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MINIREVIEW

# RECOGNITION CHEMISTRY OF ANIONIC AMINO ACIDS FOR HEPATOCYTE TRANSPORT AND FOR NEUROTRANSMITTORY ACTION COMPARED

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## Summary

Comparison of neuronal and non-neuronal membrane transport of, and neuroexcitation by, the dicarboxylic amino acids bring out provocative similarities in structural selectivity, and hence in the strategies for studying them. Parallel anomalies in stereoselectivity show for both phenomena that the recognition sites are indeed chiral, as expected for biological functions, even though both fail in special instances to discriminate between DL pairs. High and low affinity, or $Na^+$-dependence or $Na^+$-independence, are not fully reliable bases for discriminating receptor sites serving one of these functions. Tolerance of N-methylation of the amino acid serves in discriminating families of recognition sites for both phenomena, as does substitution of the sulfonate or sulfinate for the distal carboxylate group, or other structural changes. Analogs in which the functional groups of aspartate or glutamate are presented in restrained arrays serve for both, although they have so far suggested identity neither of recognition sites for transport and excitation, nor of the events consequent to binding for these two phenomena.

We call attention here to some features of the membrane transport of dicarboxylic amino acids by hepatocytes, hepatoma cells and fibroblasts that bear suggestive relations either to neurotransmittory effects or to membrane transport in neural tissue of similar amino acids. One of us suggested in 1972 that transport behavior of amino acids might be directly pertinent to the question of how neurotransmittory effects are produced (1). Ancillary relations between these two phenomena in the meantime have become more obvious, but an identity of any known transport system to synaptic signaling has by no means emerged. Possible relations between neurotransmitter receptor sites and CNS transport receptor sites have in general not been ignored; we seek here to stimulate attention to the lessons of amino acid transport outside the central nervous system, and to show that the same sort of chemical recognition may be involved, to a degree that may render continuing comparisons instructive.

The membrane transport studies considered here will exclude from consideration migrations where the dicarboxylic amino acid appears to be accepted by a neutral transport system as a neutral zwitterion (e.g. glutamic acid), usually at low pH, rather than as an anion (e.g. glutamate). This exclusion we emphasize by use of the more restrictive term, anionic amino acids, instead of the more familiar one, dicarboxylic amino acids. The distinction is necessary in transport study. Agonistic action appears also to be restricted to anionic amino acids or their analogs, although the restriction is less clear for antagonistic action. At the same time we will try to avoid the confusion that may

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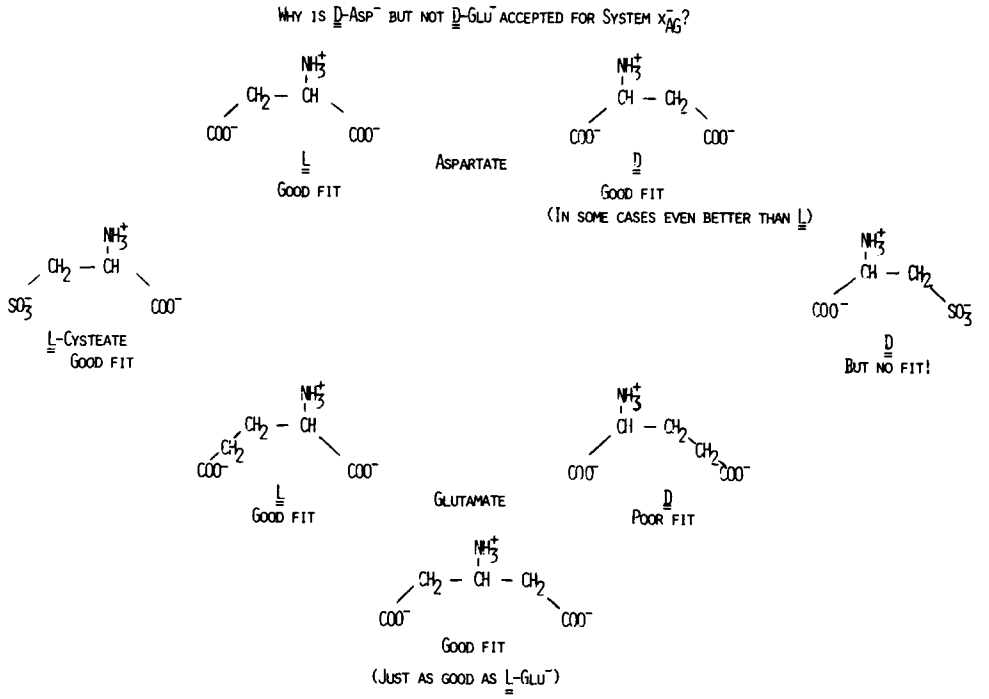
arise from use of the historic names, glutamic acid or kainic acid, when we are in fact referring to an anion, glutamate or kainate. Tests to determine the molecular species accepted for transport usually proceed by manipulating either the pH of the medium of origin of the amino acid or, by structural changes, the $pK_2'$ applying to the anionic amino acid. The first technique, that of pH variation, allowed the demonstration that in a system also transporting glutamate, cystine reacts in the form of an anion (2,3), but the technique seems to have been applied less regularly, perhaps for technical reasons, in confirming that it is indeed the anionic species of dicarboxylic amino acid analogs that affect the neuronal responses. The antagonistic action of diaminopimelate to anionic amino acid agonists seems likely to prove due selectively to the anionic species in which one of its amino groups is deprotonated, just as for the action of cystine on glutamate transport. The second technique utilizes not only dicarboxylic compounds but also sulfonic, sulfinic, phosphonic, or other low pK' analogs of aspartate and glutamate. Their low pK' values assure that the proportion of these analogs present in a transportable zwitterionic form will be very small. Evidence of the latter kind points to the anionic form as the reactive species also in producing neurotransmittory effects, although alternative possibilities again have been less uniformly excluded for antagonistic action.

