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TRANSPORT OF THE AMINO ACID ANALOG, 2-AMINOBICYCLO(2,2,1)-HEPTANE-2-CARBOXYLIC ACID, BY EHRLICH ASCITES TUMOR CELLS

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SUMMARY

1. The transport of the amino acid analogs, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH), α-aminoisobutyric acid and 1-aminocyclopentane carboxylic acid (cycloleucine), was examined in the Ehrlich mouse ascites tumor cell at 37 °C.

2. The time course of uptake of BCH was similar to that observed for cycloleucine and α-aminoisobutyric acid, and both the initial and steady-state distribution ratios were inhibited by a minimum of 60% when Na^+ in the medium was replaced by choline or K^+.

3. J_{max}, the maximal rate of BCH uptake, was markedly Na^+ dependent, and was reduced by 50% in choline-substituted media. K_m, the presumed Michaelis constant, was not altered significantly.

4. The uptake of BCH could be inhibited completely by competition with either α-aminoisobutyric acid or cycloleucine, but was at the most 15% inhibited in the presence of *N*-methyl-DL-alanine. BCH, however, did not affect the uptake of α-aminoisobutyric acid.

5. Interpreted in terms of the distinction of "A" and "L" systems for neutral amino acid transport in the Ehrlich tumor cell, these results support a dichotomy of binding sites. However, the Na^+ dependence of BCH uptake and the lack of reciprocal competitive interaction between BCH and α-aminoisobutyric acid prevent any simple dissociation of the two systems.

INTRODUCTION

The presence of separate transport sites for the basic, the acidic and, as a group, the neutral amino acids has been established for several systems including the kid-

Abbreviations: BCH, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid; cycloleucine, 1-aminocyclopentanecarboxylic acid; *N*-Me-Ala, *N*-methyl-DL-alanine.

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ney[1,2], the small intestine[3] and the Ehrlich ascites tumor cell[4-7]. This separation is based primarily on the lack of competitive interactions between members of different groups*.

On the other hand, it has been difficult to resolve the possibility of multiple independent sites for neutral amino acids, because most pairs of such amino acids demonstrate mutual competitive inhibition[5,6]. Such mutual inhibition suggests transport *via* a common carrier. However, a single such carrier seems unlikely in certain systems. For instance, in the kidney there appears to be a separate system for glycine, proline and hydroxyproline, as evidenced by kinetic data and a different rate of development of this transport capability in the newborn kidney[8,9]. In the rabbit and chicken small intestine there is an asynchrony in the ontogeny of transport for various groups of neutral amino acids[10,11]. Also, rat bone cells demonstrate different kinetic parameters and Na^+ dependence for the transport of glycine and the imino acids compared to other neutral amino acids[12].

In the case of the Ehrlich ascites tumor cell, Tenenhouse and Quastel[13] and Johnstone and Scholefield[14] suggested a dichotomy of neutral amino acid transport on the basis of a lack of competition and exchange diffusion between certain pairs of natural and artificial neutral amino acids. Oxender and Christensen[15,16] and Christensen[6,7], after a thorough examination of the uptake of several neutral amino acids, proposed the existence of multiple transport systems for these solutes. Although all pairs of neutral amino acids demonstrated competition, an analysis of the relative strength of such interactions allowed the authors to separate these amino acids initially into two groups, which were referred to as the A and L systems. The A-type amino acids had, in general, small apolar side chains and were concentrated to large steady-state gradients at slow rates. The L system preferred amino acids with long branched hydrocarbon side chains, and did not concentrate amino acids to the same degree, although the steady-state was achieved at a more rapid rate. Furthermore, the L, but not the A system, mediated exchange diffusion. Perhaps the clearest distinction between the two systems at first appeared to be the fact that the A system was Na^+ dependent and easily inhibited by changes in pH or intracellular energy stores, whereas the L was not[15,16].

Christensen and his coworkers identified α-aminoisobutyric acid and certain N-methylated amino acids such as *N*-methyl-α-aminoisobutyric acid and *N*-methylalanine (*N*-Me-Ala) as good A-system analogs[7,17,18]. However, no natural nor artificial amino acid seemed to have a primary L specificity.

Recently, Christensen *et al.*[20] and Tager and Christensen[21] have attempted to resolve this difficulty through the synthesis of a new amino acid analog, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH). These workers identified this analog as non-metabolizable and possessing the characteristics of a good L system analog: rapid entry rate, low steady-state gradient, lack of Na^+ sensitivity, rapid exchange diffusion and lack of competitive interaction with A system analogs.

We initially began to investigate the uptake of this amino acid because of the energetic implications of an amino acid which is actively transported independently of the Na^+ gradient (*cf.* ref 21). However, our initial observations revealed that

* Although competition can be shown between neutral and basic amino acids, this competition appears to require the presence of Na^+ as a cosubstrate with the neutral amino acid[7].

