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Characterization of L-threonine and L-glutamine transport in murine P388 leukemia cells in vitro. Presence of an N-like amino acid transport system

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The transport of L-threonine and L-glutamine into murine P388 leukemia cells has been characterized. Threonine appears to be a specific substrate for a Na^+-dependent amino acid transport system similar to system ASC of the HTC hepatoma cell. Threonine transport is uninhibited by 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid and α-(methylamino)isobutyric acid, shows a pattern of transport similar to that seen in HTC hepatoma cells over the pH range of 5.5–7.5, and is inhibited by L-serine and L-cysteine. Approximately two-thirds of glutamine transport into P388 cells also appears to enter P388 cells via this ASC-analogous system. However, based upon (a) inhibition studies with threonine (where the K_i of threonine inhibition of glutamine transport was 7-fold the K_m of threonine transport), (b) inhibition analysis of glutamine transport with various amino acids and amino acid analogues, and (c) different patterns of transport between threonine and glutamine over the pH range of 5.5–7.5, approximately one-third of glutamine transport can be attributed to a second Na^+-dependent amino acid transport system. This system appears to be similar to the system N of rat hepatocytes. Glutamine and threonine do not appear to enter P388 cells via systems A or L to any significant degree. P388 cells do not appear to exhibit 'adaptive regulation' of amino acid transport. Differences in 'adaptive regulation' could therefore not be utilized for comparing threonine and glutamine transport.

Introduction

The transport of neutral α-amino acids into eukaryotic cells has been investigated in a variety of cell types including Ehrlich ascites cells [1], 3T3 cells [2], human fibroblasts [3], rat hepatocytes [4–6], human lymphoblasts [7], mouse lymphocytes [8], mouse L-cells [9], Chinese hamster ovary cells [10,11], and rat pancreatic islet cells [12]. Included among the transport systems mediating the passage of neutral α-amino acids are the sodium (Na^+)-independent system L, whose strongest reactivity is with branched chain and aromatic amino acids such as L-leucine, L-valine, L-isoleucine and L-phenylalanine, and the Na^+-dependent system A which serves primarily for the transport of amino acids with short, polar and/or linear side chains such as glycine, L-alanine and α-aminoisobutyric acid. Although not as widely abundant as systems A and L, system ASC has been shown to be a major component of the Na^+-dependent transport of amino acids in the rabbit intestine [13], rabbit reticulocyte [14], pigeon erythrocyte [15], rat hepatocyte [6,16], cultured human fibroblast [3], HTC hepatoma cell [17], and Chinese hamster ovary cell [11]. It is

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Abbreviation: BCH, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid.

differentiated from system A by its greater affinity for hydroxyl- or sulfhydrylcontaining amino acids such as L-serine, L-threonine and L-cysteine, and by its intolerance to the N-methylation of substrates.

L-Glutamine is a neutral α-amino acid of great metabolic importance whose transport into cells has been shown to be via system ASC in a variety of cell types such as the pigeon erythrocyte [18], rabbit reticulocyte [19], and Chinese hamster ovary cell [11]. Glutamine transport has also been shown to be mediated by systems A, L and ASC in the Ehrlich ascites cell [1,18] and by systems L and ASC in the rat pancreatic islet cell [12]. Glutamine transport has also been shown to be mediated by a Na^+-dependent amino acid transport system designated N only in the rat hepatocyte and in hepatoma cell lines [20,21]. System N is distinguished from systems L, A and ASC by its strong reactivity with amino acids with nitrogen-containing side chains such as L-glutamine, L-asparagine and L-histidine. The transport of glutamine in a single cell type by both the N and ASC systems has not been previously described.

We have extended these studies on glutamine transport. In the present paper, we report on the transport of L-glutamine and L-threonine into murine P388 leukemia cells utilizing comparative analysis of (a) their kinetic parameters (i.e. K_m and V_{max} values), (b) their pattern of transport at various pH values and (c) their transport with various inhibitors, and by examining their respective inhibition of each other's transport.

