Kinetic Characterisation of the Plasma Membrane Transport of Excitatory Sulphur-Containing Amino Acids in Cultured Brain Cells and Isolated Nerve Endings

by

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DECLARATION

I, Angus Grieve, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

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CONTENTS

ABSTRACT

The endogenous neuroexcitatory sulphur-containing amino acids (SAAs) are generally regarded as putative transmitter substances. Of many criteria that need to be satisfied before acceptance as a transmitter, one is that a mechanism exists for termination of post-synaptic receptor activation. For amino acids such inactivation normally requires participation of a plasma membrane transport system for rapid and efficient removal of transmitter from the extracellular environment. This thesis reports on the kinetic characterisation of the plasma membrane transport of the SAAs, namely L-cysteine sulphinate (CSA), L-cysteate (CA), L-homocysteine sulphinate (HCSA) and L-homocysteate (HCA) in (i) synaptosome fractions from rat cerebral cortex and (ii) primary cultures of neurons from distinct brain regions and astrocytes from prefrontal cortex.

It was shown that each SAA acted as a substrate for the plasma membrane transporter in the different brain preparations studied with CSA and CA exhibiting a high-affinity for uptake (K_m < 100 μM) and HCSA and HCA exhibiting a low-affinity for uptake (K_m > 200 μM). The plasma membrane carrier specificity of the SAAs which was studied using cerebrocortical synaptosome fractions was established following comparison with other high-affinity neurotransmitter systems. The results obtained strongly suggest that the SAAs share a common synaptosomal transport system with L-glutamate and L- and D-aspartate.

A detailed kinetic analysis of the inhibition by the proposed selective inhibitor of HCA uptake, β-p-chlorophenylglutamate (chlorpheg), on HCA and D-aspartate uptake using (i) cerebrocortical synaptosomes and (ii) primary cultures of cerebellar granule cells and cortical astrocytes has been undertaken. The results from this kinetic inhibition study show clearly a non-selective competitive inhibition of D-

aspartate and L-HCA uptake by chlorpheg in all the brain preparations studied.

In a tangential HPLC study, the cellular localisation of HCA and HCSA was investigated using ethanolic amino acid extracts prepared from primary cultures of cerebellar neurons and astrocytes. The results suggest a predominant astrocytic, rather than neuronal, localisation of HCA and HCSA.

CHAPTER ONE

INTRODUCTION

INTRODUCTION

The acidic amino acid L-Glutamate [Glu] as well as playing a central role in brain metabolism is the major excitatory neurotransmitter mediating fast-acting synaptic transmission in the central nervous system [CNS] through distinct receptor subtypes (Collingridge and Lester, 1989). Glu lies at a branch point of numerous metabolic pathways (Fig. 1.1) and the turnover of the Glu pool in the CNS is extremely rapid.

About fifty years ago Heinnrich Waelsch suggested Glu as a therapy for epilepsy and also noted a slight improvement in mental deficiency following its administration (Waelsch, 1948). Around this time an avid uptake of Glu into brain slices was reported (Stern et al., 1949). The Japanese scholar Hayashi reported the powerful excitatory action of Glu when applied directly to the exposed dog and monkey cerebral cortex (Hayashi, 1954). Similarly Glu was observed to have an excitatory effect at the invertebrate neuromuscular junction (Van Harreveld, 1959). A possible role for amino acids in neuronal disorders was demonstrated by the neurotoxicity of Glu in the retina (Lucas and Newhouse, 1957). In the early 1960s within a series of electrophysiological studies (Curtis and Watkins, 1960; Curtis and Watkins, 1963) the pronounced excitatory effects of Glu and L-aspartate [Asp] were demonstrated when both compounds were applied ionophoretically upon the external surfaces of spinal neurons of the cat. In their initial screening of compounds structurally related to both Glu and Asp, Curtis and Watkins (1960; 1963) also observed the similar potent excitatory effects of CA, CSA and D-HCA.

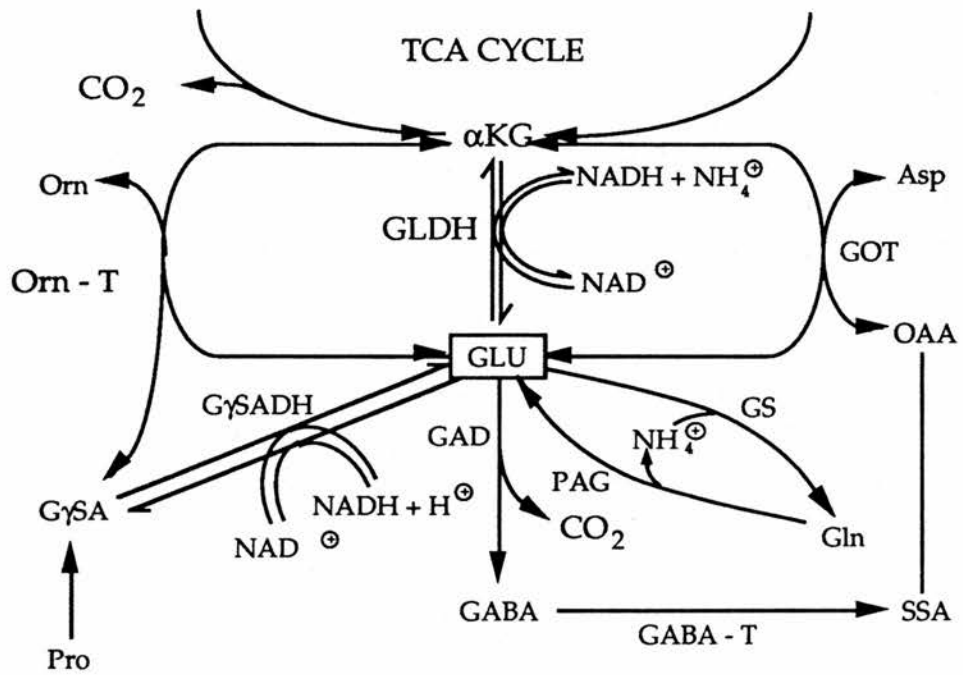


Fig. 1.1 Metabolic scheme showing reactions pertinent to metabolism of Glu and these reactions to the tricarboxylic acid [TCA] cycle.

Orn:	ornithine	GAD:	glutamate decarboxylase
αKG:	α-Ketoglutarate	PAGE:	phosphate-activated glutaminase
Asp:	aspartate		
Glu:	glutamate	GS:	glutamine synthetase
OAA:	oxaloacetate	GABA-T:	GABA transaminase
Orn-T:	ornithine aminotransferase	GγSA:	glutamate-γ-semialdehyde
GLDH:	glutamate dehydrogenase	Gln:	glutamine
GOT:	glutamic-oxaloacetate transaminase	Pro:	proline
		GABA:	γ-aminobutyrate
GγSADH:	glutamate-γ-semialdehyde dehydrogenase	SAA:	succinic semialdehyde

Glu released into the synapse is not removed enzymically (Curtis et al., 1960) unlike acetylcholine which is hydrolysed extracellularly to acetate and choline by acetylcholine esterase (Schuberth and Sundual, 1967). These initial observations cast doubt on the role of Glu as an excitatory neurotransmitter on the basis of active transport being partially responsible for the removal of Glu from the synaptic environment (Curtis et al., 1970). Further observations demonstrated the removal of Glu from the synapse by re-uptake into the neuron (Balcar and Johnston, 1972a,b). A clearer though still complicated picture of the processes likely to be involved in the transmitter function of excitatory amino acids (EAA) then began to emerge (Watkins, 1972).

Synaptic Transmission

The process of synaptic transmission by EAAs can be sub-divided into three stages:-

(1) Release of transmitter into the synaptic cleft.

(2) Interaction with postsynaptic receptors.

(3) Removal of transmitter from the synapse.

As outlined in Fig. 1.2 release of transmitter occurs upon arrival of an action potential at the nerve terminal. This local depolarization brings about an increase of intraterminal Ca^{2+}. This in turn seems to trigger release apparently by mediating fusion of neurotransmitter-containing storage vesicles and the synaptic plasma membrane. The released neurotransmitters diffuse across the synaptic cleft to interact with receptors located at the post-synaptic membrane. This process brings about changes in ion permeability of the postsynaptic membrane either directly or via a cascade of events. Termination of the signal results in the removal of the neurotransmitter from the synapse through the sodium-

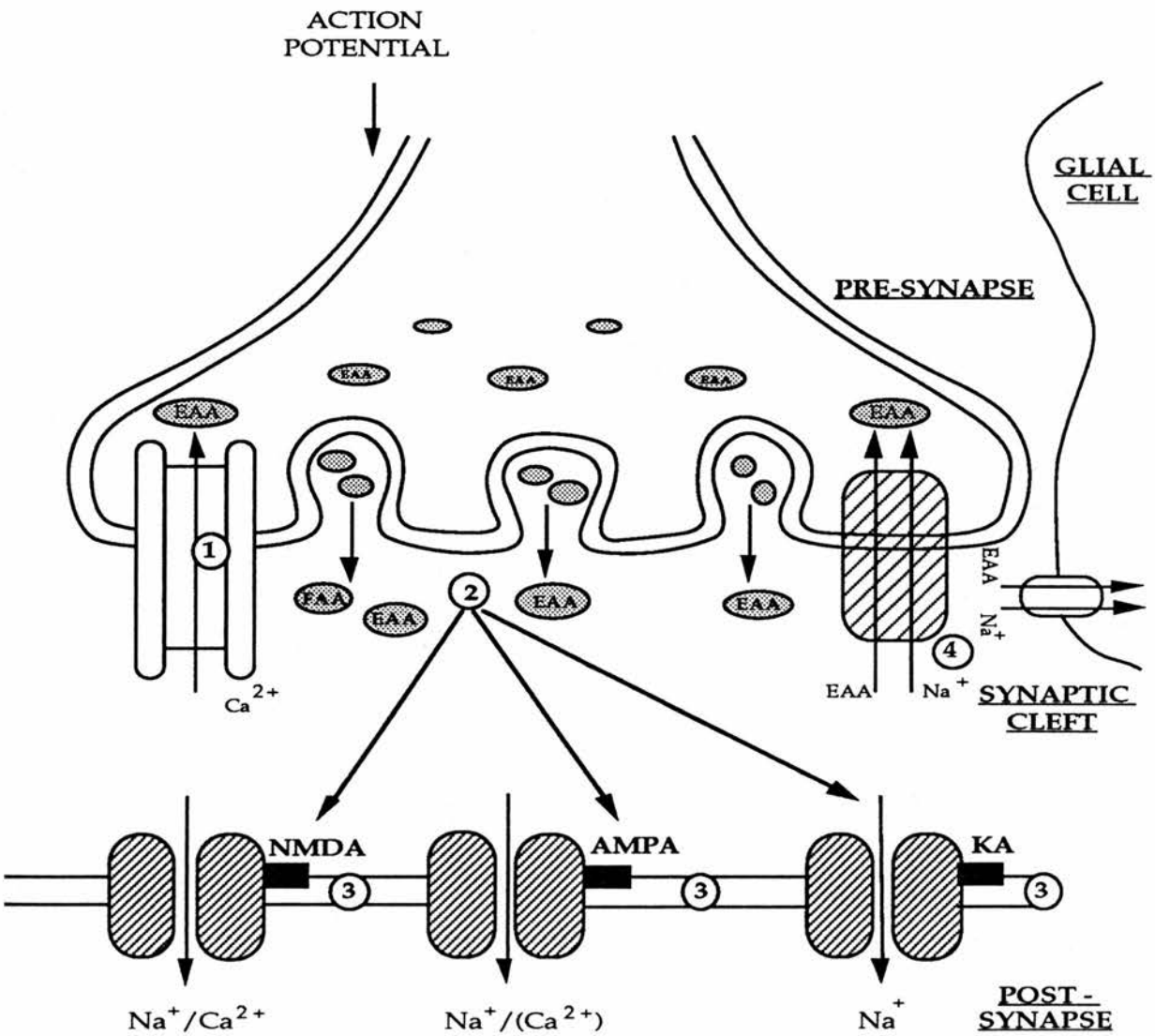


FIG. 1.2 Principle elements of EAA transmission

Depolarisation of the nerve terminal leads to activation mediated by ionotropic receptors of voltage-gated Ca^{2+}- channels (1) and subsequent exocytotic release of EAA (2) into the synaptic cleft. The EAA then diffuses to post-synaptic receptor sites where interaction with either NMDA, AMPA or KA receptors (3) leads to depolarisation by production of cationic fluxes through receptor-associated ionophores. The action of EAAs at post-synaptic sites is terminated by a high-affinity transporter (4) located pre-synaptically and on neighbouring glia elements which catalyses electrogenic Na^+ coupled uptake of EAA.

NMDA: N-methyl-D-Aspartate
AMPA: (RS)-a-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid
KA: Kainate

coupled electrogenic reuptake of transmitter to either the presynaptic nerve terminal or glial elements.

EAA Transport Kinetics

The reuptake of neurotransmitter assures that there is a constant high level of neurotransmitter in the neuron compared with a very low extracellular concentration. Under resting conditions, a considerable concentration gradient for Glu exists between the extracellular environment (<1 μM), the presynaptic cytoplasm (10 mM) and the synaptic vesicles (100 mM). The reuptake of neurotransmitters in brain preparations such as synaptosomes appears to be an active transport mechanism. This activity is observed in the presence of metabolic energy provided by glucose and is inhibited by inhibitors interfering with ATP synthesis such as cyanide and dinitrophenol. Ouabain which inhibits the plasma membrane Na^+-K^+-ATPase (Iversen and Neal, 1968) has also been shown to inhibit the uptake of neurotransmitters. Studies using membrane vesicles isolated from rat brain (Kanner and Sharon, 1978) have been instrumental in showing that as with acidic amino acid transport generally Glu uptake is driven by an inwardly directed Na^+ concentration gradient (out > in) generated by the Na^+, K^+-ATPase. As uptake of Glu shows an absolute requirement for Na^+ (Bennet et al., 1973) studies using radiolabelled [$^{22}Na^+$] show that each Glu molecule is co-transported with two Na^+ (Stallcup et al., 1979). The process is also absolutely dependent on internal K^+ and the outward oriented K^+ gradient (in > out), thus K^+ is counter-transported with Glu. This requirement of the electrogenic transport of K^+ across the membrane is demonstrated when the solubilised Glu transporter is inserted into liposomes, valinomycin causes a clear stimulation of amino acid uptake (Kanner and Sharon, 1978) when sodium chloride is the exterior medium.

The greater the magnitude of the concentration gradients $[Na^+]_{out}$: $[Na^+]_{in}$ and $[K^+]_{in}$: $[K^+]_{out}$ the larger the transmembrane gradient of the amino acid that can be generated and maintained.

Correspondingly there is a decrease in the transmembrane ion gradient when there is a rise of $[K^+]_{out}$ or fall in $[Na^+]_{out}$. This is detected as a net efflux of amino acid (Belleroche and Bradford, 1972) which in synaptosomes is especially prominent for the acidic amino acids (Erecinska and Troeger, 1986). This process for acidic amino acid transporters is reversible in that if the external concentration of Glu is increased, net uptake is observed as equilibrium is re-established. On the other hand, the action potential-induced depolarization of nerve terminals results in the release of amino acid neurotransmitters to the exterior.

As well as the existence of widely distributed Na^+-dependent Glu transporters in the brain it has been reported that Cl-dependent Glu transporters exist in the plasma membranes of glial and neuronal cells (Bridges et al., 1987; Kessler et al., 1987). The significance of the presence of Cl-dependent transport systems in neurotransmission still remains unclear.

Amino acids may penetrate across cell membranes by either of two different mechanisms: diffusion and carrier-mediated transport. Diffusion is thermodynamically dissipative and the direction and rate (v) of net transfer are governed by the concentration gradient across the membrane:-

$$v = K_{diff} ([S]_{out} - [S]_{in})$$

where K_{diff} = permeability or diffusion constant

and S = amino acid concentrations on either side of the membrane.

Although diffusion may not be the principal mode of amino acid influx in vivo it may significantly contribute to the apparent transport rates in different brain preparations studied in vitro. During transport kinetic experiments amino acid concentration gradients may change appreciably with a subsequent change in the rate and possibly direction of the net transfer of amino acid (Neame and Richards, 1972). Failure to ignore the presence of diffusion could lead to a misinterpretation of results. On the other hand a demonstration of unsaturable carrier-mediated transport can be easily misinterpreted as diffusion (Cohen, 1975). In carrier-mediated transport amino acids are translocated across the membrane by the instrumentality of carriers (transport sites) which exhibit a relative substrate specificity. The rate (v) of unidirectional mediated carrier transport is given by:-

$$v = V_{max}S/(K_m + S)$$

where

V_{max} = maximal velocity of transport and is a function of the density of the available carrier sites together with the turnover of the amino-acid-loaded and unloaded carriers in the membrane.

S = amino acid concentration on the *cis* side of the membrane.

K_m = Michaelis constant or half-saturation concentration indicating the affinity of an amino acid for the carrier.

Neurotransmitter amino acid transport into nerve cells often comprises two saturable mechanisms (Neame and Richards, 1972) one being of high-affinity (low K_m < 100 μM) and the other of low affinity (high K_m > 100 μM). Glu uptake into various CNS preparations has been characterized by a model consisting of two distinct saturable components

(Bennett et al., 1973) or by a saturable one component model (Neal and White, 1978).

From a comprehensive study of the kinetic parameters of (^{14}C) Glu synaptosomal uptake (Wheeler, 1986) it is shown that the presence of a second low-affinity carrier reported by earlier workers is incompatible for Glu uptake. These findings are the interpretation of the results from experiments where with a high concentration of extracellular Glu uptake in the absence of sodium ions is by a simple linear process while in the presence of sodium ions, uptake of Glu takes place by a carrier-mediated together with a linear process. The conclusions of the earlier studies demonstrating a low-affinity saturable component of Glu uptake usually based on the non-linearity of Lineweaver-Burke plots at high substrate concentrations of Glu could be consistent with a model demonstrating a high-affinity saturable system plus an unsaturable diffusion component.

Similarly to Glu, Asp is likely to be an excitatory synaptic transmitter in the mammalian CNS (Curtis and Johnston, 1970). The high-affinity components of D- and Asp and Glu transport show fairly similar kinetic parameters (Takagaki, 1978). A number of published apparent Km constants for Glu and Asp uptake are compiled in Table 1.

TABLE 1

K_m values (μM) for influx of L-Glu and L-Asp in various brain preparations

	L-Glu		L-Asp		
Preparation	**K_{m1}**	**K_{m2}**	**K_{m1}**	**K_{m2}**	**Reference**
Cerebral cortex	20.01	1053	16.9	368	Logan and Synder (1972)
Spinal cord	14.3	4902	21.5	3762	Logan and Synder (1972)
Cat spinal cord	12	208	13	185	Balcar and Johnston (1973)
Frog spinal cord slices	10	870	-	-	Davidoff and Adair (1975)
Rat brain slices	-	-	15.6	125	Davis and Johnston (1976)
Rat brain slices	27	650	-	-	Benjamin and Quastel (1976)
Rat cerebellar dissociated cells	10	-	-	-	East et al. (1980)
Rat synaptosomal plasma membrane vesicles	-	-	8	80	Marvizon et al. (1981)
Rat brain synaptosomes	-	-	14.6	-	Erecinska et al. (1983)
Mouse cerebral astrocyte cultures	50	-	-	-	Hertz et al. (1977)
Cultured glial cells from syrian hamster astroblasts	14.6	-	6.1	-	Balcar et al. (1977)

Specificity of EAA Transport

The uptake carrier of Glu exhibits a high degree of specificity. The high-affinity uptake of L-[^{3}H] Glu by rat brain slices was observed to be inhibited in a manner consistent with linear competitive inhibition by D-Asp and also the Asp structural analogues CSA, CA and *threo*-3-hydroxy-DL-aspartate (Balcar and Johnston, 1972). A similar study showed that the uptake of Glu and Asp were mutually competitive exhibiting a similarity of their K_i and K_m values in both cerebral, cortical and spinal cord preparations (Logan and Snyder, 1972) indicating that both compounds utilize the same uptake mechanism. A later study (Davis and Johnston, 1976) demonstrated the high-affinity Na^+-dependent uptake of D-[^{14}C]Asp in rat brain slices and sharing the same high-affinity transport system as Asp and Glu (Davis and Johnston, 1976). This active uptake of radioactive D-Asp has allowed the use of this false transmitter in the examination of the neurotransmitter roles of the EAAs. The main advantage of using D-Asp over the L-enantiomers of either Asp or Glu is that the D-form is metabolized extremely slowly < 3%/1 hour (Davis and Johnston, 1976).

Glial Transport

In addition to the well-characterized accumulation of Glu in different neuronal preparations other biochemical evidence (Benjamin and Quastel, 1972; Okamoto and Quastel, 1972) suggest that uptake into glial cells may be of greater significance in terminating transmitter action in that a release of Glu from neurons and a subsequent uptake into and conversion by, glial cells play a major role in brain metabolism. Glutamine is a major metabolite and a considerable glutamine formation has been observed in the incubated toad brain (Shank and Baxter, 1975). A characterization of the uptake of [^{14}C]Glu in rat dorsal sensory ganglia

(Roberts and Keen, 1975) exhibited two saturable components of uptake, one being of high affinity (K_m = 20.6 μM), the other exhibiting low affinity (K_m = 1130 μM). Also evidence from autoradiographic analysis (McLennan, 1976) showed the uptake of extracellular [^{14}C]Glu to be localized in glial rather than neuronal elements. The presence of an efficient high affinity sodium dependent system for [^{14}C]Glu was demonstrated for primary cultures of cortical astrocytes (Hertz et al., 1977) and also high affinity systems for Glu and Asp was shown in cultured glial cells originating from Syrian hamster astroblasts (Balcar et al., 1977). There is apparently little or no difference in the substrate and inhibitor specificity in the Glu uptake for primary cultures of neurons and glial (Balcar et al., 1987). An unequivocal differentiation between the glial and neuronal components of Glu uptake in the CNS will probably require synthesis of more potent specific Glu analogues.

Excitatory Sulphur-Containing Amino Acids

Besides Glu and possibly Asp which are widely accepted as mediators of excitatory chemical transmission in the vertebrate CNS, certain naturally-occurring acidic sulphur-containing amino acids [SAAs] have been proposed as transmitter candidates (Do et al., 1986; Griffiths, 1990; 1992; Griffiths et al., 1992). These SAAs, more precisely the sulphonic and sulphinic acid homologues, namely L-cysteic acid (CA), L-cysteine sulphinic acid (CSA) and of Glu namely L-homocysteate (HCA) and L-homocysteine sulphinate (HCSA) [see Fig. 1.3 for structures]. The SAAs were first recognised as neuroexcitant amino acids during an initial screening of various acidic amino acid analogues of Glu (Curtis and Watkins, 1960; 1963) and also as possible substrates for the Glu carrier in rat brain slices (Balcar and Johnston, 1972).

