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BIPHASIC KINETIC PLOTS AND SPECIFIC ANALOGS DISTINGUISHING AND DESCRIBING AMINO ACID TRANSPORT SITES IN S37 ASCITES TUMOR CELLS

RICHARD H. MATTHEWS, MARLINDA SARDOVIA, NEIL J. LEWIS and ROBERT ZAND

Department of Physiological Chemistry and College of Pharmacy, Division of Medicinal Chemistry, The Ohio State University, Columbus, Ohio 43210 and Biophysics Research Division, Institute of Science and Technology, University of Michigan, Ann Arbor, Mich. 48105 (U.S.A.)

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SUMMARY

Curve-fitting procedures indicated that exo-2-amino-bicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) modified V and K_m for one of two systems serving for histidine transport into the S37 ascites tumor cells. When this system was obliterated by leucine in the medium, BCH had no effect on histidine transport.

Curve-fitting procedures similarly suggest *N*-methyl-α-aminoisobutyric acid affected the K_m and V values for the other histidine-transporting system and that carboxymethylhistidine (His(Cm)) inhibited both transport systems. His(Cm) further inhibited histidine uptake into leucine-inhibited cells. K_m and V values were altered simultaneously in the presence of several inhibitory analogs.

Alanine methyl ester markedly inhibited high-concentration histidine uptake, whereas leucine methyl ester markedly inhibited low-concentration histidine uptake.

The present results confirm earlier suggestions that our high c system is Christensen's A system and our low c system his L system. We also confirm a very high degree of specificity of *N*-methyl-α-aminoisobutyric acid for the A or high c system, and of BCH for the L or low c system. We suggest the utility of combining two approaches to the study of transport system properties; use of specific analogs and modification of biphasic plots. We demonstrate that the carboxyl group is not a prerequisite molecular feature for inhibitory interaction with the A or L system.

INTRODUCTION

In 1960, Tenenhouse and Quastel [1] surmised that there were multiple transport systems for amino acid entry into Ehrlich ascites cells on the basis of differential effects of potassium and temperature upon glycine and L-*S*-ethylcysteine entry as well

Abbreviations: BCH, exo-2-amino-bicyclo-(2,2,1)-heptane-2-carboxylic acid; His(Cm), carboxymethyl histidine. c_0, extracellular concentration of a test solute; c_i, intracellular concentration of the test solute.

as patterns of competitive inhibition. In that report, a biphasic double-reciprocal plot for L-*S*-ethylcysteine uptake is presented, but the authors interpreted this to be the result of a combination of passive diffusion with mediated uptake as routes of entry. In 1962, Ahmed and Scholefield [2] proposed a systematic method of using patterns of competitive inhibition to discriminate multiple overlapping amino acid transport systems and Oxender and Christensen [3] in 1963 used patterns of competitive inhibition to divide neutral amino acid uptake into Ehrlich ascites cells into components attributable to an A (alanine-preferring) and an L (leucine-preferring) system. In 1964, Christensen [4] published a biphasic double-reciprocal plot for L-α-γ-diaminobutyric acid uptake with the interpretation that this indicated interaction with two transport systems. We subsequently found a biphasic double-reciprocal plot for histidine uptake into either Ehrlich or S37 ascites tumor cells and used its occurrence under varied conditions as a means of elucidating the properties of two transport systems of the S37 ascites tumor cell [5, 6]. Rosenberg and coworkers [7, 8] employed a somewhat similar approach in studying proline and glycine transport in kidney tubules.

Christensen and coworkers [9] have concentrated on the development of analogs which would be specific to individual transport systems as a means of distinguishing the properties of the systems, and have suggested that *N*-methyl-α-aminoisobutyric acid is specific for the A system whereas exo-2-amino-bicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) is specific for the L system. However, these two analogs are replacements for α-aminoisobutyric acid and *tert*-leucine, respectively. Also, the use of this approach led to the assertion that removal of Na^+ from the medium eliminated one system, the A system [10]. This hypothesis has been shown to be incorrect [5]. While non-metabolizable specific analogs could be useful for many purposes in studying transport systems we have thought it to be of value to subject them to an independent test of degree of specificity. To that end we have examined their effects upon biphasic double-reciprocal plots of histidine uptake. In addition to *N*-methyl-α-aminoisobutyric acid and BCH we have also employed certain other amino acid analogs for comparison purposes.