BCH has considerable Na^+ and K^+ sensitivity and a time course of uptake similar to that of α-aminoisobutyric acid. Furthermore, BCH uptake could be inhibited completely by the simultaneous presence of α-aminoisobutyric acid and to a much lesser degree by *N*-Me-Ala, but BCH had no inhibitory effect on α-aminoisobutyric acid uptake. 1-Aminocyclopentane carboxylic acid (cycloleucine), an analog which demonstrated uptake by both A- and L-type systems[15], competitively inhibited both amino acids. These results indicate that both α-aminoisobutyric acid and BCH transport are Na^+ dependent, and that, while α-aminoisobutyric acid seems to share the BCH site, the converse is not true. Thus, while the presence of two different sites for neutral amino acid transport is supported, these sites appear to have several quite similar characteristics and widely overlapping affinities which preclude conclusions as to their complete independence of one another.

METHODS

The methods used were essentially those described by Schafer and Heinz[22]. The ascites tumor, an Ehrlich–Lettré line (hyperdiploid), was kindly provided by Dr H. Hempling (University of South Carolina, Charleston, S.C.). This tumor was maintained by weekly intraperitoneal inoculation of 0.5 ml of untreated ascites from donor to recipient mouse. All mice were from Southern Animal Farms (Prattville, Ala.), and were of the ICR-Swiss genotype.

The tumor was harvested at 7–10 days, and 5–10 ml of grossly non-hemorrhagic ascitic fluid was derived per mouse. The ascitic fluid was put in 30 ml Na^+-containing Krebs–Ringer phosphate buffer (see below) with 0.1 ml sodium heparin solution (1000 units/ml; trade name, Liquaemin: Organon, W. Orange, N.J.). This suspension was run through gauze and centrifuged. The cells were resuspended in Na^+-containing Krebs–Ringer phosphate buffer at room temperature and again centrifuged. After resuspension in Na^+-containing Krebs–Ringer phosphate buffer to a cytocrit of about 0.2, the suspension was shaken at 100 cycles/s in a water bath at 37 °C for 30 min. The depth of the suspension was kept to less than 5 mm to ensure adequate aeration. After this preincubation, the suspension was centrifuged, resuspended in the desired test medium, centrifuged, and again suspended in the same medium to a cytocrit of 0.24–0.30. 2 ml of this suspension were put in the bottom of each of sixteen Erlenmeyer-like flasks with attached, tared side arms. In most cases, the side arms contained 2 ml of an amino acid solution, with radioactive label, in the same medium as were the cells. Duplicates were run for each experimental variable. The flasks and side arms were incubated at 37 °C with shaking at 100 cycles/s, generally for 5 min. The contents of the flasks and side arms were then mixed, and the mixtures were incubated for the desired length of time. At the end of the incubation the suspensions were decanted to the side arms, which were plunged into an ice–water–salt mixture (−1 to −3°C) and shaken there for 1 min. The side arms were then centrifuged for 30 min at 4 °C, 4900 rev./min, in a Sorvall Model RC2-B centrifuge with a type GSA head. The supernatant fluid was decanted and saved for analysis. The side arms were handled as described previously[22]. The pellets were lyophilized overnight and extracted in homogenizer tubes with 3 ml of distilled water for 2 h. The cell debris was then centrifuged, and the extract was used for analysis.

Computation of cellular amino acid and ion concentrations was carried out according to the method of Jacquez[23]. Sample radioactivity was measured on a Packard Model 3320 liquid scintillation spectrometer with automatic external standard employed to ascertain equal quenching among all samples. Na^+ and K^+ analyses on all supernatant fluids and some pellet extracts were made using an Instrument Laboratory Model 143 flame photometer.

Buffer solutions

The standard buffer used was a Krebs–Ringer phosphate solution containing: 124 mM NaCl, 8 mM KCl, 1.9 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 10.0 mM Na_2HPO_4. Also used were a choline buffer in which choline chloride 115 mM was substituted for NaCl and K_2HPO_4 for Na_2HPO_4, and a potassium buffer in which KCl (198 mM) was substituted for NaCl, and KH_2PO_4 was substituted for Na_2HPO_4. All buffers contained 1% bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis) and their pH was adjusted to 7.4 with a small amount of 1.0 M HCl. The KCl and choline concentrations were not substituted for Na^+ on an equimolar basis in order to provide different osmolalities. The osmolalities of the Na^+-, K^+- and choline-containing Krebs–Ringer phosphate buffers were adjusted, respectively, to 290, 400 and 270 mosM. It was found that at these osmolalities changes in cell volume were prevented for at least 15 min, and the ratio of grams of pellet intracellular water (9) to dry weight (9) was about 3.0 in each buffer.

