BINDING OF [^{3}H] L-ASPARTATE TO MEMBRANE FRACTIONS OF RAT BRAIN

by

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ABSTRACT

The concerns of the present study were to determine 1) the conditions necessary to measure displaceable [^{3}H] L-aspartate binding to membrane fractions of the rat brain, 2) whether the binding demonstrated the charcteristics of the site which is active in vivo, and 3) whether the acidic amino acid neurotransmitters aspartate and glutamate bind to identical or different sites by comparing the pharmacological specificities of the [^{3}H] L-aspartate binding with that of [^{3}H] L-glutamate.

The conditions of the [^{3}H] L-aspartate binding assay were determined in synaptosomal and total particulate fractions of whole rat brain. The reaction mixture which included the membrane fraction suspended in Tris-HCl buffer (pH 7.4) in the presence or absence of the compound under test, was incubated at 37°C for 30 minutes. The reaction was stopped by centrifugation and the radioactivity in the pellet counted by liquid scintillation spectrometry.

The [^{3}H] L-aspartate binding was characterized in total particulate fractions of rat cerebellum. The apparent dissociation constant (K_D) and maximum binding (Bmax), as determined by Scatchard analysis, are 1.64 $\pm$ 0.34 μM and 7711 $\pm$ fmol/mg protein respectively. The displaceable binding is reversible, saturable, independent of the presence of Na^+, has an affinity in the range where the neurotransmitter is active in vivo, and demonstrates a pharmacological specificity which includes stereospecificity. The compounds tested to demonstrate the pharmacological specificity were L-aspartate (IC_{50} = 1.81 μM), D-aspartate (IC_{50} = 46.6 μM), L-glutamate (IC_{50} = 1.24 μM), N-methyl-DL-aspartate (inactive), kainate (inactive), D-alpha-aminoadipate (inactive), and

L-alpha-aminoadipate (IC_{50} = 7.12 μM). The pharmacological specificity of [^{3}H] L-aspartate binding was different from that of [^{3}H] L-glutamate. When the binding data only are considered, therefore, separate receptors for aspartate and glutamate are indicated.

The pharmacological specificity of the [^{3}H] L-aspartate binding, that is the affinity of the binding site for N-methyl-DL-aspartate, D- and L-alpha-aminoadipate, however, does not correlate with the potency of these compounds derived from iontophoretic studies. L-alpha-aminoadipate is very effective while N-methyl-DL-aspartate and D-alpha-aminoadipate do not displace the [^{3}H] L-aspartate binding. In iontophoretic studies, N-methyl-D-aspartate and D-alpha-aminoadipate are very potent as compared to aspartate while L-alpha-aminoadipate is inactive. The [^{3}H] L-aspartate binding then may not represent the site which is active in vivo. The characteristics of the aspartate site in vivo, however, may not be truely represented in iontophoretic studies because of, for example, uptake of the compounds. The aspartate binding site, therefore, must be identified as that which is activated in vivo. The question of separate receptors for aspartate and glutamate then must still be resolved.

ACKNOWLEDGEMENTS

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I. INTRODUCTION

The naturally occurring amino acid L-aspartate first became of neuropharmacological interest in 1960. Curtis and coworkers (Curtis et al., 1960; Curtis and Koizumi, 1961; Curtis and Watkins, 1960; Phillis and Krnjević, 1961; Curtis and Davies, 1962) found that it excited almost all neurones in the mammalian central nervous system. It was not known if this excitation was nonspecific or if aspartate was a neurotransmitter.

Several criteria are used to establish that a substance is a neurotransmitter (Werman, 1966). The criteria are:

1. Presence of the substance in neurones, especially in nerve terminals, the site from which neurotransmitters are released.
2. Release of the substance from neurones in a calcium-dependent magnesium-antagonized manner. Release of all substances thought to be neurotransmitters has such ionic requirements.
3. Similarity of action of the exogenously applied substance with that of the naturally occurring transmitter with respect to a) change in neuronal firing rate and ionic fluxes associated with changes in membrane conductance, b) excitation or inhibition of neurones and c) the effects of various pharmacological agents.
4. Presence of an inactivating process such as degradation and/or uptake so that the effect on neurones will terminate rapidly.
5. Presence of a mechanism for transmitter synthesis.
6. Demonstration of binding to neuronal or other target membranes since neurotransmitters must bind to receptors located on the membranes to initiate their action.

None of these criteria alone is sufficient to define a substance as a neurotransmitter. Fullfillment of all of these criteria, however, provides strong evidence that a substance may be a neurotransmitter. A brief summary follows for the evidence that aspartate is a neurotransmitter. Extensive reviews have been published by Johnson (1978), DeFeudis (1979), Nistri and Constanti (1979), Puil (1981), and Watkins and Evans (1981).

Presence Aspartate is present as a metabolite in all cells. Demonstration of the presence of aspartate in a neurotransmitter role is therefore very difficult. Lesion studies in which certain neurones have been depleted, however, provide some evidence to satisfy this criterion. Comparison of the aspartate levels in intact tissues with those in tissue depleted of neurones in which there is evidence for a neurotransmitter role for aspartate can be made. If aspartate is a neurotransmitter in the neurones in question, then aspartate levels will be lower in lesioned tissues. This evidence is much stronger of course if the only amino acid level which decreases is that of aspartate. Only aspartate and glutamate levels are decreased in the olfactory cortex after olfactory bulb section (Harvey et al., 1975). Lesion studies have also demonstrated the presence of aspartate in the olivocerebellar path and dentate nucleus (Perry et al., 1977) and in the dorsal and ventral grey matter of the spinal cord (Davidoff et al., 1967).

Release Neurotransmitter release from neurones can be induced by application of a solution with a high potassium concentration (usually 50 mM) which induces depolarization or by electrical stimulation. Release has been shown with these methods from regions of the central nervous system in

vivo, and in vitro from tissue slices or synaptosomal preparations. Aspartate release has been shown, for example, by potassium stimulation from the dendate gyrus (Nadler et al., 1977), and by electrical stimulation from the spinal cord (Roberts and Mitchell, 1972). Release of aspartate has also been shown from the lateral olfactory tract (Collins, 1979 a, b), cerebral cortex (Davies and Johnston, 1976), and cerebellum.

The aspartate release in the studies above is dependent on the presence of calcium because of the following interactions. The action potential causes the influx of calcium into the nerve terminal where the calcium interacts with an actomyosin-like protein on the membranes of synaptic vesicles. These vesicles then fuse with the plasma membrane and release neurotransmitter by exocytosis. The release is antagonized by magnesium which competes with calcium for passage into the neurone and for binding sites once inside. The magnesium therefore replaces the calcium and prevents the fusion of the synaptic vesicles with the plasma membrane.

Inactivation Aspartate is inactivated by uptake into neurones and glia (Curtis et al., 1970). A low and a high affinity uptake system, with average dissociation constants of about 2×10^{-3} M and 2×10^{-5} M respectively have been demonstrated for aspartate (Cox et al., 1977; Johnson, 1978). Dissociation constants are a measure of the affinity, that is the strength of the interaction between the receptor and the particular compound under examination. Glutamate, an acidic amino acid which differs from aspartate by only one carbon atom in chain length, and which has also been well established as a neurotransmitter, uses the same transport system as aspartate (Balcar and Johnston, 1972; Storm-Mathisen, 1978).

The high affinity uptake sites are most likely those related to neurotransmitter inactivation because the aspartate and glutamate concentrations in rat cerebral spinal fluid are 1.98 and 3.6 x 10^{-5} M respectively (Clarke and Collins, 1976). At these concentrations the low affinity site is minimally occupied and therefore does not contribute significantly to uptake of neurotransmitter. The density of high affinity uptake sites also correlates with the density of glutamate binding sites and the regional distribution of intracellular glutamate concentration (Johnson, 1978). The low affinity site is probably related to general metabolic uptake (Levi and Raiteri, 1973).

The uptake systems are sodium-dependent. Uptake in the absence of sodium is negligible (Davies and Johnston, 1976). Sodium is transported with aspartate into neurones and glia (Stallcup et al., 1979).

Synthesis Aspartate is synthesized in the tricarboxylic acid cycle (Benjamin and Quastel, 1974). The two enzymes which are important in aspartate synthesis are pyruvate carboxylase which converts pyruvate into oxaloacetate and aspartate aminotransferase which converts oxaloacetate to aspartate (Perry et al., 1981). These enzymes cannot be used as markers for neurones in which aspartate is the neurotransmitter because the enzymes are the same as those present in all cells for the anabolism of aspartate.

Identity of Action Identity of the action of the substance with the naturally occurring transmitter is one of the more important criteria which must be fullfilled. It has been satisfied for aspartate in many areas of the central nervous system. Application of aspartate by ejection from glass micropipettes (iontophoresis) for example increases the firing rate

of Renshaw cells in the spinal cord (Duggan, 1974; Biscoe et al., 1976). The excitation is consistent with that induced by stimulation of the dorsal roots (Curtis et al., 1960).

Intracellular recordings of aspartate action are also consistent with those expected for an excitatory neurotransmitter. Aspartate increases membrane conductance by opening sodium channels (Curtis and Johnston, 1974).

Initially all excitatory amino acids were assumed to act on the same receptor (Curtis and Watkins, 1960; Curtis et al., 1972). The cationic amino group and the two anionic carboxyl groups of all excitatory amino acids are thought to interact in a three-point attachment to the receptor (Figure 1) (Curtis and Watkins, 1960). It was suggested that more than one of these type of receptors might exist when a differential sensitivity of neurones to aspartate and glutamate was found (Duggan, 1974). In the cuneate nucleus, for example, glutamate potently depolarises sensory terminals while aspartate has no effect (Davidson and Southwick, 1971). Comparison of the iontophonetic effects of aspartate and glutamate themselves on neurones, however, is not very informative because of the possible cross-reactivity of the two compounds with different receptors (Watkins and Evans, 1981). Many compounds have therefore been developed to try to differentiate between the actions of the two amino acids in order to discern whether they interact with the same or separate receptors. The structures of glutamate and aspartate are so similar that finding agonists and antagonists which are specific to either proposed receptor has proven very difficult.

Three receptors for the excitatory amino acids have now been characterized by their preferential interaction with N-methyl-D-aspartate, quisqualate or kainate (for reviews see: Watkins and Evans, 1981; Watkins

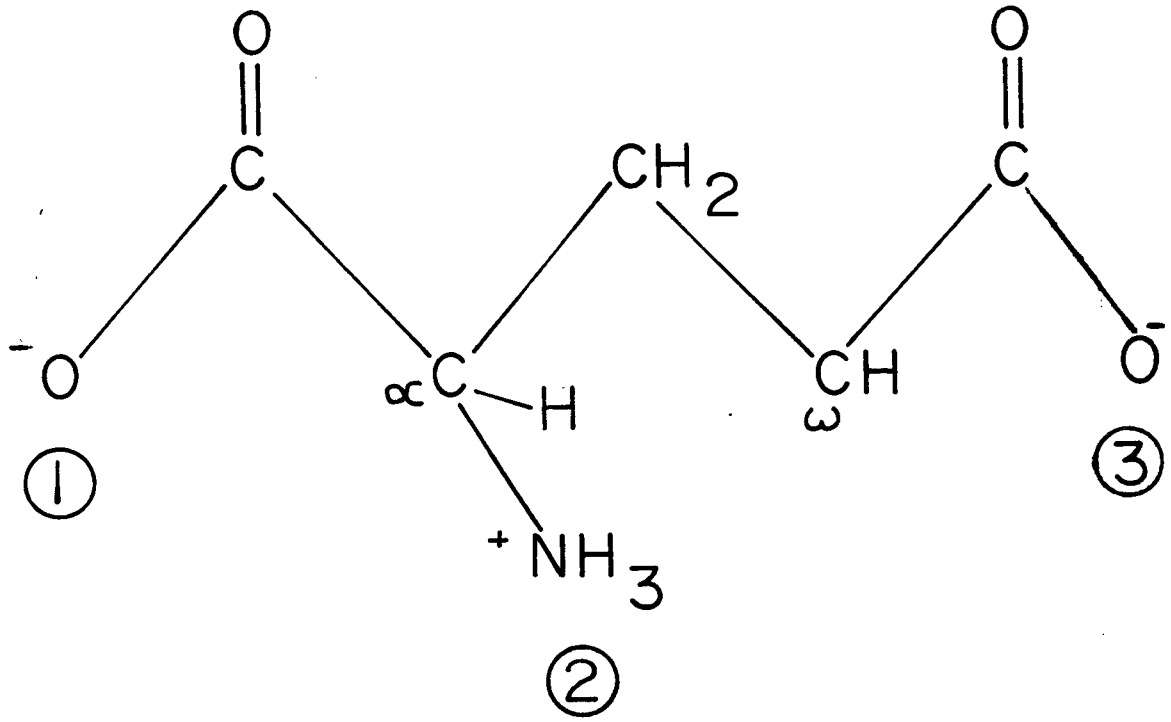
et al., 1981; Watkins, 1981). N-methyl-D-aspartate, a synthetic derivative of aspartate (Watkins, 1962) reacts very potently and specifically with the N-methyl-D-aspartate active site. Aspartate is capable of interacting with the N-methyl-D-aspartate active site. Quisqualate, isolated from seeds of a green creeping vine Quisqualis indica (Takemoto, 1978), is the most specific glutamate preferring agonist to date. Kainate is an analogue of glutamate in which the conformations of glutamate exist in the more extended form (Johnston et al., 1974).

The amino-w-carboxylate spacing appears to determine the preference of the different compounds for the receptor (Figure 1) (McLennan, 1981; McLennan et al., 1982). The N-methyl-D-aspartate-preferring receptor seems to accept molecules in which the spacing is more extended than those molecules accepted by the glutamate-preferring receptor.

Some of the evidence that there are three separate excitatory amino acid receptors is as follows. Intracellular studies with kainate demonstrate that it has a great potency, slow onset of action, produces a very large increase in membrane conductance, and an irreversible depolarization. These responses are so different from those produced by glutamate and aspartate that it seems unlikely that kainate interacts with either glutamate or aspartate receptors (Engberg et al., 1978). Separate receptors are also indicated by the fact that the dose-response curves for kainate and glutamate are not parallel (Nistri and Constanti, 1979).

The strongest evidence that N-methyl-D-aspartate and quisqualate and therefore, as has been proposed, aspartate and glutamate, act at different receptors is that 2-amino-5-phosphonovalerate, the most potent and specific N-methyl-D-aspartate antagonist described to date, substantially depresses N-methyl-D-aspartate-induced excitations while having little or no effect

Figure 1. The Three Proposed Points of Attachment of Excitatory Amino Acids



on quisqualate-induced excitations (Davies et al., 1980). Aspartate and glutamate then are mixed agonists in that both are blocked to some extent by N-methyl-D-aspartate and quisqualate antagonists (Watkins, 1981).

Magnesium is also reported to differentiate between the actions of quisqualate and N-methyl-D-aspartate (Davies et al., 1978). The quisqualate responses are not affected by magnesium while N-methyl-D-aspartate responses are inhibited by about eighty percent with the same magnesium concentration. Magnesium, however, depresses N-methyl-D-aspartate responses but not aspartate responses possibly because of the proposed interaction of aspartate with glutamate preferring receptors.

The hypothesis that separate receptors exist for glutamate and aspartate has therefore been largely based on the differential potency of glutamate and aspartate on neurones and the related agonists and antagonists. Iontophoresis, however, is not necessarily a good technique to use for the comparison of potency of various compounds. Differences in potency may be due to reasons other than the specificity of different compounds for separate receptors such as:

1. the relative numbers of different receptor types. If glutamate and aspartate have the same efficacy and there are more glutamate than aspartate receptors in a given area, glutamate might be more effective than aspartate.
2. different efficacies of the compounds at different or the same receptors. Efficacy is a measure of the ability of a compound to produce a specified response. Quisqualate for example holds ionic channels open longer than glutamate (Anderson et al., 1976; Cull-Candy et al., 1980) but this is not strong evidence that quisqualate is

specific for glutamate receptors.

3. different effective concentrations of the compounds as a result of, for example, different rates of inactivation of the compounds. Balcar and Johnston (1972) and Cox and coworkers (1977) have pointed out that most amino acid excitants are more potent than glutamate and aspartate because of differential uptake and not because of different efficacies. Another cause of different concentrations of compounds is that the concentration of compound administered can vary up to thirty percent when the same compound is iontophoresed from different barrels of the same multibarrelled electrode (Curtis and Watkins, 1963). Large errors might be encountered when compounds with different structures and charges are iontophoresed. The concentration of compound administered by iontophoresis is therefore not accurately known.
4. different sites of action of the compounds. One compound, for example, may activate sites on the dendritic tree so that the response may not be recorded in the cell body whereas another compound may activate sites on the cell body where responses are readily recorded (Biscoe et al., 1976; Davies et al., 1978; Usherwood, 1978; Watkins and Evans, 1981; Watkins, 1981). Differentiation and characterization of receptors using iontophoretic techniques is therefore extremely difficult.

In 1970, however, the application of in vitro binding techniques to neurotransmitter receptors began (Snyder, 1978). This technique consists of adding a radioactive neurotransmitter or a related compound to membrane preparations of the region of the central nervous system under investigation. The total radioactivity bound to such membranes consists of "specific" and "nonspecific" binding components. Specific and nonspecific

binding are determined by adding a large excess of the unlabelled compound which is under test to the reaction mixture. The nonspecific material bound is not displaced by the large excess of added compound and will hereafter more properly be called "nondisplaceable binding". Nondisplaceable binding consists of ionic and nonionic interactions of the compound under test with the membrane fragments. The binding which is displaced by the unlabelled compound is the "specific" binding or "displaceable binding". It consists proposedly of the binding to the neurotransmitter receptor as well as to other sites to which the radioactive compound can be displaced. The neurotransmitter binding site presumably has a higher affinity for the neurotransmitter site than any other sites from which label can be displaced. Low concentrations of radioactive neurotransmitter are therefore employed so that neurotransmitter binding sites are preferentially bound. Concentrations of label are also kept low to decrease the amount of nondisplaceable binding.

Neither the site activated by iontophoresis nor that in the binding assay are necessarily the actual neurotransmitter receptor. To distinguish between these sites, those activated by iontophoresis shall be called "the active site", those bound in the binding assay "the binding site" and those activated by the neurotransmitter "the receptor".

The binding assay is a better system than iontophoresis to determine the affinity of various compounds for the aspartate receptor for four major reasons:

1. The concentration of compound applied to the binding sites is known. A population of binding sites with, for example, a high affinity can therefore be characterized, if desired.
2. The concentration and length of time of administration of compound does

not change due to inactivation by uptake or diffusion because: a) the binding assays are performed in the absence of sodium under which conditions negligible uptake occurs (Enna and Snyder, 1975) and b) there are no problems with different diffusion rates in the binding assay because the compound is applied directly and uniformly to membrane binding sites suspended in a homogeneous mixture. The excess compound is then removed by filtration or centrifugation after a predetermined time.

3. The affinity of the compound for the receptor will be measured whether the binding site occurs on the dendrites or on the cell body of neurones.
4. Differences in efficacy do not interfere with the binding assay because the binding site-compound interaction is measured and not the response elicited after that interaction.

To demonstrate that the [^{3}H] L-aspartate is binding to the site which is active in vivo and not just binding nonspecifically, the following characteristics must be present (Burt, 1978). The displaceable binding must demonstrate:

1. Saturation; that is the presence of a finite number of binding sites.
2. Reversibility because the action of aspartate on neurones in vivo terminates and the receptors are made available for subsequent activation.
3. Stereospecificity - one isomer binding with much less affinity than the other isomer - because many biological responses demonstrate stereospecificity.
4. lack of a requirement for the presence of sodium in order to distinguish the neurotransmitter binding sites from uptake sites

5. Appropriate regional and subcellular distribution. Binding to homogenates of brain tissue, for example, should be highest in the synaptic membrane fractions and negligible in purified mitochondrial fractions.
6. Correlation with the actions seen in vivo; such as a binding affinity in the concentration range where the substance is active physiologially and displacement of binding by compounds which demonstrate an interaction with the neurotransmitter in vivo. Displacement of the neurotransmitter by a wide range of concentrations of compound also determines the affinity of the compound for the neurotransmitter binding site. The affinities with which a binding site interacts with several compounds give the pharmacological specificity. No other binding site will have the same pattern of affinities. If the pharmacological specificities for two ligands are different, then separate binding sites are indicated, while if the specificities are the same, then only one binding site is indicated.

