Basis for Substrate Preference of Amino Acid Transport System L over Amino Acid Transport System A†

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ABSTRACT: 1-Aminocyclohexanecarboxylic acid, 1-aminocycloheptanecarboxylic acid, and 1-aminocyclooctanecarboxylic acid all inhibited histidine uptake through transport system L of the S37 ascites tumor cell to similar high degrees. Although they were somewhat more effective inhibitors than 1-aminocyclopentanecarboxylic acid, and far superior to α-aminoisobutyric acid, as inhibitors of system L, these three cyclic side-chain analogues were somewhat less effective as inhibitors of histidine uptake through transport system A than were 1-aminocyclopentanecarboxylic acid and α-aminoisobutyric acid. Exchange experiments suggested that 1-aminocycloheptanecarboxylic acid bound to exchange system L more avidly than did 1-aminocyclopentanecarboxylic acid, but that the carrier was transferred more readily in combination with the latter compound. A comparison of the bond angles and transport system preferences of the compounds utilized in this study with bond angles and transport system preferences of other compounds suggested that precise steric considerations are not of paramount importance in the binding of substrates to amino acid transport systems A and L. A certain amount of hydrocarbon in the side chain is probably the primary requirement, for preferential interaction with system L, and there would seem to be a high degree of variability permitted that would still afford formation of apolar interaction at the active site of transport system L.

Amino acid uptake into ascites tumor cells has been known to occur by multiple transport systems of overlapping specificities (Tenenhouse and Quastel, 1960) and patterns of competitive inhibition were suggested as one means of differentiating the various transport systems present (Ahmed and Scholefield, 1962). Two principal amino acid transport systems which were so studied in the Ehrlich mouse ascites tumor cell were named L and A, meaning "leucine-preferring" and "alanine-preferring" (Oxender and Christensen, 1963). A series of studies of amino acid transport in the mouse sarcoma 37, or S37, ascites tumor cell described the occurrence of two transport systems for neutral amino acids resembling the two Ehrlich cell transport systems so that they have also been referred to as the L and A systems (Matthews et al., 1970, 1975; Matthews, 1972).

It has been suggested that amino acids bind to transport systems by a three-point attachment, the three points being the α-amino group, the α-carboxyl group, and the side chain (Oxender, 1965). Methylation of the amino group was found to be permissible for transport system A, but not L (Inui and Christensen, 1966). It was also found to be possible to substitute a methyl ketone or a methyl ester for the α-carboxyl group (Matthews et al., 1975), but omission of either the α-carboxyl group or the α-amino group eliminated interaction with amino acid transport systems (Oxender, 1965; Schultz et al., 1972). Within the range of naturally occurring substrates, however, it was readily apparent that the one attachment point which varied was the amino acid side chain so that it was necessarily in the interaction of the side chain with the transport system that preferences for one transport system or the other existed. An amino acid with a bicyclic hydrocarbon side chain, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH)[1] was synthesized (Christensen et al., 1969; Zand et al., 1974) and found to have a very high degree of preference for system L (Tager and Christensen, 1971; Matthews et al., 1975).

Tager and Christensen (1971) made the interpretation that interaction of the substrate with transport system L's active site was due to very precise steric considerations. They suggested, first, that BCH interacted so well with transport system L because the angle formed by the two carbon atoms adjacent to the α-carbon atom was retracted to about 103° (Apgar and Ludwig, 1972), 6° less than the angle seen in α-methylated analogues which preferred transport system A rather than L. Second, they suggested that BCH was a particularly good substrate for system L because it was a rigid structure, and that the placement of carbon atoms 1, 6, and 7 in the side chain must be particularly advantageous for interaction with system L. This was based, in part, on the drawing of structures of natural amino acids and analogues that interacted well with system L in conformations that would approximately superimpose on the one favored isomer of BCH,(-b)-BCH. One problematic feature of this interpretation, in our view, was the known broad specificity of transport system L (Oxender and Christensen, 1963; Matthews et al., 1970). For that reason, we decided to study a series of cyclic side-chain analogues along with α-aminoisobutyric acid (AIB). This series of compounds possessed differing angles in the side chain at the α-carbon atom, and also differing quantities of hydrocarbon in the side chain in addition to differing specific conformations. Our conclusion was that the angle in the side-chain carbon atoms was not critical, and that the basis for interaction with transport system L was formation of an apolar bond with loosely defined steric requirements.

