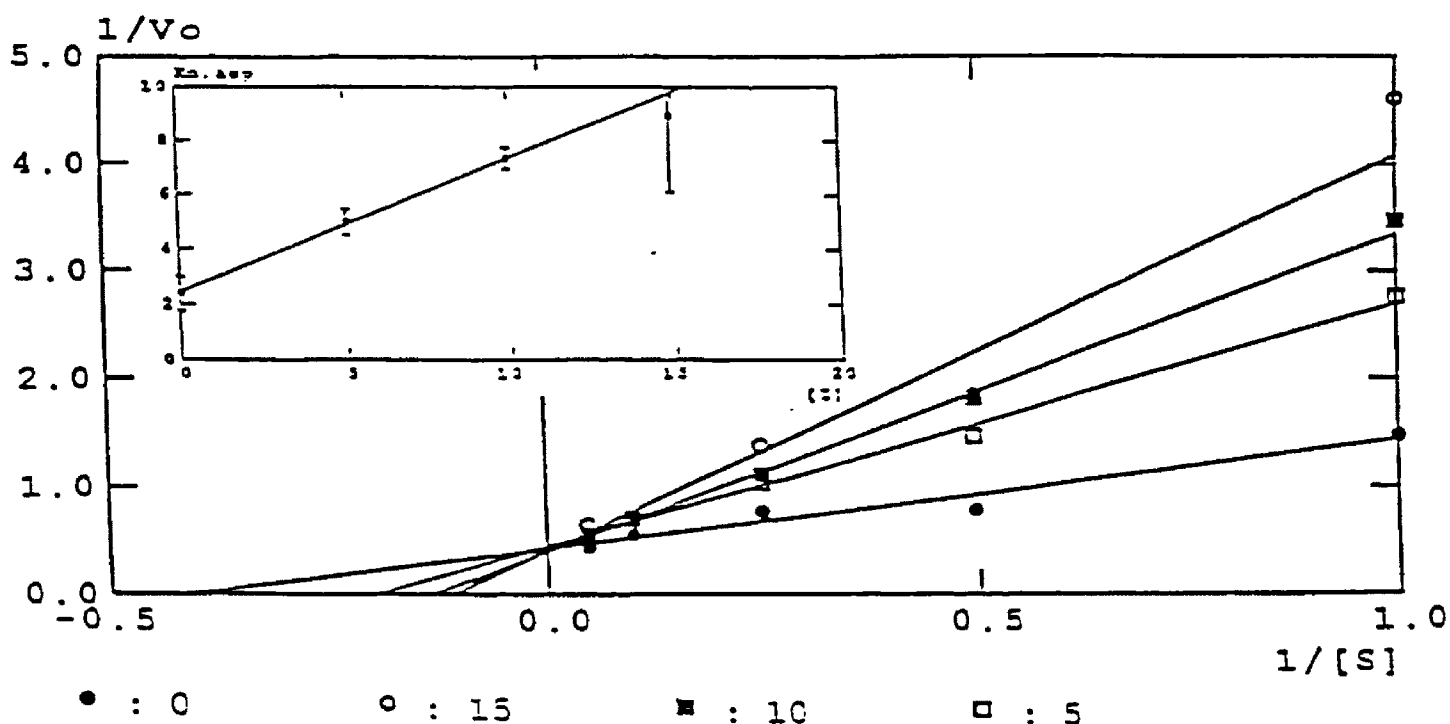
Competitive inhibition of ^3H -D-aspartate uptake into rat forebrain synaptosomes by D-DAP/+NaHCO₃ illustrated in a representative Lineweaver-Burk plot. Also shown is a replot of $K_{m, \text{app}}$ vs. D-DAP/+NaHCO₃. Values obtained from plots are: $K_m = 2.8 \mu\text{M}$ (D-aspartate), $K_I = 313 \pm 65 \mu\text{M}$, and a $V_{\text{max}} = 2.2 \text{ nmol/min/mg protein}$. Lineweaver-Burk plots and associated kinetic analysis of the transport inhibitors were carried out using k•cat kinetic program (Biometallics Inc.) with weighting based on constant relative error. K_I values were estimated on the basis of a replot of $K_{m, \text{app}}$ values.

Figure 5. Lineweaver-Burk Plot of the Inhibition of ^3H -D-Aspartate Uptake by L-DAP.



