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HETEROGENEITY OF Na^+-INDEPENDENT 2-AMINOBICYCLO-(2,2,1)-HEPTANE-2-CARBOXYLIC ACID AND L-LEUCINE TRANSPORT IN ISOLATED RAT HEPATOCYTES IN PRIMARY CULTURE

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Kinetic heterogeneity of saturable Na^+-independent transport of both leucine and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid uptake by primary cultures of isolated rat hepatocytes was demonstrated. Such heterogeneity was not evident in the transformed hepatic cell line, HTC. One of the two components of Na^+-independent transport in normal hepatocytes increased in activity with increasing time in primary culture, whereas the second component decreased in activity during the same time period. On the basis of kinetic and inhibition analysis the two components have tentatively been identified as Systems L and T. The increase in System T activity during the initial days of primary cultures of hepatocytes which is dependent on de novo synthesis of both RNA and protein, represents one of the first demonstrations of regulation for a Na^+-independent amino acid transport system.

Introduction

It is widely recognized that neutral amino acids are accumulated by eukaryotic cells via a number of unique transport systems with overlapping specificity (1). One class of transporters requires Na^+ for activity and includes Systems A, ASC, N, and Gly. A second class exists whose activity is independent of Na^+ and until recently, consisted of only one primary example, namely System L. System L activity has been reported in many eukaryotic cells and may be ubiquitous. It was originally described in the Ehrlich ascites tumor cell (2) and has been well characterized in that cell through the use of an amino acid analog, BCH[1], whose transport into Ehrlich cells is entirely Na^+-independent (3,4). Several laboratories have used BCH uptake or inhibition of transport by BCH in a variety of cell types as a direct measure of System L activity without demonstrating specificity for either condition.

We now report that BCH is not a specific substrate or inhibitor for System L activity in primary cultures of normal rat hepatocytes. The results presented demonstrate, through preferential inhibition and kinetic analysis,

[1] BCH = 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid.

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that BCH is transported by these cells via two distinct Na^+-independent agencies We believe that in addition to System L, hepatocytes accumulate BCH by a Na^+-independent neutral amino acid transport system which is similar to one recently described for the human red blood cell. In the red cell the newly discovered system, called System T, transports the aromatic amino acids, tryptophan and tyrosine, and shows some affinity for other neutral amino acids (5). We have provided evidence, to be published elsewhere[2], that System T exists in both adult and fetal hepatocytes, but may have a much broader specificity than originally reported for the red blood cell activity.

Methods

Adult rat hepatocytes were isolated by a collagenase perfusion method as described previously (6). The cells were plated on collagen coated Costar multi-well cluster dishes (12 or 24-well) in a modified Waymouth's medium as listed in Kilberg, et al (6). The cells were plated in Waymouth's medium containing 10% fetal calf serum at a density of approximately 300,000 cells/well for the 24-well trays and 675,000 cells/well for the 12-well trays. After two hours the medium was replaced with fresh medium with or without serum as indicated in the figure legends. The hepatoma cell line was maintained by the method of Handlogten, et al (7).

Transport of radioactively-labelled amino acids was measured using a rapid assay technique recently described by Gazzola, et al (8). The protein content of each well was measured by combining two modifications of the Lowry procedure (9,10). To each well of the cluster tray was added 600 µl of Cu^{++} reagent containing 0.25 g/l $Na_2Cu^{++}EDTA$, 20 g/l $NaCO_3$, 4 g/l NaOH, and 2 g/l sodium dodecyl sulfate. After a 10 min. incubation at room temperature, 60 µl of phenol reagent was added and each tray was gently shaken in a vortex-like motion. The absorbance of each sample was measured on a Bausch and Lomb 2000 spectrophotometer outfitted with an automated sipper system.

The data were calculated on a TRS-80 microcomputer utilizing software which incorporates standard statistical analyses. The kinetic analyses were handled by FORTRAN programs (11) obtained from Dr. Morris F. White, University of Michigan, designed to calculate the kinetic constants for transport data containing either one saturable and one non-saturable (K_d) components (equation 1) or two saturable and one non-saturable components (equation 2).

$$1. \quad \log v = \log\left(\frac{V_{max} \cdot S}{K_m + S} + K_d \cdot S\right)$$

$$2. \quad \log v = \log\left(\frac{V_1 \cdot S}{Km_1 + S} + \frac{V_2 \cdot S}{Km_2 + S} + K_d \cdot S\right)$$

It has been determined that the kinetic data obtained from BCH and leucine uptake by normal hepatocytes is only compatible with equation 2, whereas the kinetics of the uptake of these two amino acids by the hepatoma cells are best described by equation 1. The data are reported in picomoles or nanomoles of amino acid transported per mg protein per 30 sec. If not indicated the standard deviation was less than 10% of the average.