Stereospecificity. The hepatocyte $Na^+$-dependent transport system mainly considered for our illustrations here handles both L-aspartate and L-glutamate at nearly equal affinities. In correspondence to these balanced affinities, this system is designated $x_{AG}^-$. The designations $x_A^-$ and $x_G^-$ serve then for systems with major preferences for aspartate and glutamate, respectively, although $x_C^-$ has been adopted instead of $x_G^-$ where cystine is also a substrate. System $x_{AG}^-$ is the one also showing an anomaly in stereoselectivity in the mature hepatocyte which seems to us instructive for the interpretation of related anomalies seen in neurotransmittory effects: little transport selectivity is shown by System $x_{AG}^-$ of the hepatocyte for L-aspartate over D-aspartate, whereas L-glutamate is about ten times as transport-reactive as its D-antipode (4), not an unusual degree of stereospecificity for transport. Somewhat the same anomaly in stereoselectivity for anionic amino acid transport was seen earlier in Neurospora, for which D-aspartate is preferred over its antipode (5), later in cultured cerebellar cells (6), along with a related one for glutamic and aspartic acids in the Ehrlich cell at low pH (7). The accompanying diagram shows the several test substrates with their functional groups arranged in space to show how the components of the receptor site of such a transport-mediating system may recognize these complementary structures on the substrates. We propose that the transport receptor site is constituted with a special geometry, of course not exactly that shown here in two dimensions, and with distances between subsites such that it can accept either a formate or an acetate group on each side of the α-carbon and its charged amminium group. Hence D-aspartate can bind nearly as well as L-aspartate, but with an inverted placement of its carboxylate groups. In other words it is received as a β- rather than an α-amino acid. The case can be likened to a glove fingered so crudely that it can fit either the left or the right hand, without violating however the need for a hollow palm. L-Cysteate shows similarly high transport reactivity, whereas its enantiomorph shows much lower if any reactivity (4). The latter result shows that of the recognition points for the carboxylate groups only the distal can accept the sulfonate group in place of a carboxylate; hence stereospecificity is recovered by the sulfonate-for-carboxylate substitution. The unsuitability of the distal sulfonate may be one of bulkiness, or of the chemical mode of bonding rather than of limitations of space. The receptor site appears, however, to accept poorly the propionate group when presented to the site by the D-glutamate molecule at the "wrong" side of the α-carbon, even though the results with aspartate indicate that the acetate can be accommodated at either side of the site. We can liken glutamate to a hand on which the thumb stands too far away from the index finger for the D-isomer to fit our imaginary glove. The finding that 3-aminoglutarate (a really symmetrical hand, so to speak) is transported as well

as glutamate (4) shows that the acetate group can indeed by accommodated at either side of the recognition site, and supports the view that it is the extra space or distance required for the propionate group that allows stereoselectivity for glutamate but scarcely for aspartate. We need to note also the example of low stereoselectivity of glutamate for its membrane transport into a mouse leukemia cell (8). The system concerned in that case does not accept aspartate. It may be significant that the anomaly in stereoselectivity of transport is seen so far for the shortest identified substrate, and in the leukemia cell stereoselectivity may yet emerge between the two stereoisomers of α-aminoadipate or another homolog.