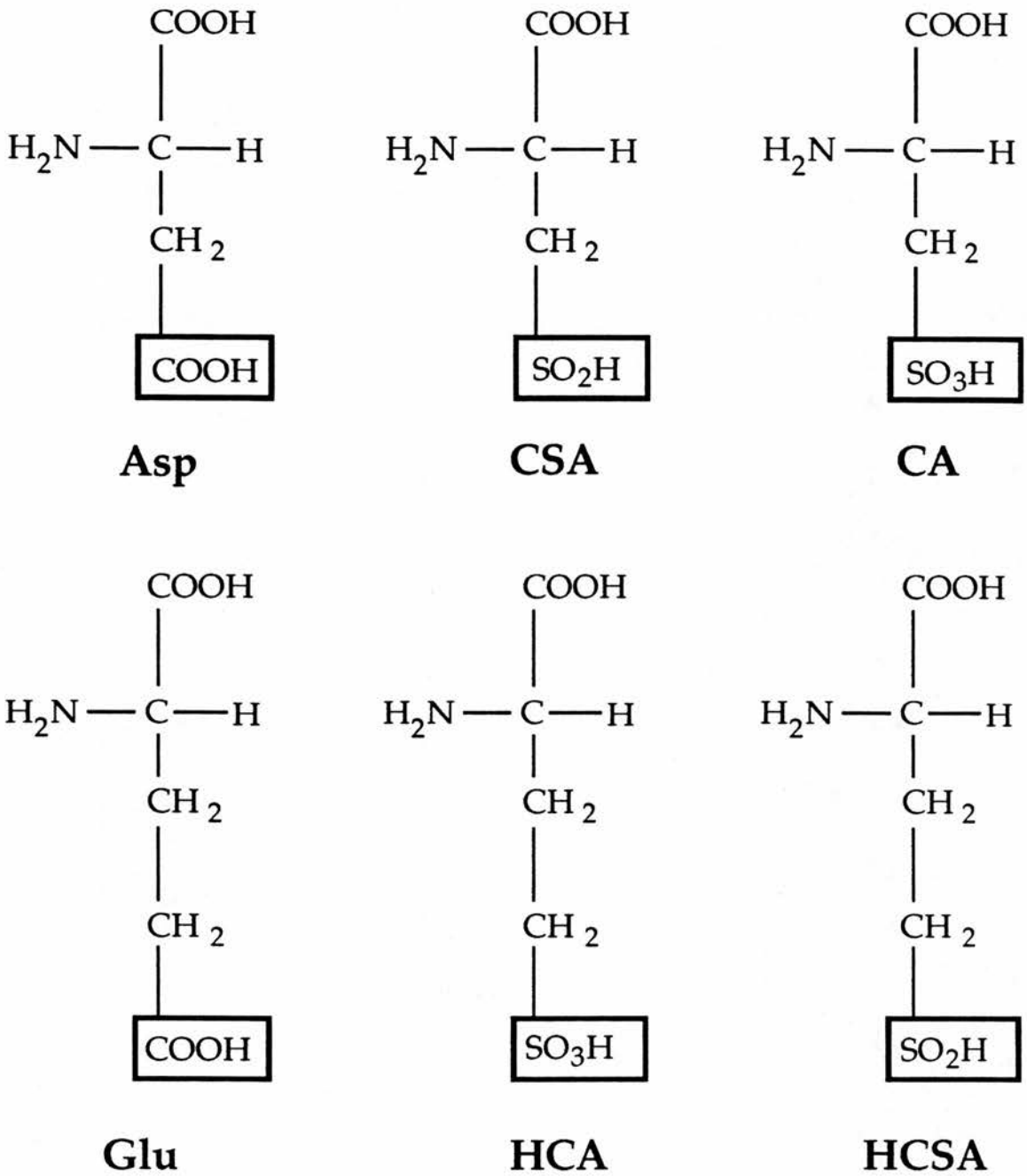


FIG. 1.3 Structures of endogenous excitotoxins

The structural similarity of the acidic excitatory SAAs is such that they differ only in the nature of the (ω-) acidic group and the number of carbon groups separating this group from the α-carbon amino and carboxyl groups.

Metabolism of SAAs

The biosynthesis of the neuroactive SAAs in mammalian brain occurs from the catabolism of the essential amino acid L-methionine (Fig. 1.4), see Griffiths 1990; 1992 for reviews.

Briefly, L-methionine is dimethylated to L-homocysteine through a series of enzymatic reactions. From this metabolic branch point, in humans about half of the L-homocysteine formed is converted via a transsulphuration pathway to L-cysteine which acts as an immediate precursor for CSA, the reaction catalysed by cysteine dioxygenase, an enzyme localised in nerve endings (Misra and Olney, 1975). Further oxidation of CSA produces CA. There is some doubt as to whether CA represents a true enzymatically formed metabolite of CSA. However, there is evidence to support the enzymatic oxidation of CSA to CA in brain tissue provided by the demonstration that [^{35}S] methionine is converted to [^{35}S] CA (Peck and Awapara, 1967). The biosynthetic routes of the Glu analogues, HCSA and HCA have not been characterised and can only be assumed by analogy to the metabolism of L-cysteine. Although the molecular steps involved are unclear, evidence that such a pathway exists is observed by the conversion of [^{35}S] methionine to [^{35}S] HCA in brain slices (Do et al., 1988). Genetic traits which effect the enzymes involved in SAA metabolism can lead to a formation and accumulation of a potentially neurotoxic compound i.e. S-sulpho-L-cysteine (SLC) which has been implicated in the neurodegenerative disease sulphite oxidase deficiency (Olney et al., 1975).

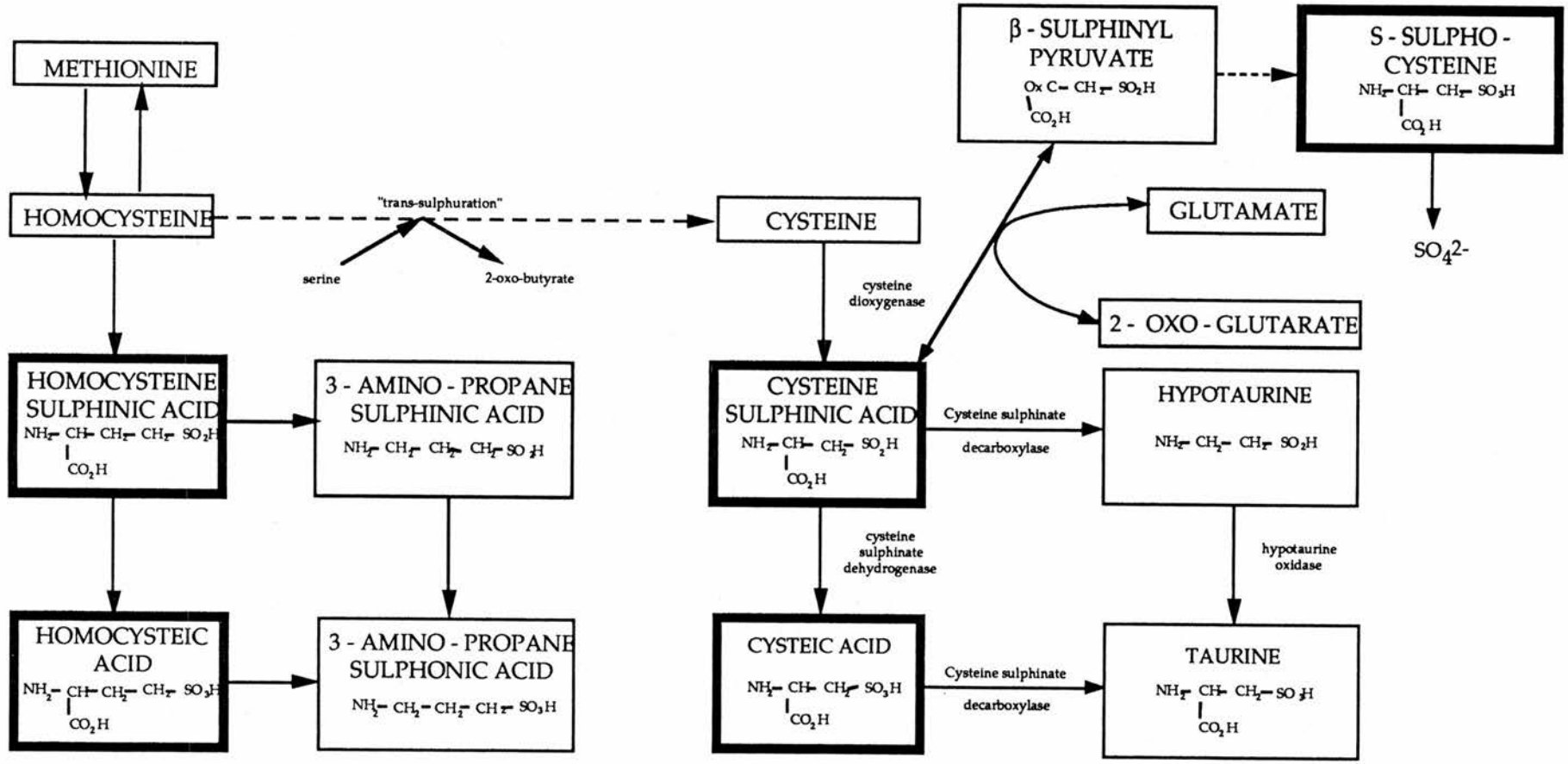


FIG. 1.4. Biosynthetic pathways for the excitatory SAAs. The excitatory SAAs are high-lighted within the bold boxes. The enzymatic conversion of cysteine to cysteine sulphinate and cysteate is discussed fully in the text, while the pathway from homocysteine to homocysteinesulphinate and homocysteate is presented only by analogy.

Endogenous Levels of SAAs in Brain

An early study utilising paper chromatography showed the presence of relative low concentrations of compounds corresponding by their respective retention times to authentic CSA and CA in rat brain (Bergeret and Chatagner, 1954). The development of a microassay specific for CSA based on the enzymatic conversion of CSA to lactate and the subsequent enzymatic cycling of NAD (Baba et al., 1980) showed the presence of CSA in different regions of rat brain with the highest value in the cerebral cortex (287 nmol CSA/g wet weight). A sensitive specific assay for the simultaneous measurement of CSA and CA in rat brain using HPLC analysis and subsequent post column o-phthalaldehyde (OPA) derivatization was developed (Ida and Kuriyama, 1982). This study showed that CSA and CA were unevenly distributed amongst the various areas of the rat brain with the highest level for both SAAs observed to be in the hippocampus and cerebellum with values of 26 nmol/g wet wt and 12 nmol/g wet wt, respectively. Further development of a sensitive HPLC method allowed the separation and simultaneous measurement of CSA, CA, HCSA and HCA at the picomole level (Do et al., 1986). This study implied the endogenous presence of all four SAAs in brain slices prepared from different regions of rat brain; as demonstrated by their release on depolarization in a Ca^{2+}-dependent manner. The Glu analogues CSA and HCSA were shown to be released from the cortex, hippocampus and mesodiencephalon with HCSA shown also to be released from the stratium. HCA release was observed from all the regions studied and was most prominent in the cortex and hippocampus. CA release was observed to be only slightly increased by depolarization in the hippocampus and mesodiencephalon. The quantification and a partial regional analysis of the SAAs in rat brain was determined by HPLC with fluorometric detection using the OPA pre-

column derivatisation procedure (Kilpatrick and Mozley, 1986). In agreement with the release studies (Do et al., 1986) this HPLC study showed that CSA was most concentrated in the prefrontal cortex (10.53 pmoles/mg wet wt). CA was most prominent in the hippocampus (5.66 pmol/mg wet wt) and HCA was not preferentially distributed within a region with values ranging from 17.34 - 22.85 pmol/mg wet wt Following reported refinements to the HPLC method for the simultaneous detection of the SAAs in conjunction with an examination of different tissue extraction medium (Waller et al., 1990) CSA was the only excitatory SAA consistently detectable (0.24 ± 0.01 pmol/mg/wet wt) in the rat spinal cord. This observation of the presence of CSA in spinal cord is in agreement with the quantification of the SAAs utilising a method based on a mild non-acidic extraction of brain tissue homogenates followed by ultrafiltration, ion-exchange solid phase extraction and OPA pre-column derivatization HPLC analysis (Do et al., 1991). Under these conditions the CSA level is most prominent in the spinal cord (0.3 pmol/mg protein). In contrast to the previous report (Waller et al., 1990) the presence of HCA was also reported with values ranging from 0.1 - 1.0 pmol/mg protein with the highest concentration recorded in the hippocampus. The HCSA levels were generally lower (0.06 - 0.2 pmol/mg protein). Using similar tissue extraction procedures and subsequent HPLC analysis the presence of CSA (30.04 ± 17.33 pmol/mg protein), HCA (29.05 ± 14.45 pmol/mg protein) and HCSA (27.78 ± 20.24 pmol/mg protein) was demonstrated in rat cortical astrocytes with similar levels for CSA, HCA and HCSA recorded in C6 glioblastoma cells (Tschopp et al., 1991). In addition undetectable amounts for HCA and HCSA were established in cerebellar granule cells. This glial localisation for HCA quantified by HPLC analysis is consistent with the findings of immunocytochemical analysis which show a strong

HCA-like immunoreactivity in the glial elements within the rat cerebellum (Grandes et al., 1991), rat cortex (Tschopp et al., 1992) and the monkey cortex (Kritzer et al., 1992). These observations for HCA are consistent with the hypothetical role for this SAA as a "gliotransmitter" (Grandes et al., 1991).

Release and Receptor Specificity

Release of preloaded [^{14}C]CSA from brain slices and synaptosomal fractions with a superfusion method was shown to be partially Ca^{2+}-dependent with depolarizing stimuli (Iwata et al., 1982). Similar release of preloaded [^{3}H]CSA, [^{3}H]Glu and [^{3}H]GABA from slices of various rat brain regions was also found to be partly Ca^{2+}-dependent with depolarizing stimuli (Recasens et al., 1984). The demonstration of the depolarization induced Ca^{2+}-dependent release of endogenous CSA, CA, HCSA and HCA from brain slices (Do et al., 1986a) and the specific release of CSA and HCA from acute hippocampal slices upon high-frequency stimulation of the Schaffer collateral commissural fibres (Klancnik et al., 1992) support a role for the SAAs in the process of synaptic transmission in the CNS. The SAAs are also able to, from a variety of brain preparations (see Griffiths, 1992 for review), evoke the release of various transmitters in the absence of additional depolarizing stimuli. In a series of papers (Dunlop et al., 1989; 1990; 1991) demonstrated a dose-dependent saturable and stereospecific partial Ca^{2+}-dependent release of [^{3}H]GABA and D-[^{3}H]Asp from primary cultures of cortical neurons and cerebellar granule cells respectively. The SAA-evoked release of these transmitters was antagonised by the selective competitive N-methyl-D-aspartate (NMDA) antagonist, 3(±-2-carboxypiperazin-4-yl)propyl-2-phosphonic acid (CPP) (IC_{50} > 50 μM) and the non-NMDA receptor antagonist, 6,7-dinitroquinoxalinedione

(DNQX) (IC_{50} range 5-50 μM). Additional evidence shows that the mechanism for the SAA evoked Ca^{2+}-independent component of release of D-[^{3}H]Asp is mediated predominantly by activation of EAA receptors resulting in a reversal of the high-affinity dicarboxylic amino acid transport system (Dunlop et al., 1992). This biochemical and pharmacological evidence demonstrating a mixed NMDA/non-NMDA receptor selectivity for the SAAs is supported by radioligand displacement studies using [^{3}H]Glu, [^{3}H]AMPA and [^{3}H]KA binding to the NMDA, AMPA and KA receptors (Mewett et al., 1983; Pullan et al., 1987). Such studies indicated that CSA exhibited a broad spectrum of activity with HCA and HCSA having a greater affinity for the NMDA sub-type. The receptor specificity of the SAAs interpreted from electrophysiological studies are somewhat contradictory depending upon whether studies were undertaken on multicellular brain preparations (e.g. slices) or single cells by "clamping" methods. There is general agreement that HCA acts predominantly as an agonist of the NMDA receptor sub-type (Do et al., 1986b; Knopfel et al., 1987; Patneau and Mayer, 1990). This is in contrast to CSA which appears to act as a preferential non-NMDA receptor agonist in multicellular studies (Herrling and Turski, 1985) but as a NMDA agonist in cultured hippocampal neurons (Patneau and Mayer, 1990) and in *Xenopus* oocytes following injection of mRNA isolated from rat brain (minus cerebellum) (Curras and Dingledine, 1991) in clamping studies.

Neurotoxicity and Neurodegeneration.

The cytoxic (Olney et al., 1971; Kim et al., 1987) and epileptogenic (Jurson and Freed, 1990) action of Glu and certain SAAs are well established, their mode of action being consistent with excessive stimulation of both NMDA and non-NMDA receptors. It is observed

during global ischemia that the extracellular levels of CA and CSA increase 1.5 to 3-fold (Andine et al., 1991b). Although the levels of these SAAs are of a magnitude of 20-30 times lower than that of Glu or Asp, this could reflect the fact that tissue levels of CA and CSA are 100-1000 times lower than that of Glu and Asp (Ida and Kuriyama, 1983; Kilpatrick and Mozley, 1986). On the return of neuronal activity (postischemic) there is an increase in the extracellular concentration of CSA which exceeds the level during ischemia (Andine et al., 1991b). This could feasibly trigger the release of Glu and Asp (Dunlop et al., 1989) leading to a postischemic excessive activation of Glu receptor with concomitant Ca^{2+} entry and indeed may be one factor in the development of delayed neuronal death (Andine et al., 1991a). The neurotoxic nature of HCA has been demonstrated following its administration at elevated doses for prolonged periods in the chick embryo retina (Olney et al., 1987), in mixed cultures of cortical neurons (Kim et al., 1987) and in primary cultures of cortex neurons (Frandsen et al., 1992). In this latter study it was shown that all SAAs were cyotoxic, with the neurotoxic effects being mediated both by their action via NMDA as well as non-NMDA receptors.

Neuronal Uptake of the SAAs

The presence of an efficient mechanism for the rapid termination of transmitter action following receptor activation is regarded as an essential criterion for transmitter status. As reported earlier, inactivation of amino acid transmitters occurs by their removal from the synaptic cleft via carrier systems located in the plasma membrane of both neurons and glia (Drejer et al., 1982). Relatively few studies have characterized SAA transport in various brain preparations. Synaptosomes isolated from rat brain accumulated [^{35}S]CA by a high-affinity transport system (Wilson

and Pastuszko, 1986) in which the kinetic constant value (K_m = 12.3 μM) is very similar with the earlier observation for uptake of [^{14}C]CSA by the P2 synaptosomal fraction of rat cerebral cortex (K_m = 12.0 μM (Iwata et al., 1982). The presence of an efficient Na^+-dependent high-affinity uptake for [^{3}H]CSA has been demonstrated in cultured neuronal and glial cells with calculated K_m values fo 5-6 μM and 20-40 μM respectively (Abele et al., 1983). Kinetic characterization of HCSA and HCA uptake is limited to the demonstration of a low-affinity (K_m approx 3 mM) uptake system for HCA in individual rat cerebral cortex slices (Cox et al., 1977). Simultaneous kinetic inhibition studies (Iwata et al., 1982; Wilson and Pastuszko, 1986) of CSA and CA uptake by Glu, Asp and CA or CSA were consistent that these acidic amino acids acted as linear competitive inhibitors, therefore suggesting that they are transported by a common system in synaptosomes. A detailed kinetic study of the inhibitory effects of the L- and D-enantiomers of the SAAs on the neuronal, astroglial and synaptosomal high-affinity glutamate transport system was undertaken using D-[^{3}H]Asp as the transport substrate (Griffiths et al., 1989). This study showed the competitive nature of the SAAs with D-[^{3}H]Asp uptake where CA and, L- and D-CSA, were potent inhibitors of the dicarboxylic acid transporter whereas the longer chain Glu analogues, HCA and HCSA, displayed a substantially weaker inhibition. This profile of inhibition of the SAAs from the in vitro kinetic studies is in general agreement with a parallel computer-assisted molecular modelling study in which volume contour maps of the SAAs were superimposed on those of D-Asp and Glu (Griffiths et al., 1989).

Glu Transport Inhibitors

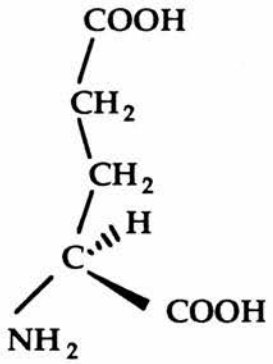
From a physiological perspective, the availability of specific inhibitors of Glu uptake are potentially useful probes for evaluating the

functional role of the plasma membrane Glu transporter in neurotransmission. Interest in the functional characteristics of this uptake mechanism has dramatically increased with the recent observation that excessive extracellular levels of Glu and of other excitatory agonists are neurotoxic and may play a significant role in a number of acute and chronic neuropathologies which include ischemia, hypoglycemia, epilepsy, Huntington's disease and Alzheimer's disease. Several compounds appear (Fig. 1.5A and B), from their structural similarities to Glu, to be potentially good substrates for the plasma membrane Glu transporter and also appear to act competitively with Glu for the uptake site and may themselves be transported:-

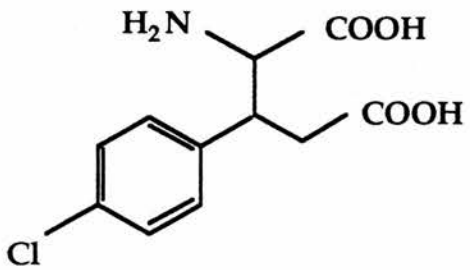
(i) Dihydrokainate is a weak neuronal excitant displaying a 500-fold lower potency than the neurotoxin kainic acid as an inhibitor of [^{3}H] kainic acid binding and at least 1000-times less potent as a convulsant (Johnson et al., 1979). Kinetic evidence shows that dihydrokainate acts as a weak non-competitive/mixed inhibitor of the Glu transporter in both primary cultures of cerebellar granule cells and synaptosomes (Dunlop et al., 1992), therefore implying that dihydrokainate is unlikely to function as a transport substrate.

(ii) The D- and L-enantiomers of *threo*-β–hydroxy-D-asparate are potent inhibitors of Glu transport in rat brain slices (Balcar et al., 1977) but may also activate to an appreciable extent EAA receptors with a special affinity for the NMDA receptors.