Materials and Methods

Materials

L-[U-^{14}C]threonine (224 mCi/mmol), L-[U-^{14}C]glutamine (280 mCi/mmol), α-amino[1-^{14}C]isobutyric acid (58 mCi/mmol), [*carboxyl*-^{14}C]inulin (3.2 mCi/mmol), and tritiated water (90 μCi/mmol) were all purchased from Amersham Corporation. α-Aminoisobutyric acid, α-(methylamino)isobutyric acid, and the unlabelled natural amino acids were obtained from Sigma Chemical Co. 2-Aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH, isomeric form b($\pm$)) was purchased from Calbiochem-Behring Corporation. McCoy's 5A modified medium, fetal calf serum, and Dulbecco's phosphate-buffered saline were supplied by Gibco; bovine serum albumin (Fraction V, powder; low-salt and salt-free fractions) from Miles Laboratories; Versilube F-50 silicone oil from Nessa Products; choline chloride, dextrose and other chemicals from Fisher Laboratories.

Harvesting of P388 leukemia cells

Male BALB/c $\times$ DBA/2F mice (hereafter called $CD2F_1$ mice) weighing 17–25 g and maintained on LAB-BLOX laboratory chow pellets and water ad libitum were used throughout this study. $CD2F_1$ mice were implanted intraperitoneally with $2 \cdot 10^6$ murine P388 leukemia cells. The mice were killed by cervical traction 4–5 days after implantation, and the P388 cells were aspirated from their abdomens. The P388 cells were suspended in either a Na^+-rich medium containing phosphate-buffered saline (pH 7.4), 0.25% dextrose, and 0.1 mM low-salt bovine serum albumin, or a Na^+-free choline-substituted medium containing 0.1% dextrose and 0.1 mM salt-free bovine serum albumin as described previously [22]. The cells were then washed twice in the appropriate medium with centrifugations at $200 \times g$ for 5 min at 23°C. All final cell suspensions were in their appropriate medium at a concentration of $4 \cdot 10^6$ P388 cells/ml.

For the amino acid starvation studies, cells were initially suspended, washed twice, and resuspended at $4 \cdot 10^6$ P388 cells/ml in either McCoy's 5A modified medium or Kreb's-Ringer bicarbonate buffer. These studies were performed in medium with or without 10% fetal calf serum. Cells were preincubated for 1 h at 37°C in their respective media when 10% fetal calf serum was added. After a 2 or 6 h incubation at 37°C and 7% CO_2, the cells were washed twice and resuspended at $4 \cdot 10^6$ cells/ml in either Na^+-rich or Na^+-free media.

Cell viability was typically 95% by Trypan blue exclusion. The percentage of red blood cells in P388 cell suspensions was always $\leqq 10\%$.

Transport experiments

Prior to the initiation of transport experiments, all cell suspensions and amino acid solutions were pre-incubated for 15 min at 37°C in a shaking water bath. Transport was initiated by the addition

of labelled amino acid in the appropriate medium to an equivalent volume of suspended P388 cells ($4 \cdot 10^6$ cells/ml) at 37°C. At indicated time points, 400-μl aliquots of the amino acid-P388 cell incubation mixture were layered onto 1 ml of Versilube F-50 silicone oil in microcentrifuge tubes in triplicate. The assay was terminated by centrifugation of the samples $12000 \times g$ for 1 min at 23°C in a Brinkmann Eppendorf microcentrifuge to separate cells from medium as previously described [23]. The supernatants were discarded and the cell pellets were solubilized in 100 μl of 0.6 M perchloric acid overnight at 4°C. The tubes were then centrifuged for 3 min at $12000 \times g$ at 23°C to pellet cellular macromolecules. The supernatants were removed and placed in scintillation vials containing 10 ml scintillation cocktail. The radioactivity was determined via scintillation counting in a Wallac 1217 Rackbeta liquid scintillation spectrophotometer and corrected for quenching.

The intracellular water space (ICW) was determined with tritiated water plus [*carboxyl*-^{14}C]inulin for all transport studies by centrifugation through versilube F-50 silicone oil to separate medium from cells as previously described [24].