Materials and Methods

Preparation of Analogues. Cyclic ketones were converted to the corresponding spirohydantoins by the addition of

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[1] Abbreviations used are: BCH, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid; AIB, α-aminoisobutyric acid; ACPC, 1-aminocyclopentanecarboxylic acid; S37, sarcoma 37 mouse ascites tumor.

aqueous potassium cyanide to alkaline ethanolic solutions of the starting materials. The amino acids were then obtained by heating the spirohydantoins with barium hydroxide (Zand et al., 1974).

Other Chemicals and Solutions. L-Histidine was obtained from Sigma Chemical Co. ^{3}H-Labeled L-histidine was obtained from New England Nuclear Co. Solutions were made fresh or stored after being sterilized by ultrafiltration using a Swinnex filter obtained from Millipore Co.

Preparation of Ascites Cells. Sarcoma 37, or S37, ascites tumor cells originated as a mouse mammary carcinoma which then underwent transformation of cell type during an early transfer (Greene and Harvey, 1968). These cells have since been used in many laboratories in diverse studies and were maintained in our laboratory by weekly intraperitoneal transplantation in male Swiss white mice weighing 20–25 g. Cells were washed in a modified Krebs–Ringer phosphate buffer (see below) and packed cells resuspended in buffer at a dilution of approximately 1:7. This suspension (0.3 mL) was injected in each mouse, and the cells were harvested 6–9 days later. Mice were sacrificed by cervical dislocation and an incision made in the abdomen through which the ascitic fluid was aspirated. The cells were washed three times prior to use by suspending them in the modified Krebs–Ringer phosphate buffer, centrifuging for 3 min at 500 rpm in a Model HN-S centrifuge (International Equipment Co.) equipped with a No. 809 head and aspirating off the supernatant liquid.

Krebs–Ringer Phosphate Buffer. The medium employed was a modification of a Krebs–Ringer phosphate-buffered salts mixture suggested by Umbreit el al. (1964). It contained 0.154 M NaCl, 0.154 M KCl, 0.11 M $CaCl_2$, and 0.154 M $MgSO_4$ (100:5:1:1, v/v). This salts solution was added to 0.1 M phosphate buffer, pH 7.4 (9:1, v/v). The KH_2PO_4 in the Umbreit formulation (1 part in the salts mixture) was replaced with 1 part of KCl. $CaCl_2$ was lowered from 3 parts to 1 part to prevent precipitation of $Ca_3(PO_4)_2$. This variation in calcium content was found not to adversely affect amino acid transport in S37 cells. The salts mixture without the phosphate buffer was prepared at 5× the stated concentrations for routine inclusion in more complex media. Use of the complete buffered salts medium rather than saline has been found to yield better results in preliminary washing of cells and in terminating incubations.

Execution of Transport Experiments. Experimental designs called for the incubation of as many as 40 samples in near simultaneous fashion. Test incubations were conducted for 2 min at 20 °C in media containing [^{3}H]histidine. Incubations were terminated by pouring the 3 mL of media into tared 12-mL centrifuge tubes containing 8 mL of chilled Krebs–Ringer buffer. Decreasing the temperature to 4–6 °C reduced measured losses of labeled material to less than 1% per min.

The large S37 cells tended to settle rapidly and also to aggregate in a variable manner. For these reasons, the assumption of uniform aliquoting from the starting cell suspension increased scatter in the data; the determination of wet weight of the cells in each individual medium improved the precision of the data over that attainable by continuously stirring the starting cell suspension and assuming uniform aliquoting. A sequenced set of centrifuge tubes which had been repeatedly cleaned and weighed to an accuracy of 0.2 mg was maintained. Cell pellets weighed about 30–60 mg.