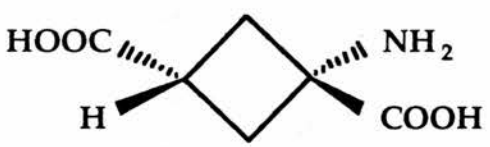
(iii) β–Methylene-D,L-asparate (β-MA) has been found to be a non-competitive inhibitor of the Glu carrier in cultured cortical astrocytes (Bender et al., 1989; Dunlop et al., 1992). However the observation that β-MA acts as a competitive inhibitor of D-[^{3}H]Asp together with a potentiation of SAA-evoked D-[^{3}H]Asp release in cerebellar granule cells is consistent with the feasibility



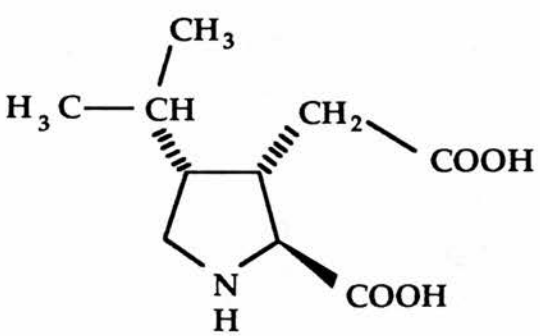
L- Glu



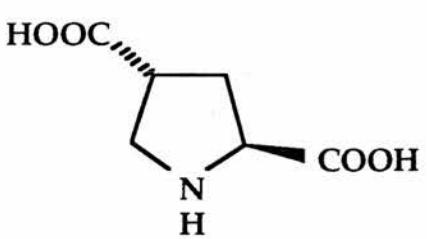
(±)-β-p - Chlorophenyl-
glutamic acid



Cis - 1 - aminocyclobutane - 1,3 -
dicarboxylic acid (cis - ACBD)

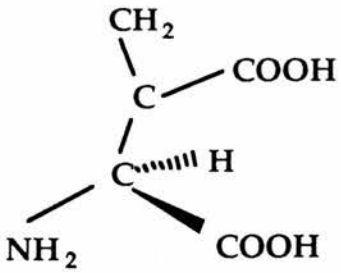


Dihydrokainic acid

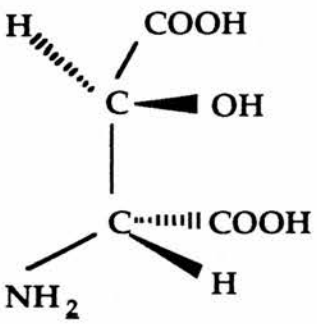


L-*trans*-2,4-pyrrolidine
dicarboxylic acid

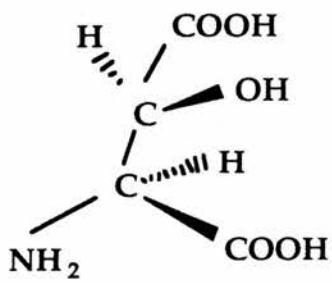
Fig 1.5A Structures of various Glu uptake inhibitors:- Glu analogues.



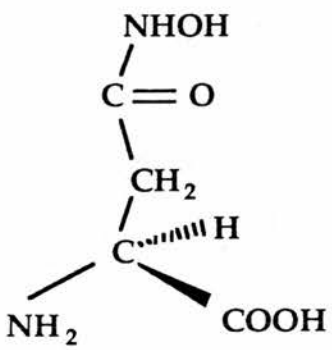
L- Asp



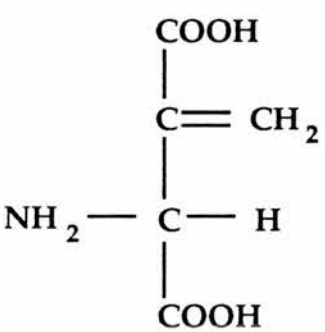
threo-3-OH-D-Asp



threo-3-OH-L-Asp



L-Aspartic acid-
β- hydroxamate



β - methylene - D,L-
Aspartic acid

Fig 1.5B Structures of various Glu uptake inhibitors:- Asp analogues.

of β-MA acting as a substrate for the Glu transporter in granule cells (Dunlop et al., 1992).

(iv) L-Aspartic-β-hydroxamate was found to be a moderately strong inhibitor of Glu uptake in synaptosomes and glia (Roberts and Watkins, 1975) and a competitive inhibitor of Glu uptake in primary cultures of neurons and astrocytes (Drejer et al., 1982).

(v) L-*trans*-Pyrrolidine-2,4-dicarboxylic acid (L-*trans*-PDC) is a highly potent competitive inhibitor of synaptosomal Glu uptake with relatively little interaction with ionotropic Glu receptors (Bridges et al., 1991). This was in agreement with a comparative study (Dunlop et al., 1993) on the selective assessment of the substrate potential of L-*trans*-PDC for the neuronal and glial transporters which showed that this compound acts as a competitive inhibitor of D-[^{3}H]Asp uptake into primary cultures of cerebellar granule cells and astrocytes. This study also showed, in the absence of any other stimulating agent, a dose-dependent release of previously accumulated D-[^{3}H]Asp, used as a non-metabolisable analogue of Glu, from the glutamatergic cerebellar granule cells following superfusion with increasing concentrations of L-*trans*-PDC. The profile of release was Ca^{2+}-independent (carrier reversal) and was unaffected by the selective NMDA/non-NMDA receptor antagonists. These observations show the potential for L-*trans*-PDC to function as a substrate for the Glu transporter.

(vi) *Cis*-1-aminocyclobutane-1,3-dicarboxylic acid (*cis*-ACBD) has been shown to be a potent competitive inhibitor of the high-affinity uptake of Glu into rat cortical synaptosomes (Fletcher et al., 1991). This observation is consistent with the competitive inhibition of *trans*-DMG of the Glu carrier in primary cultures of neurons and astrocytes (Dunlop et al., in preparation). In addition, *trans*-DMG

was observed to evoke the release of Ca^{2+}-independent carrier reversal of D-[^{3}H]Asp from primary cultures of cerebellar granule cells. This cytosolic release of D-[^{3}H]Asp from cerebellar granule cells by *trans*-DMG was observed to be in the order of a tenth of the potency observed for L-*trans*-PDC (Dunlop et al., 1993).

(vii) The Glu analogue β-p-chlorophenylglutamic acid (chlorpheg) has been reported to exert a selective potentiating effect on the responses of cat and frog spinal neurons to a range of EAA agonists, with the greatest response being to HCA whereas the response to Glu and Asp remain generally unchanged (Davies et al., 1985). A similar selective potentiating effect of chlorpheg on HCA responses was observed using rat neocortical cells (Zeise et al., 1988). By analogy with these observations the enhancement of synaptic responses evoked in CA1 rat hippocampal neurons by Schaffer collateral stimulation is consistent with the proposed role for HCA as an endogenous NMDA ligand (Ito et al., 1991). It has been recognised however that the observed selective effect of chlorpheg on HCA in comparison to Glu/Asp transport may be only apparent, being a reflection of the very different amino acid concentrations used in electrophysiological studies in comparison with their widely reported kinetic constant values for transport (Davies et al., 1985).

AIMS OF STUDY

Work from this laboratory has, over the past five years, contributed significantly to an understanding of the mechanism of action of the various SAAs. The description in this thesis is an attempt to provide further evidence to support a role for the SAAs in synaptic transmission. The principle aims of this project were as follows:-

(i) Development of a sensitive HPLC-based assay for use in characterising the transport kinetics of the SAAs; CSA, CA, HCSA and HCA using primary cultures of mouse cerebellar neurons, cerebral cortex neurons, prefrontal cortex astrocytes and rat cortical synaptosomes as model in vitro systems.

(ii) Analysis of the carrier specificity for the SAAs.

(iii) Evaluation of the proposed action of the Glu analogue β-p-chlorophenylglutamate as a selective inhibitor of HCA uptake using in vitro model systems of neuronal and glial origin.

(iv) Identification and quantitation by HPLC of the endogenous levels of certain SAAs in extracts prepared from cultured mouse cerebellar granule cells, cerebral cortex neurons and cortical astrocytes.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

Mice for cell culture were obtained from the animal quarter in the Panum Institute, University of Copenhagen and the University of St. Andrews; and 21-day male Wistar rats for synaptosome preparation were from the animal house at the University of St. Andrews.

Plastic multitest dishes for cell culture were purchased from NUNC A/S Denmark, culture media, foetal calf serum and Dulbecco's minimum essential medium (DMEM) from Gibco-Biocult Lab. (Scotland). Dibutyryl-cyclic AMP (dBcAMP), cytosine arabinoside, trypsin, soybean trypsin inhibitor, poly-L-lysine and amino acids for preparation of culture medium were obtained from the Sigma Chemical Company, Poole, Dorset, U.K. Penicillin was obtained from Leo, Denmark and insulin from NOVO-Nordisk, Denmark.

Solutions of sucrose (ARISTAR) obtained from BDH Chemicals, were routinely deionised using Dowex 50W-X8 (200-400 Mesh, hydrogen form; purchased from the Sigma Chemical Company) to prevent synaptosomal aggregation.

Ficoll 400, obtained from Pharmacia (U.K.) was exhaustively dialysed against distilled water before use.

Silicone oil (Dow Corning MS550) was purchased from BDH Chemicals, and dinonyphthalate obtained from Fluka Chemicals Ltd.

The tissue solubiliser 'Solusol' and the liquid scintillant Soluscint "O" were purchased from National Diagnostics.

D-[2,3-^{3}H] aspartic acid (specific radioactivity 15 Ci/mmol) was purchased from Du Pont (U.K.) Ltd.

Sodium acetate (HiPerSolv) for HPLC was purchased from BDH Chemicals. Methanol (HPLC grade) was purchased from LAB-SCAN Analytical Services. All solutions containing sodium acetate or methanol were prepared by using HPLC grade water purchased from Rathburn

Chemicals Ltd. and routinely filtered through 0.45μ Nylon 66 Membranes obtained from Anachem prior to HPLC.

The L- and D-enantiomers of the SAAs; CA, HCA, CSA, HCSA together with sulpho-L-cysteine, sulpho-D-cysteine, N-methyl-D-aspartate, dihydrokainate and quisqualate were purchased from Tocris Neuramin (Bristol, U.K.). All other chemicals used were of the purest grade available.

2.2 Methods

(i) Preparation of Synaptosomes (see Fig. 2.1)

Synaptosomes (isolated nerve endings) used as an in vitro model system were prepared essentially as described by Nicholls (1978) and Scott and Nicholls (1980).

Twenty-one-day-old adult male Wistar rats (200g-250g) were sacrificed by cervical dislocation. Immediately following sacrifice, the cranium was rapidly cut open, and the whole brain removed on to an ice-cold Petri-dish. The cortices were dissected from the whole brain, chopped into small pieces, and washed in ice-cold isolation medium (Appendix 1) to remove blood and other debris. The cortices were finally resuspended in ten volumes of isolation medium and the tissue was maintained at 0-4°C throughout the remainder of the preparation. The cortices were then homogenised using a Hormuth Vetter glass/teflon homogeniser (clearance 80 μm) with cooling (in an ice-bath) every 8-10 strokes. The homogenate was centrifuged at 800g (Sigma-202 MK 4x30 ml angle rotor) for 5 min. The supernatants (S1) were removed from each, combined, and stored on ice whilst the pellets (P1) were resuspended in isolation medium, mixed and recentrifuged at 800g for 5 min. The resultant supernatant (S2) was combined with (S1) and

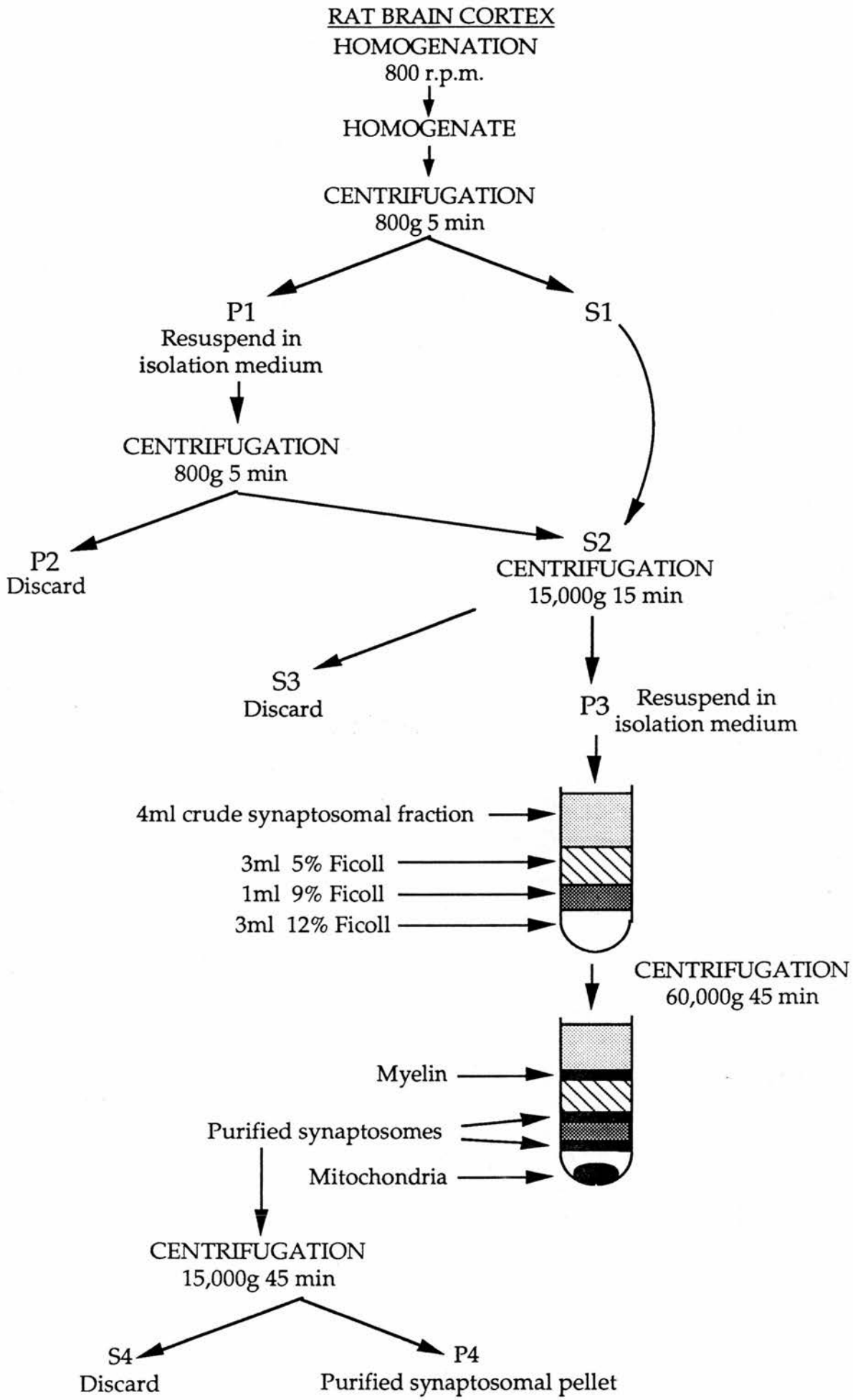


Fig 2.1 Protocol for the preparation of rat cerebrocortical synaptosomes.

centrifuged at 15,000g for 15 min using the same rotor; the pellet consisting mainly of connective tissue and white matter was discarded. After centrifugation, the final supernatant (S3) was carefully removed and the pellet (P3) comprising the crude synaptosomal fraction was resuspended in isolation medium (4 ml/cortex). Purified synaptosome fractions were then prepared by discontinuous Ficoll density-gradient centrifugation as follows. Gradients comprising 3 ml of 12%, 1 ml of 9% and 3 ml of 5% (w/v) Ficoll (Appendix 1) were prepared 1h before use and stored at 4°C. Aliquots (4 ml) of the resuspended crude synaptosomal fraction (P3) were carefully layered on to each Ficoll gradient and then centrifuged 60,000g (Beckman L8-M Ultracentrifuge, 6 x 14.5 ml swing-out rotor) for 45 min. The purified synaptosomes (Fig. 2.1) were then carefully removed by aspiration from the 9% Ficoll layer, pooled and diluted to a known volume with 250 mM Sucrose:N-tris [Hydroxymethyl]-methyl-2-aminoethane sulfonic acid (Tes) pH 7.4 (Appendix 1). All other cellular material from the crude synaptosomal fraction was discarded. At this point the total protein content of the purified synaptosomes was determined by the method of Gornall et al. (1949) (Appendix 2) using bovine serum albumin 10 mg/ml as a standard. Aliquots (25 mg) of synaptosomal protein were diluted to a final volume fo 30 ml with 250 mM Sucrose:Tes pH 7.4 and centrifuged at 15,000g (Sigma 202 MK 4 x 30 ml angle-rotor) for 45 min. The supernatants were discarded and the purified synaptosomal pellets (P4) kept on ice at 0-4°C but used within 4 hrs for appropriate assays.

(ii) Assessment of purity

Synaptosomes from a typical preparation were assessed for purity by a morphological study using electron microscopy as outlined by Dunlop (1991). This gave an indication of the proportion of

synaptosomes against the possible contaminants, myelin and free mitochondria.

(iii) Integrity of Synaptosomes

The plasma membrane integrity of the synaptosomes was routinely determined by assaying the extracellular medium for the cytosolic marker enzyme, lactate dehydrogenase (LDH).

Basis of reaction:

$$\text{pyruvate} + \text{NADH} \underset{\text{LDH}}{\rightleftharpoons} \text{lactate} + \text{NAD}^+ + \text{H}^+$$

Intact synaptosomes would release very little LDH, whereas membrane damage would result in LDH release and consequent disappearance of NADH (decrease in A_{340}) as the enzymic reaction is catalysed. LDH reagent was freshly prepared by dissolving 7.0 mg NADH, 4.0 mg sodium pyruvate, 0.6055g Tris and 10.61g sucrose in distilled water to a final volume of 100 ml with pH adjustment to pH 7.4 using hydrochloric acid. The LDH assay was carried out spectrophotometrically (λ = 340 nm) employing a heated cell carriage at 37°C. Using distilled water as a reagent blank, the absorbance of 3.0 ml of the LDH reagent was recorded over a 5 min period at a chart speed of 5 mm/min. Synaptosomes (0.18 ml; 0.2 mg/ml protein) were then added to the cuvette and the absorbance recorded for a further 5 min. An aliquot (0.02 ml) of 10% (v/v) Triton X-100 was then added to the LDH reagent/synaptosome mixture and the absorbance recorded (Fig. 2.2). This final step shows a rapid decrease in absorbance as NADH is converted due to a LDH to NAD^+ release from the lysed synaptosomes. Such assays gave an

indication of the membrane integrity of the synaptosomes (before the addition of Triton) before use for transport assays.

(iv) Transport Assays

Synaptosomal pellets were resuspended to 8-10 mg/ml protein in Elliot's 'B' incubation medium (125 mM NaCl, 3.5 mM KCl, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 0.4 mM KH_2PO_4, 1.3 mM $CaCl_2$, 20 mM NaTes and 10 mM D-glucose pH 7.4). Aliquots (150 µl) of the synaptosomal suspension were pre-incubated for 5 min at 35°C in a metabolic water-bath prior to the assay. Uptake was initiated by the addition of 100 µl pre-incubated synaptosomal suspension to 300 µl assay incubation medium containing [^{3}H]-labelled substrate amino acids in the absence and presence of unlabelled sulphur amino acid (SAA) where as appropriate.

For characterisation of the kinetic parameters of SAA transport, uptake of each SAA was measured by HPLC following their addition to the incubation medium to provide a final assay concentration range of 5 - 5000 µM. Similarly, where [^{3}H]-labelled amino acids substrates were employed, fixed amounts of [^{3}H]label were mixed with varying amounts of unlabelled amino acid to give a final assay concentation range of 5-5000 µM.

Incubation was allowed to proceed at 35°C for 2 min (kinetic assays) or 10 min (for determination of rate linearity). Uptake was terminated by withdrawing a 200 µl aliquot (0.4 mg - 0.5 mg protein) of reaction mixture and rapidly transferring to 1.5 ml capacity plastic microfuge tubes containing 100 µl of 50:50 (v/v) mixture (specific gravity 1.07) of Dow Corning MS 550 silicone oil and dinonyl phthalate followed by immediate centrifugation for 2 min at 15,000 rpm in a Beckman E

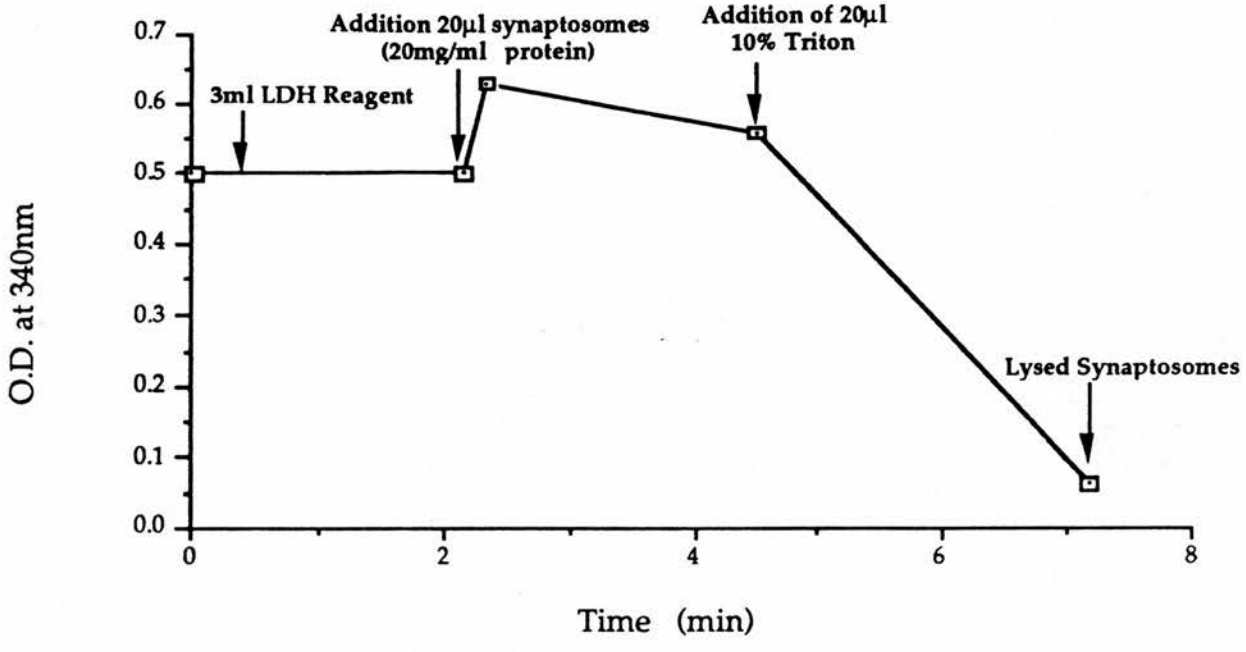


Fig. 2.2 U.V. assay for assessing membrane integrity of synaptosome fractions.

microfuge. The supernatants from each tube were aspirated and discarded, and the surface of the oil washed twice with 500 µl aliquots of distilled water prior to careful aspiration of the oil layer, the synaptosomal pellet being retained in the microfuge tube.

(v) Measurement of Uptake

(a) The uptake of ^{3}H-labelled substrates was measured by cutting the microfuge tubes ~ 5 mm from the conical tip and the synaptosomal pellet (contained in the bottom part of the tube) was solubilised with 50 µl 'Solusol' (National Diagnostics) tissue solubiliser for 1-2h at room temperature. The tubes containing the solubilised pellets were subsequently placed in Pico 'Hang-In' vials (Canberra-Packard) and 5 ml scintillation fluid (9:1 v/v) Soluscint 'O':Triton X-100 added to each vial and thoroughly mixed. Radioactivity [^{3}H] in the pellets was measured in a Packard 300 Liquid Scintillation Spectrometer at a counting-efficiency of 40%.