Estimation of g of pellet trapped volume

In order to obtain an accurate measure of the trapped volume of extracellular medium analyzed with the pellet, [^{14}C]sorbitol was used as an extracellular volume marker. In separate experiments, Ehrlich cell suspensions of varying cytocrit were placed in the incubation flasks and sorbitol was placed in the side arm. The flasks were incubated with shaking at 37 °C for 5–20 min. The flasks were chilled for 1 min and only then was the suspension mixed with the volume marker and the side arms centrifuged. The pellets and supernatant fluids were analyzed for radioactivity as above and the relation between pellet wet weight and trapped volume was determined. Several such experiments were performed with each of the buffers used. In all cases, the relation between ml of trapped volume and pellet wet weight was linear, with the following constants: trapped volume (g) = 1.0639 × pellet wet wt (g) + 0.0105 (g). The Student *t*-test revealed no difference with the various buffers.

Kinetic parameters

In order to estimate the constant for non-saturable entry of the various amino acids into the cells, the kinetic data were analyzed according to a program written by Dr John Jacquez (University of Michigan, Ann Arbor, Mich.) and designed to give the best fit to the Michaelis–Menten plot by iterative selection of the constant[19]. This program also gave estimates of K_m and J_{max} which agreed reasonably with those obtained by analysis of the flux data, corrected for non-saturable entry, according to Wilkinson[24]. All computations were carried out using a Sigma 7 computer.

Biochemicals and labeled compounds

Albumin, sorbitol, inulin, cycloleucine and *N*-Me-Ala were obtained in purest

grade from Sigma Chemical Co. (St. Louis, Mo.). α-Aminoisobutyric acid (A Grade) was obtained from Calbiochem (Los Angeles, Calif.). 1 g of BCH was generously provided by Dr. H. Christensen (Ann Arbor, Mich.), and 2 g were obtained from the New England Nuclear Corp. (Boston, Mass.; Lot Nos 600-047 and 600-081). BCH from the latter source was analyzed by Bio-Science Labs (Van Nuys, Calif.) and was reported to be 99.8% in the form of the b endomer[20]. α-[3H]Aminoisobutyric acid (Lot No. 643-273) and [*carboxy*-^{14}C]BCH (Lot Nos 600-047 and 600-081) were supplied by New England Nuclear Corp. The former had an initial specific activity of 1.21 mCi/mg and was dissolved in water to 147 μCi/ml of which 0.1 ml was used per experiment. BCH had an initial specific activity of 4.8 mCi/mg and was dissolved in water to 100 μCi/ml of which 0.1 ml was used per experiment. [*carboxy*-^{14}C]Cycloleucine was obtained from Calatomic Corp. (Los Angeles, Calif., Lot No. 015066) with a specific activity of 15 Ci/mole. This was dissolved to 100 μCi/ml, and 0.075–0.1 ml were used per experiment.

RESULTS

The preincubation of the Ehrlich cells according to the protocol described in Methods was designed merely to aerate the cells and restore them to a state in which intracellular Na^+ and K^+ were in the range 30–34 mequiv/l and 140–175 mequiv/l, respectively[22]. These concentrations were unaltered by room temperature washes with Na^+-containing Krebs–Ringer phosphate buffer prior the final incubation; however, when choline-containing Krebs–Ringer phosphate buffer was used in the washes, both intracellular Na^+ and K^+ concentrations fell by 2–6 mequiv/l during the wash procedure. Since in most cases only initial fluxes were measured, this change was not considered to alter the results in any significant way.

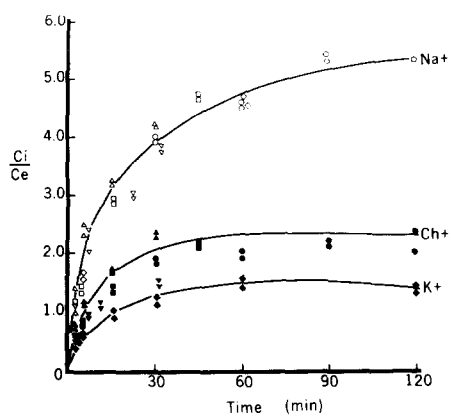

Fig. 1. Time course of BCH uptake from an initial extracellular concentration of 2 mM in Na^+-containing and Na^+-substituted buffers. The ratio of the intra- to extracellular BCH concentration (C_i/C_e) is plotted as a function of the time of incubation with BCH at 37 °C. Open symbols denote incubations conducted in media with an average extracellular Na^+ concentration of 143 mequiv/l. Closed symbols denote media in which Na^+ is replaced by choline (Ch), with the exception of the experiment designated by the symbol, ◆, in which K^+ replaced Na^+. In these media the average extracellular Na^+ concentration was 2.3 mequiv/l. Symbols of the same shape denote the same experiment and the same original cell suspension. The curves were drawn by eye to fit the respective points.