The data from binding studies have demonstrated the existence of separate binding sites for kainate and glutamate. The binding studies were performed with [^{3}H] kainate (Simon et al., 1976; London and Coyle, 1979) and with [^{3}H] L-glutamate (Roberts, 1974; Foster and Roberts, 1978; Baudry and Lynch, 1979; Biziere et al., 1980; Sharif and Roberts, 1980a, b). The pharmacological specificty of the binding sites for the two compounds in the rat forebrain are different (London and Coyle, 1979; Biziere et al., 1980). D-glutamate, for example, binds to kainate binding sites with one hundred times less affinity than it does to glutamate binding sites. Ibotenate and dihydrokainate, however, bind with much greater affinity to kainate sites than to glutamate sites. Different

receptors for glutamate and kainate are therefore indicated.

The concern of the present study was therefore: 1) to determine the conditions necessary to measure displaceable [^{3}H] L-aspartate binding to membranes prepared from rat brain; 2) to determine whether the binding demonstrated the characteristics of binding to the neurotransmitter receptor which is active in vivo; and 3) a comparison of the pharmacological specificity of the [^{3}H] L-aspartate binding with that of [^{3}H] L-glutamate in order to determine whether the two ligands interacted with separate or identical sites.

During the course of the present work Sharif and Roberts (1981) and Foster and coworkers (1981) reported that [^{3}H] L-aspartate bound displaceably to synaptosomal fractions of the cerebellum and to various subcellular fractions of the forebrain of the rat respectively. The fractions tested by Foster and coworkers were whole particulate, crude mitochondrial (P_2), microsomal, myelin, light-density synaptic plasma membrane, synaptic plasma membrane and synaptic junction.

A neurotransmitter is expected to bind to the synaptic junction membrane in order to exert its action and not to intracellular components or to areas of neurones coated with myelin. In accord with these expectations, Foster and coworkers found that only low levels of displaceable aspartate binding occurred in purified fractions of myelin and of mitochondria. The displaceable [^{3}H] L-aspartate binding also increased from whole particulate to P_2 to synaptic plasma membrane fractions and nine times more binding was seen in synaptic junction than in whole particulate fractions. The latter observation is expected for neurotransmitters because in synaptic junction fractions more binding sites occur per milligram of protein than in whole particulate fractions. The

whole particulate fractions contain a great deal more protein not related to the binding site.

84.5 $\pm$ 9.3 percent of the aspartate binding seen in synaptic plasma membrane fractions was recovered in synaptic junction fractions. This indicates that aspartate receptors are concentrated at junctional and not extrajunctional sites.

The other results obtained by Sharif and Roberts (1981) and by Foster and coworkers (1981) such as the effect of K^+, Ca^{2+} and Mg^{2+} on [^{3}H] L-aspartate binding are compared with those of the present study in the discussion.

II. METHODS AND MATERIALS

1. Preparation of Crude Synaptosomal Membranes from Whole Brain

Crude synaptosomal membranes were prepared using a modification of the method of Enna and Snyder (1975). A flow diagram appears in Figure 2. Male Wistar rats (150 - 350 gm) were killed by decapitation. The whole brain was removed and homogenized in 20 volumes (w/v) of ice-cold 0.32 M sucrose with a motor-driven teflon-glass homogenizer (Tri-R homogenizer at 1500 rpm). The membranes were kept in ice-cold solutions before use in the binding assay to prevent possible degradation of the binding sites. The homogenate was centrifuged at 1000 x g for 10 minutes (Sorval RC-5 centrifuge). The resulting pellet was discarded and the supernatant centrifuged at 20000 x g for 20 minutes. The supernatant was discarded and the pellet resuspended in 20 volumes of cold distilled water by sonication for 60 seconds (Sonic Dismembrator setting 50 on a scale of 100). This suspension was then centrifuged at 8000 x g for 20 minutes, the pellet discarded and the supernatant and buffy coat which is the upper, lighter layer of the pellet, centrifuged at 48000 x g for 20 minutes. The resulting pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5) by sonication for 30 seconds and centrifuged at 48000 x g for 20 minutes. This step was repeated and, unless otherwise stated, the final pellet was resuspended in 60 volumes of Tris-HCl buffer.

2. Preparation of the Total Particulate Fraction

Total particulate fractions were prepared using a modification of the methods of Vincent and McGeer (1980) and London and Coyle (1979). Figure 3 shows a flow diagram. Male Wistar rats (150 - 350 gm) were killed by

Figure 2 Flow Diagram for the Preparation of Crude Synaptosomal Membranes

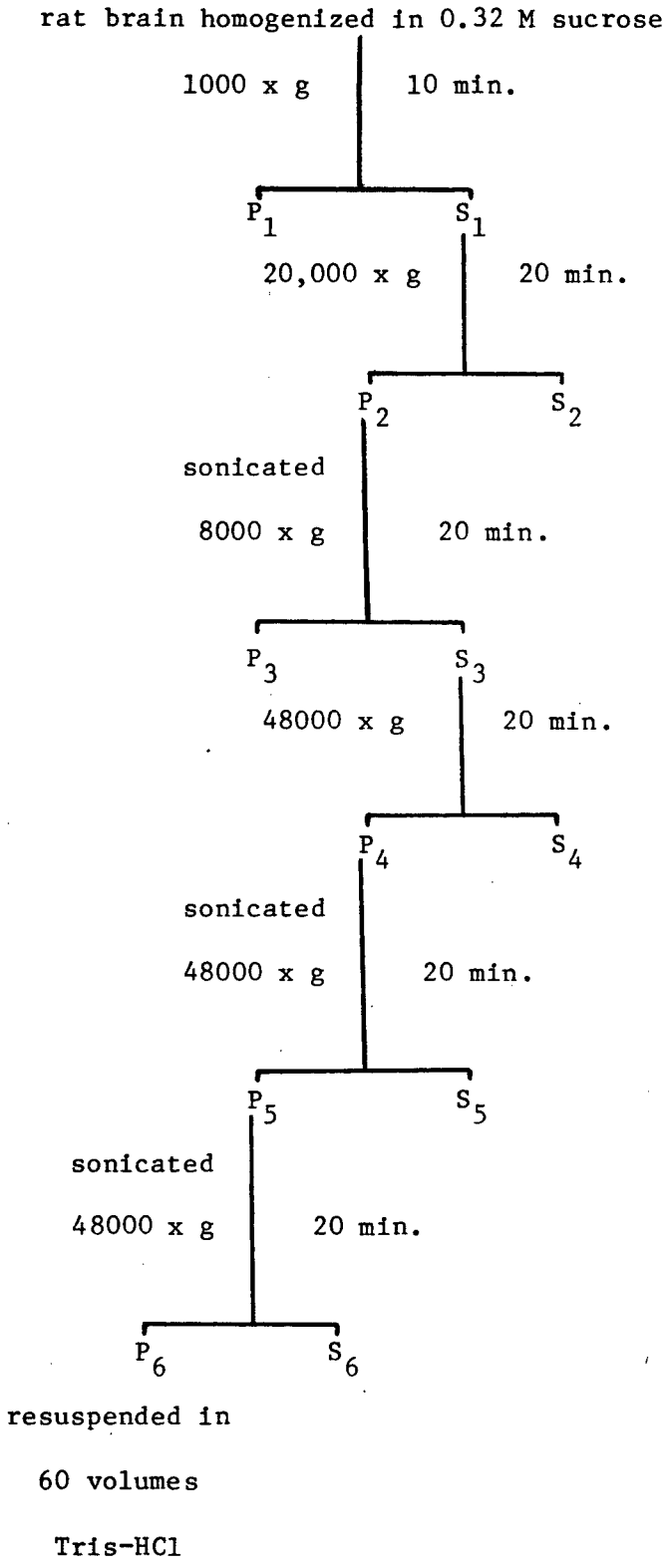
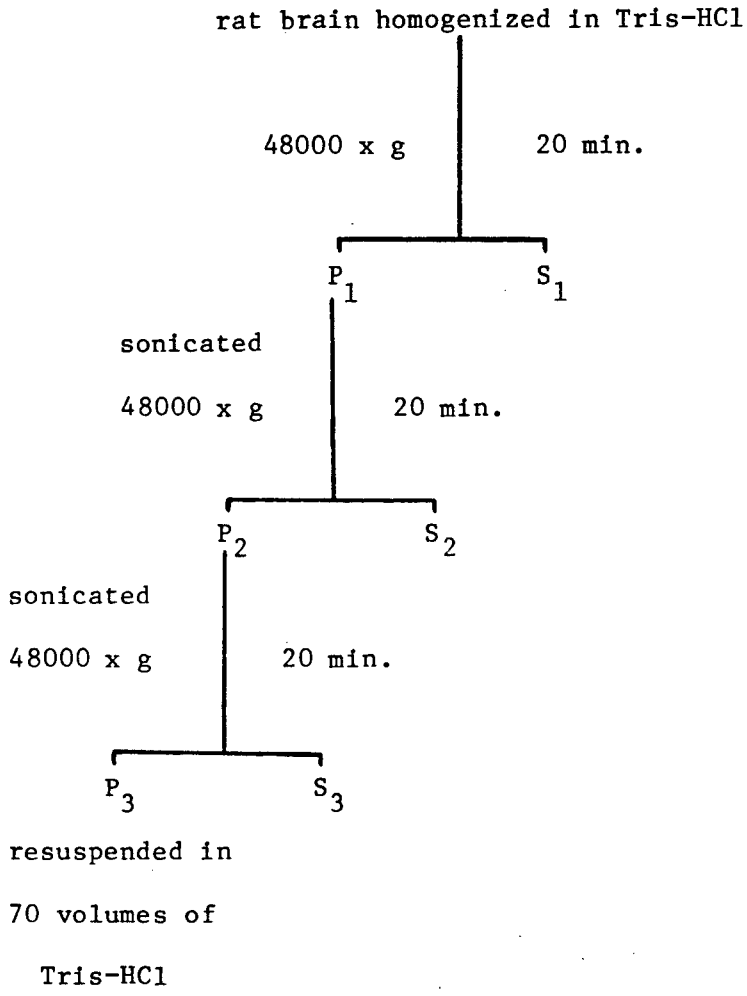


Figure 3 Flow Diagram for the Preparation of Total Particulate Fractions



decapitation. The brain region under investigation was dissected and homogenized in 20 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.5). This homogenate was centrifuged at 48000 x g for 20 minutes. The pellet was resuspended in buffer by sonication for 60 seconds and the suspension centrifuged at 48000 x g for 20 minutes. The resulting supernatant was discarded and the pellet resuspended in buffer by sonication for 30 seconds. This suspension was centrifuged at 48000 x g for 20 minutes. Unless otherwise stated, the final pellet was resuspended by sonication for 30 seconds in 70 volumes of Tris-HCl buffer.

3. [^{3}H] L-Aspartate Binding Assay

Development of the [^{3}H] L-aspartate binding assay is described in Section 1 of results. The final conditions of the binding assay were as follows. The assay mixture was contained in a total volume of 1 ml and was composed of 0.5 ml membrane preparation containing approximately 0.35 mg protein, 0.4 ml 50 mM Tris-HCl buffer (pH 7.5) or solution of the inhibitor under test, and 0.1 ml [^{3}H] L-aspartate (98 to 158 nM final concentration). [^{3}H] L-aspartate and inhibitors were prepared in Tris-HCl and the pH adjusted to 7.5

All components except the [^{3}H] L-aspartate were added to polypropylene tubes (1.5 ml) stored on ice. Binding to homogenates was determined in quadruplicate while that to blank tubes (containing no tissue) was determined in triplicate. [^{3}H] L-aspartate was then added, the solution mixed and incubated in a 37° C water bath (Haake E 1) for 45 minutes at which time the system had reached equilibrium (see Figure 6). Centrifugation for 4 minutes terminated the reaction (Beckman microfuge, Model B). The supernatant was discarded and the pellet rinsed twice with

0.1 ml of ice-cold distilled water. Excess water was blotted off the pellet and the tubes dried with Kimwipes. 1 ml of protosol was added to each tube and left at room temperature until the tissue was dissolved. The solution was then transferred to a scintillation vial and 10 ml of omnifluor (4 gm omnifluor/litre toluene) added. Vials were left at room temperature for three hours to allow chemiluminescence produced by mixing protosol and omnifluor to decrease (see Figure 4). The radioactivity present was then determined by liquid scintillation spectrometry (Beckman liquid scintillation counter). Counts were corrected for efficiency and expressed as disintegrations per minute (dpm).

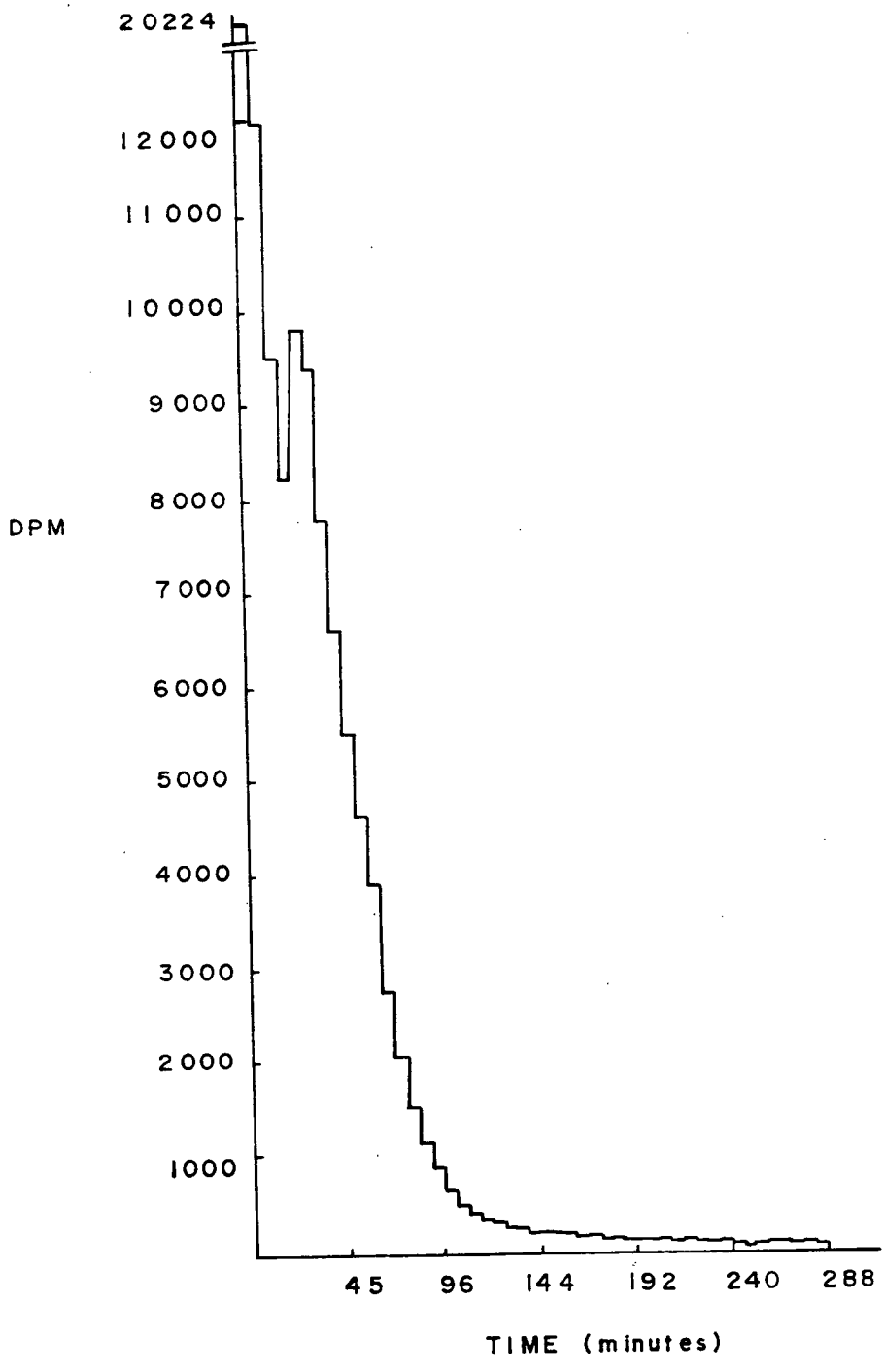
[^{3}H] L-aspartate binding to tubes was determined by replacing the membrane preparation in the binding assay with the same volume of Tris-HCl while background counts were determined by mixing 1 ml of protosol with 10 ml of omnifluor and counting the solution after three hours by liquid scintillation spectrometry. Data were corrected for background activity and [^{3}H] L-aspartate binding to tubes.

The total counts added were determined by dissolving 50 µl of the [^{3}H] L-aspartate solution used in each experiment in protosol, adding omnifluor and counting on the liquid scintillation counter. A final concentration of approximately 120 nM [^{3}H] L-aspartate was used because with this concentration displaceable binding was observed and less than 10 percent of the total radioactivity added was bound by homogenates. A valid estimate of the free concentration of [^{3}H] L-aspartate could therefore be made from the total counts added.

Nondisplaceable binding was defined as the binding to membranes in the presence of 10^{-2} M L-asparate. Displaceable binding was determined by subtracting nondisplaceable binding to membranes from the total binding

Figure 4. Decrease of Background Chemiluminescence

10 ml of omnifluor was added to 1 ml of protosol and activity in the vial continuously counted on a Beckman liquid scintillation counter. Counts per minute were measured every 6 minutes, corrected for efficiency and expressed as disintegrations per minute.



observed in the absence of inhibitor.

4. [^{3}H] L-glutamate Binding Assay

The [^{3}H] L-glutamate binding assay was performed exactly as the [^{3}H] L-aspartate binding assay except [^{3}H] L-glutamate (18 to 30 nM final concentration) replaced [^{3}H] L-aspartate and nondisplaceable binding was defined as that in the presence of 10^{-2} M L-glutamate. 45 minutes incubation was sufficiently long for the [^{3}H] L-glutamate assay as the reaction had reached equilibrium within 30 minutes (Dr. Andrew Larder, personal communication).

5. Inhibition Curves

Inhibition of [^{3}H] L-aspartate (or [^{3}H] L-glutamate) binding by various compounds was determined as follows. Increasing concentrations (0 to 10^{-2} M) of the inhibitor under test were incubated with the membrane preparation. The binding to membranes at each concentration was determined and expressed as a percentage of the total binding in the absence of inhibitor. This percentage was then plotted against the log of the inhibitor concentration.

The concentration of inhibitor at which 50 percent of the displaceable binding was inhibited, the IC_{50}, could be determined from inhibition curves of each inhibitor. Comparison of IC_{50} values, that is the affinity of each inhibitor for the binding site, could then be made.

The level at which the binding of ligand could not be further decreased by the inhibitor was taken as the nondisplaceable binding.

Drawing the line by hand through the points on the inhibition curves was somewhat arbitrary. IC_{50} values were more reproducibly calculated by

conversion of the inhibition curve to a linear graph by Hill Transformation. Data obtained from inhibition curves were used to generate the Hill plot from the equation

$$\log\frac{100-y}{y-NDB} = n \log I - \log K_D$$

where y is the percent of total binding at each concentration of inhibitor I, NDB is the percent of total binding which is nondisplaceable, n is the Hill coefficient, and K_D the apparent dissociation constant. An example of a Hill plot is shown in Figure 16. The x-intercept of the line is the IC_{50}. The slope of the line is the Hill coefficient which is an indicator of the nature of the interaction between the binding site and inhibitor and is the main reason for generating the Hill plot. When the ligand interacts with a single population of binding sites, the IC_{50} curve from 10 to 90 percent of the displaceable binding will fall between 2 log units on the x-axis. A Hill plot with a slope of 1 will be generated. When the ligand interacts with, for example, two populations of binding sites, then the IC_{50} curve from 10 to 90 percent of the displaceable binding will fall between more than 2 log units up to a maximum of 4 log units. The slope of the Hill plot generated in this instance will be less than one. If the slope is 1, therefore, ligand interaction with a single population of non-interacting sites is indicated. When the slope is less than 1, either more than one population of sites are involved, each with a different affinity for the ligand, or a single population of sites is showing negative cooperativity where binding of one molecule inhibits the binding of others. If the slope is more than 1, positive cooperativity in which binding of the inhibitor to a site enhances subsequent binding is indicated. The degree to which the Hill coefficient is positive or

negative is not relevant, only that the coefficient is positive, negative, or 1.

