AMINO ACIDS AS CENTRAL NEUROTRANSMITTERS

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INTRODUCTION

Many studies have indicated that certain amino acids, endogenous to the vertebrate CNS, may serve as neurotransmitters (1–9). Neutral amino acids (e.g. γ-aminobutyric acid, glycine, taurine) are leading candidates for roles as inhibitory transmitters, whereas acidic amino acids (e.g. glutamate, aspartate) may serve as excitatory transmitters. If these amino acids are transmitters, they perhaps function in a greater proportion of central synapses than other possible transmitters (e.g. norepinephrine, dopamine, acetylcholine, serotonin) because they are present in much higher concentrations in the CNS and can alter the excitability of most neurones. However, these amino acids are heavily involved in general metabolism and, except for γ-aminobutyric acid (GABA) and taurine, are substrates for protein synthesis. Consequently, it seems imperative to devise methods to separate transmitter from nontransmitter compartments and glial from neuronal functions for these amino acids.

Early studies indicated that changes in the cerebral concentrations of some of these amino acids might be associated with seizures or other neurological dysfunctions (10, 11). More recent evidence has revealed that these substances may be involved in the functions of cerebral cortical, cerebellar, hippocampal, medullary, spinal, and extrapyramidal systems (2, 4, 5, 12–18). Studies on the in vivo release of these amino acids from various central structures have provided additional support for their proposed roles as transmitters (19–23), and they have been collected from various cerebral structures (24) in labeled form after synthesis with U-^{14}C-D-glucose as precursor. With regard to physiological mechanisms of action, membrane hyperpolarizations produced by iontophoretic applications of GABA, glycine, or taurine to central neurones appear to be mediated by changes in Cl^-

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permeability (4, 5) whereas the depolarizing actions of acidic amino acids might be caused by increased entry of Na^+ and Ca^{2+} into neurones (25–29).

This review focuses on recent advances which have been made using in vivo (iontophoretic) and in vitro (uptake, binding, and release) approaches. The search for specific antagonists of the pharmacological actions and of the inactivation (reuptake) of these active amino acids in the CNS is particularly emphasized.

MICROIONTOPHORETIC STUDIES

Cerebral Cortex

Krnjević & Phillis (30) demonstrated generalized, highly effective, and reversible actions of GABA and glutamate on single feline cortical units, which indicated that these substances might be the natural transmitters. In further experiments, extracellular applications of GABA hyperpolarized pericruciate cells and lowered their resistance in accord with the synaptic inhibitory effect, and the actions of both GABA and the surface-evoked inhibitory postsynaptic potential (IPSP) were reversed at a similar membrane potential, or by injecting Cl^- into cells (13, 31, 32). However, no specific antagonist to this inhibitory action on GABA was found (33, 34). Later, Curtis et al (35, 36) reported that bicuculline reversibly antagonized the depressant action of GABA in cerebral cortex without modifying that of glycine, but this agent lacked specificity since it also blocked the depressant effects of β-alanine and taurine, and often elevated neuronal firing rates (36). Also, since iontophoretically applied strychnine reduced the depressant actions of glycine, β-alanine, and taurine, its action in the cortex is also not highly specific (36–38). Further experiments on cortical units indicated that the large iontophoretic currents of bicuculline that had to be used to block the depressant action of GABA sometimes increased neuronal excitability, and it was concluded that bicuculline did not appear to be useful for critical tests of GABA-mediated inhibition in the cortex (39, 40). It was shown also that bicuculline could either potentiate or antagonize the depressant action of GABA, depending on the relative concentrations of the two substances that were present (41). The potentiating effect of bicuculline on GABA could be due to its direct nonspecific action on the postsynaptic membrane, while its antagonism of GABA could be mediated by either a pre- or postsynaptic action (41).

Hill et al (42) showed that, upon iontophoretic application, (+)-tubocurarine was a potent GABA-antagonist, picrotoxin and bicuculline were less potent, strychnine and penicillin were only weakly active, and metrazol was inactive. All substances studied produced changes in the neuronal firing rate that did not correlate with their antagonism of GABA, and both bicuculline and strychnine could potentiate the depressant action of GABA. Strychnine, when applied in large amounts, also exerted some antagonistic action on the effects of GABA in the cortex (38, 42, 43), a finding which, when taken together with evidence that strychnine can antagonize the depressant actions of acetylcholine (ACh), 5-hydroxytryptamine (5-HT), and norepinephrine (NE) on certain central neurones (44, 46), indicates that this agent

is not specific to glycine-operated synapses. (+)-Tubocurarine can also antagonize the depressant effects of ACh, 5-HT, and NE (47).

1-Hydroxy-3-aminopyrrolid-2-one (HA-966) may exert a direct depressant action on cells of the cortex, since upon iontophoretic application to cells of the feline pericruciate cortex this agent reduced excitation induced by glutamate or aspartate while not markedly affecting spontaneous firing or ACh-induced excitation (48). This depressant action of HA-966 might not be "GABA-like" or "glycine-like" because it was relatively unaffected by either bicuculline or strychnine, and because both GABA and glycine depress spontaneous and ACh-evoked firing in th as effectively as they depress amino acid-induced excitation (48–50). The differential potency of HA-966 on amino acid- and ACh-induced excitation of cortical units was shown to be greater than those of L-glutamate diethyl ester and L-methionine-DL-sulfoximine, substances that also exert antagonistic effects on amino acid-induced excitation (28, 51). However, it has been suggested that more systematic testing is required to show that HA-966 does not possess a GABA-like action (5, 52).

Cerebellar Cortex and Lateral Vestibular (Deiters') Nucleus

Both basket and Golgi cell inhibitions, which are strychnine-resistant (54, 55), can be blocked by intravenous picrotoxin or bicuculline (35, 53). GABA, γ-amino-β-hydroxybutyric acid, and imidazole-4-acetic acid appeared to be about equally effective in depressing the firing of Purkinje cells, while β-alanine and glycine were much weaker (36, 56). In contrast to previous observations (56), iontophoretically administered strychnine reduced or abolished reversibly the actions of glycine and β-alanine on cerebellar neurones while not affecting the action of GABA, and iontophoretically administered bicuculline reduced the depressant action of GABA, while not altering that of glycine (53).

Taking as clues the findings that cerebellar Purkinje cells do not appear to be influenced by strychnine (54, 57) and that Deiters' neurones receive inhibitory synapses directly from Purkinje cells (58, 59), Obata et al (14) studied both the pharmacological properties of the postsynaptic inhibition by Purkinje cell axons and the action of GABA on Deiters' neurones. Although strychnine, picrotoxin, metrazol, and β-methyl-β-ethylglutarimide did not affect this inhibition, GABA depressed both IPSPs and excitatory postsynaptic potentials (EPSPs) and often blocked spike potentials, increased membrane conductance, and produced membrane hyperpolarization in Deiters' neurones (14). In a further study (60), it was shown that Purkinje cell inhibition of Deiters' neurones could be blocked by larger intravenous doses of picrotoxin (5–10 mg/kg) than were previously used (14). [High doses of picrotoxin were also required to block the inhibition of oculomotor neurones elicited by stimulating secondary vestibular neurones (61) and the inhibition of cells of vestibular nuclei caused by stimulation of the flocculus (62).] Since picrotoxin effectively blocked both Purkinje cell (synaptic) inhibition and the action of GABA on Deiters' neurones, it was concluded that GABA might be the inhibitory transmitter in this system (60). ten Bruggencate & Engberg (63) showed, also on Deiters' neurones, that GABA, glycine, and δ-aminovaleric acid all hyperpolarized the cell membrane, increased membrane conductance, and depressed post-

synaptic potentials, and reversal of the hyperpolarizing effects of these amino acids was correlated with the reversal of IPSPs evoked by stimulation of cerebellar Purkinje cells. Although the depressant action of glycine was much less pronounced than that of GABA on Deiters' neurones (60, 63, 64), bicuculline (40–150 nA) generally blocked the action of GABA without reducing the effects of glycine, whereas strychnine generally abolished the depressant action of glycine without affecting that of GABA (36). Hence, picrotoxin, bicuculline, and strychnine, though not appearing to be very specific antagonists, have been useful in showing that the inhibitory transmitter released from Purkinje cell axon terminals is likely to be GABA.