(b) Uptake of all SAAs was measured by HPLC following preparation of samples from the synaptosomal pellets as follows. The pellets were acidified by the addtion of 200 µl 1% (w/v) $HClO_4$, the suspension mixed and left at 4°C before centrifugation at 15,000 rpm for 15 min. Aliquots (150 µl) of the resulting supernatants were neutralised by addition of 15 µl of a solution containing 1.5M K_2CO_3/0.5M NaTes and kept on ice for 1h to precipitate $KClO_4$ before finally being centrifuged at 15,000 rpm for 15 min as before. Aliquots (135 µl) of the resulting neutralised supernatants were mixed with 15 µl of the appropriate internal standard and the combined sample stored at -20°C prior to HPLC analysis.

2.3 Brain Cell Cultures

(i) Preparation of Cerebellar Granule Cells

Primary cultures of cerebellar granule cells were prepared essentially as described by Drejer and Schousboe (1988) and Schousboe et al. (1989). Cerebella from seven-day-old mice were removed following animal sacrifice by decapitation, and placed in freshly prepared Puck's solution (137 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 5 mM glucose pH 7.4). The tissue was then cut into small pieces and cells dissociated by mild trypsinisation (0.1% (w/v) trypsin; 37°C for 10 min). This was followed by rapid centrifugation to pellet dissociated tissue. The supernatant was discarded and the pellet resuspended in 5 ml of Puck's solution before gentle passage (repeat 3 times) through a stainless steel syringe (2 mm inner diameter) in order to dissociated tissue clumps. To this suspension, 15 ml of modified Eagle's minimum essential medium (MEM; for full details of tissue culture medium see Appendix 4) supplemented with KCl (24.5 mM), glucose (30 mM), p-aminobenzoic acid (7 μM) and insulin 200 mU/L (neuronal-medium) was added. Small clumps remaining in the suspension were allowed to settle for 5 min and then removed. The cell suspension was centrifuged at 500g for 10 min to pellet the cells, before resuspension in neuronal-medium containing 10% (w/v) fetal calf serum. Cells were seeded into poly-L-lysine coated 3-cm Petri dishes or 24-place multi-well plates. The antimitotic agent cytosine arabinoside (40 μM) was added to cultures after 4 days, a treatment which led to virtual disappearance of glial elements. Experiments were carried out on the cultures after 8-10 days in vitro (DIV).

(ii) Preparation of Cortical Neurons

Primary cultures of cerebral cortex neurons were prepared from sixteen-day-mouse foetuses as described by Dichter (1978) and Hertz et al., (1978a). Following tissue dissection of cerebral cortex, tissue was trypsinised by exposure to 0.5% trypsin (w/v) in Puck's solution and subsequently centrifuged for 10 min at 900g. The pelleted cells were resuspended (1.2×10^6 cells/ml) in a modified Eagle's MEM containing 20% (w/v) inactivated fetal calf serum and 20% (w/v) horse serum. Cells were seeded in poly-L-lysine coated 3-cm or 24-well Petri-dishes. Following a 15 min incubation at 37°C, unattached cells were removed together with the medium which was then replaced by an analogous medium containing additionally 24.5 mM KCl, 7 mM glucose, 7 µM p-aminobenzoic acid and 100 mU/L insulin. After 4 days in culture, cells were exposed to 20 µM cytosine arabinoside to prevent glial cell proliferation. Following this treatment the medium was changed to one without the mitotic inhibitor and the cells were used for experiments at 8-10 DIV. Primary cultures of either cortical neurons or cerebellar granule cells have been shown to be minimally contaminated with either glial elements or other neuronal cell types (Schousboe et al., 1985).

(iii) Preparation of Cortical Astrocytes

Primary cultures of cortical astrocytes were prepared essentially as described by Hertz et al. (1982, 1989(b)). Prefrontal cortices were dissected from cerebral hemispheres from newborn (0-24h) mice, cut into 1 mm cubes and vortexed at maximum speed. The suspension was then passed through sterile nylon sieves (80 µm and 10 µm pore size) into a modified Eagle's minimum essential medium containing 20% (v/v) inactivated foetal calf serum and inoculated into the individual wells of Nunclon 24-well multi-test dishes. The cultures were grown for a total of

three weeks at 37°C in atmospheric air and CO_2 mixture (95%/5%; v/v) with a relative humidity of 90%. The culture medium was exchanged two days after inoculation and subsequently three times a week. After two weeks of culture, when the cells are confluent (due to a rapid proliferation) 0.25 mM dibutryl cyclic adenosine monophosphate (dBcAMP) was added to the cultures. This addition of dBcAMP causes a pronounced morphological and some biochemical differentiation of the cells. In addition, treatment further reduces the small number (< 5%) of macrophages, the major non-astroglial cell population present in these cultures. Under these conditions, the cultures consist of well-differentiated astrocytes and have shown to be devoid of neurons.

(iv) Transport Assay for SAA Aptake

The cell culture medium in each well was aspirated and dried free of growth medium. Aliquots (500 µl) of HEPES-buffered saline (10 mM HEPES, 135 mM NaCl, 5.0 mM KCl, 1.0 mM $CaCl_2$, 0.6 mM $MgSO_4$ and 6 mM glucose, pH 7.4) were added to each well and the cells preincubated for 3 min at 37°C. Subsequently this medium was exchanged with HEPES buffered saline (HBS) containing differing concentrations (0-5000 µM) of CSA, CA, HCSA and HCA and incubated for 2 min at 37°C. The medium was removed by a rapid evacuation and the cell monolayer carefully washed twice with 500 µl aliquots of HBS.

(v) Measurement of Uptake

As for synaptosomes, the uptake of CSA, CA, HCSA and HCA was measured by HPLC following preparation of samples from each well within a 24-well plate as follows. Following aspiration of the final wash, 500 µl aliquots of ice-cold 70% (v/v) ethanol was added to each well. The cells were removed by scraping and the suspension transferred to

Eppendorf tubes prior to centrifugation at 15,000 rpm for 3 min. Aliquots (300 µl) of the supernatants were lyophilized then resuspended in a fixed volume of 5 µM homoserine (which acted as an appropriate internal standard and stored at -20°C for HPLC analysis. The remaining supernatant was carefully removed and the dried cell pellets incubated at 37°C for 1h with 200 µl 1.0M KOH. After this time the solubilised pellet volume was adjusted to 500 µl by addition of distilled water. Aliquots (25 µl) were taken from each sample for measurement of protein content using the method of Lowry et al. (1951) (Appendix 3) with bovine serum albumin (100 µg/ml) as the external standard.

2.4 High Pressure Liquid Chromatography (HPLC) of Amino Acids

(i) Introduction

The last ten years has seen the development of an alternative to the classical ion-exchange methods (Moore et al., 1958) for the quantitative estimation of free amino acids by HPLC (Ettre, 1981). The use of precolumn fluorogenic derivatization in combination with reversed phase liquid chromatography has simplified and improved the separation of amino acids and is more sensitive and potentially faster than ion-exchange methods (Lindroth and Mopper, 1979).

(ii) o-Phthaldialdehyde (OPA)

The use of OPA for the quantitation of primary amino acids was originally reported by Roth (1971). In the presence of a reducing agent 2-mercaptoethanol (2MCE) the OPA reacts on alkaline medium with primary amines to form highly fluorescent thioalkyl substituted isoindoles (Simons and Johnson, 1976) (Fig. 2.3). Precolumn OPA/2MCE

derivatization is ideally suited to amino acid analysis by reversed-phase chromatography because:-

(1) The fluorescent products have a high quantum yield that results in high sensitivity allowing the determination of extremely low amino acid concentrations in the sub-picomole range.

(2) The OPA is water-soluble, non-fluorescent, the derivatives are rapidly formed at room temperature and are less polar than the free amino acids.

(3) The chemistry of OPA derivatization is apparently insensitive to most matrix interferences (Quereshi et al., 1984).

(iii) Internal Standards

Internal standards were included with all samples for HPLC analysis as a routine check for correct derivatization and pipetting. For samples derived from cell culture uptake experiments, homoserine was used as the internal standard which eluted between serine and glycine with no interference from any endogenous peak contained in the sample. For samples from synaptosomal uptake experiments, one of the acidic SAAs, CA or CSA was substituted for homoserine since this latter compound co-chromatographed in the synaptosomal extracts with an endogenous peak.

(iv) Chromatography

Prior to chromatography, aliquots of amino acid standard solution, supernatant samples from cell culture neutralised synaptosomal extracts and the OPA/2MCE reagent were individually filtered into clean microfuge tubes using disposable 0.45 μm Acro LC3A filter discs (Gelman Sciences). A high performance liquid chromatograph (LKB) comprising

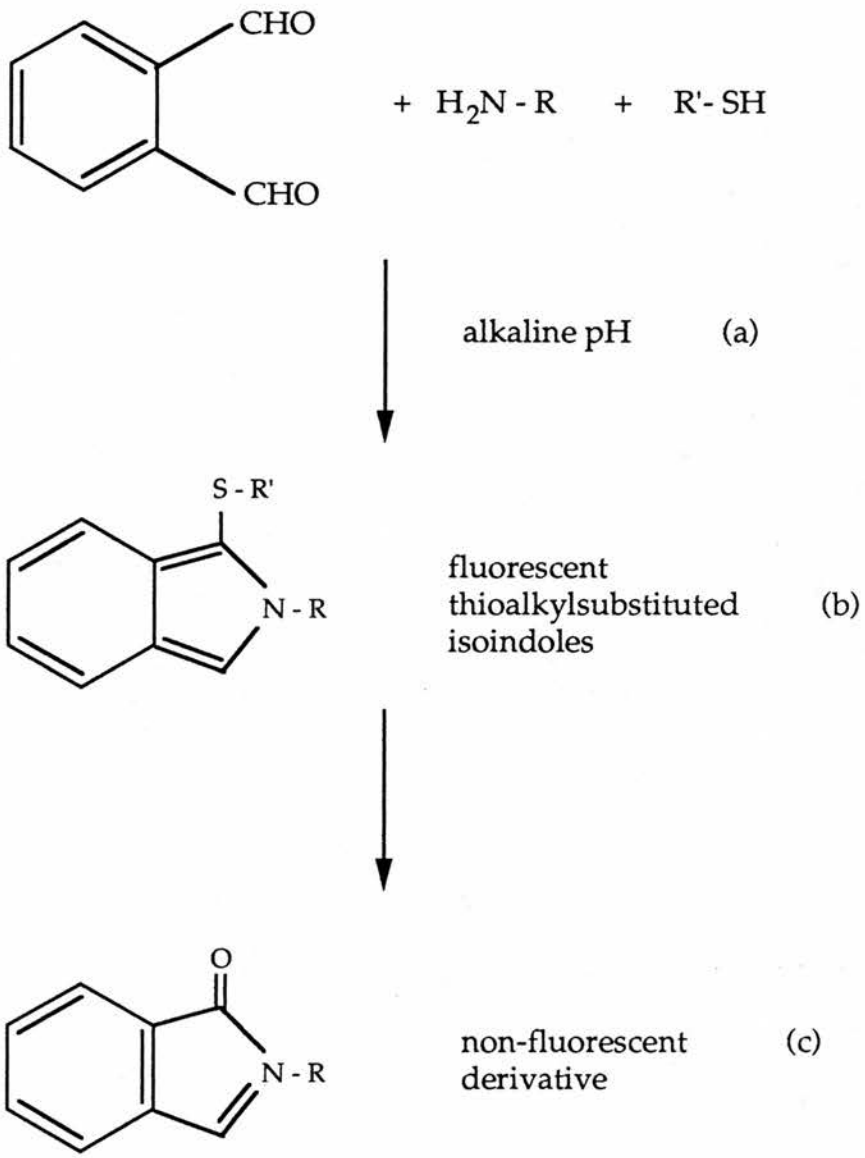


Fig 2.3 Derivatisation of primary amino acids with the o-phthaldialdehyde (OPA) reagent.
Under conditions of alkaline pH and in the presence of a strong reducing agent (a), OPA reacts with the primary amino acids to form highly fluorescent thioalkylsubstituted isoindoles (b). These derivatives are unstable and by chemical rearrangement revert to non-fluorescent products (c).

two Model 2150 pumps with a Model 2151 LC Controller was used throughout the study. A 20 µl aliquot of standard amino acid (5 µM) or sample was derivatized automatically via a Model 2157 Autosampler (LKB) by mixing with 40 µl OPA/2MCE reagent (Roth, 1971) for a period of 90 sec. Derivatized samples (40 µl) were injected via the autosampler directly to a Chromspher C-18 (Chrompack, UK) 5 µm partical diameter reversed-phase cartridge column 100 x 4 mm (i.d.).

This column was protected by using in conjunction with a Chrompack (5 µm particle diameter) guard column 10 x 4 mm (i.d.). This column removes any strongly retained contaminants within the sample matrix. Finally to extend the life of the analytical column a solvent saturation column (30 x 4 i.d.) packed with Spherisorb 10 µm particle size free silica was situated before the injector.

For the kinetic studies, the mobile phase for separation of CSA, CA and HCSA comprised the aqueous component (A) 100 mM sodium acetate, pH 5.55 and the organic component (B) methanol/H_2O (9:1; v/v). For the resolution of HCA, the pH of the 100 mM sodium acetate was raised to 5.8. The gradient programme for the analysis of all SAAs comprised an isocratic hold at 5% B, 0-4 min (0.75 ml/min); followed by a gradient 5-20% B, 4.7 min (1.0 ml/min); 20-50%, 7-20 min (1.0 ml/min); 50-100% B, 20-23.5 min (1.0 ml/min). The column was re-equilibrated between samples by going from 100% - 5% B over a 10 min period at a flow rate of 1.0 ml/min.

The amino acids eluted from the column were detected using a Merck-Hitachi F1000 variable wavelength Fluorescence Spectrophotometer (excitation wavelength 340 nm; emission wavelength 450 nm) fitted with a 12 µl volume flow cell.

To quantify the endogenous levels of the SAAs in the samples derived from cultured neurons and astrocytes a gradient programme was

devised essentially as described by Waller et al. (1991) which gave a complete separation of CA, CSA, HCA, HCSA together with a variety of neuroactive and other amino acids. The mobile phase solvent A comprised a mixture of 75 mM KH_2PO_4 solution and 10% (v/v) methanol (adjusted to pH 5.85 with NaOH) and the organic component B 100% methanol. The gradient program comprised an isocratic hold at 0% B, 0-2 min; 0-20% B, 2-28 min. The column was re-equilibrated by a rapid step to 100% B over 30 sec, held for 1 min, finally returning to 0% over 5 min. The flow rate throughout the gradient was 1 ml/min.

(v) HPLC Data Analysis

Peak areas of individual SAAs were stored and quantified using a TriVector Trio Chromatography Computing Integrator. The SAAs with other neuroactive amino acids were tentatively identified by the direct comparison of their eluted retention time with the corresponding retention time of an eluted authentic SAA within an external standard mixture analysed under exactly the same conditions. Identification of individual SAAs within samples derived from uptake experiments was further substantiated by analysing duplicate sample aliquots together with a sample aliquot 'spiked' with authentic SAA. 'Millipore' water samples were routinely analysed so as to eliminate any carry-over of any amino acid between experimental samples. For each individual sample containing the appropriate internal standard homoserine or CA/CSA the concentration of the SAA within a given sample was calculated by reference to a previously analysed standard chromatogram containing the same internal standard.

Calculation Example

$$\frac{\text{Sample Peak Area SAA}}{\text{Sample Peak Area Int. Std}} \times \frac{\text{STD Rf (SAA)}}{1} \times$$

pmol int. std. x dilution factor

= pmol/min/mg protein

The kinetic parameters K_m and V_{max} of transport were calculated by weighted non-linear regression analysis, and the correlation relationships of the inhibition profiles by linear regression analysis, using the ENZFITTER software program (Leatherbarrow, 1987). The neuronal, astrocytic and synaptosomal uptake of the various SAAs was found to best-fit the equation:-

$$v = V_{max}[1/(1+K_m/S)] + kS$$

where v and V_{max} indicate the initial and maximum velocities, respectively; K_m, the Michaelis-Menten constant; k, the rate constant of the non-saturable influx component, and S, the substrate concentration (Neame and Richards, 1972). In addition, all uptake data (for estimation of the transport constants) were also analysed by fitting to the Michaelis-Menten equation assuming either a single saturable site or two non-interacting saturable uptake sites of differing affinities.

CHAPTER THREE

RESULTS

3.1 HPLC Calibration Curves for the SAAs

Construction of calibration curves using HPLC for each of the authentic SAAs, viz. CSA, CA, HCA and HCSA (Fig. 3.1) shows that for each of these compounds the measured amount of OPA-derivatized SAA loaded on to the C-18 5 μm reversed-phase column is directly proportional to the corresponding peak area on the chromatogram only up to 0-400 pmol. Therefore prior to HPLC quantitative measurements used in the kinetic analysis, all samples derived from cell culture and synaptosomes containing any of the above SAAs were subsequently diluted with HPLC-grade water until the corresponding amount of measurable SAA eluted from the column was within the range 0-400 pmol. The minimum detection column limits for each SAA in conjunction with the utilisation of the different sensitivity parameters of the fluorescence spectrophotometer was about 200 fmol.

3.2 Time Course of SAA Uptake

Valid use of the Michaelis-Menten equation requires that *initial* rates of uptake are measured. Consequently, the time course of neuronal and astrocytic uptake of CSA, CA, HCSA, HCA and D-[^{3}H] Asp was determined for each compound at an assay concentration of 100 μM and 2500 μM at 35°C over a 20-min incubation period. The net uptake for each compound was found to deviate from linearity following 3.0 - 3.5 min incubation at 35°C. A typical progress curve, in this case for CSA uptake into synaptosomes is shown in Fig. 3.2. For all subsequent kinetic studies of SAA uptake, assays using cultured neurons and astrocytes and synaptosomal preparations were terminated following a 2-min incubation period at 35°C.

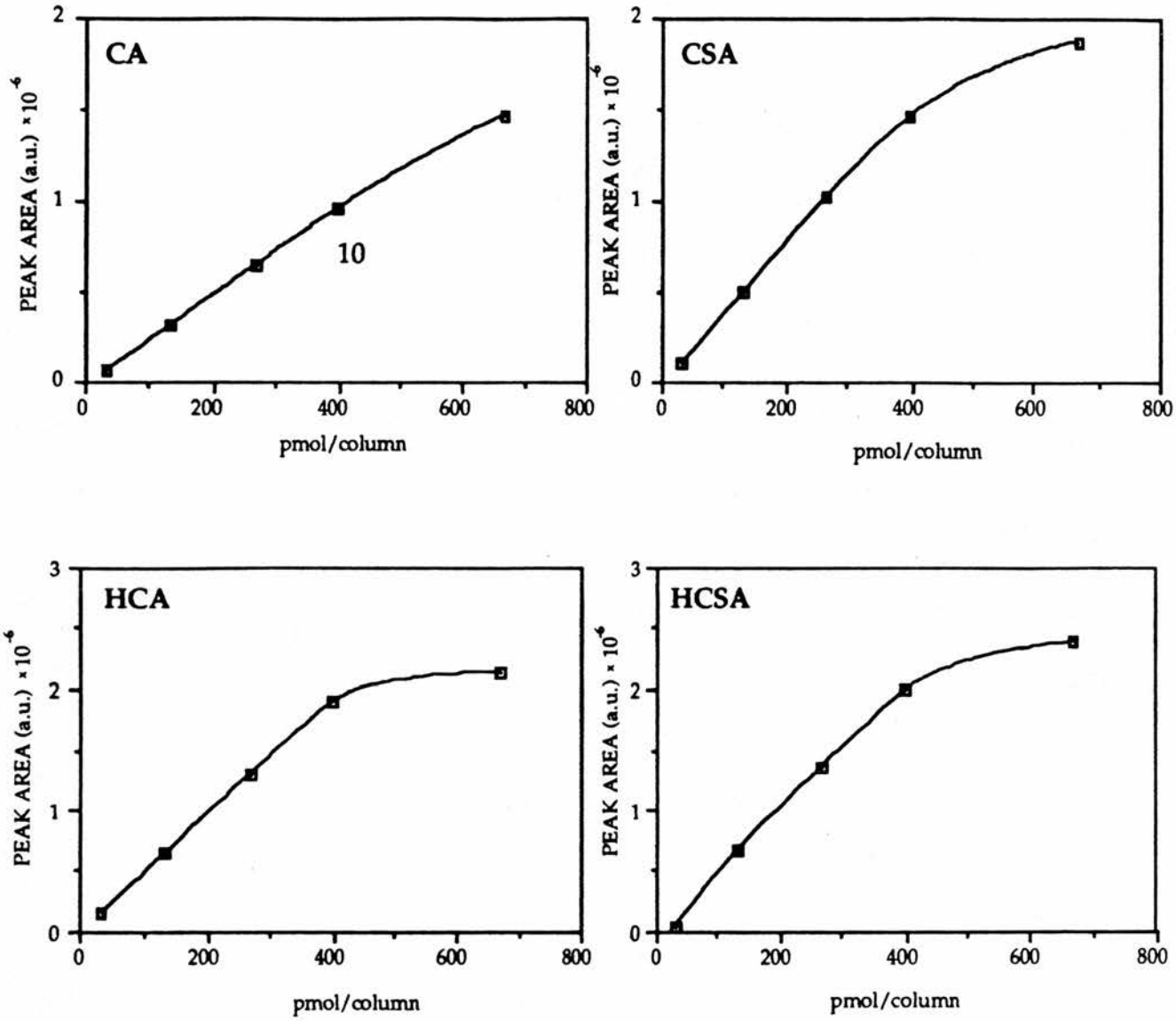


FIG. 3.1 HPLC calibration curves for CA, CSA, HCA and HCSA

The procedure for the HPLC analysis of varying amounts of o-phthaldialdehyde derivatized authentic CA, CSA, HCA and HCSA was as described in Methods. Representative graphs in which the measured amount of CA, CSA, HCA and HCSA loaded on to the 5 mm C-18 column is directly proportional to the corresponding peak area only in the range 0-400 pmol. Subsequently all standards and samples prior to HPLC analysis were diluted with HPLC water until the amount of o-phthaldialdehyde derivatized amino acid eluted from the column was within this range.

