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INTERACTIONS BETWEEN THE CO-SUBSTRATES OF A NA^+-DEPENDENT NEUTRAL AMINO ACID TRANSPORT SYSTEM

by

Edwin Lee Thomas

A dissertation submitted in partial fulfillment
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ABSTRACT

INTERACTIONS BETWEEN THE CO-SUBSTRATES OF A
NA^+-DEPENDENT NEUTRAL AMINO ACID TRANSPORT SYSTEM

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Chairman: Halvor N. Christensen

Interest in further studies of the Na^+-dependent neutral amino acid transport system designated as the ASC system resulted from studies of the Ly^+ system for cationic amino acids. Na^+ and neutral amino acids of appropriate structure inhibited the Ly^+ system, taking the place of both portions of the natural substrate and permitting a study of the Ly^+ binding site and the interactions between Na^+ and polar groups on the amino acid side chain. Cationic amino acid inhibition of the ASC system suggested that the Na^+ binding site might lie in the same relation to the bound amino acid as in the Ly^+ system. Study of the results of interaction of amino acid and cation analogues with the pigeon erythrocyte ASC system was undertaken to gain knowledge of its physical nature and operation.

Uptake of isotopically labeled amino acids and $^{22}Na^+$ was measured by incubating cells in their presence, centrifuging to separate the cells from the medium, and extracting the radioactivity from the cells for measurement by liquid scintillation spectrometry. Exodus was studied following an incubation to load the cells with a labeled substrate.

Points of binding for the α-amino and carboxyl groups were proposed to explain the inhibitory properties of glycine and the stereospecificity of binding. For amino acids with side chains, three separate

effects were shown to change the apparent affinity, as expressed by the K_i or K_m values. Affinity increased with the apolar mass of the side chain up to a length limitation defined by norleucine, so that an apolar binding region of limited dimensions was proposed. In the presence of Na^+, an increase in apparent affinity was observed for amino acids having side chains without hydrocarbon branches and not exceeding norvaline in length. The translocation process appeared responsible for this change because the extent of the decrease in K_m with Na^+ concentration was well correlated with the maximal rate of uptake of the amino acid and with its maximal ability to stimulate exodus of amino acids by exchange.

A polar group on the β- or γ-carbon produced an additional increase in apparent affinity and raised the ratio of Na^+ to amino acid entry. Polar groups in other positions lowered the apparent affinity perhaps through repulsion by the apolar binding region. In this way, a relatively small polar binding region was shown to lie close to the points of α-amino and carboxyl group binding. We have concluded that this region is the point of Na^+ binding, in analogy to the highly similar effects observed with the $\underline{Ly^+}$ system, and with the correlation of reduced reactivity with features of amino acid side chains that would prevent the access of Na^+ to this region. A type of bonding may be formed between the co-substrates by way of the unshared electrons of the polar group. The differing reactivity of the stereoisomers of γ-hydroxyproline showed that the point of Na^+ binding is positioned $\underline{trans}$ to the bound carboxyl group.

Although the transport process had a nearly total specificity for Na^+, other monovalent cations inhibited uptake by competing with Na^+ binding. Only Na^+ appeared capable of producing the changes secondary

to binding in the transport mediating structure which are required for translocation. The stereospecificity of cationic amino acid binding showed that these inhibitors were bound in the manner of neutral amino acids. The ω-guanidino amino acids were more effective inhibitors than were the diamino acids, consistent with the greater ability of the guanidinium ion to compete with Na^+ binding, relative to ammonium ion. In this way, cationic amino acid inhibition provided more evidence for the position of Na^+ binding and for the essentiality of Na^+ in the transport process.

Evidence was obtained that the ASC systems of differing cell types show only minor differences, as is the case with the Ly^+ system. The similarity of the ASC and Ly^+ binding sites was also noted and separate evolutionary development from a common precursor was suggested as the basis for their similarity.

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INTRODUCTION

In the nineteenth century, differences were observed in the access of substances to the cell interior, and were attributed to the selective permeability of the cell membrane. Overton (1,2) proposed that the barrier to entry of hydrophilic substances was composed of lipid. Höber (3) first suggested that the entry of solutes was due to the action of specific components of the membrane rather than to differences in solubility. The distinction between these two aspects was expressed by the terms physiological permeability and physical permeability.

The concept of a mobile carrier within the membrane developed over an extended period. Classic contributions were made by Osterhout (4) in 1935, by Widdas (5) in 1952, and by T. Rosenberg and Wilbrandt (6) in 1955. The latter authors provided a review of the carrier concept and a mathematical analysis in 1961 (7). The mobile carrier was believed to be a small molecule which shuttled between the faces of the membrane, binding and releasing the transported solute. In order to accomplish net transport, the carrier was held to be capable of movement when the substrate was not bound. Transport against a concentration gradient could be accomplished by coupling carrier movement to a source of energy. The carrier concept developed as a result of the frequent observations that neither solubility differences nor diffusion processes could account for the selectivity and kinetic features of transport.

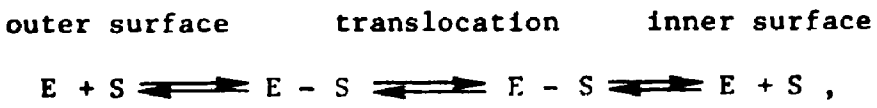
In 1902, Nagano (8) observed that glucose and galactose were absorbed more quickly by the intestine than were xylose or arabinose. The pentoses would be expected to diffuse more rapidly on the basis of their solubility and size. The most convincing evidence of this kind was ob-

tained for the glucose transport system of the human erythrocyte. Linear (9) and cyclic (10,11) polyalcohols were found to have low rates of uptake compared to sugars, despite the expected similarity of their physical permeability. Differing rates of uptake of enantiomorphic pairs of sugars were also observed (12). In 1958, LeFevre and Marshal (13) demonstrated a systematic correlation of sugar reactivity with relative ability to assume a particular conformation of the pyranose ring structure. This study revealed the importance of the shape of the molecule, an effect which is best explained by transient binding during the translocation event. In this way, the preferred conformation also provided information about the spatial properties of the sugar binding site. Further support for the binding hypothesis was obtained by Gibson and Wiseman (14) in 1951. These workers observed differing rates of uptake of the D and L forms of amino acids in the rat intestine. Such specificity can arise only by way of a three-point binding to an asymmetric site.

In 1913, Van Slyke and Meyer (15,16) observed the uptake of amino acids against apparent concentration gradients, an effect which can not be explained by simple diffusion. In addition to confirming these results, other workers found evidence of saturation and competition effects in transport. Saturation was first observed by Cori (17) in 1925 for intestinal sugar transport. Under the conditions of high concentration which he used, the rate of uptake appeared independent of concentration. Also, the presence of a second sugar was observed to decrease the rate of uptake (18). The findings of Jacobs, Glasman, and Parpart (19) in 1935, on glycerol permeability in red blood cells were interpreted by Danielli (20) in 1943 to indicate that a component of the entry could be described by diffusion kinetics and that the remaining component was

due to specialized portions of the membrane that permitted rapid entry. Competition among amino acids for reabsorption in the kidney was observed by Kirk (21) in 1936. In 1943, Pitts (22,23) observed the non-linear relationship of uptake and amino acid concentration in this tissue. Improved methods of study permitted Fisher and Parsons (24) in 1953 to demonstrate the applicability of the Michaelis-Menten equation to intestinal sugar transport. The high rates of transport in the Ehrlich mouse ascites tumor cell permitted this type of kinetic description for a component of the uptake and accumulation of amino acids (25-29).

Whereas transport by way of a fixed site or a pore in the membrane will be affected by the local concentration of the solute, a mobile carrier will respond sequentially to the concentrations present in the inner and outer phases. Evidence for such sequential effects and therefore for the mobility of the carrier was first obtained from the studies of Ussing (30,31), and of Heinz and Walsh (32) of the phenomena which have come to be called <u>competitive exchange diffusion</u> and <u>accelerative exchange diffusion</u> (33). If saturating levels of substrates are present on both sides of the membrane, the rate of net transport from one phase to another will decrease, since under these circumstances the carrier exists almost entirely in the loaded form (E - S, where E is the carrier, S the substrate) and net transport requires the movement of free carrier. A rapid exchange of labeled substrates from one phase with unlabeled substrates in the other phase may be observed, however, because the rate of exchange depends on the process:



without any translocation of free E. Although net transport is inhibited by product, exchange requires the presence of product. This phenomenon is described as competitive exchange diffusion. If the level of substrate in one phase is not saturating, the rate of movement of label from one phase to the other will depend on the translocation of free E. The most completely characterized example of this phenomenon is probably the glucose transport system of the human erythrocyte (34-37). For some systems the rate of movement of label depends on the level of substrate in the phase into which movement is measured even when non-saturating levels of substrates are used. In this case the rate of translocation of E - S is greater than that of E, so that the rate of movement of label is determined by the rate of return of E - S. This second phenomenon is described as accelerative exchange diffusion, in that the binding of substrate increases the apparent mobility of the carrier. Translocation need not be rate-limiting for these effects to occur; the rate of return of E or E - S may be determined by the rates of association and dissociation of the complexes. Accelerative effects have been observed with many transport systems, including the human erythrocyte glucose transport system when studied at low temperatures (38). In some cases, E is incapable of translocation so that the movement of label is observed only when the substrate is present in both phases. This special case of accelerative exchange diffusion is described as compulsory exchange diffusion (33), because no net transport can occur and only exchange diffusion is observed. These phenomena require that the carrier be available to the substrate first at one side, then at the other side of the barrier to solute movement.

In 1960 Christensen (39) called attention to the necessity of a macromolecular structure to provide the observed specificity of substrate

binding. This requirement along with the success in identifying proteins as components of transport systems (40) has led to the current concept of transport systems as macromolecular structures which bind the substrate and release it to another phase by way of a reversible change in conformation or orientation. Such changes may be induced by the binding of substrate as indicated by the studies of accelerative exchange diffusion. Current concepts of the carrier have therefore come to resemble the contractile proteins proposed by Goldacre (41) in 1952 and the rotating macromolecules proposed by Lundegardh (42) in 1954. Vidaver (43) has shown that the mobility inherent in these models is kinetically equivalent to that of the original carrier model.

The concept that different membrane components are responsible for transport of different classes of solutes has rarely been questioned. The relative lack of mutual inhibition of uptake of cationic, neutral, and anionic amino acids, compared to the competition among members of the same class (44-46), led to the suggestion that separate transport systems existed for these three classes (46). In animal cells, neutral amino acids of widely differing structures were found to compete for transport. Evidence that a single system could not account for neutral amino acid uptake in the Ehrlich cell was obtained by Tenenhouse and Quastel (47) in 1960 and by Johnstone and Scholefield (48) in 1961. Two systems, designated as A and L, were described for the Ehrlich cell by Oxender and Christensen (49,50) in 1963. The two systems had somewhat different specificities but their requirements were not so strict as to exclude any naturally occuring amino acid.

Two general approaches were developed in order to distinguish separate systems with overlapping specificities. Ahmed and Scholefield (51) pointed out that a study of K_i and K_m values might serve to exclude the

possibility that all the uptake of two solutes occured by way of the same system. Inui and Christensen (52) developed a kinetic test to determine whether two solutes shared the same system. Such kinetic tests have been refined in subsequent reviews (53,54). In Christensen's laboratory, model amino acids were designed so as to eliminate reactivity with all but one system (52,55-58). Such tests and model compounds permitted the description of other neutral amino acid transport systems (59,60), including a system for β -amino acids, discovered by Kromphardt (61) in the pigeon erythrocyte and confirmed for the Ehrlich cell by Christensen (62). A system almost completely specific to glycine was discovered in the pigeon erythrocyte by Vidaver, Romain, and Haurowitz (63), but this system is not present in the Ehrlich cell.

Amino acid and monosaccharide transport systems similar to those described for a particular cell type were often found in other tissues (64) confirming the view that these systems represent genetic and evolutionary entities. The heritability of transport defects was first noted for cystinuria (65,66) in 1948. Doudoroff (67) in 1951 suggested that the defects observed in some bacterial mutants were mutations in transport processes, a view which has been frequently substantiated. Proteins that appear to be substrate-binding portions of transport systems for sulfate (68), amino acids (69), and sugars (70-72) have been isolated from bacteria.

Christensen, Riggs, and associates first demonstrated the decrease in neutral amino acid accumulation in the Ehrlich cell (26) and avian erythrocyte (73) when other alkali metal cations were substituted for Na^+ in the medium . The same replacement technique was used by Riklis and Quastel (74) to demonstrate the dependence of intestinal glucose uptake on Na^+ concentration. Csaky and Zollicoffer (75) showed that this effect could be reversed upon adding back Na^+. The use of brief incubation periods by Kromphardt, et. al. (76), proved that the effect of extracellular Na^+

on the accumulation of amino acids was due to changes in the rate of uptake. Since the recognition of these effects in the early 1950's, the majority of the transport systems for neutral substrates have been found to be Na^+-dependent.

The effect of Na^+ on these systems appears to be separate from the process that maintains high intracellular K^+ and low intracellular Na^+ levels, relative to the external medium. Ouabain usually inhibits the Na^+- and K^+- dependent ATPase (77,78) believed responsible for this process, but does not directly inhibit the substrate or Na^+ movements associated with other Na^+-dependent systems (79-81). Csaky, Hartzog, and Fernald (82) reported in 1961 that ouabain inhibited intestinal sugar transport, but these results were obtained using long incubation periods, during which the normal Na^+ and K^+ gradients may have been lost (83). The importance of these gradients was demonstrated by Christensen, Riggs, and associates (84, 85). They reported that the concentrative uptake of glycine by the Ehrlich cell was accompanied by a net gain of Na^+ inside the cell, and a net loss of K^+. In 1958 Riggs, Walker, and Christensen (86) proposed that the concentrative feature of glycine uptake was the result of coupling glycine uptake to movements of Na^+ or K^+ or both down their concentration gradients, and proposed a complex of the carrier, glycine, and monovalent cation. In the course of studies of intestinal sugar transport, Crane subsequently developed a similar view for the involvement of the Na^+ and K^+ gradients, stated in the most complete form in 1965 (87). He was able to show the active efflux of glucose from the intestine when the Na^+ gradient was reversed (88). Similarly, Eddy and his associates (89-91) have shown that the intracellular level of glycine in the Ehrlich cell could be controlled by manipulating the Na^+ and K^+

gradients.

For the well-defined Na^+-dependent systems uptake is abolished in the absence of Na^+. Inui and Christensen (52) reported that the portion of methionine uptake inhibited by the removal of Na^+ was the same as the portion inhibitable by the fully competitive substrate of the A system, α-(methylamino) isobutyric acid. In their studies of Na^+ movements associated with the A system, Shafer and Jaquez (92-95) found no evidence for either Na^+ or amino acid transport by way of this system in the absence of the other substrate. Bihler, Crane, and their associates (94, 95) have reported that both the concentrative, energy-dependent portion and the non-concentrative portion of intestinal sugar transport are totally dependent on Na^+.

As suggested by the observations of net cation movements, Na^+ has been found to behave as a co-substrate for all the Na^+-dependent systems for which studies of Na^+ movement have been made. In 1963, Bosakova (96) used $^{22}Na^+$ movements to demonstrate the influx of Na^+ associated with intestinal glucose uptake. Vidaver (97) subsequently reported that the rate of glycine-dependent Na^+ entry as measured by $^{22}Na^+$ movements is twice that of the Na^+-dependent glycine entry for the pigeon erythrocyte glycine system. His interpretation is that two Na^+ ions are bound to the carrier per glycine molecule bound, which is held to be consistent with the second-order dependence of glycine uptake on Na^+ concentration. The stoichiometry of Na^+ and substrate movements need not reflect the kinetic form of the Na^+-dependence, however. Wheeler and Christensen (81,98) confirmed Vidaver's results using the rabbit reticulocyte, and also reported that the ratio of the rates of uptake of Na^+ and taurine, a substrate of the pigeon erythrocyte β-amino acid system, is close to one, which does

not reflect the higher order dependence of this system on Na^+. As seen below, an even more complex kinetic and stoichiometric relationship was also observed in these studies. A one-to-one ratio was also reported by Schafer and Jacquez (92) for the uptake of Na^+ and α -aminoisobutyric acid, by way of the Ehrlich cell A system. Exact stoichiometry has not been obtained for intestinal sugar transport. Nevertheless, Semenza (99) has developed a kinetic analysis of this system, concluding that a one-to-one binding of Na^+ and sugar to the carrier was required to yield the hyperbolic dependence of sugar transport on Na^+ concentration (100-103), and of the change in the potential difference across the membrane on sugar concentration (104-106). This conclusion is in agreement with Crane's proposal of 1962 (107), that intestinal sugar transport occurs by way of the usual carrier model, with an additional binding site for cations.

The Na^+-dependent system designated as the ASC system was first demonstrated for the Ehrlich cell by Christensen, Liang, and Archer (60). This system showed a preference for neutral amino acids with short side chains, such as alanine, serine, and cysteine, and for their four-carbon homologues. Uptake of these substrates by this system was not inhibited by amino acids having two hydrocarbon substituents on the α -carbon such as α -aminoisobutyric acid, or by N-methylated amino acids such as N - methyl alanine, but was inhibited by some amino acids which did not appear to be taken up by this system. The ASC system also differed from the A system in being more stereospecific, and in having the capacity to exchange. A mode of uptake similar to the Ehrlich cell ASC system in its substrate requirements and Na^+-dependence was discovered in the pigeon erythrocyte by Eavenson and Christensen (108) and was recognized to

correspond to the ASC pattern seen in the Ehrlich cell. Such a system was also demonstrated for the rabbit reticulocyte bv Winter and Christensen (109) and by Wheeler and Christensen (81), although this system was lost upon maturation to the erythrocyte stage (109, 110). Systems with somewhat similar properties have been described for the rat kidney by Scriver and Wilson (111) and the rabbit kidney by Hillman, Albrecht, and Rosenberg (112). Studies of the kidney have placed emphasis on the reactivity of glycine and the imino acids with this system, which may correspond more closely to a biological function of this system than to a description of its substrate specificity. The human disease of iminoglycinuria (113, 114) in which hydroxyproline and glycine are excreted, appears to result from an inherited defect in a renal transport system similar to the ASC system in its higher affinity for proline and hydroxyproline than for glycine (113).

The pigeon erythrocyte and rabbit reticylocyte ASC systems showed a hyperbolic dependence on Na^+ concentration (81,98,115). The rate of Na^+ entry was found to be proportional to amino acid entry over a wide range of concentrations, but the ratio of the rates had a different, characteristic value for each amino acid tested. Koser and Christensen (116) extended this study to determine the ratios of Na^+ to amino acid entry for a number of ASC substrates, using the pigeon erythrocyte. Amino acids having side chains with polar substituents showed high values of this ratio.

The pigeon erythrocyte ASC system appeared to operate only by exchange diffusion so that uptake was balanced by the loss of endogenous Na^+ and amino acids (98,108). The Ehrlich cell ASC system, however, appears capable of net transport at least in the outward direction (117),

although the rates of exchange are greater. As pointed out by Eavenson and Christensen (108), the alkali metal gradients may serve to drive the accumulation of amino acids, but concentrative uptake is not an essential feature of Na^+-dependent systems, in contrast to the suggestion of Csaky (118).

Our interest in further studies of the ASC system developed as a result of recent work in this laboratory. In the study of the transport system (Ly^+) for cationic amino acids in the Ehrlich cell by Christensen and Liang (119), inhibition of lysine uptake by neutral amino acids was observed. The rate of lysine uptake measured in the presence of phenylalanine increased when Na^+ was replaced by the choline cation. Christensen and Antonioli (120) found that homoserine was a remarkably strong inhibitor of the rabbit reticulocyte Ly^+ system and that the inhibitory action was Na^+-dependent. Following an extensive study of these phenomena, we reported that neutral amino acids of appropriate structure together with a monovalent cation such as Na^+ could react with the Ly^+ system in three cell types (121-124). Na^+ and the neutral amino acid could be shown to act together as a competitive inhibitor of cationic amino acid uptake, or to be exchanged across the membrane for cationic amino acids (123,125). The neutral amino acid appeared to take the position ordinarily occupied by the neutral portion of the cationic amino acid, with Na^+ taking the position usually occupied by the cationic ω-amino or guanidino group. The inhibitory properties of several homologous series of neutral amino acids demonstrated the requirement for an apolar region of the amino acid side chain, the interaction of polar groups with the bound cation, and a chain length requirement which showed good agreement with the expected position of binding of the cationic portion of the substrate, relative to

the position of α-amino and carboxyl group binding.

In the course of the studies of the $\underline{Ly}^+$ system, we observed the converse phenomenon of cationic amino acid inhibition of the ASC transport system. We can be certain that these effects do not occur by way of the same system, in that the $\underline{Ly}^+$ system has a much greater preference for cationic amino acids than for Na^+ and neutral amino acids, and the ASC system has the opposite preference. Furthermore, neutral amino acid inhibition of the $\underline{Ly}^+$ system could be demonstrated in the rabbit erythrocyte, in which the ASC system is absent, and inhibition of the ASC system could be seen in the pigeon erythrocyte in which the $\underline{Ly}^+$ system has very low rates of uptake of cationic amino acids, relative to that observed in other cell types.

The inhibition of the ASC system by cationic amino acids suggested that cationic amino acids might bind in place of both neutral amino acid and Na^+, showing that the Na^+ binding site is probably physically close to the amino acid binding site. The techniques used in the study of the $\underline{Ly}^+$ system have been extended to the ASC system in the present work, using a variety of cationic and neutral amino acids and monovalent cations, in order to study the binding sites and overall operation of the ASC system.

EXPERIMENTAL DETAIL

I. General statement of methods.

Pigeon erythrocytes were separated from the other components of the blood, then incubated for specified periods in isotonic media containing amino acids and Na^+ in known concentrations, and radioisotopically labeled amino acids or $^{22}Na^+$ in known specific activities. The cells were then separated from the medium and washed, followed by extraction and measurement of the intracellular concentration of radioisotope. The times of incubation were sufficiently short so that movement of the isotope in one direction was measured. The movement of amino acids or of Na^+ was calculated from the movement of isotope.

All of the techniques used were the same as or derived from established procedures. Heinz (27) and Christensen and co-workers (25) developed the procedures for use of labeled amino acids and incubation in defined, artificial media for the Ehrlich cell. Winter and Christensen (126) modified the procedure for the mammalian erythrocyte. The procedures used in the present work were most directly derived from the work of Eavenson (108) and Wheeler (98) on the pigeon erythrocyte, and on the use of the labeled cation, $^{22}Na^+$.