Medulla

MEDULLARY RETICULAR FORMATION Studies on brain stem neurones indicated that GABA and β-alanine exert depressant actions while glutamate has an excitant action (65, 66). In unanesthetized, decerebrate cats, DL-homocysteate was a more potent excitant than L-glutamate and L-aspartate on most bulbar reticular neurones tested, and glycine, β-alanine, and GABA (in decreasing order of potency) depressed most neurones tested (67). Iontophoretically applied strychnine reversibly blocked the actions of glycine and β-alanine, but not that of GABA, while iontophoretically administered picrotoxin slightly reduced the depression caused by glycine and GABA on less than half of the neurones tested (67). Bicuculline (at high ejection currents) often reversibly reduced the depression caused by GABA, but it also reduced the effects of glycine on some neurones (68).

Studies in which strychnine and bicuculline (or picrotoxin) were used to block the depressant actions of glycine and GABA, respectively, indicated that both glycine and GABA could serve as inhibitory transmitters in the medullary reticular formation, if they are released from axon collaterals of two different populations of inhibitory reticulospinal neurones (69). Also, inhibition of medullary reticular neurones by various afferent inputs was blocked reversibly by strychnine, which also antagonized the depressant action of glycine on these neurones (70). Other synaptic inhibitions in this region can be antagonized by strychnine, but not by bicuculline (43, 71). However, some inhibitions in this region (69) and in other medullary regions can be antagonized by picrotoxin (72, 73) or by bicuculline (74, 75). Iontophoretically applied strychnine reversibly reduced the depressant actions of taurine and glycine on medullary neurones, but not that of GABA; whereas iontophoretic bicuculline [as in spinal neurones (76)] generally exerted no action on taurine-induced depression of the same neurones on which it clearly reduced or blocked the depressant action of GABA (77). These results are in accord with the concept of Curtis et al (37, 78) of GABA-like and glycine-like inhibitions.

CUNEATE NUCLEUS Bicuculline and picrotoxin both antagonize synaptically evoked postsynaptic inhibition and the depressant action of GABA on cuneate neurones (72, 74, 75, 79), evidence that supports the suggestion that GABA is a postsynaptic inhibitory transmitter in this region (80). Other evidence obtained in anesthetized rats has indicated a dual action for GABA in the cuneate nucleus, i.e.

of depressing the excitability of postsynaptic neurones and of increasing the excitability of primary afferent terminals (81, 82). Therefore, GABA could serve as both a postsynaptic and a presynaptic inhibitory transmitter in the cuneate region (81–83, but see 5, 72). Davies & Watkins (50) showed that HA-966 reduced both synaptic excitation and glutamate- or aspartate-induced excitation of cuneate neurones.

Spinal Cord

Both GABA and glycine have actions comparable to that of an inhibitory transmitter on spinal neurones (15, 37, 84, 85). The finding that glycine is present in higher concentration in ventral gray matter than in other regions of the cord (86, 87) does not provide convincing evidence in support of a transmitter role for glycine rather than GABA in the cord, as regional concentrations of amino acids are not reliably reflected in iontophoretic or binding studies (see below). Also, as suggested by Curtis et al (37), if the intraspinal distribution of glycine is indicative of a transmitter role (86, 87), then α-alanine and serine, which also exert strychnine-sensitive inhibitory effects, and which are also present in higher concentration in spinal gray than in spinal white matter (88), should also be considered as candidates. However, physiological studies (15, 84, 85, 89) have provided good evidence that glycine may be a spinal transmitter. Fulfillment of the criterion of "pharmacological identity" (90) by showing that the depressant action of glycine, but not that of GABA, was blocked by strychnine, an antagonist of some spinal postsynaptic inhibitions, made a strong case for glycine (37).

Werman et al (15) showed that iontophoretically administered glycine reduced or abolished the firing of most spinal cells tested, whether these were spontaneously firing, activated by peripheral inputs, or excited by application of excitatory amino acids. Glycine appeared to be slightly more effective than GABA on motoneurones. Although Werman et al (15) could not show an effect of glycine on Renshaw cells, Curtis et al (37) showed, in a series of over 170 Renshaw cells, that spontaneous or induced firing of all but one was readily depressed by glycine (0–40 nA). Glycine blocked spike invasion into neuronal somata, caused membrane hyperpolarization and reduced the size of EPSPs, changes that closely mimicked the conductance change produced by glycine (15). Both the IPSP and the glycine-induced membrane hyperpolarization had similar equilibrium potentials; both became depolarizing responses when Br^- and I^- were injected into motoneurones (15). After it was reported that the depressant action of glycine on spinal interneurones could be reversibly blocked by strychnine (37), Davidoff & Aprison (91) reported that iontophoretically applied picrotoxin ***consistently blocked*** the depressant action of glycine on spinal interneurones, but these results have not been confirmed (34, 67).

Although picrotoxin appears to be a specific GABA antagonist at crustacean inhibitory neuromuscular junctions (92–95), high intravenous doses are usually required to block postsynaptic inhibitions in the CNS (see above), and iontophoretically applied picrotoxin, though effective in reducing the depressant actions of GABA on cuneate (72) and spinal (96) neurones, never exerted a clear-cut effect. This led Curtis et al (35, 76) to study the action of bicuculline on spinal neurones.

Bicuculline reversibly antagonized the depressant actions of GABA and other GABA-like amino acids when applied iontophoretically, and when administered intravenously (in convulsant doses) it did not affect strychnine-sensitive inhibition of spinal motoneurones but did reduce the prolonged inhibition of these cells (which are also sensitive to picrotoxin) and peripherally evoked dorsal root potentials (76). It was suggested that GABA might be released at inhibitory axo-dendritic or axo-axonic synapses, and therefore be responsible for presynaptic inhibition (see also 81, 83). Hence, at least two types of spinal inhibitory interneurone, glycine- and GABA-releasing, might be involved in the control of firing of motoneurones (76). In this relation, the demonstration of strychnine- and picrotoxin-sensitive recurrent inhibitions of spinal motoneurones (97) is of interest.

Other Regions of the CNS

Iontophoretically applied GABA consistently depressed the activity of hippocampal neurones, by an action which was more effective than those glycine or β-alanine (12, 98, 99). Iontophoretically applied bicuculline (80–150 nA), which usually enhanced neuronal firing of pyramidal cells of the hippocampus, generally blocked or reduced the depressant actions of GABA and β-alanine while not altering that of glycine (36). In contrast, iontophoretically administered strychnine (10–30 nA) reduced, but never abolished, the effects of all three amino acids on hippocampal neurones, without exerting any obvious selective action on glycine (36). GABA, β-alanine, and glycine (in descending order of potency) depressed most medial geniculate neurones tested, and iontophoretically administered strychnine blocked the depressant action of glycine but not that of GABA (46). However, as strychnine also reduced or abolished the depressant actions of ACh and monoamines on medial geniculate neurones (44–46), it did not appear to be a competitive antagonist to glycine action. In neurones of the ventrobasal thalamus, bicuculline usually reduced or abolished the actions of GABA and β-alanine while not affecting that of glycine, whereas strychnine usually reduced or blocked the depressant actions of glycine and β-alanine while not affecting that of GABA (36). Davis & Huffman (100) found glycine to be more potent than GABA as a depressant of red nucleus cells of the baboon. But in more recent studies it was shown that though both GABA and glycine increased the membrane conductance of neurones of the feline red nucleus, GABA was clearly more potent than glycine (101).

UPTAKE, BINDING, AND RECEPTOR INTERACTIONS OF ACTIVE AMINO ACIDS IN VITRO

The metabolism of slices of CNS tissues is largely intact when a suitable medium is used, and their rates of O_2 consumption can be increased to levels which approximate the resting rate of cerebral O_2 consumption in situ by increasing the K^+ concentration of the medium or by electrical stimulation (102–104). Synaptosomes also maintain functional integrity in that they respire; produce lactate, ATP, and phosphocreatine; and synthesize amino acids from glucose (105–109).