[2] L. Weissbach, M.E. Handlogten, H.N. Christensen and M.S. Kilberg, manuscript submitted for publication.

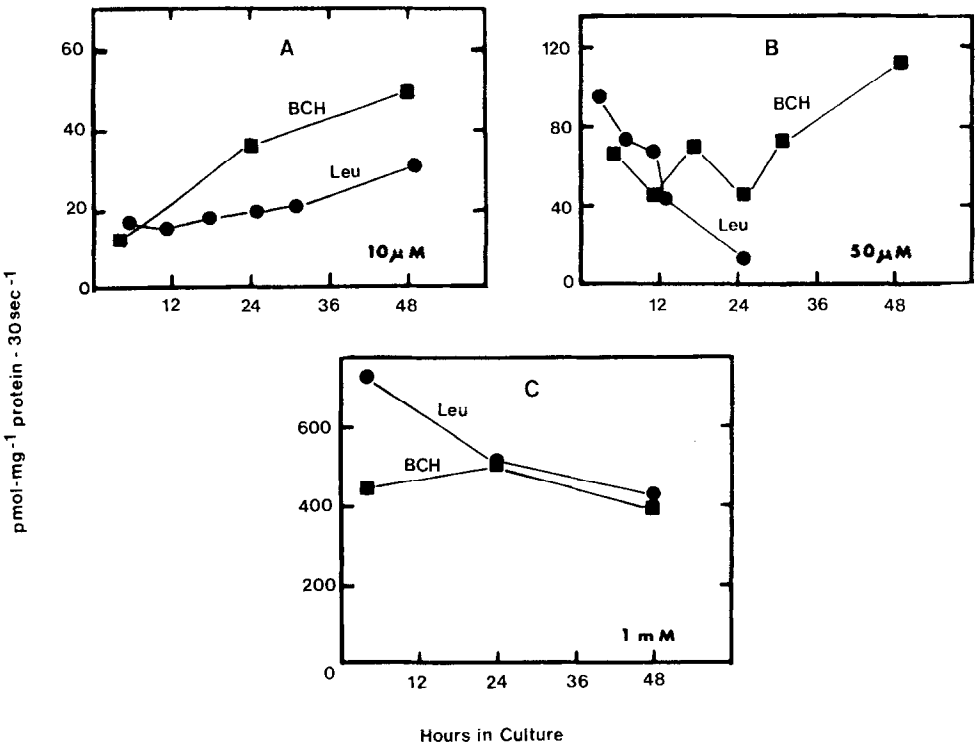

Figure 1. At the indicated time after establishing primary cultures of rat hepatocytes, the Na^+-independent uptake of either BCH or leucine was measured for 30 sec. The results shown represent only the saturable uptake, determined by the addition of 10 mM unlabelled amino acid to the assay. One hour prior to the uptake assays the cells were placed in Na^+-free Krebs-Ringer phosphate buffer to allow depletion of intracellular amino acids. The data are the averages of at least 4 determinations and are the composite of two different hepatocyte preparations. The standard deviation was typically less than 10% of the mean.

Results

The Na^+-independent uptake of either leucine or BCH with respect to time after establishing primary cultures of adult rat hepatocytes is shown in Figure 1. At low concentration, such as 10 μM, the uptake rates of both amino acids were increased during the 48 hr. period studied (Figure 1A), although the increase of leucine uptake was somewhat less than that for BCH. At a somewhat higher concentration of substrate (Figure 1B), namely 50 μM, an apparent dichotomy begins to appear. During the initial 24 hr. the BCH uptake is variable but remains essentially unchanged overall, however, between 24 and 48 hr. the transport of BCH is increased by two-fold (Figure 1B). In contrast, the uptake of 50 μM leucine begins to decline immediately after starting the hepatocyte cultures and continues to do so for the next 24 hr. (additional experiments have confirmed that this process continues to decline between 24 and 48 hr. as well). Finally, if one assays the transport of these two amino acids at a concentration of 1 mM (Figure 1C), leucine uptake decreases, while 1 mM BCH transport stays