To determine whether each inhibitor was interacting with the same population of sites as those affected by L-aspartate, tubes containing L-aspartate alone (10^{-2} M) or L-aspartate (10^{-2} M) plus the inhibitor under test (10^{-2} M) were assayed with each inhibition curve. If the combination of L-aspartate plus inhibitor reduced binding more than L-aspartate alone, then some of the inhibition was of the so-called nondisplaceable membrane binding sites. This displacement must then be from a different population of sites than that for compounds inhibiting only the displaceable membrane binding defined by 10^{-2} M L-aspartate alone. Comparisons of inhibitor affinity could not accurately be made in those circumstances. None of the compounds tested in the present experiments, however, displaced more [^{3}H] L-aspartate than did unlabelled L-aspartate. Binding to the same population of receptors was therefore indicated so that comparison of all the compounds tested could be made.

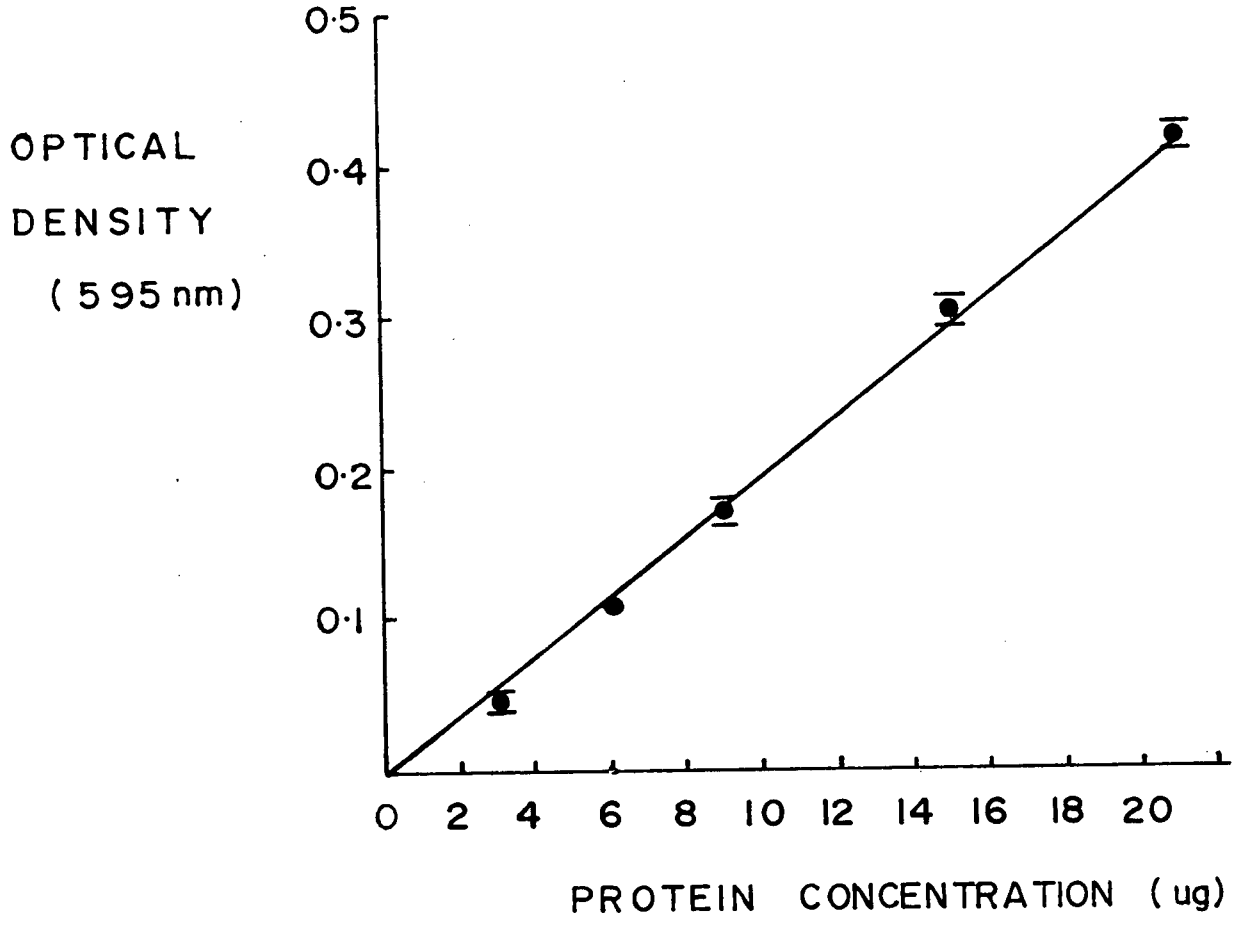
6. Bio-Rad Protein Assay

Samples of homogenate from each experiment were stored frozen until assayed for the protein concentration by the Bio-Rad microassay (Bio-Rad Laboratories, 1979).

The total volume of the assay mixture was 1 ml and consisted of 0.8 ml of diluted homogenate or standard protein solution, and 0.2 ml of concentrated Bio-Rad dye reagent. Blank tubes used to zero the spectrophotometer contained 0.8 ml of distilled water and 0.2 ml of concentrated dye reagent.

Figure 5. Bio-Rad Protein Standard Curve

Points are means ± standard error of the mean (SEM) from 10 experiments.



Dilutions of a 100 μg/ml standard solution (Bovine plasma gamma globulin) were made with distilled water to generate a standard curve ranging from 3 to 21 μg/ml protein. Homogenates from experiments were diluted with distilled water so that the protein concentration fell on the standard curve. When dilutions of all samples and standards had been made in duplicate, concentrated dye reagent was added and the solution immediately mixed. After 5 minutes incubation at room temperature, the absorbance of the samples at 595 nm was measured (SP6-500 uv spectrophotometer). Figure 5 shows the standard curve obtained from the average of 10 experiments. Protein concentrations of the samples were determined from the standard curve and expressed in fmol/mg protein.

7. Materials

L-[2, 3-^{3}H] aspartic acid (5.0 to 20 Ci/mmol) was purchased from Amersham, New England Nuclear, or ICN. L-[G-^{3}H] glutamic acid was obtained from Amersham, and protosol and omnifluor from New England Nuclear. The following chemicals were also used: L-aspartic acid (K and K Laboratories); D-aspartic acid (Calbiochem); D-α-aminoadipic acid, N-methyl-DL-aspartic acid, kainic acid, and Trisma base (Sigma Chemical Co.); and Standard I-Bovine plasma gamma globulin and Bio-Rad dye reagent concentrate (Bio-Rad Laboratories). All other reagents were from Fisher Scientific Co.

III. RESULTS

1. Development of the [^{3}H] L-Aspartate Binding Assay

1a. Initial experiments

The initial [^{3}H] L-aspartate binding assays were performed on synaptosomal preparations of whole brain. The procedure was as described in the methods except that the incubation time was 30 minutes and the displacing compound L-aspartate (10^{-2} M) was dissolved in Tris-HCl (pH 7.5) with no further pH adjustment. The average displaceable binding observed (Table I) was 3229 dpm per 0.106 mg protein, 0.106 mg being the average amount of protein per tube. (See Appendix I for a discussion of the low dpm obtained).

1b. Effect of cations on the initial [^{3}H] L-aspartate binding

The low displaceable binding observed in the initial experiments was not considered adequate for a reliable assay. In an attempt to increase the displaceable binding, a buffer composed of 50 mM Tris-HCl (pH 7.5), 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ was tested in the assay. The displaceable binding was increased 3.6 times in the buffer containing these salts (Table II).

To identify which ions were responsible for the increased displaceable binding, the effect of each ion alone was compared to that in the absence of ions or in the presence of all three ions. The results in Table II indicate that Ca^{2+} appeared mainly responsible for the increased displaceable binding. The increased binding in the presence of Ca^{2+}, however, occurred only under the initial buffering conditions of the [^{3}H]

Table I Displaceable [^{3}H] L-Aspartate Binding to Crude Synaptosomal Membranes

Crude synaptosomal membranes suspended in 50 mM Tris-HCl (pH 7.5) were incubated with [^{3}H] L-aspartate (120 nM final concentration) in the presence or absence of 10^{-2} M L-aspartate, and the displaceable binding determined. Each value is the mean ± SEM of a single experiment performed in quadruplicate.

Experiment	Displaceable [^{3}H] L-Aspartate Binding (dpm/average mg protein concentration in the tube)
1	3296 ± 397 / 0.0715 mg protein
2	4002 ± 506 / 0.092 mg protein
3	2389 ± 266 / 0.15 mg protein
Mean	3229 ± 467 / 0.106 mg protein

Table II Effect of Cations of [^{3}H] L-Aspartate Binding

Displaceable [^{3}H] L-aspartate binding was determined in the presence of 50 mM Tris-HCl buffer (pH 7.5) or the same buffer containing 5 mM KCl, 2 mM $CaCl_2$, and/or 1 mM $MgCl_2$ as specified below. Values are the mean ± SEM from 3 experiments performed in quadruplicate. S = significantly different from binding in the absence of salts, $p<0.05$; NS = not significantly different.

	Displaceable Binding (fmol/mg protein)		
Experiment #:	1	2	3
Salts in Buffer			
None	689 ± 69	342 ± 116	476 ± 58
KCl + $MgCl_2$ + $CaCl_2$	1256 ± 152 S	1398 ± 117 S	2308 ± 186 S
KCl	232 ± 23 NS	268 ± 65 NS	389 ± 24 NS
$MgCl_2$	673 ± 42 NS	309 ± 64 NS	553 ± 74 NS
$CaCl_2$	1380 ± 110 S	588 ± 44 NS	1718 ± 146 S

L-aspartate assay (see Results section 1f for data and discussion). The displaceable binding observed with K^+ or Mg^{2+} alone was not significantly different from that observed with Tris buffer (Students t-test). Experiments in sections 1c to 1f of Results were therefore performed with Tris-HCl plus 2mM $CaCl_2$ buffer (pH 7.5). Experiments after section 1f, however, were performed using Tris-HCl for the reasons outlined in that section.

1c Time course of the association of [^{3}H] L-aspartate binding

Analysis of the data from binding assays assumes that the reaction is at equilibrium when incubation is terminated. To determine whether the displaceable aspartate binding to synaptosomes of whole brain was at equilibrium after the 30 minute incubation initially chosen, binding was measured after 0, 5, 10, 15, 20, 30, and 45 minutes incubation. The average binding to tubes (56 dpm) determined from previous experiments was negligible and therefore not determined in subsequent experiments (except in section 1f of Results).

The nondisplaceable binding was maximal in 5 minutes and did not change with longer incubation times (Table III). Figure 6 shows that displaceable binding was at equilibrium by 30 minutes incubation. Lower concentrations of [^{3}H] L-aspartate, however, require a longer incubation time. To ensure that equilibrium was reached for all [^{3}H] L-aspartate concentrations used, a 45 minute incubation time was chosen.

1d Determination of nondisplaceable [^{3}H] L-aspartate binding

The nondisplaceable binding of [^{3}H] L-aspartate to synaptosomes of whole brain was determined from an inhibition curve with [^{3}H] L-aspartate

Table III Time Course of Nondisplaceable [^{3}] L-Aspartate Binding

Nondisplaceable [^{3}H] L-aspartate binding (142 nM) to crude synaptosomes of whole brain was determined in the presence of 10^{-2} M L-aspartate at various incubation times. Values are the mean ± SEM of one experiment performed in quadruplicate. NS = not significantly different from the nondisplaceable binding at 5 minutes; s = significantly different $p<0.05$.

Incubation Time (minutes)	Nondisplaceable Binding (dpm)	
5	8216 ± 202	
10	8173 ± 359	NS
15	10096 ± 686	S
20	8558 ± 139	NS
30	8514 ± 276	NS
45	8511 ± 212	NS

Figure 6. Time Course of [^{3}H] L-Aspartate Binding

Binding to synaptosomal fractions of whole brain incubated for various times was determined. o, ●, and ▲ represent the results from 3 different experiments each performed in quadruplicate. The SEM of each point ranged from 4% to 63% with the majority below 10%.

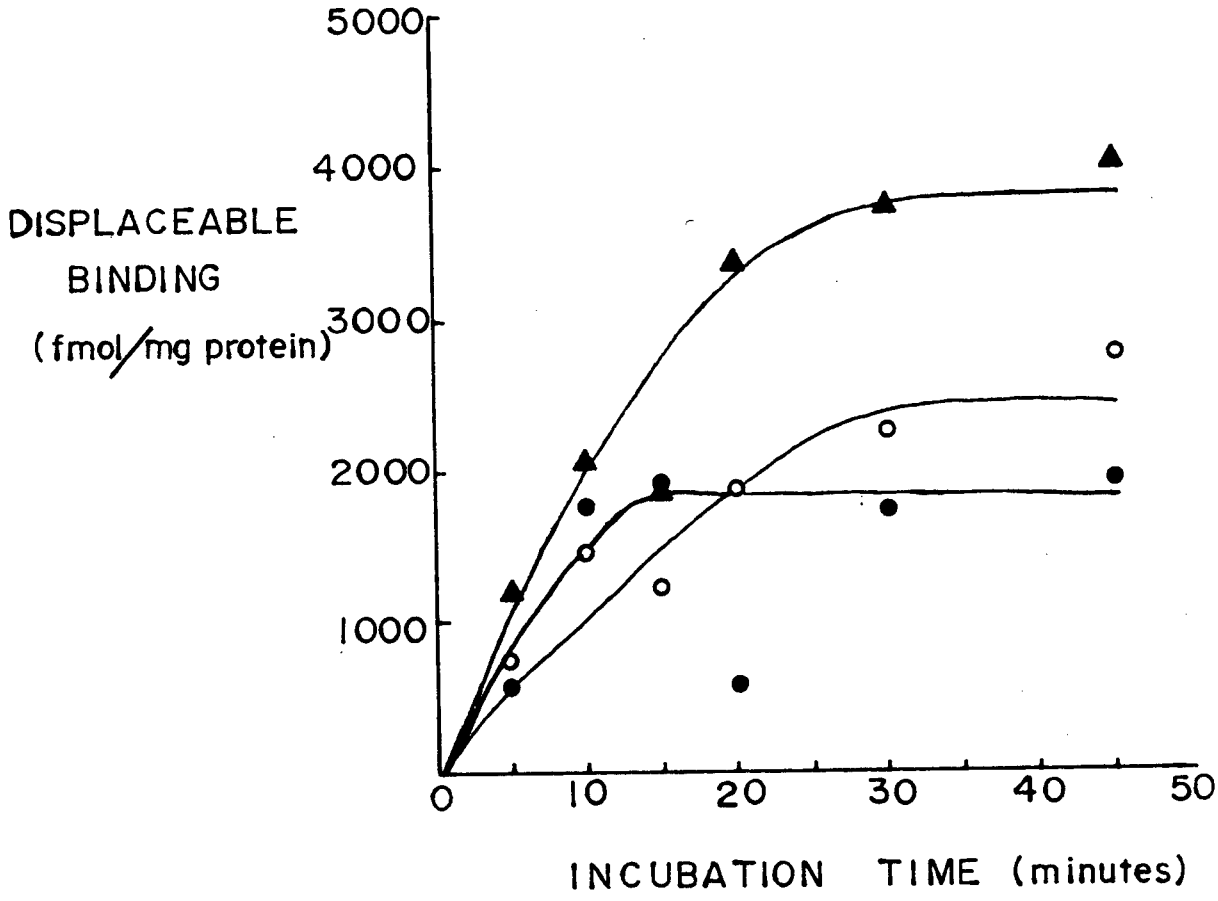
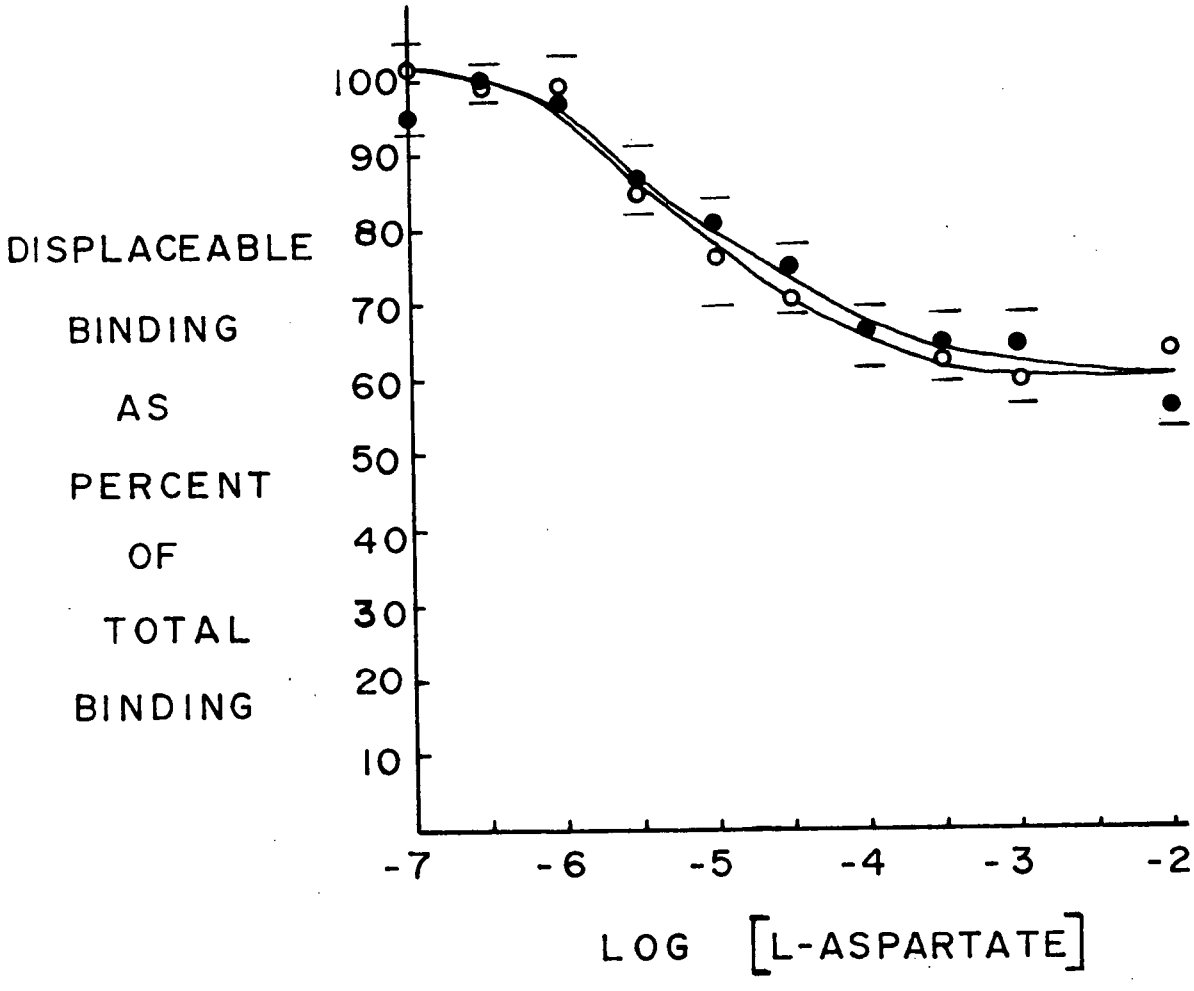


Figure 7. [^{3}H] L-Aspartate Binding to Crude Synaptosomes and to Total Particulate Fractions of Whole Brain

Membrane preparations were suspended in Tris-HCl plus 2 mM $CaCl_2$ (pH 7.5). Points are the mean ± SEM of 4 experiments. Crude synaptosomal preparations (•), [^{3}H] L-aspartate = 146 nM final concentration. Total particulate fractions (o), [^{3}H] L-aspartate = 148 nM final concentration.



as the inhibitor. Figure 7 (closed circles) shows that increasing concentrations of L-aspartate decreased [^{3}H] L-aspartate binding up to 3×10^{-4} M L-aspartate after which the binding was not further displaced. Nondisplaceable binding comprised 58 percent of the total binding while the displaceable binding at 10^{-2} M L-aspartate was 4370 fmol per mg protein. The IC_{50} obtained from Hill plot analysis of Figure 7 was 11.0 μM. The Hill coefficient was 0.79.