Na^+-Dependent Uptake and Binding

UPTAKE It is commonly believed that Na^+ is essential for the cerebral uptake of GABA and other amino acids. However, studies by Margolis & Lajtha (110) revealed that a "physiological" Na^+ gradient between tissue and medium is not essential for the accumulation of amino acids by brain slices.

Using slices of cerebral cortex, Balcar & Johnston (111) showed that though the "high-affinity" uptakes of both GABA and glutamate (10^{-8} *M*) are Na^+ dependent, at external Na^+ concentrations $>$ 30 meq/liter the uptake of glutamate was less sensitive to external Na^+ than was the uptake of GABA. Glutamate uptake was also less sensitive than GABA to drugs such as tetrodotoxin, strophanthidin, amiloride, and protoveratrines that modify Na^+ movements (111). These data (111) are in accord with data that have indicated that the binding of GABA is more dependent on Na^+ than that of glutamate (112, 113). In homogenates, the high- but not the low-affinity uptakes of glutamate and aspartate in cerebral cortex, and of glutamate, aspartate, and glycine in spinal cord of rats, required Na^+ (114). Inhibition of Na^+/K^+-ATPase activity by ouabain reduced the high-affinity uptakes of glutamate and aspartate in homogenates of cortex and cord and of glycine in cord (115). In adult rats glycine uptake was much less dependent on Na^+ than were the uptakes of aspartate or glutamate, and GABA and glutamate uptakes were markedly affected by Na^+ in all ages of rats examined (115), in agreement with results obtained in recent binding studies (113).

Martin & Smith (116) found that the Na^+-dependency curve for GABA uptake by cerebral cortical synaptosomes was markedly sigmoidal, suggesting a role of allosteric effects on GABA uptake. Allosteric interactions were also apparent between glycine and Na^+ in spinal homogenates (115). The Na^+-dependent uptake of GABA by synaptosomes followed simple saturation kinetics with respect to GABA, and there were no heterotropic, cooperative effects of GABA on kinetics observed with Na^+ (117). Hence, GABA uptake could proceed by a mechanism involving only one GABA site, though more complex mechanisms are possible (116, 117). At 27°C, the V_{max} for GABA uptake by synaptosomes at 19 meq/liter Na^+ was only about 12% of its value at 95 meq/liter Na^+, whereas the apparent K_m was increased only about twofold (117). In other studies, the uptakes of glycine and α-alanine into rat cerebral cortical synaptosomes at 37°C were found to be only partially dependent on Na^+ in both immature and adult animals, whereas the uptake of aspartate exhibited an absolute Na^+ requirement in both adult and immature animals (118). Synaptosomal uptake of aspartate in Na^+-containing medium increased progressively with aging of the animal, and Na^+ significantly enhanced the uptake of glycine in fractions of newborn and adult rats but had only a slight effect in fractions prepared from 12–17-day-old rats (119). This synaptosomal uptake of glycine proceeded by two systems, one of which was totally dependent on external Na^+, while the other was independent of Na^+ (119). Interestingly, the Na^+-independent system for energy-mediated glycine uptake was most active during days 12–17 of development, the period during which maximal rate of synaptogenesis (120) and maximal brain excitability (121) also occur in rats.

Using Hill plots, Martin (117) showed that a minimum of three cooperatively interacting Na^+ sites are involved in GABA transport in synaptosomes, whereas a complete interaction of two Na^+ ions appears to be involved in the activation of glycine transport (119). In studies with homogenates, Hill plots gave slopes of about 1.0 for glutamate uptake by rat cerebral cortex and for glycine uptake by spinal cord (115).

In recent studies it was shown that the high-affinity uptake of GABA ($3–5 \times 10^{-7}$ *M*) by Purkinje, stellate, and interstitial neurones of cultures of rat cerebellum is Na^+ dependent (122, 123), and that the rates of uptake of both glutamate (4.8×10^{-8} *M*) and glycine (2×10^{-10} *M*) into cultures of chick spinal cord were increased by increasing the Na^+ concentration of the medium (124). Also, the high-affinity uptakes of GABA by cultures of rat astrocytoma and mouse neuroblastoma exhibited Na^+ dependency (125). These observations are of interest in that the Na^+ requirement for amino acid uptake was demonstrated in CNS tissues maintained under artificial conditions for rather long periods of time.

Serious criticisms apply to most of the above studies on amino acid uptake. For instance, Bowery & Brown (126) showed that GABA uptake by rat sympathetic ganglia, which contain negligible amounts of GABA, GAD, and GABA-T (127, 128), exhibited a Na^+-dependent high-affinity system. This uptake could not have been related either to endogenous GABA content or to synapses and was likely due to glial cells. Other recent studies have also indicated that high-affinity uptake processes for amino acids occur in glial cells (125, 129–131).

BINDING It is apparent from the results discussed above, that it is difficult indeed to show any amino acid to be a neurotransmitter substance by using the Na^+ dependency of energy-mediated uptake as the criterion. Studies of the Na^+-dependent binding of amino acids, which is not energy-dependent (e.g. 132, 133), may provide better clues regarding the identity of amino acid transmitters. When brain tissue is homogenized in isosmotic sucrose solutions containing Na^+, maximal binding of GABA occurs at about 40 meq/liter Na^+ (112, 134), and this binding occurs mainly to synaptosomal particles (134, 135). However, the optimal Na^+ concentration for GABA binding is much higher (e.g. 100–200 meq/liter) when brain particles are first isolated and then exposed to radioactive GABA in media containing Tris buffer (e.g. 132, 136–140).

In a recent study in which mouse brain was homogenized in sucrose solutions containing various concentrations of Na^+ plus ^{3}H-GABA, it was found that at concentrations of $Na^+ > 10$ meq/liter the percentage of the total ^{3}H-GABA which sedimented in synaptosome-enriched P_2 fractions was decreased; however, GABA binding, in terms of dpm/mg protein, was maximal at about 40 meq/liter Na^+ (134). The decrease in the percentage of ^{3}H bound to P_2 fractions at $>$ 10 meq/liter Na^+ was caused by "clumping" effects on the particles by the excess salt (141). With high salt concentrations, many particles possessing high affinity for GABA binding sedimented in crude nuclear (P_1) rather than in P_2 fractions (141), but by monitoring the sucrose distributions throughout all operations it was shown that changes in neither the constitution of particulate fractions nor in the rates at which the particles

were sedimented could account for the increase in GABA binding caused by Na^+ (134). In other studies, using homogenizing fluids which either contained 40 m*M* NaCl or were Na^+ free, it was shown that the binding of ^{3}H-GABA to both P_1 and P_2 fractions of rat brain was increased by 40 m*M* NaCl and that both processes were age-dependent (113). The binding of GABA to P_1 fractions increased with aging of rats up to 21–22 days, after which it remained constant, whereas GABA binding was maximal to P_2 fractions of the brains of 11- and 15-day-old rats, and then declined. The binding of glutamate and glycine exhibited age dependency similar to that of GABA (113). The binding of GABA to synaptosome-enriched fractions of mouse brain was decreased by subjecting the mice to prolonged periods of individual housing; this type of study provided a measure of the changes in nerve endings that can be caused by the environmental modification (142).

It is noteworthy that rather identical values have been reported for the K_B for GABA binding to cerebral cortical synaptosomal fractions [1.8×10^{-5} *M* (133, 143)], the K_D for bicuculline-sensitive GABA binding to cerebellar cortical synaptosomes [2.1×10^{-5} *M* (144)], and the K_m for high-affinity GABA uptake into small slices of cerebral cortex [2.2×10^{-5} *M* (145)]. Although the above binding constants for GABA were determined at 0-4°C and the K_m for its uptake was measured at 25°C, it seems possible that the same process (or processes) was being measured, since GABA binding is nearly complete in 10 min (132), the incubation time used for determining these K_m (145) and K_B (133) values.