MATERIALS AND METHODS

His(Cm) preparation

Carboxymethylhistidine (His(Cm) or 4-imidazolyl-3-amino-2-butanone) was prepared in our laboratories according to the method of Smissman and Weis [11] from L-histidine · HCl · H_2O (Sigma), acetic anhydride and pyridine by the Dakin-West reaction followed by the acid hydrolysis of the intermediate amidoketone. Synthetic His(Cm) was purified by fractional crystallization and was analytically pure. Preliminary tests had been carried out with small quantities of His(Cm) supplied by McNeil Laboratories, Inc., Fort Washington, Pennsylvania.

N-methyl-α-aminoisobutyric acid preparation

N-Methyl-α-aminoisobutyric acid, or *N*-2-dimethylalanine, used for these studies was prepared from acetone, KCN and methylamine hydrochloride via a modified Strecker amino acid synthesis [12–14] and compared with literature physical parameters. The intermediate amino nitrile was separated from cyanohydrin by HCl

precipitation of the amine salt from ether extracts. The amino nitrile was converted to the pure amino acid by hydrolysis with 8 M H_2SO_4 and was purified by fractional crystallization.

BCH

To a solution of 110 g (1.0 mol) of nonbornanone in 250 ml of methanol in a 1-l round bottom flask was added 55 g (1.0 mol) of NH_4Cl and 65.2 g (1.0 mol) of KCN in 250 ml of distilled water. The flask was stoppered and placed in a water bath at 80–85 °C for 8 h. After cooling to room temperature, the contents were transferred to a 3-l beaker in a hood and concentrated HCl was added until a pH of 1.0 was reached. The solution was transferred to a 2-l round bottom flask and concentrated on a rotary evaporator until nearly dry. The collected amino nitrile hydrochloride was recrystallized rapidly from boiling HCl containing some Norite. Three recrystallizations yielded a white powder of m.p. 198–200 °C (decomposed).

The recrystallized aminonitrile hydrochloride was dissolved in 1 l of 6 M HCl and autoclaved at 120 °C for 24 h. The solution was filtered through a sintered glass funnel while still hot, the filtrate distilled until the original volume was reduced by 70 % and the remaining liquid then removed on a rotary evaporator. The residual powder was dried at 110 °C overnight and triturated repeatedly (at least six times) in methanol/ether (1/1, v/v). The residue was redissolved in water, taken to dryness and the trituration repeated. The combined methanol/ether extracts were taken to dryness and the residue dissolved in 500 ml of boiling water, filtered and cooled rapidly in an ice bath. Upon adjusting the pH to 5.5, amino acid precipitated. Analysis of the product on the amino acid analyzer indicated it to be at least 99 % isomer a, m.p 321–323 °C (decomposed).

General procedures

Curve fitting was done using a general purpose non-linear curve-fitting program (Matthews, R. H. and Alben, J. O. unpublished). Coefficients returned by this program were converted to kinetic parameters by a short routine prepared for a programmable calculator. Some indication of the reliability of the fitting procedures is given in the following section. Other procedures are as reported previously [5, 6].

RESULTS

Reliability of the curve-fitting procedure

To test the curve-fitting programs, three sets of ideal data were generated from arbitrarily chosen values for the parameters V_1, V_2, K_1 and K_2 according to the equation

$$v = \frac{V_1 \cdot c_0}{K_1 + c_0} + \frac{V_2 \cdot c_0}{K_2 + c_0} \tag{1}$$

These sets of ideal data were processed and the values returned for the four kinetic parameters agreed within 1 % of the original values in all cases.