Competitive inhibition of ^3H -D-aspartate uptake into rat forebrain synaptosomes by L-DAP illustrated in a representative Lineweaver-Burk plot. Also shown is a replot of $K_{m, app}$ vs. L-DAP. Values obtained from plots are: $K_m = 3.3 \mu\text{M}$ (D-aspartate), $K_I = 120 \pm 7 \mu\text{M}$, and a $V_{max} = 1.8 \text{ nmol/min/mg protein}$. Lineweaver-Burk plots and associated kinetic analysis of the transport inhibitors were carried out using k•cat kinetic program (Biometallics Inc.) with weighting based on constant relative error. K_I values were estimated on the basis of a replot of $K_{m, app}$ values.

Figure 6. Lineweaver-Burk Plot of the Inhibition of $^3\text{H-D-Aspartate}$ Uptake by L-DAP/+NaHCO₃ (20 mM).



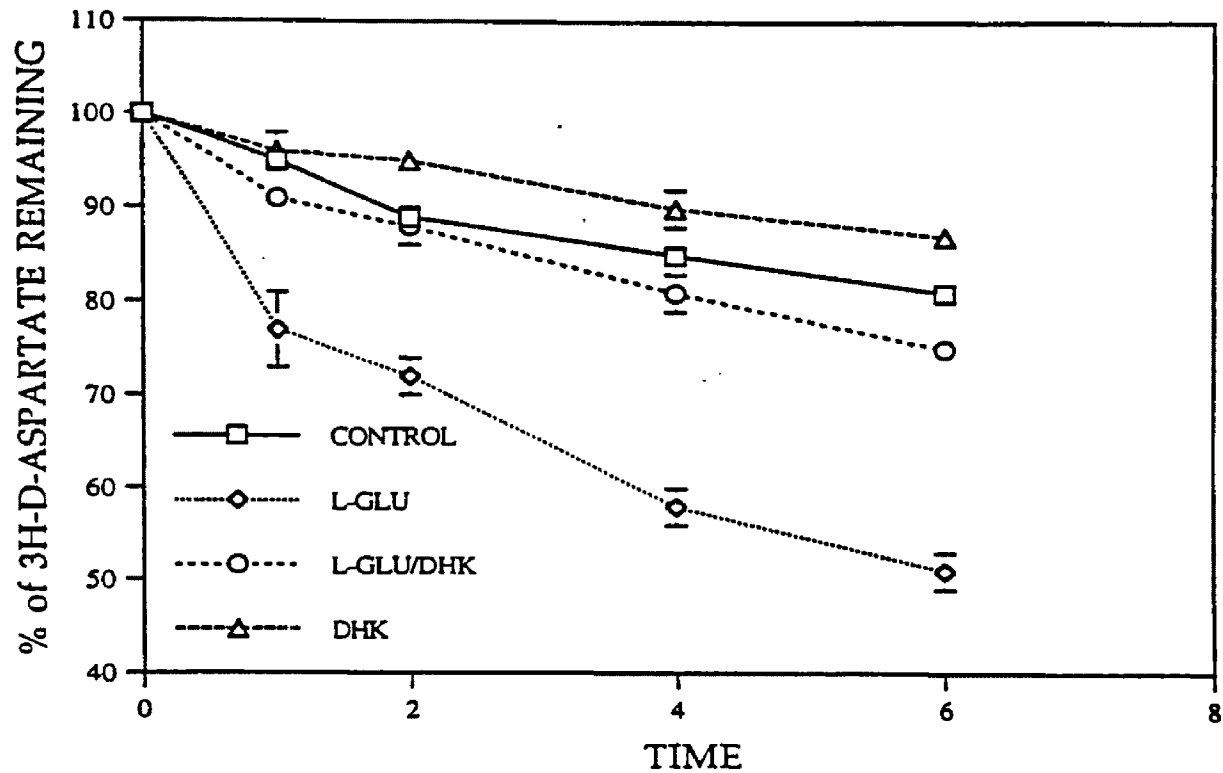
Competitive inhibition of $^3\text{H-D-aspartate}$ uptake into rat forebrain synaptosomes by L-DAP/+NaHCO₃ illustrated in a representative Lineweaver-Burk plot. Also shown is a replot of $K_{m,app}$ vs. L-DAP/+NaHCO₃. Values obtained from plots are: $K_m = 2.4 \mu\text{M}$ (D-aspartate), $K_I = 5.1 \pm 0.34 \mu\text{M}$, and a $V_{max} = 2.4 \text{ nmol/min/mg protein}$. Lineweaver-Burk plots and associated kinetic analysis of the transport inhibitors were carried out using k•cat kinetic program (Biometallics Inc.) with weighting based on constant relative error. K_I values were estimated on the basis of a replot of $K_{m,app}$ values.