Kuriyama et al (139) showed, in media containing 50 m*M* Tris buffer, that optimal GABA binding to a synaptic vesicle fraction of mouse brain occurred in the presence of about 130 meq/liter Na^+, and though GABA binding to synaptic vesicle fractions was enhanced by Na^+, the binding of ACh was decreased. About 10–20% of putative amino acid transmitters (i.e. GABA, taurine, aspartate, glutamate, glycine) have been shown to be associated with synaptosomal-mitochondrial fractions of guinea pig cortex in the absence of added Na^+ (146, 147), and substantial portions of these were bound to synaptic vesicle fractions (147). But as these amino acids were not released by hypo-osomotic conditions, it was concluded that "transmitter" amino acids may not be stored in synaptic vesicles, as is ACh (147). Studies with synaptic vesicles of rat cerebral cortex indicated that of the amino acids measured taurine and glutamate were bound in the most significant amounts and these vesicular pools were also not sensitive to osmotic shock (148).

Other Ion Requirements for Uptake and Binding

Since the release of amino acids appears to be intimately related to their re-uptake, Ca^{2+} perhaps plays a significant role in uptake and binding of amino acids. Mechanisms for the active accumulation of amino acids also appear to be coupled to K^+ movements via the Na^+/K^+ pump as these mechanisms are inhibited by ouabain (e.g. 115, 145, 149). It has been shown that removal of K^+ from the medium bathing brain slices caused a marked inhibition of amino acid (e.g. glutamate, aspartate) uptake that varied according to the Na^+ concentration and the amino acids studied (110). That the presence of both Na^+ and K^+ in the incubation medium is essential for sustained uptake of GABA by cerebral cortical synaptosomes has also been

demonstrated (116). However, the cooperativity of GABA transport with Na^+ (117) was relatively unaffected by increasing concentrations of K^+. Ca^{2+} strongly enhanced the initial rate of GABA uptake into synaptosomes at low Na^+ concentrations, but had little effect at Na^+ concentrations $>$ 100 meq/liter; Ca^{2+} did not support GABA uptake in the absence of Na^+ (116). In other studies (150), all anion substitutions for Cl^- significantly reduced GABA (1.6×10^{-5} *M*) uptake into synaptosomal fractions. The major requirement for the binding of GABA to brain particles appears to be Na^+ (132), but it should be noted that substantial amounts of Ca^{2+} are present in particulate preparations themselves, even if Ca^{2+}-free media are used.

Regional Uptake and Binding (High- and Low-Affinity Processes)

High-affinity components of amino acid uptake processes are believed by some workers to provide a method for identifying the inhibitory and excitatory transmitters within various structures of the CNS (e.g. 114, 151–155). Actually the strongest argument in support of this idea is based on studies of the uptake of glycine. But, although it has been maintained that a high-affinity mechanism for glycine uptake occurs in the cord, but not in the cerebral cortex (114, 151–153), even this has been recently questioned (119; see below). Also, recent studies (157) have indicated that high- and low-affinity systems for the *binding* of both GABA and glycine occur in synaptosomal fractions of both spinal cord and cerebral cortex at 0°C. As high-affinity mechanisms for GABA uptake have been shown in spinal cord, cerebral cortex, and many other CNS structures (155, 156), it can only be concluded, from the above, that GABA must be a transmitter in all of these structures. This area of research has been further confounded by the different concentration ranges used by various investigators. As examples, Blasberg (158) failed to observe high-affinity mechanisms for amino acids in brain slices because the lowest concentration he used was 10^{-4} *M;* other workers (153, 159) failed to observe the low-affinity mechanism for glycine in rat cord as their highest concentration was 5×10^{-4} *M;* and Logan & Snyder (151, 152) showed both low- and high-affinity systems because their concentration range was 5×10^{-6}–2×10^{-3} *M,* but they did not use lower concentrations to test whether another system was present.

Rather identical K_m values for the uptake of GABA and glutamate by slices of rat cerebral cortex have been shown, though these amino acids do not share a common transport system. High-affinity GABA uptake at 25° had a K_m value of about 2.2×10^{-5} *M* in cortex slices and a V_{max} of about 0.115 μmol/min/g of cortex (145), and glutamate uptake into similar preparations had a K_m of about 2×10^{-5} *M*, and a V_{max} of 0.24 μmol/min/g (160). However, the above K_m value for GABA uptake has not been reproduced in other types of preparations. For instance, Henn & Hamberger (161) reported values of 0.72, 0.42, and 0.27×10^{-6} *M* for the K_ms of GABA uptake into neuronal, synaptosomal, and glial fractions, respectively, of rabbit brain, and Martin (117) reported a K_m for GABA uptake by cerebral cortical synaptosomes of 0.4×10^{-5} *M* (at 95 m*M* Na^+). These differences may have been due to differences in incubation conditions and/or to the possibility that the dominant system for GABA uptake by brain slices is neuronal or glial rather than synaptosomal (117).

Glycine uptake by slices of rat spinal cord, incubated at 37°C, had a K_m of about 3.1×10^{-5} M and a V_{max} of about 0.48 μmol/min/g of cord (159). In slices of cat spinal cord, both the high-affinity K_m for glycine uptake (1.4×10^{-5} M) and its V_{max} (0.008 μmol/min/g) were lower than Neal's (159) values, and the high-affinity uptakes of glutamate, asparatate, and glycine exhibited nearly identical K_m values that were about half as great as that found for GABA; V_{max} values for glutamate and aspartate were about twice as high as those for GABA and glycine (155). Low-affinity K_m values for slices of cat cord were very similar for aspartate, glutamate, and glycine, and GABA did not show a low-affinity mode of uptake (155). In general, as shown by Logan & Snyder (151, 152) in homogenates of rat spinal cord, glycine, glutamate, and aspartate were taken up by two kinetically different systems: a low-affinity system with $K_m \cong 1\text{–}4 \times 10^{-3}$ M, and a high-affinity system with $K_m \cong 2 \times 10^{-5}$ M. Although only a low-affinity system ($K_m \cong 8 \times 10^{-4}$ M) was reported for glycine uptake by cortical homogenates (151, 152), Peterson & Raghupathy (119) found a K_m of about 2.5×10^{-5} M when using a glycine concentration range of 1.2–21.2 μM with synaptosomal fractions of rat cerebral cortex, and with a range of 0.1–1 mM glycine their data indicated the presence of a system of lower substrate affinity. The uptake of taurine by rat cortex slices, measured at 37°C, occurred with a K_m of 5×10^{-5} M and a V_{max} of 0.03 μmol/min/g (149). These latter results were confirmed by Lahdesmaki & Oja (162), who showed the presence of one nonsaturable plus two saturable transport systems for taurine in rat brain slices. It is noteworthy that the K_m values for taurine high-affinity uptake (149, 162) were about 2–3 times greater than those reported for aspartate, glutamate, glycine, and GABA uptakes by slices of cord or cortex.

Geometric factors may affect the determination of kinetic constants for amino acid uptake. With glycine and glutamate, (at external concentrations of 0.2–2.0 mM), lower values were obtained for intact mouse olfactory bulbs than for slices of olfactory bulb for both initial rates of uptake and final steady-state levels (163). Although the V_{max} for glutamate uptake was about fourfold higher in slices of bulb than in intact bulbs, the affinity of this amino acid for its "carrier" (K_m value) was similar in both preparations, an indication that the same transport process was being measured in both cases (163). Using dorsal and ventral spinal roots of the rat, in which this geometric factor is to a large extent controlled, Roberts & Keen (164) showed that the K_m for dorsal roots was significantly lower than that for ventral roots and that the V_{max} for dorsal roots was nearly twice that for ventral roots. These results are in accord with those of Hammerschlag (165), who showed that the V_{max} for glutamate uptake by the dorsal region of rat spinal cord was about twice that for ventral cord. High-affinity glutamate uptake appeared to occur in the ventral root (164), which has not been suggested as a region in which glutamate might serve a transmitter function. The retina also possesses high- and low-affinity uptake mechanisms for the transmitter candidates, glutamate, aspartate, taurine, GABA, glycine, and α-alanine, and also for the nontransmitter candidate, valine (166).