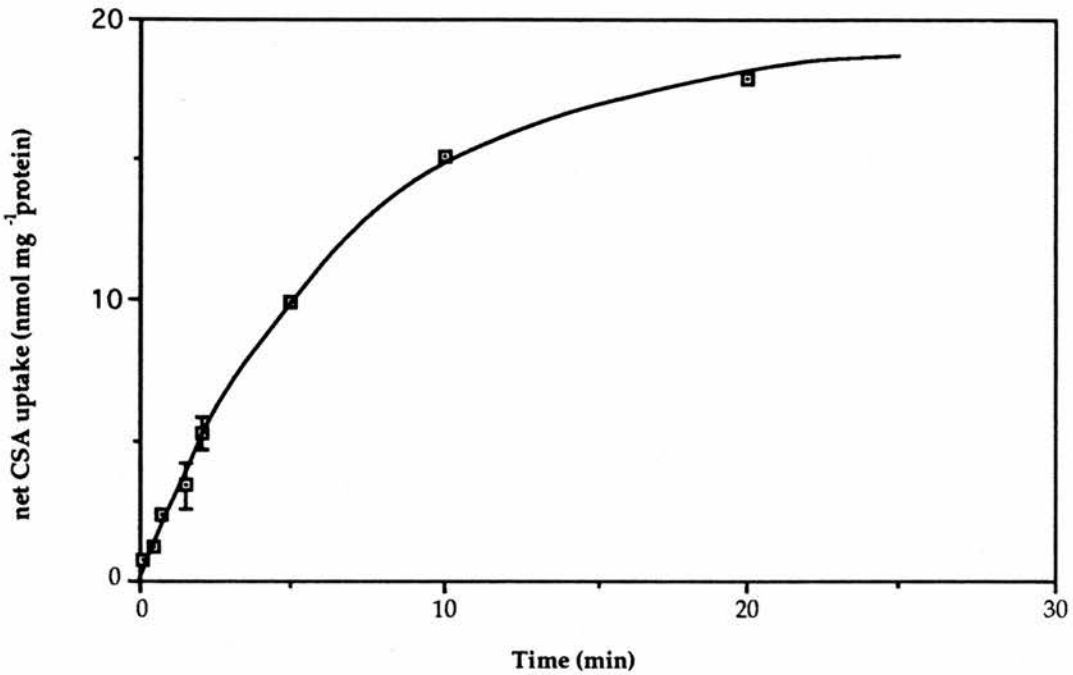


FIG. 3.2 Time course of CSA uptake by rat cerebrocortical synaptosomes

Synaptosomes (8-10 mg/ml protein) were added at 35°C to incubation medium which contained an assay concentration of 100 μM CSA. Uptake was terminated at varying time intervals (0-20 min) by the withdrawal of an aliquot (200 ml) from the reaction mixture followed by immediate centrifugation through a 50:50 (v/v) mixture of Dow Corning MS 580 silicone oil and dinonylphthalate for 2 min at 15000g. Synaptosomal extracts were prepared and subsequently measured by HPLC analysis as described in Methods. Results from a representative experiment show synaptosomal uptake of CSA was found to deviate from linearity following 25-30 min at 35°C. Values for each time point are the mean ± SEM of triplicate assays which in turn were subjected to HPLC analysis undertaken in duplicate.

3.3 Effect of Na^+ on SAA Uptake

Total uptake of all the SAAs was partially sodium-dependent in cultured neurons and astrocytes and synaptosomes. A representative experiment is illustrated in Fig. 3.3 in which using fixed concentrations of HCSA (100 - 5000 μM) synaptosomal uptake was performed in the presence and absence of extracellular sodium (135 mM), sodium ions replaced with an equimolar concentration of lithium ion. At 100 μM HCSA, in the absence of extracellular sodium, uptake (sodium-independent component) was typically < 20% of the total uptake. The sodium-independent component of uptake showed a marked increase with increasing assay concentrations such that at HCSA concentrations of 2500 μM and 5000 μM it was about 60-80% of total uptake.

3.4 Kinetic Characterisation of SAA Uptake

In order to determine the kinetic parameters, K_m and V_{max}, the total uptake of the SAAs, viz, CSA, CA, HCSA and HCA by primary cultures of cerebellar granule cells, cortical neurons, cortical astrocytes and cerebrocortical synaptosomes was measured over a substrate concentration range of 0-5000 μM. The uptake of each SAA, quantified by HPLC analysis, exhibited a substrate concentration dependency over this range. This is illustrated in Figs. 3.4 - 3.8 which show a series of representative HPLC chromatograms obtained for each of the SAAs in cultured neurons, astrocytes and synaptosomes as described in Methods. In Fig. 3.4, the separation of a standard amino acid mixture is presented in which the resolution of CA and HCA from other amino acids is clearly shown. Compounds of neurochemical interest i.e. Asp, Glu, glutamine, glycine, taurine and GABA were routinely included in the standard amino acid mixture, together with the appropriate internal standard

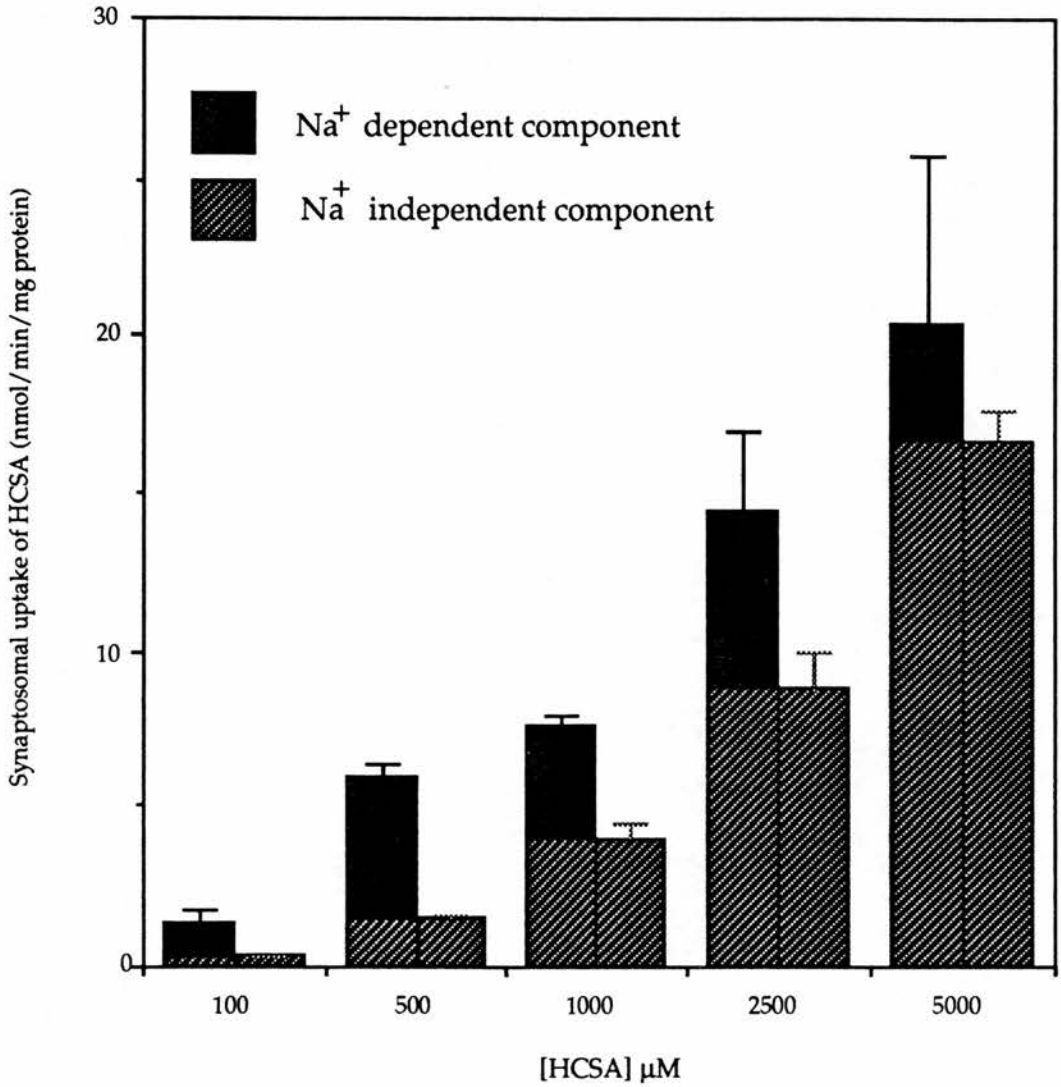


FIG. 3.3 Effect of the Na^+ ion on HCSA uptake in rat cerebrocortical synaptosomes

Synaptosomes (8-10 mg/ml protein) were incubated at 35°C for 2 min in incubation medium containing varying concentrations of HCSA (100-5000 μM) and in the absence and presence of extracellular sodium (135 mM). Uptake of HCSA was measured by HPLC analysis and is expressed as nmol/min/mg protein. Values are the mean ± SEM of triplicate assays with HPLC analysis undertaken in duplicate. Results from a representative experiment show the increase of the Na^+-independent uptake component with increasing HCSA concentration. At 100-500 μM HCSA, the Na^+-independent component of uptake is in the order of 20-24%, while at 2500-5000 μM HCSA the Na^+-independent component of uptake is 60-81%.

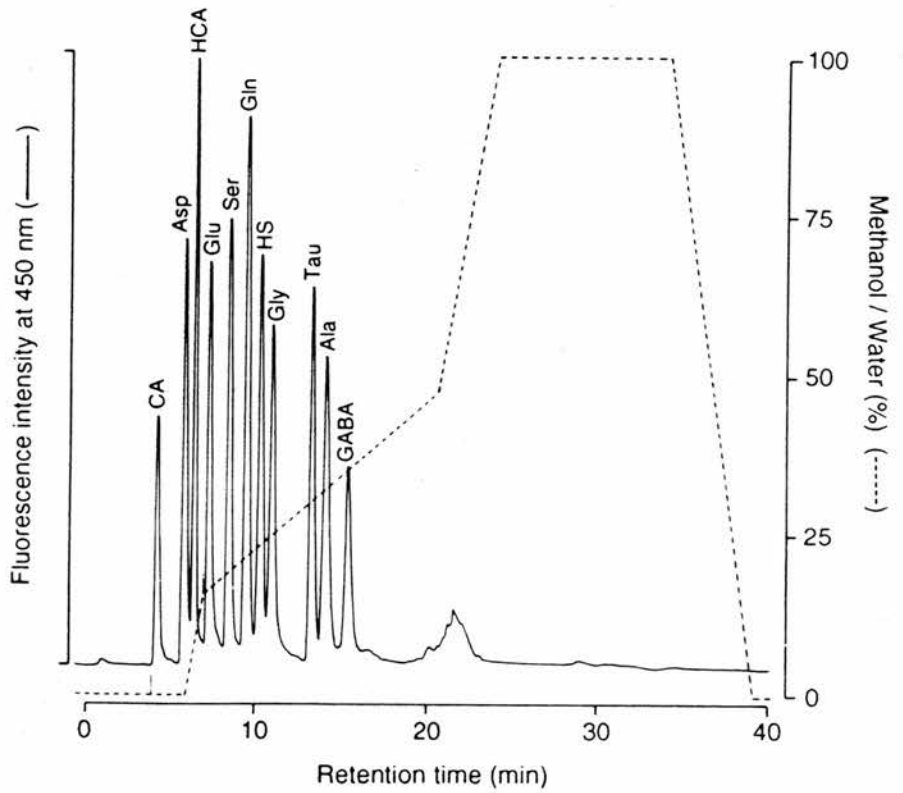


FIG. 3.4 HPLC analysis of *o*-phthaldialdehyde-derivatised amino acids representing a 5 mM standard amino acid mixture

The procedure for HPLC separation and detection of derivatised amino acids using the 0.1M Na acetate pH 5.8: methanol gradient is as described in Methods. A representative chromatogram is presented which illustrates the separation of a number of authentic neuroactive amino acids. The amino acids in the mixture eluted with their respective retention times in the order CA = cysteic acid, ASP = aspartate, HCA = homocysteic acid, GLU = glutamate, SER = serine, GLN = glutamine, HS = homoserine, TAUR = taurine, ALA = alanine, GABA = γ-aminobutyric acid. Each amino acid peak is the equivalent to 33.3 pmoles eluted from the 5 μm C-18 column. For calibration purposes each peak was normalised to the appropriate added internal standard CA or HS.

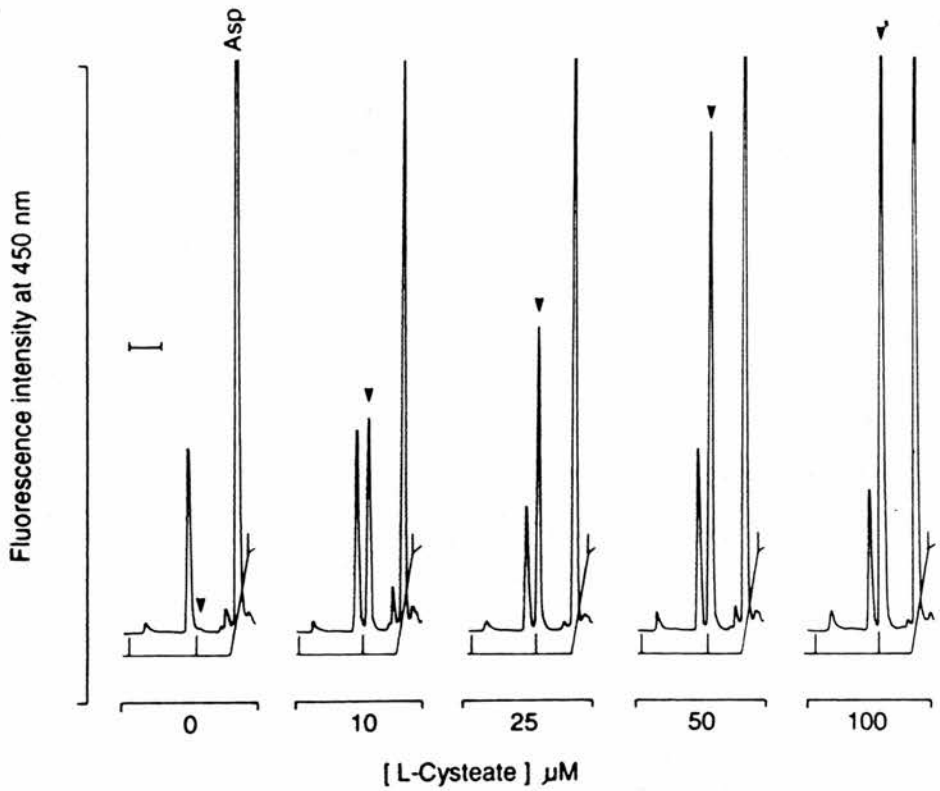


FIG. 3.5 HPLC analysis of L-cysteate transport in cerebellar granule neurons

Primary cultures of cerebellar granule cells derived from mouse brain were incubated for 2 min at 35°C with varying concentrations of CA. Following termination of uptake and subsequent ethanolic extraction, free amino acids in the cells were determined by HPLC analysis as described in Methods. A representative series of partial chromatograms is presented which illustrates the accumulation of CA (elution position indicated by the vertical arrow) in cerebellar granule cells as a function of its extracellular concentration (0 - 100 µM). The concentration-dependent uptake of CA can be observed as an increase in the fluorescence intensity of 450 nm. The horizontal bar (-) represents a time scale (1 min).

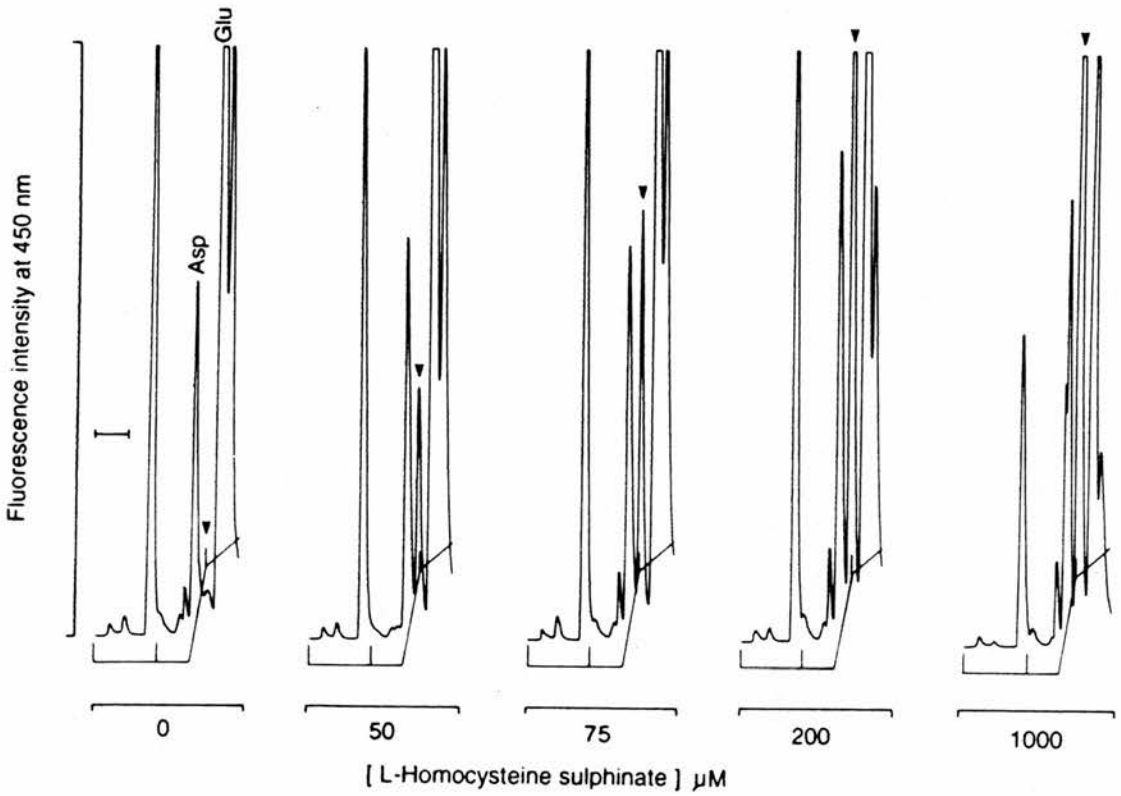


Fig. 3.6 HPLC analysis of L-homocysteine sulphinate transport in cortical astrocytes

Primary cultures of cerebral cortex astrocytes derived from mouse brain were incubated for 2 min at 35°C with varying concentrations of HCSA. Following termination of uptake and subsequent ethanolic extraction, free amino acids in the cells were determined by HPLC analysis as described in Methods. A representative series of partial chromatograms is presented which illustrates the accumulation of HCSA (elution position indicated by the vertical arrow) in cortical astrocytes as a function of its extracellular concentration (0 - 1000 μM). The concentration-dependent uptake of HCSA can be observed as an increase in the fluorescence intensity at 450 nm. The horizontal bar (-) represents a time scale (1 min).

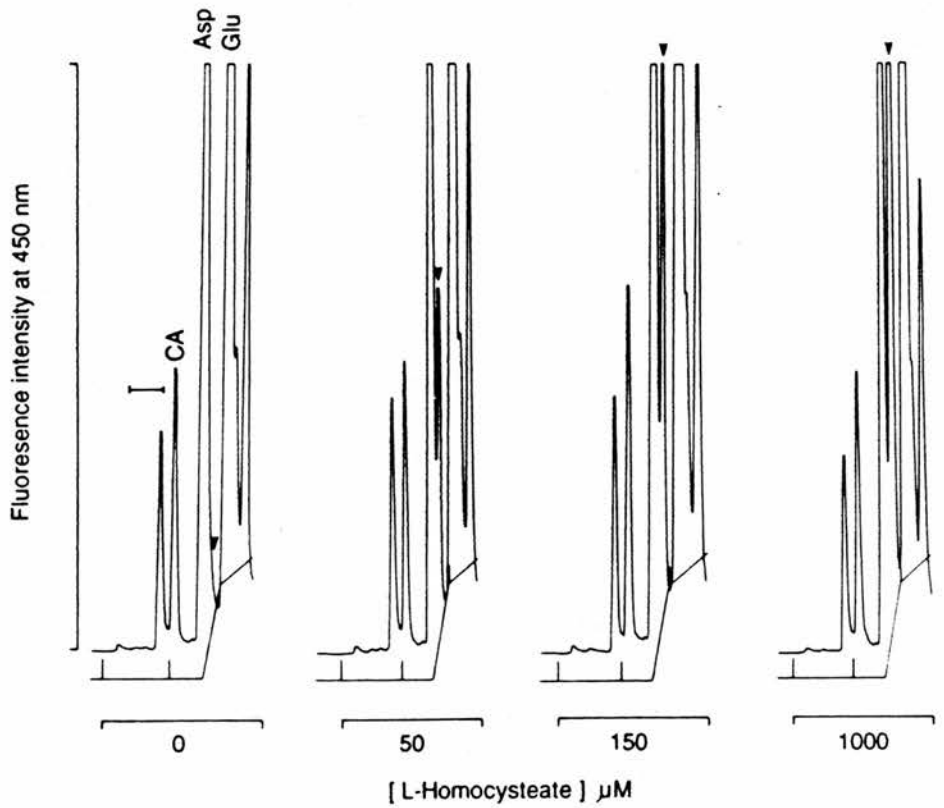


FIG. 3.7 HPLC analysis of L-homocysteate transport in cerebrocortical synaptosomes

Cortical synaptosomes derived from rat brain were incubated for 2 mins at 35°C with varying concentrations of HCA. Following termination of uptake and subsequent PCA extraction, free amino acids in the synaptosomal extracts were determined by HPLC analysis as described in Methods. A representative series of partial chromatograms is presented which illustrates the accumulation of HCA (elution position indicated by the vertical arrow) in cortical synaptosomes as a function of its extracellular concentration (0 - 1000 μM). The concentration-dependent uptake of HCA can be observed as an increase in the fluorescence intensity at 450 nm. The horizontal bar (-) represents a time scale (1 min).

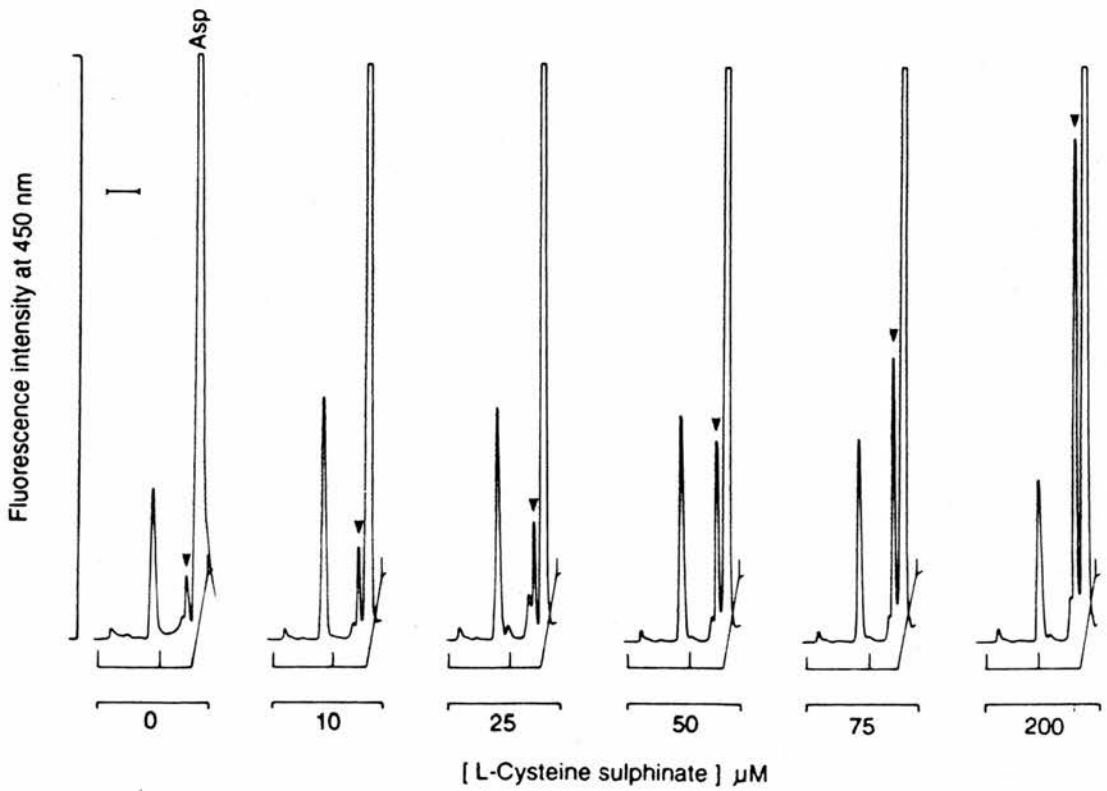


FIG. 3.8 HPLC analysis of L-cysteine sulphinate transport in cerebrocortical synaptosomes

Cortical synaptosomes derived from rat brain were incubated for 2 mins at 35°C with varying concentrations of CSA. Following termination of uptake and subsequent PCA extraction, free amino acids in the synaptosomal extracts were determined by HPLC analysis as described in Methods. A representative series of partial chromatograms is presented which illustrates accumulation of CSA (elution position indicated by the vertical arrow) in cortical synaptosomes as a function of its extracellular concentration (0 - 200 μM). The concentration-dependent uptake of CSA which is observed as an increase in the fluorescence intensity at 450 nm was calculated after the subtraction of an endogenous peak corresponding by its retention time to authentic CSA which was present in synaptosomes. The horizontal bar (-) represents a time scale (1 min).