II. Materials.

Chemicals were obtained from commercial sources and were of reagent grade, or the highest grade available. Radioisotopically labeled amino acids were obtained from Calbiochem, Schwartz, and New England Nuclear. Radiopurity of these compounds as determined by paper chromatography was 99% or greater.

Sodium chloride in aqueous solution, labeled with $^{22}Na^{+}$, was obtained from New England Nuclear, and had a minimum radiopurity of 99.9%.

III. Preparation of isotonic media.

The media used throughout this work were modified forms of the Krebs-Ringer phosphate medium described by Vidaver (79). Calcium salts and glucose were not included in the media, since Eavenson (108) showed that their elimination had no apparent effect on transport or cellular integrity. All experiments were performed in isotonic media. Increases in sodium chloride concentration were compensated by lowering the concentration of choline chloride. When concentrations of neutral amino acids greater than 5 mM, or concentrations of cationic amino acids greater than 2.5 mM, or other cations were used, the choline chloride concentration was reduced.

The composition of the media used in this work was as follows:

NaCl + choline chloride	118 mM
KCl	5
$MgSO_4$	1
KH_2PO_4	1
phosphate, as choline or sodium phosphate	20 mM

Intermediate concentrations of Na^{+} were obtained by mixing the desired volumes of 118 mM choline chloride (Na^{+}-free) medium with 118 mN sodium chloride media. Media containing compounds other than sodium chloride in replacement of part of the choline chloride were obtained by mixing the appropriate volumes of 118 mN Na^{+} media, the Na^{+}-free medium, and a medium without sodium or choline chloride, but containing the other salts and buffering agent as well as the desired amount of the replacement compound. This medium contained amino acids or other compounds in sufficient concentration to give the same osmolarity as the Na^{+}-free and 118 mN Na^{+} media.

For experiments in which low Na^+ concentrations were desired, 20 mM choline phophate was used as the buffering agent. Choline phosphate buffer was prepared by mixing an excess of an aqueous solution of choline bicarbonate with a measured volume of 85% phosphoric acid. The resulting solution was boiled to remove carbon dioxide, then diluted to yield 0.1 M phosphate. The pH was adjusted to 7.5 with 0.1 M phosphoric acid. The 0.1 M choline phosphate solution was stored frozen. Twenty ml of this solution were added per 100 ml final volume in the preparation of isotonic media.

For experiments in which higher Na^+ concentrations were desired than those obtainable using media buffered with choline phosphate, sodium phosphate was used as the buffering agent. Sodium phosphate buffer containing 0.1 M phosphate was prepared using a 6.5 to 1 molar ratio of disodium to monosodium phosphate. When diluted to yield 20 mM phosphate, this buffer had a pH of 7.5, and a Na^+ concentration of 37.3 mN. The presence of KH_2PO_4 in the salts added in preparation of the isotonic medium resulted in a final pH of 7.4, when either choline phosphate or sodium phosphate at pH 7.5 were used.

IV. Preparation of the individual incubation mixtures.

The desired volumes of the media described above were pipetted into 25 ml Ehrlenmeyer flasks to yield a total volume of 4.6 ml. The amino acids were added as serial dilutions in one of the solutions added. The concentrations of amino acids and of Na^+ used were such that they gave the desired concentration on dilution of the medium with the Na^+-free medium contained in the cell suspension.

In most experiments, a constant amount of isotopically labeled amino acid or $^{22}Na^+$ was added to each flask, such that the activity per ml was constant throughout the experiment. The protocol for each experiment was designed

so that the isotope could be added in diluted form, contained in one of the solutions added in equal volume to all, or to a large number of the individual flasks. The specific activities of the labeled materials used were high enough that their addition did not add significantly to the total concentration.

A 0.2-ml sample of the solution containing the labeled material was taken for measurement of radioactivity per ml, prior to the experiment. The activity per ml in each of the flasks was then calculated by multiplying the activity per ml of the 0.2-ml sample by the ratio of the volume of radioactive solution added per flask to the final volume obtained during incubation. For experiments using $^{22}Na^{+}$, a 1:10 dilution of the solution containing the isotope was made with 5% (w/v) trichloroacetic acid (TCA) in water. The original solution and this diluted solution were sampled as above, and the radioactivity per ml was determined. $^{22}Na^{+}$ showed a counting efficiency in 5% TCA about 1.23 times the efficiency in phosphate media, using the liquid scintillation system described below. A 1:10 dilution was sufficient to obtain the maximum increase in counting efficiency. This ratio of efficiency was determined for the solutions used in all experiments that utilized $^{22}Na^{+}$. All $^{22}Na^{+}$ radioactivity measurements were corrected to the efficiency expected in 5% TCA before final calculations were made. This correction was not found to be necessary for ^{14}C or ^{3}H labeled materials.

The individual flasks containing all the components described were stored frozen between the time of their preparation and the time of their use in the incubation procedure.

V. Collection and preparation of pigeon erythrocytes.

White, male pigeons were obtained from commercial sources. For each experiment, a pigeon was lightly anesthetized with ether to facilitate handling,

then decapitated, and the blood was allowed to flow into a beaker containing 25 ml of cold Na^+-free medium containing about 10^3 units of heparin. Four to eight grams of packed cells were usually obtained from a single pigeon. The diluted blood was passed through two layers of cheesecloth to remove any clots or down, then diluted to 225 ml with the cold buffer, and centrifuged in six, 40-ml plastic centrifuge tubes for 2.5 minutes at about 3000 RPM. This centrifugation and all centrifugation steps were performed in an International Refrigerated Centrifuge at 4^oC. The plasma and buffer were removed by aspiration. The cells were resuspended in the Na^+-free medium to about 75 ml total volume, and centrifuged for 2.5 minutes at about 3000 RPM in six, 15-ml plastic conical centrifuge tubes. The buffer and buffy coat were removed by aspiration. The cells were resuspended in the Na^+-free medium to yield about 0.2 to 0.3 grams of packed cells per ml of cell suspension.

The experiments using rabbit reticulocytes differed only in the procedure for collection of cells. Reticulocytosis was induced in rabbits by phenylhydrazine injection (120) on the first and third days, and blood was collected from the marginal vein of the ear on the eighth day. Transport into the Ehrlich cell was studied by other workers in the laboratory, using techniques similar to those described here. All experiments other than those referred to in Results Section IX were made on pigeon erythrocytes.

VI. Incubation procedure.

The solutions and flasks were brought to 37^o C in a controlled-temperature water bath equipped to shake the flasks. The incubation step was initiated by adding 0.5 ml of cell suspension. After the desired period, which was usually three minutes, the incubation was terminated by pouring the incubation mixture into twenty ml of ice-cold, Na^+-free buffer, contained in a

forty-ml centrifuge tube. The equipment designed by Eavenson (108) was used, so that the initiation and termination of incubation could be carried out simultaneously for six flasks. The centrifuge tubes containing the buffer and cell samples were then centrifuged for 30 seconds at a maximum of 3000 RPM, not counting the time taken to reach 2500 RPM or the decceleration time. During the decceleration period, incubation was initiated in the next set of six flasks.

The buffer was removed by aspiration, and the 40-ml tubes containing the packed cells were placed in an ice bath. Usually, four sets of six flasks were used in each experiment. On conclusion of all the incubations, 10 ml of cold buffer was added to each of the 40-ml centrifuge tubes, and the packed cells were resuspended using glass stirring rods. The resuspended cells from each tube were then transferred to chilled, previously weighed, 15-ml conical centrifuge tubes, and centrifuged for four minutes at about 3000 RPM.

The two-stage washing procedure described above was adapted from the technique of Wheeler (98). The first step diluted the radioactivity and the concentrations of amino acids and Na^+ in the medium, but was used primarily for the purpose of rapid cooling of the cells, so as to lower the amino acid and Na^+ movements to negligible levels. The purpose of the second washing was to lower the radioactivity in the supernatant solution, so as to minimize the contamination of the packed cells by radioactivity from the diluted incubation media. This two-step procedure was found to be necessary for experiments measuring $^{22}Na^+$ movements, and was used in all the experiments in the present work so as to employ a consistent method, and to aid in the measurement of low rates of uptake of labeled amino acids. Wheeler found that losses of radioactivity from the cells were negligible under similar conditions of washing. The radioactivity per ml of the second wash fluid was proportional

to the activity per ml in the incubation mixture, and did not vary with the Na^+ or amino acid concentration used, nor with the radioactivity per ml obtained within the cells.

The periods of incubation used were the shortest that allowed sufficient movement of isotope to yield reproducible measurements. Three-minute incubation periods were used for the majority of the experiments. Five-minute periods were used for most $^{22}Na^+$ measurements, and for relatively slow moving amino acids such as proline. Eavenson (108) reported that movements measured during 10 minute incubations gave an approximation of the initial rates. Koser (116) reported that Na^+ and amino acid movements remained proportional during incubation periods of up to 10 minutes.

After the cells were packed by centrifugation, a sample of the supernatant fluid was removed for measurement of the concentration of the radioisotope. The supernatant fluid was removed by aspiration, and pointed pieces of folded filter paper were used to remove the remaining fluid. The tubes were then reweighed, and the weight of the packed cell sample obtained by difference. One ml of 5% TCA was then added to each tube, and the contents mixed immediately with a Vortex Jr. mixer. The TCA-insoluble material was sedimented by centrifugation. Aliquots of the cell extract and of the supernatant fluid of 0.2 ml were measured into glass vials, 10 ml of a toluene-ethanol-scintillator mixture (127) were added, and the radioactivity in the samples was measured using a Packard Tricarb Liquid Scintillation Spectrometer.

Before the start of each experiment, 0.5 ml of the cell suspension was added to each of four flasks, two containing ^{14}C-inulin in 10 ml cold Na^+-free buffer, and two containing 10 ml of the buffer without labeled materials. The suspensions were poured into chilled, previously weighed, 15-ml centrifuge tubes and centrifuged for four minutes at about 3000 RPM. Portions of

the supernatant fluids containing ^{14}C-inulin were removed with Pasteur pipets, and the remaining fluid removed by aspiration. The tubes containing the radioactive samples were weighed and extracted as described for the incubated samples. The other two samples were weighed, then dried to constant weight in an oven at 105°C. The weight of water per gram of packed cells was determined from the ratio of (wet wt - dry wt)/ dry wt. For the pigeon erythrocyte this value usually fell in the range of 0.65 to 0.70 grams water per grams wet weight, with an average value of 0.67.

The calculation of extracellular space was based on the activity per ml of ^{14}C-inulin obtained in the extract of the cells exposed to inulin in the cold. Inulin is not expected to enter the cells, so that the activity obtained in the extract represents the activity in the extracellular space. From the activity per ml in the supernatant fluid the activity in the extract could be converted to ml of extracellular water contained in the packed cells.

$$\left[\frac{1.0\ \text{ml} + \left[\frac{\text{wet wt}}{\text{wet wt}}\right] \cdot \left[\frac{\text{g water}}{\text{g wet wt}}\right]}{\text{wet wt}}\right] \cdot \left[\frac{\frac{\text{activity}}{\text{ml extract}}}{\frac{\text{activity}}{\text{ml supernatant fluid}}}\right] = \frac{\text{g extracellular water}}{\text{g wet wt}}$$

Values for extracellular space varied from 0.12 to 0.22 grams extracellular water per gram of packed cells, with an average value of 0.17.

On several occasions the TCA insoluble material in each tube was dried to constant weight in an oven at 105° C. No significant difference was observed between the value of grams of water per gram of wet cells obtained in this way and the value obtained from the two samples which were dried

without extraction. For this reason, all calculations were based on the wet weight of packed cells, assuming the ratios of total water and extracellular water to be constant for all the samples in a given experiment.

The 0.5 ml of cell suspension added to each flask was assumed to contain 0.4 ml of buffer available to dilute the incubation medium and 0.1 ml unavailable volume composed of intracellular water and cellular solids. The addition of 0.5 ml of cell suspension to 4.6 ml incubation medium was therefore assumed to yield a final volume of 5.0 ml. If the cell suspension contained 0.24 grams of packed cells per ml of suspension, then a volume of exactly 5.0 would be obtained, assuming a value of 0.17 for grams extracellular water per gram of packed cells. In practice, the weight of cells used throughout an experiment often exceeded 0.12 grams per 0.5 ml suspension, but it can be shown that adding twice this amount of cells in a volume of 0.5 ml results in less than a 2% error in the calculated concentrations of components of the incubation mixture.

A slightly different procedure was used in experiments in which the loss of isotopically labeled materials from the cells during an incubation period was measured. The cells were washed by the usual procedure. The cells were resuspended in 20 ml of a medium containing an amino acid and radioisotope in known concentration and activity per ml. This suspension was then incubated for 30 minutes at 37°C. The suspension was then diluted to 70 ml with cold Na^+-free buffer and centrifuged at about 3000 RPM for 2.5 min. The buffer was removed by aspiration, and the cells were resuspended to a volume of 70 ml with cold buffer, centrifuged, and the buffer removed as before. The cells were resuspended in the Na^+-free medium to yield about 0.2 to 0.3 grams of packed cells per ml of cell suspension, then used as described above for the unincubated cell suspension. Two 0.5-ml aliquots of the suspension were

added to 10 ml cold Na^+-free buffer, transferred to 15-ml tubes, and centrifuged for four minutes at about 3000 RPM. These samples were weighed and extracted as were the other samples, to measure the radioactivity present in the cells prior to incubation.

VII. Calculations.

The calculation of amino acid and Na^+ concentration and of radioactivity per ml in the incubation medium was made as described above, based on the known concentration of these components in the 4.6 ml added to each flask and the final volume obtained during incubation, which was taken to be 5.0 ml.

The intracellular concentration of radioactivity was determined from the activity per ml in the cell extract, corrected for the weight of cells extracted and the contribution to the activity from the extracellular water.

$$\frac{\left[\frac{\text{activity}}{\text{ml extract}}\right]\left[1.0\text{ ml} + \left[\text{wet wt}\right]\left[\frac{\text{g water}}{\text{g wet wt}}\right]\right] - \left[\frac{\text{g extracellular water}}{\text{g wet wt}}\right]\left[\text{wet wt}\right]\left[\frac{\text{activity}}{\text{ml supernatant fluid}}\right]}{\left[\text{wet wt}\right]\left[\frac{\text{g intracellular water}}{\text{g wet wt}}\right]}$$

$$= \frac{\text{activity}}{\text{g intracellular water}}$$

Under the experimental conditions used in these studies, the amount of substances entering the cells was sufficiently small so that the extracellular concentrations were unchanged. Thus the ratio of the activity per ml inside the cells to the activity per ml in the incubation medium times the concentration of the substance in the medium gives the rate of movement per weight of intracellular water. This rate of movement is expressed in units of

mmoles/min/Kg intracellular water.

$$V = \frac{\frac{\text{activity}}{\text{ml intracellular water}}}{\frac{\text{activity}}{\text{ml incubation medium}}} \cdot \frac{\text{mmoles substrate}}{\text{ml incubation medium}} \cdot \frac{1000 \text{ ml}}{\text{Kg intracellular water}} \cdot \frac{1}{\text{time of incubation}}$$

In those experiments in which losses of labeled substances from previously incubated cells were measured, the percent loss for the time interval of incubation is reported, based on the radioactivity per ml inside the cells before and after incubation. Experience has indicated that this procedure is more reliable than measuring the rate of appearance of the label in the incubation medium (117).

RESULTS

Section I. The effect of Na^+ on the ASC system.

Section II. Half-saturation constants of neutral amino acid substrates and inhibitors.

Section III. Rates of uptake of neutral amino acid substrates.

Section IV. Rates of exchange produced by neutral amino acid substrates.

Section V. The ratio of half-saturation constants.

Section VI. Stimulation of Na^+ uptake.

Section VII. Correlation of neutral amino acid structure with ASC reactivity.

Section VIII. Inhibition by cationic amino acids.

Section IX. The ASC system in other cell types.

I. The effect of Na^+ on the ASC system.

The uptake of amino acids by way of the ASC system depends on the concentration of Na^+ in the external medium. Figure 1 shows the effect of Na^+ on the kinetic parameters of serine and alanine uptake. In the range of Na^+ concentrations between 25 and 118 mN, the value of V_{max} remains fairly constant, whereas the K_m decreases by a factor of about three. Plots of the reciprocal of serine uptake vs. the reciprocal of the Na^+ concentration yield a family of straight lines, one for each concentration of serine, as shown in figure 2. The point of intersection on the abcissa changes with the concentration of serine, showing that the concentration of Na^+ which produces half-maximal stimulation of serine uptake depends on the serine concentration. The lines intersect at a point that may give, under an assumption to be discussed later, an indication of the affinity of Na^+ for the carrier in the absence of amino acids, that is, the equilibrium constant for the step, $E + Na^+ \rightleftharpoons E - Na^+$. In figure 3 the area in which the lines intersect has been enlarged by plotting lines drawn between the values of the points of intersection on the ordinate and abscissa of individual 1/v vs. $1/Na^+$ plots for each concentration of serine. Some of the data on which this graph is based are shown in figure 2. The apparent equilibrium constant falls in the range of 140 to 170 mN Na^+, in three such graphical determinations with serine as substrate, and one such determination with alanine.

As mentioned in the Introduction and treated further in the Discussion, the rate of uptake of Na^+ is proportional to the rate of uptake of the amino acid substrate at all concentrations of Na^+ and the amino acid, so that a reciprocal plot of serine uptake and serine concentration will take the place of a reciprocal plot of Na^+ uptake and serine concentration for the

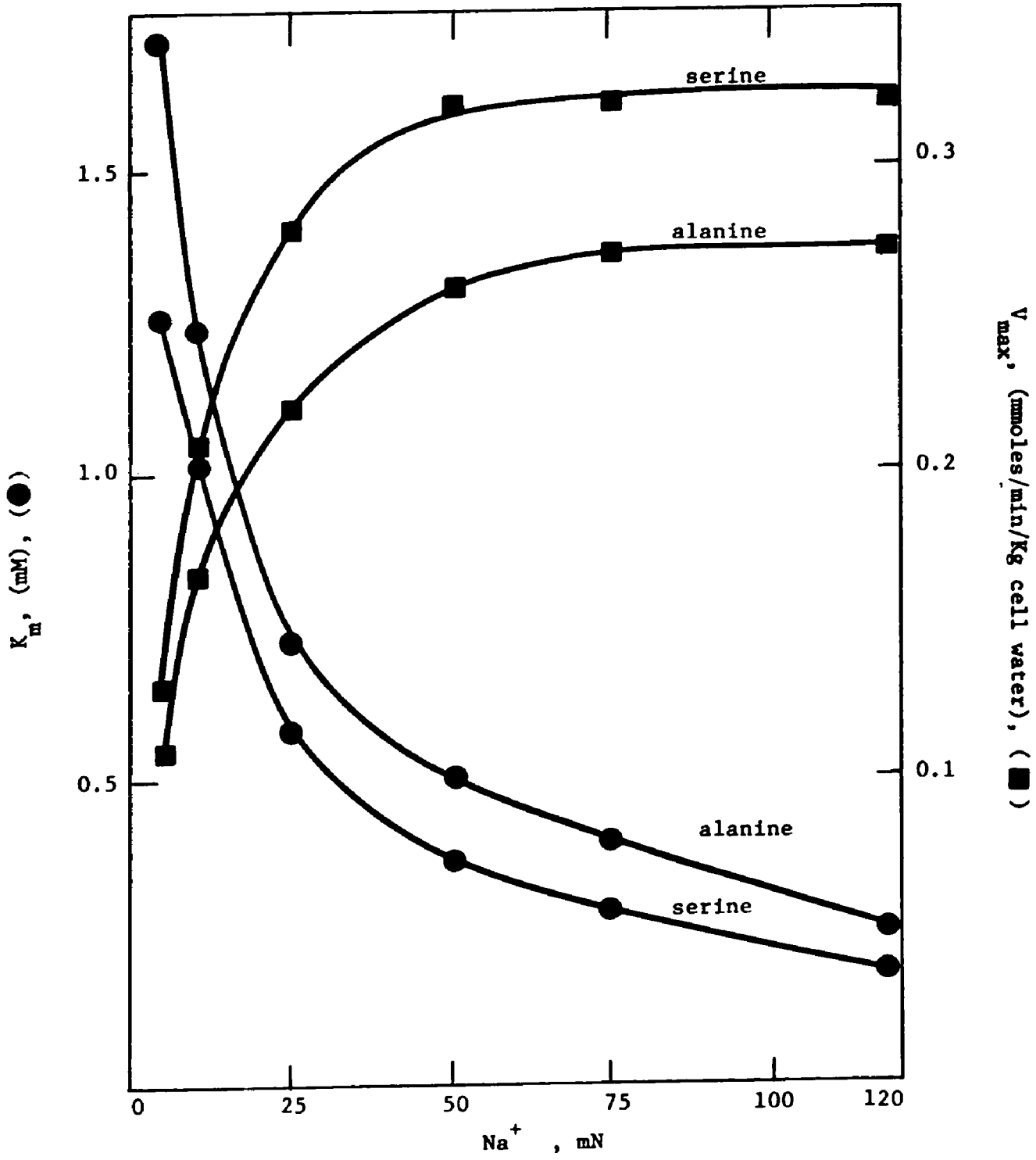


Figure 1. Effect of Na^+ concentration on the kinetic parameters of alanine and serine uptake.

Legend for Figure 1.

The K_m (● , left scale) and V_{max} (■ , right scale) values for the Na^+-dependent uptake of alanine and serine are plotted vs. Na^+ concentration. K_m and V_{max} values were obtained from separate Dixon plots for each Na^+ concentration. The rates were corrected for the non-saturable rates of entry, measured in the Na^+-free medium. The concentrations of amino acids were 0.05, 0.1, 0.2, and 0.8 mM. Na^+ concentrations were 5, 10, 25, 50, 75, and 118 mN.