Anomalous stereoselectivity applies not only for neural excitation by aspartate and its higher homologs (9), but also for transport into neural tissues (e.g. 10,11) from various species and anatomical sites. We select a recent study to illustrate the transport anomaly (12). In this case, the rate of uptake of L-aspartate by vesicles prepared from rat brain synaptosomes was decreased to 4% by excess L-aspartate, and to 6% by the same excess of either D-aspartate or L-glutamate. But D-glutamate at the same level was only weakly inhibitory, no more so than glycine. Neuronal transport of D- and L-aspartate, and of L-, but scarcely D-glutamate, has been well verified to occur by a common mediating system by showing consistency of $K_i$ and $K_m$ values (13). When we turn to the neuroexcitatory effects, the D-isomers of both aspartate and glutamate are effective at the so-called NMDA (N-methyl-D-aspartate) population of neuroreceptors (9; see reviews 14-16). 3-Aminoglutarate has also been found neuroexcitatory (9). Along with the isomers of the dicarboxylic amino acids both L- and D-homocysteate (along with L-cysteate) take their places in that order in the list of antagonists effective at these receptors, whereas only their L-isomers appear in the list for another class of receptors for neuroexcitation (14-17).

We have already seen from the case of anionic amino acid transport in

Neurospora (5) that an unevenly expressed chirality may be built into transport receptor sites of the $x^-_{AG}$ type in a manner to favor the D rather than the L isomer. Hence a preference for D isomers in neurotransmittory action would not violate the precedents as to discrimination of these isomers seen in membrane transport. Nor does the neurotransmitter activity seen for both stereoisomers of anionic amino acids in which the anionic groups are more than 4 carbon atoms apart need to raise doubts about applicability of the explanation of our diagram. Whereas L-aminoadipate is a neuroexcitant, D-α-aminoadipate and its analog, D-2-amino-5-phosphonovalerate, have been identified as strong, rather specific antagonists for the NMDA population of neuroreceptors (14-17). Also antagonistic is γ-D-glutamylglycine, which rather than being a typical dipeptide is in fact an amino acid analogous in structure to the antagonistic D-α-aminopimelate, with the peptide N in the γ-dipeptide interpolated for one of the methylene groups in the α-aminopimelate (14). Given that the receptor site provides enough space for the side chains of longer analogs to curve into conformations appropriately stabilized by environmental structures, the two anionic groups can be brought as close or closer together on binding as are the two carboxylate groups of aspartate, a possibility that might apply for one molecule but not for a slightly different one. Given that chirality may favor either the D or the L isomer, on occasion D-glutamate or D-homocysteate may be more reactive with a receptor than their antipodes or than D-aspartate, a relation not yet illustrated by our $x^-_{AG}$ model. Our experiments have generally observed only a single optimum for substrate molecular length at the hepatic transport receptor sites.

The threo and erythro isomers of 3-hydroxyaspartate demonstrate another point of chirality in a $Na^+$-dependent, high-affinity transport into rat brain slices. In fact, the relation between the positions taken by the 3-hydroxy and the 2-amino groups proved more critical than the absolute configuration on carbon 2 (18). The high transport activity achieved with it allows the threo isomer to intensify the excitatory effect of glutamate by inhibiting its removal by transport. It is not a new phenomenon in membrane transport for chirality to be intensified by features of the substrate molecule absent in most of the substrates of the given transport system. For example, System L in the Ehrlich cell shows rather weak preferences between the stereoisomers of its ordinary neutral amino acid substrates, such as phenylalanine. Stereospecificity is, however, sharply decreased for amino acids with certain unrequired side chain structures that cause unusually steep accumulation. The System L uptake of the L isomers of azaleucine, thialysine and ε-hydrolysine is far faster and more concentrative than that of their D isomers (19).

The generalization extracted here is that the chirality inherent in biological affinity and recognition may not be fully and uniformly signaled in the recognition process. Is it not at the same time a significant similarity that at least one receptor site for transport and one for neural excitation show much the same incompleteness of stereoselectivity? In any case, the doubts expressed on the latter account about the role of aspartate and glutamate in neural transmission (e.g. 20) appear unjustified. An experimental advantage contributed by a D amino acid over L, its antipode, namely a greater stability to enzymatic attack, could also contribute to apparently stronger and more prolonged action. The possibility has also been considered that an analog might mimick agonistic effects by trans stimulation of exodus of an endogenous agonist if it thereby increased the level of the agonist in the synaptic cleft (21)

The horizontal cells of the goldfish retina are depolarized by either L-glutamate or L-aspartate, but only at millimolar concentrations. The effects of L-glutamate are, however, potentiated 15-fold by exposure of the tissue to D-aspartate, which also augments their electrical response to light (22). D-aspartate is able to block uptake of both of the dicarboxylic amino acids; so the potentiation of the effect of L-glutamate might arise from protection of its

supply from depletion, or from other effects on its distribution. The rods of the goldfish retina accumulate L-glutamate by a high affinity system with a 30-fold preference over aspartate. Hence L-glutamate might serve as a specific neurotransmitter in this cell type. Red-sensitive and green-sensitive cones instead accumulate both L- and D-aspartate, also L-glutamate but not D-glutamate, with little selectivity among the first three (23). This result bears an obvious parallelism to the transport behavior of the hepatocyte and of vesicles prepared from synaptosomes. The blue-sensitive cones show relatively little uptake of any of these anionic amino acids. Blockade with 2,3-piperidinedicarboxylate has served to differentiate two classes of synaptic receptors that mediate cone influence in the outer retina in Necturus (24). These results support further the view that the endogenous transmitter is an excitatory amino acid.