Calculations

Kinetic parameters were determined by linear-regression analysis and were equivalent when plotted on either Lineweaver-Burk or Eadie-Hofstee plots. For Fig. 5, the K_i was determined utilizing non-linear regression analysis of the initial part of the curve (i.e., up to 1 mM threonine). All uptake data are expressed in nanomoles or picomoles (as indicated) of tested amino acid per μl of intracellular water per min.

Results

Characterization of L-glutamine and L-threonine uptake

The time-course of the Na^+-dependent uptake of L-glutamine and L-threonine by P388 leukemia cells at 37°C is shown in Fig. 1. The transport of glutamine and threonine at both 0.01 and 0.40 mM was linear up to at least 1 min. On the basis of these results, an incubation time of 30 s was utilized throughout this study to approximate the initial rate of glutamine or threonine uptake. Threonine uptake approached steady-state levels at about 10 min for both concentrations. Fig. 2 shows how glutamine and threonine saturate their own Na^+-dependent uptake into P388 leukemia cells. Lineweaver-Burk plots for the kinetics of both glutamine and threonine uptake were linear over the concentration range utilized, 0.01 to 0.40 mM. The observed K_m for glutamine was 0.28 mM while the V_{max} was 4.35 nmol $\cdot$ (μl ICW)$^{-1} \cdot$ min^{-1}. The apparent K_m for threonine transport, was 0.11 mM while the observed V_{max} was 5.61 nmol $\cdot$ (μl ICW)$^{-1} \cdot$ min^{-1}.

General survey of inhibitory action on the transport of L-threonine and L-glutamine

The uptake of 0.1 mM threonine and 0.1 mM glutamine were tested for possible inhibition by a 100-fold excess of various amino acids and amino

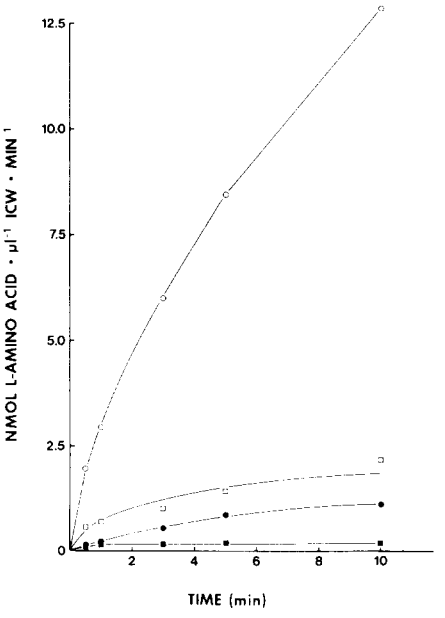

Fig. 1. Time-course of 0.01 mM or 0.4 mM L-threonine and 0.01 mM or 0.4 mM L-glutamine by P388 leukemia cells in suspension. The cells were incubated with 0.01 or 0.4 mM labelled L-threonine and 0.01 or 0.4 mM labelled L-glutamine at 37°C for a period of up to 10 min, with aliquots removed from the incubation mixture at specified time points (see Materials and Methods), (■) 0.01 mM L-[^{14}C]threonine; (□) 0.40 mM L-[^{14}C]threonine; (●) 0.01 mM L-[^{14}C]glutamine; (○) 0.40 mM L-[^{14}C]glutamine. The data shown are the Na^+-dependent uptake rates only. The data are the averages of three determinations.

acid analogues. Table I shows that BCH, a non-metabolizable amino acid analogue known to enter cells specifically by the Na^+-independent system L, showed little inhibition of threonine transport. Consistent with this finding is the observation that threonine transport was reduced to 15% of control in Na^+-deficient media. As well, α-(methylamino)-isobutyric acid, a non-metabolizable amino acid analogue known to enter cells specifically by the Na^+-dependent system A, showed no inhibition of threonine transport. Threonine transport was inhibited to 50% of control by 100-fold excess histidine. Leucine, serine, cysteine, asparagine and glutamine were the most effective inhibitors, reducing threonine transport to $\leq 26\%$ of control.