(homoserine, CA or CSA). A 5 μM standard mixture was employed equivalent to the loading of 33.3 pmol of each amino acid onto the 5 μm C-18 HPLC column. Figs. 3-5 - 3.8 represent typical profiles of neuronal, astrocytic and synaptosomal free amino acids following incubation with varying concentrations of the SAAs and either ethanolic or PCA extraction. The uptake of [^{3}H]D-Asp in synaptosomes quantified by liquid scintillation spectrometry also exhibited a substrate concentration dependency over the concentration range 0-5000 μM.

For all the brain preparations the rate of uptake for each of the SAAs comprised a distinct non-saturable component and a saturable carrier-mediated transport component. This is illustrated in Fig. 3.9 which clearly demonstrates the significant contribution of the non-saturable component of uptake in particularly at high substrate concentrations. Representative illustration of 'corrected' velocity-substrate curves are shown in Fig. 3.10 for respectively CSA uptake in cultured neurons and astrocytes and CSA, CA, HCSA and HCA uptake in synaptosomes (Fig. 3.11). The calculated kinetic constants, K_m and V_{max} for all the SAAs together with Glu are presented in Table 3.1. In each neuronal preparation CSA and CA exhibited a single high-affinity uptake system with K_m values ranging from 14-40 (μM) thus displaying a higher affinity for the carrier than in cultured astrocytes (K_m = 88 - 101 μM). The magnitude of the kinetic constants describing CSA and CA uptake in the different brain preparations closely resemble those of Glu transport, the only anomaly being the observed lower V_{max} values for the astrocytic uptake of CSA and the synaptosomal uptake of CSA and CA (see Table 3.1). The V_{max} of CA uptake in cultured neurons and astrocytes is signficantly higher than that of CSA uptake.

HCSA and HCA in cultured neurons and astrocytes, and synaptosomes are also seen to be transported by a single monophasic

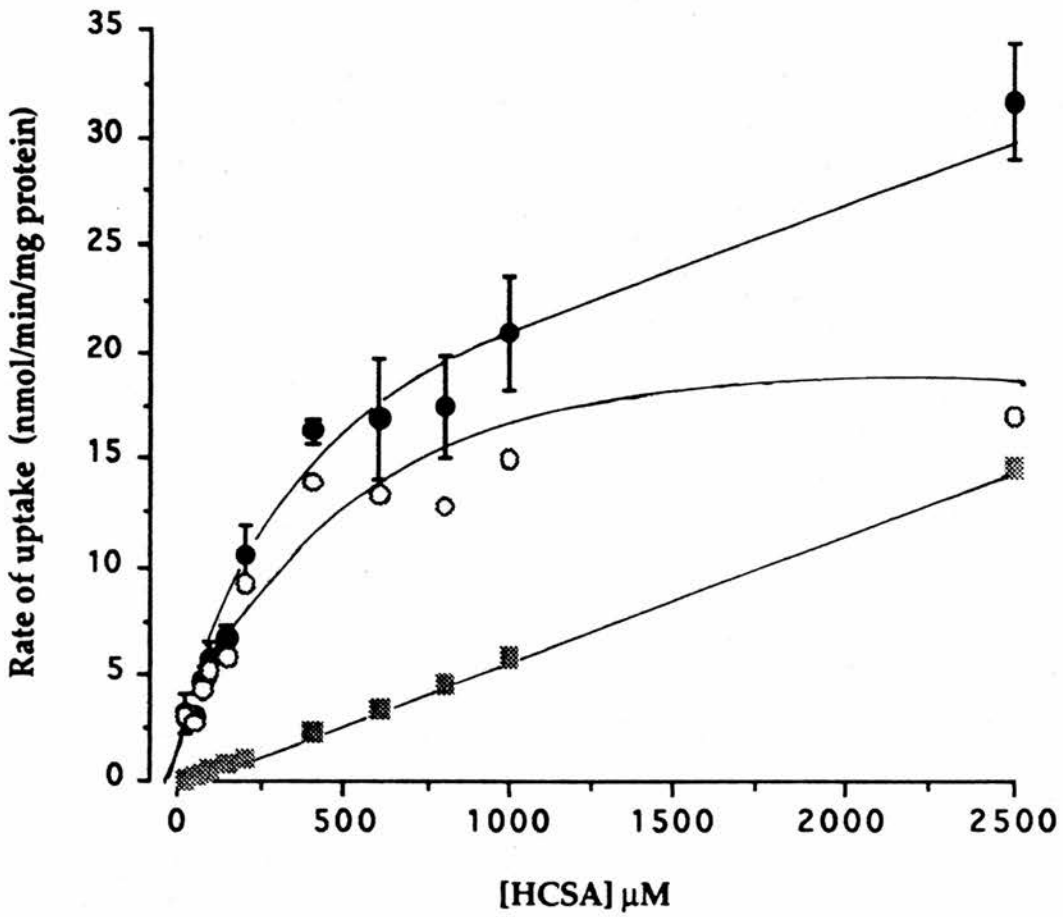


FIG. 3.9 Transport of HCSA by cultured astrocytes

The measured uptake of HCSA [●] was biphasic and could be most appropriately separated into a non-saturable influx component [▩] and a saturable carrier-mediated component [○]. Uptake was measured by the following equation:-
$v = V_{max}S/(K_m + S) + kS$ where v and V_{max} are the initial and maximum velocities respectively and K_m the Michaelis constant for saturable uptake: k is the rate constant for non-saturable influx. Data points are the mean ± S.E.M. of 2 experiments in which assays were carried out in quadruplicate. Zero time controls were also subtracted to correct for extracellular trapping.

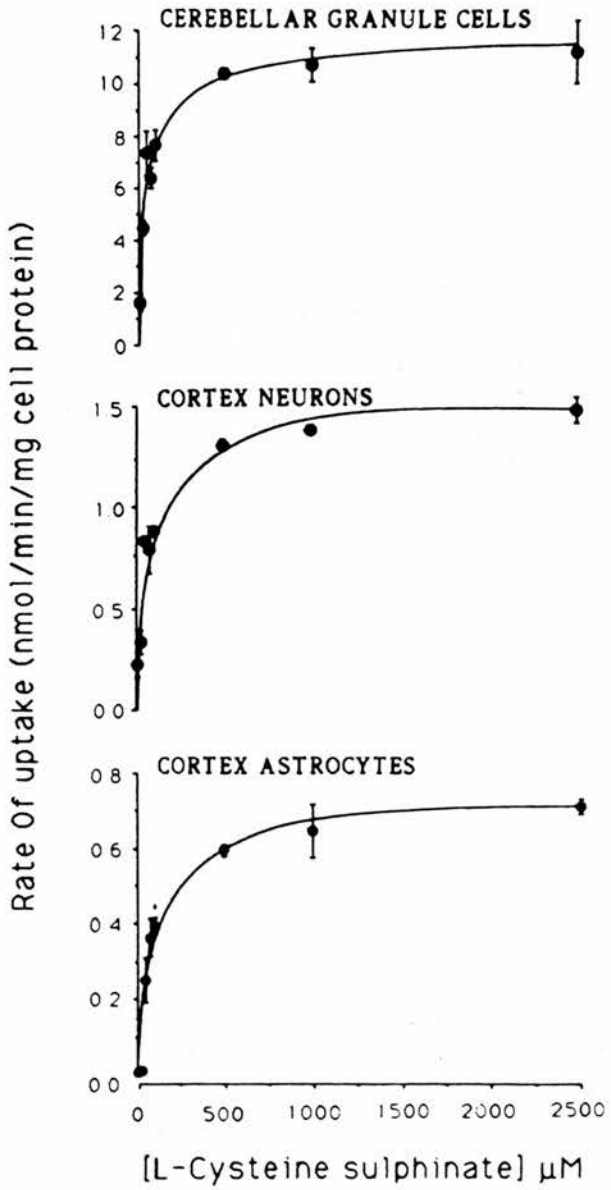


FIG. 3.10 Transport of L-cysteine sulphinate in primary cultures of neurons and astrocytes

Primary cultures of (a) cerebellar granule cells, (b) cortical neurons and (c) cortical astrocytes were incubated at 35°C for 2 min with varying (0-5000 µM of CSA. Only uptake values ≤ 2500 µM CSA have been illustrated). The rates of uptake have been corrected for the non-saturable influx component. Data points are the mean ± S.E.M. of 8 replicates in which assays were carried out in quadruplicate in which HPLC analysis was undertaken at least in duplicate.

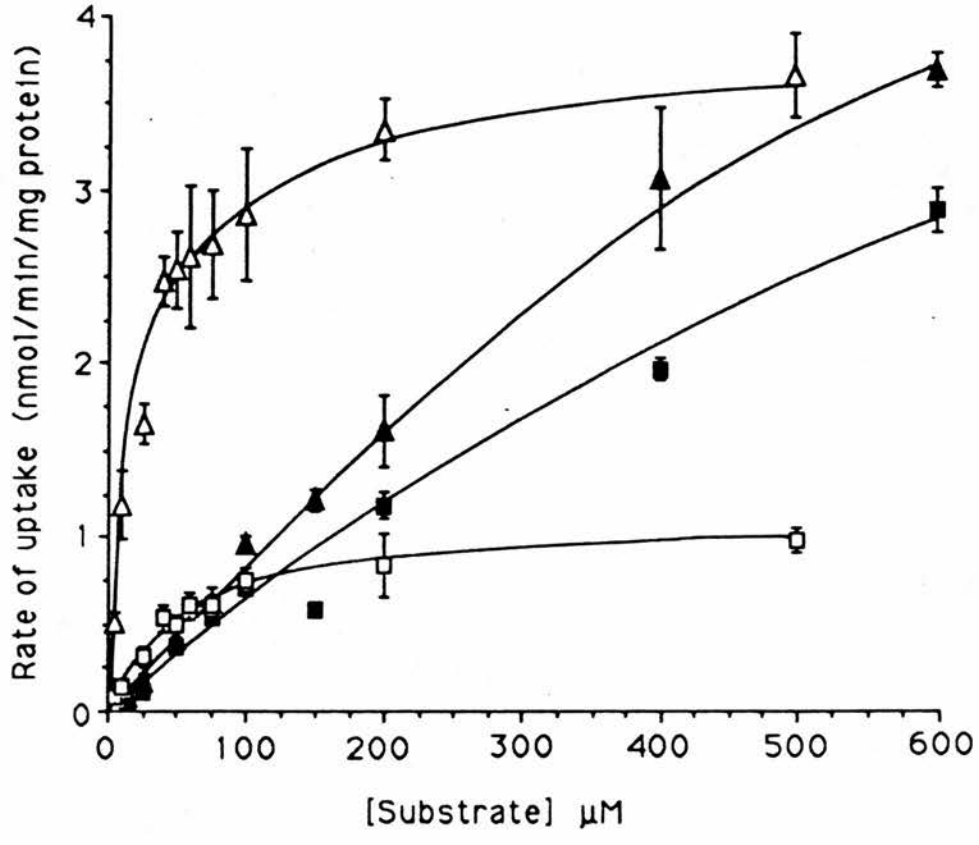


FIG. 3.11 Transport of CSA, CA, HCSA and HCA in rat cerebrocortical synaptosomes

Synaptosomal fractions were incubated at 35°C for 2 min with varying concentrations of 0-5000 µM of CSA [□], CA [△], HCSA [■] and HCA [▲]. The rates of uptake have been corrected for the non-saturable influx component. For purposes of clarity only uptake values of ≤ 600 µM have been illustrated. Data points represent the mean ± S.E.M. of 8 replicates. HPLC analysis of each replicate was undertaken at least in duplicate.

TABLE 3.1

KINETIC CONSTANTS FOR SAA UPTAKE BY PRIMARY CULTURES OF NEURONS, ASTROCYTES AND CEREBROCORTICAL SYNAPTOSOMES

	K_m (μM)				V_{max} (nmol/min/mg protein)			
Amino Acid	**Cx**	**Gn**	**Ast**	**Syn**	**Cx**	**Gn**	**Ast**	**Syn**
CSA	58 ± 8	41 ± 10	101± 8	57 ± 6	1.5 ± 0.2	11.1 ± 0.9	0.7 ± 0.1	1.2 ± 0.1
CA	14 ± 4	41 ± 8	88 ± 12	23 ± 3	5.4 ± 0.3	25.2 ± 1.7	27.2 ± 2.9	2.6 ± 0.1
HCSA	525 ± 159	486 ± 110	225 ± 57	502 ± 152	3.0 ± 0.4	10.8 ± 1.0	18.3 ± 2.5	6.1 ± 1.3
HCA	1210 ± 101	941 ± 62	901 ± 334	1550 ± 169	3.8 ± 0.3	15.7 ± 0.5	31.8 ± 10.9	10.3 ± 1.1
*GLU	43 ± 11	42 ± 4	67 ± 7	8.6 ± 1.5	5.9 ± 0.7	10.2 ± 0.5	14.9 ± 0.6	15.4 ± 1.8

Primary cultures of cortical neurons (Cx), cerebellar granule cells (Gn), cortical astrocytes (Ast) and rat cortical synaptosomes (Syn) were incubated for 2 min at 35°C in the presence of increasing concentrations (0 - 5000 μM) of the SAAs. The kinetic parameters K_m, V_{max} represent mean values (± S.E.M.) of 4 - 6 determinations. Data were analysed by a non-linear regression curve fitting program (ENZFITTER, Leatherbarrow, 1987).

*Values for the kinetic constants of Glu for cell culture are taken from Drejer et al. (1982).

system. In direct comparison with their respective lower homologues CSA and CA however, HCSA and HCA exhibit a low affinity for the uptake system with K_m values ranging from 225 - 1550 μM. The V_{max} values of HCSA and HCA uptake for each of the preparations used are essentially similar to the reported values for Glu uptake. The respective V_{max} values for HCSA and HCA uptake are signifciantly higher in cultured astrocytes than that meaured in cultured neurons (see Table 3.1). In primary cultures the SAAs, together with Glu, exhibit greater V_{max} values (10.2 - 25.2 nmol/min/mg protein) in cerebellar granule cells than in cortical neurons (1.5 - 5.9 nmol/min/mg protein). These values are in accord with the known glutamatergic role of cerebellar granule cells in comparison to cortical neurons, which are recognised as mainly GABAergic (Drejer and Schousboe, 1988).

3.5 Specificity of SAA Transport

Synaptosome fractions derived from rat cerebrocortex were resuspended in incubation medium and incubated at 35°C together with fixed concentrations of CSA, CA, HCSA and HCA at an assay concentration which reflected their respective K_m values of synaptosomal transport as recorded in Table 3.1. Identical assays were performed for each of the SAAs but in the presence of a fixed concentration of various inhibitors (Fig. 3.12). Taking into account the significant concentration of passive diffusion to SAA transport (Fig. 3.9) especially for the low-affinity HCSA and HCA transport, preliminary uptake assays for each of the SAAs, both in the absence and presence of each inhibitor, were also performed in the total absence of extracellular sodium which was replaced by an equimolar (135 mM) concentration of lithium ions. None of the inhibitors employed exerted any effect on the Na^+-independent contribution to uptake. The degree of inhibition of uptake calculated in

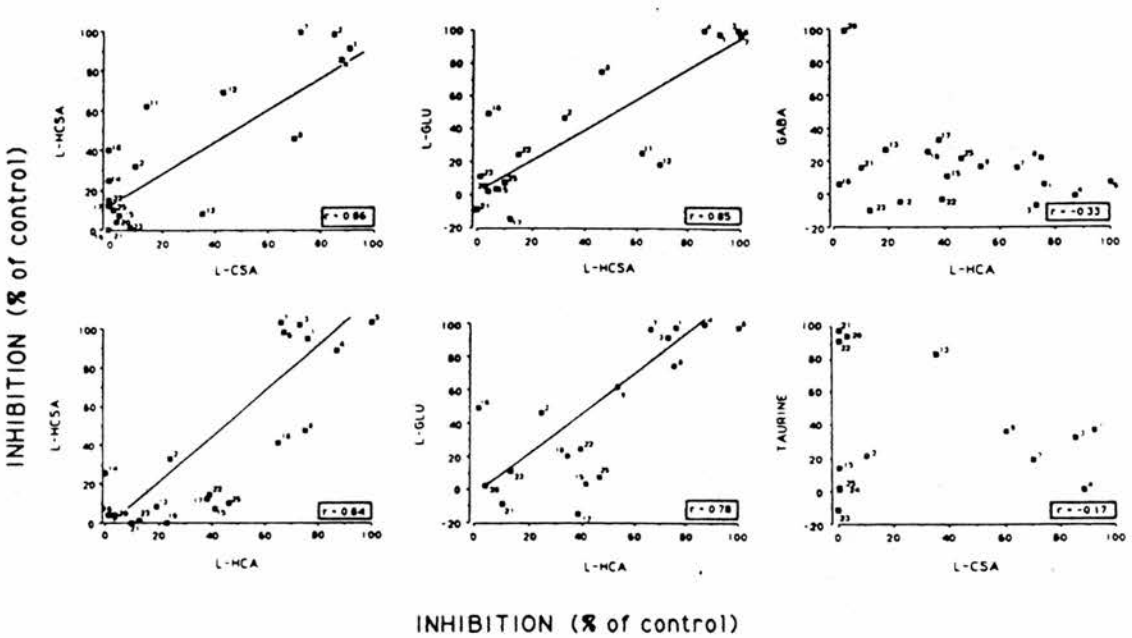


FIG. 3.12 Correlation analysis of the inhibition of synaptosomal SAA uptake of various neuroactive amino acids

Cerebrocortical synaptosome fractions were incubated at 35°C with various SAAs substrates at a fixed concentration which reflected their respective K_m values for synaptosomal transport (Table 3.1). The concentration of all inhibitors was fixed at 200 µM. The figure shows representative inhibition profiles of some of the data provided in Table 3.2. The data points represent the mean ± S.E.M. of three experiments in which assays were carried out in triplicate. The variation of inhibition did not exceed 15% (error bars have been omitted for clarity of viewing).
1 = L-Glu; 2 = D-Glu; 3 = L-Asp; 4 = D-Asp; 5 = L-CSA; 6 = D-CSA; 7 = L-CA; 8 = D-CA; 9 = L-HCSA; 10 = D-HCSA; 11 = L-HCA; 12 = D-HCA; 13 = S-sulpho-L-cysteine; 14 = S-sulpho-D-cysteine; 15 = kynurenate; 16 = kainate; 17 = N-methyl-D-aspartate; 18 = dihydrokainate; 19 = quisqualate; 20 = GABA; 21 = taurine; 22 = β-alanine; 23 = L-methionine; 24 = L-serine; 25 = L-glycine; r = correlation coefficient.

subsequent specificity studies for each of the inhibitors against individual SAA transport relates therefore to the inhibition of the Na^+-dependent component of uptake. This was based on a control value calculated from net difference between the uptake measured in the presence and absence of extracellular sodium, quantified by HPLC.

The nature of the specificity for each of the individual SAA transport in synaptosomes was determined by the correlation between any two substrates following the calculation of the inhibition of uptake of each SAA by a range of similar inhibitory compounds (Fig. 3.12). The square of the correlation (r^2) presented in Table 3.2 measures the extent to which two variables are linked to each other. The inhibition profile of CSA uptake showed a significant positive correlation with respect to that of CA uptake ($r = 0.97$, $P < 0.001$) and also to that of HCSA ($r = 0.86$, $P < 0.001$) and HCA ($r = 0.76$, $P < 0.001$). Similarly a significant positive correlation ($r = 0.84$, $P < 0.001$) was observed between the inhibition profiles of the Glu analogues HCA and HCSA. From Table 3.2 and Fig. 3.12 it can also be seen that the inhibition profiles of CSA, CA, HCSA and HCA exhibit a significant positive correlation ($r = 0.78$ - 0.98, $P < 0.001$) when directly compared with the inhibition profiles of the excitatory dicarboxylic amino acids L-Glu and both L- and D-Asp. This is in contrast to the inhibition profiles of CSA, CA, HCSA and HCA when compared to the inhibition profiles of the inhibitory neuroactive amino acids GABA and taurine ($r = 0.03$ - 0.17) (Table 3.2) showing clearly that there is very little association between the transport systems for the different classes of neuroactive amino acids.

The specificity constants (V_{max}/K_m) (Table 3.3) calculated from the kinetic values of Table 3.1 for all the SAAs together with Glu give an indication of the degree of specificity of these compounds as substrates for the plasma membrane transporter illustrating the efficiency at which

TABLE 3.2

CORRELATION OF THE INHIBITION OF UPTAKE OF SAAs AND OTHER NEUROACTIVE AMIO ACIDS*

Uptake of amino acid$_1$ vs.	amino acid$_2$	Correlation coefficient (r)	r^2	P value[a]
L-CSA	L-CA	0.97	0.94	$P < 0.001$
	L-HCSA	0.86	0.74	$P < 0.001$
	L-HCA	0.76	0.58	$P < 0.001$
	L-Glu	0.95	0.95	$P < 0.001$
	L-Asp	0.98	0.96	$P < 0.001$
	D-Asp	0.96	0.92	$P < 0.001$
	GABA	-0.23	0.05	$P > 0.2$
	Taurine	-0.17	0.03	$P > 0.2$
L-Glu[b]	L-HCSA	0.95	0.72	$P < 0.001$
	L-HCA	0.78	0.61	$P < 0.001$
	L-CA	0.96	0.92	$P < 0.001$
	L-Asp	0.99	0.98	$P < 0.001$
	D-Asp	0.98	0.96	$P < 0.001$
	GABA	-0.41	0.17	$P < 0.2$
	Taurine	-0.31	0.10	$P < 0.2$
L-HCA	L-HCSA	0.84	0.71	$P < 0.001$
	GABA	-0.33	0.11	$P < 0.2$
	Taurine	-0.19	0.04	$P > 0.2$

* Correlation coefficients were determined for the inhibition of uptake (by the compounds identified in the legend of Fig. 3.12 of one neuroactive amino acid (amino acid$_1$) vs. a second neuroactive amino acid (amino acid$_2$).

[a] P values refer to statistical significance of r.

[b] correlation coefficient values (r) are taken from Blicharski (Honours thesis, 1988).