TABLE I

Inhibition of $Na^+$-Dependent L-Glutamate Uptake Via System $x^-_{AG}$ into H35 Hepatoma Cells by Amino Acid Analogs

| Inhibitor | $K_i$ values mM ± S.E. | Inhibitor | v, % of control +1 mM | +5 mM |
|---|---|---|---|---|
| L-Aspartate | 0.012 ± 0.002 | α-Methyl-DL-aspartate | 28 | 22 |
| D-Aspartate | 0.0093 ± 0.0015 | α-Methyl-DL-glutamate | 75 | 97 |
| L-Cysteate | 0.0058 ± 0.0007 | N-Methyl-DL-aspartate | 37 | 26 |
| D-Cysteate | 0.46 ± 0.10 | N-Acetyl-L-aspartate | 62 | 29 |
| L-Cysteinesulfinate | 0.0083 ± 0.0012 | N-Methyl-L-glutamate | 105 | 120 |
| L-Glutamate | 0.029 ± 0.004 | L-Aspartate-β-hydroxamate | 12 | 1.1 |
| D-Glutamate | > 5 mM | L-Glutamate-γ-hydroxamate | 100 | 100 |
| L-Homocysteate | > 5 mM | 1-Aminocyclohexane-1,2-trans-dicarboxylic acid | 49 | 33 |
| D-Homocysteate | > 5 mM | Same, cis isomer | 78 | 93 |
| L-α-Aminoadipate | 0.76 ± 0.07 | 3-Aminoglutarate* | 0 | 0 |
| D-α-Aminoadipate | > 5 mM | (±)-Cis 2,5-piperidine-dicarboxylate (2,5-PDA) | 100 | 72 |
| DL-α-Aminopimelate | 1.8 ± 0.4 | (±)-Cis 2,6 PDA | 110 | 85 |
| L-Cystine | > 1 mM | Trans-α-carboxycyclopropylglycine* | 96 | Not det. |
| L-Threonine | 1.3 ± 0.2 | Same, cis isomer* | 5 | Not det. |
| | | Kainate | 0 | 0 |

Left, $K_i$ values in approximate order of increasing chain length, determined using 4 selected glutamate and 6 selected inhibitor concentrations by computer fit to the equation for competitive inhibition. Right, velocity of uptake of 25 μM L-glutamate as per cent of uninhibited rate. *Contrasting results on Systems $x^-_A$ and $x^-_C$, M. Makowske and H. N. Christensen, to be published.

Chain length and N-methylation. Table I, left, shows the effect of chain length on the $K_i$ values of anionic amino acids as inhibitors of glutamate uptake via System $x^-_{AG}$ of the hepatoma cell line H4-11-EC,3, here referred to as H35. This cell line has been selected here for its greater ease of study than the mature rat hepatocyte and because of the similarity of its glutamate transport to that of the hepatocyte (4), in contrast with the fetal hepatocyte and the hepatoma cell line, HTC (3). The transport system under study, $x^-_{AG}$, seems likely to be the same one as the $Na^+$-dependent, high-affinity system detected by Logan and Snyder in their search for an agency by which the hypothetical neuronal transmitter pools of aspartate and glutamate may be generated. Synaptosomes prepared from rat cerebral cortex showed in their study a $K_m$ of 16.9 μM for aspartate uptake, and a $K_m$ of 36 μM for glutamate uptake (25), values very similar to those seen for $x^-_{AG}$ in H35 (Table I, left), and hence carrying no implication of unique service in the neurone. Low half-saturation levels are seen for fibroblasts (26) and various other cells, and appear not to justify separate

treatment of amino acid uptake in neural and non-neural tissues. Highest affinity binding sites no longer tend necessarily to be accorded top significance for neural transmission. The right hand panel shows further comparisons of analog inhibition of glutamate uptake by the same system. We have reported elsewhere similar tests of various other hepatoma cell lines, and of fetal and mature rat hepatocytes (3,27,28). Ineffective inhibitors of the uptake of 25 μM glutamate in H35 during 1 min will show readings of nearly 100 in both columns of Table I, right, i.e. at both 1 and 5 mM. Reservations as to possible effects of trace impurities may need to be entertained for higher concentrations. We note first by the inhibition of glutamate uptake that the transport reactivity of DL-aspartate is retained after its N-methylation, whereas for L-glutamate the same step causes loss of essentially all inhibitory action. For the other $Na^+$-dependent system for anionic amino acid uptake by hepatocytes, $x_A^-$, N-methylation of either aspartate or glutamate leaves no inhibitory action on uptake of the preferred amino acid substrate, cysteinesulfinate (3,27,28). Similarly, for $Na^+$-independent System $x_C^-$ as tested in hepatoma line HTC, neither retains inhibition of glutamate transport on N-methylation. Such inter-system differences in tolerance to N-methylation are already familiar for the neutral amino acid transport systems, two being highly tolerant (A and Gly) and at least two entirely intolerant (ASC and L). N-Methylation of amino acid substrates of transport has accordingly long (29) been an important strategy for systems discrimination (30). Neuroscientists have speculated that the N-methyl group introduced into the D-aspartate molecule, by diminishing the extent to which the carbon chain tends to fold, intensifies the agonistic action with the NMDA population of neuronal receptors.