BCH and α-(methylamino)isobutyric acid showed little or no inhibitory action on glutamine transport (Table I). Histidine and leucine inhibited glutamine trnasport by approx. 50% while threonine reduced glutamine transport to about one-third of control. Cysteine and asparagine were the most effective inhibitors of glutamine transport and, like threonine, glutamine was not transported effectively by P388 leukemia cells in Na^+-deficient media.

The inhibition of glutamine uptake was examined in the presence of either 100-fold excess threonine or glutamine alone, or together in combination, in Na^+-rich and Na^+-poor media (results not shown). No significant difference in glutamine transport was observed in Na^+-deficient media with or without excess unlabelled threonine or glutamine alone or in combination (i.e. $\leq 12\%$ of control). Threonine was not as effective an inhibitor as was excess unlabelled glutamine in blocking glutamine uptake in Na^+-rich media. Threonine decreased glutamine transport to 28% of control as compared to 12% by excess unlabelled glutamine. When excess unlabelled threonine and glutamine were added together to the incubation mixture, the effect was the same as that of excess glutamine alone.

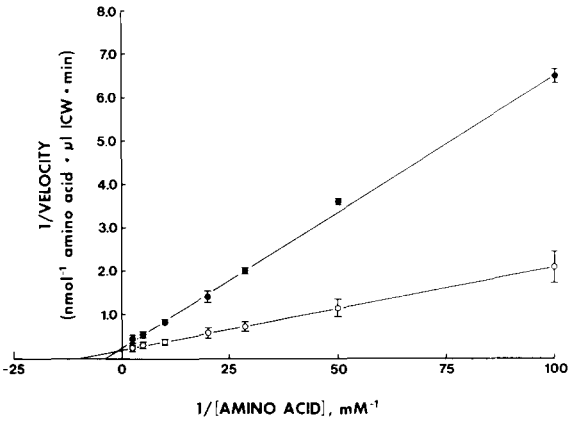

Fig. 2. Lineweaver-Burk plot of kinetics of L-threonine and L-glutamine at 0.01 to 0.40 mM were tested for 30 s at 37°C as described under Materials and Methods. (○) L-[^{14}C]threonine; (●) L-[^{14}C]glutamine. The observed velocities in the absence of Na^+ have been subtracted so that only the Na^+-dependent velocities are shown. The data are the mean ± S.E. of five determinations for L-threonine and three determinations for L-glutamine and were analyzed via linear-regression analysis.

Effect of external pH

The uptake rates of both glutamine and threonine into P388 leukemia cells at several pH values between 5.5 and 8.0 are shown in Fig. 3. The uptake of 0.1 mM threonine was not significantly suppressed by raising the $[H^+]$ to pH 6.0

TABLE I

INHIBITION OF L-THREONINE AND L-GLUTAMINE UPTAKE BY A VARIETY OF NATURAL AND ARTIFICIAL AMINO ACIDS

Transport of 0.1 mM labelled L-threonine and 0.1 mM labelled L-glutamine into P388 leukemia cells in suspension was measured in the presence or absence of 10 mM unlabelled inhibitor. The uptakes were for 30 s at 37°C as described under Materials and Methods. The data are expressed as the mean ± S.E. for two to seven determinations. The uninhibited velocities in Na^+-rich media were 3.36 ± 0.51 nmol·(μl ICW)$^{-1}$·min^{-1} for L-threonine and 2.20 ± 0.59 nmol·(μl ICW)$^{-1}$·min^{-1} for L-glutamine. MeAIB, α-(methylamino)isobutyric acid.

Inhibitor tested, 10 mM	Uptake (% of control)	
	L-threonine	L-glutamine
None	100	100
BCH	91 ± 2	120 ± 6
MeAIB	104 ± 15	93 ± 10
L-Leucine	21 ± 3	54 ± 4
L-Serine	14 ± 1	–
L-Cysteine	19 ± 7	15 ± 4
L-Asparagine	14 ± 3	23 ± 1
L-Histidine	51 ± 4	46 ± 4
L-Glutamine	26 ± 9	12 ± 2
L-Threonine	16 ± 3	32 ± 7
In Na^+-free media (− inhibitor)	21 ± 3	11 ± 2