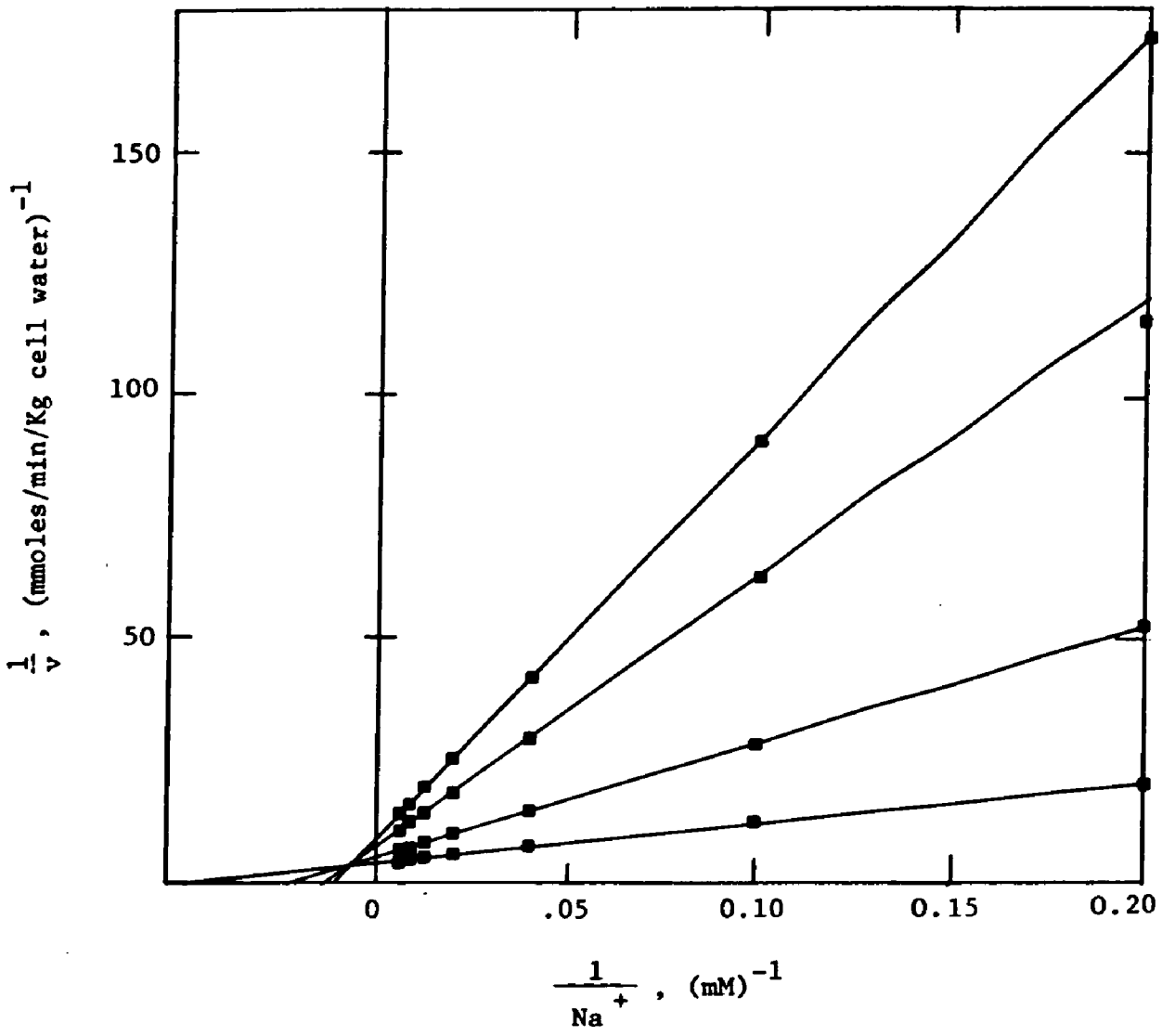


Figure 2. Linear relationship of the reciprocal of serine uptake to the reciprocal of Na^+ concentration. The reciprocal of the rate of serine uptake during a 3-minute incubation, corrected for the non-saturable component, is plotted vs. the reciprocal of the Na^+ concentration. The results shown here were obtained in a different experiment than those shown in figure 1. Only four of the five lines obtained from this experiment are shown here. Serine concentrations of 0.05, 0.075, 0.20, 0.75, and 2.5 mM were used at each of the seven concentrations of Na^+, which were 5, 10, 25, 50, 75, 118, and 143 mN.

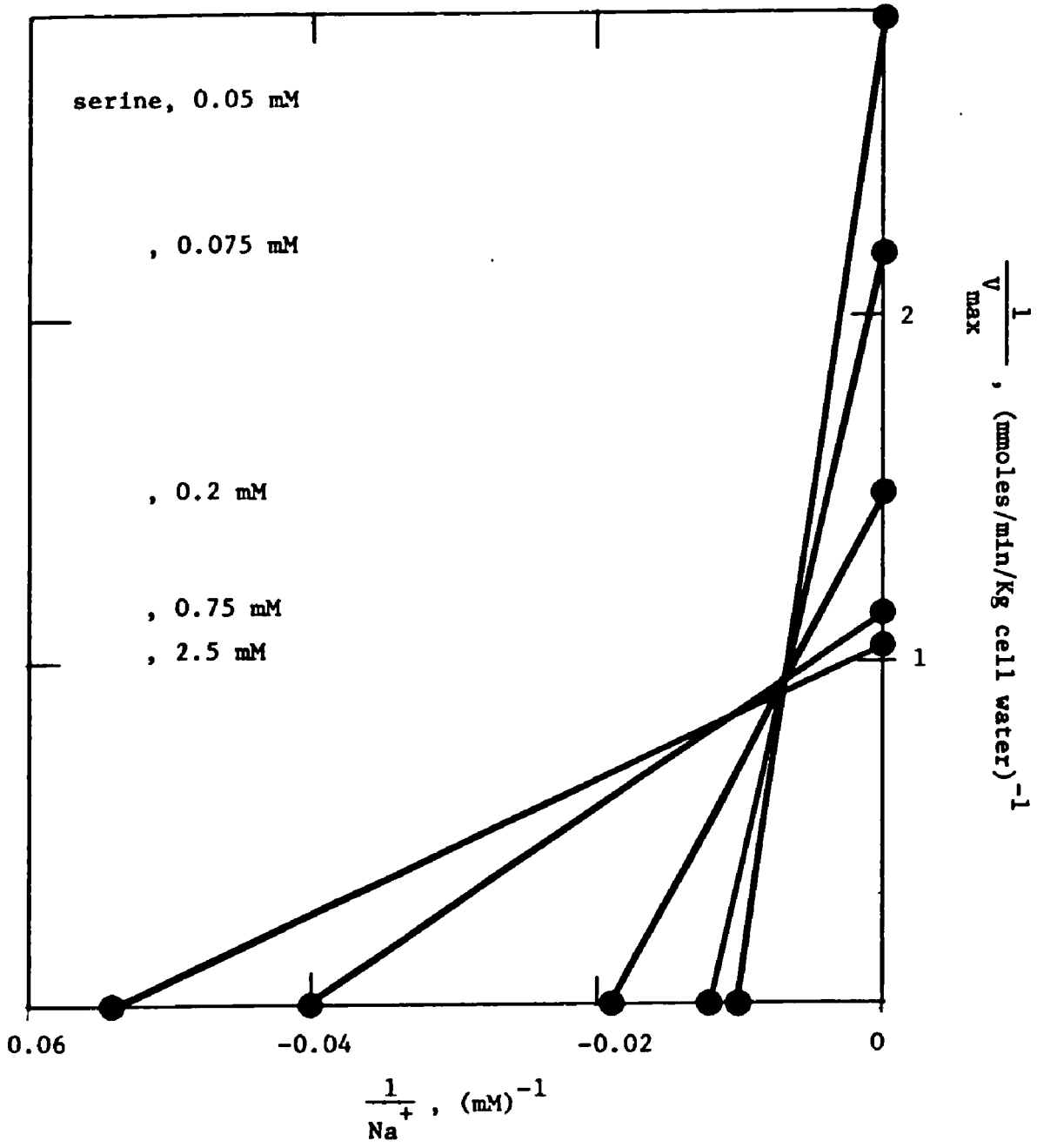


Figure 3. The area of intersection of reciprocal plots of the rate of serine uptake and the Na^+ concentration. The values of the points of intersection on the ordinate and abcissa of plots of 1/v (serine) vs. $1/Na^+$ concentration have been replotted and the points connected. This figure represents an enlargement of the area of intersection of the lines in figure 2. The point of intersection is taken to have a $1/Na^+$ value of -0.07, representing a Na^+ concentration of about 140 mN.

purpose of determining the point of intersection. Such a plot is shown in figure 4, using a portion of the data shown in figure 2. Enlargement of the area of intersection has been obtained in figure 5, by the same technique as in figure 3. The value obtained, which may be the equilibrium constant for the step $E + S \rightleftharpoons ES$, falls in the range of 1 to 2 mM for both serine and alanine.

The K_m for Na^+ in the presence of infinite serine concentration, and the K_m for serine in the presence of infinite Na^+ concentration are not easily obtained from the data in figures 2 through 5, but the slowed rate of change of the parameters at high concentration gives evidence that these values approximate the maximal values. The K_m for serine would be expected to fall in the range of 0.05 to 0.1 mM, and the concentration of Na^+ producing half-maximal uptake of infinite serine would be about 5 to 10 mN. These constants may be the equilibrium constants for the formation of a ternary complex.

$$E - Na^+ + \text{serine} \rightleftharpoons E - Na^+ - \text{serine}$$

$$E - \text{serine} + Na^+ \rightleftharpoons E - Na^+ - \text{serine}$$

As shown in later sections, not all amino acids show this change in half-saturation constants, and in Section VII, an attempt will be made to correlate amino acid structure with the differences observed.

The half-saturation constants have been interpreted as indicating the affinity of the substrates for the carrier. This is the meaning they would possess if the kinetics of the ASC system could be treated as above by the equilibrium analysis devised by Michaelis and Menten (128), and extended to two-substrate systems by Haldane (129). A more complete analysis is included in the Discussion, where it is shown that the kinetics of the ASC system do not permit the equilibrium assumption. For those amino acids which have K_i values independent of Na^+ concentration, we may reasonably

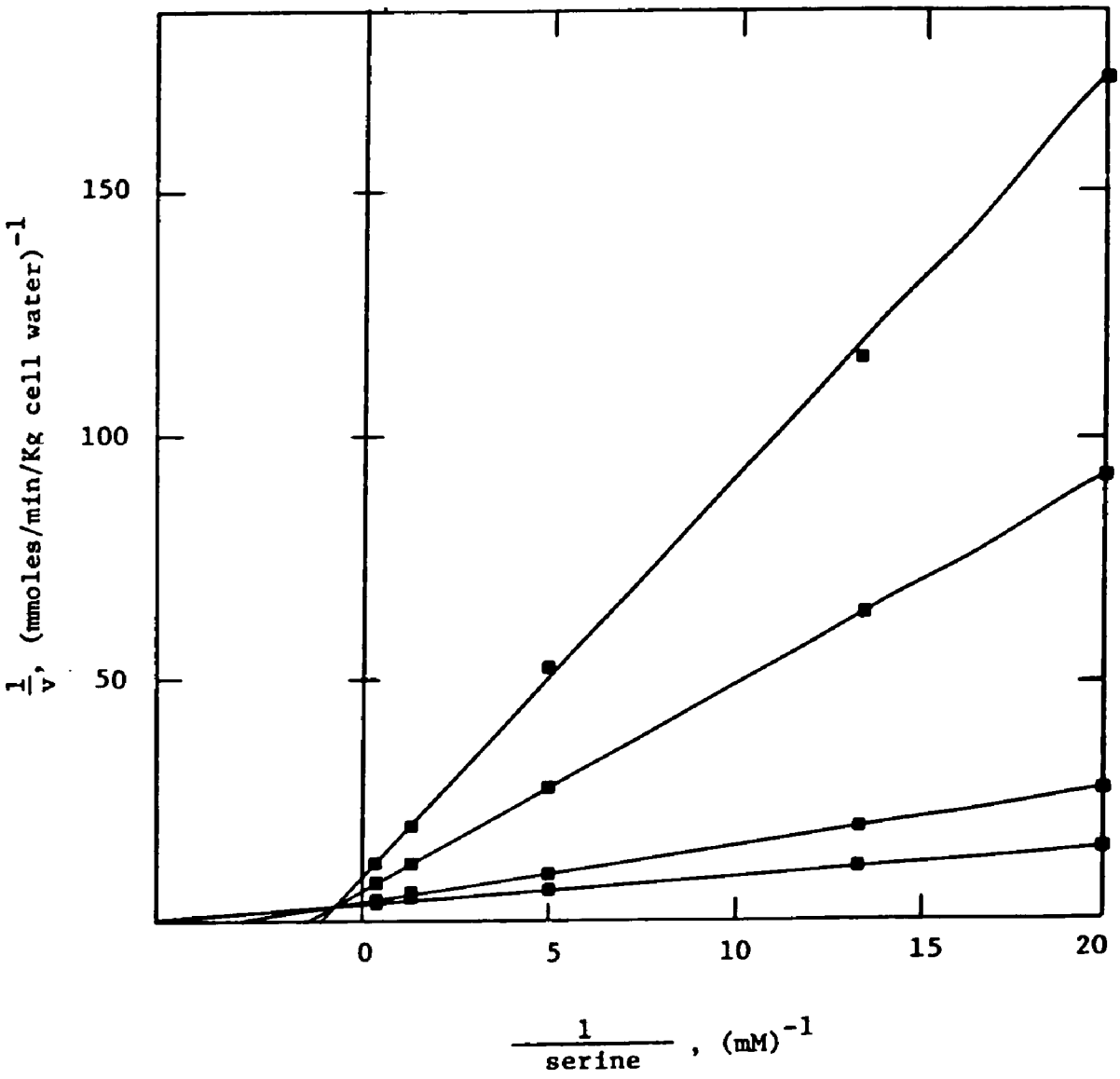


Figure 4. Linear relationship of the reciprocal of serine uptake to the reciprocal of serine concentration. The reciprocal of the rate of serine uptake during a 3-minute incubation, corrected for the non-saturable component, is plotted vs. the reciprocal of serine concentration. Five concentration of serine, ranging from 0.05 to 2.5 mM were employed at each of seven Na^+ concentrations ranging from 5 to 143 mN. Only four of the seven lines obtained from this experiment are shown here. The data plotted here are the same as those partially shown in figures 2 and 3.

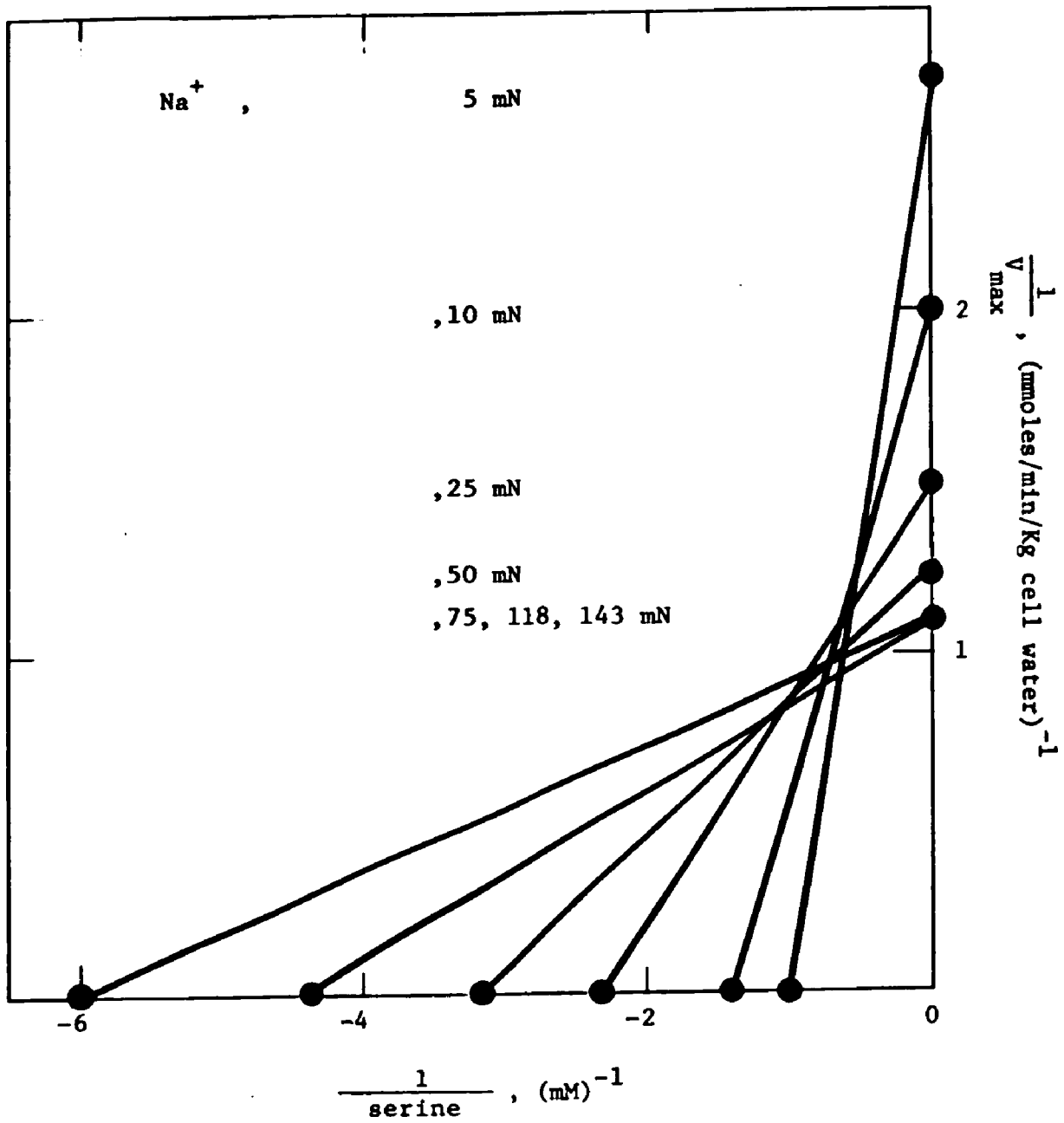


Figure 5. The area of intersection of reciprocal plots of the rate of serine uptake and serine concentration. The values of the points of intersection on the ordinate and abcissa of plots of 1/v (serine) vs. 1/ serine concentration have been replotted and the points connected. This figure represents an enlargement of the area of intersection of the lines in figure 4. The area of intersection is taken to have a 1/serine value of -0.5 to -1, representing a serine concentration of 1 to 2 mM.

assume that the K_i equals an association constant for the carrier, because no transport takes place for these amino acids and therefore there is no depletion of the E - I complex. The change in K_i or K_m observed for serine and other substrates may arise from the transport process, rather than by an effect of Na^+ on the equilibrium constants for substrate binding.

Transport of Na^+ or of amino acids by way of the ASC system is not believed to occur unless both substrates are present. The movement of amino acids that does occur in the absence of Na^+ can be shown to occur by way of other transport systems and by non-saturable processes (130,131). We can be confident that the non-saturable component does not represent a low-affinity uptake by way of an ASC E-S complex, because the non-saturable component is not inhibited by methionine or arginine, which do not require Na^+ to inhibit the ASC system. To obtain the K_i values for methionine, arginine, and other amino acid inhibitors, the uptake of substrate in the Na^+-free medium has been subtracted from all the rates of uptake. Similarly, Na^+ uptake in the absence of amino acids in the external medium does not occur by way of an ASC E - Na^+ complex, because this uptake is not inhibited by Li^+ or homoarginine, which do inhibit the amino acid-dependent Na^+ uptake. Table 1 shows the rate of Na^+ entry measured in the presence and absence of inhibitors of the ASC system.

The pigeon erythrocyte ASC system appears to be highly specific for Na^+ as the co-substrate. Table 2 shows the rates of serine uptake in the presence of a number of salts in replacement of part of the choline chloride. The rates of serine uptake in the presence of cations other than Na^+ are approximately linear with respect to the concentration of serine, and are therefore equal to the non-saturable component. Li^+ was found not to stimulate the uptake of serine, alanine, α-amino-n-butyric acid, or asparagine. A possible exception was obtained with the highly atypical substrate, proline. Table 3

Table 1. Uptake of Na^+ from media containing inhibitors of the ASC system, in the absence of substrates of the ASC system.

Na^+ concentration: 50 mN		Na^+ concentration: 10 mN	
Homoarginine concentration, mM	Na^+ influx, meq/3 min/Kg cell water	Li^+ concentration mN	Na^+ influx meq/5 min/Kg cell water
0	1.2	0	0.36
5	1.4	25	0.37
10	0.9	50	0.34
25	1.2	75	0.38
50	1.1		

Table 2. Uptake of serine from media containing chloride salts in replacement of choline chloride.

	Serine influx, mmoles/3 min/Kg cell water		
Concentration of compound replacing choline chloride	0.05 mM serine	0.2 mM serine	0.8 mM serine
85 mM NaCl	0.153	0.426	0.983
85 mM LiCl	0.006	0.025	0.107
85 mM KCl	0.005	0.023	0.088
85 mM RbCl	0.005	0.021	0.079
85 mM NH_4Cl	0.005	0.020	0.094
170 mM sucrose	0.006	--	--
(choline chloride) medium	0.006	0.025	0.102

The rates of serine uptake shown are the rates obtained during 3-min incubation. The linear component of serine uptake has not been subtracted.

shows the difference in the effect of Li^+ on proline and asparagine uptake. In this case, 85 mN Li^+ gives about half the stimulation of uptake of 5 mM proline as given by 10 mN Na^+. The inhibitory effect on asparagine uptake shows that Li^+ is not inert with respect to the ASC system, although it does not appear to fulfil a co-substrate role with any amino acid other than proline. Figures 6 and 7 show the inhibition of serine uptake produced by Li^+ in media containing 10 and 25 mN Na^+. The apparent K_i of Li^+ appears to increase as the Na^+ concentration increases, indicating that Li^+ competes for the Na^+ binding site. Li^+ inhibition of serine-stimulated Na^+ uptake is shown in figure 8. Na^+ uptake remains proportional to amino acid uptake in the presence of inhibitors of the ASC system.

II. Half-saturation constants of neutral amino acid substrates and inhibitors.

K_m values were obtained from Dixon plots for those amino acids which were available in isotopically labeled form, and which had measurable uptake by way of the pigeon erythrocyte ASC system. The reciprocal of the rate of uptake at a given Na^+ concentration minus the rate in the Na^+-free medium was plotted vs. the reciprocal of the amino acid concentration. The apparent affinity of other amino acids were determined by obtaining K_i values from plots of 1/v vs. inhibiting amino acid concentration, with serine or alanine as substrate. In all cases in which both parameters could be measured, the K_m was found to equal the K_i, and the K_i was independent of the substrate against which inhibition was tested. The K_i values for a number of neutral amino acids (K_m values in the case of alanine and serine) are listed in table 5. The two values reported for each amino acid were obtained within the same experiment, using the same preparation of cells. K_i values obtained using two

Table 3. Effect of Li^+ on proline and asparagine uptake.

		Amino acid influx, mmoles/10 min/Kg cell water	
Na^+ concentration mN	Li^+ concentration mN	5 mM proline	0.8 mM asparagine
0	85	0.09	--
10	0	0.18	0.19
10	50	0.31	--
10	75	--	0.12

The uptake of 5 mM proline and 0.8 mM asparagine during 10 min. incubations was measured in the presence of varying concentrations of Li^+ and Na^+. These rates have been corrected for the rates of uptake in the absence of Na^+ and Li^+.