Hydroxamates. Next in Table I, right, we note the contrast between the effects of converting L-aspartate and L-glutamate to their ω-hydroxamates. This conversion presumably raises the pK' of the distal anionic group (31), probably an unfavorable effect, and lengthens by one nitrogen atom the separation of the two anionic groups. These compounds also bear structural relations to the transmitter antagonist HA-966 (32). For aspartate this conversion to its hydroxamate does not greatly decrease reactivity with transport via System $x_{AG}^-$ of H35, whereas the reactivity of glutamate appears to be eliminated by conversion to its γ-hydroxamate. From the comparison with the effects of chain length on $K_i$ seen in Table I, left, the former molecule should logically not have become too long, whereas the latter molecule might well have become long for System $x_{AG}^-$. In contrast, the action of L-glutamate on transport by System $x_C^-$ persists after its conversion to the γ-hydroxamate as tested in hepatoma line HTC, a predictable effect because this system has room for longer amino acid chains as in α-aminopimelate and cystine, although apparently not for the extreme length of homocystine (3). The inappreciable reactivity of aspartate with the same System $x_C^-$ in HTC remains scarcely appreciable on lengthening to the β-hydroxamate (Table II in ref. 3).

Only weak neuroexcitation has been found for the ω-hydroxamates of L-glutamic and DL-aspartic acid, whereas these analogs were among the stronger inhibitors of the high-affinity uptake of L-glutamate by rat dorsal root glia and cerebral synaptosomes (32,33). In contrast, substances that antagonize amino acid-induced excitation of central neurones were found without effect on glutamate uptake. In another test L-glutamate-γ-hydroxamate at 5 mM was as effective as L-glutamate at 1 mM in stimulating neuronal efflux of previously loaded $^{22}Na^+$ (34). This behavior could have a number of explanations, including a transmembrane exchange of amino acid and $Na^+$ together across the plasma membrane by a $Na^+$-dependent transport system.

Restrained analogs in discriminating receptor sites. Ring formation is often used to restrain the positions that the chemical groups of amino acids can take in space for receptor recognition. The second and third populations of agonistic sites, namely the quisqualate- (35) and kainate-responsive

populations, are presumably discriminated by these agents on that basis. Among three piperidine dicarboxylates, the 2,3-dicarboxylate (2,3-PDA), already mentioned for retinal photoreceptors (24), identifies the NMDA receptors. 2,5-PDA is in turn fairly selective for the quisqualate population (36,37), although that group is better identified by its sensitivity to blockade by iontophoretically applied glutamic acid diethylester. Although unfortunately we did not have access to 2,3-PDA, neither 2,5-PDA nor 2,6-PDA showed appreciable inhibition of $x^-_{AG}$ transport in H35 (Table I, right). This table also compares the inhibition of glutamate uptake via System $x^-_{AG}$ by the two conformers of aminocyclohexane-1,2-dicarboxylic acid (7), analogous to 2,3-PDA. Comparison of the acid-base properties of these isomers places the amino group of one between the two carboxylate groups, which thus lie in a trans relation. This trans conformer is a fairly effective inhibitor of $x^-_{AG}$, whereas the cis conformer appears devoid of inhibition (Table I). Neither conformer has appreciable action on transport systems $x^-_A$ and $x^-_C$, tested with their preferred substrates, $CySO^-_2$ and glutamate.

The sharpest inter-system contrast we obtained among restrained glutamate analogs is seen with the α-carboxycyclopropylglycine isomers of Fowden et al. (38). The cis form is a potent inhibitor of glutamate transport by $x^-_{AG}$ (Table I), whereas the trans isomer is ineffective. System $x^-_C$ shows a sharply opposite selectivity (unpublished results, M. Makowske and H. N. Christensen). This contrast tells us that the subsite recognizing the distal anionic group of amino acid substrates falls in entirely different positions in the two systems. Such differences serve strikingly then to discriminate receptors both for transport and neuroexcitation. Although without effect on either $x^-_A$ or $x^-_C$, kainate proved a potent inhibitor of $x^-_{AG}$ in H35 (Table I) - conceivably its $K_i$ may prove low enough to correspond to its intense agonistic potency in neurones. Along with quisqualate, kainate has offered the hope of showing binding related only to its pharmacologic action. Its high transport reactivity with $x^-_{AG}$ may occasion some rethinking, and intensifies our interest in the neurochemical function of this widely distributed transport system. Perhaps the $Na^+$-independent neuronal binding of kainate may prove more homogeneous, although two high-affinity components have already been detected (39).