1e. Comparison of [^{3}H] L-aspartate binding to synaptosomes and total particulate fractions of whole brain

Only 25 percent of the initial tissue wet weight was recovered after preparation of crude synaptosomal membranes compared to the 90 percent recovery obtained with the total particulate fraction. It was therefore desirable to use total particulate fractions because of the better recovery. Before total particulate fractions could be used, however, it had to be shown that the [^{3}H] L-aspartate binding sites were the same in both synaptosomes and total particulate fractions. The inhibition curve in total particulate fractions of whole brain (Figure 7, open circles) showed similar nondisplaceable binding (60 percent), IC_{50} (5.20 μM), and Hill coefficient (0.60) as that observed using crude synaptosomal membranes. IC_{50} values differing by less than a factor of 10 were considered to be similar. Differences may have been due to differences in the membrane fraction used.

The displaceable binding was increased 2.5 times from 1730 to 4370 fmol/mg protein in crude synaptosomal preparations as compared to total particulate fractions indicating that the binding sites may be associated with synaptic junctions.

1f. Effect of pH and Ca^{2+} on [^{3}H] L-aspartate binding

In the experiments described so far, L-aspartate was dissolved in Tris-HCl buffer already adjusted to pH 7.5. The pH of a 10^{-2} M L-aspartate solution prepared in this way, however, was found to be 3.5. When the aspartate was added to the binding assay reaction mixture, a final pH of 4.25 was observed.

Receptors in a binding assay where the pH is acid may not display the characteristics seen in vivo where the pH is approximtely neutral. Ca^{2+} in the previous experiments may then have acted by stabilizing receptors in an acid environment. The increased binding in the presence of Ca^{2+} may then not be a characteristic seen in vivo.

To determine whether displaceable binding would be increased at a more neutral pH (pH 7.5) in the absence of Ca^{2+} and whether Ca^{2+} increased the displaceable binding further at pH 7.5 the following experiment was performed. Four experimental conditions of nondisplaceable binding to synaptosomal membranes were compared. 10^{-2} M L-aspartate with the pH of the final reaction mixture either 4.2 or 7.5 was dissolved in either Tris-HCl or Tris-HCl plus 2 mM $CaCl_2$. The results are shown in Table IV. Synaptosomal preparations were used again in the experiments of this section, so that comparisons could be made with previous data.

Homogenates incubated with L-aspartate in Tris buffer at a final pH of 4.2 served as controls. Increasing the pH or adding Ca^{2+} increased the displaceable binding but increasing the pH in the presence of Ca^{2+} had no additional effect on displaceable binding. Ca^{2+} may therefore have acted to stabilize receptors under conditions which were too acid.

It was preferable to use solutions buffered with Tris-HCl (final pH 7.5) in the absence of Ca^{2+} because of the possibility that Ca^{2+} might

Table IV Effect of pH and Ca^{2+} on [^{3}H] L-Aspartate Binding to Synaptosomal Fractions of Whole Brain

Values are the means ± SEM of 3 experiments except * which are the results from 2 experiments each performed in quadruplicate. [^{3}H] L-aspartate = 152 nM final concentration; NDB = nondisplaceable binding; DB = displaceable binding. S = significantly different from binding at pH 4.2 ($p<0.05$); NS = not significantly different.

Buffer	Displaceable Binding (fmol/mg protein)
Tris-HCl final pH 4.2	585 ± 65, NDB>DB*
pH 7.5	1155 ± 207 S
Tris-HCl + 2 mM $CaCl_2$	
final pH 4.2	1237 ± 366
pH 7.5	1237 ± 187 NS

substitute for Na^+ and thus activate Na^+-dependent aspartate uptake sites. The inhibition curve generated with L-aspartate solutions adjusted to pH 7.5 using total particulate fractions of whole brain is shown in Figure 8. The curve with 62 percent nondisplaceable binding, an IC_{50} of 2.12 μM and Hill coefficient of 0.38 was similar to the curve generated in the presence of Ca^{2+} at a final reaction mixture pH of 4.2 (Figure 7 open circles). All subsequent experiments used total particulate fractions of the brain region under investigation with all solutions buffered with Tris-HCl, with no Ca^{2+} present, and the final pH adjusted to 7.5.

1g Effect of washing the surface of the membrane pellet on displaceable [^{3}H] L-aspartate binding

To determine the effect of washing the surface of the pellet on the [^{3}H] L-aspartate binding, the pellet was washed 0, 2, or 4 times with 100 μl of ice-cold distilled water and binding in the presence and absence of 10^{-2} M L-aspartate determined (Table V).

With no washes, the displaceable binding was very high, as was the standard error of the mean. After 2 washes, displaceable binding was much lower and the SEM was reduced to 7 percent of the displaceable binding. The results obtained after 4 washes were not significantly different from those with 2 washes. Two washes were therefore performed for all other experiments.

In summary then, the conditions for the measurement of [^{3}H] L-aspartate binding utilized the total particulate fractions of the brain region under investigation with all solutions made up in Tris-HCl and the final reaction mixture pH adjusted to 7.5. Two washes of the pellet

Figure 8. Inhibition of [^{3}H] L-Aspartate Binding to the Total Particulate Fraction of Whole Brain

Membranes were suspended in Tris-HCl buffer (pH 7.5). [^{3}H] L-aspartate = 152 nM. Values are the means ± SEM from 3 experiments each performed in quadruplicate.

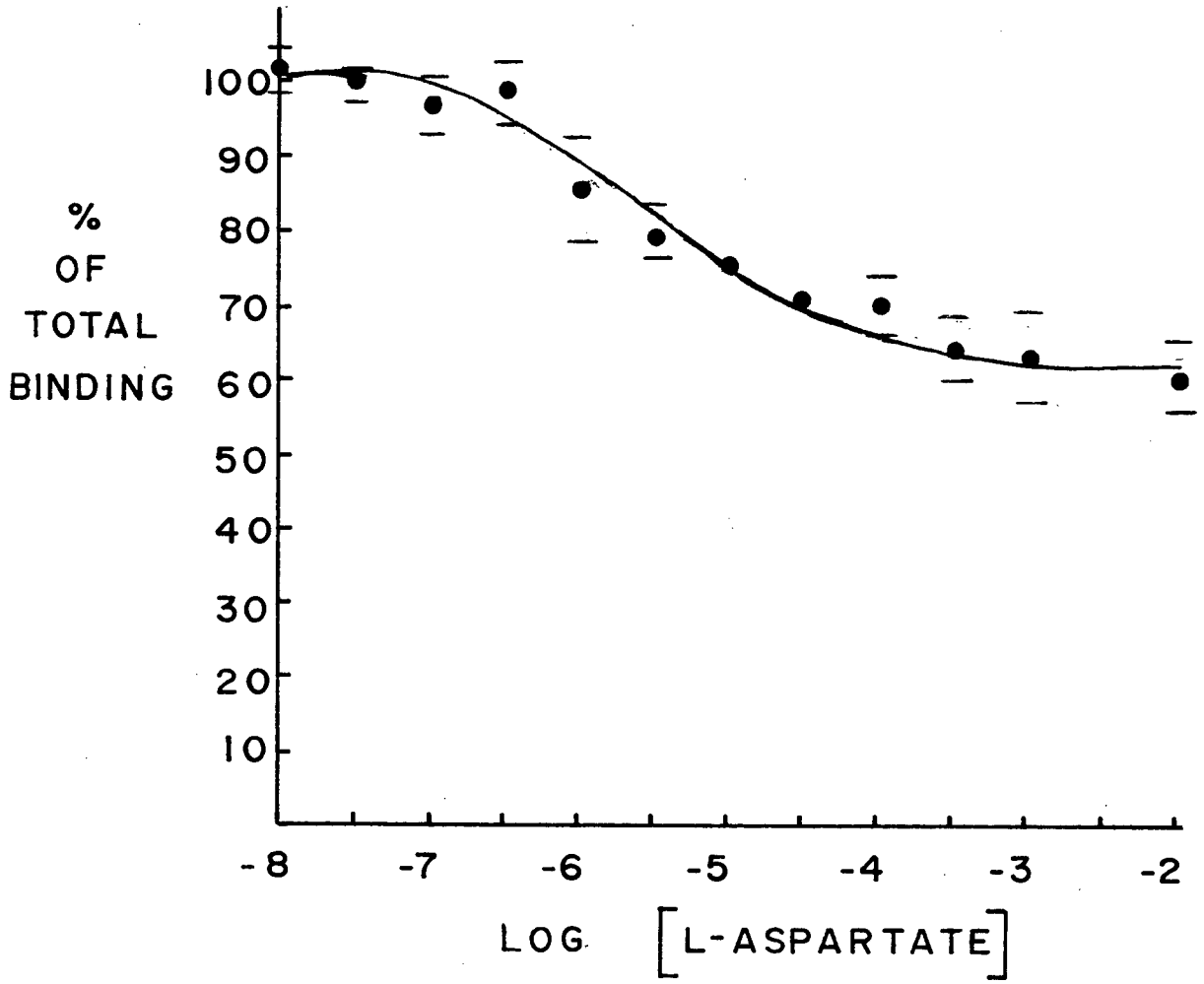


Table V Effect of Washing the Surface of the Membrane Pellet on Displaceable [^{3}H] L-Aspartate Binding

The pellet was washed various times with 100 µl of ice-cold distilled H_2O and the displaceable binding of [^{3}H] L-aspartate (145 nM final concentration) to total particulate fractions of whole brain determined. Values are the mean ± SEM from a single experiment performed in quadruplicate. NS = not significantly different from 2 washes, $p>0.05$.

# Washes	Displaceable Binding (dpm)	
0	8593 ± 3153	
2	5524 ± 387	
4	4661 ± 541	NS

obtained after incubation of the reaction mixture were routinely performed.

2. Characterization of [^{3}H] L-Aspartate Binding in the Cerebellum

The cerebellum was chosen for the characterization of [^{3}H] L-aspartate binding for three major reasons:

1. There is strong evidence that both aspartate and glutamate are neurotransmitters in the cerebellum (Storm-Mathisen, 1978; Stone, 1979; Perry et al., 1981). Aspartate is most likely the neurotransmitter in the climbing fibers (Nadi et al., 1977) while glutamate is most likely that in the parallel fibers of granule cells.
2. Dissection of the cerebellum is rapid and very reproducible. Differences between experimental results caused by variations in the brain region dissected are therefore negligible.
3. The cerebellum is a large region. Large numbers of rats are therefore not required to do each experiment.

2a. [^{3}H] L-aspartate binding in the cerebellum

The displaceable binding of [^{3}H] L-aspartate to total particulate fractions of cerebellum (730 $\pm$ 31 fmol/mg protein, n=9) was significantly greater than in whole brain preparations (592 $\pm$ 33 fmol/mg protein, n=5, $p<0.05$). The average displaceable binding to cerebellar membranes was 4468 dpm/0.225 mg protein, 0.225 mg of protein being the average amount of protein in each tube.

In an attempt to increase the displaceable [^{3}H] L-aspartate binding to cerebellar membranes, two procedures were tested: 1. preincubation of the membrane preparation because preincubation increased [^{3}H] L-glutamate

binding (Sharif and Roberts, 1980b) and 2. a filtration assay.

2a i. Effect of preincubation on [^{3}H] L-aspartate binding to cerebellar membranes

Preincubation was performed during the preparation of the total particulate fraction as follows. The cerebellum was homogenized in ice-cold Tris-HCl (pH 7.5) and centrifuged at 48000 x g for 20 minutes. The pellet, resuspended by sonication for 60 seconds, was incubated in a 37° C water bath for 30 minutes. The suspension was then centrifuged at 48000 x g for 20 minutes and the membrane preparation continued as in methods (section 2).

Preincubation decreased the displaceable binding by approximately 28 percent (Table VI) so this step was not included in subsequent experiments. Denaturation of the binding site during preincubation may have been the cause of the decreased binding.

2a ii. Measurement of [^{3}H] L-aspartate binding using a filtration assay

The fraction of total binding that is displaceable can sometimes be increased by decreasing the nondisplaceable binding with the use of filtration instead of centrifugation as a means of separating bound from unbound label. The membrane preparation is spread over the filter instead of being compacted into a pellet. Less label is then nonspecifically trapped in the filtration method. The technique is also very fast so less dissociation of the ligand from the binding site may occur than when centrifugation is used as the method of separation (Bennett, 1978).

A filtration assay of [^{3}H] L-aspartate binding to total particulate fractions of the cerebellum was performed (kindly by Dr. Andrew Larder) as

Table VI Effect of Preincubation on Displaceable [^{3}H] L-Aspartate Binding to Cerebellar Membranes

Values are the mean ± SEM from 2 experiments each performed in quadruplicate. Preincubation significantly decreased binding in both experiments (Students t-test, $p<0.05$).

	Displaceable Binding (fmol/mg protein)	
Experiment #:	1	2
No preincubation	712 ± 46	681 ± 80
Preincubation	520 ± 75	486 ± 55

follows. The reaction mixture and incubation temperature and time were identical to those in the centrifugation assay (Methods section 3). At the end of incubation, samples were filtered under reduced pressure (12 chambered Millipore Filtration Manifold) and the glass-fibre filters (GF/C filters) washed one to three times with 5 ml each time of ice-cold buffer. The trapped radioactivity was measured by soaking the filter in protosol (1 ml) for 2 hours, adding omnifluor (10 ml) and counting by liquid scintillation spectrometry. Binding to the filters in the presence and absence of L-aspartate (10^{-2} M) was also assayed by substituting the membrane preparation with the same volume of buffer. For comparison, a centrifugation assay was performed simultaneously with the same membrane preparation used in the filtration assay.

After subtracting filter binding, the displaceable binding in the filtration assay performed with one wash was no different from that in the centrifugation assay. The standard error of the mean of the filtration assay, however, was high. Two or three washes of the filter reduced the displaceable binding significantly below that seen in the centrifugation assay (Students t-test, $p<0.05$). Centrifugation therefore continued to be the procedure used to separate bound from unbound ligand.

2b. Time course of the association of [^{3}H] L-aspartate binding to cerebellar membranes

To determine whether displaceable binding to total particulate fractions of cerebellum was at equilibrium after the 45 minute incubation time, total and nondisplaceable [^{3}H] L-aspartate binding was measured after 0, 5, 10, 15, 20, 30, 45 or 60 minutes incubation.

Displaceable binding increased as incubation time increased from 0 to

20 minutes after which no further increase occurred (Figure 9). Binding was therefore at equilibrium at the 45 minute incubation time chosen for [^{3}H] L-aspartate binding studies. The nondisplaceable binding was instantaneous and did not change significantly with time (Table VII).

The time course of binding to the total particulate fraction of cerebellum was similar to that in synaptosomal fractions of whole brain performed in the presence of Ca^{2+} (see Figure 6). These data infer that the binding in cerebellum would be the same in total particulate fractions and synaptosomal preparations.

2c. Increase of [^{3}H] L-aspartate binding with protein concentration

The protein concentration of homogenates varied somewhat between experiments because membranes were resuspended in buffer according to the wet weight of the pellet. Different amounts of water were trapped in pellets after the last centrifugation step. To compare results expressed as the binding per milligram of protein from different experiments, the binding to homogenates must increase linearly with the protein concentration. Total binding, nondisplaceable binding (that observed in the presence of 10^{-2} M L-aspartate) and displaceable binding to the cerebellar total particulate fraction were therefore determined at various protein concentrations. Figure 10 demonstrates that the displaceable binding increased linearly over the range of protein concentrations encountered routinely in experiments (about 0.25 to 0.45 mg protein/ml).

2d. Inhibition of [^{3}H] L-aspartate binding to cerebellar membranes by L-aspartate

An inhibition curve of [^{3}H] L-aspartate displaced by L-aspartate was

Figure 9. Time Course of Association of [^{3}H] L-Aspartate Binding to Total Particulate Fractions of Cerebellum

Values are the mean ± SEM of a single experiment performed in quadruplicate. [^{3}H] L-aspartate = 140 nM final concentration.

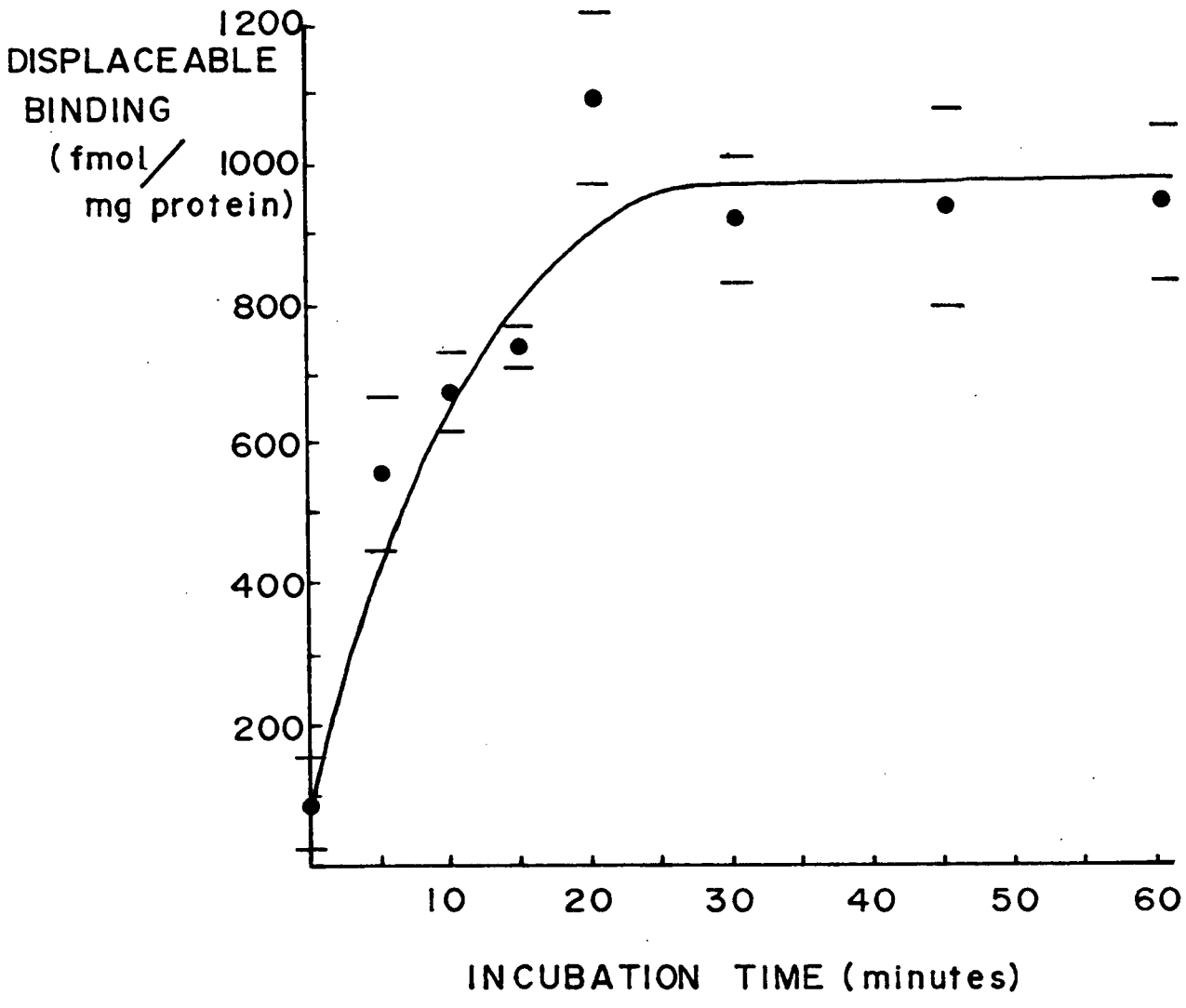


Table VII Nondisplaceable Binding of [^{3}H] L-Aspartate to Cerebellar Membranes

Values are the mean ± SEM of quadruplicates from a single experiment. All values, except that at 5 minutes, were not significantly different from that at 0 minutes, $p>0.05$.