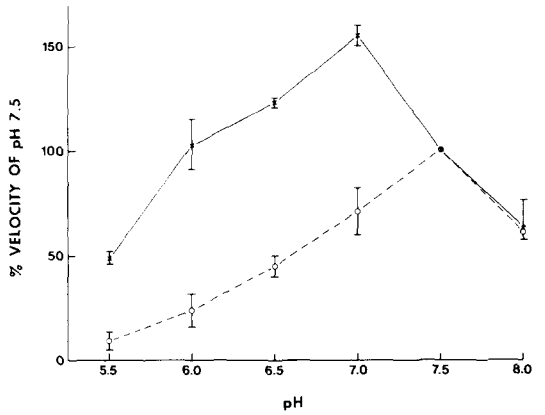

Fig. 3. The relation between pH of the uptake medium and the uptake of labelled L-threonine and labelled L-glutamine in P388 leukemia cells. P388 leukemia cells in suspension were assayed at varying pH values between 5.5 and 8.0, (×) 0.1 mM L-[^{14}C]threonine; (○) 0.1 mM L-[^{14}C]glutamine. The data presented are rates for the Na^+-dependent component only at each of the pHs examined. The rates (pmol·(μl ICW)$^{-1}$·min^{-1}) at pH 7.5 were 383 for L-threonine and 566 for L-glutamine. Uptakes were measured for 30-s incubations at 37°C as described under Materials and Methods. The data are the averages ± S.E. of at least three determinations.

from pH 7.5. Glutamine uptake was, however, significantly decreased as the [H^+] increased, and glutamine was significantly more sensitive than threonine at all pH values treated except for pH 8.0.

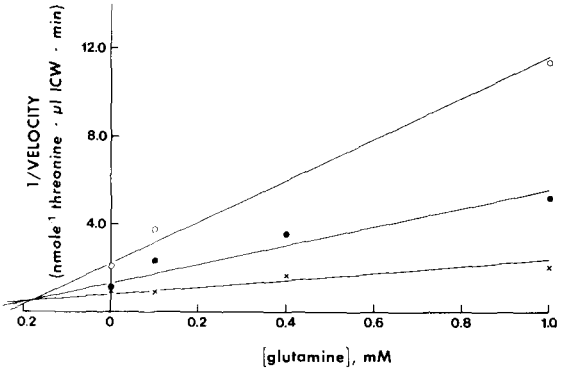

Fig. 4. A Dixon plot of L-glutamine inhibition of L-threonine transport. The 30 s transport of 0.025, 0.075, and 0.15 mM labelled L-threonine in media containing 0.1 to 1 mM labelled L-glutamine were measured in P388 leukemia cells at 37°C as described under Materials and Methods. (○) 0.025 mM L-[^{14}C]threonine; (●) 0.075 mM L-[^{14}C]threonine; (×) 0.15 mM L-[^{14}C]threonine. The results represent the Na^+-dependent component of L-threonine uptake only and are the averages of triplicate determinations. The uninhibited velocities of 0.025, 0.075, and 0.15 mM labelled L-threonine were 0.48, 0.9 and 1.11 nmol L-threonine·(μl ICW)$^{-1}$·min^{-1}, respectively.