"Excitotoxic" effects. Several neuroexcitatory analogs behave like glutamate in producing lesions when applied iontophoretically to specific brain areas. Certain retinal (40) and other CNS (41) regions outside the blood-brain area may be vulnerable in neonatal rodents to high oral glutamate intake. Although kainate blocks glutamate uptake weakly, thereby perhaps leading to some persistence of elevated glutamate levels in the synaptic area, this effect seems not to explain the toxic action of kainate. The resulting brain lesions cause effects mimicking Huntington's chorea and epileptic attacks. Since the toxic effects apparently result from activation of central excitatory receptors for amino acids, these receptors have come to be seen as potential points for pharmacological intervention. The neurotoxic action appears, however, to arise largely through activation of presynaptic receptors on nerve termini, stimulating release of endogenous agonist, although direct excitatory effects of kainate are considered postsynaptic (42). When kainate application produces lesions to the striatum or the retina, it also causes major losses in their subsequently measured high-affinity kainate-binding sites. Furthermore, cell types resistant to that injury, including retinal rods, carry low densities of such receptors (43). Nevertheless excitatory responses to kainate persist after cortical lesions have been produced by it (44,45).

Sulfonate and sulfinate analogs; diamino-dicarboxylic acids. We have mentioned the use of D-cysteate to show that D-aspartate transport binding involves an inverted recognition of the two carboxyl groups. We spoke also of the usefulness of the analog, cysteinesulfinate. We will complete briefly here the

story of the substitution of the sulfonate or sulfinate group for the distal carboxylate. The increasing reactivity with System $x_A^-$ in hepatoma line HTC takes the sequence, L-aspartate < L-cysteate < L-$CySO_2^-$ (28, Fig. 5). The advantage of the latter analog over cysteate we attribute tentatively to the similarity of the space requirement for the $-SO_2^-$ group to that for the carboxylate group. Similar results apply to the fetal hepatocyte and to hepatoma cell line H35. Homocysteate presents an ion distinctly too long to be inhibitory to this transport system, as shown with various other long-moleculed analogs (Table I; Table III in ref. 28), including L-glutamate. The cysteate and cysteinesulfinate ions are in turn too short for the sodium-independent transport System $x_C^-$, for which aspartate is also almost inactive as a substrate. Homocysteate is an effective inhibitor of System $x_C^-$, and hence the pair, cysteate and homocysteate serve impressively to show the difference in length of the amino acid molecules accommodated by the two systems, $x_A^-$ and $x_C^-$. For System $x_{AG}^-$, however, the absence of homocysteate inhibition is somewhat anomalous, since other anionic amino acids of similar length are accommodated. The sulfonate group may be too bulky for this recognition site. Among these three systems, only $x_C^-$ has proved responsive to amino acid depletion, and that only after several hours (46).

Aspartate and cysteate, and particularly $CySO_2^-$, served for the surprising observation that System $x_A^-$ on deprotonation becomes effective for the still $Na^+$-dependent transport of neutral amino acids of similar chain length. Conversely we observed that protonation of System ASC, when serving for transport of neutral amino acids, increases its susceptibility to aspartate or cysteinesulfinate inhibition of that transport, also to the transmembrane exchange between neutral and anionic amino acids. In this way we showed the two transport systems to be in fact one and the same (28). The strange result is that the anionic $CySO_2^-$, when applied at pH 5.5 or 6, is so far our best model substrate for System ASC. Furthermore an interconvertibility to serve as System $x_A^-$ appears to be a widespread characteristic of the apparently ubiquitous System ASC.

With regard to neurotransmission, homocysteate is a strong antagonist for both the quisqualate- and the NMDA-preferring receptors, both the D and the L-isomer serving in the latter case. Although we have noted that the D isomers and the N-methyl derivatives of the anionic amino acids offer technical advantages, both as agonists and antagonists, for discriminating and studying the NMDA population of neuroreceptor sites, these are artificial analogs, and presumably no one supposes them to serve endogenously as neurotransmittory agents. Ultimately their structures may bring attention to unknown endogenous agonists. In contrast, cysteinesulfinate ($CySO_2^-$) has been suspected of endogenous action as a neurotransmitter (47,48), even though it also was first introduced as an artificial analog to mimick endogenous amino acids, both for membrane transport (e.g. 3,26,27) and for the production of neurotransmittory effects. $CySO_2^-$ is asymmetrically distributed in the rat brain (47), and the enzymes involved in both its synthesis (via cysteine oxidase) and its degradation are localized in synaptosomes (49-51). Furthermore taurine as a product of its metabolism may act as an inhibitory synaptic transmitter, thus contributing further to the possible significance of $CySO_2^-$. In addition, $CySO_2^-$ at low levels increased the cyclic AMP concentrations more strongly than did glutamate in brain slices of guinea pigs (52).