Incubation Time (minutes)	Nondisplaceable Binding (dpm)	n
0	6840 ± 383	4
5	8587 ± 455	4
10	7178 ± 266	4
15	6806 ± 142	4
20	6236 ± 351	4
30	7318 ± 323	3
45	8528 ± 512	3
60	7675 ± 184	3

performed on the total particulate fraction of cerebellum (Figure 11). Increasing concentrations of L-aspartate from 10^{-8} to 3×10^{-5} M increasingly inhibited [^{3}H] L-aspartate binding to the membrane preparation while higher concentrations produced no further inhibition. The nondisplaceable binding defined as that occurring at 10^{-2} M L-aspartate was 64 percent of the total binding. The IC_{50} calculated from the Hill plot was 1.81 μM while the Hill coefficient was 0.60. These data indicate that [^{3}H] L-aspartate binding in the cerebellum and in whole brain are similar.

The apparent dissociation constant (K_I) for an inhibitor can be calculated from the equation

$$K_I = \frac{IC_{50}}{1 + \frac{L}{K_D}} \qquad (1)$$

where K_D is the dissociation constant of the ligand-receptor complex and L is the ligand concentration. When the inhibitor is the same compound as the ligand, $K_I = K_D$. Equation (1) becomes

$$IC_{50} = K_D + L$$

and rearranging

$$K_D = IC_{50} - L$$

substituting the IC_{50} and L values obtained from the inhibition curve of [^{3}H] L-aspartate displaced by L-aspartate, the K_D can be calculated. The K_D value for L-aspartate was approximately equal to the IC_{50} value of 1.81 μM.

2e. <u>Saturation analysis of [^{3}H] L-aspartate binding to cerebellar membranes</u>

To determine whether [^{3}H] L-aspartate binding was saturable,

Figure 10. Increase of [^{3}H] L-Aspartate Binding with Protein Concentration

Total (●), nondisplaceable (o), and displaceable binding (▲) to the cerebellar total particulate fracion were determined at various protein concentrations. Values are the mean ± SEM from one experiment performed in quadruplicate. [^{3}H] L-aspartate = 120 nM final concentration.

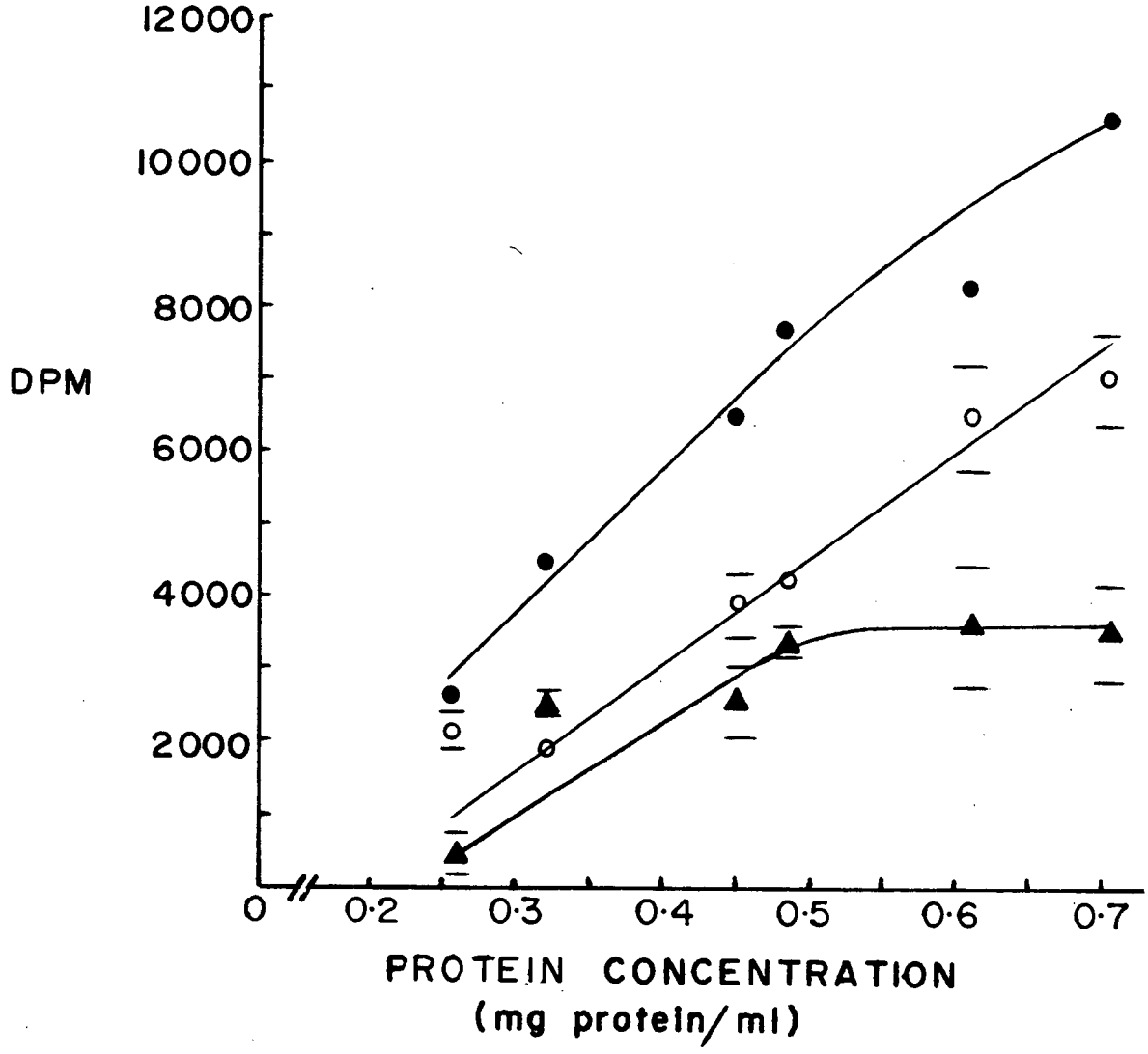
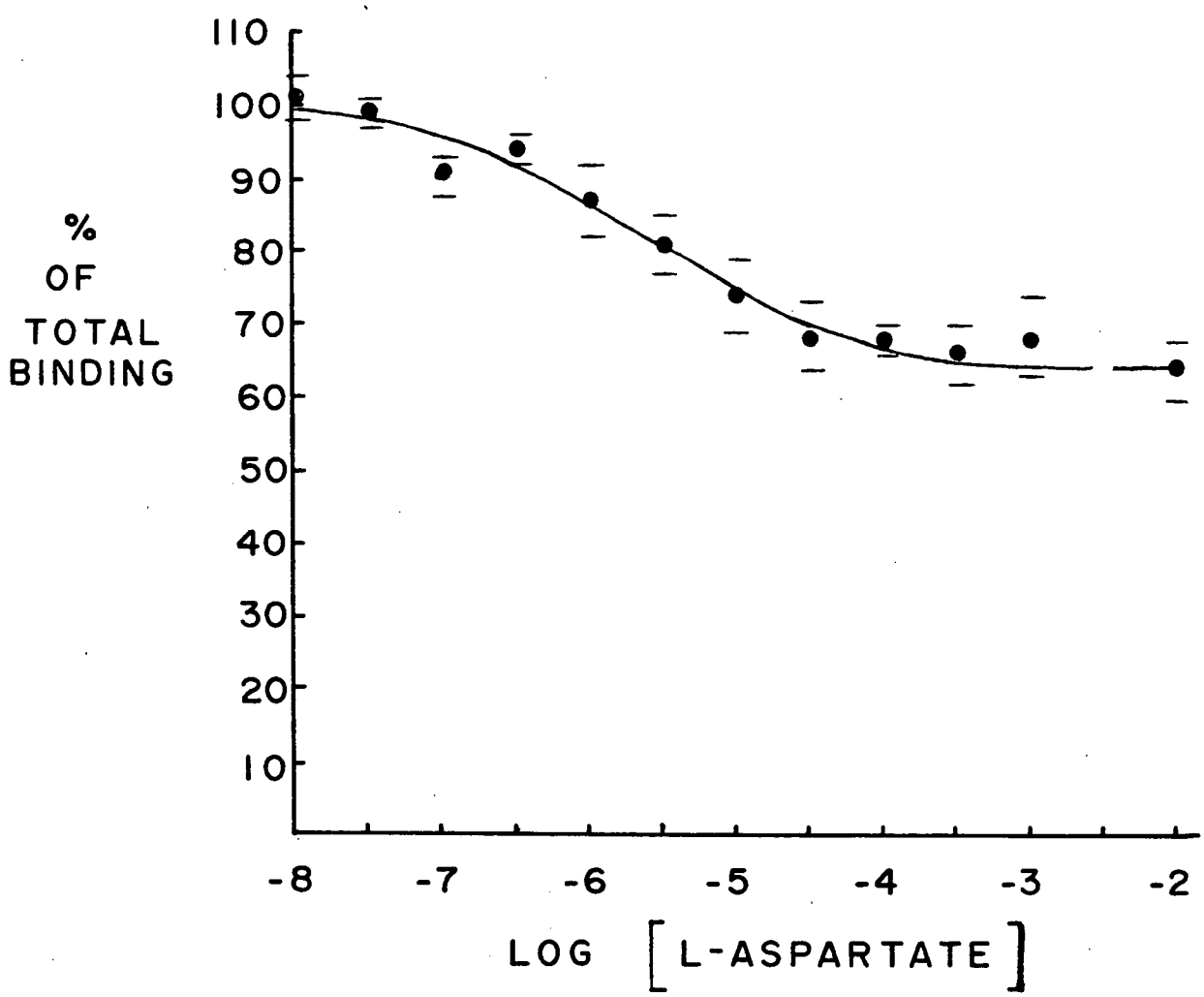


Figure 11. Inhibition of [^{3}H] L-Aspartate Binding to the Total Particulate Fraction of Cerebellum by Unlabelled L-Aspartate

Membrane preparations were suspended in Tris-HCl buffer (pH 7.5) [^{3}H] L-aspartate = 143 nM. Values are the means ± SEM from 4 experiments each performed in quadruplicate.



cerebellar membranes were incubated with increasing concentrations of [^{3}H] L-aspartate in the presence and absence of 10^{-2} M L-aspartate. The displaceable binding observed was plotted as a function of the [^{3}H] L-aspartate concentration (Figure 12).

The displaceable binding in two experiments was saturable while the concentration range in a third experiment was not sufficiently wide to show saturation of displaceable binding.

The displaceable binding divided by the free concentration of [^{3}H] L-aspartate was plotted as a function of the free concentration of [^{3}H] L-aspartate to form a Scatchard plot. The Scatchard equation is

$$\frac{B}{F} = \frac{Bmax - B}{K_D} \qquad (2)$$

where B is the amount of ligand bound, F is the concentration of ligand, Bmax is the maximum number of binding sites, and K_D is the apparent dissociation constant. K_D can be derived by rearranging equation 2

$$K_D = \frac{Bmax - B}{\frac{B}{F}}$$

which is the negative inverse of the slope of the Scatchard plot. Bmax is the x-intercept of the Scatchard plot. The major advantage of using Scatchard analysis is that in a saturation curve the plateau and therefore Bmax are difficult to determine.

Scatchard analysis of the data from these three experiments (Figure 13 and Table VIII) gave a mean dissociation constant (K_D) of 1.64 μM for the displaceable [^{3}H] L-aspartate binding and a mean maximum number of binding sites (Bmax) of 7711 fmol/mg protein. The apparent dissociation constant determined from Scatchard analysis and from the L-aspartate inhibition curve are therefore identical.

Figure 12. Saturation Analysis of [^{3}H] L-Aspartate Binding to Total Particulate Fractions of Cerebellum

Values are the means of quadruplicates. The SEM of each point was between 5 and 36 percent with the majority less than 15 percent. The line is the average of results from 3 separate experiments (●, o, ▲).

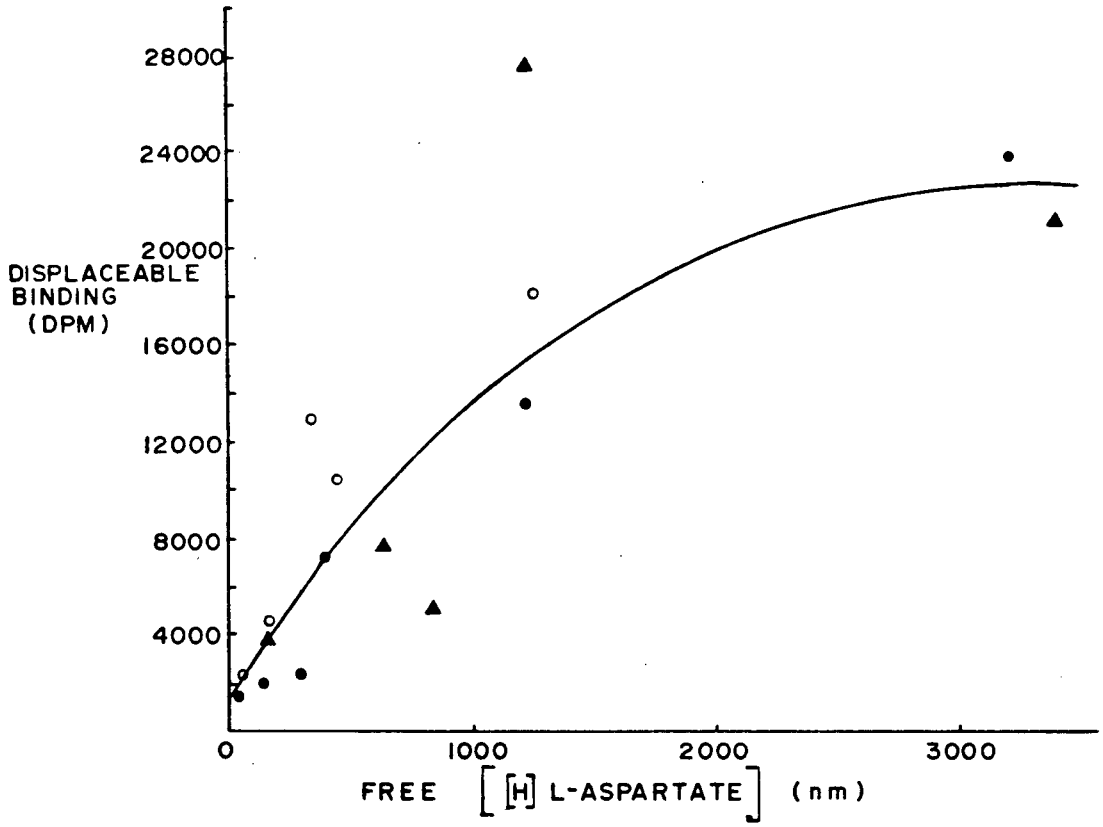


Figure 13. Scatchard Analysis of [^{3}H] L-Aspartate Binding to Total Particulate Fractions of Cerebellum

Points are the means of quadruplicates from three separate experiments while the line through the points is drawn using the average K_D and Bmax obtained from the three experiments.

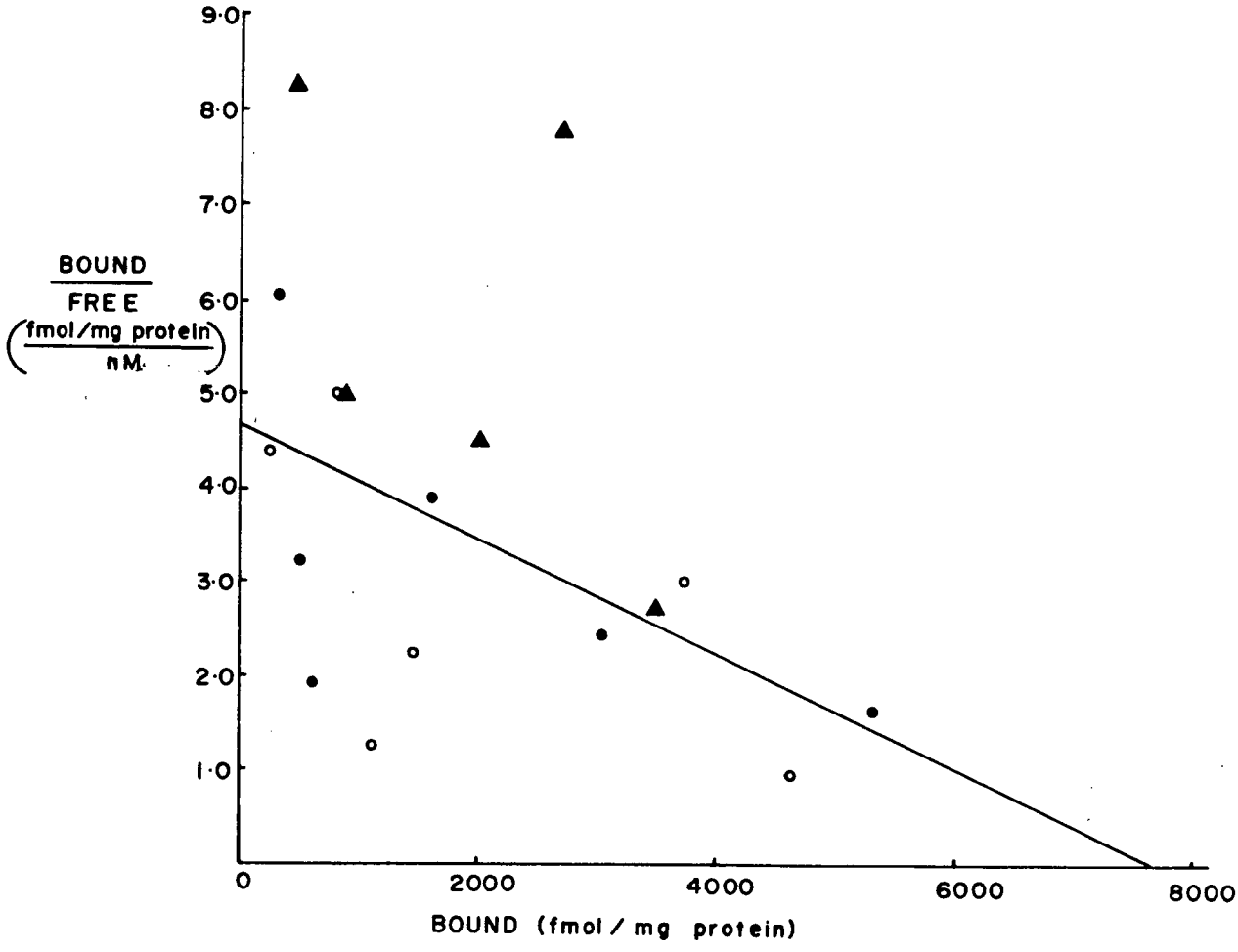


Table VIII Summary of Scatchard Analysis Results Shown in Figures 12 and 13

Experiment #	K_D(μM)	Bmax (fmol/mg protein)
1	0.971	7358
2	1.87	7245
3	2.07	8531
Mean	1.64 ± 0.34	7711 ± 411

3. Pharmacological Specificity of [^{3}H] L-Aspartate and [^{3}H] L-Glutamate Binding to Cerebellar Membranes

It is possible that [^{3}H] L-aspartate and [^{3}H] L-glutamate bind to the same population of binding sites. To test this hypothesis and to characterize the [^{3}H] L-aspartate binding, the ability of a range of concentrations of each of L- and D-aspartate, L-glutamate, N-methyl-DL-aspartate, kainate, D- and L-alpha-aminoadipate to inhibit [^{3}H] L-aspartate binding to cerebellar membranes was compared with their ability to inhibit [^{3}H] L-glutamate binding. Inhibition for these compounds displacing [^{3}H] L-aspartate or [^{3}H] L-glutamate are shown in Figures 14 and 15 respectively. Where possible, IC_{50} values and Hill coefficients were determined using the Hill Transformation (Table IX and X).