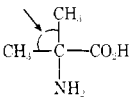
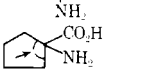
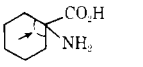
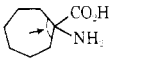
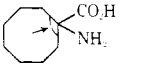
After incubations were terminated by mixing the media with chilled Krebs–Ringer buffer in centrifuge tubes, the samples were centrifuged for 1 min at 1940*g* (Model HN-S centrifuge, International Equipment Co., equipped with a No. 809 head). The supernatant solution was removed by siphoning, and extracellular radiolabel entrained in the pellet was removed by resuspending the pellet in 8 mL of chilled Krebs–Ringer buffer and repeating the centrifugation. After siphoning off the second supernatant, a third 1-min centrifugation at 1940*g* was used for the final packing of the cell pellet. The external surfaces of the tubes were wiped immediately, and the tubes were inverted to drain for 15 min. Moisture on the interior surface of the centrifuge tubes was removed using lint-free tissues and glass rods. The wet weight of the cell pellets was determined by difference. Under these conditions, the cell pellets have been found to consist of 15.8% solids, measured by dry weight fraction, 17.8% extracellular space, determined by sulfate label, and 66.4% intracellular water space, estimated by difference.

[^{3}H]Histidine was released by suspending each cell pellet in 5 mL of 95% ethanol for 30 min, stirring the suspension vigorously, and then centrifuging at 1940*g* for 1 min. A 0.5-mL aliquot of each ethanolic extract was taken for liquid scintillation counting.

Alternative Experimental Designs. Our experience indicated that the maximum number of samples which could be handled effectively in any one experimental design was approximately 40. To most clearly elucidate the effects of analogues or other experimental conditions in differential fashion on uptake by the A and L systems, we examined the deflection of a biphasic double-reciprocal plot obtained using histidine as the labeled test substrate (Matthews et al., 1970, 1975; Matthews, 1972). Since the control series required ten concentrations as did any analogue series, only one analogue could be employed in a design when replicates at each concentration were performed, or a maximum of three analogues could be compared without replicates. To compare a larger number of compounds or conditions for effect on transport systems A and L, 0.1 mM L-[^{3}H]histidine uptake was used in duplicates or triplicates as a test for system L activity, and 10 mM L-[^{3}H]histidine uptake was employed as a test for system A activity. At the extremes of the concentration range most usually employed, uptake from a 0.1 mM histidine medium was usually about 85% representative of system L activity, whereas uptake from a 10 mM histidine medium was usually about 80% representative of system A activity (Matthews et al., 1975). In measuring the effects of a variety of analogues upon uptake through the A and L amino acid transport systems, the experimental design was therefore varied depending upon the number of analogues to be employed or the degree of precision with which the effects of transport systems A and L were to be disentangled.

In experiments in which exchange was to be measured, more regular results were obtained when exchange efflux rather than exchange uptake was measured. This may have been due to the rapidity with which an exchange uptake process would bring cellular concentrations to a level at which flow in the reverse direction was a significant process. In exchange efflux experiments, the much greater volume of the extracellular space than the intracellular space aided in minimizing any reversed-flow problem. In the present case, S37 cells were first incubated 30 min at 37 °C with 1 mM L-[^{3}H]histidine present. Incubations were terminated by pouring the cells into ice-cold Krebs–Ringer phosphate buffer, centrifuging them for 1 min at 1940*g*, combining the cells, and resuspending them in chilled Krebs–Ringer phosphate buffer. The cells were centrifuged and resuspended in chilled Krebs–Ringer phosphate buffer twice more prior to using them in incubations in which they were exposed to varying concentrations of unlabeled amino acids. The second incubations were conducted for 5 min at 20 °C. The difference in concentration, Δ, between cells exposed

TABLE I: Inhibitory Effects of Analogues upon Activities of Transport Systems A and L, and Side-Chain Angles about the α-Carbon Atoms.

Angle value[a] (deg) (authority)	Structure[a]	Analogue[b]	System A uptake as % control[c]	System L uptake as % control[c]
109 (Tager and Christensen, 1971)		AIB	76 (71, 81)	83 (80, 86)
103–104 (Chandrasekharan et al., 1968; Mallikarjunan et al., 1972)		ACPC	74 (69, 79)	14 (12, 16)
112–114 (Chacko et al., 1971b; Varughese et al., 1975)		1-Aminocyclohexane-carboxylic acid	83 (81, 85)	8 (7, 8)
115 (Chacko et al., 1971a)		1-Aminocycloheptane-carboxylic acid	81 (79, 82)	8 (7, 9)
116 (Srikrishnan et al., 1971)		1-Aminocyclooctane-carboxylic acid	79 (77, 80)	9 (8, 10)

[a] The angle referred to is the angle formed by the bonds attaching side-chain carbon atoms to the α-carbon atom. [b] Analogues were present at a concentration of 5 mM and incubations were conducted for 2 min at 20 °C; other conditions were as described in the Materials and Methods section. [c] The system A uptake test was 10 mM L-[^{3}H]histidine; the system L test was 0.1 mM L-[^{3}H]histidine, as described in more detail under Materials and Methods. The percent of control uptake was an average of replicates, and was determined on two separate occasions (individual results of the experiments in parentheses).