Because the high-affinity K_m values are quite similar for the "active" amino acids (except perhaps for taurine), regardless of the tissue studied (see above), it is difficult to pick one amino acid over another as a transmitter using uptake as the criterion. Perhaps the only regional differences that may indicate specificity of transmitter

function are those in which either a high- or low-affinity mechanism appears to be missing from a given tissue, i.e. the cases for glycine and GABA uptakes in cortex and cord (e.g. 151, 152, 155; but see above). In any case, all studies on GABA uptake, and perhaps those performed on the uptakes of other amino acids, are subject to the criticism of Bowery & Brown (126), who showed in rat sympathetic ganglia an apparent high-affinity K_m for GABA uptake of 4×10^{-7} M (after 5 min incubations) and 7×10^{-6} M (for 30 min incubations). The high-affinity system for GABA uptake by this non-"GABA-ergic" system is more potent than it is in any of the central structures in which GABA is endogenously present in high concentration. Another criticism against using the criterion of high-affinity uptake systems to identify possible transmitter substances derives from the findings that such systems have been described in non-nervous tissues such as kidney, bacteria, and yeast (167, 168; see 169). Until these criticisms are overcome, regional differences in the uptakes of putative amino acid transmitters will not be very meaningful.

These uptake studies have also not established whether the high-affinity mechanisms represent a net inward flux or an exchange of amino acid with endogenous stores. Jones & McIlwain (170) concluded from their studies on slices of guinea pig cerebral cortex that uptakes, especially of small amounts of amino acid ($< 10^{-5}$ M) are influenced by exchange processes. This type of effect could be the basis of explaining these "double-affinity" systems: i.e. at low concentrations ($< 10^{-5}$ M) of added amino acid, an exchange mechanism could lead to a significant increase in the amount of labeled amino acid entering the particles (or slices) and this would be observed as a system of higher affinity. Also, though it is well known that both amino acid exit and uptake can be observed in brain slices (e.g. 170, 171), in most studies with slices, uptake alone has been discussed, while the net accumulations (uptakes) of the amino acids that were measured really represented the sum of three systems (uptake, exit, and exchange). If high-affinity mechanisms are predominantly exchange processes, then there is reason to believe that they are not involved in the inactivation of released transmitters. Binding systems that exist at 0°C may be related to carrier and/or receptor interaction, and it is expected that less exchange would occur in washed particles at 0°C than in unwashed homogenates or slices at 22–37°C. Hence, studies of preferential binding of amino acids might be expected to show differences more closely related to re-uptake and/or receptor processes than have been shown in uptake studies.

Using isosmotic media containing 32 mM NaCl and concentrations of 10^{-7}–10^{-4} M ^{3}H-GABA and ^{14}C-glycine, the order of potency of binding to synaptosome-enriched fractions at 0°C was: GABA of cortex $>$ GABA of cord $\cong$ glycine of cord $>$ glycine of cortex (133). These differences in the binding of GABA and glycine to synaptosomal fractions were in accord quantitatively with estimates of the relative iontophoretic potencies of GABA and glycine in the feline spinal cord and cerebral cortex (15, 37, 172) and cannot be explained merely by differences in the endogenous regional distribution of these amino acids (127, 159, 173). For example, although glycine is present in much higher concentration than GABA in the cord (127), the binding of these two amino acids (133), like their iontophoretic potencies (15, 37), is about the same in cord. In more recent studies, it was shown that

although more glycine binding sites than GABA binding sites may be present in synaptosomal fractions of the cord, GABA binding occurs with greater affinity (157). Also, the binding capacity of GABA in rabbit retina is about 70% that of whole rabbit brain though the GABA content of retina is only about 20% that of whole brain (174), which provides additional evidence that the binding capacity of GABA may not be related to its endogenous concentration. In order to resolve further the possible transmitter-inactivation and receptor-interaction components of binding, future studies should perhaps be conducted on neuronal and glial preparations (e.g. 125, 175) with more refined methods for the isolation and purification of neuronal, intracellular, synaptosomal, and postsynaptic membrane fractions (176, 177).

Relationships Between Uptake or Binding and Endogenous Amino Acid Concentration

Parallelisms have been shown to exist between the endogenous glycine contents of various regions of rat CNS and glycine uptake (151), and strychnine binding (178). However, the report by Bowery & Brown (126) refuted the idea that there might exist a positive correlation between GABA uptake and its endogenous tissue content, as had been proposed from studies on slices (179) and subcellular particles (e.g. 180–183). Other evidence against this type of parallelism has been provided in studies of amino acid binding (133). Perhaps the only real parallelism which does exist is that subcellular particles prepared from various CNS regions reflect the total endogenous amino acid contents of these regions. For example, synaptosomes prepared from spinal cord contained more glycine that cerebral cortical synaptosomes (109), though glycine was not enriched in spinal cord synaptosomes when its concentration was compared with whole tissue levels of glycine (109, 184). In the study of DeBelleroche & Bradford (148) only taurine was associated more with synaptic vesicles of rat cerebral cortex than could be deduced from its total endogenous content. Regional differences in amino acid content may reflect mainly the differences in gray/white matter ratios, or in metabolism, or in total tissue water content, which exist among various CNS tissues. Hence, it seems apparent that though striking differences in amino acid content exist among gross structures of the CNS, simple schemes for the neurochemical basis of transmitter actions based on these data (87) have provided very little support for results obtained with iontophoretic, or binding methods.

Do Different Synaptosomal Populations Exist?

Autoradiographic studies have indicated that GABA, taken up by brain slices or homogenates, might be associated with specific populations of neurones (179, 185–187). Iversen & Bloom (187) showed that about 70% of the ^{3}H activity of ^{3}H-GABA taken up by slices or homogenates of rat cerebral cortex, spinal cord, and other brain regions was localized in nerve terminals. The proportion of terminals labeled by ^{3}H ranged from 13% in homogenates of cerebellum to 42% in homogenates of hippocampus. Perhaps their most interesting finding (187) was made with spinal cord homogenates in which it was shown that either ^{3}H-GABA or ^{3}H-glycine

labeled about 25% of the nerve terminals, but that simultaneous exposure to a mixture of ^{3}H-GABA plus ^{3}H-glycine labeled about 50% of all identified synaptosomes. On this basis it was suggested that GABA and glycine were accumulated by different populations of nerve terminals in spinal homogenates. However, in this study the control experiment of doubling the concentration and the amount of ^{3}H of either amino acid was not performed. These studies (187) indicated that a regional dependency exists for GABA uptake (plus metabolism) by synaptosomes (see also 188, 189).

The rationale for the use of linear sucrose gradients for studying synaptosomal populations is in part based on the idea that exogenous GABA accumulated by slices of rat cerebral cortex has a subcellular distribution that resembles very closely that of endogenous GABA (181). However, it has been shown (190) that radioactive GABA, either added to homogenizing fluids or accumulated by cortex slices. distributes rather identically after homogenization of the slices. In any case, this type of approach does allow the characterization of various subcellular particles in terms of their affinities for GABA or other substances, and has been useful in showing that GABA binding occurs to a greater extent to synaptosomal, than to other particles (135, 153, 155, 181–183).

Using homogenates of rat spinal cord, Johnston & Iversen (153) showed that synaptosomes labeled with ^{3}H-GABA could be partially separated from those labeled with ^{14}C-glycine by linear gradient centrifugation, but in more recent studies with homogenates of cat spinal cord, this finding was not confirmed (155). In other studies (182), performed on homogenates of slices of rat corpus striatum incubated with ^{14}C-norepinephrine plus ^{3}H-GABA, it was shown that NE-binding synaptosomes localized in a denser region of gradients than GABA synaptosomes. However, as the extent of separation of NE from GABA synaptosomes was independent of incubation time, these workers (182) were perhaps observing mainly the binding of ^{14}C-NE and ^{3}H-GABA to the particles that occurred during the process of homogenization, rather than their processes of uptake and/or storage, which would have been related to incubation time. Wofsey et al (191), using homogenates of rat cerebral cortex incubated with 5×10^{-7} *M* glutamate or aspartate, or with 10^{-6} *M* GABA, observed that radioactive glutamate and aspartate sedimented with particles that were localized in a denser region of sucrose gradients than GABA. Although these workers (191) suggested that leakage of aspartate and glutamate during the passage of particles through the gradient might account for their different localization, the control study of adding the radioactive amino acids directly to homogenates was not performed.