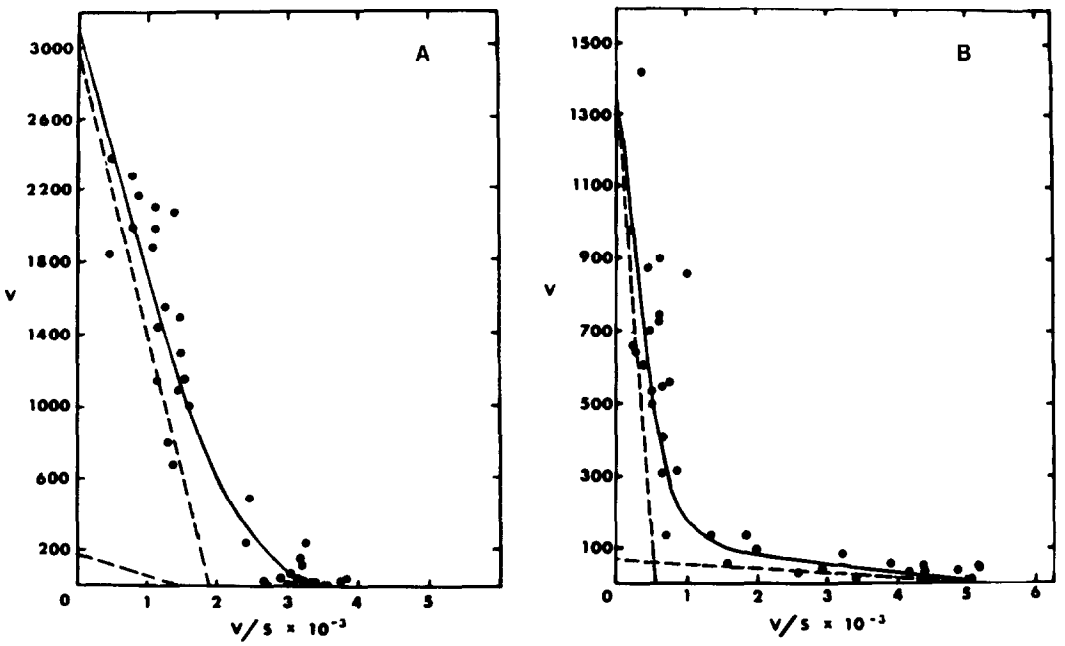

Figure 2. The Na^+-independent uptake of BCH, early (4 hr., Part A) or late (48 hr., Part B) in culture, was measured over the concentration range of 0.001 to 20 mM. The cells had been depleted of amino acids for 1 hr. prior to the 30 sec. uptake. The kinetic constants, calculated by computer analysis as described in the text, are summarized in Table I. The units for velocity (saturable uptake only) and substrate concentration were pmol·mg^{-1} protein·30 sec^{-1} and mM, respectively. The solid line represents the computer-derived best fit for the data and the dashed line shows the calculated intercept values after resolution of the two components.

relatively unchanged. The specificity of such changes in transport activity with time in culture is demonstrated by the fact that the activity of System A remains constant during this 48 hr. period (data not shown). Furthermore, the cells have been depleted of exchangable intracellular amino acids by incubation for 1 hr. in Na^+-free Krebs-Ringer bicarbonate buffer prior to the transport assays.

The relation between substrate concentration and uptake velocity for BCH is shown in Figure 2. The Eadie-Hofstee plot is biphasic regardless of whether one assays BCH transport early (prior to 12 hr.) or late (after 24 hr.) in culture. These results indicate a heterogeneity of Na^+-independent uptake of BCH by the rat hepatocyte. Analysis of the data with the aid of computer programs as described in the Methods section yields the kinetic constants shown in Table I. The changes seen for the two components are in agreement with the data of Figure 1 in that rate constant (V_{max}/Km) of the high affinity agency increases (3 to 4-fold) with time, while that of the low affinity component decreases (2 to 3-fold) over the same time period. Interestingly, the change in component I is due to an alteration in Km, whereas that of component II is

TABLE I

Time in Culture / Substrate	Component I			Component II		
	K_m, mM	V_{max}, pmol·mg^{-1} protein·30 sec^{-1}	Rate Constant, V_{max}/K_m	K_m, mM	V_{max}, pmol·mg^{-1} protein·30 sec^{-1}	Rate Constant, V_{max}/K_m
Early						
BCH	0.125	174	1392	1.6	2989	1868
Leucine	0.007	8	1143	0.98	2219	2264
Late						
BCH	0.014	71	5071	1.9	1326	698
Leucine	0.012	36	3000	0.37	511	1381

Hepatocytes were cultured as indicated in the text. At 2 to 6 hr (early) or 24 to 48 hr (late) after establishing the primary cultures the medium was changed to Na^+-free Krebs-Ringer phosphate for 1 hr to deplete the intracellular amino acid pools prior to the 30 sec. uptake assays. Choline phosphate and choline chloride osmotically replaced the corresponding sodium salts in the Na^+-free buffer. The data, shown in Figures 2 and 3, were analyzed with the aid of computer programs as described in the text.

the result of a change in V_{max}. Three different hepatocyte preparations have given essentially the same results.