Fig. 1 illustrates the time course of uptake of BCH from an extracellular concentration of 2 mM, in the three different suspending media at 37 °C. It should be noted that, under these conditions, the rate of approach to the steady state for BCH uptake does not differ from that of cycloleucine (Fig. 2) or α-aminoisobutyric acid (Fig. 3), and all three amino acids achieve one half the steady-state distribution in 8–10 min. The substitution of choline or K^+ for Na^+ brings about a marked decrease in BCH uptake over the whole time course of uptake. Table I summarizes the average distribution ratios of BCH after incubation for 1 and 30 min to 2 h in

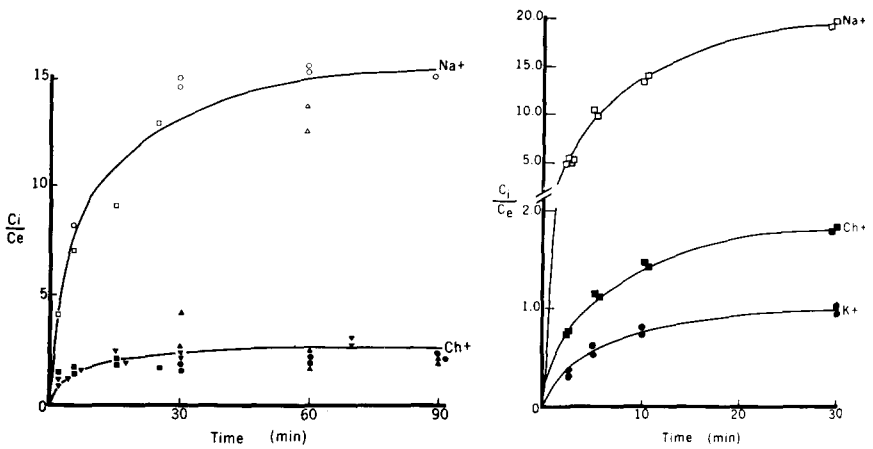

Fig. 2. Time course of uptake of cycloleucine from an initial extracellular concentration of 2 mM in Na^+-containing and Na^+-substituted buffers. The ratio of the intra- to extracellular concentration is plotted as a function of the time of incubation with cycloleucine at 37 °C. The symbols have the same meaning as described for Fig. 1. In Na^+-containing Krebs–Ringer phosphate buffer the average extracellular Na^+ concentration was 135 mequiv/l, whereas in the choline (Ch)-containing Krebs–Ringer phosphate buffer it was 3.6 mequiv/l.

Fig. 3. Time course of uptake of α-aminoisobutyric acid from an initial extracellular concentration of 2 mM in Na^+-containing and Na^+-substituted buffers. The plot and meaning of the symbols are the same as explained in the legends of Figs 1 and 2. Ch, choline.

TABLE I

COMPARISON OF INITIAL AND STEADY-STATE DISTRIBUTION RATIOS OF BCH IN VARIOUS BUFFER SOLUTIONS

The distribution ratio is given as the mean ratio of intra- to extracellular BCH concentration *plus* or *minus* the standard deviation with the number of determinations in parentheses. All differences seen between Na^+-replete and Na^+-substituted buffers were significant with $P < 0.001$. The extracellular BCH concentration was 2 mM.

Time (min)	*BCH distribution ratio (C_i/C_e)*		
	Na^+ buffer	*Choline buffer*	*K^+ buffer*
1	1.30 ± 0.19 (8)	0.67 ± 0.13 (12)	0.27 (2)
30–120	4.71 ± 0.43 (11)	2.03 ± 0.30 (11)	1.49 ± 0.02 (4)

Na^+, choline and K^+ buffers. Additional uptake studies were conducted at extracellular BCH concentrations near or below its K_m. Section I of Table II presents the average uptakes of BCH from choline and Na^+ buffers at low extracellular concentrations of BCH. Section II of the same table presents the results of three experiments in which the uptake of BCH was measured in these two buffers within the same experiment, using the same batch of cells. In this case, the period of chilling in the ice bath and centrifugation after the incubation were reduced, respectively, to 30 s and 10 min. Taken together the data presented in Fig. 1 and Tables I and II indicate clearly that, under a number of different experimental conditions, there were two components for BCH uptake in these cells, one Na^+ dependent and the other Na^+ independent.