TABLE 3.3

SPECIFICITY CONSTANTS FOR THE SAAs AND GLU IN CULTURED NEURONS, ASTROCYTES AND SYNAPTOSOMES

	Specificity Constant V_{max}/K_m			
Amino Acid	**Cx**	**Gn**	**Ast**	**Syn**
CA	0.385	0.615	0.309	0.157
CSA	0.025	0.270	0.007	0.021
HCA	0.003	0.017	0.035	0.007
HCSA	0.006	0.023	0.081	0.012
Glu	0.137	0.242	0.222	1.79

Specificity constants (V_{max}/K_m) were calculated from the kinetic parameters in Table 3.1. Abbreviations for neuronal preparations are as defined in Table 3.1.

the SAAs are removed from the extracellular synaptic environment in comparison to Glu.

In all the brain preparations studies Glu and CA show clearly the greater specificity as substrates for the plasma membrane transporter when compared to CSA, HCSA and HCA. An exception to this was the finding that CSA in the 'glutamatergic' granule cell preparation exerted a similar specificity to that of Glu. From Table 3.3 it can also be seen that HCSA and HCA demonstrate a 2-4 fold greater specificity as substrates for the plasma membrane transporter in astrocytes compared to neurons.

3.6 Inhibition by Chlorpheg of HCA and D-Asp Uptake in Neurons, Astrocytes and Synaptosomes.

In a separate series of experiments the kinetic characterisation of HCA and D-[^{3}H]Asp uptake in the absence and presence of chlorpheg was evaluated in cultured cerebellar granule cells and cortical astrocytes and in rat cortical synaptosomes. The substrate assay concentrations of HCA and D-Asp varied from 1 μM - 10 mM in the absence and presence of 7.5 mM chloropheg. The uptake profile of both HCA and D-Asp was similar to that obtained in previous kinetic experiments demonstrating a saturable carrier-mediated component of uptake superimposed with a non-saturable (passive diffusion) component which was more evident at millimolar substrate concentrations. D-Asp exhibited a high affinity for the transporter with K_m values of 69 ± 5 μM, in astrocytes, 42 ± 3 μM, in granule cells and 7 ± 1 μM in synaptosomes (Table 3.4). In contrast HCA displayed a low-affinity for the carrier with calculated K_m values of 468 ± 75 μM, 654 ± 78 μM and 1550 ± 169 μM in respectively astrocytes, granule cells and synaptosomes. Chlorpheg inhibited only the saturable carrier-mediated uptake component, an observation noted in previous transport experiments involving the presence of inhibitors (data not

TABLE 3.4

KINETIC CHARACTERIZATION OF D-ASP AND L-HCA UPTAKE AND INHIBITION BY CHLORPHEG

		K_m(μM)		V_{max}(nmol/min/mg)		
Preparation	**Substrate**	**- CPG**	**+ CPG**	**- CPG**	**+ CPG**	**K_{ei} (μM)**
Astrocytes	D-Asp	69±5	160±9	9.1±0.1	10.4±0.2	5322
	L-HCA	468±75	2187±42	34.1±1.6	32.4±1.6	2081
Granule Cells	D-Asp	42±3	76±7	13.0±0.3	13.3±0.4	9183
	L-HCA	654±78	4485±4898	23.5±1.9	18.2±2.1	1344
Synaptosomes	D-Asp	7±1	12±2	7.2±1.4	7.8±1.8	11980
	L-HCA	1550±169	4138±244	10.3±1.1	9.4±1.4	4520

Kinetic constants (presented as mean values ± S.E.M.) were obtained by computer-assisted non-linear regression analysis of the uptake data using the Enzfitter software package (Leatherbarrow, 1987). The values of the inhibitor constant (K_{ei}) were calculated from the expression:
$K_m^{app} = K_m[1 + I/K_{ei}]$ where K_m and K_m^{app} are respectively the Michaelis constant determined in the absence and presence of inhibitor (I) and K_{ei} is the inhibitor dissociation constant describing competitive inhibition.

shown). The kinetic data presented in Table 3.4 demonstrates clearly that chlorpheg acts as a competitive inhibitor of D-Asp and HCA uptake as seen by the signficant increase of the K_m value for each of the brain preparations with no apparent change in the corresponding V_{max}. In each preparation chlorpheg proved to be essentially a weak inhibitor of both HCA uptake (with K_{ei} values ranging from 1.3 - 4.5 mM) and D-Asp uptake (with K_{ei} values ranging from 5.3 - 12.0 mM).

3.7 Endogenous Levels of HCA and HCSA in Astrocytes and Neurons Measured by HPLC

Ethanolic extracts of free amino acids from cultured cortical astrocytes and cerebellar neurons were screened by HPLC for the presence and quantification of HCA and HCSA. Resolution of HCA and HCSA was achieved by the use of the stepped methanol: 75 mM KH_2PO_4, pH 5.85 chromatogram gradient (Waller et al., 1990) as described fully in Chapter Two.

A partial chromatogram for the elution of authentic HCA and HCSA together with that of Asp and Glu is illustrated in Fig. 3.13(A). This chromatogram shows the clear resolution of HCA and HCSA from Asp and Glu. When ethanol-extracted free amino acids from the astrocytic samples were analysed by HPLC under similar chromagraphic conditions peaks with the same retention times as authentic HCA and HCSA were observed (Fig. 3.13(B)). With the high sensitivity of the chromatography in the HPLC analysis i.e. detection of peaks eluted from the column between 350 - 1100 fmol a mean quantitation value for the peaks with retention times corresponding to HCA and HCSA which relate to background noise in HPLC grade water blanks was calculated. In conjunction with external standards the quantitation of the peaks relating to HCA and HCSA after substraction of the background noise

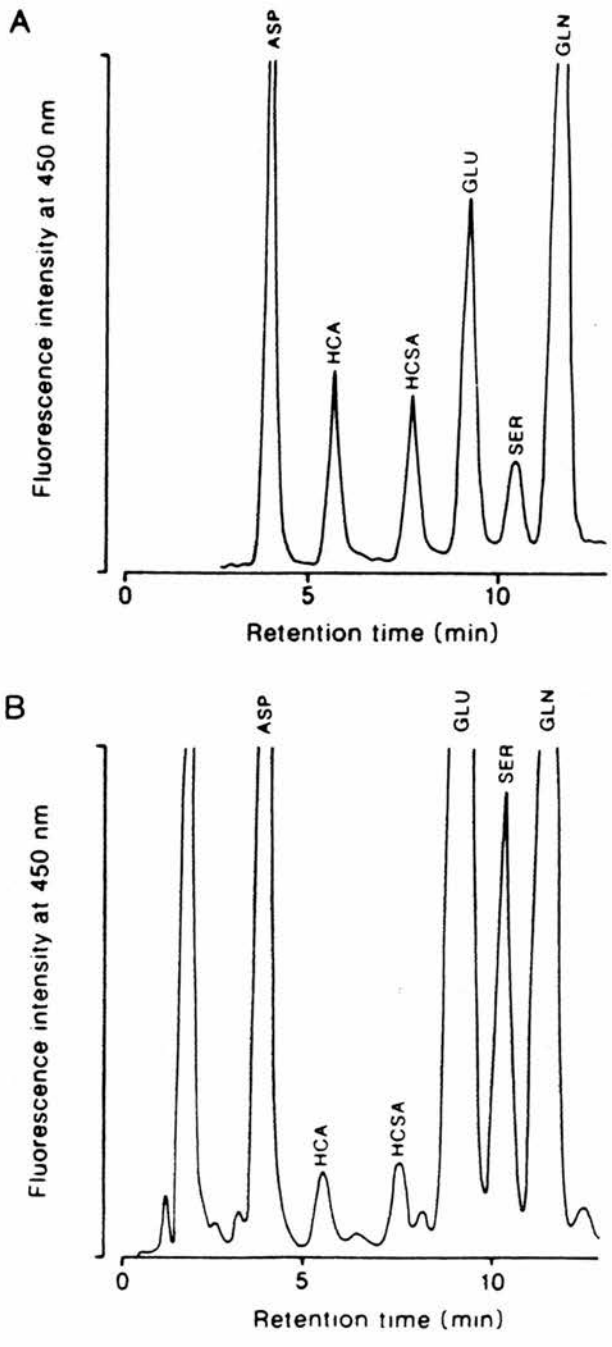


FIG. 3.13 HPLC separation of HCA and HCSA

An aliquot (40 µl) of o-phthaldialdehyde derivatized authentic amino acid mixture (A) or astrocytic extract (B) was injected at zero time. The procedure for the ethanolic extraction of the free amino acids in the astrocytes and subsequent HPLC analysis using a 75 mM KH_2PO_4 pH 5.85: methanol gradient is as described in Methods. The partial chromatogram (A) is representative of 1.33 pmol of each amino acid injected on to the column. The detection of HCA and HCSA in the partial chromatogram (B) was made on the basis of matching retention times. Resolved free amino acids presented are: Asp, aspartate; HCA, HCSA, Glu, glutamate; Ser, serine; Gln, glutamine. Traces presented have been redrawn from those obtained on an EPSON LX-80 printer which were unsuitable for direct reproduction. The scale of VDU screen expansion of the computing integrator for A and B are not identical and comparison of profiles are valid only for viewing synchrony of retention time. Consequently the scale of fluorescence intensity at 450 nm is only of relative value.

value gave calculated values of 72.3 ± 33.7 pmol/mg astrocytic protien and 49.4 ± 27.7 pmol/mg astrocytic protein respectively.

A further substantiation of the positive identificaiton of HCA and HCSA in the ethanolic extracts derived from astrocytes was achieved by sample 'spiking'. This was accomplished by the addition of a calculated amount of authentic HCA and HCSA which corresponded to a total of 3.5 pmol to different samples of each SAA eluted from the column. The partial profile of a 'spiked' chromatogram is shown in Fig. 3.14 which clearly shows an increase in the peak area only in the peaks which correspond by their respective retention times to HCA and HCSA.

For the 24 ethanolic extracts derived from cerebellar neurons only a slight unmeasurable peak of below 200 fmol corresponding to the retention time of HCA was observed above the peak relating to background noise. There were no recorded presence of HCSA in any of the ethanolic extracts derived from cerebellar neurons.

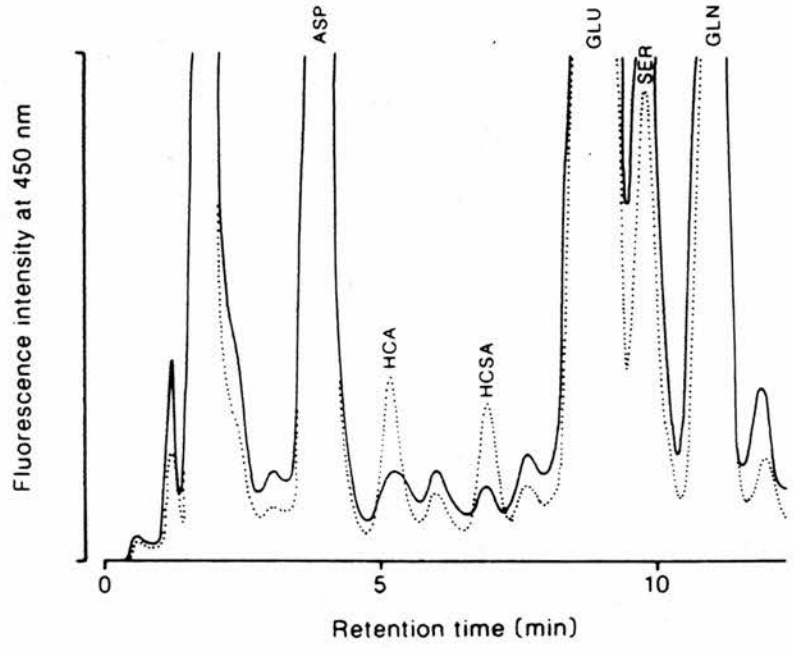


FIG. 3.14 Identification by 'spiking' of HCA and HCSA in ethanol extracts of astrocyte cultures

The solid line is the elution profile of an aliquot of o-phthaldialdehyde-derivatized astrocyte extract. The dotted line is the 'spiked' chromatogram and represents the same astrocytic extract to which 3.5 pmol each of authentic HCA and HCSA has been added. Only in the case of HCA and HCSA does a peak corresponding to each of these authentic amino acids appear to be increased in comparison to the 'unspiked' extract. The scale of VDU screen expansion for each chromatogram is the same.

CHAPTER FOUR

DISCUSSION

A major part of this thesis deals with the demonstration of uptake and characterisation of transport of a group of neuroexcitatory sulphur-containing amino acids (SAAs), namely CSA, CA, HCSA and HCA, using presynaptic nerve terminals (synaptosomes) and primary cultures of glutamatergic (granule cells) and GABAergic (cortex) neurons as well as astrocytes as model in vitro systems. Of the four neuroactive SAAs studied, CSA (Recasens et al., 1982; Griffiths, 1990) and HCA (Cuenod et al., 1986; Do et al., 1986a,b, 1988; Lehman et al., 1988) have strongest support as transmitter candidates while less evidence is available for CA and HCSA (Mewett et al., 1983).

This study shows that CSA and CA are transported by a high affinity system in cultured cerebellar granule cells and cortical neurons and also rat cortical synaptosomes and with a lower affinity by cultured cortical astrocytes. The kinetic parameters of CSA and CA uptake in cultured neurons and astrocytes (Table 3.1) are similar to those of Glu uptake in similar cell types (Drejer et al., 1982). Also in cortical synaptosomal preparations the kinetic parameters of CSA and CA uptake is of the same order as those reported in a number of earlier studies employing a similar brain preparation (Iwata et al., 1982; Wilson and Pastuszko, 1986). In addition, the K_m values for CSA and CA uptake in this study are essentially similar in magnitude to their respective K_{ei} values as competitive inhibitors of D-[^{3}H]Asp uptake in primary cultures of neurons and astrocytes and also synaptosomes (Griffiths et al., 1989). A number of workers in 'single-point' studies have shown that CSA and CA are potent inhibitors of D-Asp, Asp and Glu uptake (Balcar and Johnston, 1972; Balcar et al., 1977, 1980; Davis and Johnston, 1976; Iwata et al., 1982; Mewett et al., 1983). There is general agreement that Glu and D-Asp are transported by a common high-affinity system in cultured neurons and astrocytes (Drejer et al., 1983). Taken together these

observations are in agreement with mutual-inhibition kinetic analysis which demonstrates a common high affinity transporter for CSA, CSA, Glu and Asp in rat cortex synaptosomes (Wilson and Pastuzko, 1986). In this thesis correlation analysis of the inhibition profiles for CSA and CA in synaptosomes results in correlation coefficient (r) of 0.97 ($p < 0.001$) (Table 3.2). From Table 3.2 it can be seen that the value of r^2 which is a measure of the closeness of association for inhibition of CSA versus CA is 0.94 (i.e. 94% of the variation in inhibition of CSA uptake can be explained as completely associated with variation in inhibition of CA uptake by the same compounds, whereas 6% is residual variation independent of inhibition of uptake). From Table 3.2 similar r^2 values for inhibition of CSA uptake versus Glu, Asp and D-Asp are observed. Such a result provides further evidence to support the involvement of a single carrier for these compounds.

High affinity uptake of CSA has been demonstrated by a number of earlier workers using a variety of different preparations, viz. synaptosomal P_2 fraction (Iwata et al., 1982), rat synaptic membrane vesicles (Recasens et al., 1982) and, primary cultures and clonal cell lines (Abele et al., 1983). The kinetic constants in neuronal primary cultures (K_m = 41 μM (granule cells); K_m = 58 μM (cortex)) and cortical synaptosomes (K_m = 57 μM) reported in this study are higher than those reported in cortical synaptosomes or membrane vesicles (see above) where the K_m varies between 12-27 μM, although V_{max} values are in good agreement. Moreover Abele et al. (1983) reported K_m values for CSA of 5-6 μM in neurons (region unspecified) and 20-40 μM in astrocytes. The slight variations between the kinetic parameters determined in the present study compared with other earlier studies may be a result of a combination of differences which include brain preparation, species differences and methodological approach.

Less attention has been given to the transmitter status of CA which was one of the first amino acids tested for excitatory activity (Curtis and Watkins, 1960, 1963) and is equipotent with Glu (Mewett et al., 1983. Wilson and Pastuszko (1986) first showed that rat brain synaptosomes accumulate [^{35}S]CA by a high affinity transport system (K_m = 12 μM, V_{max} = 2.5 nmol $min^{-1}mg^{-1}$). Koyama et al. (1989) reported that [^{35}S]CA selectively detects a chloride-dependent Glu transporter (exhibiting a K_m of 5.4 μM) of unknown significance to the process of neurotransmission.

The measured kinetic parameters for uptake of synaptosomal CA in this study are in general agreement with Wilson and Pastuszko (1986) and also correlate well with inhibition kinetic studies which show CA to competitively inhibit D-[^{3}H]Asp uptake (1) in synaptosomes with K_{ei} values equipotent with CSA and (2) in cultured neurons and astrocytes where CA exhibits a potency twice that of CSA (Griffiths et al., 1989). In vitro autoradiography has also been employed to localise and quantify sodium-dependent [^{35}S]CA binding sites (Parsons and Rainbow, 1984). These workers showed the anatomical distribution of [^{35}S]CA to be heterogeneous and the pharmacological specificity to differ from D-[^{3}H]Asp, which was used as a marker for Glu/Asp transport sites. It can be seen therefore that both CSA and CA display certain properties which reflect transmitter functions. The high-affinity observed for the uptake systems of both CSA and CA located within the plasma membrane of neurones and glial could describe a mechanism for termination of their activity within the synaptic cleft. Such a mediation of an inactivation mechanism would appear to be essential in respect to their strong depolarising actions and neuronal cytotoxicity (Olney et al., 1971; Pullan et al., 1977; Frandsen et al., 1990, 1992).

The two Glu analogues HCSA and HCA are also regarded as transmitter candidates. In particular the depolarization-induced calcium-

dependent release from brain slices (Do et al., 1986a) and its ability to elicit release of other neurotransmitters following activation of Glu receptors (Dunlop et al., 1989, 1990; Lehman et al., 1988). Other factors consistent with a role of HCA as an endogenous excitatory amino acid ligand are its role as a preferential endogenous NMDA ligand (Olverman et al., 1988; Schwarz et al., 1990), and as a preferential agonist at synapses such as the hippocampal Schaffer collateral-CA1 synapse (Ito et al., 1991; Provini et al., 1991). However some observations of the transmitter role for HCA are not in accordance with the classical concept of neurotransmission (1) chlorpheg has a central role as a reportedly selective inhibitor of HCA uptake in the assessment of HCA as a preferential excitatory amino acid agonist at certain central synapses (Davies et al., 1985; Ito et al., 1991; Provini et al., 1991; Zeise et al., 1988). However in this thesis chlorpheg was found to be essentially equipotent as a competitive inhibitor of D-Asp and HCA carrier-mediated uptake in primary cultures of neurons and astrocytes and rat brain synaptosomes and importantly the description of chlorpheg as a selective blocker of HCA uptake is incorrect. (2) The localization of HCA-like immunoreactivity appears to be almost exclusively localised in the glial cells of the cerebellum (Grandes et al., 1991), cortex (Tschopp et al., 1992), and hippocampus (Streit et al., 1990). (3) The uptake of HCA characterised kinetically by transport in rat cerebral cortex slices (Cox et al., 1977) and in primary cultures of neurons and astrocytes and also synaptosomes from this study can be attributed mainly to a low-affinity component and (4) when a nerve pathway is activated a high-frequency TTX-sensitive release of endogenous HCA can only be collected following a relatively long delay (Klancnik et al., 1992).

The observation described earlier, that an antibody against HCA revealed most immunoreactivity in astroglial cells raises the possibility of

the presence and hence storage of HCA in astrocytic cultures which are devoid of neuronal contamination. In a HPLC-based study within this thesis it is demonstrated that in primary cultures of mouse cortical astrocytes maintained in vitro for 21-24 days the levels of HCA and HCSA were 72.3 ± 33.7 pmol/mg protein and 49.4 ± 28.7 pmol/mg protein respectively with no HCA or HCSA detected in the neuronal cultures. These calculated levels for HCA in astrocytes although slightly higher are in general agreement with Tschopp et al., 1992, who calculated levels of 30-50 pmol/mg protein in rat cortical astrocytes maintained in vitro for 15-50 days; which was 30 to 50 times higher than in extracts of rat cerebral cortex. Furthermore, clusters of astrocytes in culture showed a very strange HCA-like immunoreactivity (Tschopp et al., 1992). Taken together these observations demonstrate that astrocytes at least in culture are able to generate and store HCA in the absence of neurons and support the suggestion that in nervous tissue HCA need not to originate primarily from neurons.

The glial localization of HCA-like immunoreactivity has been interpreted in one of two ways (Cuenod et al., 1991). Thus (1) under physiological conditions, HCA could be located in nerve terminals (operating as a neurotransmitter?) and during immunohistochemical processing, HCA could leak from nerve terminals and be taken up by glial cells. However, it can be seen from the K_m values (Table 3.1) that both neurons and astrocytes exhibit a similar low-affinity uptake for HCA. Moreover, the uptake of HCA in synaptosome fractions exhibits a similar low-affinity system. Based on this evidence it is unlikely therefore that HCA leakage from nerve terminals would result in preferential accumulation by glial cells. Alternatively, (2) HCA is indeed stored in glial elements and that its efflux from this compartment is either by depolarization or by exchange with Glu during the uptake process. This

mechanism could explain why in climbing fibre-deprived cerebellum, the calcium-dependent release of HCA is abolished although HCA remains present in glial elements (Vollenweider et al., 1990). It would also be consistent with the delayed efflux of HCA following high-frequency stimulation of the Schaffer collaterals in hippocampal slices (Klancnik et al., 1991). In cortex, immunochemical localization of HCA has been observed mainly in astrocytic endfeet (Cuenod et al., 1990). Such endfeet may abutt neuronal soma or dendrites, and also blood capillaries. The predominant localization of HCA to astrocytes could therefore be a reflection of direct uptake of its ultimate precursor, methionine, from the blood and its subsequent metabolism in astrocytes. The data presented in this thesis shows that HCA exhibits a low but similar affinity for astrocytic and neuronal uptake in cortex. However the capacity for uptake for HCA in astrocytes is ~ 10-fold that in neurons, an observation of some interest in view of the immunochemical studies. According to these observations the hypothesis that HCA belongs to a class of "gliotransmitters" could be considered.

The results of this study show that HCA and HCSA are transported by a low-affinity, high capacity system in cultured granule cells and cortical astrocytes, but appear to display low-affinity and lower capacity for cultured cortical neurons and cortical synaptosomes. The magnitude of their K_m values which may be particularly significant when compared to the low levels of release of endogenous HCSA and HCA (Do et al., 1986a) does not readily support a transmitter role. The K_m values of HCA calculated from this study (K_m 900 μM - 1550 μM) are appreciably lower than the only other published value (K_m > 3 mM) reported by Cox et al. (1977). However, similar values (K_m > 3 mM) could be obtained when primary culture or synaptosomal data were fitted to the Michaelis-Menten equation in which no allowance was made

for diffusion. It is possible that the magnitude of the diffusion component for the markedly different brain preparations employed could explain the variations between the two sets of calculated kinetic parameters. Cox et al. (1977) also reported the possible existence of a minor contributory high-affinity uptake of HCA. In this study no evidence for such uptake of HCSA or HCSA could be obtained.