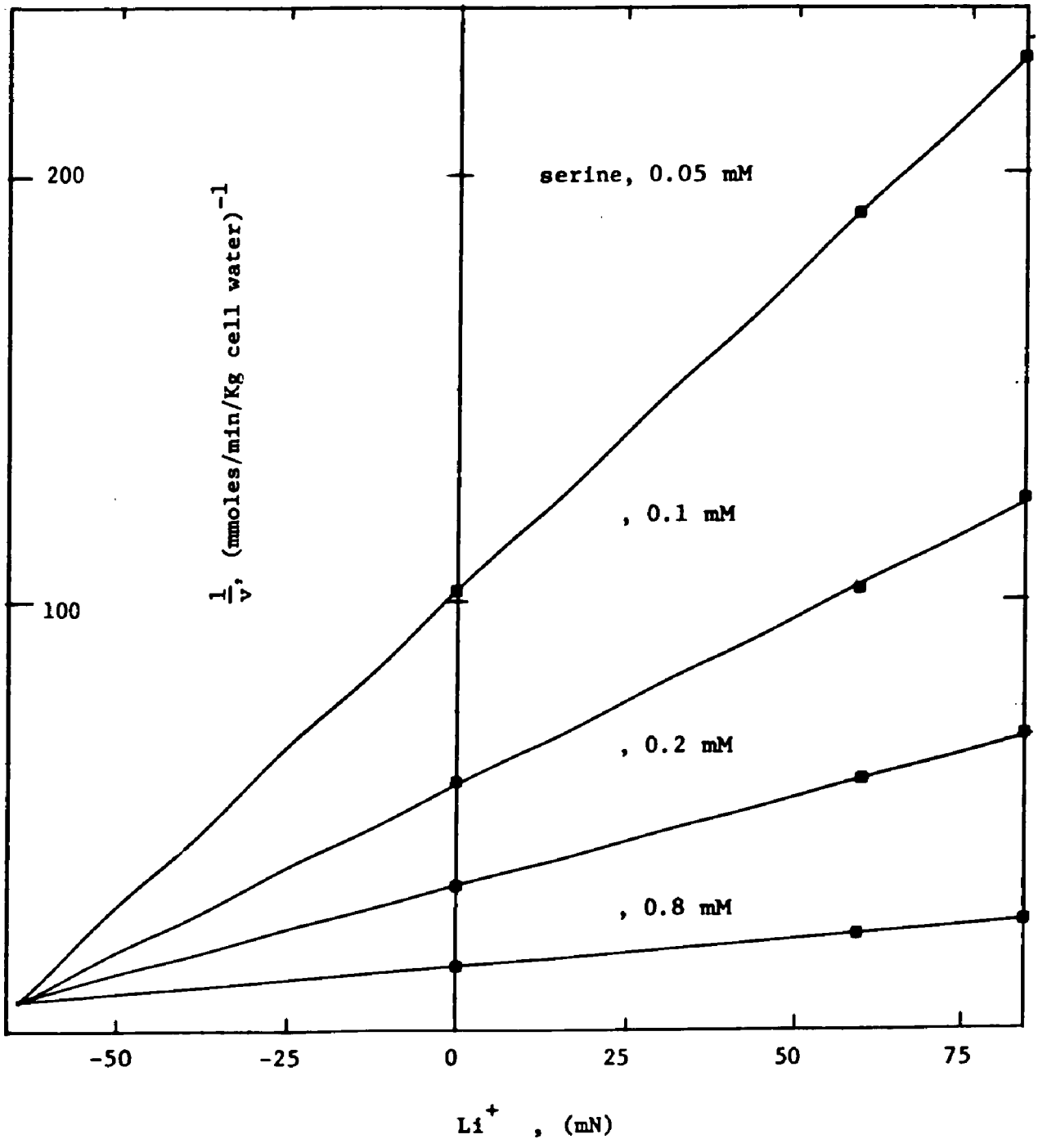


Figure 6. Inhibition of serine uptake by Li^+ in a medium containing 10 mN Na^+.

Legend for Figure 6.

The reciprocal of the rate of serine uptake during a 3-minute incubation in the presence of 10 mN Na^+, corrected for the non-saturable component measured in the Na^+-free medium, is plotted *vs.* the concentration of the inhibiting cation, Li^+. The concentrations of the substrate were 0.05, 0.1, 0.2, and 0.8 mM serine, and the concentrations of the inhibitor were 0, 60, and 85 mN Li^+. The K_i of Li^+ was taken to be about 65 mN, at this concentration of Na^+.

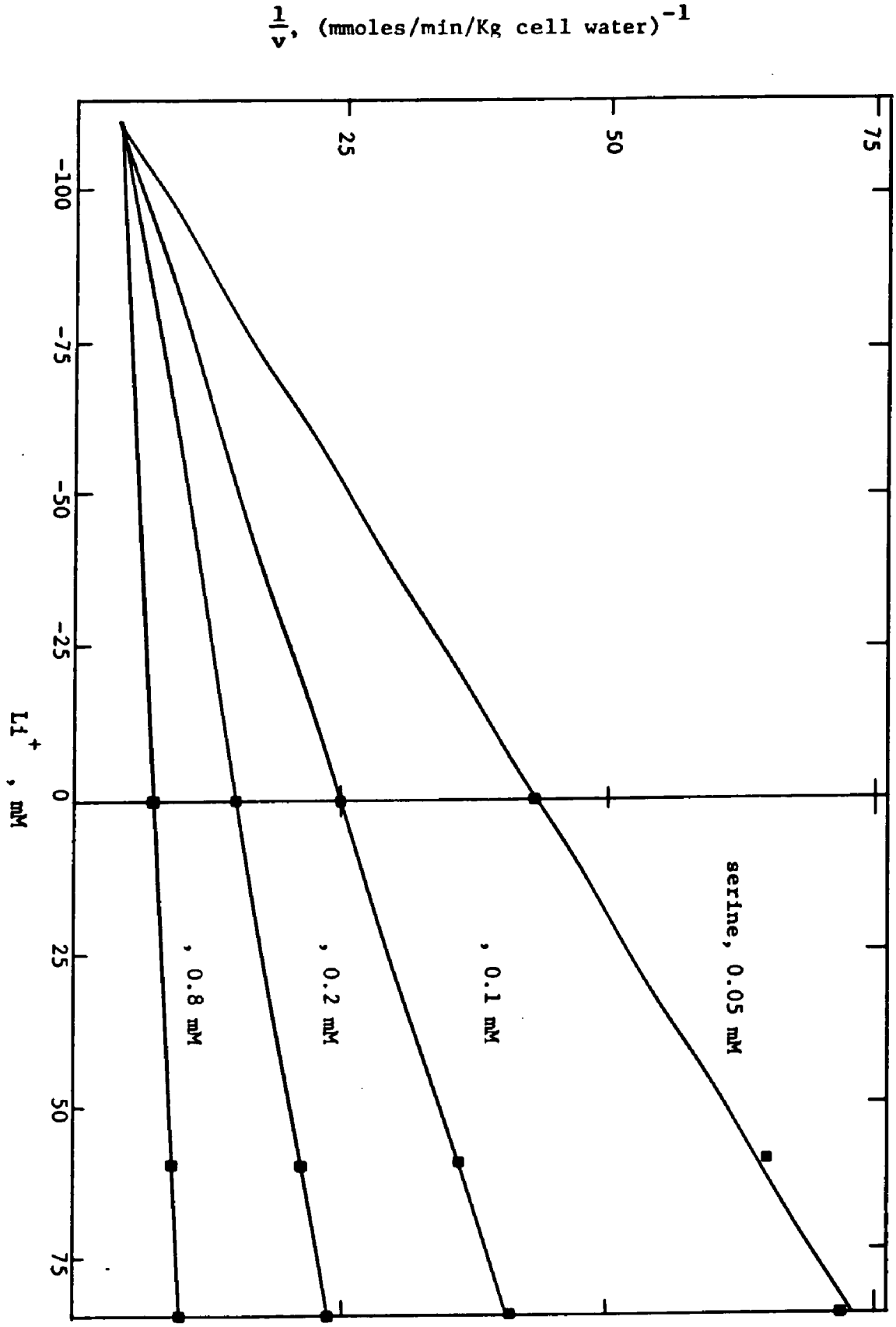


Figure 7. Inhibition of serine uptake by Li^+ in a medium containing 25 mN Na^+.

Legend for Figure 7.

The reciprocal of the rate of serine uptake during a 3-minute incubation in the presence of 25 mN Na^+, corrected for the non-saturable component measured in the Na^+-free medium, is plotted *vs.* the concentration of the inhibiting cation, Li^+. The concentrations of the substrate were 0.05, 0.1, 0.2, and 0.8 mM serine, and the concentrations of the inhibitor were 0, 60, and 85 mN Li^+. The K_i of Li^+ was taken to be about 110 mN, at this concentration of Na^+. The data shown in figures 6 and 7 were obtained within the same experiment.

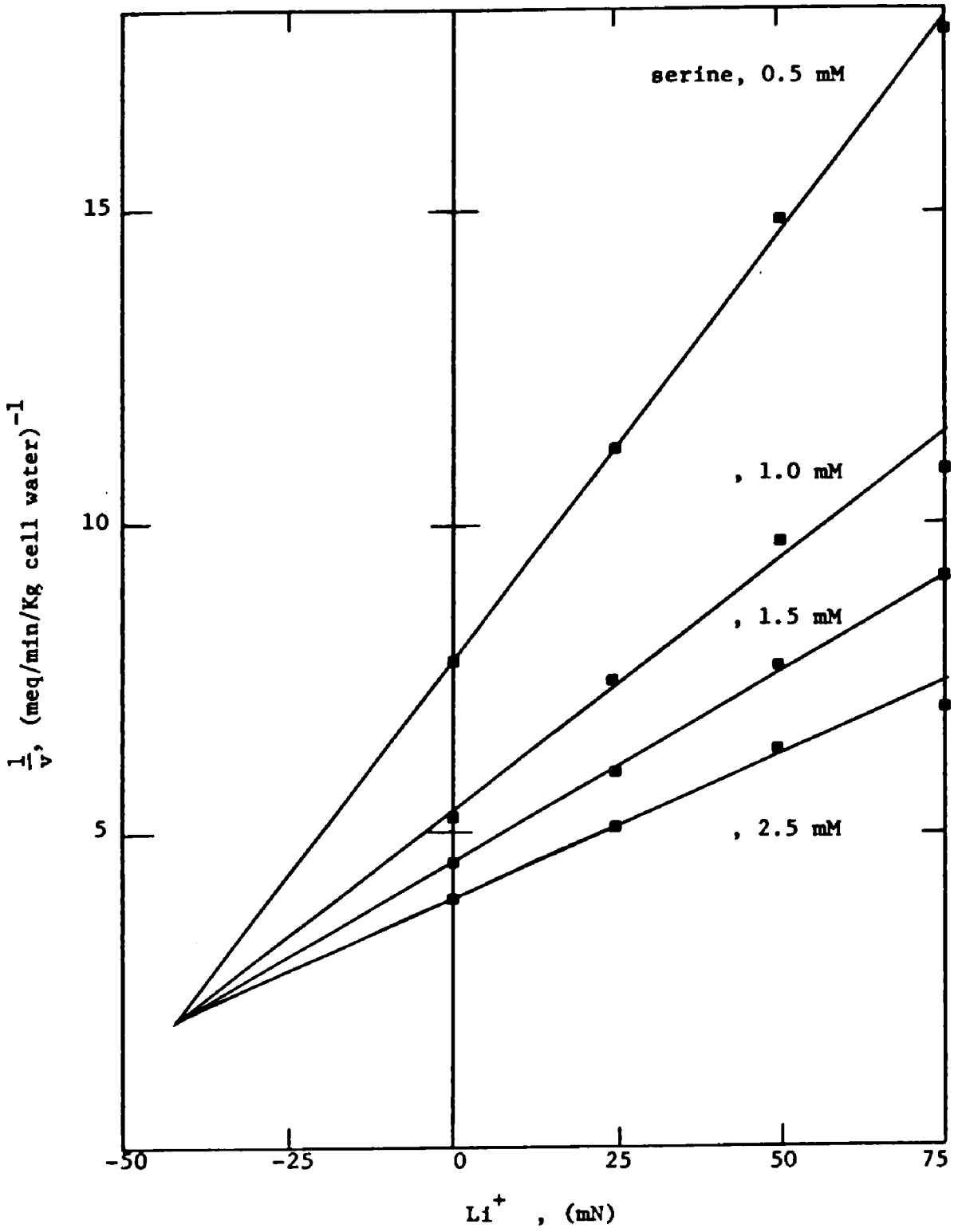


Figure 8. Inhibition of serine-stimulated Na^+ uptake by Li^+ in the presence of 10 mN Na^+.

Legend for Figure 8.

The reciprocal of the rate of Na^+ uptake from a medium containing 10 mN Na^+ during a 5-minute incubation in the presence of serine concentrations of 0.5, 1.0, 1.5, and 2.5 mM, corrected for the rate of Na^+ entry in the absence of serine, is plotted vs. the concentration of the inhibiting cation, Li^+. The concentrations of Li^+ were 0, 25, 50, and 75 mN. The K_i of Li^+ was taken to be about 40 to 45 mN, at this concentration of Na^+.

different concentrations of Na^+ were found to be both increased or both decreased when cells obtained from different animals were used, but the values were highly reproducible when tested within the same experiment. In cases in which K_i values were found to be similar for two or more amino acids, their K_i values were determined within the same experiment so as to eliminate the variation between experiments. The K_i values obtained in 25 and 118 mN Na^+ media were taken as an indication of the affinity for the carrier, and of the extent of the effect of Na^+ on the constants, because the effect of Na^+ in this range of concentrations was principally to change the K_m, with little effect upon the V_{max}.

δ-Hydroxynorvaline and ε-hydroxynorleucine were used as the unresolved DL mixtures. K_i values obtained with these preparations were divided by two to approximate the K_i of the L form. The K_i values of a number of pure D isomers were measured, and were found to be invariant with Na^+ concentration and at least six times greater than the K_i of the L forms.

III. Rates of uptake of neutral amino acid substrates.

Values for the maximal rates of uptake in media containing 118 mN Na^+ were obtained from Dixon plots for those amino acids available in isotopically labeled form, as described above. V_{max} values are listed in table 5, as $V_{max,\ 118mN\ Na^+}$. A variation of at least thirty-fold in the maximal rates can be noted for the amino acids tested.

IV. Rates of exchange produced by neutral amino acid substrates.

The amino acids listed in table 5 are arranged in order of their ability to stimulate exchange by way of the ASC system in the presence of 118 mN Na^+.

Measurement of the ability to stimulate the exodus of previously accumulated ^{14}C-alanine from the pigeon erythrocyte did not depend on the availability of isotopically labeled substrates. Also, the rates of exchange could be determined within the same experiment, overcoming the problem of differences among lots of cells. Since alanine had no detectable entry or exodus other than by the ASC and non-saturable routes, the percentage loss of ^{14}C-alanine during an incubation in the presence of 20 mM extracellular amino acid, minus the percentage loss in the absence of extracellular amino acids, provides a measure of the ability of an amino acid to move by way of the ASC system. This value is listed in table 5 as Δ % exodus. The amino acid concentration of 20 mM was well in excess of the K_i or K_m for most of the amino acids tested, so that only the maximal rates of stimulated exodus are reflected in the Δ % exodus results. The results can be seen to parallel the maximal rates of uptake. In table 4 the percentages of loss of alanine stimulated by selected amino acids in the presence of 118 and of 143 mN Na^+ are shown. One may see that near-maximal rates of exchange are obtained in the presence of 118 mN Na^+ for all the substrates of the ASC system.

V. The ratio of half-saturation constants.

A comparison of the K_i (K_m) values of the amino acids listed in table 5 with their maximal rates of uptake and the maximal rates of alanine exodus stimulated by them, shows that the K_i values do not parallel the relative abilities of the amino acids to move by way of the ASC system. On the other hand, the ratio of the K_i measured in the presence of 25 mN Na^+ to that in 118 mN Na^+ is proportional to the maximal rates observed, so that those amino acids which are the best substrates also have K_i values which vary the most with Na^+ concentration. Amino acids such as methionine,

Table 4. Effect of Na^+ concentration on the rate of exchange.

	Δ % exodus of alanine; % loss in presence of amino acid— % loss in absence of amino acids, 5 minute incubation.	
Amino acid stimulating exodus (20 mM)	118 mN Na^+	143 mN Na^+
serine	35.2	30.4
alanine	29.3	28.3
norvaline	13.8	13.2
proline	5.0	5.0
methionine	1.0	1.0

Cells were preincubated for 30 min. in the presence of 0.5 mM alanine labeled with ^{14}C-alanine, and 143 mN Na^+. An internal concentration of 1.0 mM alanine was obtained. The cells were washed, then incubated for 5 min. in the presence of 20 mM amino acid, and either 118 or 143 mN Na^+. The percent loss of preloaded alanine has been corrected for a 2.8% loss in the absence of extracellular amino acids. This amino acid-independent loss was the same in 118 and 143 mN Na^+ media.

with very little ASC uptake, have ratios within experimental error of one, despite the fact that the K_m of the substrate against which inhibition was tested was simultaneously changing by a factor of about three over this range of Na^+ concentrations. The ratios of the K_i values are listed in table 5 as $K_{i\ 25}/K_{i\ 118}$.

VI. Stimulation of Na^+ uptake.

Since Na^+ is an obligatory co-substrate of the ASC system, the rate of Na^+ uptake stimulated by an amino acid should provide an additional measure of the ability of an amino acid to serve as a substrate. The rate of Na^+ uptake from a 50 mN Na^+ medium, labeled with $^{22}Na^+$, in the presence of 5 mM amino acid, minus the rate in the absence of extracellular amino acids, is listed in table 5 as ΔV_{Na^+}. This concentration of amino acid is well in excess of the K_i value for most of the amino acids listed, and the V_{max} in the presence of 50 mN Na^+ is sufficiently close to that at 118 mN Na^+, so that

$$\Delta V_{Na^+} \approx r \cdot V_{max,\ 118\ mN\ Na^+},$$

where r is the characteristic ratio of the rate of Na^+ entry to the rate of entry for a particular amino acid. If the rates of Na^+ entry are divided by the maximal rates of amino acid entry, r values are obtained similar to those obtained by others in this laboratory with the reservation that precise values of r are obtained only when both movements are measured within the same experiment.

In figure 9, the inhibition of serine-stimulated Na^+ uptake by methionine is shown. Although methionine appears to have a detectable rate of uptake by way of the ASC system, and must therefore have an associated rate of Na^+ uptake, this effect is sufficiently small so that only the inhibition of Na^+ uptake is observed in figure 9.

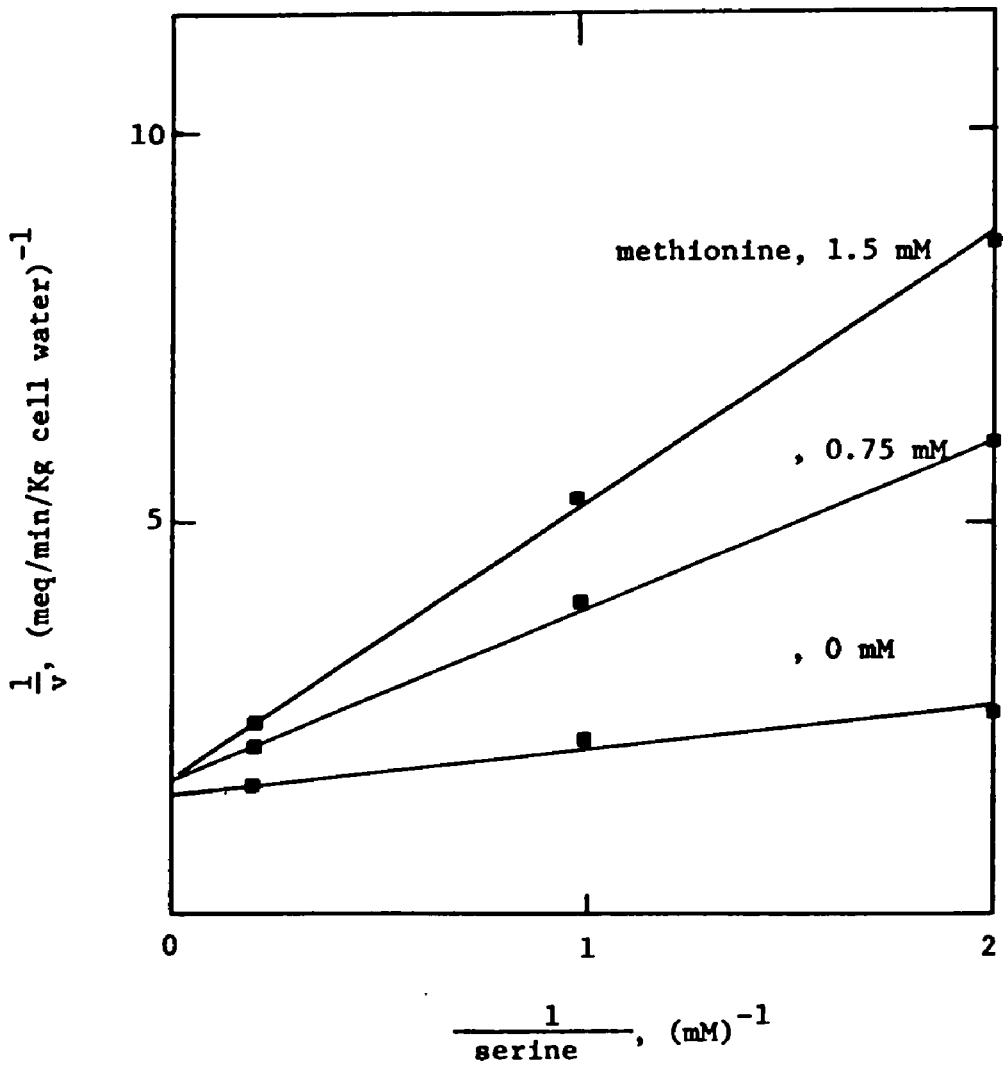
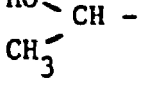
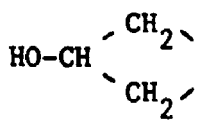
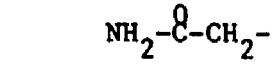
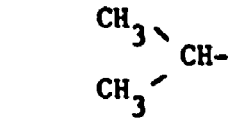
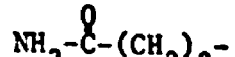
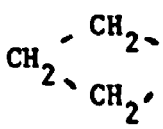
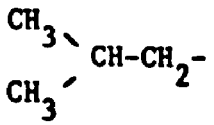
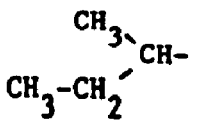


Figure 9. Inhibition of serine-stimulated Na^+ uptake by methionine. The reciprocal of the rate of Na^+ uptake from a medium containing 50 mN Na^+ during a 5-minute incubation in the presence of serine concentrations of 0.5, 1.0, and 5.0 mN, and methionine concentrations of 0, 0.75, and 1.5 mM, corrected for the rate of Na^+ entry in the absence of amino acids, is plotted vs. the reciprocal of serine concentration.