Studies by Bennett et al (114) indicated that Na^+ was required for high- but not for low-affinity uptakes of glutamate and aspartate by homogenates of rat cerebral cortex, and of glutamate, aspartate, and glycine in homogenates of rat spinal cord. Although these workers suggested that Na^+-dependent amino acid uptake systems labeled "unique synaptosomal fractions," no studies were conducted at 0°C to see whether this was Na^+-dependent binding rather than energy-mediated uptake. The shift of the synaptosomal peaks toward lower densities in sucrose gradients run with samples previously incubated in media containing 143 m*M* NaCl, as compared with

those incubated in Na^+-free media (which contained Tris, choline, or Li^+, substances that would interfere with Na^+-dependent mechanisms), could have been due to "clumping" effects (141), since these effects seem to occur in the presence of as little as 10 meq/liter Na^+ (134). Also, the greater effect of Na^+ of enhancing glycine retention by spinal cord than by cerebral cortical preparations found by these workers (114) could be related to the recent finding that about twice as many glycine binding sites exist in synaptosomal fractions of cord than of cortex (157). Balcar & Johnston (155), using homogenates of cat spinal cord, could not separate out any specific populations of synaptosomes for the uptake (or binding) of aspartate, glutamate, or glycine using either equilibrium or nonequilibrium density gradient fractionation. In a more recent study (192), in which ten-step discontinuous sucrose gradients were used to analyze the distributions of ^3H-glutamate and ^3H-glycine, after their uptake by slices of dorsal and ventral regions of cat spinal cord, a partial separation of synaptosomal subpopulations was observed.

Many of the pitfalls of autoradiographic methods have been discussed by Hökfelt & Ljungdahl (179, 193). With regard to the use of gradient fractionation techniques, the problems caused by redistribution of substances during homogenization and subsequent separation, and by salt-induced clumping effects have been discussed above. It should also be noted that upon exposure of synaptosomes to low external concentrations of amino acids in the presence of Na^+, the binding mechanism is in full play but transport and intrasynaptosomal storage mechanisms are not, and thus one cannot say, until equilibrium (steady-state) conditions are achieved, that exogenously applied amino acids label a pool that is different from the endogenous pool.

Inhibitors of Uptake and Binding

METABOLIC INHIBITORS Ouabain and related glycosides or aglycones may be metabolic inhibitors as these agents block the Na^+/K^+ pump, which is known to depend upon metabolism and which perhaps drives metabolism. Alterations in the levels of any of the ions that govern neuronal excitability (i.e. Na^+, K^+, Ca^{2+}, Mg^{2+}, or Cl^-) may also inhibit metabolism and hence amino acid uptake, as these ions affect metabolic reactions linked to the maintenance of transmembrane electrochemical gradients.

Cherayil et al (171) showed that the *exit* of some amino acids from mouse brain slices could be increased by metabolic inhibitors [e.g. 2,4-dinitrophenol (DNP), cyanide, anoxia, or Na^+-free media] but not by *p*-chloromercuribenzoate (2.5×10^{-4} *M*). It was suggested (171) that addition of metabolic inhibitors to the medium bathing brain slices might alter membrane structure, which could account for their actions on amino acid exit. GABA uptake by rat cerebral cortex slices (145) and the uptakes of glutamate, aspartate, and glycine by homogenates of rat cortex and cord were inhibited by ouabain or strophanthidin (10^{-4}–10^{-5} *M*) (111, 115). Inhibition of the uptakes of glutamate, GABA, glycine, and taurine were also caused by other drugs (at 10^{-5}–10^{-4} *M*), including electron-transport inhibitors, such as DNP and chlorpromazine (e.g. 145, 149, 153, 154, 194, 195). Mercurials (at 10^{-5}–10^{-4} *M*) inhibited the high-affinity uptake of glutamate by slices of rat cerebral cortex (111, 160) and that of glycine by slices of rat cord (159, 196). In retina, both high-

and low-affinity mechanisms for glutamate and aspartate uptakes were inhibited by *p*-hydroxymercuribenzoate (*p*-HMB) at 10^{-5} *M* and were abolished at 0°C and in Na^+-free medium (169). It is of interest that the potentiation of the inhibitory action of glycine and of the excitatory action of glutamate on spinal neurons by mercurials (37, 197) might be explained by the inhibitory actions of these agents on amino acid uptake (111, 159, 196). However, the action of *p*-HMB is not specific to putative transmitters since it also reduced the uptake of lysine by slices of spinal cord (197). Also, Sano & Roberts (132) had shown earlier that *p*-HMB (5×10^{-4} *M*), when preincubated with particulate fractions of mouse brain, inhibited the Na^+-dependent binding of GABA (10^{-5} *M*). Therefore, in addition to their actions as enzyme inhibitors, mercurials may act by reducing the affinity of transport systems for Na^+ or for the amino acid, by their reactions with sulfhydryl groups that may be in the regions of binding or uptake sites.

OTHER AMINO ACIDS AND THEIR DERIVATIVES Blasberg & Lajtha (198) showed that concentrative uptake of glycine (2 m*M*) by mouse brain slices was significantly inhibited by dicarboxylic amino acids (10 m*M*), and that concentrative uptake of GABA (2 m*M*) was inhibited by glycine, β-alanine, and dicarboxylic amino acids (all at 10 m*M*). However, the intial uptake of glycine (6×10^{-7} *M*) by slices of rat spinal cord was not affected by large molar excesses of dicarboxylic amino acids (159). Also, the uptake of GABA (at 5×10^{-8} *M*) appears to be more structurally specific than indicated in the above study (198) as, in slices of rat cerebral cortex, it was unaffected by large molar excesses of glycine, glutamate, aspartate, or β-aminobutyrate, but was inhibited by α-alanine, histidine, γ-amino-β-hydroxybutyric acid, and β-guanidinopropionic acid (145). Conformational specificity exists for GABA uptake by rat cortex slices since 11 conformationally restricted GABA analogues (at 10^{-4} *M*) significantly inhibited GABA (1.25×10^{-8} *M*) uptake (199). [In these studies (199), the tissue was preincubated for 15 min in the presence of analogues at 25°C, and uptake was measured 10 min after addition of radioactive GABA.] As certain GABA analogues of restricted ("extended") conformation (i.e. *trans*-4-aminocrotonic acid, DL-*cis*-aminocyclohexane-1-carboxylic acid, and 4-aminotetrolic acid) were competitive inhibitors of GABA uptake in rat brain slices, it was suggested that this conformation might be important in the initial binding of GABA to its transport carrier (199). DL-2-Fluoro-GABA, L-2,4-diaminobutyric acid (DAB), and muscimol were all found to be noncompetitive inhibitors of GABA uptake into brain slices (154, 200). Further studies indicated that 2-hydroxy-, 2-chloro-, and 4-methyl-GABA (δ-aminovaleric acid) were competitive inhibitors of GABA uptake in rat cerebral cortex slices (201). 2-Hydroxy-GABA was the most potent inhibitor of GABA uptake, but no specific inhibitor of GABA uptake was found.

In relation to these studies on possible uptake inhibitors, Simon & Martin (202) found that DAB served as a competitive inhibitor of GABA uptake into synaptosomal fractions of cerebral cortex during "short-term" exposure of these fractions to the inhibitor (i.e. no preincubation with inhibitor), but that this same agent acted as noncompetitive inhibitor when preincubated with fractions for 20 min. It was suggested that the noncompetitive inhibition of GABA that occurred after pretreat-

ment with DAB could have been caused by its uptake, which could then have exerted its effect from within the synaptosomes (202). These results obtained by preincubation of synaptosomes with DAB (i.e. noncompetitive inhibition of GABA uptake) are in accord with those of Iversen & Johnston (154) who preincubated slices of various regions of rat CNS with DAB. However, in other studies in which preincubation methods were used, some GABA derivatives served as competitive inhibitors of GABA uptake (199, 201).