Substitution of one "bad data point" in the ideal data led to a fitting that did not return the original parameters. It was found that expressing the activity of the two systems in terms of V_1/K_1 and V_2/K_2 minimized this effect somewhat.

When the parameters obtained by fitting 24 data sets from separate experiments and utilizing different S37 cell preparations were compared, there was no minimization of dispersion gained by considering V/K ratios as opposed to V or K for either transport system. We, therefore, consider the utilization of V/K ratios to be a means of minimizing experimental error with a given preparation of cells rather than a manipulation which minimizes the variations in functional properties of the cells.

In deciding whether or not an analog had an effect on a transport system we have divided V/K for a control series by V/K for the experimental series. A quotient close to 1.0 would indicate no effect on the transport system; a quotient much larger than 1.0 would indicate an inhibitory effect.

BCH specificity

BCH is much more inhibitory than His(Cm) is of histidine uptake in the low-concentration region, but cross-over occurs so that His(Cm) is more inhibitory in the high-concentration region. Uptake of histidine in the presence of BCH gives a linear double -reciprocal plot which approaches control values at the highest histidine concentrations studied. This is a first indication of the specificity of BCH for the L system (low c system).

A second indication that BCH is specific to the L (low c) system was gained by fitting a curve for the concentration-dependent uptake of histidine in the presence and absence of BCH (Table I). It will be noted that in most cases the fitting procedures

TABLE I

INHIBITION OF HISTIDINE UPTAKE BY BCH

Incubations were conducted for 2 min at 20 °C. v_{obs} is one-half the observed intracellular concentration in mM units at termination of incubation (average of two determinations). v_{fit} is the result of the curve-fitting procedure. c_0 is the predetermined histidine concentration of the medium.

c_0	Control values		With 13 mM BCH present	
	v_{obs}	v_{fit}	v_{obs}	v_{fit}
0.10	0.401	0.389	0.160	0.180
0.125	0.428	0.421	0.196	0.210
0.167	0.465	0.465	0.266	0.253
0.25	0.528	0.532	0.345	0.328
0.50	0.638	0.681	0.534	0.508
2.00	1.349	1.297	1.263	1.263
2.50	1.557	1.457	1.419	1.456
3.33	1.591	1.692	1.634	1.733
5.00	2.036	2.070	2.319	2.170
10.00	2.800	2.780	2.922	2.960
V_1		0.468		0.237
V_2		4.13		4.59
K_1		0.039		0.108
K_2		7.88		6.84
V_1/K_1		12.0		2.2
V_2/K_2		0.52		0.67
$\frac{V_{control}}{V_{exptl}} \times \frac{K_{exptl}}{K_{control}}$		for system 1: 5.47 for system 2: 0.78		

TABLE II

VARIATION OF KINETIC PARAMETER DETERMINATION FOR UNINHIBITED HISTIDINE UPTAKE

Incubations were for 2 min at 20 °C. Ten concentrations of labeled histidine ranging from 0.10 to 10.0 mM were employed. Four series of such uptakes were conducted using the same pool of cells and the resulting internal concentrations averaged by pairs and compared as indicated in the table.

	Averaging series					
	1+2	3+4	1+3	2+4	1+4	2+3
V_1	0.26	0.19	0.19	0.25	0.17	0.27
V_2	1.22	1.56	1.17	1.76	1.26	1.62
K_1	0.16	0.10	0.09	0.15	0.07	0.16
K_2	7.37	8.49	5.26	12.28	5.57	11.88
V_1/K_1	1.6	1.9	2.1	1.7	2.4	1.7
V_2/K_2	0.17	0.19	0.22	0.15	0.23	0.14
$\frac{V_{control}}{V_{exptl}} \times \frac{K_{exptl}}{K_{control}}$ of paired figures						
for system 1:			0.86	1.27	1.44	
for system 2:			0.90	1.55	1.66	

agree with experimental data to within 5 % and that there is only one point where disagreement is as great as 10 %. When V/K ratios and control/experimental quotients are calculated, the results in the presence of BCH suggest that only system 1, the L (low c) system, is affected (compare with the variation upon repetition with the same cells, Table II).