Table 5. The reactivity of neutral amino acids with the pigeon erythrocyte ASC system.

	Structure of side chain	A Δ % exodus	B V_{max}, 118 mN Na^+	C ΔV_{Na^+}	D K_i 25/ K_i 118	E K_i', 25 mN Na^+	F K_i', 118 mN Na^+
serine	$HO-CH_2-$	35.2	.32	.75	2.7	0.43	0.16
alanine	CH_3-	29.3	.27	.63	2.8	0.83	0.30
threonine		24.1	.14	.72	2.6	0.22	0.085
α -amino-n-butyric acid	CH_3-CH_2-	24.1	.24	.51	2.7	0.30	0.11
homoserine	$HO-(CH_2)_2-$	18.3	.10	.71	2.3	0.085	0.037
cysteine	$HS-CH_2-$	--	.10	.20	2.0	0.094	0.047
hydroxy-proline		14.6	.09	.24	1.7	0.27	0.16
norvaline	$CH_3-(CH_2)_2-$	13.8	--	.12	1.8	0.28	0.16
asparagine		12.5	.14	.07	1.9	0.54	0.28
hydroxy-norvaline	$HO-(CH_2)_3-$	11.3	--	.14	1	1	1

S-methyl-cysteine	CH_3-S-CH_2-	6.1	--	.04	1.6	0.45	0.29
valine		5.9	.06	.05	1.1	1.2	1.1
glutamine		5.7	.07	.02	1	3.4	3.4
proline		5.0	.08	.08	1.1	2.0	1.8
glycine		4.7	--	--	1	4.5	4.5
hydroxy-norleucine	$HO-(CH_2)_4-$	2.1	--	.03	1	2.5	2.5
citrulline		1.2	--	--	1	9-10	9-10
leucine		1.2	.01	-.01	1.0	0.49	0.48
isoleucine		1.0	--	-.02	1.0	0.63	0.65
methionine	$CH_3-S-(CH_2)_2-$	1.0	.01	0	1.0	0.50	0.50
ethionine	$CH_3-CH_2-S-(CH_2)_2-$	0.2	--	--	1	1	1
norleucine	$CH_3-(CH_2)_3-$	-0.4	.06	.01	1.0	0.44	0.44

Legend for Table 5

Column A. Percent loss of preloaded ^{14}C-alanine in the presence of neutral amino acids and 118 mN Na^+. Cells were preloaded with ^{14}C-alanine by means of a 30 minute incubation in a medium containing 0.5 mM alanine labeled with ^{14}C-alanine, and 143 mN Na^+. An internal concentration of 1.0 mM alanine was achieved. The percent loss of ^{14}C-alanine from these cells was measured during a 5 minute incubation in the presence of 20 mM amino acid and 118 mN Na^+. The loss in the absence of extracellular amino acids was 2.8%, and this value was subtracted from all the values shown. DL-hydroxynorvaline and hydroxynorleucine were present in 20 mM total concentration, and thus 10 mM in the L form.

Column B. Maximal rate of uptake of amino acids in the presence of 118 mN Na^+. V_{max} values were obtained from Dixon plots of the reciprocal of the rate of uptake in 118 mN Na^+ minus the rate in the absence of Na^+ vs. the reciprocal of the concentration of the amino acid. The concentrations of the amino acids spanned the region of the K_i as determined from inhibition studies. Incubation times were 3 minutes in the case of alanine and serine, and 5 minutes for the other amino acids. The rates have been divided by the time of incubation to give one-minute rates.

Column C. Stimulation of Na^+ uptake. The rate of Na^+ uptake was measured using either a 3- or 5-min. incubation period, from media containing 5 mM amino acid and 50 mN Na^+, labeled with $^{22}Na^+$. The concentrations of DL-hydroxynorvaline and hydroxynorleucine were 10 mM, and thus 5 mM in the L form. The rates have been divided by the time of incubation.

Column D. The ratio of the K_i determined in the presence of 25 mN Na^+ to that determined in the presence of 118 mN Na^+. The K_i values from which this ratio was obtained are listed in columns E and F.

Columns E and F. The K_i determined in the presence of 25 mN Na^+ (E) and the K_i determined in the presence of 25 mN Na^+ (F). The K_i values were obtained from plots of the reciprocal of the rate of serine or alanine uptake vs. the concentration of the amino acid inhibitor. The concentration of the substrate were chosen to span the K_m values of serine in the presence of 25 and 118 mN Na^+. The concentrations of amino acid inhibitor were chosen to span the K_i values, based on the results of preliminary experiments. Three-minute incubation periods were employed. The values shown for serine and alanine are K_m values, obtained from Dixon plots as described for column B. The two values shown for each amino acid were determined within the same experiment using the same cell preparation.

VII. Correlation of neutral amino acid structure with ASC reactivity.

The collected results in table 5 show that the $V_{max,\ 118mN\ Na^+}$, $\Delta\%$-exodus, $K_{i\ 25}/K_{i\ 118}$, and ΔV_{Na^+} values provide a consistent means of evaluating the ability of an amino acid to move by way of the ASC system. In contrast, the affinity of an amino acid for the receptor site as indicated by the K_i values, is not consistent with the maximal rate observations. The structural requirements that an amino acid must meet in order to have significant activity as a substrate are somewhat different and more restrictive than the requirements for binding to the ASC site. By observing the effect of differences in structure of the amino acids, some conclusions can be drawn about the factors that contribute to binding affinity and the factors that contribute to substrate activity. Some of the results are organized in graphic form below to assist in such comparisons. Results not presented in graphic form are contained in table 5, or subsequent tables.

The effect of increasing chain length upon the ability of amino acids to move by way of the ASC system is presented in figures 10 and 11. In these figures, the Δ % exodus (■, left scale), the ΔV_{Na^+} (●, left scale/20), and $K_{i\ 25}/K_{i\ 118}$ (▲, right scale) are plotted vs. the number of carbon atoms in the chain for the homologous series of amino acids with straight, apolar side chains (figure 10), and for the homologous series of amino acids with a terminal hydroxyl group (figure 11). The lines in each figure are similar, except that the line representing the Δ% exodus values shows less of a discontinuity than do the other lines. The Δ % exodus value is probably the best measure of the ability of an amino acid to serve as a substrate. The ratio of K_i values is an arbitrary measure of the effect of Na^+ on the K_i; a better correlation would probably be obtained if the ratio of the K_i in the absence of Na^+ to that in the presence of infinite Na^+ could be obtained. Such

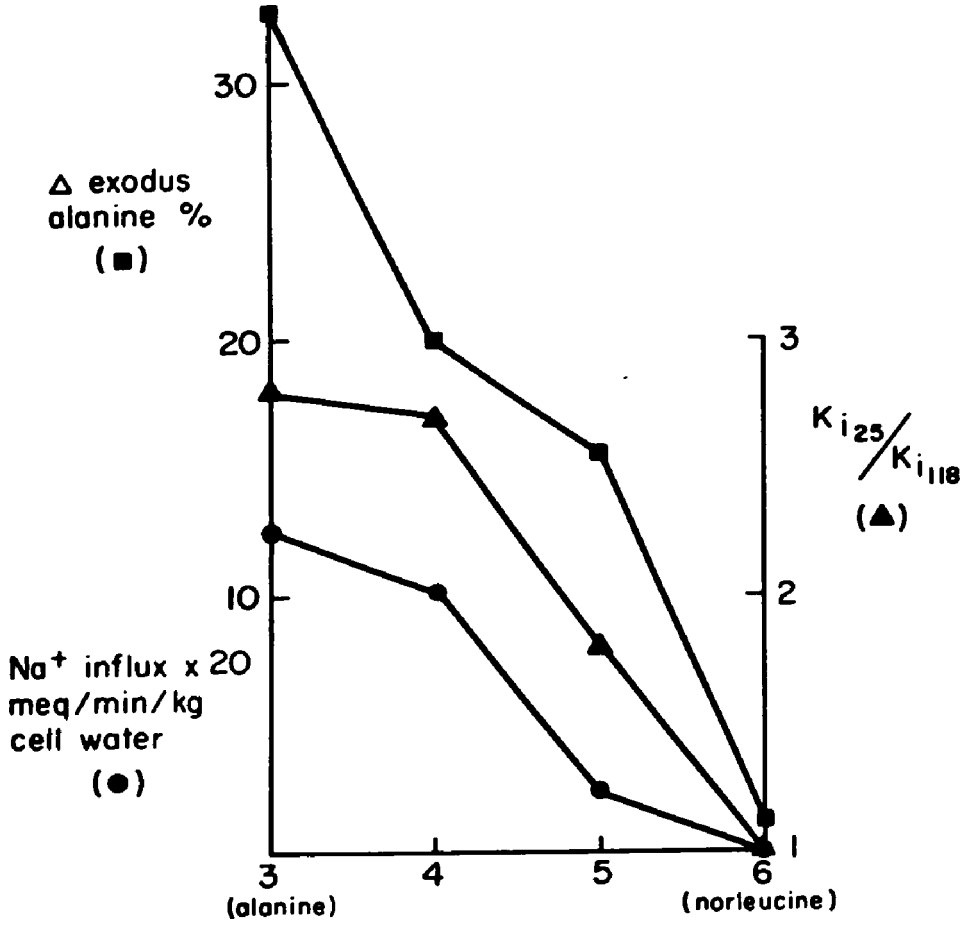


Figure 10. Effect of length of amino acid side chains on ability to serve as a substrate of the ASC system.

Legend for Figure 10.

The percent loss of previously accumulated ^{14}C-alanine, corrected for the percent loss in the absence of extracellular amino acids (3.0%), is plotted (■) vs. the chain length of the amino acid present in the incubation medium. Cells were incubated for 30 minutes in the presence of 0.5 mM alanine, labeled with ^{14}C-alanine, and 143 mN Na^+. An intracellular concentration of 1.4 mM alanine was obtained. After washing, the cells were incubated for 5 minutes in media containing 118 mN Na^+, and 20 mM amino acid. The values shown are the average of duplicate determinations.

The ratio of the K_i or K_m determined in the presence of 25 mN Na^+ to that in the presence of 118 mN Na^+ is plotted (▲) vs. chain length. K_m values obtained from Dixon plots were used for alanine. K_i values were obtained from plots of the reciprocal of alanine uptake vs. the concentration of the inhibitor. All incubations were of 3 minutes. The values shown are the same as listed in table 5.

The rate of Na^+ uptake, multiplied by 20, from media containing 50 mN Na^+ and 5 mM amino acid is plotted (●) vs. chain length of the amino acid responsible for the stimulated rate of Na^+ uptake. The rate of Na^+ uptake in the absence of amino acids has been subtracted from all the values. Incubations were of 5 minutes. The values shown are the average of duplicate determinations within the same experiment, and are the same as listed in table 5.

All values have been plotted vs. the number of carbon atoms in the chain of the series of neutral amino acids with straight, apolar side chains (alanine, α-amino-n-butyric, norvaline, and norleucine).

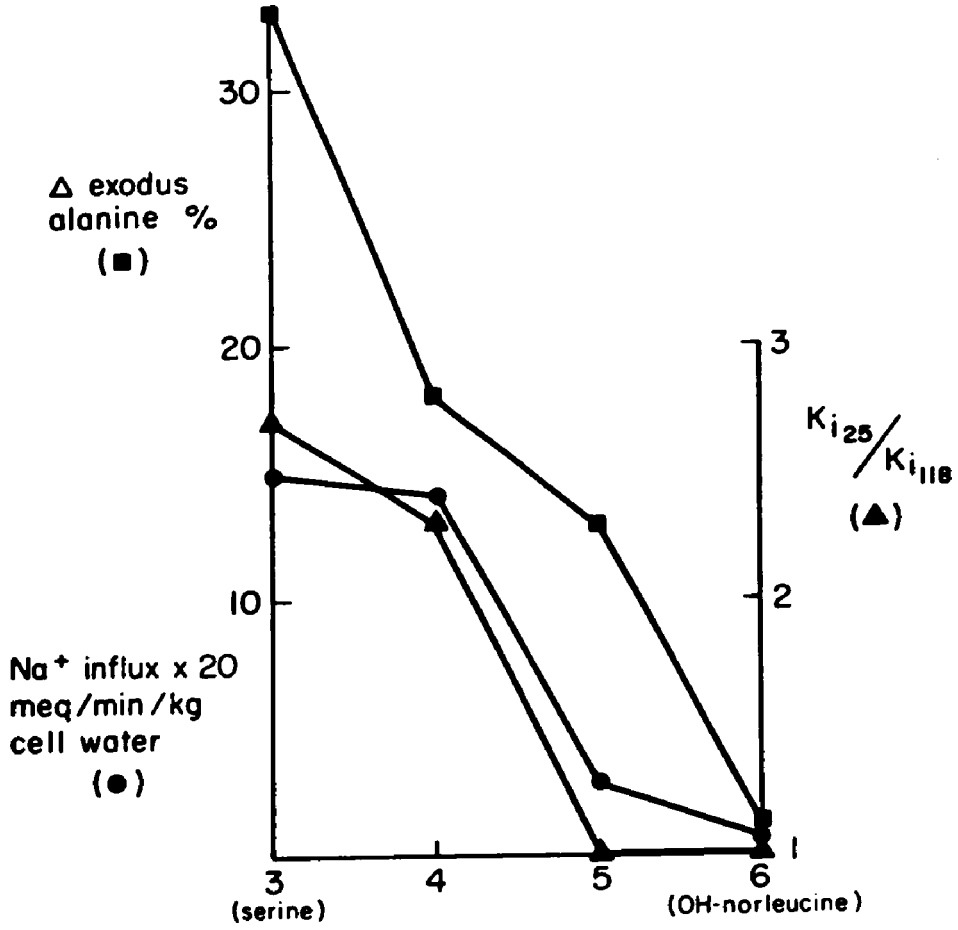


Figure 11. Effect of length of amino acid side chains on ability to serve as a substrate of the ASC system.

Legend for Figure 11.

The percent loss of previously accumulated ^{14}C-alanine, corrected for the percent loss in the absence of extracellular amino acids (3.0%), is plotted (■) vs. the chain length of the amino acid present in the incubation medium. Cells were incubated for 30 minutes in the presence of 0.5 mM alanine, labeled with ^{14}C-alanine, and 143 mN Na^+. An intracellular concentration of 1.4 mM alanine was obtained. After washing, the cells were incubated for 5 minutes in media containing 118 mN Na^+, and 20 mM amino acid. DL-hydroxynorvaline and hydroxynorleucine were 10 mM in the L form. The values shown are the average of duplicate determinations.

The ratio of the K_i or K_m determined in the presence of 25 mN Na^+ to that in the presence of 118 mN Na^+ is plotted (▲) vs. chain length. K_m values obtained from Dixon plots were used for serine. K_i values were obtained from plots of the reciprocal of serine uptake vs. the concentration of the inhibitor. All incubations were of 3 minutes. The values shown are the same as listed in table 5.

The rate of Na^+ uptake, multiplied by 20, from media containing 50 mN Na^+ and 5 mM amino acid is plotted (●) vs. chain length of the amino acid responsible for the stimulated rate of Na^+ uptake. The rate of Na^+ uptake in the absence of amino acids has been subtracted from all the values. Incubations were of 5 minutes. DL-hydroxynorvaline and hydroxynorleucine were 5 mM in the L form. The values shown are the average of duplicate determinations within the same experiment, and are the same as listed in table 5.

All values have been plotted vs. the number of carbon atoms in the chain of the series of neutral amino acids with straight side chains with an ω -hydroxyl group (serine, homoserine, hydroxynorvaline, hydroxynorleucine).

an extrapolation to the limiting values is shown for serine in Results Section I, but this extrapolation can not be made routinely or precisely. As shown in Results Section VI, the stimulation of Na^+ entry equals the product of the rate of amino acid entry and the r value. The maximal rate of amino acid entry in the presence of 118 mN Na^+ probably decreases with chain length, just as the ability to stimulate alanine exodus decreases. The value of r, however, appears to be a complex function of the K_m, a parameter which does not decrease in parallel to the stimulation of exodus. Despite the differences in the shapes of the lines, a dramatic decrease with chain length can be observed for all the values based on maximal rates.

In figure 12, the reciprocals of the K_i values measured in 25 (■) and 118 (●) mN Na^+ media are plotted vs. the number of carbon atoms in the chain for the series of amino acids beginning with glycine and adding a straight hydrocarbon side chain. Glycine is a poor substrate, and the value of its K_i sets a maximal limit for the affinity of the binding site for the α-carbon and its substituents other than the side chain. Alanine and α-amino-n-butyric are good ASC substrates. Na^+ produces a large change in the K_i values of these amino acids. They also have similar values of the other indicators of substrate activity. Norvaline has a limited ability to serve as a substrate, but its apparent affinity in low Na^+ media is greater than that of alanine. Norleucine has little activity as a substrate, but is an effective inhibitor with a K_i value independent of Na^+ concentration. The relation between S-methyl-cysteine and methionine is very similar to that between norvaline and norleucine. The ASC binding site has considerable affinity for the α-amino and carboxyl portions and for the apolar side chains of methionine and norleucine, but the length or bulk of the side chains is such as to interfere in some way with the process by which Na^+ lowers the K_i for amino acid substrates. We have concluded that there is a region of the binding site that shows

affinity for the apolar side chains, and that the effect of Na^+ is superimposed on this effect. The apolar interaction increases with chain length, whereas beyond alanine the effect of Na^+ decreases with chain length. The rates of change of these two effects are such that α-amino-n-butyric acid shows the greatest apparent affinity at high Na^+ concentrations and norvaline shows the greatest apparent affinity at low Na^+ concentrations. Norleucine would probably have the greatest affinity if its K_i could be measured in Na^+-free media. If a method were available by which K_i values could be measured in the absence of Na^+, a complete separation of the requirements for binding and for transport could presumably be achieved.

Figure 13 shows a similar plot of the reciprocals of the K_i values measured in 25 (■) and 118 (●) mN Na^+ media vs. the chain length for the series of ω-hydroxy amino acids. The difference in scale between figures 12 and 13 shows that the polar group confers greater apparent affinity upon serine and homoserine, relative to alanine and α-amino-n-butyric acid, despite the presence of an apolar binding region. This result is very similar to the results observed in the studies of the Ly^+ system, and has led us to propose that the Na^+ binding site lies in a position close to the amino acid binding site, such that the amino acid side chain may interact directly with the bound Na^+ ion. A type of bonding may be formed between amino acid and cation by way of the unshared electrons of the oxygen atom. Since the $K_{i\ 25}/K_{i\ 118}$ ratio is about the same for serine and alanine, and the same is true of homoserine and α-amino-n-butyric, the enhancing effect of the polar group is seen at Na^+ concentrations below 25 mN, as is shown in figure 1. With the exception of the comparison of proline and hydroxyproline, the ratio of K_i values does not reflect the

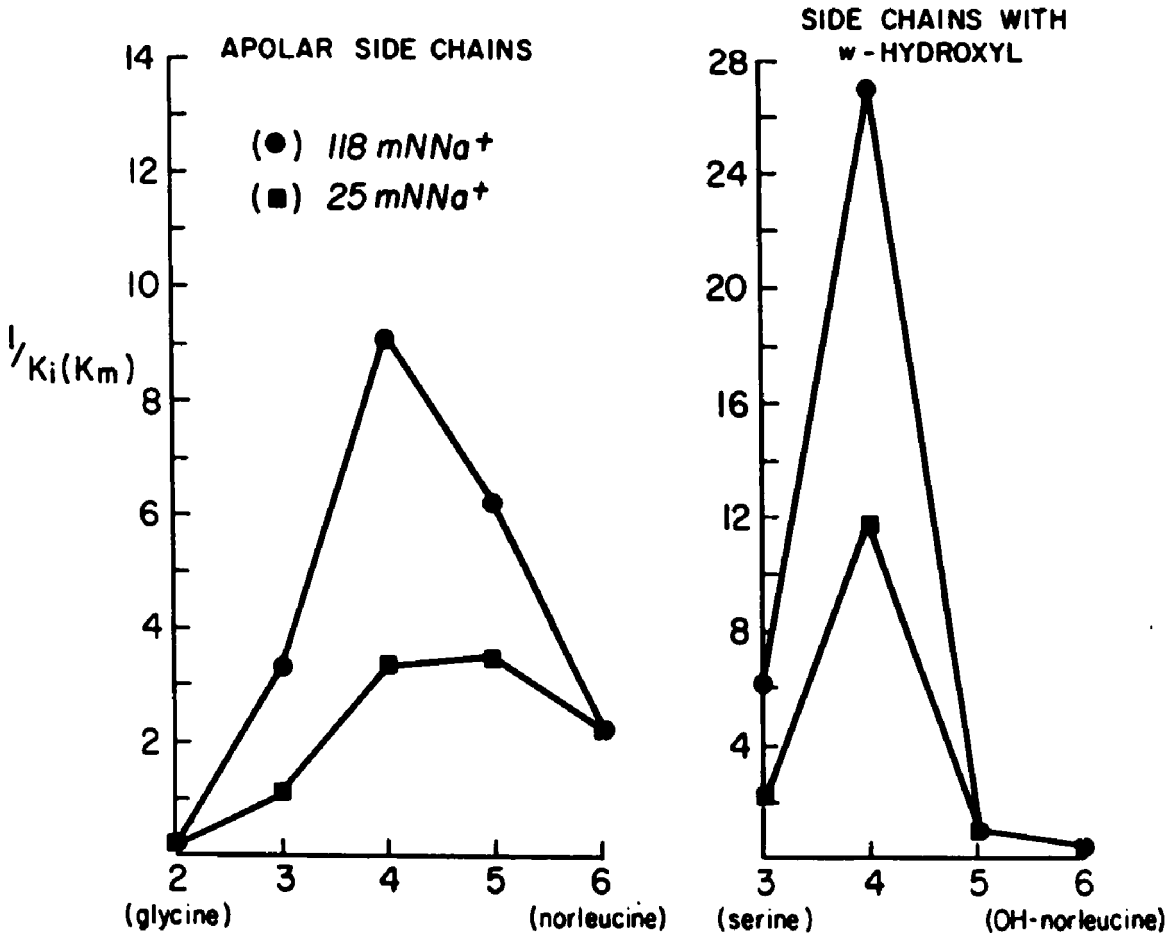


Figure 12. Effect of chain length on apparent affinity of neutral amino acids with apolar side chains.