Whether or not HCA shares a common transporter with Glu or other SAAs is unclear. Correlation analysis of the inhibition profiles for HCSA and HCA in synaptosomal tissue show a highly significant positive correlation ($r = 0.84 : p < 0.001$) (Table 3.2, Fig. 3.12) with each other and a value for r^2 which shows that 70% of the variation in inhibition of HCA uptake is accounted for by variation in inhibition of HCSA uptake. These findings are consistent with both amino acids being transported by the same carrier. When the inhibition profiles of high-affinity CSA or CA uptake were subjected to correlation analysis against low-affinity HCSA or HCA uptake, a significant positive correlation was exhibited by each pairing ($r \geq 0.76$; $p < 0.001$) (Table 3.2, Fig. 3.12) suggesting that all four excitatory SAAs may share the same transporter. The r^2 value for inhibition of CSA uptake versus HCA uptake (Table 3.2) indicates however a 58% association between the respective transport systems, which is similar to the r^2 value of 61% association for Glu uptake versus HCA uptake. Cox et al. (1977) showed that Glu, L-Asp, D-Asp, HCSA, CSA and CA all at 1 mM concentration inhibited uptake of 0.1 mM [^{35}S]HCA ($K_m > 3$ mM) in cortical slices by $\leq 50\%$. These observations imply that although HCA may share a common transport system with Glu, Asp, D-Asp, CSA, CA and HCSA, an independent HCA carrier mechanism cannot be ruled out.

Kinetic evidence has been presented which demonstrates that CSA and CA also utilize the same transporter as Glu and Asp in cortical

synaptosomes. The implication therefore is that the dicarboxylic amino acids, Glu, Asp and D-Asp are transported by a common carrier which is also able to transport amino acids of similar chain length and which possess ω -sulphinic and sulphonic acid moieties in place of an ω-carboxylic acid group. Correlation analysis of the inhibition profiles of any two of the excitatory amino acids - namely CSA, CA, HCSA, HCA, Glu, Asp or D-Asp - results in a coefficient ($r \geq 0.76$; $p < 0.001$) and r^2 values of > 58% indicating a significant positive correlation (Table 3.2) and therefore supporting the view of a common carrier which exhibits group specificity. The suggestion of a common carrier at least for Glu, Asp, D-Asp, CSA, CA is supported by kinetic evidence which shows that not only do the amino acids exhibit essentially similar kinetic parameters for transport in various brain preparations (Drejer et al., 1983; Debler and Lajtha, 1987) but the K_m value for any one amino acid closely resembles the K_{ei} vlaues for inhibition of its uptake by the remaining amino acids (Wilson and Pastuszko, 1986; Griffiths et al., 1989). Furthermore the K_{ei} values for HCSA and HCA as competitive inhibitors of D-Asp uptake in different brain preparations (Griffiths et al., 1989) are very similar to their calculated K_m values from this study (Table 3.1). Taken together these observations provide support consistent with a common carrier for CSA, CA, HCSA and HCA and also the dicarboxylic amino acids.

In previous correlation analyses the inhibition profiles (Debler and Lajtha, 1987) show that the high affinity Glu/Asp synaptosomal membrane carrier exhibited an insignificant negative correlation compared with the high-affinity carriers for the neutral neuroinhibitory amino acids, taurine and GABA, thus illustrating the existence of distinct transport systems for these classes of amino acid. Although the importance of taurine to CNS function is undisputed, the significance of its neuroinhibitory actions remain highly speculative (Huxtable, 1989).

Taurine is a metabolite and close structural analogue both of CSA and CA and therefore might be expected to exhibit some overlapping substrate specificity for transport. The results from this thesis show, however that all the excitatory SAAs exhibit a negative, insignificant correlation when compared with inhibition profiles of high-affinity taurine or GABA transport, with r^2 values indicating only a 3-11% association (Table 3.2, Fig. 3.12), observations consistent with the excitatory and inhibitory SAAs being transported predominantly by distinct carrier systems. The removal of the α-carboxylic acid group of Glu, CSA, and CA to give respecitvely GABA, hypotaurine, and taurine appears sufficient to render the latter compounds non-substrates for the "di-acidic" amino acid carrier, thus supporting the group specific nature of the plasma membrane amino acid carriers.

The kinetic data provided in this thesis provides further support to an already considerable body of biochemical and pharmacological evidence (Robinson et al., 1993) indicating that distinct differences exist between Glu transporters derived from either different brain regions (cortex versus cerebellum) or cell type (neuronal versus glial). Major recent advances in the cloning of Glu transporters has established that Glu transport is accomplished by a multigene family distinct from the gene family that includes all other neurotransmitter transporters isolated so far (Storck et al., 1992; Pines et al., 1992; Kanai and Hediger, 1992). The molecular characterization of neurotransmitter carriers began with the purification, amino acid sequencing and cloning of a GABA transporter and was consolidated when the expression cloning of the noradrenaline transporter demonstrated that a transporter gene family exists. Since then a growing list of neurotransmitter transporters have been isolated by methods that rely on the sequence conservation within the 12 transmembrane motifs of this gene family (Amara and Kuhar, 1993).

Members of the family which includes transporters for the biogenic amines, GABA, glycine, choline, proline and taurine, all seem to link substrate influx to the cotransport of Na^+ and Cl^- ions across the plasma membrane. Glu transport however has appeared mechanistically distinct because amino acid influx is ocupled to the cotransport of Na^+ and the countertransport of K^+, with no dependence on Cl^-. Such functional distinction are now explained by the demonstration that Glu transporters represent a very different structural class of amino-acid carriers. Further support for the kinetic data is forthcoming from the result of recent molecular biology studies in which cDNAs have been cloned which enclode structurally-related EAA transporters. These studies show that the Glu transporters exhibit differences based on their cell origin (neuronal (Kanai and Hediger, 1992) versus glial (Pines et al., 1992). It should not be long before molecular biology techniques allows a more complete understanding of the molecular mechanisms and characteristics of Glu transport in the CNS.

In summary, this thesis mainly shows that four excitatory SAA transmitter candidates - namely CSA, CA, HCSA and HCA are taken up in neurons, astrocytes and cortical presynaptic nerve terminals possibly by a common transport system which is shared by the excitatory dicarboxylic amino acids, Glu and Asp. In addition, an independent carrier may exist for HCA. The high-affinity uptake of CSA and CA is consistent with the presence of a rapid inactivation mechanism for termination of any post-synaptic action and provides functional evidence to support a neurotransmitter role. However the low-affinity for transport exhibited by HCSA and HCA does not appear compatible with a transmitter status when based on this criteria alone although an alternative mechanism may be a possibility. The description of chlorpheg as a selective blocker of HCA uptake is incorrect, a fact which should be

recognised before further use of this compound for attempting to identify pathways using HCA as a neurotransmitter. Further studies are also required to endorse the proposal of HCA acting as a "gliotransmitter" in its suggested role as an endogenous excitatory amino acid transmitter.

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PUBLICATIONS ARISING FROM THIS THESIS

A. Full Papers

(i) Grieve A., Dunlop J., Schousboe A. and Griffiths R. (1991) Kinetic characterization of sulphur-containing excitatory amino acid uptake in primary cultures of neurons and astrocytes. *Neurochem. Int.* **19**, 467-474.

(ii) Grieve A., Butcher S.P. and Griffiths R. (1992) Transport of excitatory sulphur amino acid transmitter candidates in cortical synaptosomes: a kinetic and substrate specificity study. *J. Neurosci. Res.* **32**, 60-68.

(iii) Grieve A. and Griffiths R. (1992) Simultaneous measurement by HPLC of the excitatory amino acid transmitter candidates homocysteate and homocysteine sulphinate supports a predominant astrocytic localisation. *Neurosci. Lett.* **145**, 1-5.

(iv) Griffiths R., Grieve A., Allen S. and Olverman H.J. (1992) Neuronal and glial plasma membrane carrier-mediated uptake of L-homocysteate is not selectively blocked by β-p-chorophenylglutamate. *Neurosci. Lett.* **147**, 175-178.

B. Transactions

(i) Grieve A., Cameron D. and Griffiths R. (1990) Characterization of cysteine sulphinate transport by intact rat brain cerebrocortical synaptosome fractions. *Biochem. Soc. Trans* . **18**, 426-427.

(ii) Grieve A., Dunlop J., Schousboe A. and Griffiths R. (1991) Kinetic characterisation of excitatory sulphur amino acid transport on synaptosomes and in primary cultures of different brain cells. *Biochem. Soc. Trans.* **19**, 5s.

APPENDICES

APPENDIX I

Synaptosome Preparation Solutions

(i) Isolation Medium

Dilute 1 part Medium A with 5 parts Millipore water to give:-

310 mMSucrose	Adjust to pH 7.4
5 mM NaTes	with 2N NaOH
0.5 mM Na EDTA	

Medium A

1.86 M Sucrose	636.6 g	
30 mM NaTes	6.87 g	Adjust to pH 7.4
3 mM Na EDTA	1.17 g	

* The sucrose was dissolved in Millipore water and deionized using Dowex-50W (regenerated) to prevent synaptosomal aggregation.

Final volume 1.0 litre with Millipore water.

(ii) 250 mM Sucrose/Tes pH 7.4

Dilute 50 ml Medium A to 372 ml with Millipore water. Add 0.0341g NaTes and adjust to pH 7.4.

(iii) Ficoll Solutions

(a) Preparation of Dialysis Tubing

(1) Boil tubing in 2.0 litres EDTA solution (37.2 g/2..0 litres water).

(2) Wash tubing with distilled water.

(3) Boil tubing in 2.0 litres 0.05M sodium carbonate solution.

(4) Wash tubing (x 3) with distilled water and store +4^{o}C with distilled water.

(b) Preparation of 18% Ficoll

(1) Dissolve Ficoll 400 (Pharmacia) to give an overall concentration of approximately 50%. Add gradually, with continual mixing on magnetic stirrer.

(2) Dialyse Ficoll solution using pre-washed dialysis tubing removing air bubbles against distilled water for 4.5 h.

(3) Determine specific gravity (S.G.) of the dialysed Ficoll solution using a hydrometer in a 50 ml tall-form graduated measuring cylinder.

* Make sure Ficoll solution is bubble free and that the hydrometer does not touch the sides of the measuring cylinder.

(4) Calculate the % of the Ficoll solution from the standard graph and finally dilute to 18% with millipore water making a final measurement of S.G. of the final solution using the hydrometer.

(c) Preparation of 12%, 9%, 5% Ficoll

% Ficoll	Total volume (ml)	Medium A (ml)	Distilled H_2O (ml)	18% Ficoll (ml)
12	30	5.0	5.0	20.0
9	30	5.0	10.0	15.0
5	30	5.0	16.67	8.33

APPENDIX 2

Protein Determination

Biuret Method (Gornall et al., 1949)

Reagents

(i) Biuret reagent

0.1 g $CuSO_4$ $5H_20$

0.6 g Potassium sodium tartrate

Dissolve in 50 ml distilled water.

Add 3.0 g NaOH.

Final volume 100 ml with distilled water.

(2) Standard protein solution

10 mg/ml Bovine serum albumin (Fraction V).

(iii) Sodium Dodecyl Sulphate (SDS) - 0.6%

Tube	Dist. H_20	STD/Sample	0.6% SDS	Biuret Reagent
Blank	300 µl	0	100 µl	600 µl
Standard	220 µl	80 µl	100 µl	600 µl
Sample	220 µl	80 µl	100 µl	600 µl

Assay in triplicate.

Vortex tubes and read O.D. at 540 nm after 15 min.

APPENDIX 3

Protein Determination (Lowry et al., 1951)

Reagents

(A) 3 volumes distilled water

1.5 volumes 2% w/v $CuSO_4$ $5H_20$

1.5 volumes 4% w/v Potassium sodium tartrate

60 volumes 10% w/v Sodium carbonate in 0.5 M NaOH

Method

To duplicate 25 µl samples add 225 µl distilled water. To the 250 µl sample/standard add 250 µl freshly prepared Reagent A and incubate for exactly 10 min at room temperature. To this mixture add 750 µl freshly prepared Reagent B and incubate for 20 min at 37°C. The resultant blue colour is then measured at 550 nm using distilled water as the reagent blank. The total protein (µg) of each well is then calculated:-

$$\frac{\text{O.D. Sample}}{\text{O.D. 100 µg/ml Std}} \times \frac{25\ (\mu g\ \text{std P})}{1} \times \frac{500\ [\text{well volume µl}]}{25\ [\text{volume sample µl}]}$$

APPENDIX 4

TISSUE CULTURE MEDIUM

STOCK SOLUTIONS FOR MEM 5 X CONCENTRATED

(The amount is equivalent to 30 litres of complete medium)

1. Solution of Amino Acids

Double distilled water	350 ml	
6N HC1	45 ml	
	(Sigma Cat No.)	
L-Arginine	A-5006	2.5200g
L-Cystine	C-8755	1.4400g
L-Histidine	H-8125	1.2600g
L-Glycine	G-7126	0.9000g
L-Isoleucine	I-2752	3.1500g
L-Leucine	L-8000	3.1500g
L-Lysine, HCL	L-5626	4.3800g
L-Phenylalanine	P-2126	1.9800g
L-Serine	S-4500	1.2600g
L-Threonine	T-8625	2.8500g
L-Tryptophan	T-0254	0.4800g
L-Tyrosine	T-3754	2.1600g
L-Valine	V-0500	2.8200g
L-Methionine	M-9625	0.9000g

When all the amino acids have been solubilized, add double distilled water to a total of 500 ml. Freeze in 10 aliquots of 50 ml.

2. Vitamin Solution

Double distilled water	450 ml
6N NaOH	0.400 ml

		(Sigma Cat. No.)	
	D-Ca-Pantothenate	P-2250	0.1200g
	Choline Chloride	D-1879	0.1200g
	Folic Acid	F-7876	0.1200g
	I-Inositol	I-5125	0.2160g
	Niacinamide	N-3376	0.1200g
	Pyridoxal, HCl	P-9130	0.1200g
	Riboflavin	R-4500	0.0120g
	Thiamine, HCl	T-4625	0.1200g

When all the vitamins have been solubilized add double distilled water to a total of 600 ml. Freeze in 10 aliquots of 60 ml.

Solutions of Salts etc.

3.	$CaCl_2.2H_20$	6.0g
	6N HCl	0.250 ml
	Double distilled water to	60 ml
	Freeze in 10 aliquots of 6 ml	
4.	$MgSO_4, 7H_20$	6.0g
	Double distilled water to	40 ml
	Freeze in 10 aliquots of 4 ml	
5.	NaH_2PO_4	3.75g
	Double distilled water to	40 ml
	Freeze in 10 aliquots of 4 ml	
6.	Double distilled water	1500 ml
	Phenol Red	0.60g
	$Fe(NO_3)_3, 9H_20$	0.0030g
	KCl	12.0g
	NaCl	192.0g
	Glucose, H_20	35.64g

When everything has been solubilized add double distilled water to a total of 2000 ml.

Freeze in 10 aliquots of 200 ml.

MEM 5 x concentrated (600 ml). Mix one portion of each of the solutions 1-6. Add double distilled water to a total of 600 ml. Keep at 4°C/No need to be sterile. Bench life approximately 2 months at 4°C.

Other Buffers and Solutions Required:-

Krebs buffer	NaCl	7.07g	(120.90 mM)
	KCl	0.36g	(4.83 mM)
	KH_2PO_4, x H_20	0.166g	(1.22 mM)
	$NaHCO_3$	2.140g	(25.50 mM)
	Glucose	2.57g	(13.00 mM)
	Phenol red	0.015g	

Dissolve in 1 litre double distilled water. Keep at 2-4°C.

$MgSO_4$ solution	$MgSO_4$, $7H_20$	0.37g	(150 mM)

Dissolve in 10 ml double distilled water.

Tyrode solution	NaCl	8.00g	(136.90 mM)
	KCl	0.20g	(2.68 mM)
	$CaCl_2$, $2H_20$	0.20g	(1.36 mM)
	$MgCl_2$, $6H_20$	0.10g	(0.49 mM)
	NaH_2PO_4, H_20	0.05g	(0.36 mM)
	Na_2HPO_4, $2H_20$	0.102g	(0.57 mM)

Dissolve in 1 litre double distilled water and filter under sterile conditions. Keep at 2-4°C.

Poly-L-lysine solution	10 mg poly-L-lysine (Sigma Cat. NO. P-1524, polymerization degree 1700, mol wt. 3500.00)

	in 1000 ml double distilled water. The solution is filtered under sterile conditions. Keep at 2-4°C.		
Buffer for insulin solution	$Na_2HPO_4, 2H_20$	0.577g	(32.4 mM)
	NaH_2PO_4, H_20	0.105g	(7.6 mM)
	Albumin	1.00g	
Dissolve in 100 ml double distilled water.			
Insulin Solution A	Dissolve 1 mg insulin (25 i.u., cruptal ox) in 100 µl 0.01 N HCl. Add buffer (see above) to a total of 10 ml.		
Insulin Solution B	Dilute 100 µl A with 10 ml buffer (see above). Keep at -20°C in small aliquots. Make up 100 ml at a time		
Penicillin Solution 5×10^5 units/ml	Add 2.0 ml sterile double distilled water to a glass of lyophilized penicillin (10^6 units).		
p-Aminobenzoic (pABA)	p-Aminobenzoic acid (1 mg/ml) in double distilled water. Keep in small aliquots at -20°C. (Prepare 25 ml at a time).		
Cytosine arabinoside (cytarabin)	2 mM cytarabin: 4.85 mg cytosine arabinoside in 10 ml double distilled water. Filter under sterile conditions and keep at -20°C. Stable for 3 months.		

DNAse	Dissolve DNAse in double distilled water (10 mg/ml). Pipette at 0°C 80 µl in small tubes and freeze at -20°C.
KG-MEM	200 ml 5 x conc. MEM. 700 ml double distilled water. Adjust to pH 7.0 with 6N NaOH. Then add:- 2.2000g $NaHCO_3$ (26.2 mM) 1.4200g KCl (19 mM) 4.9450 g glucose (25 mM) 1 ml penicillin solution Add double distilled water to 1.00 litre. Filter under sterile conditions and keep at 2-4°C. Stable for 4-6 weeks.
Neuronal MEM + 10% serum	For preparation of 500 ml neuronal MEM (NMEM) the following procedure is recommended:- Add to 20 ml KG-MEM: 0.0585g L-glutamine (G-3126) (0.8 mM) 2000 µl insulin B, 500 µl pABA. Filter this solution under sterile conditions into 430 ml KG-MEM and add 50 ml serum. Stable for approximately 2 weeks at 4°C.
Serum	Newborn calf serum, foetal calf serum or horse serum are suitable. It is, however, necessary to select the right 'batch'. Thaw serum in a water bath at 37°C. Before use, the serum must be inactivated (with regard to complement) at 56°C, 30 min.

Aliquot (50 ml) to sterile tubes in laminar hood. In each new 'batch' of serum the pH is adjusted as follows (only for newborn calf and horse): a serum-aliquot of 25 ml is equilibrated with 5% CO_2 in 95% atmospheric air at 37°C. Adjust pH to 7.3 with 1N HCl or 1N NaOH. Note the actual volume of acid or base added. Calculate the correct amount of sterile acid or sterile base, and add this to the inactivated serum. After this procedure the serum is ready for use.

Solutions for processing the cells. Prepare just before use.

1. 80 ml Krebs + 0.24g BSA + 0.62 ml 150 mM $MgSO_4$-solution.
   Weigh BSA and place on the surface of the Krebs-Mg solution. (Do not stir!).
   The BSA will dissolve within 5 minutes.
2. 20 ml 1. + 5 mg Trypsin.
3. 20 ml 1. + 80 ml DNAse solution + 10.4 mg SBTI + 0.2 ml 150 mM $MgSO_4$-solution.
4. 3.2 ml 3. Add solution 1. to a total of 20 ml.

BSA: Bovine Serum Albumin (Sigma A-4503)

SBTI: Soybean Trypsin Inhibitor (Sigma T-9128)

Trypsin: Sigma T-0134

DNAse: Sigma D-5025

The solutions are filtered under sterile conditions and subsequently transferred to 100 ml sterile bottles. Equilibrate with 5% CO_2 by blowing gas (5% CO_2 + 95% atmospheric air) into the bottles with a sterile Pasteur pipette equipped with cotton. Screw on the

lid quickly. Always make sure that the media are equilibrated with CO_2 before and during use. Ensure pH O.K. before starting experiments. Red-orange colours c.f. red-blue.

Coating with poly-L-lysine

Preferably, the surfaces of the culture dishes are incubated at room temperature with 10 mg/1 poly-L-lysine 24h before use. If necessary, the incubation can be done at 37°C for 2 h before seeding the cells.

Matrix	Volume
Multidish	1 ml/well
Petrti dish 35 mm	2 ml
Flasks 25 cm^2	4 ml
Flasks 80 cm^2	10 ml

After incubation, replace with equivalent volumes of Tyrode solution. Before seeding the cells, the Tyrode solution should be completely removed by suction.

APPENDIX 5

ADDRESS OF SUPPLIERS

Anachem	Anachem Limited, 20 Charles Street, Luton, Beds., LU2 OEB.
BDH	BDH Merck Limited, Burnfield AVenue, Thornliebank, Glasgow, G46 7TP.
Burke Electronics	Burke Electronics Limited, 4 Park Gardens, Glasgow G3 7YE.
Chromacol	Chromacol Limited, Glen Ross House, Summers Row, London, N12.
Chrompack	Chrompack UK Limited, Unit 4, Inderscon Court, Millharbour, London, E14 9TN.
Du Pont	Du Pont (UK) Ltd., NEN Research Products, Wedgewood Way, Stevenage SG1 4QH.
Fluka	Fluka Chemicals Limited, The Old Backyard, New Road, Gillingham, Dorset, SP8 4JC.
Gelman	Gelman Sciences, Brackmills Business Park, Caswell Road, Northampton, NH4 OEZ.
Gibco/Biocult Lab	Life Technologies Limited, 3 Wasington Road, Paisley, PA3 4EP.
National Diagnostics, c/o B.S.&S. (Scotland) Limited) Laboratory	
LAB-SCAN	Supplies, 5/7 West Telferton, Portobello Industrial Estate, Edinburgh, EH7 6UL.
Novo	Novo Nordisk, Sydmarken J, DK-2860 Soeberg, Denmark.
Nunc A/S	Kamstrupvej 90, DK-4000 Roskilde, Denmark.
Packard	Canberra Packard Limited, Brook House, 14 Station Road, Pangbourne, Berks, RG8 7DT.

Pharmacia	Pharmacia Biosystems (U.K.) Limited, Davey Avenue, Knowlhill, Milton Keynes, MK5 8PH.
Phase Separation	Pharse Separation Limited, Deeside Industrial Park, Queensferry, Clwyd, Wales.
Rathburn	Rathburn Chemicals, Caberston Road, Walkerburn, Peebleshire, EH43 6AU.
Sigma	Sigma Chemical Company Limited, Fancy Road, Poole, Dorset, BH17 7NH.
Tocris	Tocris Neuramin Limited, Churchill Building, Langford, Bristol, BS18 2DY.
Trivector	c/o N.E. Technology, Bessell Lane, Stapleford, Nottingham, N69 7BX.