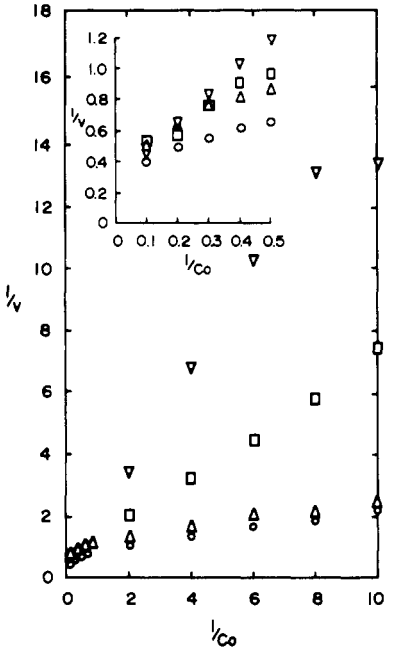


FIGURE 1: Inhibitory effects of various analogues upon L-[^{3}H]histidine uptake through amino acid transport systems A and L. Incubations were conducted for 2 min at 20 °C; v represented ½ the intracellular concentration attained after 2 min. C_0 represented the extracellular concentration of L-[^{3}H]histidine. Either 1-aminocycloheptanecarboxylic acid (▽), ACPC (□), AIB (△), or no analogue (○) was present at a concentration of 2.5 mM. Repetition of the experiment yielded similar results.

to no other amino acid and cells exposed to a given analogue at a given concentration represented the exchange efflux induced by the amino acid analogue.

Liquid Scintillator. The scintillator employed was a modification of one proposed by Patterson and Greene (1965). It formed stable solutions with aqueous or ethanolic samples of 1 mL or less and gave counting efficiencies as high as 46% with ^{3}H. The solvent consisted of ethanol, Triton X-100, and reagent grade toluene (1:2:4, v/v). The scintillator was prepared by adding 100 g of naphthalene, 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 10 g of 2,5-diphenyloxazole to each liter of the mixed solvent. The scintillator was stirred overnight to dissolve the 1,4-bis[2-(5-phenyloxazolyl)]benzene.

Results

In examining relative affinities for transport systems A and L within the complete series of analogues considered in this study, AIB was seen to have very little effect upon use of transport system L by the test substrate, L-[^{3}H]histidine, ACPC had considerably greater effect upon system L, and the remaining three analogues, 1-aminocyclohexanecarboxylic acid, 1-aminocycloheptanecarboxylic acid, and 1-aminocyclooctanecarboxylic acid, all exhibited a similar and higher degree of inhibition of histidine uptake under conditions favoring usage of system L (Table I). At the same time, AIB was a relatively effective inhibitor of histidine uptake through system A, as was ACPC. The three larger analogues were relatively ineffective as inhibitors of uptake through transport system A. The initial experiment therefore suggested that AIB was relatively specific for interaction with transport system A, and that ACPC interacted with both transport system A and transport system L. The three analogues with larger cyclic side chains appeared to be similar in showing specificity for transport system L.

To confirm the suggestions of the first experiment, a second type of experiment was conducted in which deflection of biphasic kinetic plots was examined. Because of the need to restrict the number of analogues employed in this design (see Materials and Methods), 1-aminocycloheptanecarboxylic acid was chosen as representative of the three larger cyclic side-chain analogues used in the first type of experiment. AIB raised and deflected the limb of the double-reciprocal plot nearest the origin, which was associated with dominance of transport system A, but the limb distant from the origin associated with system L was only displaced slightly upward and remained parallel to the control uptake. This behavior would suggest that the small degree of inhibition seen at low histidine concentration was largely due to a contribution from transport system A in the region dominated by system L. ACPC evidently inhibited transport system L as the limb of the plot in the low concentration region was displaced upward in position and