Table 5. Summary of K_I Values for the Identified Transport Inhibitors.

Compound (μM)	Competitive Inhibition of $^3\text{H-D-aspartate}$ K_I Values
D-Aspartate	2.83 \pm 0.39 (Km)
L-Glutamate	4.9 \pm 2.6 (4)
L-DAP	171 \pm 15 (5)
L-DAP / +NaHCO ₃	3.4 \pm 1 (5)
D-DAP	> 1,000*
D-DAP / +NaHCO ₃	268 \pm 23 (3)
DHK	28.7 \pm 0.5 (2)

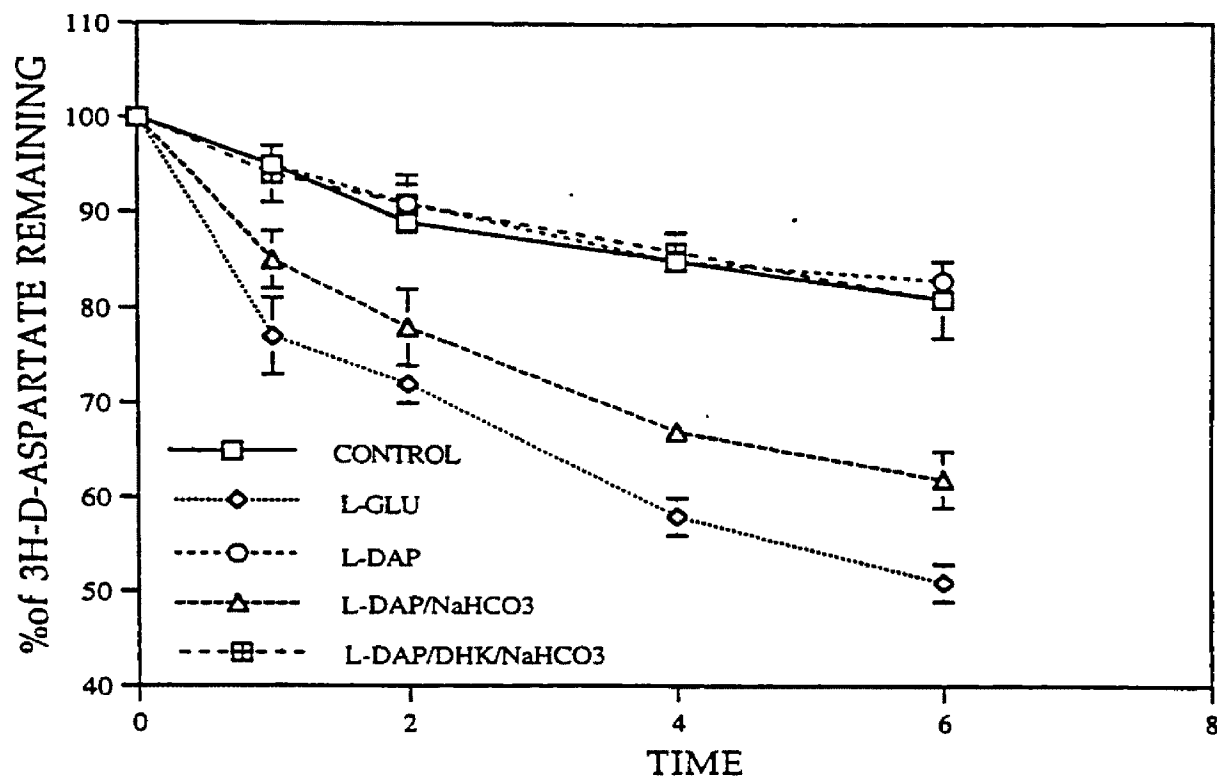
The compounds listed were measured for degree of competitive inhibition of $^3\text{H-D-aspartate}$ into synaptosomes. Values are reported as $K_I \pm \text{S.D.}$ (n=number of duplicate assays). *Value was calculated from screening data using the Michaelis-Menten equation.