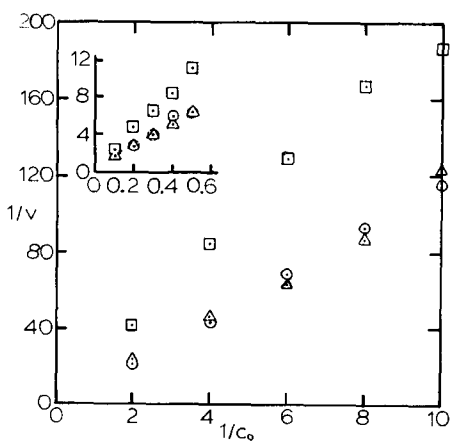

Fig. 1. Leucine-inhibited uptake of histidine in the presence of BCH or His(Cm). Uptakes of labeled histidine were for 2 min at 20 °C and v was calculated as half the intracellular concentration (mM) of labeled histidine obtained following termination of the incubation. 10 mM leucine was present in all cases. In addition, 8 mM sucrose (0), 8 mM BCH (△) or 4 mM His(Cm) (□) was present. The insert indicates the continuation of observed trends in the high-concentration region, which is very near the origin in the double-reciprocal plot. Repetition of this experiment yielded similar results.

An additional indication of the suggested specificity is obtained by examining added effects of analogs upon leucine-inhibited cells. Leucine is known to have a high affinity for the L (low c) system [5] but only moderate affinity for the A (high c) system [6]. At a concentration of 10 mM leucine, the biphasic double-reciprocal plot for histidine uptake is replaced by a single straight line from which a rather high K_m value may be obtained. When 8 mM BCH is added (Fig. 1) there is no change from the control, obtained by addition of 8 mM sucrose. In contrast to this, 4 mM His(Cm) addition caused a deflection of the double-reciprocal plot which we infer is the result of an interaction with the remaining portion of A (high c) system activity.

Examining Table I for an indication of the nature of the inhibition elicited by BCH we note that this analog lowers V_1 and also increases the apparent K_1 obtained with the given cell preparation. We have no explanation to offer for this dual effect at this time.

N-methyl-α-aminoisobutyric acid

By the criterion of change in the ratio of V/K for an individual transport system there is no effect of *N*-methyl-α-aminoisobutyric acid upon the L (low c) system, but a pronounced inhibitory effect upon the A (high c) system (Table III). Examining V_2 and K_2 individually, it would again appear that the inhibitor decreases V at the same time that it markedly increases K_m for the system.

TABLE III

INHIBITION OF HISTIDINE UPTAKE BY *N*-METHYL-α-AMINOISOBUTYRIC ACID

Incubations were conducted for 2 min at 20 °C. v_{obs} is one-half the observed intracellular concentration of labeled histidine in mM units upon termination of incubation (average of two determinations). v_{fit} is the result of the curve-fitting procedure. c_0 is the predetermined extracellular concentration of labeled histidine.

c_0	Control values		With 20 mM *N*-methyl-α-aminoisobutyric acid present	
	v_{obs}	v_{fit}	v_{obs}	v_{fit}
0.10	0.117	0.108	0.081	0.081
0.125	0.125	0.123	0.091	0.088
0.167	0.139	0.143	0.094	0.097
0.25	0.166	0.172	0.106	0.108
0.50	0.222	0.230	0.130	0.125
2.00	0.452	0.423	0.159	0.164
2.50	0.456	0.471	0.178	0.174
3.33	0.546	0.543	0.188	0.189
5.00	0.648	0.660	0.219	0.218
10.00	0.895	0.891	0.290	0.290
V_1		0.20		0.131
V_2		1.36		0.846
K_1		0.112		0.066
K_2		9.67		42.77
V_1/K_1		1.79		1.99
V_2/K_2		0.141		0.0198
$\frac{V_{control}}{V_{exptl}} \times \frac{K_{exptl}}{K_{control}}$		for system 1: 0.90 for system 2: 7.11		