The results of a series of kinetic experiments, designed to illustrate the partial Na^+ dependence of BCH uptake, are illustrated in Table III. In these studies, the 1-min BCH flux was measured from 8 different extracellular concentrations in each experiment, in either Na^+-containing or choline-containing Krebs-Ringer phosphate buffer. In order to obtain somewhat higher Na^+ concentrations than normally present in choline-containing Krebs-Ringer phosphate buffer, additional Na^+-containing Krebs–Ringer phosphate buffer was added to the choline-containing Krebs–Ringer phosphate buffer suspension in some cases. The maximal influx rate, J_{max}, averaged 10.45 μmoles per g dry wt per min in Na^+-containing Krebs–Ringer phosphate buffer but only 5.32 μmoles per g dry wt per min in choline-containing Krebs–Ringer

TABLE II

INITIAL UPTAKE RATES OF BCH FROM LOW EXTRACELLULAR CONCENTRATIONS

Section I gives the overall average of all 1-min BCH uptakes from choline and Na^+ buffers. The comparisons between fluxes from Na^+ and choline were not obtained within the same experiment. The methods were the same as described in the text. Section II provides a comparison of BCH initial uptakes in choline and Na^+ buffers within the same experiment. In this case, the uptake was for 30 s, the period of chilling in ice to stop uptake was reduced to 30 s, and the suspension was centrifuged for only 10 min. Values are given as μmoles per min per g dry wt.

Expt No.	*Extracellular BCH concn (mM)*	*Na^+ buffer*	*Choline buffer*
I. Average of all 1-min flux data			
	0.125	3.34 ± 0.15 (6)	1.57 ± 0.46 (4)
	0.25	5.15 ± 0.04 (8)	2.49 ± 0.78 (7)
	1.00	6.87 ± 0.67 (5)	4.57 ± 1.32 (8)
II. Additional 30-s uptake experiments			
81	0.1	6.04 ± 0.09 (4)	3.78 ± 0.03 (4)
	0.2	—	5.77 ± 0.04 (4)
82	0.1	4.26 ± 0.01 (4)	3.37 ± 0.05 (4)
	0.2	8.73 ± 0.33 (4)	5.24 ± 0.06 (4)
83	0.1	5.26 ± 0.09 (4)	3.70 ± 0.04 (4)
	0.2	8.17 ± 0.06 (4)	5.94 ± 0.03 (4)

TABLE III

DEPENDENCE OF THE KINETIC PARAMETERS OF BCH UPTAKE ON THE EXTRACELLULAR Na^+ CONCENTRATION

J_{max} and K_m were determined from the saturable component of 1-min BCH fluxes at 37 °C in Na^+-containing Krebs–Ringer phosphate buffer or choline-containing Krebs–Ringer phosphate buffer, by means of the Wilkinson[24] analysis. In each case, the following BCH extracellular concentrations were used: 20 or 10, 8, 5, 2, 1, 0.5, 0.25, 0.125 mM. The constant of nonsaturable entry was always less then 0.05 cm^3 per g dry wt per min.

$[Na^+]_e$ (*mequiv/l*)	K_m (*mM*)	J_{max} (*μmoles/min per g dry wt*)
139	0.14	9.94
135	0.24	9.59
135	0.16	11.52
140	0.19	10.75
12.0	0.13	8.23
4.5	0.17	4.44
4.2	0.13	4.16
4.0	0.09	6.74
2.0	0.20	4.70
2.3	0.17	3.67

phosphate buffer. The difference was significant with $P<0.001$. The apparent K_m averaged 0.18 mM in Na^+-containing Krebs–Ringer phosphate buffer and 0.15 mM in choline-containing Krebs–Ringer phosphate buffer, but the difference was not statistically significant. The marked dependence of J_{max} on the extracellular Na^+ concentration is shown in the lower portion of Fig. 4 and is compared to the lack of dependence evidenced for the K_m. Table IV presents similar data for cycloleucine. The removal of Na^+ reduced J_{max} and K_m both to a significant extent. This same effect has also been observed by Jacquez, J. A. (personal communication).

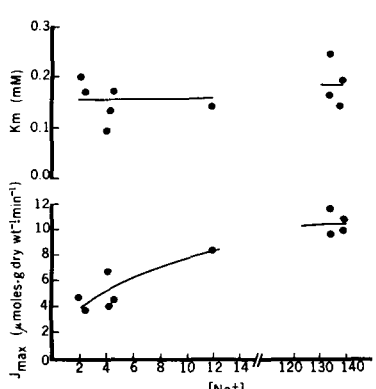

Fig. 4. Dependence of the kinetic parameters of BCH uptake on the extracellular Na^+ concentration. K_m and J_{max} values for BCH uptake are those presented in Table III. The buffers used were either Na^+-containing Krebs–Ringer phosphate buffer or choline-containing Krebs–Ringer phosphate buffer. The latter in some cases had varying amounts of Na^+-containing Krebs–Ringer phosphate buffer added.