Figure 7. Time Course of Substrate Stimulated Exodus of $^3\text{H-D-Aspartate}$ from Synaptosomes.



Average exchange values are shown as % of $^3\text{H-D-aspartate}$ remaining in synaptosomes (load value= 1505 ± 58 (12) pmol/mg protein). L-Glutamate's ability to act as a substrate is blocked by dihydrokainate (DHK), a weak inhibitor of the glutamate uptake system.

Figure 8. Time Course of Substrate Stimulated Exodus of $^3\text{H-D-Aspartate}$ from Synaptosomes.



Average exchange values are shown as % of $^3\text{H-D-aspartate}$ remaining in synaptosomes (load value= 1505 ± 58 (12) pmol/mg protein). The ability of L-DAP/+NaHCO₃ as a substrate of the glutamate transporter can be seen.

Table 6. Summary of Synaptosomal $^3\text{H-D-Aspartate}$ Exodus Rates for a Series of Transport Inhibitors.

Compound	Concentration (μM)	% of Control
Control		100 (27)
DHK	500	64 \pm 11 (6)
L-Glutamate	10	** 289 \pm 36 (11)
L-Glutamate/DHK	10/500	** 119 \pm 26 (4)
L-DAP	10	79 \pm 13 (4)
L-DAP/+NaHCO ₃	10	** 246 \pm 28 (8)
L-DAP/+NaHCO ₃ /DHK	10/500	*** 77 \pm 14 (5)
D-DAP	10	89 \pm 9 (3)
D-DAP/+NaHCO ₃	10	117 \pm 5 (3)
D-DAP/+NaHCO ₃ /DHK	10/500	86 \pm 6 (3)
NaHCO ₃		103 \pm 22 (12)

The above compounds were evaluated as substrates of the glutamate transport system. Synaptosomes were loaded with 1485 \pm 44 (19) pmol/mg $^3\text{H-D-aspartate}$. The control rate of exodus of $^3\text{H-D-aspartate}$ from synaptosomes was 107 \pm 11(26) pmol/mg/min. Data is presented as % of Control \pm SEM (n=number of duplicate assays). Comparisons were made between experimental points and the control value (*). Compounds in the presence of added DHK were compared with corresponding compounds in the absence of DHK (*). Levels of significance are denoted by *P<0.05, **P<0.01, ***P<0.001.

Table 7. Relative Synaptosomal $^3\text{H-D-Aspartate}$ Exodus Rates for a Series of Transport Inhibitors.

Compound	K_I	Concentration (μM)	% of Control
Control			100 (27)
L-Glutamate	4.9 ± 2.6 (4)	10	** 289 ± 36 (11)
DHK	28.7 ± 0.5 (2)	100	61 ± 20 (4)
L-DAP	171 ± 15 (5)	500	* 249 ± 51 (5)
L-DAP/+NaHCO ₃	3.4 ± 1 (5)	10	** 246 ± 28 (8)
D-DAP/+NaHCO ₃	268 ± 23 (3)	800	** 391 ± 38 (4)
NaHCO ₃			103 ± 22 (12)

The above compounds were evaluated as substrates of the glutamate transport system at concentrations of three times the K_I of the compound. Synaptosomes were loaded with 1505 ± 58 (12) pmol/mg $^3\text{H-D-aspartate}$. The control rate of exodus of $^3\text{H-D-aspartate}$ from synaptosomes was 107 ± 11 (26) pmol/mg/min. Data is presented as % of Control \pm SEM (n=number of duplicate assays). Comparisons were made between experimental points and the control value (*). Levels of significance are denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

DISCUSSION

Studies over the past 30 years have identified L-glutamate as both the primary excitatory neurotransmitter in the mammalian CNS and a potent neurotoxin. As both of these actions are mediated through EAA receptors, it is not surprising that these receptors have been the central figures in most investigation of EAA physiology and pathology. However; it is important to remember that a number of factors control the amount of glutamate in the synaptic cleft and that the disruption of any one of these processes could potentially contribute to excitotoxic damage. In the present investigation we have examined a number of novel EAA excitotoxins for their ability to inhibit and serve as substrates of the high-affinity Na⁺-dependent glutamate transporter system in rat brain synaptosomes. The potential interactions of these compounds with the glutamate transporter could significantly influence their excitotoxic actions.