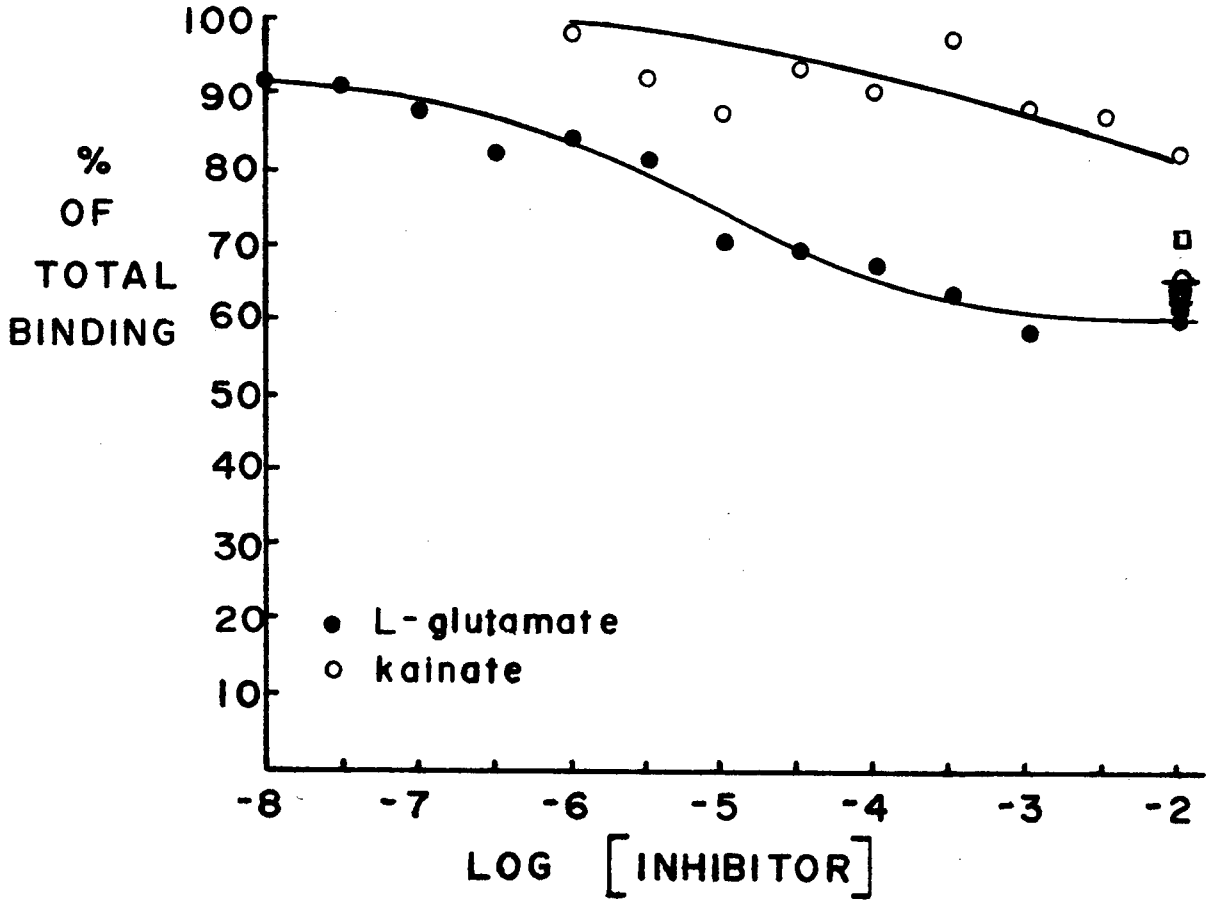
When [^{3}H] L-aspartate was the ligand, L-aspartate and L-glutamate were equally active with IC_{50} values of 1.81 and 1.24 μM respectively and were the most potent inhibitors of [^{3}H] L-aspartate binding. L-alpha-aminoadipate was slightly less potent (IC_{50} = 7.12 μM) while D-aspartate with an IC_{50} of 46.6 μM was 10 times less potent. Kainate and N-methyl-DL-aspartate were very weak inhibitors with detectable [^{3}H] L-aspartate displacement only at concentrations greater than 10 and 300 μM respectively. D-alpha-aminoadipate did not inhibit binding even at a concentration of 10000 μM. At no concentration did kainate or N-methyl-DL-aspartate inhibit 50 percent of the displaceable [^{3}H] L-aspartate binding. IC_{50} values therefore could not be determined for kainate, N-methyl-DL-aspartate, or D-alpha-aminoadipate.

When the ligand was [^{3}H] L-glutamate, L-glutamate, D-alpha-aminoadipate, and L-aspartate with IC_{50} values of 2.25, 2.51, and

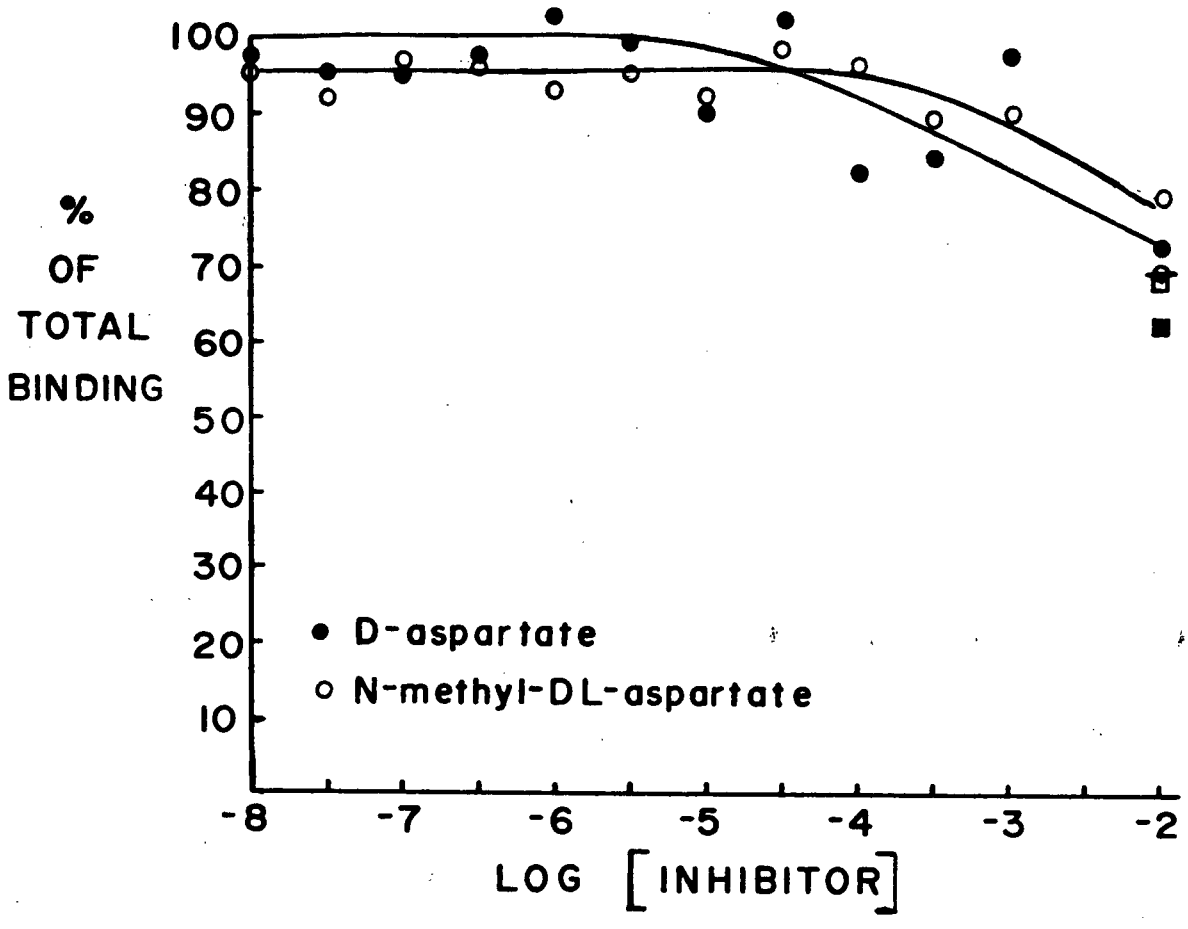
Figure 14. Inhibition of [^{3}H] L-Aspartate Binding to Cerebellar Membranes by Various Inhibitors

Values are the average of quadruplicates from 2, 3, or 4 experiments as indicated in Table X. The SEM was between 0 and 11 percent with the majority below 5 percent. The final concentration of [^{3}H] L-aspartate varied between 102 and 153 nM. The squares represent the % of total binding in the presence of 10^{-2} M L-aspartate while the circles with lines through them represent the % of total binding in the presence of L-aspartate (10^{-2} M) plus inhibitor (10^{-2} M) for a) L-glutamate (■, ●) and kainate (□, o), b) D-aspartate (■, ●) and N-methyl-DL-aspartate (□, o), and c) D-alpha-aminoadipate (■, ●)and L-alpha-aminoadipate (□, o).

a)



14 b)


100
90
80
70
60
50
40
30
20
10
% OF TOTAL BINDING
D-aspartate
N-methyl-DL-aspartate
-8
-7
-6
-5
-4
-3
-2
LOG [INHIBITOR]

14 c)

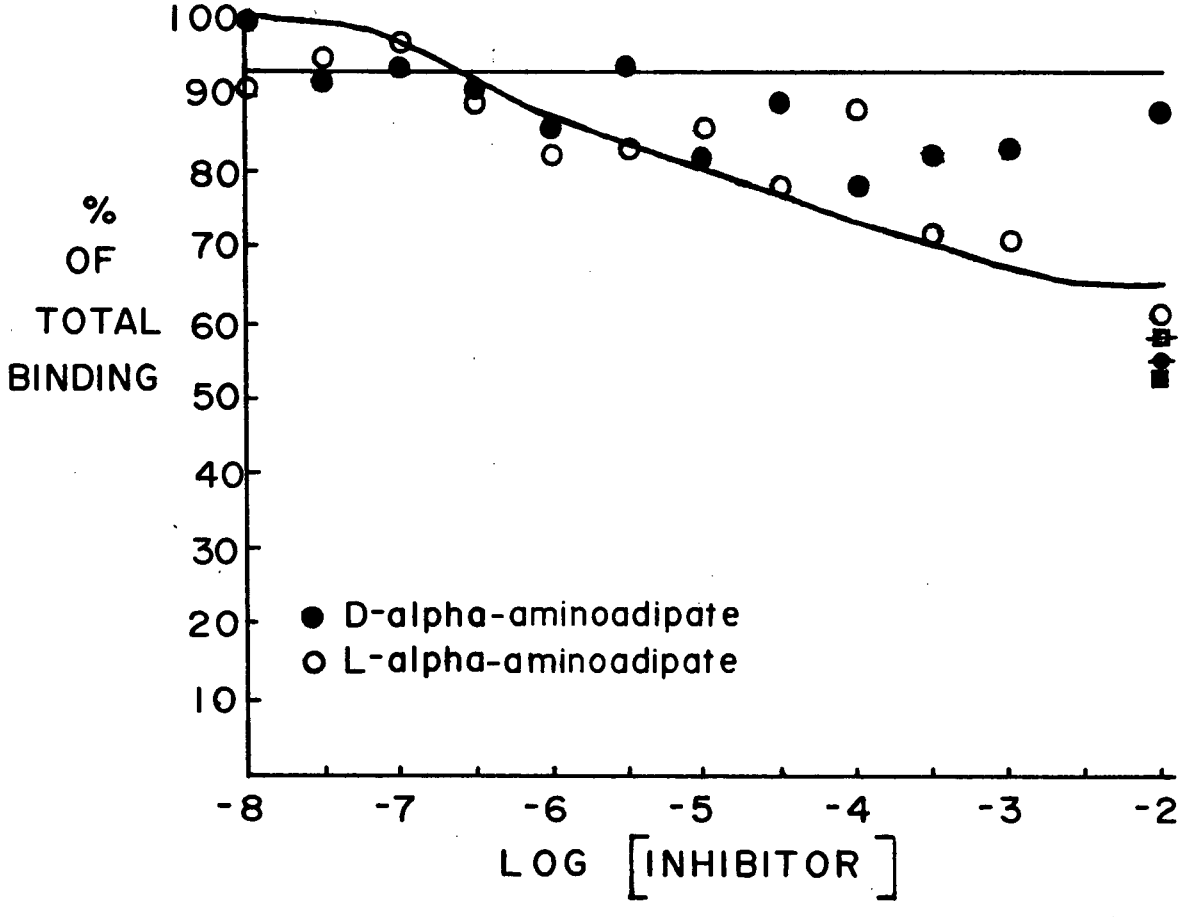
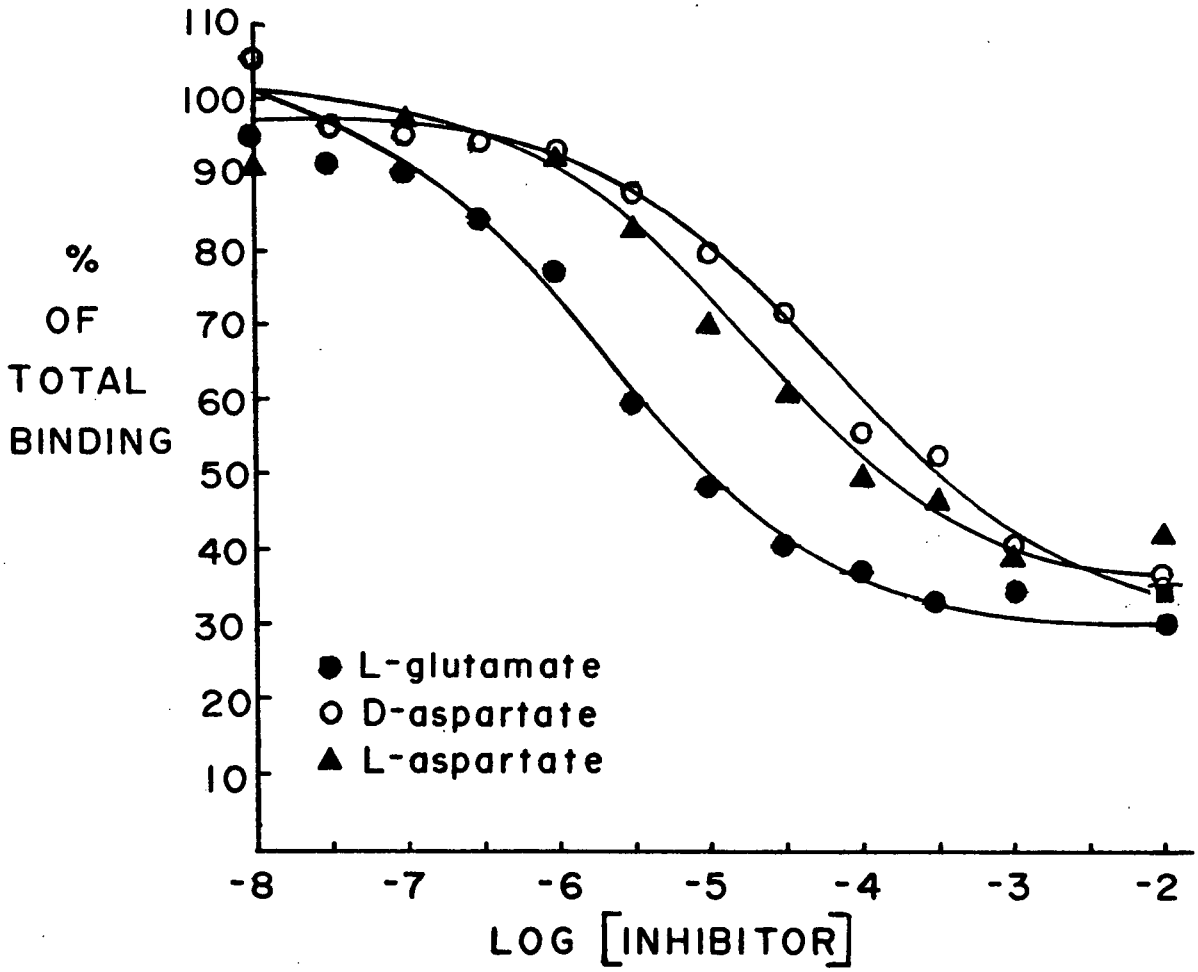

100
90
80
70
60
50
40
30
20
10
%
OF
TOTAL
BINDING
D-alpha-aminoadipate
L-alpha-aminoadipate
-8
-7
-6
-5
-4
-3
-2
LOG [INHIBITOR]

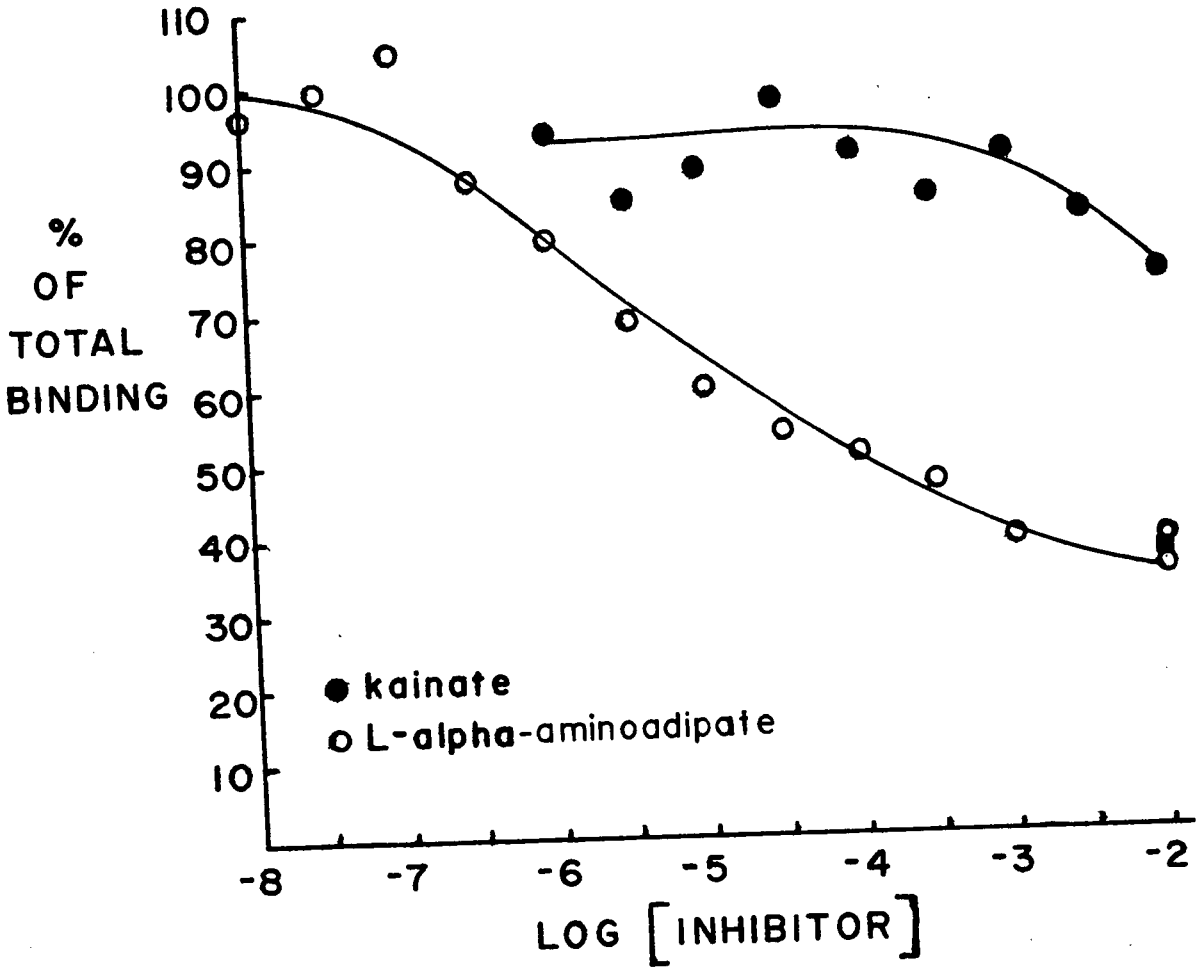
Figure 15. Inhibition of [^{3}H] L-Glutamate Binding to Cerebellar Membranes by Various Inhibitors