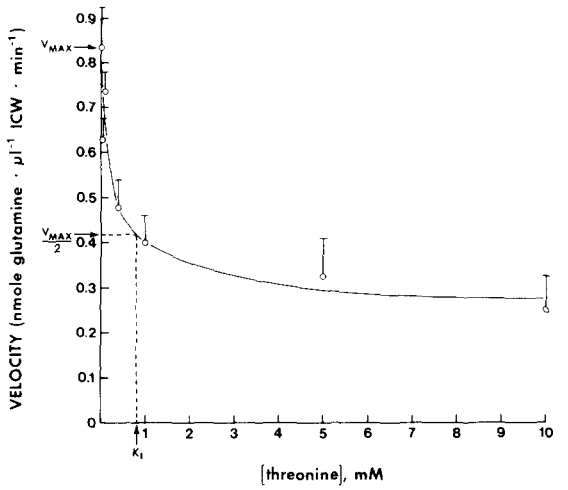

Fig. 5. A plot of [L-threonine] versus velocity of L-glutamine uptake. The 30 s uptake of 0.1 mM labelled L-glutamine in media containing 0.05 to 10 mM unlabelled L-threonine was measured in P388 leukemia cells at 37°C as described under Materials and Methods. The results represent the Na^+-dependent component of L-glutamine uptake only and are the means ± S.E. of three determinations. The uninhibited velocity of labelled L-glutamine was 0.835 nmol·(μl ICW)$^{-1}$·min^{-1}.

Dixon plot analysis of glutamine and threonine transport

Fig. 4 is a Dixon plot showing the effect of increasing levels of external glutamine on the uptake of 0.025, 0.075, and 0.15 mM labelled threonine. The results appraoch a competitive-type inhibition and yield a K_i of 0.18 mM. This value is close to the glutamine concentration which half-saturates its own uptake (i.e., K_m of 0.28 mM; see Fig. 2).

Fig. 5 shows the uptake of labelled 0.10 mM glutamine into P388 leukemia cells under inhibition by unlabelled threonine. A computer-derived best fit rectangular hyperbola similar to that seen for both [^{3}H]cysteate in rat hepatocytes and [^{3}H]aspartate in human fibroblasts [25] was not justified since glutamine transport was not completely inhibited by excess unlabelled threonine. A baseline appears to be reached at a velocity of glutamine uptake of about 0.27 nmol·(μl ICW)$^{-1}$·min^{-1}. The observed K_i (see Materials and Methods) of threonine inhibition of glutamine uptake was 0.8 mM, which is approximately 7-fold the K_m of threonine transport of 0.11 mM (see Fig. 2). The velocity of glutamine uptake corre-

sponding to the observed K_i was 0.42 nmol · (μl ICW)$^{-1}$ · min^{-1}.

Effects of amino acid starvation on transport activity

The effects of amino acid starvation on the transport activity of α-aminoisobutyric acid in P388 cells were investigated. α-Aminoisobutyric acid was shown to be a specific, non-metabolizable amino acid analogue for system A in P388 cells (results not shown). The uptake of 0.1 mM labelled α-aminoisobutyric acid was subjected to inhibition analysis after the P388 cells were cultured for 2 or 6 h in either amino acid-rich (McCoy's 5A modified) or amino acid-free (Kreb's-Ringer bicarbonate) medium with or without 10% fetal calf serum. α-Aminoisobutyric acid transport remained relatively unchanged in Na^+-rich medium after 2 h of amino acid starvation in media without fetal calf serum (i.e., 1.39 nmol · (μl ICW)$^{-1}$ · min^{-1} for cells cultured in amino acid-rich medium versus 1.40 nmol · (μl ICW)$^{-1}$ · min^{-1} for cells cultured in amino acid-free medium) but showed a 65% decrease in Na^+-rich medium after 6 h in the same amino acid-free medium (i.e. 2.65 nmol · (μl ICW)$^{-1}$ · min^{-1} for cells cultured in amino acid-rich medium versus 0.92 nmol · (μl ICW)$^{-1}$ · min^{-1} for cells cultured in amino acid-poor medium). The addition of 100-fold excess unlabelled alanine, α-(methylamino)isobutyric acid, or α-aminoisobutyric acid to the incubation mixture did not change this pattern. When choline replaced Na^+ as the cation in the incubation mixture, a decrease in α-aminoisobutyric acid transport was seen after both 2 and 6 h of amino acid starvation. Thus, there was no evidence of stimulation of α-aminoisobutyric acid transport after 6 h of amino acid starvation. A similar pattern of transport was seen after 6 h of amino acid starvation when 10% fetal calf serum was added to the medium.

A similar pattern was seen for the transport of both glutamine and threonine (results not shown). After a 6-h amino acid starvation period in media with or without 10% fetal calf serum, a general decrease in the transport of both amino acids was observed comparable to that seen for α-aminoisobutyric acid.