deflected upward in angle relative to control (Figure 1). When only ACPC was compared to control, it was apparent that some residual activity of both systems remained under the conditions of the experiment since the biphasic character of the plot was retained (Figure 2). However, the limb of the plot nearest the origin was somewhat deflected also, indicating that there was an effect upon system A as well as system L. 1-Aminocycloheptanecarboxylic acid deflected the limb of the plot distant from the origin much more than ACPC had; this suggested a greater affinity of the larger analogue for transport system L (Figure 1). A replot of the data for 1-aminocycloheptanecarboxylic acid alone was very nearly linear with the exception of one aberrant data point, so that transport system L had apparently been almost completely obliterated by the strong interaction with the larger analogue. Convergence of this line toward the control plot at high concentrations (insert of Figure 1) suggested that 1-aminocycloheptanecarboxylic acid had little effect upon transport system A. These results confirmed in greater detail those of the first experiment: AIB was reasonably specific in its interaction with system A; 1-aminocycloheptanecarboxylic acid was reasonably specific in its interaction with system L; ACPC interacted with both principal neutral amino acid transport systems.

Augmentation of L-[^{3}H]histidine efflux from S37 cells in the presence of increasing concentrations of extracellular analogues appeared to be a saturable phenomenon and the reciprocal of the difference engendered by the presence of the analogue was plotted vs. the reciprocal of extracellular analogue concentration. This was a simplistic approach to the exchange phenomenon in that previous theoretical treatments suggest a more complex kinetic expression (Jacquez, 1961, 1964). However, the available data did not justify an attempt to fit to kinetic expressions having a larger number of parameters. In accord with prior experience (Matthews et al., 1970), these reciprocal plots gave no evidence of a biphasic character. More scatter was evident in these data than in data for the previous experiments reported in this study. The greater difficulty in retaining precision in experiments of this type may be associated with several factors. First, there are an increased number of manipulations of the ascites cells. Second, the dependent variable is a difference of two experimental values in this case so that its magnitude is less than either and its variability is dependent upon experimental error in the determination of either of two values. The significance of the precision problem to the results obtained is that in the cases of ACPC and 1-aminocycloheptanecarboxylic acid, there may be some uncertainty in the precise values of the graphically determined kinetic parameters to a presumed saturation equation of the Michaelis–Menten type, but there is no doubt that one system has functioned with a behavior that would be characteristic of an exchange system. In the case of AIB, however, the data were equivocal and there was a sense of arbitrariness in plotting a line. The V_{max} values for the exchange process indicated that the magnitude of any possible exchange effect involving AIB was of a lower order of magnitude than that seen with ACPC or 1-aminocycloheptanecarboxylic acid, and these data have been deleted from further consideration due to its equivocal nature. Another point was that the K_m value determined for 1-aminocycloheptanecarboxylic acid, 0.17 mM, was much smaller than the K_m value for the process when ACPC was used, 1.3 mM. This suggested stronger interaction of the larger analogue with the exchange system, which would be consistent with the stronger inhibitory action on system L (Figure 1) and the previous finding that system L was the exchange system in S37 cells (Matthews et al., 1970). Nevertheless, the V_{max} for the exchange process was consistently lower for 1-aminocycloheptanecarboxylic acid than it was for ACPC, averaging 3.6 mM/min as opposed to 5.2 mM/min for ACPC. This would be consistent with a more rapid transfer of the carrier binding ACPC than the carrier binding 1-aminocycloheptanecarboxylic acid.

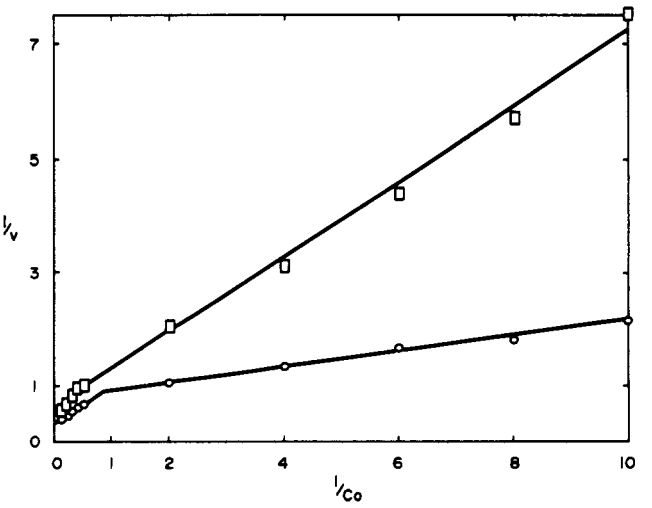


FIGURE 2: Inhibitory effects of ACPC (□) upon L-[^{3}H]histidine uptake through amino acid transport systems A and L. Details as for Figure 1.