Similarly, the kinetics of leucine uptake yield biphasic Eadie-Hofstee plots (data not shown). Leucine uptake by component I increased in activity at the longer culture times, while the lower affinity component II declined in velocity between 4 and 48 hr. These results are summarized in Table I which lists the kinetics values for both components. In contrast to the results for BCH, the changes in leucine uptake by the two transport systems are primarily due to an increase and decrease in V_{max} for components I and II, respectively.

The BCH and leucine kinetics for Na^+-independent uptake in the hepatoma cell line, HTC, are shown in Figure 3A (BCH) and Figure 3B (leucine). In sharp contrast to the results from the normal hepatocyte, the Eadie-Hofstee plots are linear over the entire concentration range of 0.001 to 5.0 mM. The computer calculated kinetic constants suggest the uptake occurs by a high affinity system whose maximal velocities are significantly higher than those found in cultured normal hepatocytes.

Discussion

The results presented here demonstrate a heterogeneity in the Na^+-independent transport of both leucine and BCH by normal adult rat hepatocytes in primary culture. The uptake of these amino acids is generally regarded as a System L-specific test when measured in the absence of the sodium ion; both have been used to monitor System L in hepatocytes (6, 12-15). Although others have cautioned that linear kinetics

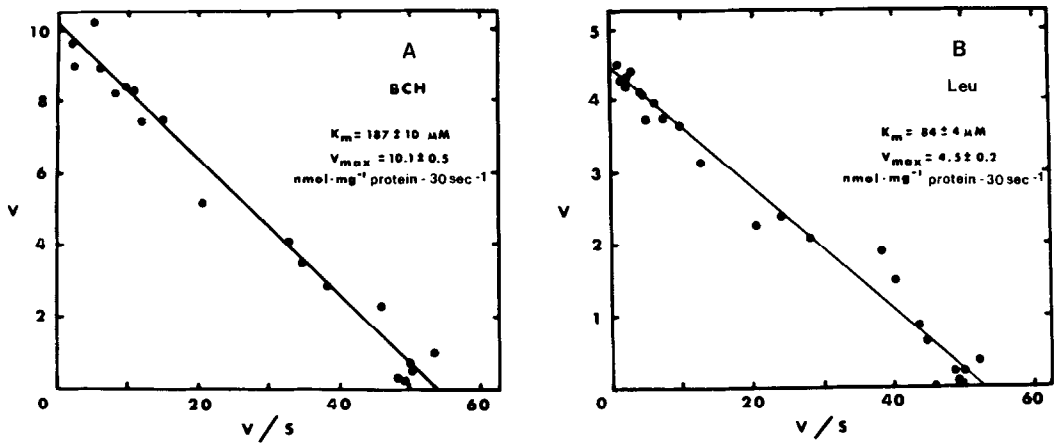

Figure 3. The transport kinetics of either BCH (Part A) or leucine (Part B) were measured in HTC cells over the concentration range of 0.001 to 5.0 mM. In the same manner used for the hepatocytes, the cells were depleted of amino acids for 1 hr. prior to uptake by incubation in Na^+-free medium. Only the saturable, Na^+-independent transport is given (v = nmol·mg^{-1} protein·30 sec^{-1} and s = mM); the non-saturable component having been calculated and subtracted by computer fitting (11). The correlation coefficients for the experimental data were 0.976 and 0.978 for BCH and leucine, respectively.

alone do not justify automatic assignment of the uptake to one system (16), it is less likely that biphasic kinetics with such large differences in Km values would arise from artifacts. Furthermore, the uptake by component I can be preferentially inhibited to a significantly greater extent than component II by tryptophan, tyrosine, and histidine[2]. This relative specificity of inhibition can be contrasted with the HTC cell in which reciprocal inhibition analysis supports the hypothesis that only one Na^+-independent system exists (data not shown).

Recently, Rosenberg, et al (17) described a newly discovered Na^+-independent amino acid transport system in human red blood cells. The system, designated System T, transports the aromatic amino acids tryptophan, tyrosine, and to a limited extent phenylalanine. It exists in the human RBC along with System L, yet the two systems can be distinguished on the basis of inhibition and kinetic analyses (17). We have tentatively assigned the nomenclature of System T to component I and System L to component II on the basis of information which will be published elsewhere[2]. Regardless of the final assignment of nomenclature, the results demonstrate that the Na^+-independent uptake of either leucine or BCH by normal rat hepatocytes should not be attributed exclusively to System L, although such specificity may be valid for HTC cells.

Acknowledgement

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