Values are the average of quadruplicates from 2, 3, or 4 experiments as indicated in Table X. The SEM was between 1 and 12 percent with the majority below 5 percent. The final concentration of [^{3}H] L-glutamate varied between 18 and 30%. The squares represent the % of total binding in the presence of 10^{-2} M L-glutamate while the circles with lines through them represent the % of total binding in the presence of L-glutamate (10^{-2} M) plus inhibitor (10^{-2} M) for a) D-aspartate (■, ●) and L-aspartate (□, o), b) kainate (■, ●) and L-alpha-aminoadipate (□, o), and c) D-alpha-aminoadipate (■, ●) and N-methyl-DL-aspartate (□, o).

a)



15 b)


110
100
90
80
70
60
50
40
30
20
10
% OF TOTAL BINDING
kainate
L-alpha-aminoadipate
-8
-7
-6
-5
-4
-3
-2
LOG [INHIBITOR]

15 c)

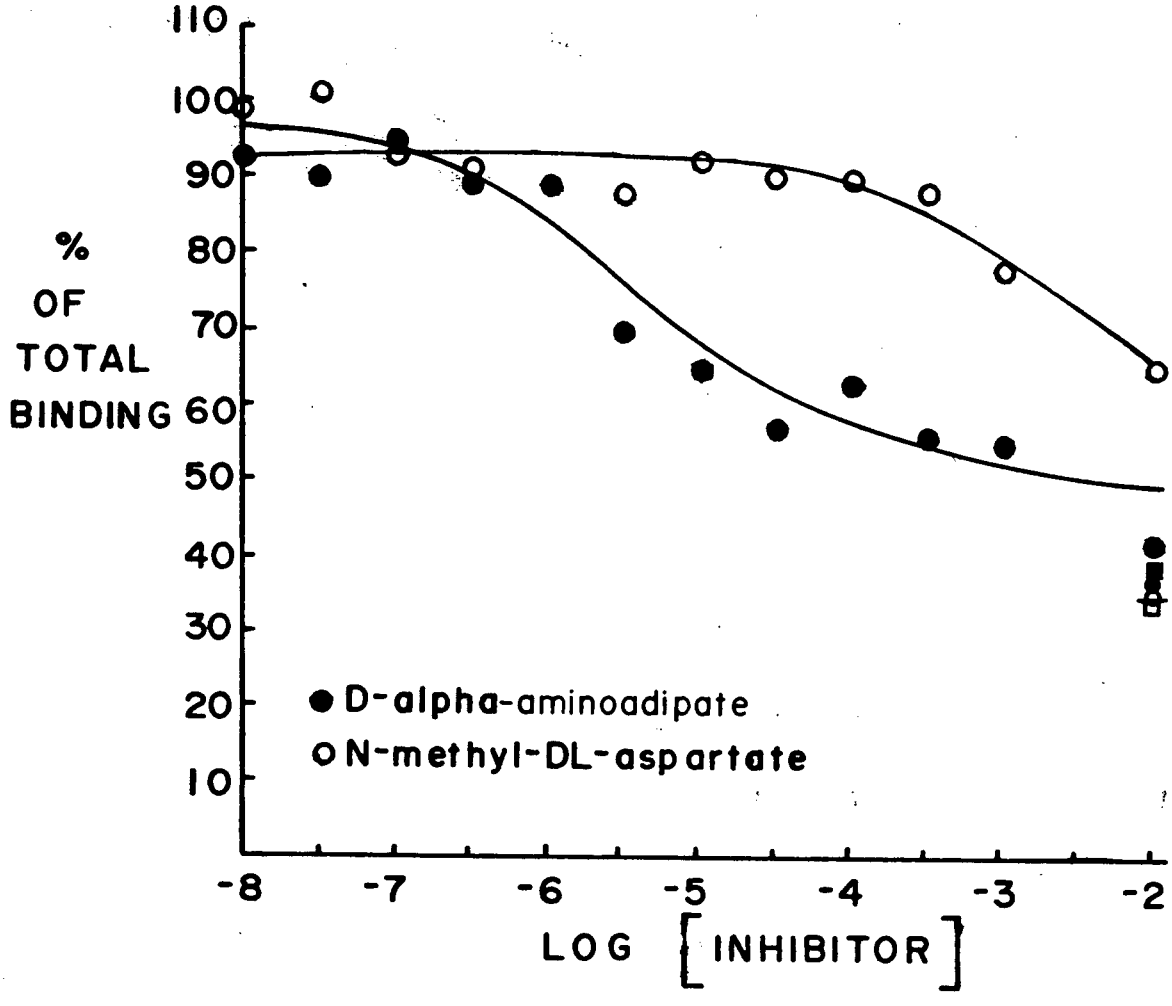

110
100
90
80
70
60
50
40
30
20
10
% OF TOTAL BINDING
D-alpha-aminoadipate
N-methyl-DL-aspartate
-8
-7
-6
-5
-4
-3
-2
LOG [INHIBITOR]

Table IX The IC_{50} Values of Various Compounds Displacing [^{3}H] L-Aspartate and [^{3}H] L-Glutamate Binding to Cerebellar Membranes

IC_{50} values were calculated by linear regression analysis of the Hill plot and were averages from 2, 3, or 4 experiments as shown in Table X.

Inhibitor	IC_{50} (μM) [^{3}H] L-Aspartate	[^{3}H] L-Glutamate
L-aspartate	1.81	3.84
D-aspartate	46.6	36.8
L-glutamate	1.24	2.25
NMDLA	>1000	>1000
Kainate	>1000	>1000
D-alpha-aminoadipate	>10,000	2.51
L-alpha-aminoadipate	7.12	5.28

Table X Hill Coefficients for Various Compounds Displacing [^{3}H] L-Aspartate and [^{3}H] L-Glutamate Binding to Cerebellar Membranes

Values are averages from 2, 3, or 4 experiments (χ). Dashes represent compounds for which there was insufficient inhibition of ligand for IC_{50} value determinations.

	Hill Coefficients			
Inhibitor	[^{3}H] L-Aspartate	χ	[^{3}H] L-Glutamate	n
L-aspartate	0.60	4	0.60	3
D-aspartate	0.70	2	0.53	3
L-glutamate	0.29	3	0.69	3
NMDLA	-	2	-	3
Kainate	-	2	-	2
D-alpha-aminoadipate	-	3	0.59	3
L-alpha-aminoadipate	0.23	3	0.44	3

3.84 μM respectively were the most potent inhibitors of [^{3}H] L-glutamate binding while D-aspartate with an IC_{50} value of 36.8 μM was an order of magnitude less potent. Kainate and N-methyl-DL-aspartate were very weak inhibitors displacing [^{3}H] L-glutamate binding only at concentrations greater than 100 and 1000 μM respectively.

The Hill plot for the L-aspartate inhibition curve is shown in Figure 16. All the Hill coefficients for the inhibition of [^{3}H] L-aspartate and [^{3}H] L-glutamate binding to cerebellar membranes were less than one (Table X). Negative cooperativity or occupancy of more than one population of sites each with a different affinity for the ligand was therefore indicated.

In order to compare data where different ligand concentrations are used, IC_{50} values are generally converted to dissociation constants for each inhibitor by equation (1)

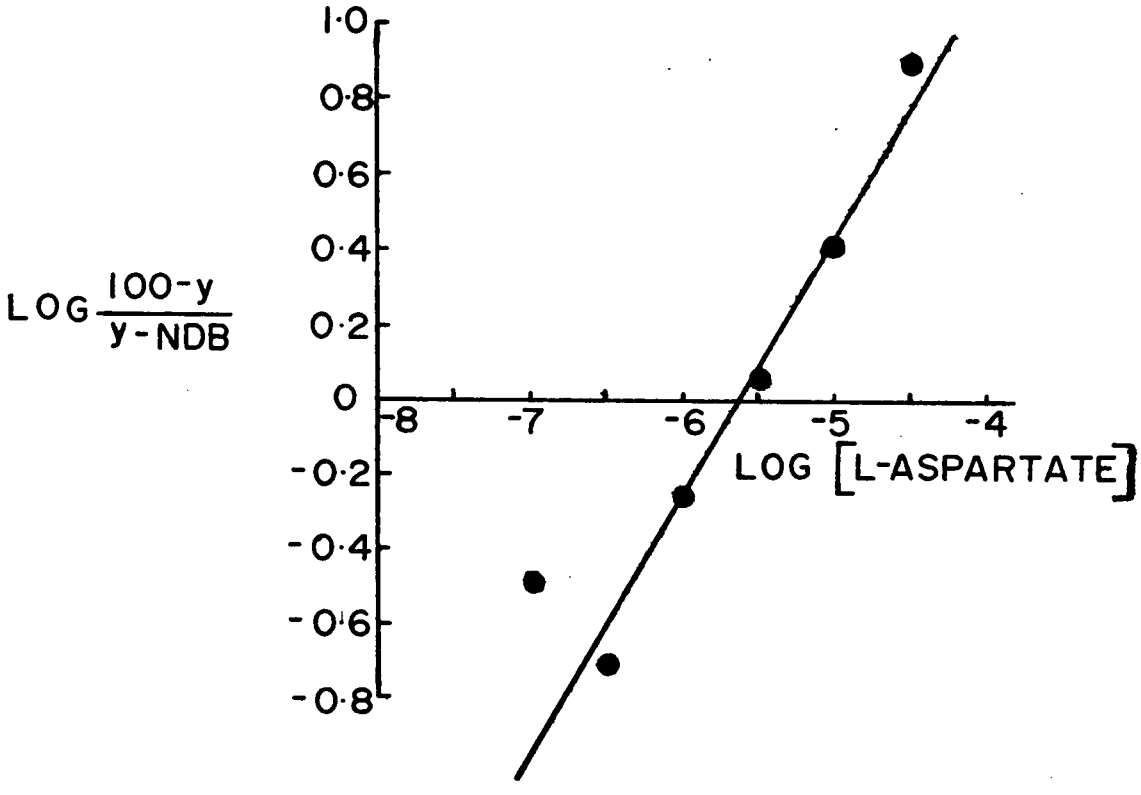
$$K_I = \frac{IC_{50}}{1 + \frac{L}{K_D}} \qquad (1)$$

IC_{50} values in the present experiments, however, could not be converted to true K_I values as the conversion assumes that one population of non-interacting sites is bound. The ligand concentration used in the inhibition experiments, however, was low (120 nM) compared to the K_D obtained from Scatchard analysis. The IC_{50} values for the various compounds therefore approximate the K_D values.

L- and D-aspartate, L-glutamate and L-alpha-aminoadipate then inhibited both [^{3}H] L-aspartate and [^{3}H] L-glutamate binding with similar potencies. N-methyl-DL-aspartate and kainate did not inhibit either [^{3}H] L-aspartate or [^{3}H] L-glutamate binding.

Figure 16. Hill Plot for the Inhibition of [^{3}H] L-aspartate Binding to Cerebellar Membranes by L-Aspartate

Values are obtained from the average of 4 experiments. NDB = Nondisplaceable Binding; y = the percent of total binding at each concentration of L-aspartate.



D-alpha-aminoadipate, however, inhibited [^{3}H] L-glutamate binding much more effectively than [^{3}H] L-aspartate.

The results from the limited number of compounds used to identify the pharmacological specificity of [^{3}H] L-aspartate and [^{3}H] L-glutamate binding indicate the interaction of each ligand with different sites. Testing more compounds would help to strengthen this conclusion.

IV. DISCUSSION

The displaceable [^{3}H] L-aspartate binding to total particulate fractions of the cerebellum in the present experiments demonstrates many of the characteristics of a physiological receptor. The binding is reversible, saturable, independent of the presence of Na^+, has an affinity in the range where according to iontophoretic studies (Hösli et al., 1973) the neurotransmitter is active in vivo, demonstrates a pharmacological specificity which includes stereospecificity, and, as discussed earlier, has a subcellular distribution appropriate for that of a neurotransmitter binding site. These characteristics and the effects of ions on the displaceable [^{3}H] L-aspartate binding are discussed in more detail below.

The displaceable [^{3}H] L-aspartate binding was reversible in that large quantities of cold aspartate displaced the bound [^{3}H] L-aspartate. Sharif and Roberts (1981) and Foster and coworkers (1981) also found that [^{3}H] L-aspartate binding to synaptosomal fractions of the cerebellum and to membrane fractions of the forebrain was saturable, reversible, and independent of Na^+.

The apparent dissociation constants for aspartate binding are in the range of the minimum concentration of aspartate (10^{-5} or 10^{-6} M) needed to stimulate neurones (Hösli et al., 1973). Binding of [^{3}H] L-aspartate to synaptic receptors which are active in vivo is therefore indicated.

The apparent dissociation constant (K_D) for [^{3}H] L-aspartate obtained by Scatchard analysis in the present experiments (1.64 ± 0.34 μM) is in agreement with the 0.874 μM obtained in crude synaptosomal preparations of cerebellum by Sharif and Roberts (1981). The

K_D of 0.556 ± 0.062 μM obtained by Foster and coworkers (1981) in purified synaptosomal preparations of the forebrain may have been lower than that obtained in the cerebellum because of the Triton X-100 treatment of the membranes of the forebrain and consequent possible partial degradation of the aspartate binding site. The difference may also be due to regional variation in the affinity of [^{3}H] L-aspartate for its binding site.

There is good correlation between the apparent dissociation constant for [^{3}H] L-aspartate binding in the cerebellum obtained by the author from Scatchard analysis and that calculated from the IC_{50} value obtained from the inhibition curve. The two apparent dissociation constants obtained by Sharif and Roberts, however, do not agree. The apparent dissociation constant calculated from the IC_{50} value (5.0 μM) was 4.68 μM while that obtained from Scatchard analysis was 0.874 μM. The dissociation constant calculated from the IC_{50} value obtained by Sharif and Roberts may have displayed a lower affinity than that obtained from Scatchard analysis because large concentrations of L-aspartate (up to 10^{-2} M) were used in experiments from which the IC_{50} values were determined. The Scatchard analysis, however, encompassed a lower range of ligand concentrations (from the absence of ligand up to 1000 nM) so that sites with higher affinities (lower K_D values) were measured. These data indicate that at least two [^{3}H] L-aspartate binding sites exist, one of lower affinity than the other.

The maximum binding can also be calculated from the data by Scatchard analysis. The maximum binding to total particulate fractions of cerebellum was found in these experiments to be about six times (7.71 pmol/mg) less than that found by Sharif and Roberts in synaptosomal

fractions (44 pmol/mg). More binding is expected in synaptosomal preparations because neurotransmitter receptors are concentrated in this fraction.

The pharmacological specificity of [^{3}H] L-aspartate binding was shown by the IC_{50} values obtained for the displacement of the binding by several compounds. L-aspartate inhibited [^{3}H] L-aspartate binding 10 times more effectively than D-aspartate did. The [^{3}H] L-aspartate binding was therefore stereospecific, a characteristic expected for naturally occurring reactions. Sharif and Roberts (1981) also found stereospecificity of [^{3}H] L-aspartate binding. L-aspartate in their experiments was 100 times more effective than D-aspartate. The IC_{50} values of L-aspartate, L-glutamate, N-methyl-DL-aspartate, and kainate for [^{3}H] L-aspartate binding found in this study were similar to those found by Sharif and Roberts (Table XI). The IC_{50} for the racemic mixture DL-alpha-aminoadipate obtained by Sharif and Roberts, however, was 100 times less effective than that obtained by the author for either isomer alone.

The discrepancy between the IC_{50} values of D-aspartate, and alpha-aminoadipate obtained in the present study and those found by Sharif and Roberts may be because, in the experiments of Sharif and Roberts, the inhibition curves are not well defined. Only four different concentrations of from 10^{-5} M to 10^{-3} M of the various compounds were tested (Roberts et al., 1980). As can be seen from Figures 14 and 15 of the present experiments, a great deal of inhibition has already occurred at 10^{-5} M of the various compounds under test and 10^{-3} M of some compounds, such as D-aspartate, did not fully inhibit the [^{3}H] L-aspartate binding. The inhibition curves derived from such abbreviated

Table XI Comparison of the Pharmacological Specificity of [^{3}H] L-Aspartate Binding Obtained by the Author with that Obtained by Sharif and Roberts

The IC_{50} values (in μM) of the author were taken from Table X but with the use of "inactive" defined by Sharif and Roberts (1981) as an IC_{50} value greater than 10^{-3} M. *Sharif and Roberts used N-methyl-D-aspartate while the author used N-methyl-DL-aspartate.

Inhibitor	Sharif and Roberts	Author
L-aspartate	5.0	1.81
D-aspartate	457.0	46.6
L-glutamate	2.0	1.24
N-methylaspartate*	inactive	inactive
Kainate	inactive	inactive
DL-alpha-aminoadipate	360.0	
D-alpha-aminoadipate		inactive
L-alpha-aminoadipate		7.12

data are most likely shifted to the right, producing higher IC_{50} values in some instances in the work of Sharif and Roberts (1981) than those obtained in the present studies.

The differences in production of inhibition curves may be the reason for the discrepancy between the Hill coefficients and, consequently, the number of binding sites observed in the present experiments as compared to those of Sharif and Roberts. In the present experiments more than one L-aspartate binding site is indicated by the Hill coefficient of less than one. The Hill coefficient of unity obtained by Sharif and Roberts suggest a homogeneous population of receptors.

The Hill coefficients for the displacement of [^{3}H] L-aspartate by compounds other than L-aspartate were not determined by Sharif and Roberts. Binding of each of D-aspartate, L-glutamate, and L-alpha-aminoadipate to more than one site, however, is indicated in that the Hill coefficients obtained in the present studies are less than one.

The different number of binding sites observed by the two investigations may also have been because total particulate fractions were used in the present study while synaptosomal fractions were used by Sharif and Roberts. The total particulate fractions may have contained extra-junctional receptors as well as those found in synaptosomal fractions. The differences in the IC_{50} values for L-aspartate and alpha-aminoadipate may also have been due to measurement of a lower affinity binding site in the experiments of Sharif and Roberts as discussed earlier.

The effects of K^+, Mg^{2+}, and Ca^{2+} on the [^{3}H] L-aspartate binding in synaptosomal fractions of whole rat brain obtained in the present study do not agree with those found by Sharif and Roberts (1981) or by Foster and coworkers (1981). Sharif and Roberts found that binding to

crude synaptosomal membranes of rat cerebellum was enhanced by K^+ concentrations of about 2 to 5 mM while concentrations higher than 5 mM inhibited the binding. The present study found no effect of 1 mM K^+ in a final reaction mixture of pH 4.5. Sharif and Roberts also found that lower concentrations of Ca^{2+} from about 2 to 20 mM enhanced binding while higher concentrations were inhibiting. These results for the effects of Ca^{2+} agree with experiments described in this report in which the final pH was 4.5. At pH 7.5, however, it was found that Ca^{2+} had no effect on the displaceable aspartate binding. In regard to Mg^{2+}, Foster and coworkers (1981) found that a concentration of 2.4 mM increased [^{3}H] L-aspartate binding to whole particulate fractions of forebrain 2.5 times. In the present study however, Mg^{2+} had no effect on displaceable aspartate binding. These discrepancies could be explained by differences in the ion concentration, pH, the membrane fraction, or the brain region investigated. Differences between membrane subfractions have been shown by Foster and coworkers (1981). The enhancement of [^{3}H] L-aspartate binding in the presence of Ca^{2+} (2.5 mM) decreased from whole particulate fractions of cerebral cortex to purified synaptosomal preparations.

Correlation of the ionic effects on binding with those seen on depolarization in vivo are difficult because, in vivo, the ions affect sites other than the synaptic receptor. Decreased extracellular K^+, for example, enhances depolarization induced by aspartate, a response which is consistent with the decreased aspartate uptake seen at lower K^+ concentrations (Evans et al., 1977) or the hyperpolarization of neuronal membranes in low K^+ concentrations.