A $Na^+$- and $Ca^{2+}$-dependent high-affinity uptake system for $CySO_2^-$ uptake by the synaptosome preparation has been described (47). Mitochondria purified from the same preparation failed to take up $CySO_2^-$. The uptake by synaptosomes was inhibited by L-glutamate, D- and L-aspartate, and L-cysteate, but not by N-methyl-DL-aspartate. The inhibition by the three L forms was competitive, although that by D-aspartate was not tested for this feature. Depolarization by $K^+$ at 56 mM or by veratridine at 10 μM stimulated a somewhat $Ca^{2+}$-dependent release of previously accumulated [$^{14}$C]-$CySO_2^-$ (48).

In separate experiments from the same laboratory (53), a $Na^+$-independent specific binding site for $CySO_2^-$ was shown in crude synaptic membranes. Both the $Na^+$-dependent and $Na^+$-independent sites were studied with [$^{35}$S]-cysteate. The $Na^+$-independent site was considered probably different from that shown for glutamate. It is noteworthy that effects of raising and lowering the pH from 7.4 were tested, binding being sharply decreased both at pH 9 and pH 5. One wishes such tests might be reported more often for neural work, particularly when tests are made on analogs for which we may expect changes in state of charge that might serve to identify the charged species producing effects. We can illustrate the problem by speculating whether some of the effects of the cationic glutamic acid diethyl ester as a specific inhibitor of the quisqualate-preferring receptors might arise from its exceptionally rapid passage across cellular membranes at neutral pH, a characteristic of amino acid esters exploited to load cells with say leucine, or lysosomes with cystine (54). Subsequent cleavage within the neurone might then plausibly yield free intracellular glutamate to contribute in some unknown way to the action of this antagonist. Concern has been expressed that this agent shows different effects when it is supplied in the bath rather than iontophoretically. Conceivably electrophoresis could tend to release selectively say a monoethyl ester from the iontophoretic electrode.

This section has shown that the same sulfur analogs serve for the study of the two phenomenon our title proposed to compare.

Low affinity transport into glia as a possible means of terminating excitation. Although interest has often focused on high-affinity transport receptors for anionic amino acids in relation to neurotransmitter action, attention has also fallen on low-affinity, high-capacity transport systems. Such transports have frequently been seen as appropriate for a glial uptake that might serve to terminate the effects of amino acid excitants in the synaptic cleft (55). The question was asked whether N-methyl-D-aspartate produces a prolonged excitation because of its rather slow uptake via a glial transport system. A potent inhibitor of that uptake was reported however not to influence the excitant action (56). Histidine potentiates the neurotransmitter effect of quisqualate on the rat spinal cord, presumably by retarding its transport from the synaptic region (57). The neurone as indicated earlier may itself participate in the recapture of released amino acids. Glutamate captured by the glial cells is believed to be converted by them to glutamine, which then may migrate back to the neurone for cleavage to free glutamate in the mitochondria (58,59). The glutamate may, according to this hypothesis, serve again in a cyclic fashion. Even disregarding this proposed interconversion, we may face a competition among three immediate fates for an excitatory amino acid entering the synaptic cleft: a perhaps reversible binding at postsynaptic receptors by which a message is transmitted; recapture by a presumably high-affinity presynaptic transport system that maintains the neuronal pool, e.g. after a second transport step into Naito and Ueda's vesicle; and its capture by the presumably low-affinity system whereby glial cells bring the amino acid below exciting concentrations. It is inherently unlikely that each of these three processes come to equilibrium. Instead, excitatory amino acids probably sustain a low steady state flow with these fluxes all increased on stimulation (cf. 60). Glial capture may not, however, play a major role in these events. Tissue cultures grown under conditions that suppress glial multiplication show a time course for synaptic excitation little changed from that seen in vivo or in slices. In any case membrane transport retains an essential role in the sequence of events.