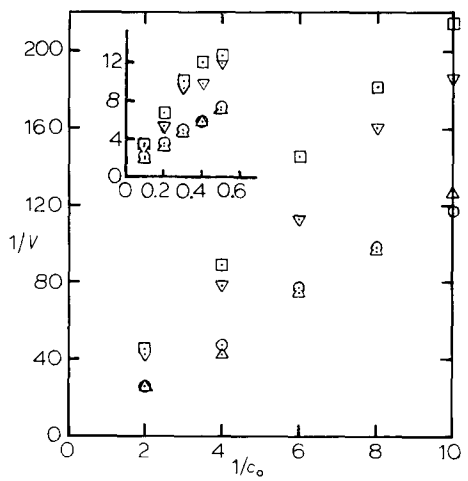

Fig. 2. Leucine-inhibited uptake of histidine in the presence of BCH, *N*-methyl-α-aminoisobutyric acid or proline. Uptakes of labeled histidine were for 2 min at 20 °C and v was calculated as half the intracellular concentration (mM) of labeled histidine obtained following termination of the incubation. 10 mM leucine was present in all cases. In addition, 15 mM sucrose (○) or 8 mM BCH (△) or 10 mM *N*-methyl-α-aminoisobutyric acid (□) or 10 mM proline (▽) was present. The insert indicates the continuation of observed trends in the high-concentration region, which is very near the origin in the double-reciprocal plot.

The effect of *N*-methyl-α-aminoisobutyric acid on leucine-inhibited cells was compared with the effect of proline and the effect of BCH (Fig. 2). The data indicate that BCH has no further effect upon cells in which the L (low c) system is functionally obliterated by leucine, hence no effect on the A (high c) system as has previously been indicated. Concentrations of *N*-methyl-α-aminoisobutyric acid and proline only slightly greater than the BCH concentration employed give marked effects on the leucine-inhibited cells, and the relative positions of the data points indicate that *N*-methyl-α-aminoisobutyric acid has a slightly greater inhibitory effect than proline on the A (high c) system, although proline is one of the more effective inhibitors of the A (high c) system [6].

His(Cm) specificity

His(Cm) has an appreciable affinity for both the A (high c) system and the L (low c) system. Several lines of evidence support this. Line-segment slopes are increased over those of control uptake in both high- and low-concentration ranges in a double-reciprocal plot. Although BCH shows a greater effect in the low-concentration range, His(Cm) shows greater inhibition in the high-concentration range, indicating an effect upon the A (high c) system. Uptake of histidine into leucine-inhibited cells is further inhibited by His(Cm), but not by BCH (Fig 1). This also indicates interaction with the A (high c) system as the L (low c) system has been functionally obliterated.

Uptake of histidine at c_0 of 0.1 mM in a sodium-free medium for 2 min at 20 °C represents uptake by the L or low c system quite predominantly. 30 mM proline and 30 mM *N*-methyl-α-aminoisobutyric acid (300 times the test solute concentration)

TABLE IV

INHIBITION OF HISTIDINE UPTAKE BY HIS(Cm)

Incubations were conducted for 2 min at 20 °C. v_{obs} is one-half the observed intracellular concentration of labeled histidine in mM units upon termination of incubation (average of two determinations). v_{fit} is the result of the curve-fitting procedure. c_0 is the predetermined extracellular concentration of labeled histidine.