Assuming from the previous evidence that the sites active in binding aspartate in vitro are those activated in vivo, the binding assay provides

evidence for separate aspartate and glutamate receptors. Four pieces of evidence which are discussed in more detail below include the different pharmacological specificities, maximum amount of binding, effects of ions on the binding, and also the Hill coefficients of less than one.

First, differentiation between L-aspartate and L-glutamate binding is based primarily on differences in pharmacological specificities. The apparent dissociation constants for the various inhibitors tested against [^{3}H] L-aspartate binding in these experiments agreed with those observed in cerebellar synaptosomal fractions by Sharif and Roberts (1981) and in synaptosomal preparations of the hippocampus by Baudry and Lynch (1979).

Different pharmacological specificities were obtained for [^{3}H] L-aspartate and [^{3}H] L-glutamate. The two pharmacological specificities were found in the present experiments to differ in affinity for D-alpha-aminoadipate. Sharif and Roberts (1981) found that [^{3}H] L-aspartate and [^{3}H] L-glutamate binding also differed in the affinities for quisqualate, DL-2-amino-4-phosphonobutyrate, DL-homocysteate, L-glutamate diethylester, 1-hydroxy-3-aminopyrrolidone-2(HA-966), D-aspartate and somewhat for ($\pm$)-ibotenate.

Second, Sharif and Roberts also found that the maximum binding of glutamate to synaptosomal fractions of the cerebellum was three times higher than that of aspartate to the same membrane preparation. These data indicate that aspartate and glutamate bind to separate receptors.

Third, Foster and coworkers (1981) found that certain ions affected aspartate binding differently to glutamate binding. Na^+ increased aspartate binding more than glutamate binding, while Ca^{2+} and Mg^{2+} increased glutamate more than aspartate binding. The differential effects of ions on aspartate and glutamate binding lend more evidence to the

argument for separate receptors.

Fourth, the Hill coefficients of less than one obtained in the present study for both [^{3}H] L-aspartate and [^{3}H] L-glutamate binding indicate the interaction of each amino acid with more than one site. [^{3}H] L-aspartate may interact with an aspartate and a glutamate site.

How do the data from binding studies correlate with those from iontophoresis? The relative affinities of L-aspartate, D-aspartate, L-glutamate, and kainate for the [^{3}H] L-aspartate binding site are in accord with the relative potencies of these compounds as seen in iontophoretic studies. D-aspartate and L-glutamate excite neurones more, less, or as well as L-aspartate depending on the region of the brain tested (Curtis and Watkins, 1960, 1963; Hall et al., 1979). Kainate had little effect on the [^{3}H] L-aspartate binding, which is consistent with the iontophoretic evidence that kainate acts at a separate receptor (Engberg et al., 1978; Nistri and Constanti, 1979).

There are major disagreements, however, between the iontophoretic and binding data for N-methyl-D-aspartate and D- and L-alpha-aminoadipate. Iontophoretic data strongly indicate that aspartate can interact with N-methyl-D-aspartate receptors, although N-methyl-D-aspartate is much more potent and specific (Evans and Watkins, 1978; Davies and Watkins, 1979) and that D-alpha-aminoadipate has a strong and preferential antagonistic action against N-methyl-D-aspartate (Biscoe et al., 1978; McLennan and Hall, 1978). The D isomer of alpha-aminoadipate itself is not excitatory while the L-isomer is weakly excitatory. The L-isomer is not an antagonist of aspartate or glutamate excitation (Biscoe et al., 1977; Lodge et al., 1978). The binding data indicate opposite effects. N-methyl-D-aspartate and D-alpha-aminoadipate were ineffective while L-alpha-aminoadipate

inhibited the [^{3}H] L-aspartate binding a great deal. Both D- and L-alpha-aminoadipate inhibited [^{3}H] L-glutamate binding more than [^{3}H] L-aspartate binding.

There are several possible reasons for the discrepancy between the iontophoretic and binding data. The interaction of aspartate with N-methyl-D-aspartate active sites determined by iontophoresis may be incorrect; alternatively, the binding experiments may not measure the interaction with synaptic receptors in the state and conditions in which they occur in vivo.

N-methyl-D-aspartate may appear to be more potent than L-aspartate because of differential uptake of the two compounds. The uptake of L-aspartate (K_I = 1.6 μM) is much greater than that of N-methyl-D-aspartate (K_I = 28 μM) in crude synaptosomes of rat hippocampus (Baudry and Lynch, 1979). After iontophoresis of the same concentrations of L-aspartate and N-methyl-D-aspartate, N-methyl-D-aspartate would therefore be more abundant and would diffuse over a greater area than L-aspartate. N-methyl-D-aspartate would therefore activate more receptors and appear to be more potent than aspartate.

N-methyl-D-aspartate, however, may not bind to the receptor as well as L-aspartate, as shown by the results of Garthwaite and Balazs (1981) in adult rat cerebellar slices. N-methyl-D-aspartate was less effective than L-aspartate in increasing cGMP levels in cell suspensions of rat cerebellum. Only three or four data points, however, were used to determine the dose-response curves from which these conclusions were drawn. Expansion and confirmation of the data is therefore required.

N-methyl-D-aspartate, on the other hand, may not interact with the aspartate receptor but activate a different receptor. The cGMP production

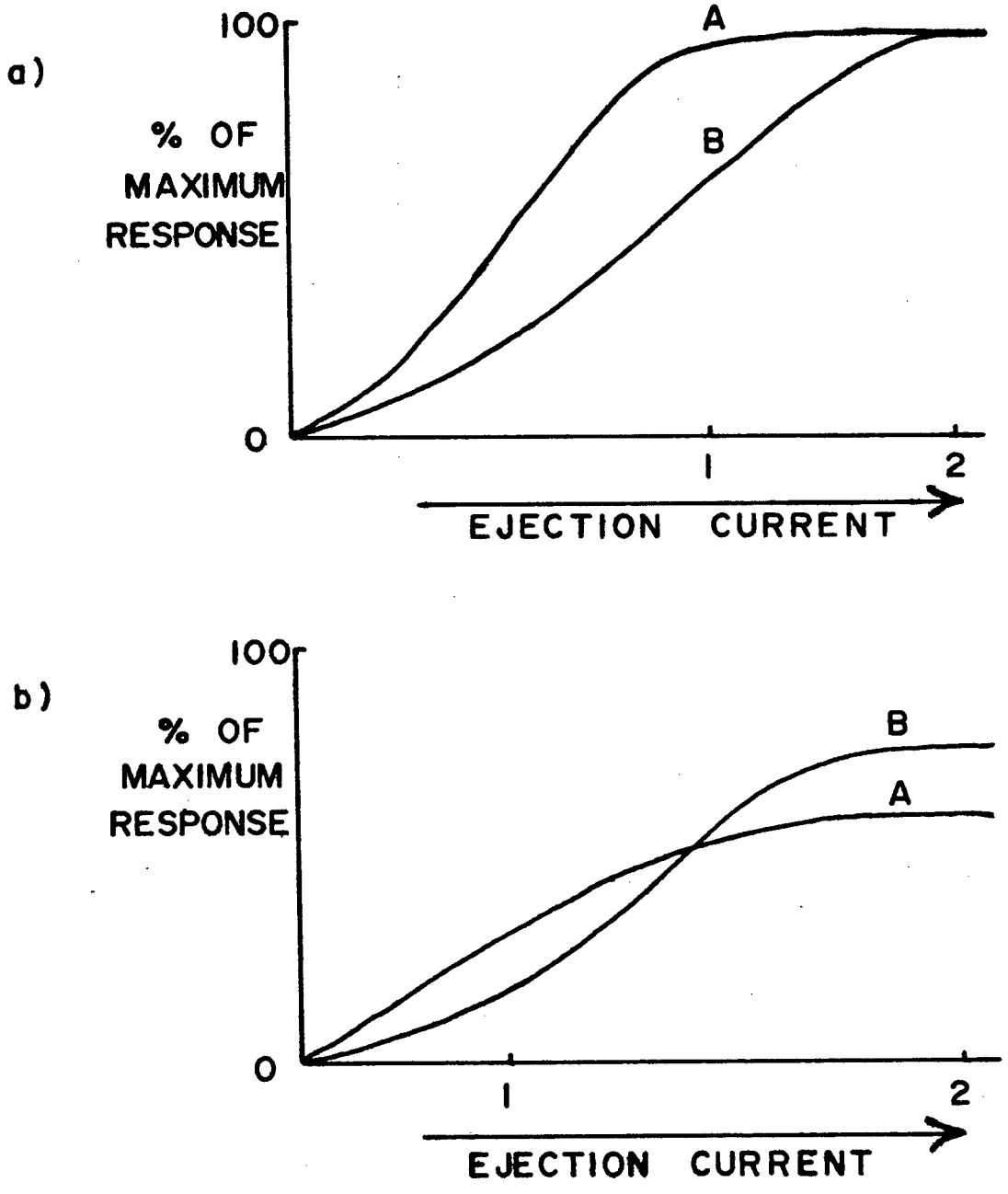
induced by L-aspartate and L-glutamate was resistant to D-alpha-aminoadipate while N-methyl-D-aspartate-induced stimulation of cGMP was inhibited (Garthwaite and Balazs, 1981). Intracellular responses are also different for N-methyl-D-aspartate and L-aspartate. L-aspartate produced a small increase in membrane conductance while N-methyl-D-aspartate produced a very large decrease (Engberg et al., 1978). These data are substantiated by the results of the present experiments in which N-methyl-D-aspartate had little effect on [^{3}H] L-aspartate binding. Neither the iontophoretic nor the binding studies, however, provide definitive evidence for different receptors. The probable activation of both glutamate and N-methyl-D-aspartate sites by aspartate, for example, may account for the different intracellular responses of aspartate and N-methyl-D-aspartate.

Another problem with the potencies determined by iontophoresis is that in the majority of instances only one effective dose of, for example, aspartate and N-methyl-D-aspartate are compared. The effective dose is determined as follows. A range of ejection currents for aspartate are tested and a current chosen which produces about 50 percent of the maximum response (ED_{50}). The potency of N-methyl-D-aspartate as compared to aspartate is then estimated from the intensity of the iontophoretic current required to elicit an equal response. Only a single dose of N-methyl-D-aspartate is then compared with the ED_{50} of aspartate.

In order properly to determine the potencies of various compounds, the ED_{50} values must be compared. The reason for the necessity of ED_{50} values is as follows. Examples of possible dose-response curves for two compounds are shown in Figure 17. At an ejection current, 2 (Figure 17a) for example, two compounds, A and B, may elicit the same response. At a

Figure 17. Possible Dose-Response Curves

A and B are two compounds iontophoretically ejected at currents 1 and 2.



lower ejection current, 1, compound A may still exhibit the maximum response while compound B exhibits only 70 percent of the maximum response. The dose-response curves may also be as shown in Figure 17b where, at ejection current 2, compound A is less potent than compound B but at a much lower ejection current, 1, compound B is less potent than A. Comparison of the potency of various compounds can therefore only be made after several ejection currents of the compounds have been tested, ED_{50} curves drawn, and the ED_{50} values determined. It is not possible, however, to obtain ED_{50} values in a large number of instances because in determining the maximum response the neurone is inactivated (Curtis and Watkins, 1960). Relative potencies of various compounds must therefore be viewed with caution.

Several problems therefore exist in the interpretation of the iontophoretic data. Some of the problems with the binding studies are as follows. In vitro studies create an artificial environment for neuronal membranes in which the aspartate receptors may be altered, destroyed, or unable to react properly because of ionic or other deficiencies. The [^{3}H] L-aspartate and the [^{3}H] L-glutamate may therefore not have bound to the sites which are activated in vivo or to sites in the same state as those in vivo.

The aspartate synaptic receptors may, on the other hand, have been intact and operable in the binding assay but because of the low concentration of [^{3}H] L-aspartate employed, only the high affinity sites were characterized. Aspartate may interact with lower affinity to the N-methyl-D-aspartate binding site so displacement of [^{3}H] L-aspartate by N-methyl-DL-aspartate would not be observed in the present experiments. In iontophoretic studies low affinity sites may have been measured.

The disagreement between the data from iontophoretic and binding studies may be because of the measurement of aspartate uptake sites in the binding studies. Roberts and Kuriyama (1968); Zukin et al., (1974); and Enna and Snyder (1975) showed that in the presence of Na^+ the neurotransmitter gamma-aminobutyric acid (GABA) bound to uptake sites while in the absence of Na^+ neurotransmitter sites were activated. Binding to putative synaptic neurotransmitter receptors is therefore routinely done in the absence of Na^+. The binding of aspartate to neurotransmitter sites in the absence of Na^+, however, has not been substantiated. Na^+-dependent [^{3}H] L-aspartate binding has not been measured, so no correlation has been established between Na^+-dependent binding and uptake and those differentiated from Na^+-independent binding. Two arguments, however, indicate that it is unlikely that the aspartate binding in these experiments was to uptake sites.

First, the apparent dissociation constants for [^{3}H] L-aspartate uptake and Na^+-independent binding were different. The K_D for the high affinity [^{14}C]L-aspartate uptake in rat cortical slices was 1.6×10^{-5} M (Davies and Johnston, 1976) while that of Na^+-independent aspartate binding in the present study was 10^{-6} M. [^{3}H] L-aspartate binding to the uptake site in the absence of Na^+ would be less than optimal and the affinity therefore less than 10^{-5} M. Binding to uptake sites would also have been negligible in the present experiments because of the low concentration of [^{3}H] L-aspartate employed (120 nM) compared to the uptake K_D.

Second, the pharmacological specificity of [^{3}H] L-aspartate and [^{3}H] L-glutamate uptake sites were found by Balcar and Johnston (1972) to be the same. The pharmacological specificity of [^{3}H] L-aspartate binding

in the present experiments, however, was different from that of glutamate as well as of aspartate uptake sites. In uptake studies [^{3}H] L-aspartate was inhibited somewhat more by D-aspartate than by L-aspartate (Balcar and Johnston, 1972). In the present binding studies L-aspartate inhibits the binding much more than D-aspartate does.

There are therefore many possible reasons for the discrepancy between the binding and iontophoretic data. Correlation is very difficult because the former measures a response which is sensitive to efficacy and inactivation, for example, while the latter measures only the affinity of various compounds for a binding site which is unrelated to the cellular responses, that is to efficacy. The [^{3}H] L-aspartate binding does not demonstrate the characteristics of the aspartate actions seen in vivo such as the strong potency of N-methyl-D-aspartate and therefore may not represent the site which is activated in vivo. The in vivo studies, however, may not truely represent the characteristics of the aspartate site because of, for example, uptake of the compounds iontophoresed. No final conclusion as to whether separate receptors exist for aspartate and glutamate can therefore be made at this time. More data are required from both techniques before a more accurate correlation can be made.

From iontophoresis, the data required are intracellular recordings, dose-response curves, and more specific agonists and antagonists. The binding studies require correlation with a cellular response which is close in time to the receptor compound interaction. Aspartate bound nonspecifically to sites other than the neurotransmitter site would interfere much less with these type of data. The data required from iontophoresis are discussed first.

The specificity of most agonists and antagonists, as mentioned

earlier, is based on extracellular measurements of changes in firing rate. Confirmation of the specificities are required from the more subtle measurements of intracellular recordings such as reversal potentials which give information as to which ionic channels are opened. Differences in intracellular measurements may differentiate between compounds which were previously not distinguished by the measurement of changes in the firing rate of neurones.

More specific agonists and antagonists for aspartate and glutamate are required to differentiate between the actions of the two compounds. Conductance changes produced by aspartate itself, for example, may be masked if more glutamate than aspartate receptors are present because of the interaction of both amino acids with the two possible receptors. Four types of experiments which would help to relate the binding studies to an appropriate physiological receptor are outlined below.

First, characterization of the N-methyl-D-aspartate binding site may substantiate whether aspartate binds to N-methyl-D-aspartate sites. Unfortunately, Olverman and Watkins (unpublished observations, Watkins and Evans, 1981) and the author could not reproduce the work of Snodgrass (1979) in which aspartate displaced [^{3}H] N-methyl-D-aspartate binding to membrane fractions of rat brain. Further experiments are therefore necessary for better identification of the receptors for N-methyl-D-aspartate as well as for D-alpha-aminoadipate with aspartate receptors because, as Evans and Watkins (1981) state, "no compelling evidence is yet available to confirm or refute the possibility that N-methyl-D-aspartate receptors are aspartate transmitter receptors."

A second type of study which could be performed using the binding assay itself is one in which lesions have been made to deplete various

neuronal cell types. The cerebellum is an excellent region in which to study the effects of lesions because animals are available in which certain cerebellar cell types or fibers are absent. Climbing fibers, for example, can be depleted with the use of 3-acetylpyridine (Desclin and Escubi, 1974).

Third, neurones in tissue culture may be a good environment in which to study binding because the receptors would not be subject to possible digestion by enzymes released during membrane preparation. This system, however, may be complicated by the inactivation of the compounds under test by uptake. These studies therefore await development of a compound which blocks uptake without blocking the synaptic receptors.

The fourth type of experiment is the direct measurement of a cellular response in vitro such as cGMP levels and Na^+ fluxes. Garthwaite and Balazs (1981) measured cGMP levels in slices of rat cerebellum in response to the application of L-glutamate, L-aspartate, N-methyl-D-aspartate and kainate. They found that the in vitro stimulation of cGMP levels mimics that seen in vivo. Increases in cGMP were, for example, seen after in vivo stimulation of parallel or climbing fibers in the cerebellum (Rubin and Ferrendelli, 1977; Biggio and Guidotti, 1976; Evans et al., 1979). Different efficacies of various compounds in altering the cGMP levels may differentiate between compounds previously not distinguished. Many compounds and regions of the central nervous system have yet to be tested in the cGMP system. It will be interesting to correlate the results with potencies obtained from iontophoretic studies.

The measurement of ion fluxes such as Na^+ induced by various compounds is a technique which looks promising for future work. Glutamate, for example, changed the Na^+ fluxes in striatal slices (Luini et al., 1980). Iontophoretic studies suggest that the glutamate receptor, as well

as aspartate, are linked with Na^+ channels (Curtis and Johnston, 1974). Expansion of these studies with the testing of more compounds and the production of dose-response curves would be very interesting since the reactions of ionic channels are more immediately associated with the receptor-compound interaction and therefore more accurately reflect the interaction.

Of all the experiments mentioned above, those which hold the most promise of correlation between data from in vitro and in vivo experiments are the measurement of dose-response curves for Na^+ fluxes both in vitro in brain slices and in vivo.

The binding assay, assuming that [^{3}H] L-aspartate binds to the receptor which is active in vivo and that agonists and antagonists specific to the site are found, could provide a great deal of information. The distribution, regulation, development, alteration during disease or aging, and the screening of various drugs for effects on the aspartate receptor could be studied. Distribution and lesion studies may help to determine the pathways in which aspartate may be a neurotransmitter. The localization of aspartate receptors at pre- or post-synaptic, junctional or extrajunctional sites could be determined by autoradiography or histochemistry. The point during formation of the synapse at which receptors become evident and any changes which may occur during development could also be studied with binding assays, particularly Scatchard analysis of the binding at each stage of development. The possible alteration of receptors during prolonged administration of certain drugs could also be determined from binding studies. These data may be correlated with changes in physiological responses such as Na^+ fluxes or cGMP levels. Before any of these studies can proceed, however, identification of the aspartate

binding site with the receptor which is active in vivo is required.

APPENDIX I

The disintegrations per minute obtained for [^{3}H] L-aspartate binding were low because of the low specific activity of the label used. The free concentration of [^{3}H] L-aspartate could not be estimated when concentrations in which greater than 10 percent of the total radioactivity bound to membrane preparations were used. Higher concentrations of label also dramatically increase the nondisplaceable binding so that accurate measurements of the displaceable binding, which is determined by subtracting the nondisplaceable from the total binding, would be very difficult. Higher concentrations of this label could therefore not be used to increase the disintegrations per minute obtained.

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