Mode of release, excitatory action, and termination of action of the presumed neuroexcitatory amino acids. Substantial support is available then for roles for glutamate and aspartate, perhaps also for cysteinesulfinate as endogenous neurotransmittory agents. In approaching the question of the mode of

their presynaptic release, writers may have been impressed unduly by an analogy with the release of hormones through their accumulation in vesicles, followed by exocytosis; also by analogy with an imperfectly established mode of acetylcholine release. Until recently, little evidence has been available for the accumulation of any of these amino acids into vesicles within the neurone, although synaptic vesicles had been detected. Now, however, Naito and Ueda have developed an ingenious immunologic procedure for isolating from the bovine cerebral cortex a class of endogenous synaptic vesicles able to accumulate L-glutamate with remarkable specificity in an ATP-dependent, $Na^+$-independent manner (61). The ability to isolate these vesicles came about from their recognition of a kinase-phosphorylated protein characteristically marking the surface of these vesicles (62). This protein is an endogenous substrate for both cAMP-dependent and calcium-calmodulin-dependent synaptic protein kinases. Antibodies to this Protein I then served to isolate vesicles of this class. This finding allows us to suppose that the contents of this vesicle may be accumulated for abrupt release into the synaptic cleft by exocytosis. The specific release step may thus at last come to be proved a vesicular transport.

Finally comes the harder question, how does the release of aspartate or glutamate, or the introduction of their agonistic equivalents into the synaptic region, produce its physiological effect? What we have seen as to the specificity of action of various anionic amino acid analogs allows the conclusion that one or another of the different populations of receptor sites recognizes them by binding them, and that this recognition bears important similarities to that initiating transport of amino acids. For both of these processes, given that the bound amino acid meets certain structural requirements beyond those allowing binding, a second step follows, namely translocation in the one case and neural stimulation in the other case. We have for analogy the contrast between the binding of leucine or 2-aminonorbornane-2-carboxylic acid for membrane transport, and its recognition for the stimulation of insulin release from the beta cells (63). Despite detailed geometric correspondence in the recognition of the latter molecule, the response of the membrane is not the same. Conceivably gene duplication has led to roles apparently so different for sites so similar. Furthermore, the similarity is too great to encourage interpreting a receptor-mediated endocytosis as the mode of response.

From an early time depolarization attributed to a $Na^+$ influx was associated with neurostimulatory action, and hyperpolarization with neural inhibitory action. We quote from Curtis and Watkins in 1960 (9):

> "The most satisfactory explanation of the difference between the actions of the acidic and neutral amino acids is that only the former results in the net entry of sodium ions into the cell, thus accounting for the observed depolarization."

This net entry of $Na^+$ was not supposed to occur as a stoichiometric consequence of dicarboxylic amino acid uptake (i.e. by cotransport), but by a membrane activation producing an unspecified $Na^+$ permeability. In current terms, do such neurotransmitters act by modulating inorganic ion channels, and if so, how? Conductance rises in some instances, but falls in others; and excitatory amino acids may not always act by modulating ion channels. To add to the complexity, anionic amino acids increase cyclic AMP levels in cerebral slices from guinea pigs or rats (52). Observations of $Ca^{2+}$ uptake concurrent with excitation add to the interest. By the use of a patch-clamped electrode, the gating by a glutamate ion has been anatomically identified with a single ion channel in the locust neuromuscular junction (64). Could the apparatus conduct the receptor-bound glutamate inward quickly enough to account for channel closing? "... the possibility exists that the induction of a response by glutamate-receptor interaction may be closely linked to an uptake system" (65). Whether the

excitatory amino acid molecule gives rise to a signal on binding without itself crossing the membrane is unknown. Could membrane transport have anything further to offer in explaining the effect? It is not known how an amino acid might open an ion channel. We note a weak precedent for a possible relation: a single amino acid molecule can serve for the inflow of numerous sodium ions because the System ASC carrier may dissociate its bound amino acid from a ternary complex less frequently than its sodium ion (66). An extreme case of that behavior might only with difficulty be distinguished from action through a reversible static surface binding of the excitatory amino acid. The ability of System ASC to serve for anionic amino acid transport counsels us not to ignore such possibilities. Lee and Vidaver have speculated on a signaling function of transport by System ASC, in which case cellular $Ca^{2+}$ uptake is catalyzed (67). Snodgrass closes a discussion with these words: "Studies of mechanism have much to explain in the realm of excitatory responses" (16).

Conclusion. The information so far available suggests that at least three separate populations of receptor sites may serve directly in postsynaptic recognition of amino acids to produce excitation. The information also suggests that perhaps a similar number of amino acid transport systems have supportive functions to the neurotransmittory action at several points. These membrane transport activities are often much more similar than has been supposed to those in non-neural tissues. The evidence indicates further that the chemistry which allowed nature to design specific receptor sites for these amino acids for transport, and specific receptors for neural impulses, is to a large degree one and the same chemistry, both as to the substrates and as to the complementary membrane structures recognizing these substrates. Furthermore, the discrimination of different populations of synaptic receptor sites involves much the same complex strategy as the discrimination of distinct transport systems. Our comparisons and the speculations arising from them perhaps have sufficient merit to allow us to urge continued comparison of these classes of recognition sites on various cell surfaces.

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