c_0	Control values		With His(Cm) present (5 mM)	
	v_{obs}	v_{fit}	v_{obs}	v_{fit}
0.10	0.202	0.194	0.062	0.059
0.125	0.221	0.214	0.074	0.071
0.167	0.239	0.240	0.086	0.090
0.25	0.265	0.281	0.123	0.121
0.50	0.354	0.368	0.187	0.190
2.00	0.744	0.712	0.394	0.390
2.50	0.820	0.800	0.426	0.437
3.33	0.928	0.927	0.521	0.508
5.00	1.078	1.129	0.632	0.638
10.00	1.516	1.499	0.980	0.979
V_1		0.254		0.290
V_2		2.15		6.150
K_1		0.054		0.464
K_2		7.24		77.64
V_1/K_1		4.72		0.62
V_2/K_2		0.297		0.079
$\frac{V_{control}}{V_{exptl}} \times \frac{K_{exptl}}{K_{control}}$		for system 1: 7.53 for system 2: 3.75		

caused inhibitions of only 10 and 11 % of the control uptake, respectively. 15 mM His(Cm) caused an inhibition of 88 % of the control uptake, while 15 mM BCH caused 92 % inhibition. This indicates His(Cm) is an inhibitor of the L (low c) system. Further evidence for the interaction of His(Cm) with both the A (high c) and L (low c) transport systems is provided by Table IV, which indicates by curve-fitting procedures that the activity of both transport systems is noticeably inhibited by the presence of 5 mM His(Cm). We conclude that, although the relative affinity of His (Cm) for the L (low c) system may not be as high as for the A (high c) system, there is still a very detectable interaction of this analog with both systems on an inhibitory basis.

Methyl esters of leucine and alanine as inhibitors

The interaction of His(Cm) with both neutral amino acid transport systems prompted us to test other analogs lacking the free carboxyl group for transport system inhibitions. The methyl esters of leucine and alanine were selected because they were readily available, represented modifications which retained a polar group but not a great change in bulkiness, and were analogs of substrates with known preferences for the individual systems. Control incubations for 2 min at 20 °C yielded a c_i value of 0.152 mM from a medium with $c_0 = 0.1$ mM labeled histidine; c_i was 1.40 when $c_0 =$

10 mM. 16 mM sucrose was present in the control incubations. When 10 mM alanine methyl ester was substituted for the sucrose, c_i was 0.109 when $c_0 = 0.1$ and c_i was 0.90 when $c_0 = 10$ mM. This means that there was a 35 % inhibition of what was predominantly A (high c) system uptake as opposed to 28 % inhibition of what was predominantly L (low c) system activity at $c_0 = 0.1$ mM even though the ratio of inhibitor to substrate was 1 : 1 for the A (high c) test and 100 : 1 for the L (low c) test. This result suggests a preferential interaction of alanine methyl ester with the A (high c) system, in accord with the preference of the natural substrate alanine. When 10 mM leucine methyl ester was employed instead of alanine methyl ester, c_i was only 0.036 when $c_0 = 0.1$ mM: c_i was 1.58 when $c_0 = 10$ mM. This represents a 76 % inhibition in the region of L (low c) system dominance and a modest increase over control in the region of A (high c) system dominance. We, therefore, conclude that the methyl esters of alanine and leucine interact with the L (low c) and A (high c) transport systems in an inhibitory manner and that they exhibit the system preferences of the parent amino acids.

DISCUSSION

Role of the carboxyl group in transport system recognition

Our finding that analogs lacking a free carboxyl group can function as inhibitors of amino acid transport systems may seem to be in conflict with suggestions of other workers that the carboxyl group is an absolute requirement for interaction with an amino acid transport system. Oxender [15] found isobutylamine to be a relatively ineffective inhibitor of L-valine uptake into Ehrlich ascites cells. Schultz et al. [16] concluded that "both the α-amino and α-carboxylate groups appear to be essential for interaction with the transport mechanism" in brush border, on the basis of experiments employing analogs in which one of the two groups was completely deleted. Our studies differ from those cited in that a polar group, either a ketone in the case of His(Cm) or an ester in the case of leucine methyl ester and alanine methyl ester, was substituted for the free carboxyl group of the natural substrates. Our disagreement may be more substantial with the results of Paine and Heinz [17], who reported that glycine methyl ester did not inhibit glycine uptake, but glycine uptake probably occurs by a transport system other than those which we have examined [9].