Discussion

Tager and Christensen (1971) suggested that α-methylation of an analogue which might otherwise have shown preference for system L prevented uptake through that route. Since BCH had a methylene group attached at the α-carbon but yet showed a very high specificity for system L (Tager and Christensen, 1971; Matthews et al., 1975) it would seem to have been a logical rationalization to suggest that diminution of the angle formed by side-chain substituents about the α-carbon atom from a value of about 109° for AIB or other α-methylated analogues to 103° in the case of BCH was of cardinal importance in permitting interaction with system L. Tager and Christensen (1971) extended their argument for specificity in very precise steric terms by suggesting that certain other substrates, such as leucine or isoleucine, that preferred system L could be fit to the conformation of (-b)-BCH. One might at this point ask, how is it that methionine, phenylalanine, and tryptophan, all good L system substrates (Oxender and Christensen, 1963; Matthews et al., 1970), are selected for interaction with system L? We now note that the value for the side-chain angle is very similar for ACPC and BCH, but that the reactivities of the two analogues with transport systems differ markedly. Whereas BCH is very specific to system L, ACPC appears to be a moderately effective inhibitor of system L which also interacts with system A (Figures 1 and 2; Table I). We further note that 1-aminocyclohexanecarboxylic acid, 1-aminocyclopheptanecarboxylic acid, and 1-aminocyclooctanecarboxylic acid all have side-chain angles slightly larger than AIB, and these analogues all exhibit a strong specificity for system L (Table I; Figure 1). Crystallographic studies carried out on the six-, seven-, and eight-membered ring analogues yield somewhat varying structures which would not seem to superimpose readily with BCH (Chacko et al., 1971a,b; Srikrishnan et al., 1971; Varughese et al., 1975). We also note that both glycine and alanine are poor substrates for system L; they prefer system A (Oxender and Christensen, 1963; Matthews, 1972). We therefore conclude that the selection of transport system L is not primarily on the basis of precise steric considerations, and is not so much on the basis

of the lack of a methyl group on the α-carbon atom as on the presence of an adequate amount of apolar material in the side chain.

In attempting to define what an adequate amount of hydrocarbon is, we would suggest that minimal amounts for interaction with system L would be that present in ACPC or the natural substrate valine (Oxender and Christensen, 1963; Matthews, 1972). This would consist of a group of three carbon atoms, not counting a second side-chain attachment to the α-carbon atom, as a methylene group attached to a polar moiety probably makes little or no contribution to hydrophobic effects (Tanford, 1973). A more optimal interaction with system L is apparently reached with addition of at least one more CH_2 group, as in the case of 1-aminocyclohexanecarboxylic acid or leucine.

A semiquantitative examination of the nature of the interaction may be made as follows. If we make the assumption that the rate constants for translocation steps in membrane transport or exchange are small compared with the rate constant for the dissociation of carrier-substrate complex, then the K_m values of Table I may approximate dissociation constants for the carrier-analogue complexes. Applying the relationship $\Delta G° = -RT \ln K_{eq}$ to the K_m values found for exchange, the $\Delta G°$ value for dissociation of ACPC-carrier complex is +3.87 kcal/mol, whereas $\Delta G°$ for dissociation of the 1-aminocycloheptanecarboxylic acid-carrier complex is +5.05 kcal/mol. Adding two CH_2 groups to the substrate has therefore increased bonding of the analogue to the L system carrier by −1.18 kcal/mol. Tanford (1973) suggested that the transfer of alkanes or fatty acids from an aqueous phase to an apolar phase was associated with a free-energy change of −884 or −825 cal/mol CH_2. In view of the assumptions made, and the possibility that binding to the transport site does not completely remove the analogue from the aqueous phase, we consider the agreement in free-energy change values quite satisfactory. Preference for system L is primarily dictated by a side chain having about 4–6 carbon atoms in a reasonably compact but variable arrangement. It is quite possible that the apolar bonding that takes place involves some flexibility in the active site as well as the substrate.

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