In studies with homogenates of rat cerebral cortex and spinal cord, Logan & Snyder (152) showed that the mutual inhibition of glutamate and aspartate high-affinity uptakes was competitive. In other studies (160) it was shown that the uptake of L-glutamate (10^{-3} or 10^{-8} *M*) by slices of cerebral cortex was not inhibited either by excitant amino acids that exert greater depolarizing actions than L-glutamate on cortical neurones (e.g. D-homocysteate) or by antagonists of L-glutamate-induced excitation. However, the inactive L-isomer of homocysteate (with respect to iontophoretic studies) and D-aspartate, L-aspartate, L-cysteate, and L-cysteinesulfinate were potent inhibitors of glutamate uptake. It was suggested that these latter compounds might act by releasing glutamate or by preventing its re-uptake, whereas strong excitants (e.g. D-homocysteate) might have low affinity for the L-glutamate uptake system and high affinity for postsynaptic receptors (160). Evidence based on inhibition of high-affinity uptake by structural analogues (111, 160), on kinetic analyses (151, 152), and on subcellular fractionation (191) all support the notion that L-glutamate and L-aspartate possess comparable affinity for the same high-affinity uptake system in the rat CNS.

Regarding the uptake of ^{35}S-taurine (5×10^{-7} *M*) by slices of rat cerebral cortex, Kaczmarek & Davison (149) showed that β-alanine (10^{-3} *M*) was the only amino acid among those tested that produced significant inhibition, and of the analogues tested, 2-aminoethylsulfinic acid (10^{-3} *M*) was the most potent inhibitor of taurine (1.5×10^{-6} *M*) uptake. These inhibitors are the carboxyl and sulfinic derivatives of taurine. Other studies on rat cortex slices (162) have indicated that hypotaurine, β-alanine, GABA, N-methyltaurine, and L-cysteic acid all acted as competitive inhibitors of taurine transport. Therefore, carrier sites for taurine appear to recognize, to an equal degree, strongly ionized electropositive and electronegative ends of an acceptable molecule separated by two or three carbon atoms (162). Using slices of rat spinal cord, Neal (159) showed that α-alanine and leucine (10^{-3} *M*) reduced glycine (6×10^{-7} *M*) uptake by about 25% during 10 min incubations at 37°C, whereas glutamate, aspartate, and GABA had no effect. Studies on cat spinal cord indicated further that considerable structural specificity exists for these uptake systems (155).

Recent studies have not been focused on inhibition of amino acid binding mechanisms by other amino acids or their analogues. However, a considerable degree of structural specificity for GABA binding was indicated in the early work of Sano & Roberts (132). Binding and high-affinity uptake processes for amino acids may occur by very similar, if not identical, mechanisms (133, 203).

RECEPTOR ANTAGONISTS Although recent iontophoretic studies have indicated that picrotoxin, bicuculline, and benzyl penicillin exert antagonistic affects on the

postsynaptic inhibitory action of GABA and that strychnine and related agents antagonize the actions of glycine-like amino acids, these agents (at 10^{-4} *M*) have no effect on the high-affinity uptakes of GABA and glycine by slices of rat CNS tissue (e.g. 153, 154, 197, 201). High-affinity uptakes of glutamate and aspartate by slices of cat spinal cord were also not inhibited by postsynaptic antagonists, such as L-glutamate diethyl ester (155).

Since the actions of these in vivo antagonists could not be shown by uptake methods, it was necessary to study these mechanisms using binding methods. Synaptosomal or synaptic membrane fractions of rat cerebellar cortex, pretreated with 0.5 m*M* chlorpromazine (at 0–4°C, 10 min) and then incubated at 0–4°C for 1 hr in the presence of GABA, were shown to bind GABA, and this binding was competitively inhibited by bicuculline (144, 204). The K_D for specific binding of GABA to synaptic membranes was about 1.6×10^{-5} *M*, and additions of bicuculline ($0.5–1.0 \times 10^{-4}$ *M*) to the incubation medium inhibited this binding with a K_i about 3–6 times greater than the K_D for GABA (204). Excesses of strychnine or picrotoxin did not affect this binding of GABA, which was shown in the absence of added Na^+ (204). Snyder et al (178) showed that glycine and glycine-like amino acids (at 10^{-5}–10^{-3} *M*) inhibited the binding of ^{3}H-strychnine (4×10^{-9} *M*) to synaptic membrane preparations of CNS tissues and that the potency of these amino acids in displacing strychnine paralleled closely their relative iontophoretic potencies (37). Specific strychnine binding was not affected by replacement of Na^+ with Tris.

If, as indicated by the above-mentioned results, the receptor binding mechanisms for GABA and glycine do not require Na^+, then it follows that Na^+-dependent amino acid binding mechanisms may be related rather exclusively to their transport systems. In the latter studies (178) the affinity of glycine for its proposed receptor was about three orders of magnitude less than that of strychnine, which is roughly in accord with neurophysiological data that have indicated that strychnine is more potent than glycine on glycine receptors. The difference in potency of the inhibitor in these studies (144, 178, 204) seems to be due to the different procedures used. Schaeffer et al (204) added bicuculline to the incubation medium simultaneously with labeled GABA, while Snyder et al (178) tested glycine displacement of strychnine binding (a closer mimic of in vivo studies in which glycine was applied iontophoretically after iv strychnine).

GABA might act on postsynaptic receptors or with its transport carrier in "extended" (35, 199, 205, 206) and/or "folded" (207, 208) conformations. As muscimol functions as a GABA analogue with respect to its inhibitory action on central neurones (205) and as GABA and muscimol can assume similar conformations as zwitterions with charged centers of about 5–6 Å apart (206), GABA might interact with its receptor in extended conformation. 4-Aminotetrolic acid, which has an acetylenic bond that fixes its charge separation at 5.2–5.8 Å, also possesses a GABA-like action on spinal interneurones that is antagonized by bicuculline but not by strychnine (209). However, further studies are necessary before the steric requirements of the antagonism of GABA by bicuculline can be discussed (210). van Gelder (208) proposed, after studying molecular models of GABA and glutamate, that the

steric dimensions of the α-carboxyl group of glutamate or the γ-hydrogen of GABA (in the comparable position), represent the information necessary to induce conformational changes in the membrane that coincide with the hydrated diameters of Na^+ and Cl^-, the ions that when mobilized elicit the appropriate physiological responses. In this manner a space could be created in the membrane, which is 5.7–6.3 Å for glutamate and 3.7–4.5Å for GABA, values in good agreement with the diameters of hydrated Na^+ and Cl^-. Hence it was suggested that both GABA and glutamate could combine with their receptors in folded form (208). It seems apparent that before further progress can be made on amino acid receptor mechanisms, the receptor actions must be separated from those linked with transport, or nonreceptor binding processes. Although ionophores, binding, and active transport all require ions, it may be that receptor interaction itself is not heavily dependent on ions (178, 204).

Some Quantitative Estimates of GABA Content and Binding

Both GABA and GAD are specifically localized in terminals of Purkinje cell axons (16, 211), and pharmacological evidence has indicated that these terminals may be "GABA-ergic" (14, 60). Feline Purkinje cells and cells of the vestibular and cerebellar deep nuclei contain high levels of GABA (about 6–6.6 m*M*) whereas spinal motoneurones have low GABA levels (about 0.9 m*M*) (211–213). The high GABA concentrations (about 50–100 m*M*) of Purkinje nerve terminals may account for the high GABA contents of cells of vestibular and cerebellar deep nuclei (214). These demonstrations that cells and nerve terminals associated with inhibitory mechanisms are enriched with GABA have provided good evidence that GABA is the inhibitory transmitter. In another recent study, the amount of GABA bound per nerve ending of rat cerebral cortex was estimated to be about 0.16×10^{-18} mol at saturation of its binding mechanism at 0°C, in the presence 32 m*M* NaCl (133). This binding mechanism appears to be potent enough either to account for the inhibitory effect of iontophoretically applied GABA or to inactivate the amount of GABA required to produce cortical inhibition (133).