We agree with the suggestions of Oxender [15] and Paine and Heinz [17] that the carboxyl group is probably a point of attachment for the normal substrate to the transport system but we consider that it is possible for the carboxyl group to be replaced by some other polar group. We interpret these observations as an indication that binding to the transport system occurs by non-covalent linkages: most probably the carboxyl group or the analogous ketone or ester group attaches to the transport system by hydrogen bonding.

Our data suggests that the above behavior of the carboxyl group is common to both the A (high c) and L (low c) system. Leucine methyl ester apparently interacts with the L (low c) system while alanine methyl ester does so with the A (high c) system. His(Cm) interacts with both systems, although relative to the affinities displayed by histidine it would appear to be a better analog for the A (high c) system than for the L (low c) system. We, therefore, suggest that the free carboxyl group may be slightly more difficult to replace in the case of the L (low c) system than in the case of the A (high c) system.

Our experience with alanine and leucine methyl esters leads us to agree with Oxender [15] and Paine and Heinz [17] that preference of a substrate for a transport system may be largely associated with the nature of the side-chain interactions between substrate and transport system.

It is noteworthy that, at the same meeting in which a preliminary report of our His(Cm) data was made [18], other workers reported the interaction of phenylalanine chloromethylketone with a bacterial transport system for amino acids [19].

Degree of specificity of analogs and the identity of transport systems

Our results with BCH and *N*-methyl-α-aminoisobutyric acid provide confirming evidence for the specificity of these analogues which has been suggested [9, 20]. Our data indicates that BCH interacts with the L (low c) system but not with the A (high c) system and that *N*-methyl-α-aminoisobutyric acid interacts with the A (high c) system but not with the L (low c) system. The association of specificities for the two systems with these two analogues adds further support to the suggestion that our high c is Christensen's A system and our low c his L system [6].

Potential usefulness of combining specific analogues with low and high concentrations of histidine in exploring transport system properties

Uptake of 0.1 mM histidine has previously been used as a test situation for activity of the low c system and uptake of 10 mM histidine as a test for activity of the high c system [5, 6]. It has been apparent that the low c system makes the major contribution to uptake of 0.1 mM histidine while the A (high c) system predominates at $c_0 = 10$. The curve-fitting procedures permit us to quantitate the contributions made by both systems at selected concentrations. Use of the parameters given in Tables I–IV for the control uptake reveals that the low c system typically accounts for about 85 % of the influx observed at $c_0 = 0.1$ mM whereas the high c system accounts for about 80 % of the influx observed at $c_0 = 10$ mM.

It has always been possible to use some substrate which favors the low c system, leucine for example, to study the high c system in comparative isolation, if one does not object to the loss of some A (high c) system activity. An example of this type of experiment is provided in Figs 1 and 2 of the present work. The parameters listed in Table I permit us to estimate that 1.3 mM BCH, which does not inhibit the A (high c) system, reduces the share of the L (low c) system at $c_0 = 10$ mM from 17 to 8 % of the total influx. Similarly, the parameters listed in Table III permit us to estimate that the contribution of the A (high c) system to total influx at $c_0 = 0.1$ mM is reduced from 13 to 2 % by 20 mM *N*-methyl-α-aminoisobutyric acid, which does not inhibit the L (low c) system. This finding is especially important in that it has proven difficult to remove completely the activity of the A (high c) system so as to study the L (low c) transport system activity in isolation. We, therefore, suggest that the activity of the two neutral amino acid transport systems may be conveniently studied by using the test substrate histidine at $c_0 = 0.10$ mM in the presence of 20 mM *N*-methyl-α-aminoisobutyric acid for the L (low c) system and histidine at $c_0 = 10$ mM in the presence of modest concentrations of BCH (5–10 mM) for the A (high c) system. In this manner a commercially available labeled amino acid can serve as the test substrate and only the two unlabeled analogues need be synthesized.

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