Figure 13. Effect of chain length on apparent affinity of neutral amino acids with a terminal hydroxyl group.

Legend for Figure 12.

The reciprocal of the K_i determined in the presence of 118 mN Na^+ (●) and 25 mN Na^+ (■) are plotted *vs.* the chain length of the series of amino acids beginning with glycine and adding a straight, apolar side chain. The higher values represent greater apparent affinity. K_m values obtained from Dixon plots were used for alanine. K_i values were obtained from plots of the reciprocal of alanine uptake, corrected for the non-saturable component, *vs.* the concentration of the amino acid inhibitor. All incubations were of 3 minutes. The values used are those listed in table 5.

Legend for Figure 13.

The reciprocal of the K_i determined in the presence of 118 mN Na^+ (●) and 25 mN Na^+ (■) are plotted *vs.* the series of amino acids with straight side chains and an ω-hydroxyl group. K_m values obtained from Dixon plots were used for serine. K_i values were obtained from plots of the reciprocal of serine uptake, corrected for the non-saturable component, *vs.* the concentration of the amino acid inhibitor. Other conditions were as indicated for figure 12.

effect of a polar group on ASC reactivity. The effect of a polar group is observed in the K_i values and also in the r values, as noted in the Introduction. A comparison of the rates of Na^+ uptake in figures 10 and 11 shows that the ω-hydroxy amino acids are more effective in stimulating Na^+ uptake.

In the increase in length between homoserine and hydroxynorvaline, the limit is exceeded beyond which the polar group can no longer interact with the bound Na^+ ion. This same length limitation is defined, with the same result, by asparagine and glutamine. The high K_i values of hydroxynorvaline and hydroxynorleucine illustrate that a polar group detracts from the affinity if it is not in a position in which it can interact with the bound Na^+ ion. If the polar group can not reach the Na^+ ion, it may be repelled by the apolar binding region. Ethionine, with a side chain longer than methionine or norleucine, has a higher K_i than these two amino acids, even though its K_i is also invariant with Na^+ concentration. Therefore methionine and norleucine appear to define the limit of the apolar binding region, such that apolar side chains longer than those of methionine and norleucine detract rather than add to the affinity. Citrulline, with an even longer chain and a polar substituent, has an even lower affinity than glycine, which has no side chain. The apolar binding region extends only to a distance from the α-amino and carboxyl binding sites that can be reached by the side chain of norleucine, and the Na^+ binding site lies within an area that can be reached by either the γ-hydroxyl group of homoserine or the β-hydroxyl group of serine.

Although an appropriately positioned polar group lowers the K_i, this is not the principal means by which Na^+ affects the K_i values of ASC substrates. A change in K_i with increasing Na^+ concentration is observed for amino acids with apolar side chains not exceeding that of norvaline

in length. This effect is poorly understood, in that it seems unlikely that an apolar side chain could interact directly with a bound Na^+ ion. One could possibly explain this effect in terms of the equilibrium constants, by proposing that the binding of Na^+ produces a conformational change that increases the extent of interaction between the amino acid and the binding sites proposed above. Alternatively, this change in K_i may be related to the translocation process, so that the values change for any amino acid that does not interfere with transport as do methionine, norleucine, and the branched-chain amino acids.

Chain branching does not decrease the affinity for the receptor site, as shown by comparing the K_i values of norleucine, leucine, and isoleucine. The affinities of these amino acids appears to be about that expected considering the total hydrocarbon mass of the side chain, although the straight chain may be slightly preferred. Branching does, however, greatly decrease the ability of an amino acid to serve as a substrate, as shown by the comparison of valine with either norvaline or α-amino-n-butyric acid. The four indicators of substrate activity are much lower for valine. The K_i values are also much higher, but this effect reflects the lack of change in K_i with Na^+ concentration. The affinity of norvaline would be expected to be about that shown by valine if Na^+ had no effect on the K_i. The disadvantage produced by the branched structures is consistent with the view that the branch projects into the polar binding region, which is thought to be the Na^+ binding site. The effects of branching are disadvantageous only when both of the branches are apolar, as all the values shown for threonine in table 5 fall between those of serine and homoserine.

Proline may be considered as the cyclized form of norvaline, but it

appears to be much less reactive than norvaline. This decreased reactivity may be due to the secondary nitrogen atom and the attached, cyclized hydrocarbon structure. As mentioned in the Introduction, N-methylated amino acids have a low reactivity with the ASC system; the cyclic structure of proline apparently pulls the methylene carbon attached to the nitrogen atom away from the position where it would interfere completely with activity. The hydroxyl group of hydroxyproline confers an advantage to the proline structure, such that hydroxyproline and norvaline are very similar in their ASC reactivity. The hydroxyl group of hydroxyproline is on the γ-carbon, as is the case with homoserine. This is the position in which a polar group has the greatest effect on the K_i. The addition of the hydroxyl group also increases the ability of the proline structure to serve as a substrate, an effect not seen with homoserine and α-amino-n-butyric acid.

In contrast to the behavior of natural hydroxyproline, the allo isomer of hydroxyproline is a poor substrate; indeed it may well be less reactive than proline (table 6). This is the first example known in which an asymmetric center other than the α-carbon affects the reactivity of an amino acid with a transport system. The rigid structure of proline appears to be essential to this difference in reactivity, presumably by preventing free rotation to the reactive conformation. DL-allo-threonine and DL-allofree threonine, which are capable of rotation, were indistinguishable in their reactivity with the ASC system, as shown in table 6.

The preparations of hydroxyproline and allo-hydroxyproline used in these studies were indistinguishable by paper chromatography in n-butanol: acetic acid: water ::12:3:5, but they were clearly separated from proline. Proline and the hydroxyproline isomers gave the color response with ninhydrin characteristic of the proline structure.

Table 6. Reactivity of hydroxyproline and threonine isomers.

	A Δ% exodus	B K_i 25/K_i 118	C K_i, 25mN Na^+	D K_i, 118mN Na^+
proline	5.0	1.1	2.0 mM	1.8 mM
hydroxyproline	14.6	1.7	0.27	0.16
allo-hydroxyproline	0.7	1	2	2
allo free threonine	24.1	2.6	0.22	0.085
DL-allo threonine	22.1	2.4	0.47	0.20
DL-allo free threonine	22.6	2.7	0.54	0.20

Cells were preincubated in media containing 0.5 mM alanine and ^{14}C-alanine, and 143 mN Na^+. The cells were washed and then incubated for 5 minutes in the presence of the amino acids shown, each at a concentration of 20 mM. The percent loss of ^{14}C-alanine minus the rate of loss in the absence of external amino acids is shown in column A. The DL-threonine isomers were 10 mM in the L form. The K_i values in the presence of 25 and 118 mN Na^+ were obtained from plots of the reciprocal of serine uptake vs. the concentration of the inhibitor and are shown in columns C and D. The ratio of the K_i values is shown in column B. The K_i values observed for the DL-threonine isomers have not been divided by two.

The differences observed between the hydroxyproline isomers gives further evidence that the position in space of the components of the side-chain is crucial in determining the reactivity of an amino acid. The decreased reactivity of allo-hydroxyproline, relative to proline, is consistent with my conclusion that a polar group detracts from reactivity if it is not in a position in which it can interact with the bound Na^+ ion. The structure of hydroxyproline, with the hydroxyl group trans to the carboxyl group, must give an approximation of the conformation adopted by homoserine when it binds to the ASC site. This structure also serves to define further the location of Na^+ binding, relative to that of the α-amino and carboxyl groups.

VIII. Inhibition by cationic amino acids.

Cationic amino acids do not serve as substrates of the pigeon erythrocyte ASC system, but they act as competitive inhibitors with K_i values independent of Na^+ concentration. Under the same conditions as indicated for the alanine exodus experiments, arginine produced a Δ % exodus of -0.2%, which is within experimental error of zero. A plot of 1/v vs. 1/s for serine uptake in a 50 mN Na^+ medium in the presence of homoarginine is shown in figure 14. The same data are plotted as 1/v vs. homoarginine concentration in figure 15. Figures 16 and 17 show the same types of plots for homoarginine inhibition in the presence of 118 mN Na^+. The K_i value is the same at the two Na^+ concentrations. In table 7 a number of cationic amino acids are listed in order of their effectiveness as inhibitors of Na^+-dependent serine uptake.

Although the inhibition of the ASC system by cationic amino acids was the phenomenon that first turned our attention to the possibility of a detailed study of the ASC binding site, it has not proved to be as informa-

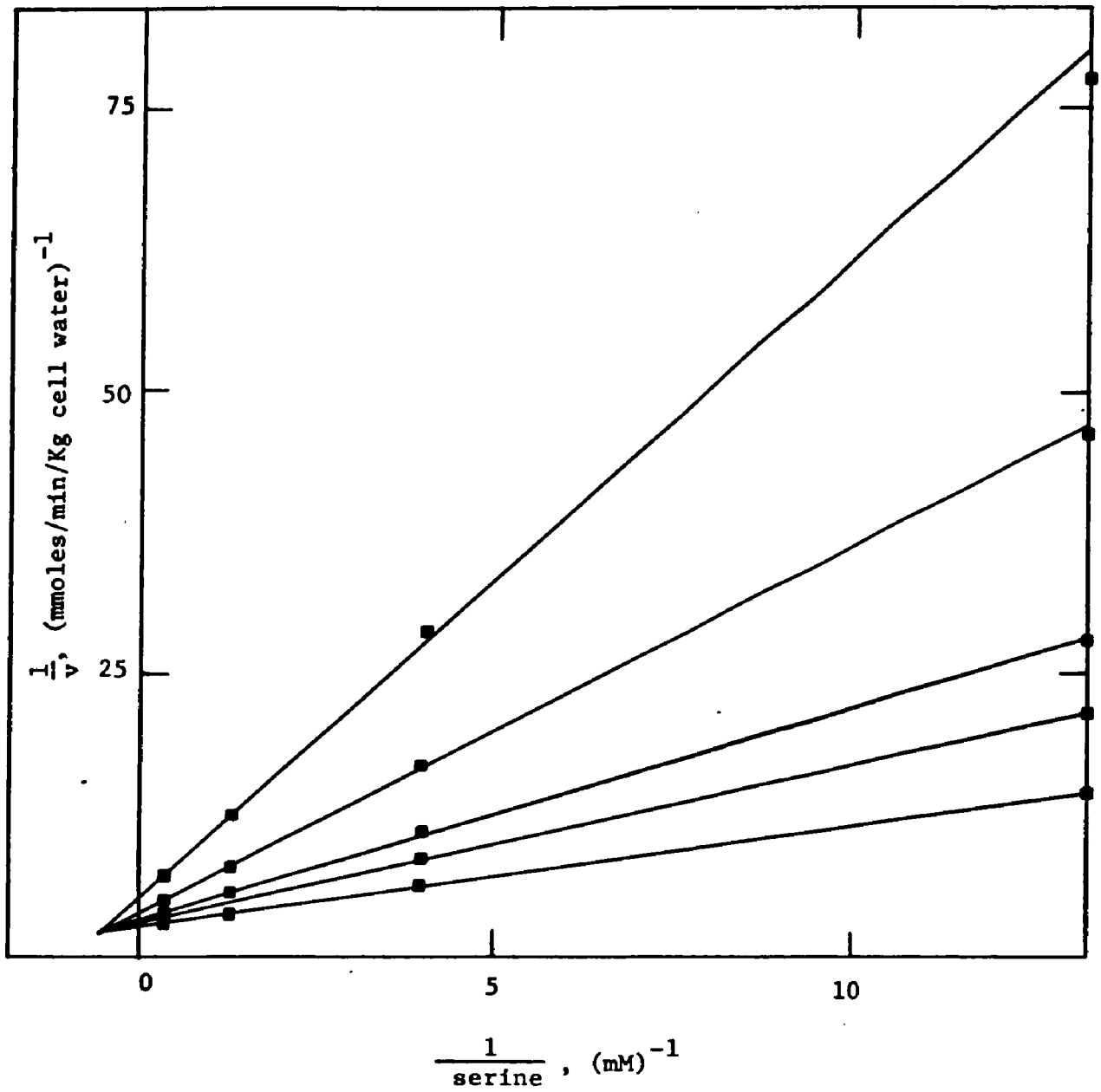


Figure 14. Inhibition of serine uptake by homoarginine in the presence of 50 mN Na^+. The reciprocal of the rate of serine uptake during a 3-minute incubation in the presence of 50 mN Na^+ and 0, 5, 10, 25, or 50 mM homoarginine is plotted vs. the reciprocal of the concentration of serine. All the rates have been corrected for the non-saturable rate of entry measured in the Na^+-free medium. The concentrations of serine were 0.075, 0.25, 0.75, and 2.5 mM.

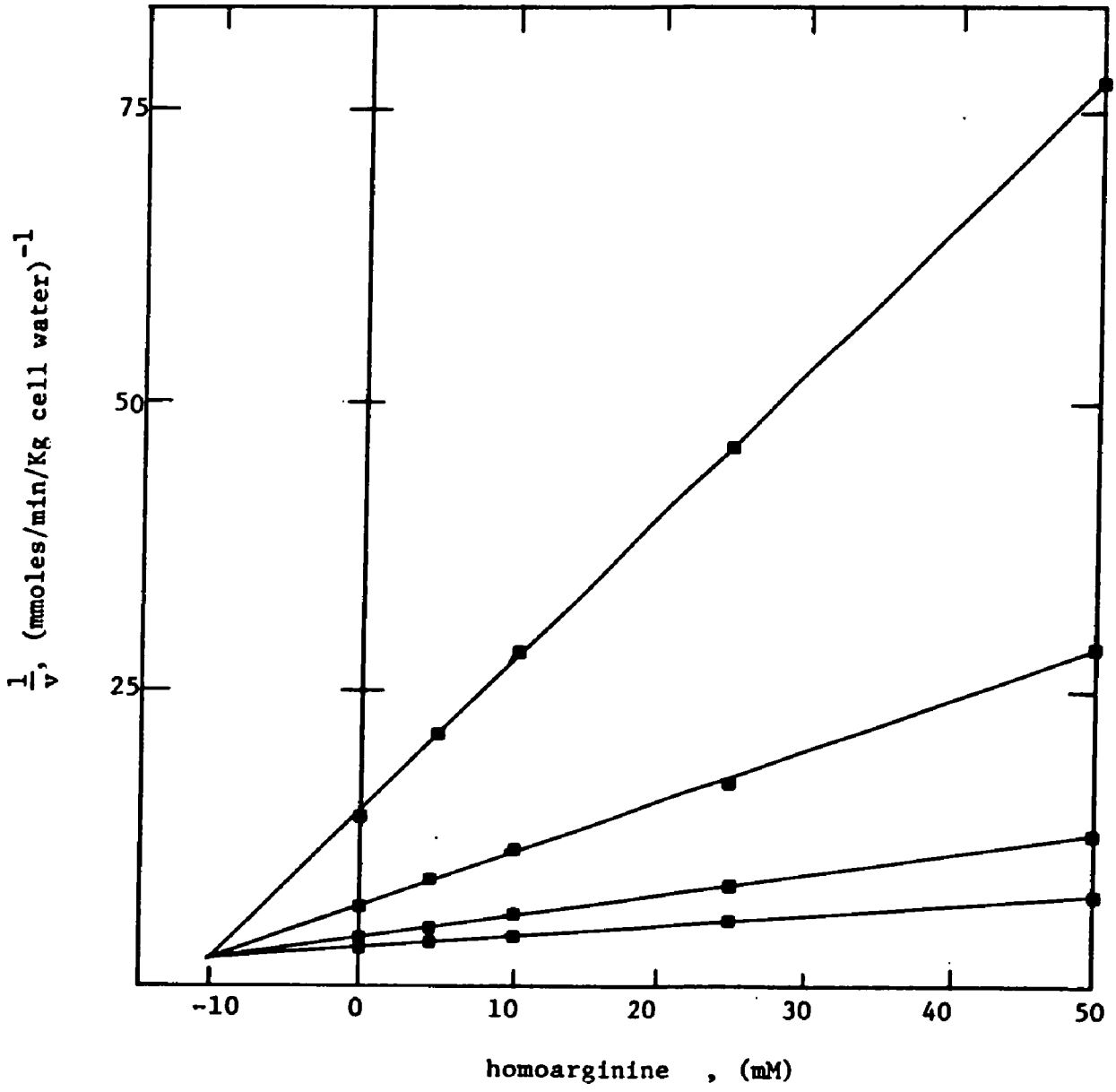


Figure 15. Determination of the K_i of homoarginine inhibition of serine uptake in the presence of 50 mN Na^+. The reciprocal of the rate of serine uptake during a 3-minute incubation in the presence of 50 mN Na^+ minus the non-saturable component measured in the Na^+-free medium, is plotted vs. the concentration of the inhibitor, homoarginine. The concentrations of serine were 0.075, 0.25, 0.75, and 2.5 mM, and the homoarginine concentrations were 0, 5, 10, 25, and 50 mM. The data are the same as plotted in figure 14. The K_i is taken to be about 10 mM.

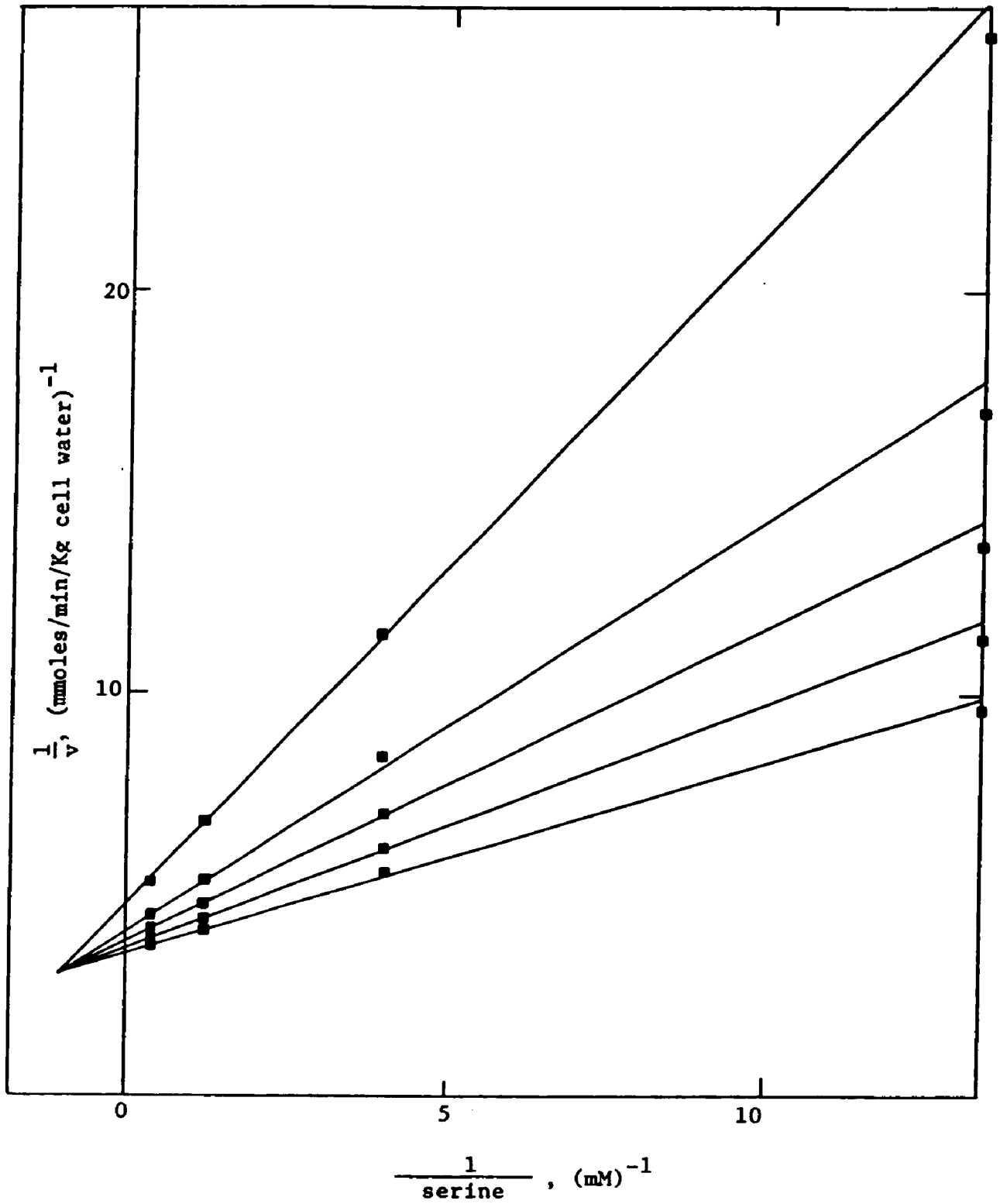


Figure 16. Inhibition of serine uptake by homoarginine in the presence of 118 mN Na^+.

Legend for Figure 16.

The reciprocal of the rate of serine uptake during a 3-minute incubation in the presence of 118 mN Na^+ and 0, 2, 5, 10, or 25 mM homoarginine is plotted vs. the reciprocal of the concentration of serine. All the rates have been corrected for the non-saturable rate of entry measured in the Na^+-free medium. The concentrations of serine used were 0.075, 0.25, 0.75, and 2.5 mM.