The excitotoxins included in the present study (*e.g.*, BMAA, L-cysteine, DAP, and DAB) are unusual in that they are presumed to act as EAA receptor agonists only in the presence of NaHCO₃, where they can form carbamates. Thus, while the parent compounds are monocarboxylic amino acids, the resulting carbamates contain a second carboxyl group that make them structurally more similar to glutamate. The existence of the carbamates, as well as kinetic studies of their formation, has been established in a number of studies (Nunn, *et.al.*, 1987; Spencer, *et.al.*, 1987; Weiss and Choi, 1988; Weiss,

et.al., 1989; Olney, *et.al.*, 1990; Kisby *et.al.*, 1992). The most detailed studies have been carried with BMAA, where NMR spectra indicate that at physiological concentrations of bicarbonate (25 mM), carbamate formation may be as much as 25% in solutions of BMAA (Nunn and O'Brien, 1989).

The formation of a carbamate by BMAA, and its resemblance to glutamate, has been critical to elucidating the neurotoxic properties of this compound. BMAA is found in *Cycas* species, native plants which serve as a food source to people of Guam (Vega and Bell, 1967; Weiss and Choi, 1989a). Consumption of the flour made from the seeds of these plants has been linked to Guam amyotrophic lateral sclerosis (ALS)-parkinsonism-dementia complex (PD) (Nunn, *et.al.*, 1987). Using murine cortical cultures, Choi and his group demonstrated that BMAA-mediated toxicity was bicarbonate-dependent. When BMAA was added to the cultures in the presence of bicarbonate (10-12 mM), it induced the neuronal degeneration with an IC_{50} of about 1 mM (24 hr exposure) (Weiss, *et.al.*, 1989b). As this excitotoxicity could be attenuated by D-AP5 (a competitive antagonist of NMDA receptors) the investigators concluded that the toxin was acting at NMDA receptors (Weiss, *et.al.*, 1989b). Additional experiments demonstrated that chronic exposure (1-3 days) of the cultures to much lower concentrations of BMAA, also led to the selective damage of a subgroup of neurons known to be especially vulnerable to non-NMDA agonists (Weiss, *et.al.*, 1989b). Later studies by this same group demonstrated that in electrophysiological preparations, BMAA acted as an NMDA agonist in the presence of bicarbonate (Weiss, *et.al.*, 1989a). Together

with subsequent NMR studies on carbamate formation, these findings suggest that the carbamate formed by BMAA is the active agent in the excitotoxicity mediated by this amino acid.

Similar studies have also been carried out with L-cysteine, a common sulfur-containing amino acid found in all cells (Olney, *et.al.*, 1990). Like BMAA, the administration of bicarbonate in combination with L-cysteine dramatically enhanced its toxic actions within an embryonic chick retina preparation (Olney, *et.al.*, 1990). Increasing the concentration of bicarbonate present in the retinal media from 4 to 24 mM, decreased the concentration of L-cysteine necessary to induce significant damage from >1 mM to about 200 μ M (Olney, *et.al.*, 1990). Consistent with an action at NMDA receptors, the toxicity induced at low concentrations of L-cysteine/bicarbonate was attenuated by the addition either D-AP5 or MK-801 (Olney, *et.al.*, 1990). Higher concentration of L-cysteine (> 2mM) appeared to exhibit some cross reactivity with the non-NMDA receptors (Olney, *et.al.*, 1990). In related experiments with neonatal rats, subcutaneous injections of L-cysteine produced neuronal damage in several brain regions that could be protected by pre-treatment with MK-801 (Olney, *et.al.*, 1990). Analogous to BMAA, this evidence indicates that the excitotoxic actions of L-cysteine are most likely attributable to its carbamate.