TABLE IV

DEPENDENCE OF THE KINETIC PARAMETERS OF CYCLOLEUCINE UPTAKE ON THE EXTRACELLULAR Na^+ CONCENTRATION

J_{max} and K_m were determined as in Table I, from the same extracellular concentrations given there.

$[Na^+]_e$ (*mequiv/l*)	K_m (*mM*)	J_{max} (*μmoles/min per g dry wt*)
139	0.69	20.85
140	0.93	23.61
3.0	0.31	13.83
2.7	0.40	12.70

TABLE V

THE EFFECT OF OTHER AMINO ACIDS ON THE KINETIC PARAMETERS OF BCH UPTAKE

Kinetic analyses of the 1-min flux of BCH from various extracellular concentrations were conducted as described in Table I; however, in this case a second amino acid, indicated in the first column was present. The K_m so computed is referred to as the apparent K_m, $K_{|m|}$, of the BCH uptake. From this parameter and the known K_m of BCH uptake (Table I), the inhibitor constant, K_i, was computed as:

$$K_i = I\left(\frac{K_m}{K_{|m|} - K_m}\right).$$

Competing amino acid	$[Na^+]_e$ (*mequiv/l*)	*Apparent* K_m (*mM*)	J_{max} (*μmoles/min per g dry wt*)	K_i (*mM*)
20 mM α-aminoisobutyric acid	139	1.14	10.24	3.78
	130	1.78	14.07	2.27
	1.5	0.78	8.63	4.68
	5.0	0.95	5.67	3.68
	2.0	1.40	4.89	2.36
	3.0	1.20	4.95	2.81
10 mM cycloleucine	138	4.97	13.61	0.38
	2.0	2.19	4.82	0.72
	3.2	2.40	2.97	0.66
20 mM cycloleucine	3.0	9.59	5.67	0.32

Table V gives the results of kinetic studies conducted on BCH uptake in the presence of high concentrations of α-aminoisobutyric acid or cycloleucine as competitors. It is obvious that both α-aminoisobutyric acid and cycloleucine increase the apparent K_m of BCH uptake without lowering its J_{max}. We have computed the inhibitory constant to be in the range 2.3–4.6 for α-aminoisobutyric acid, and 0.4–0.7 for cycloleucine. Fig. 5 presents a slightly different method of computing

K_i, suggested by Christensen[25]. The 1-min uptake of BCH from an extracellular concentration of 0.5 mM is plotted against the extracellular concentration of the inhibitor amino acid α-aminoisobutyric acid. Note that the entire BCH uptake can be inhibited by the higher concentrations of α-aminoisobutyric acid. The curves drawn through the points are the theoretical curves predicted for an inhibitory constant, K_i, of 2.9 mM. However, *N*-Me-Ala reduced the BCH flux by a maximum of only 15% in either Na^+-containing Krebs–Ringer phosphate buffer or choline-containing Krebs–Ringer phosphate buffer, even at 100 mM extracellular concentrations (Fig. 6). N-Me-Ala had a K_i of approx. 0.5 mM for that portion of BCH uptake which it inhibited.

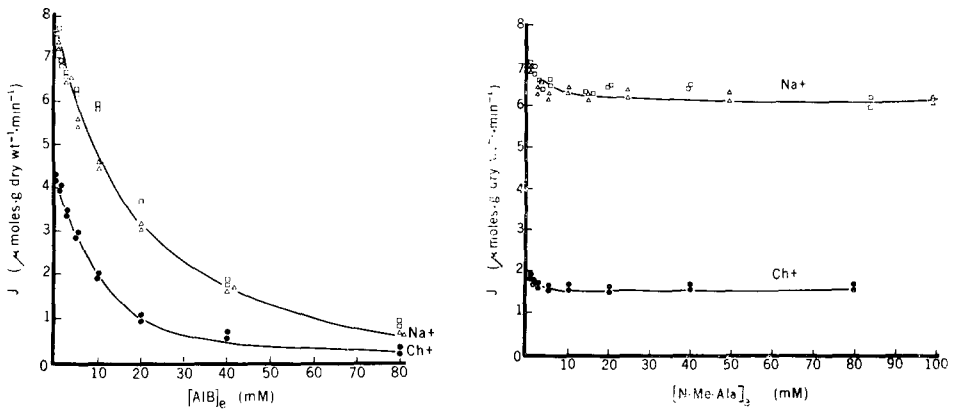

Fig. 5. Inhibition of BCH flux by α-aminoisobutyric acid (AIB). BCH flux was measured from an extracellular concentration of 0.5 mM. The experiments designated by the open symbols were performed in Na^+-containing Krebs–Ringer phosphate buffer (average extracellular Na^+ concentration, 112 mequiv/l) whereas the experiment designated by the closed symbols was performed in choline-containing Krebs–Ringer phosphate buffer (average extracellular Na^+ concentration, 2.1 mequiv/l). α-Aminoisobutyric acid was replaced isosmotically by choline (Ch)-containing Krebs–Ringer phosphate buffer in both media. The curves were drawn from the theoretical relationship:

$$J = \frac{J_{max} C_e}{K_m \left(\frac{1+I}{K_i}\right) C_e}$$

where: J_{max} and K_m are taken from Table III; I is the inhibitor concentration; C_e is the extracellular α-aminoisobutyric acid concentration, 0.5 mM; and K_i is taken to be 2.9 mM to give the best fit to the data.

Fig. 6. Inhibition of BCH flux by *N*-Me-Ala. BCH flux was measured from an extracellular concentration of 0.5 mM. The experiments designated by the open symbols were performed in Na^+-containing Krebs–Ringer phosphate buffer (average extracellular Na^+ concentration, 97 mequiv/l), whereas the experiment designated by closed symbols was performed in choline (Ch)-containing Krebs–Ringer phosphate buffer(average extracellular Na^+ concentration, 3.4 mequiv/l). *N*-Me-Ala was replaced isosmotically by choline-containing Krebs–Ringer phosphate buffer at the lower concentrations in both media. The curves were drawn by eye to fit the data.

Table VI presents the kinetic parameters of α-aminoisobutyric acid uptake both alone and in the presence of 10–25 mM BCH. BCH had no effect on the apparent K_m of α-aminoisobutyric acid except possibly to decrease it.

TABLE VI

KINETIC PARAMETERS OF α-AMINOISOBUTYRIC ACID UPTAKE

The kinetic parameters were estimated as described in Table I, but from extracellular concentrations of 25, 15, 10, 8, 4, 2, 1.0 and 0.5 mM. The constant for nonsaturable entry ranged from 0.3 to 0.6 cm^3 per g dry wt per min.

Competing amino acid	$[Na^+]_e$ *(mequiv/l)*	*Apparent* K_m *(mM)*	*V (μmoles/min per g dry wt)*
None	140	1.59	21.80
None	138	1.73	27.87
None	1.0	4.20	7.75
10 mM BCH	141	0.95	24.57
20 mM BCH	137	0.90	18.60
25 mM BCH	142	1.43	23.81

DISCUSSION

Many of the results of our studies are in agreement with those of Christensen and co-workers[20,26] regarding BCH accumulation by the Ehrlich ascites cell. First, the time course of uptake (Fig. 1), although somewhat slower is similar to that of Christensen *et al.*[20] and reaches nearly the same steady-state distribution ratio. Second, our values of the kinetic constants K_m and J_{max} (Table III) agree, within experimental error, with those of Christensen *et al.*[20]. Third, we are in agreement with these authors in finding no effect of high concentrations of BCH on α-aminoisobutyric acid uptake, and little effect of *N*-Me-Ala on BCH uptake. However, there are other results which contrast with those of Christensen and co-workers[20,26]. It is clear that both the initial influx of BCH (Tables I, II, and III, Figs 1 and 4) and its steady-state distribution ratio (Fig. 1, Table I) are markedly dependent on the extracellular Na^+ and K^+ concentrations. Further, in contrast to the report of Christensen *et al.*[20], we find that α-aminoisobutyric acid is able to inhibit completely the uptake of BCH and that this interaction is competitive (Table V, Fig. 4).

Much of this apparent contradiction can be resolved in light of the experimental techniques used by Christensen and his co-workers and ourselves. The importance of these techniques is in part attributable to the fact that BCH is capable of exchange with intracellular amino acids[20,26]. The Ehrlich ascites cell contains a large (greater than 25 mM) pool of endogenous amino acids. Potashner and Johnstone[27] have shown that methionine, an amino acid with at least moderate L affinity[15], exchanges rapidly with this intracellular pool. Furthermore, Christensen *et al.*[20] have interpreted their data to indicate a rapid exchange of extracellular BCH with endogenous amino acids. The important point is that this exchange is of relatively short duration, providing a maximal contribution in the first 30 s of BCH uptake (see especially ref. 20, Fig. 21). Since exchange diffusion for many amino acids has been shown to be independent of extracellular Na^+ (refs 19, 27, 28), most of the 30-s uptake of BCH could be expected to be Na^+ independent. In this regard, it should be noted that whereas the J_{max} for the 1-min uptake of BCH was reduced by 50% in the Na^+-free media, the 30-s uptake from a 0.1 mM extracellular concentration was