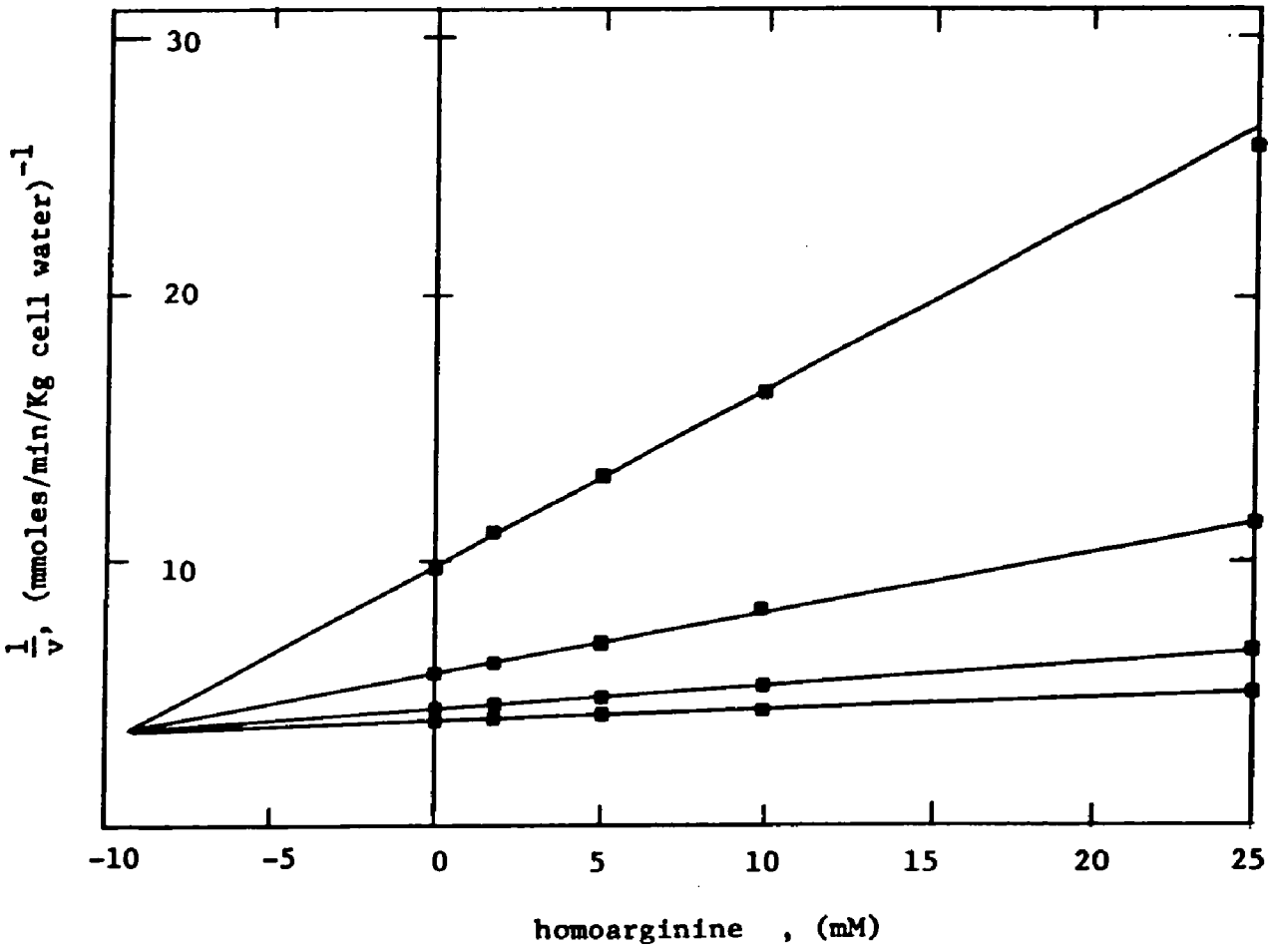


Figure 17. Determination of the K_i of homoarginine inhibition of serine uptake in the presence of 118 mN Na^+. The reciprocal of the rate of serine uptake during a 3-minute incubation in the presence of 118 mN Na^+ minus the non-saturable rate measured in the Na^+-free medium, is plotted vs. the concentration of the inhibitor, homoarginine. The concentrations of serine were 0.075, 0.25, 0.75, and 2.5 mM, and the concentrations of homoarginine were 0, 2, 5, 10, or 25 mM. The data are the same as those plotted in figure 16. The K_i is taken to be 9 to 10 mM.

Table 7. Cationic amino acids in order of their effectiveness as inhibitors of the ASC system.

cationic amino acid inhibitor	Structure of side chain	K_i, (mM)
canavanine	$NH_2 - \overset{\overset{NH_2}{\|}}{C} - NH - O - (CH_2)_2 -$	5-6
α-amino- β-guanidinopropionic acid	$NH_2 - \overset{\overset{NH_2}{\|}}{C} - NH - CH_2 -$	8
arginine	$NH_2 - \overset{\overset{NH_2}{\|}}{C} - NH - (CH_2)_3 -$	8
homoarginine	$NH_2 - \overset{\overset{NH_2}{\|}}{C} - NH - (CH_2)_4 -$	9-10
methionine methyl sulfonium ion	$\begin{matrix} CH_3 \\ \diagdown \\ \quad S - (CH_2)_2 - \\ \diagup \\ CH_3 \end{matrix}$	25
DL- δ-hydroxy-lysine	$NH_3 - CH_2 - \overset{\overset{HO}{\|}}{CH} - (CH_2)_2 -$	70 ($K_i/2 = 35$)
ornithine	$NH_3 - (CH_2)_3 -$	85
lysine	$NH_3 - (CH_2)_4 -$	85

Legend for Table 7

K_i values were obtained from plots of the reciprocal of serine uptake corrected for the non-saturable component measured in the Na^+-free medium *vs.* the concentration of the cationic amino acid. K_i values for the first five amino acids on the list were determined in the presence of 118 mN Na^+, and the highest concentration of inhibitor used was 25 mM. K_i values for the other amino acids were determined in the presence of 93 mN Na^+, with 50 mM as the highest inhibitor concentration.

tive as the converse study of the Ly^+ system using neutral amino acids. It has not proved possible to determine the location of Na^+ binding by observing a change in affinity of cationic amino acids when the ω-amino or guanidino group was in a position to bind at the Na^+ binding region of the ASC site. The range of structures of cationic amino acids available for study has so far been more limited than in the case of neutral amino acids. Most of the cationic amino acids studied exceed the limit at which length becomes a disadvantage. The homologous series of ω-guanidino amino acids, which span the proposed region of Na^+ binding, have the disadvantage that the positive charge may be distributed over the three nitrogen atoms of the guanidino group, such that a dramatic change in affinity with increasing chain length would not be expected. In addition, the three- and four-carbon diamino acids have a substantial neutral component at the pH values used in this work. In fact, it remains to be demonstrated that cationic amino acids show greater affinity than might be predicted from their structures, disregarding an interaction with the Na^+-binding portion of the ASC site.

We can say, however, that cationic amino acids show a greater affinity for the ASC system than they show for any other neutral amino acid system. For the Ehrlich cell, homoarginine has a K_i of 3 mM for the ASC system, whereas its inhibition is hardly detectable for the A and L systems. The K_i values for cationic amino acid inhibition of three Na^+-dependent neutral amino acid transport systems of the pigeon erythrocyte in the presence of 118 mN Na^+ are shown in table 8. The K_i values of cationic amino acids for the ASC system are independent of Na^+ concentration and independent of the substrate against which inhibition was tested. This independence was not tested for the glycine or β-amino acid systems.

Table 8. Inhibition of pigeon erythrocyte Na^+-dependent systems by cationic amino acids.

K_i, (mM)

System		ASC			glycine	β - amino acid
Substrate		alanine	serine	proline	glycine	taurine
cationic amino acid inhibitor	homoarginine	9-10	9-10	10-15	40-45	20
	lysine	85	--	80	300	100
	α - γ -diaminobutyric acid	55	--	--	45	25

Legend for Table 8

K_i values were obtained from reciprocal plots of the rate of substrate entry during incubations of 3 minutes for serine and alanine and 10 minutes for proline, glycine, and taurine, minus the rates of entry in the absence of Na^+, vs. the concentration of the amino acid inhibitor. The Na^+ concentration was 118 mN. The highest concentration of inhibitor used was 25 mM.

Also, comparisons can be made between the K_i values of cationic amino acids and those neutral amino acids of similar structure. For the Ehrlich cell ASC system, a number of neutral amino acids other than the N-methylated and α-disubstituted amino acids have K_i values greater than that of homoarginine (60). For the pigeon erythrocyte ASC system, only L-methionine-D,L-sulfoxide with a K_i of 18 mM was found to have a K_i clearly higher than those found for a number of cationic amino acids. The neutral amino acid citrulline has a structure highly analogous to that of arginine, and the two have about the same K_i (9-10 mM and 8 mM, respectively). Hydroxynorvaline and ornithine, also with analogous structures, have K_i values of 1 and 85 mM, respectively. This contrast suggested that the guanidino group might have greater affinity for the Na^+ binding portion of the ASC site than does the positively charged amino group, an expectation that was confirmed by comparing the inhibition of serine uptake by guanidine hydrochloride and ammonium chloride, as shown in table 9.

The inhibition of serine uptake by guanidine hydrochloride was consistent with competitive inhibition, and similar to the inhibition produced by Li^+. Guanidine hydrochloride has been widely used as a protein-denaturing agent, and therefore as a non-competitive inhibitor of enzyme activity, but this disruptive property does not appear to be the cause of inhibition of the ASC system at the low concentrations used in these studies.

The difference in affinity of ω-amino and ω-guanidino amino acids can therefore be explained by the difference in affinity of the cationic groups for the Na^+-binding portion of the ASC site. This difference would provide evidence that some cationic amino acids can interact with both the Na^+-binding and amino acid-binding portions of the site, when they are bound in the manner of neutral amino acids. We should note, however, that guanidino amino acids are also more effective inhibitors of glycine and

Table 9. Inhibition of serine uptake by monovalent cations.

		Percent inhibition of 0.05 mM serine influx		
Na^+ concentration		25 mN	25 mN	10 mN
Inhibitor concentration		50 mM	60 mM	75 mM
Inhibitor	LiCl	20%	34%	48%
	KCl	2	--	14
	NH_4Cl	1	1	21
	guanidine hydrochloride	--	30	41
	methylguanidine hydrochloride	--	--	30

The percent inhibition of the rate of uptake of 0.05 mM serine, corrected for the non-saturable component, was measured using the Na^+ and inhibitor concentrations shown.

taurine uptake. This result may suggest that the Na^+ binding sites of the glycine and β -amino acid systems lie in a position similar to that of the ASC site, or that in these systems, the cationic amino acids compete with Na^+ binding as simple cations.

Although cationic amino acids have a net charge of one, inhibition of the ASC system by cationic amino acids differs from that shown by simple monovalent cations. The K_i of Li^+ increases with increasing Na^+ concentration, indicating that Li^+ and Na^+ compete for the same site. The K_i of homoarginine appears independent of Na^+ concentration, indicating that homoarginine competes principally for the amino acid binding portion of the ASC site. The K_i of D-arginine is about twice that of L-arginine. This stereospecificity could arise only if arginine were subject to binding at multiple points, as are the neutral amino acids. The slight deviation from competitive kinetics in figures 14 and 16 may indicate that homoarginine also competes with Na^+ for binding. At infinite serine concentration, homoarginine inhibition would still be seen if homoarginine also had affinity for the Na^+ binding region. Therefore, the reciprocal plots would not be expected to intersect exactly on the ordinate. A slight deviation from the kinetics of competitive inhibition is also observed for Li^+ inhibition of serine uptake.

The results obtained with Li^+ clarify some aspects of the inhibition produced by cationic amino acids. Since no cation other than Na^+ appears capable of stimulating uptake by the ASC system, it might be thought that the cation binding region is too highly specific to show affinity for an amino or guanidino group. Alternatively, if some affinity for the cationic group is accepted, it might be expected that cationic amino acids would take the place of both co-substrates and be transported by the ASC system.

The results obtained with Li^+ show that the specific requirement for Na^+ is not entirely due to binding specificity; other cations show some affinity for the Na^+ site, but transport does not result.

IX. The ASC system in other cell types.

Table 10 shows the stimulation of Na^+ uptake by a number of amino acids by way of the rabbit reticulocyte ASC system. These results are similar to those given in table 5 for the pigeon erythrocyte. Results obtained by Miss Mary Handlogten in this laboratory (table 11) indicate that the pattern of substrate specificity and inhibitor affinity of the Ehrlich cell ASC system is similar to that found for the pigeon erythrocyte ASC system (table 5). The K_i values are generally three to five times higher for the Ehrlich cell, but the ratios of the K_i values measured in the presence of 20 and 120 mN Na^+ are close to the $K_{i\ 25}/K_{i\ 118}$ ratios obtained for the pigeon erythrocyte. No Na^+-stimulated uptake of norvaline or norleucine by the Ehrlich cell could be detected by chemical analysis when the A system was blocked by a high concentration of α-(methylamino) isobutyric acid. These studies provide more evidence for the detailed similarity of the ASC system in differing cell types, and lends support to our belief that the ASC system represents an evolutionary entity.

Table 10. Amino acid-stimulated Na^+ influx for the rabbit reticulocyte ASC system.

amino acid(5 mM) stimulating Na^+ influx	amino acid-dependent Na^+ influx (meq/min/Kg cell water)
alanine	0.59
α -amino-n-butyric	0.53
norvaline	0.14
norleucine	0.11
serine	0.56
homoserine	0.55
S-methyl-cysteine	0.23
methionine	0.13

The rate of Na^+ uptake was measured during a 5 min-incubation period, from media containing 5 mM amino acid and 50 mN Na^+, labeled with $^{22}Na^+$. The observed rates have been divided by the time of incubation.

Table 11. The reactivity of neutral amino acids with the Ehrlich cell ASC system.

	A	B	C
	Ratio of the K_i values	K_i, 20 mN Na^+ (mM)	K_i, 120 mN Na^+ (mM)
alanine	2.1	2.0	0.95
α -amino-n-butyric	2.9	2.4	0.83
norvaline	1.3	1.2	0.91
norleucine	1.0	2.2	2.2
S-methyl-cysteine	1.6	2.5	1.6
methionine	1.0	2.0	2.0

The ratio of the K_i value measured against alanine uptake in the presence of 20 mN Na^+ (B) to that in the presence of 120 mN Na^+ (C) is given in column A. The values shown for alanine are K_m values, obtained from Dixon plots. The K_i values were obtained from plots of the reciprocal of the rate of alanine uptake vs. the concentration of the amino acid inhibitor. Results provided by Miss Mary Handlogten of this laboratory.

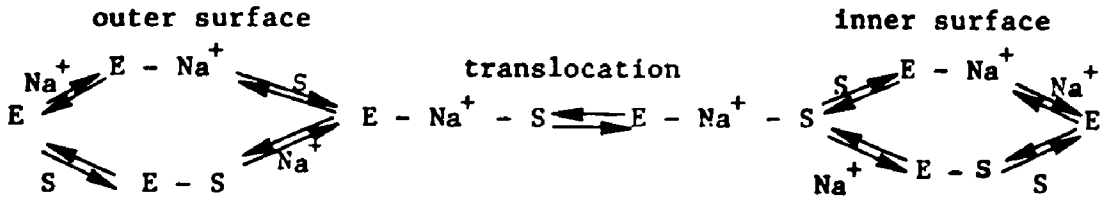
DISCUSSION

For the present, the ASC system appears unique among the well defined transport systems in providing many approaches to a detailed understanding of the binding specificity and the transport mechanism. The system shows many surprising features that can be studied separately and which must be incorporated into any proposed model for the system. The transport process has a nearly total specificity for Na^+, which is not reflected in the specificity of cation binding. Cationic amino acids show differences in affinity, but do not act as substrates. Many neutral amino acids with high affinity for the binding site are among the least effective substrates. Among the neutral amino acids which do serve as substrates, wide differences are observed in K_m and V_{max} values, and these parameters vary independently with changes in amino acid structure and changes in Na^+ concentration. Also, striking differences occur in the stoichiometry of Na^+ and amino acid fluxes. Any comprehensive model we may develop for the operation of this system is therefore subject to many tests of consistency and completeness.

Since the requirements for transport differ from the requirements for binding, it has proved possible to evaluate these aspects separately. A binding site of considerable complexity has been proposed. The combination of this model for the binding site with the model for overall operation of an exchanging system appears capable of explaining the observed differences in affinity, uptake, and stoichiometry.

The kinetic analysis of the pigeon erythrocyte ASC system is based on the model (below) proposed by Wheeler and Christensen (98). Contributions have been made to our current understanding of this system by extensive discussion amon members of this laboratory, and by consultations with members

of the thesis committee.



No step involving translocation of free E has been included, based on the evidence that only the ternary complex is capable of the translocation step. Neither Na^+ nor amino acids appear to be taken up by way of the ASC system in the absence of the other co-substrate. Also, alanine shows little or no exodus from this cell by way of the ASC system in the absence of extracellular amino acids, despite the presence of both co-substrates inside the cell. The lack of exodus implies that the carrier cannot recycle unless both co-substrates are present on both sides of the membrane. The apparent rates of Na^+ and amino acid entry appear to be rates of exchange of external labeled substrates with internal unlabeled substrates, and not rates of accumulation. If the system is capable of net transport, the rates of exchange are so much greater that they prevent observation of the rate of that transport. Indeed, the ASC system would probably not be observed in cells or cell preparations such as the pigeon erythrocyte "ghosts" prepared by Vidaver (97), unless Na^+ and amino acid substrates were present intracellularly. The ASC system of the pigeon erythrocyte appears to be a compulsory exchange-diffusion system.

Four equilibria are assumed to be involved in the formation of the ternary complex.

$$E + Na^+ \underset{}{\overset{K_{Na}^+}{\rightleftharpoons}} E - Na^+$$

$$E + S \overset{K_S}{\rightleftharpoons} E - S$$

$$E - Na^+ + S \overset{K_S'}{\rightleftharpoons} E - Na^+ - S$$

$$E - S + Na^+ \overset{K_{Na}'^+}{\rightleftharpoons} E - Na^+ - S$$

The four equilibrium constants describe these interactions, and are related by the equation below.

$$K_{Na}^+ \times K_s' = K_s \times K_{Na}'^+$$

In applying this basic model to two-substrate transport processes, the translocation step is usually assumed to be rate-limiting, so that all the complexes shown on each side of the membrane are in equilibrium. The rate of uptake therefore depends on the concentration of the ternary complex. For any constant concentration of one substrate, variation of the concentration of the other substrate results in a hyperbolic relationship of the rate and substrate concentration. If K_{Na}^+ equals $K_{Na}'^+$ and K_s equals K_s', then the true equilibrium constants equal the K_m values obtained from plots of 1/v *vs.* 1/ substrate concentration at any concentration of the second substrate. In order to deal with the more general case in which affinity for a substrate may be changed by the prior binding of the other substrate, Florini and Vestling (132) have developed graphical methods to determine these constants. Plots of 1/v *vs.* 1/ substrate (S) concentration for differing concentrations of the other substrate (Na^+), intersect at a point that has the horizontal coordinate of $-1/K_{Na}^+$. The equilibrium constants for formation of the ternary complex (K_s' and $K_{Na}'^+$) are equal to the K_m values of the substrates, each in the presence of an infinite concentration of the other substrate. If the affinity of either substrate is affected by the prior binding of the other

substrate, then the apparent affinity of each substrate varies with the concentration of the other substrate. For example, as the Na^+ concentration is increased, S comes to react more frequently with E - Na^+ than with E, so that the value of the K_m tends toward K'_s.

The results in Section I shows that the uptake of serine by the pigeon erythrocyte ASC system is consistent with these expectations. The kinetics of Na^+ and serine uptake are hyperbolic, and the families of reciprocal plots intersect to give values for the affinity of serine and Na^+ for the unloaded carrier. Also, the values suggested for the equilibrium constants are consistent with the equation below.

$$K_{serine} \times K'_{Na^+} = K_{Na^+} \times K'_{serine}$$

Other amino acids have K_i values which appear invariant with Na^+ concentration. For these amino acids we may assume that K_s equals K'_s, and from the equation above, K_{Na^+} equals K'_{Na^+}.

Since serine uptake by the ASC system seems to fit the equilibrium analysis, this analysis has been used in order to assign a simple physical interpretation to the K_m and K_i values, describing them as apparent affinity constants. As seen below, however, the equilibrium assumption is not justified. The best view appears to be that those K_i values that are invariant with Na^+ concentration represent affinity constants for the carrier, and the change in K_m observed for amino acids such as serine is a result of the translocation event rather than a change in affinity.

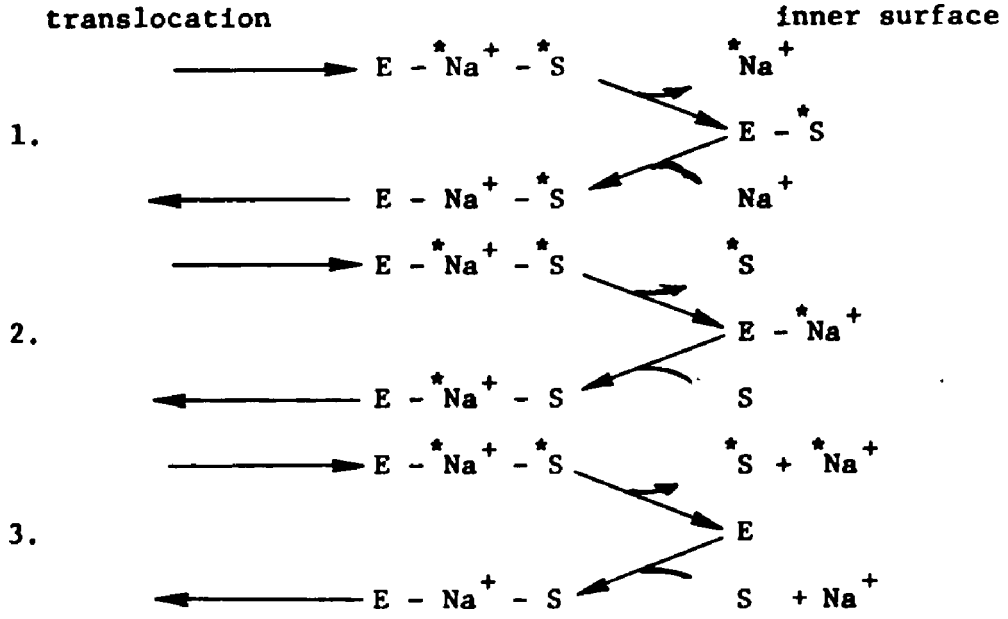
There are five conditions any of which will lead to hyperbolic kinetics in two-substrate systems. The first condition is that the translocation step is the rate-limiting step. This possibility has been discussed above

and seems not to explain the variable stoichiometry of Na^+ and amino acid movements. If Na^+ and the amino acid substrate are bound in a one-to-one ratio and translocation is rate-limiting, then the translocation and the observed rates must proceed with a one-to-one stoichiometry. The second condition is that no ternary complex is formed (133). Such mechanisms have characteristic kinetic features that have not been observed for any transport system. The third condition is that there is an obligatory order of formation of the ternary complex (134). An obligatory order would require that either the rate of Na^+ entry or of amino acid entry would always exceed the other regardless of the amino acid used as substrate, and this is not the case. The fourth and fifth conditions are special cases of the steady state analysis of a random-order mechanism. Dalziel (135) has demonstrated that hyperbolic kinetics are obtained if the rates of the forward reaction for the formation of the ternary complex are much slower than the rates of breakdown of the binary complexes. This situation will usually be obtained only when the concentrations of the substrates are low, since the rate of each forward reaction is the product of the rate constant and the concentration of substrate. Ferdinand (136) demonstrated that hyperbolic kinetics may be obtained at higher concentrations, as is observed for the ASC system. The conditions which lead to non-hyperbolic kinetics are that the concentrations of substrate must exceed the limits proposed by Dalziel, and that the substrate which reacts faster in the formation of a binary complex must react slower in the formation of a ternary complex, such that one pathway to the ternary complex is kinetically preferred. Wheeler and Christensen (98) proposed that such a preference may account for the non-hyperbolic kinetics of the pigeon erythrocyte glycine and β-amino acid systems.