In the present project, we have further investigated the potential actions of BMAA, cysteine, and their respective carbamates, within the EAA system by assessing their ability to inhibit the high affinity glutamate

transporter. We found that at concentrations as high as 1 mM, BMAA and L-cysteine, in the presence or absence of NaHCO_3 , produced little if any inhibition in the uptake of $^3\text{H-D-aspartate}$ by rat forebrain synaptosomes. Importantly, the finding that these compounds do not bind to the transporter, indicates that BMAA and L-cysteine are not substrates of the uptake system. Consequently, these excitotoxic compounds can not be rapidly cleared from the synaptic cleft by this system, as occurs with substrates such as glutamate. Furthermore, our demonstration that cystine is a significantly more potent inhibitor of $^3\text{H-D-aspartate}$ uptake than cysteine, raises the question as to whether or not any of the *in vivo* actions of cysteine may be attributable to cystine. The inability of BMAA and L-cysteine to be removed from the synaptic cleft by the transporter could have a significant influence on the half-lives of these compounds within the synaptic cleft, increasing their potential to induce excitotoxic damage.

In contrast to the lack of activity observed for BMAA and cysteine, the carbamate of L-DAP appeared to be a very potent inhibitor of the synaptosomal glutamate transporter. DAP is found as a metabolite in a variety of plants, including those that contain BMAA (Bell, 1979). While no evidence has been put forward to identify DAP as a neurotoxin in laboratory animals, work by Choi's group identify it as an excitotoxin (Weiss, *et.al.*, 1989a). In these studies, murine cortical cultures were used to demonstrate that DAP could both induce excitotoxic damage and activate NMDA receptor-channels in a bicarbonate-dependent manner (Weiss, *et.al.*, 1989a). The

enhanced neurotoxic damage produced by the addition of NaHCO_3 with DAP could be blocked by the co-addition of kynurenic acid, a non-specific EAA receptor antagonists (Weiss, *et.al.*, 1989a). The more selective NMDA antagonist MK-801 was capable of attenuating only a portion of the neuronal damage (Weiss, *et.al.*, 1989a), suggesting that the DAP carbamate acted as an excitotoxin at both NMDA and non-NMDA receptors.

When evaluated as an inhibitor of the uptake of $^3\text{H-D-aspartate}$ into rat brain synaptosomes, we also observed a significant enhancement in activity when combined with NaHCO_3 . Thus the $K_{I,app}$ for inhibiting the sodium-dependent high affinity transporter decreased from about 170 μM in the absence of bicarbonate, to 3 μM in the presence of 20 mM bicarbonate. These results support the conclusion that the carbamate of L-DAP is a potent inhibitor of the uptake system. Further studies using heteroexchange to assess substrate suitability, demonstrated that the L-DAP carbamate mimicked the action of glutamate in its ability to stimulate the exodus of $^3\text{H-D-aspartate}$ previously loaded into synaptosomes. Importantly the heteroexchange stimulated by either glutamate or the L-DAP carbamate could be blocked by the non-transportable inhibitor DHK. These results indicate that the L-DAP carbamate is not only an inhibitor, but an excellent substrate of the transporter.

Comparison of K_I values and the concentration-dependence of heteroexchange suggests that the activity of the L-DAP carbamate is

comparable to that of the endogenous substrate L-glutamate. Indeed, if the results of carbamate formation studied with BMAA are extrapolated to approximate carbamate formation with DAP, then the true carbamate concentration in these studies may be only about 25% of the total DAP concentration. Thus, the K_I value for transporter inhibition may actually be significantly lower than the 3 μM value we report. Furthermore, the potency with which the carbamates acts at the transporter, raises the possibility that some of the activity observed with L-DAP (as an inhibitor or substrate) in the absence of added NaHCO_3 might still be attributable to endogenously formed carbamate. Thus, even though the assay solutions were bubbled with nitrogen to reduce carbon dioxide levels, the possible formation of carbamates from residual carbon dioxide cannot be ruled out.

The fact that L-DAP carbamate can inhibit the glutamate transport system, as well as serve as a substrate, undoubtedly influences its action as an excitotoxin. Our results suggest that the L-DAP carbamate, unlike BMAA or cysteine, can bind to the transporter and reduce its ability to clear other agonists from the synaptic cleft. Thus, in addition to the excitotoxic action of the L-DAP carbamate itself, reduced transporter activity would increase the half life of any endogenous glutamate in the synaptic cleft and increase the likelihood that it would also induce some excitotoxic damage. On the other hand, its suitability as a substrate suggests that a mechanism exists that can remove the excitotoxic L-DAP carbamate from the extracellular environment. Interestingly, the metabolic or toxic consequences of internalizing the L-DAP

carbamate into neurons or glia have yet to be explored.