Discussion

It has been previously shown that threonine is a specific substrate and inhibitor of system ASC in the HTC hepatoma cell [17]. The results presented here suggest that two neutral amino acid transport systems other than systems A and L are present in murine P388 leukemia cells, and that threonine appears to be a model substrate for one of these systems. The uptake of threonine was essentially unaffected by the addition of BCH or α-(methylamino)isobutyric acid and was greatly decreased when choline replaced Na^+ in the incubated mixture suggesting that threonine does not enter P388 cells by systems L or A. Threonine transport was also greatly reduced by excess unlabelled serine and cysteine, both of which have been known to enter a variety of cell types by system ASC [6,12,26,27]. As well, threonine transport was not suppressed by increasing $[H^+]$, a pattern similar to that observed for cysteine transport by system ASC in the rat hepatocyte [20]. Furthermore, the pattern of threonine transport observed in P388 cells in the pH range of 5.5–7.5 is remarkably similar to that seen over the same pH range in the HTC hepatoma cell line for system ASC. These data suggest that threonine transport into P388 cells is via a single Na^+-dependent system analogous to system ASC.

Glutamine transport was also relatively unaffected by the addition of either excess unlabelled BCH or α-(methylamino)isobutyric acid and was greatly decreased in Na^+-deficient media, suggesting that glutamine, like threonine, does not enter P388 cells by systems L or A. However, excess unlabelled threonine only reduced glutamine transport to one-third of control. This differed from the effect observed when both excess unlabelled threonine and glutamine were added together, or when glutamine was added alone to the incubation mixture. These results suggest that approximately two thirds of glutamine uptake is via the same Na^+-dependent ASC-analogous system by which threonine enters P388 cells. The apparent discrepancy between the effects of cysteine and threonine on glutamine transport can be explained by the ability of cysteine to bind non-competitively with transport sites of amino acid transport systems other than system ASC, as seen for the rat hepatocyte [6] and the HTC hepatoma cell [17].

System ASC showed less sensitivity to increases in $[H^+]$ than system A in the Ehrlich cell [26] and

system A and system N in the rat hepatocyte [20]. The results presented here show that glutamine uptake was more sensitive to increasing $[H^+]$ than threonine uptake in P388 cells, again suggesting that at least a portion of glutamine transport into P388 cells differs from that of threonine. Inhibition studies support this conclusion. The 7-fold discrepancy between the K_i of threonine inhibition of glutamine transport and the K_m of threonine uptake into P388 cells is not consistent with a completely shared transport between glutamine and threonine. It appears as if glutamine enters P388 cells by two separate Na^+-dependent amino acid transport systems: one which also transports threonine (i.e., the ASC-analogous transport system), and another as yet unidentified system similar to the system N of the rat hepatocyte. Glutamine transport in P388 leukemia cells differs from other mammalian cells in that it appears to be transported by both the ASC and N systems.

The activity of system N in the rat hepatocyte [20] and system A in a variety of cell types [28–34] has been shown to increase when the cell has been incubated in an amino acid-free medium. This enhancement of transport, or 'adaptive regulation', has been shown to depend on the synthesis of protein and RNA resulting from derepression of the synthesis of one or more proteins involved in amino acid translocation [35]. We have shown, however, that P388 cells do not exhibit 'adaptive regulation' for amino acids. α-Aminoisobutyric acid showed no change in transport after a 2-h incubation of cells in an amino acid-free medium, and in fact showed a decrease in transport after a 6 h incubation. This was also true for both glutamine and threonine in P388 cells. Thus, differences in adaptive regulation for the transport of threonine and glutamine for comparison purposes could not be utilized.

In conclusion, threonine appears to be a specific substrate for a Na^+-dependent ASC-analogous amino acid transport system in P388 leukemia cells. Glutamine apparently shares this system to a large extent. Glutamine also appears to be transported by a second Na^+-dependent system not inhibited by α-(methylamino)isobutyric acid. This system appears to be similar to the system N of the rat hepatocyte. Therefore, glutamine transport via the N system appears to occur in P388 leukemia cells as well as in rat hepatocytes and hepatoma cell lines.

Acknowledgements

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