It is likely therefore, that the overall operation of the pigeon

erythrocyte ASC system may best be described by the model in which (a) translocation is not rate-limiting, (b) there is no kinetically preferred pathway, and (c) as seen below, the rate constants for the formation and breakdown of the complexes are such that little or no free E is present when exchange is occuring.

Since the observed rates for the entry of the labeled amino acid and $^{22}Na^+$ differ, there must be two rate-limiting steps, one for each substrate. The carrier may become reloaded and therefore able to return by way of three different sequences or by a statistical combination of these sequences.



The substrates labeled with asterisks represent isotopically labeled substrates from the incubation medium. If E - *Na+ - *S were in equilibrium with E at the inner surface, complete equilibration would occur so that only unlabeled substrates would be returned to the outer surface under initial rate conditions, as is shown in sequence 3. Since the apparent rate of Na^+ entry usually exceeds that of the amino acid substrate (i.e., $r > 1$), sequence 1 occurs with greater frequency.

The r value of an amino acid will be determined by the relative rates of dissociation of E - $^{*}Na^{+}$ - $^{*}S$ to E - $^{*}Na^{+}$ and to E - $^{*}S$ at the inner surface, and by the relative rates of reformation of E - $^{*}Na^{+}$ - S or E - Na^{+} - $^{*}S$ from the binary complexes. The rate constants for the breakdown of E - $^{*}Na^{+}$ - $^{*}S$ will depend on the nature of $^{*}S$. The rate of the reassociation step E - $^{*}S$ + Na^{+} ⟶ E - Na^{+} - $^{*}S$, depends on the nature of $^{*}S$ and the intracellular concentration of Na^{+}. The rate of the step E - $^{*}Na^{+}$ + S ⟶ E - $^{*}Na^{+}$ - S depends on the intracellular concentrations and the structures of the amino acids making up the intracellular pool. During a brief incubation, the intracellular concentrations of Na^{+} and amino acids will not change because the ASC system operates by exchange. Also the composition of the intracellular pool will not change greatly under initial-rate conditions. The only variable factor is the structure of $^{*}S$, so that each amino acid has a characteristic value of r, which is invariant with respect to extracellular concentrations.

The intracellular concentrations of Na^{+} and amino acids, and the composition of the intracellular amino acid pool appear to be reproducible for freshly-washed lots of cells (98 ,108), so that the same values of r are obtained in different experiments. During long incubation periods, the Na^{+} and amino acid concentrations may increase, as entry occurs by routes other than the ASC system, and the composition of the intracellular amino acid pool will change as exchange occurs. Koser found that r values could be changed by a prior incubation of the cells in the presence of various amino acids and Na^{+} (125).

Those amino acids with high r values probably decrease the rate constant of the step E - $^{*}Na^{+}$ - $^{*}S$ ⟶ E - $^{*}Na^{+}$ + $^{*}S$, and increase the rate constant of the step E - $^{*}S$ + Na^{+} ⟶ E - Na^{+} - $^{*}S$. Both of these changes imply that the property of $^{*}S$ which produces high r values increases the rate of

formation and decreases the rate of breakdown of the ternary complex. This increased reactivity would be likely to result in a decrease in the rate of the step $E - {}^{*}Na^{+} - {}^{*}S \longrightarrow E - {}^{*}S + {}^{*}Na^{+}$, although this would lower rather than raise the $\underline{r}$ value. Obviously the $\underline{r}$ value is a complex function that depends on relative changes in the rate constants. This complexity accounts for the observation that the $\underline{r}$ values are not well correlated with either maximal rate or binding affinity observations.

As noted previously, amino acids with appropriately positioned polar groups have high $\underline{r}$ values. The addition of a polar group may also increase the apparent affinity (comparing homoserine to α -amino-n-butyric), and/or increase the maximal rate observations (comparing serine to alanine, or hydroxyproline to proline), but these changes are distinct from the process by which Na^{+} usually affects the K_m and V_{max}, which is described by the $\underline{K_{i\ 25}/K_{i\ 118}}$ ratio. Also, high $\underline{r}$ values can not be correlated with true affinity, because amino acids with long side chains, such as norvaline, have low $\underline{r}$ values. We conclude that the distinction between the special effect of a polar group, and the more general effect of Na^{+} on the kinetic parameters is due to the direct interaction of the polar group with the bound Na^{+} ion, an interaction that is helpful but not essential to binding and transport.

Implicit in the foregoing discussion is the assumption that observations based on maximal rates, such as $\underline{\Delta\ \%\ exodus}$, and $\underline{V_{max,\ 118mN\ Na^{+}}}$, describe the maximal ability of an amino acid to move by way of the ASC system. The effect of serine and Na^{+}, each to lower the concentrations that result in half-maximal uptake of the other, will insure that maximal rates are obtained at the upper limits of Na^{+} and serine concentrations used in this work. Those amino acids with K_m values less dependent on Na^{+} concentration may not lower

the K_m for Na^+, so that maximal rates may not have been obtained. We can be sure, however, that similar rates would not be obtained for all amino acids under conditions of infinite Na^+ and amino acid concentrations. In the alanine exodus experiments, the Na^+ concentration was in the same range as the K_m for Na^+ at low concentrations of serine, and the extracellular amino acid concentration was usually at least ten times the K_i of the amino acid at this Na^+ concentration. Under these conditions, we can assume that nearly-maximal rates are obtained, an assumption that was confirmed by raising the Na^+ concentration even further (table 4). The increase from 118 to 143 mN Na^+ did not increase the rates, nor for example, did it greatly increase the rate for methionine relative to serine. Maximal rates are obtained for all substrates at low Na^+ concentrations, the effect of Na^+ at higher concentrations being principally to change the K_m (figure 1). The differences in maximal rates cannot be due to differing rates of translocation, because translocation was shown not to be rate-limiting. No amino acid has been observed to have an $\underline{r}$ value of one, which would be required if translocation were slowed so as to become the rate-limiting step. The maximal rate therefore depends on the rate of formation of the ternary complex. The amino acids with low rates of uptake may have lower rates of binding, or may frequently displace the co-substrate in the course of binding.

$$E - Na^+ + S \longrightarrow E - S + Na^+$$

$$E - S + Na^+ \dashrightarrow E - Na^+ + S$$

By observing the effects of amino acid structure on the rates of uptake, some evidence for the latter possibility has been obtained. Amino acids which when bound have side chains branched in the proposed region of Na^+ binding have low rates of uptake, and effect that could be due to displacement of the Na^+ ion by the branched structure.

The use of branched-chain amino acids to clarify an aspect of the mechanism illustrates the principal goal of this work, which was to provide an analysis of the physical interaction of amino acids and Na^+ with the ASC system, and thus to gain knowledge of the physical nature of the transport mediator and the transport process. The conclusions which may be drawn from this analysis are discussed extensively under Results Sections VII and VIII and are summarized below.

Because the pigeon erythrocyte ASC system appears to have affinity for the substituents of the α-carbon of amino acids, apart from the affinity for the side chain, points of binding have been proposed for the α-amino and carboxyl groups. Affinity for the site increases with the hydrocarbon mass of the side chain up to a length limitation defined by norleucine. Therefore an apolar binding region of finite dimensions has been proposed. A decrease in K_m in the presence of Na^+ is observed for amino acids having side chains without hydrocarbon branches and not exceeding norvaline in length. This phenomenon may be attributed either to a Na^+-induced change in the mediating structure which increases the interaction between the amino acid and the binding regions proposed above, or to kinetic rather than thermodynamic effects.

Amino acids having side chains with an appropriately placed polar group show an additional decrease in K_m values. We have proposed a direct interaction between the bound cation and the polar group. The point of Na^+ binding would therefore be close to the region of binding of the α-amino and carboxyl groups, not exceeding a distance defined by homoserine. This result provides the first evidence for the location of Na^+ binding for a transport system which is a member of the class of systems specific to Na^+ and neutral substrates. The spatial relation of the Na^+ and amino acid substrate would be such that a localized conformational change might be sufficient to carry out

translocation of both substrates.

The finding that other cations may bind in place of Na^+ without stimulating amino acid uptake is consistent with the view that Na^+ produces specific changes in the mediating structure. These changes may be equivalent to the conformational change which we suppose results in translocation. The inhibition of the ASC system by cationic amino acids provides more evidence for the essentiality of Na^+ in the translocation process, and for the location of Na^+ binding.

The differences in reactivity of the hydroxyproline isomers gives an indication of the conformation adopted by amino acids when they are bound, and further defines the relations of the binding regions of the ASC site. In figures 18-20, these relations are represented diagramatically. Hydroxyproline and norleucine are shown filling the proposed ASC site.

Some of the evidence for the similarity of the ASC system in different cell types has been presented above. The Ly^+ system also appears to be an evolutionary entity, with highly similar properties in each cell in which it has been studied. Although the ASC and Ly^+ systems are clearly distinct, the studies of these systems have revealed striking similarities. Both systems reject amino acids with two apolar substituents on the α-carbon and N-methylated amino acids. Both systems appear to have sites with points of binding for α-amino and carboxyl groups, for apolar side chains, and for cations. The spatial relations of these binding regions are such that the neutral amino acids that are the best inhibitors of the Ly^+ system are often the best substrates of the ASC system. The ASC system of the pigeon erythrocyte and the Ly^+ system of the rabbit reticulocyte show a decided discontinuity in reactivity at the point between homoserine and hydroxynorvaline, and between asparagine and glutamine. The cation binding site of the Ehrlich cell Ly^+ system seems farther removed from the region of binding of the α-amino and

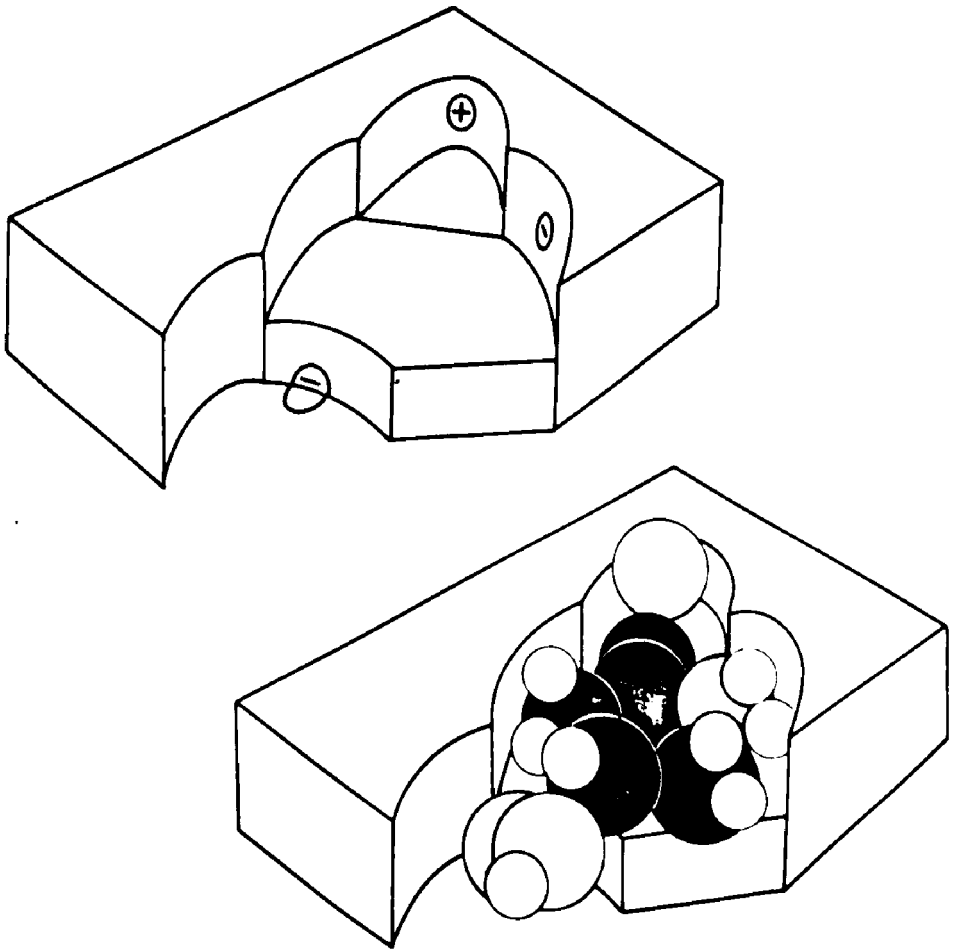


Figure 18. Diagrammatic representation of the spatial relations of the proposed binding regions of the ASC site.

Figure 19. Binding of hydroxyproline and $Na^{\oplus}$ to the proposed ASC site.

Legend for Figure 18.

The trans structure of hydroxyproline has been used to define three planes, the highest plane for carboxyl group binding (⊕), the middle plane for amino group (⊖) and apolar binding, and the lowest plane for Na^+ binding (⊖). The Na^+ ion has been placed in accordance with the ability of the β-hydroxyl of serine and the γ-hydroxyl of homoserine to interact with Na^+. The scalloped structure on the left is intended to represent the limited apolar binding region.

Legend for Figure 19.

Representations of the hydroxyproline molecule and of Na^+ ion are shown filling the proposed ASC binding site.

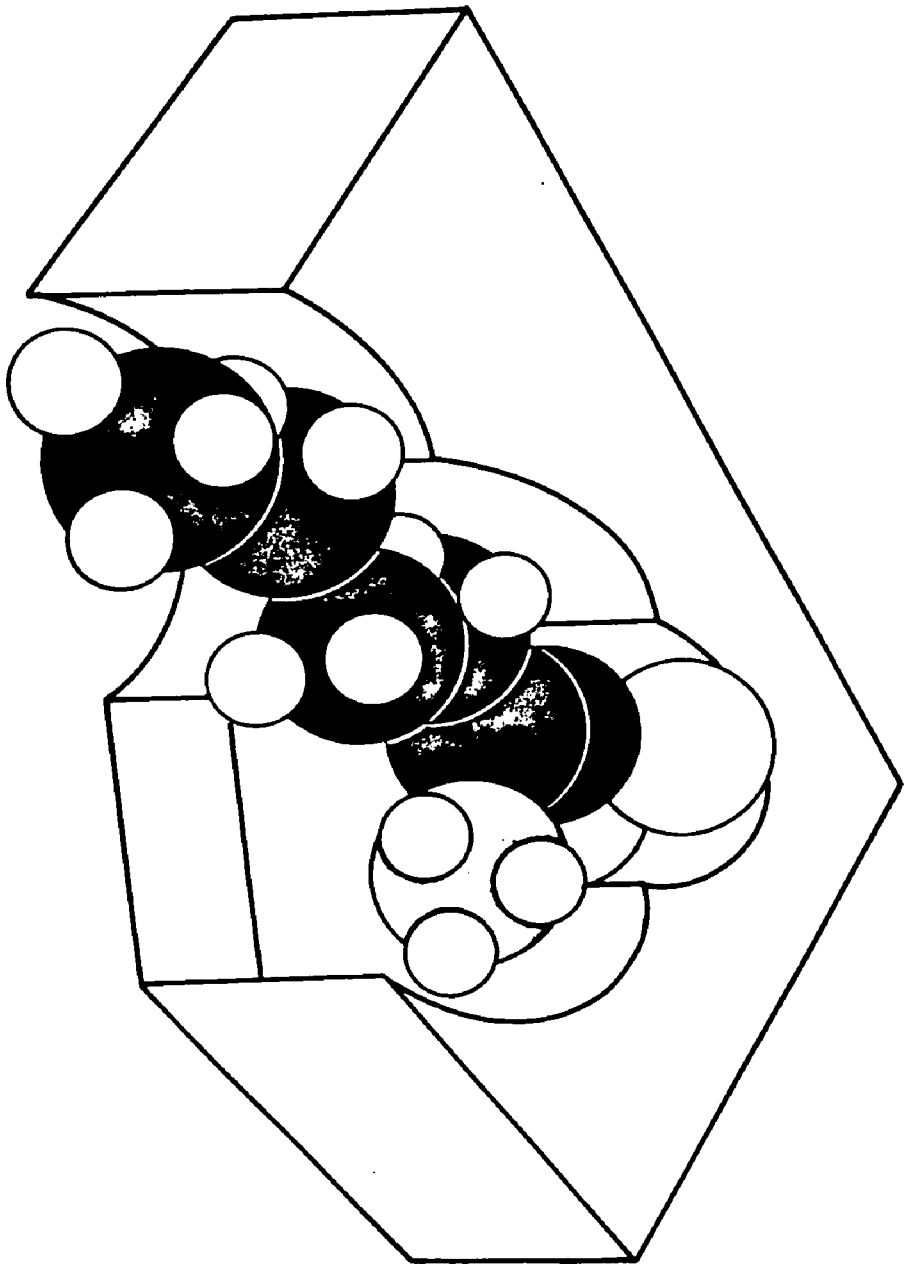


Figure 20. Binding of norLeucine to the proposed ASC site.

Legend for Figure 20.

A representation of the norleucine molecule is shown filling the proposed ASC binding site. The β- and γ-carbons have been placed in accordance with the structure of hydroxyproline. The length of the side chain defines the limit of the apolar binding region, and is intended to show obstruction of Na^+ binding.

carboxyl groups, such that the inhibitory action of neutral amino acids drops off beyond a point defined by hydroxynorleucine and glutamine. Further experimentation should reveal whether the length limitation of the Ehrlich cell ASC system is the same as the Ehrlich cell Ly^+ system, or shorter as in the pigeon erythrocyte ASC system.

For the Ly^+ and ASC systems of all the cell types studied, effects have been observed that appear to result from a direct interaction between a bound cation and a polar group on the neutral amino acid side chain. The binding of either an amino acid of appropriate structure or of Na^+ lowers the concentration of the other which is required to produce half the maximal effect. This decrease in K_i or K_m is observed for amino acids with apolar side chains, but is especially notable for amino acids having a polar group on the side chain. Such interactions may provide significant clues to the way in which specific cation binding sites are generated in proteins, perhaps with particular significance to alkali metal transport.

Under certain conditions the systems appear to be functionally as well as chemically similar. The Ly^+ system can be caused to act as a Na^+ and neutral amino acid transport system, by measuring exchange into a phase containing cationic amino acids. The Na^+ and amino acid fluxes obtained in this way are not tightly coupled, however; low fluxes are observed in the absence of one of the two co-substrates.

The ASC and Ly^+ systems would be expected to be similar, because of the similar nature of their substrates. We have made use of the same substrates and inhibitors in studying both systems. The similarity of the systems may be more than a co-incidence, however. Both systems may have evolved from a common precursor by gene duplication followed by separate development, as has been suggested for the chains of hemoglobin (137), and other proteins (138).

Bovine α -lactalbumin and egg white lysozyme provide an example of duplication and separate evolution leading to proteins with clearly different functions but similar substrate specificity (139). Such a development of several functions from a common precursor appears to be a more efficient means of increasing genetic content and cellular complexity than is a separate development of each function.

The studies of the $\underline{Ly}^+$ system and the present study of the interaction of amino acid and cation analogues with the ASC system have provided evidence for the structures of the binding sites, and for some of the features of the transport mechanism. We hope and expect that the means will become available to isolate these transport structures, so as to permit a study of their chemical properties and similarities.

SUMMARY

Earlier observations were confirmed that the rates of Na^+ and amino acid uptake show a hyperbolic dependence on concentration, and that the ratio of the rates of their uptake had a differing, characteristic value for each amino acid. The amino acid substrate and Na^+ each increased the maximal rate of uptake and decreased the concentration producing half-maximal uptake of the other. Amino acids which inhibited the system but which did not serve as substrates produced half-maximal inhibition at a concentration independent of Na^+ concentration. Kinetic observations and the essentiality of both co-substrates for transport were consistent with translocation by way of a ternary complex of Na^+, amino acid, and the transport mediating structure. The differing rates of Na^+ and of amino acid entry showed that translocation was not rate-limiting and that the observed rates of entry or exodus were rates of exchange of labeled substrates with unlabeled substrates in the other phase.

By studying the effect of Na^+ on the kinetic parameters of amino acid uptake, the requirements for binding to the ASC site could be evaluated separately from the requirements for transport. Points of binding for the α-amino and carboxyl groups were proposed to explain the inhibitory properties of glycine and the stereospecificity of binding. For amino acids with side chains, three separate effects were shown to change the apparent affinity, as expressed by the K_i or K_m values. Affinity increased with the apolar mass of the side chain up to a length limitation defined by norleucine, so that an apolar binding region of limited dimensions was proposed. In the presence of Na^+, an increase in

apparent affinity was observed for amino acids having side chains without hydrocarbon branches and not exceeding norvaline in length. The translocation process appeared responsible for this change because the extent of the decrease in K_m with Na^+ concentration was well correlated with the maximal rate of uptake of the amino acid and with its maximal ability to stimulate exodus of amino acids by exchange.

A polar group on the β- or γ -carbon produced an additional increase in apparent affinity and raised the ratio of Na^+ to amino acid entry. Polar groups in other positions lowered the apparent affinity perhaps through repulsion by the apolar binding region. In this way, a relatively small polar binding region was shown to lie close to the points of α-amino and carboxyl group binding. We have concluded that this region is the point of Na^+ binding, in analogy to the highly similar effects observed with the $\underline{Ly^+}$ system, and with the correlation of reduced reactivity with features of amino acid side chains that would prevent the access of Na^+ to this region. A type of bonding may be formed between the co-substrates by way of the unshared electrons of the polar group. The differing reactivity of the stereoisomers of γ -hydroxyproline showed that the point of Na^+ binding is positioned <u>trans</u> to the bound carboxyl group.

Although the transport process had a nearly total specificity for Na^+, other monovalent cations inhibited uptake by competing with Na^+ binding. Only Na^+ appeared capable of producing the changes secondary to binding in the transport mediating structure which are required for translocation. The stereospecificity of cationic amino acid binding showed that these inhibitors were bound in the manner of neutral amino acids. The ω-guanidino amino acids were more effective inhibitors

than were the diamino acids, consistent with the greater ability of the guanidinium ion to compete with Na^+ binding, relative to ammonium ion. In this way, cationic amino acid inhibition provided more evidence for the position of Na^+ binding and for the essentiality of Na^+ in the transport process.

Evidence was obtained that the ASC systems of differing cell types show only minor differences, as is the case with the Ly^+ system. The similarity of the ASC and Ly^+ binding sites was also noted and separate evolutionary development from a common precursor was suggested as the basis for their similarity.

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