reduced by only about 30%. Also, Christensen *et al.*[20] report their 30-s fluxes for BCH in units of mmoles per kg cell water per min. Since Ehrlich cells have been reported[22] to shrink in isotonic choline buffers, such a reduction in intracellular water may well account for an overestimation of the BCH flux in choline media. We have avoided this problem by using slightly hypotonic choline buffer in which the ratio of intracellular water to cell dry weight is the same as in Na^+ buffer, and by expressing our fluxes in terms of cell dry weight. Another reason for the lack of Na^+ dependence evidenced by Christensen *et al.*[20] may lie in the rather acute dependence of the maximal uptake rate for BCH, J_{max}, on the extracellular Na^+ concentration (Fig. 4). Because of this phenomenon, even an extracellular Na^+ concentration as low as 12 mequiv/l can give a J_{max} 80% as large as the J_{max} observed at a Na^+ concentration of 140 mequiv/l.

Although the absolute percent inhibition of BCH uptake achieved in choline or K^+-substituted media may not at first appear to be as dramatic as for cycloleucine or α-aminoisobutyric acid, (Figs 1–3), it should be noted that replacement of Na^+ with other ions results in approximately the same steady-state distribution ratio for all three amino acids, *i.e.* 2.0–2.5 for choline replacement, and 1.0–1.5 for K^+ replacement of Na^+. Rather, the differences among these amino acids lie in the steady-state gradients achieved by them in the presence of Na^+. This does not imply that the residual uptake observed for BCH in the absence of Na^+ is not mediated by a separate transport system. BCH uptake did demonstrate saturation kinetics in the complete absence of Na^+, whereas A system amino acids such as α-aminoisobutyric acid and *N*-methylaminoisobutyric acid show almost no saturable uptake in the absence of Na^+ (refs 18, 29). However, the degree to which the Na^+-independent system is able to actively transport amino acids is questionable. Although a distribution ratio of about 2.0 was obtained from an extracellular concentration of 2 mM BCH in choline buffer, this distribution ratio was only about 1.0 in the K^+ buffer.

The second problematic finding is that high concentrations of α-aminoisobutyric acid inhibit completely the uptake of BCH (Fig. 5). Although the K_i for α-aminoisobutyric acid inhibition is about twice its own K_m, the degree of interaction is evident. Cycloleucine, as expected, also inhibited BCH uptake with a K_i value similar to its own K_m. One possible explanation is that α-aminoisobutyric acid is a very poor A analog and possesses considerable L reactivity. Although α-aminoisobutyric acid is no longer considered to be as good an A system analog as certain N-methylated analogs[17,18], nevertheless almost all the saturable uptake of α-aminoisobutyric acid is Na^+ dependent, and α-aminoisobutyric acid does not exchange with either endogenous or exogenous intracellular amino acids[22,29]. Furthermore, BCH does not inhibit α-aminoisobutyric acid uptake. On the other hand, our results when *N*-Me-Ala is used as a competitor of BCH are almost identical to those of Christensen *et al.*[20] using *N*-methylaminoisobutyric acid, and indicate that at most 15% of the BCH uptake is inhibitable by these A analogs.

In view of these findings, it seems unfeasible to exclude completely the existence of two binding sites. It would be impossible for BCH to have no effect on α-aminoisobutyric acid uptake and yet share its transport site if the availability of that site were rate-limiting to α-aminoisobutyric acid entry. Furthermore, the data of Christensen *et al.*[20] do establish that BCH competes most readily with L-type amino acids,

and that it exchanges with endogenous amino acids. Also, the Na^+-independent portion of BCH uptake is saturable. However, the total independence of these sites is called into question by the effect of removing Na^+ on BCH transport, which is quite similar to that for α-aminoisobutyric acid, as well as by the ability of aminoisobutyric acid to completely inhibit BCH uptake. Although it might appear that the degree of interaction between these sites could be evaluated in terms of their kinetics, it should be recognized that in a system in which the rate-limiting steps have not been satisfactorily elucidated, and in which the number of steps involved is unknown, the interpretation of K_m and K_i becomes tenuous at best. Furthermore, the lack of reciprocal competitive inhibition between α-aminoisobutyric acid and BCH suggests that the interactions between these amino acids involve more than simple enzyme kinetics.

In regard to the distinction between A and L transport systems, the L system may indeed be Na^+-independent and the primary vehicle for exchange diffusion. However, the present experiments as well as those of Jacquez *et al.*[19] show that all known L-type amino acids have a significant Na^+-dependent component in their uptake, and that this overlapping specificity makes a rigid distinction of the sites difficult at best.

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