In addition to the insight our results provide into the potential toxic mechanisms of these excitotoxins, our data also provides valuable information on the pharmacology of the high affinity Na⁺-dependent glutamate transporter. At the most basic level, we have demonstrated that the carbamate of L-DAP can bind to the substrate site on the transporter protein. The finding that NaHCO₃ by itself exhibits little if any activity as an inhibitors, indicates that the carbamate forms prior to binding. Structure activity data indicate that this binding is stereoselective, as D-DAP carbamate was a much weaker inhibitor ($K_{I,app} = 268 \mu\text{M}$). This finding is consistent with the stereoselectivity observed with glutamate, where again the principal activity resides with the L-enantiomer.

Losses in activity were also observed with a corresponding increase in carbon backbone length, as L- and D-DAB were inactive, both in the presence and absence of NaHCO₃. An analogous structure-activity relationship is seen in the progression from glutamate to α -aminoadipate, where considerable inhibitor activity is also lost. The lack of activity of the DAB-carbamates, as either transport inhibitor or substrate, is of particular note because it has also been reported to exhibit excitotoxic properties (Weiss, *et.al.*, 1989a). In parallel with the characterization of DAP, Choi's laboratory demonstrated DAB's ability to depolarize as well as induce excitotoxic damage in murine cortical cells in a bicarbonate dependent manner (Weiss, *et.al.*, 1989a). Although the DAB carbamate was less potent than DAP, its effects were limited to the

NMDA receptor, as the toxicity could be attenuated by MK-801. Our present results would group DAB in a class with BMAA and L-cysteine, in that its carbamate-mediated excitotoxicity can not be reduced by rapid uptake from the synaptic cleft through the high affinity glutamate transporter.

In summary these studies demonstrate that selected amino acid carbamates (*e.g.*, L-DAP) can inhibit and serve as substrates of the high affinity Na⁺-dependent synaptosomal glutamate transporter. In addition to providing a more detailed picture of transporter pharmacology, these results also increase our understanding of the mechanisms by which these excitotoxic carbamate induce CNS damage. On one hand, acting as inhibitors of the uptake process (*e.g.*, L- and D-DAP carbamates), these toxins can slow the rate of clearance of other agonists, such as glutamate, and increase the chances of excitotoxic damage. On the other hand, those carbamates that are not inhibitors (and consequently not substrates) may have significantly longer half lives in the synaptic cleft, as they cannot be cleared from the extracellular space by the glutamate uptake system. In either case, our study highlights the important role of transport within the EAA system.

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APPENDIX A

SYNAPTOSOMAL PREPARATION SOLUTIONS

**I. 12% FICOLL:
(500 mLs)**

60 g Ficoll
440 mls 0.32 M Sucrose
100 μ L 20 mM K⁺ EDTA
pH to 7.4 with very dilute KOH
bring up to final volume with 0.32 M Sucrose

**II. 7.5% FICOLL:
(500 mLs)**

37.5 g Ficoll
4462.5 mls 0.32 M Sucrose
100 μ L 20 mM K⁺ EDTA
pH to 7.4 with very dilute KOH
bring up to final volume with 0.32 M Sucrose

**III. HOMOGENIZATION BUFFER:
(1 L)**

1.211 g Tris
bring up to 800 mLs with 0.32 M Sucrose
pH to 7.4 with Glacial Acetic Acid
bring up to final volume with 0.32 M Sucrose

**IV. 2 M SUCROSE:
(1 L)**

**684.6 g Sucrose
bring up to 1 L with Nanopure water
heat gently until dissolves**

**V. 0.32 M SUCROSE:
(1 L)**

**160 mLs 2 M Sucrose
bring up to 1 L Nanopure water**

APPENDIX B

SYNAPTOSOMAL ASSAY SOLUTION

I. CONTROL BUFFER 2X:**(1 L)**

3.604 g D-Glucose

15.018 g NaCl

0.744 g KCl

0.414 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (monobasic)0.492 g MgSO_4

2.422 g Tris

pH to 7.4 with Glacial Acetic Acid

0.588 g CaCl_2 (anhydrous)**II. 100 mM HEPES:****(1 L)**

26.03 g HEPES

bring up to 800 mLs with Nanopure water

pH to 7.4 with 12 N HCl

bring up to final volume with Nanopure water

III. 50 mM HEPES:**(2 L)****Mix 1 L of 2X Control Buffer and 1 L of 100 mM HEPES**