IN VITRO STUDIES ON THE RELEASE OF CENTRALLY ACTIVE AMINO ACIDS

Release of Amino Acids from Tissue Preparations

Strychnine (0.2 m*M*), but not picrotoxin or bicuculline, has been shown to reduce the spontaneous efflux of previously accumulated ^{14}C-GABA from slices of rat cerebral cortex (230). Crnic et al (215) showed that the effluxes of GABA and glutamate from rat cerebral cortex slices were highly specific and were accelerated by structurally similar amino acids which either depress or excite motoneurones. It was suggested that the inhibitory effects of certain iontophoretically applied amino acids (e.g. β-aminobutyric acid, δ-aminovaleric acid, and DAB) could be mediated by their acceleration of GABA release to the extracellular space, and that the excitatory effects of aspartate could be caused by its acceleration of glutamate release. In another study with slices of cerebral cortex, a rapid release of previously

accumulated ^{35}S-taurine was caused by electrical stimulation of the slices, and its rate was shown to be Ca^{2+} dependent (149).

Although some experiments (216) led to the notion that ischemic lesioning that caused a loss of spinal interneurones did not decrease GABA levels in the spinal cord, more recent studies (217) have indicated that such lesioning produced a *specific and localized loss of GABA* from the dorsal horns of cat spinal cord accompanied by failure of the dorsal root potentials. The release of previously acquired, labeled GABA from slices of rat spinal cord by depolarizing stimuli (218, 219) has also been demonstrated. Roberts & Mitchell (220) showed that although stimulation of spinal roots had no effect on amino acid efflux from the spinal cord, a result not in accord with the finding of Aprison (221) on glycine efflux, direct high-frequency electrical stimulation of frog or toad spinal cord produced large Ca^{2+}-dependent increases in the effluxes of previously accumulated ^{3}H-GABA, ^{14}C-glycine, ^{14}C-glutamate, and ^{14}C-aspartate, but not of "control" amino acids (serine, threonine, and leucine), mannitol, or urea. Collins (23) showed further that the spontaneous efflux of previously accumulated radioactive GABA from isolated hemisected frog spinal cord could be significantly increased by ouabain, *p*-HMB, and DAB, agents that also reduce GABA uptake (see above). Strychnine, but not picrotoxin or bicuculline, reduced the spontaneous release of GABA from frog spinal cord, whereas electrically evoked GABA release was inhibited by ouabain, *p*-HMB, and picrotoxin, and was potentiated by bicuculline (23). These effects cannot be readily explained by the actions of these drugs on the uptake and binding of amino acids (see above). Collins (23) indicated further that maximal evoked release of ^{3}H-GABA occurred at relatively low (5–30 Hz) rates of electrical stimulation, whereas rates of about 100 Hz were needed to release ^{14}C-glycine. This demonstration that glycine was not released under conditions favoring the Ca^{2+}-dependent release of GABA is consistent with the suggestion that GABA serves a transmitter function in the spinal cord.

Many criticisms apply to the above-mentioned "release" studies, especially when these are performed with exogenously applied amino acids (5). With regard to the electrically evoked release of amino acids, it was necessary to use very high frequencies in many studies, and Jones & McIlwain (170) have shown that electrical stimulation increased the efflux of leucine from slices of guinea pig cerebral cortex; leucine is usually considered to be a control amino acid in this type of study. Also, exchange processes would play a greater role in influencing experimental results as the amount of radioactive amino acid used to "load" the tissue is decreased (170). Furthermore Bowery & Brown (126) showed that rat sympathetic ganglia, which are not "GABA-ergic," can accumulate labeled GABA that can then be released by electrical- or high K^{+}-induced depolarization.

Using the phenomenon of "spreading depression" of the chicken retina, characterized by a change in its transparency (222), Van Harreveld & Fifkova (223) demonstrated that when the retina was allowed to accumulate ^{14}C-glutamate and then stimulated with unlabeled glutamate, KCl solution, or electrically (DC), a transient increase occurred in its transparency which coincided with a marked

increase in glutamate release. It was suggested that that this release of glutamate might be the primary event involved in spreading depression and that subsequent glutamate-induced depolarization could then cause K^+ release (223, 224). Several amino acids or analogues (e.g. L-glutamate diethyl ester, DL-α-methyl glutamate, DL-homocysteate, glutamine, and cysteine) changed retinal transparency and increased glutamate release (225). It is noteworthy that glutamate, aspartate, and DL-homocysteate, which exert potent excitatory effects on CNS neurones and alter retinal transparency, also increased glutamate release (226). DL-α-methyl glutamate and glutamate diethyl ester have also been shown to antagonize the action of glutamate on central neurones (51), but this appears to be a nonspecific action (28, 29).

Release of Amino Acids from Subcellular Particles

Differential release of previously acquired radioactive GABA, glutamate, and aspartate caused by electrical or high-K^+ stimulation of cortical synaptosomal "beds" was abolished by omission or chelation of Ca^{2+} of the media (108, 227). The spontaneous releases of aspartate, glutamate, GABA, α-alanine, and glycine could also be increased by including β-aminobutyric acid or DAB in the medium at 1 m*M*, the greatest increase in release occurring with GABA (108). These analogues also prompted a greater release of GABA by electrical, or high-K^+ stimulation, whereas N-acetylglutamate (1 m*M*) increased only glutamate release (108).

Bradford et al (228) showed further that sheep and rat hypothalamic synaptosomes and rabbit, sheep, and rat cortical synaptosomes exhibited respiratory and glycolytic responses to electrical stimulation that occurred in parallel with a Ca^{2+}-dependent differential release of radioactive GABA, glutamate, and aspartate; there was only a small effect on glycine. High K^+ (55 meq/liter) also released radioactive aspartate, glutamate, and GABA from these synaptosomal preparations (228), an effect previously shown only for GABA in slices of cerebral cortex (229). Using suspensions and beds of medulla/spinal cord synaptosomes, Osborne et al (109) showed that differential release of radioactive glutamate, aspartate, GABA, and glycine occurred during both electrical, and high-K^+ stimulation, and that during electrical stimulation of synaptosomal suspensions GABA was released to a greater extent than glutamate, aspartate, or glycine.

Levy et al (227) devised a method for monitoring the stimulus-induced release of previously acquired radioactive GABA from rat brain synaptosomes, in which the synaptosomes were immobilized on a membrane filter and then rapidly perfused. Since fractions could be collected at 20 sec intervals before and after exposure of the particles to various media, a rapid profile of GABA efflux could be obtained. In preparations of synaptosomes perfused with Ca^{2+}-free media containing 56 meq per liter K^+, which depolarized synaptosomes (108), the introduction of 4 meq per liter Ca^{2+} caused an increase in the efflux rate of GABA that occurred within 40 sec. This effect was reversed by reexposing the particles to Ca^{2+}-free medium. Because such short time intervals can be used with this method, it has many further applications in the study of release mechanisms.

CONCLUSIONS

From the foregoing discussion it is apparent that although much evidence exists to support roles for the active amino acids as CNS transmitters, many of the pitfalls that have been encountered have not yet been overcome. Iontophoretic studies have indicated that certain agents can serve as antagonists to the actions of active amino acids, and strychnine, picrotoxin, and bicuculline have been useful for the characterization of inhibitory mechanisms in various regions of the CNS. The strongest argument for a central transmitter role can be made for GABA, the only candidate that is present only in the CNS and that exerts potent depressant effects on cells of all regions of the CNS. In vitro studies have added little support to the in vivo actions demonstrated for amino acid antagonists, except perhaps for recent binding studies in which strychnine and bicuculline have been shown to compete, with glycine and GABA respectively, for membrane binding sites (144, 178, 204). It seems likely that further studies of amino acid binding with preparations from different regions of the CNS may yield valuable information concerning the identification of amino acid transmitters and that both in vivo and in vitro studies of the release of newly synthesized amino acids (from precursor U-^{14}C-D-glucose) will also be fruitful. Further attempts to separate out receptor-binding from carrier-binding mechanisms for amino acids will be limited until more specific receptor antagonists become available.

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