Evidence for Two Na\(^+\)-independent Neutral Amino Acid Transport Systems in Primary Cultures of Rat Hepatocytes

TIME-DEPENDENT CHANGES IN ACTIVITY*

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Adult rat hepatocytes placed in primary culture contain at least two distinct Na\(^+\)-independent transport systems for neutral amino acids. The characteristics of the two systems do not allow assignment to previously described Na\(^+\)-independent agencies, so we have tentatively termed the two processes Systems L1 and L2. Uptake by System L1 is substantially inhibited by cysteine, valine, isoleucine, leucine, methionine, histidine, tryptophan, tyrosine, phenylalanine, and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid. In contrast, System L2-mediated transport is completely inhibited by isoleucine, leucine, phenylalanine, and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid. Amino acids transported by both systems show biphasic kinetics yielding \(K_c\) values for the System L1 component in the micromolar range, whereas the corresponding values for System L2 are an order of magnitude higher. In freshly isolated hepatocytes, the activity of System L2 is relatively high and declines over the initial 24 to 48 h of culture. The Na\(^+\)-dependent Systems N and ASC also show a significant decay in activity during this time period. In contrast to the decrease in uptake by System L2, transport by System L1 increases during culture following an initial lag period of 12 to 24 h. The increase in System L1 activity can be blocked by the addition of either cycloheximide or actinomycin D. System L1 appears to be present also in fetal hepatocytes, although, in the hepatoma cell line, HTC, the Na\(^+\)-independent component appears to be homogeneous as though one of the two systems present in the normal adult hepatocyte is not expressed in these transformed cells.

Rat hepatocytes used either as freshly isolated cell suspensions or as primary cultures have been shown to contain neutral \(\alpha\)-amino acid transport Systems A, ASC, N, Gly, and a system thought to be System L (1-6). Of these, only the latter component is Na\(^+\)-independent (2-6). In fact, until recently, System L was provisionally taken to be the only Na\(^+\)-independent transport system for neutral amino acids detected in mammalian cells. Both Systems A and N have the potential to undergo adaptive regulation, a process measured as a stimulation of transport activity after maintaining the

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1 L. Weissbach and M. S. Kilberg, unpublished results.
2 The abbreviation used is: BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid.
medium lacked the serum during the remainder of the incubation period. Cell viability, judged by trypan blue exclusion, was better than 85% immediately after isolation. Only viable cells appear to remain attached to the trays after the final rinses. There is a continuous loss of total protein in each well during the 24- to 48-h period studied, such that the protein content of each well of a 24-well tray may typically be 150 to 200 mg of protein at 2 h after plating. Before 24 to 48 h, this value may decline to 40 to 60 mg. HTC cells were maintained in Waymouth's medium containing 10% fetal calf serum as described previously (5). Hepatocytes from rat fetuses, near term, were isolated by the collagenase-dispersion method of Leffert et al. (14). The transport kinetics were analyzed with computer programs designed to calculate kinetic constants from uptake data reflecting the presence of either one saturable and one nonsaturable component or two saturable and one nonsaturable component (15).

Uptake of radioactively labeled amino acids was measured according to the method of Gazzola et al. (16) with a few minor modifications. Prior to the uptake assays, the cells were depleted of intracellular amino acids by two successive incubations in Na+-free Krebs-Ringer phosphate buffer, the first for 15 min and the second for 45 min. In the various Na+-free buffers used, choline chloride, choline bicarbonate, or choline phosphate replaced the corresponding sodium salts. The uptake period is intended to eliminate possible trans-effects due to concurrent changes in the cellular amino acid content. Following the transport assays, the cells were completely solubilized by the addition of 0.2 ml of a 0.2 n NaOH solution containing 0.2% sodium dodecyl sulfate to each well of a 24-well tray. After a 15-min incubation at room temperature, 0.1 ml of the extract was transferred to scintillation vials for determination of radioactivity. The remaining 0.1 ml was left in each well for protein determination by a combination of two modifications of the Lowry procedure (17, 18). Briefly, 600 μl of CuCl2 reagent were added to each well, followed by the addition of 60 μl of phenol reagent after a 10-min incubation. The absorbance of each well was determined after 35 to 40 min with the use of a Bausch and Lomb 2000 spectrophotometer outfitted with an automated sipper system.

RESULTS

During studies in Ann Arbor involving histidine transport by freshly isolated and primary cultures of rat hepatocytes, it was discovered that histidine uptake by cells in suspension occurred predominately by the Na+-dependent System N (4), yet, after culturing hepatocytes (from the same preparation of cells) for 24 h, the per cent uptake by System N had decreased and the contribution by saturable2 Na+-independent uptake had increased significantly (Table I).

When the research was reinitiated in Gainesville, the activity of each major neutral amino acid transport systems was measured at specific intervals during the first 24 h after establishing primary cultures of normal hepatocytes. The Na+-dependent systems, as shown in Fig. 1A, either remained the same (System A) or declined in activity by varying amounts (System ASC or N). System ASC activity decreased whether assayed by Na+-dependent uptake of leucine or of cysteine (3), and both System N-specific substrates, glutamine and histidine,4 showed similar decreases (Fig. 1A). Note that the rate of change of the two systems is different over the course of the experiment.

The Na+-independent, BCH-inhibitable uptakes of 50 μM cysteine, glutamine, histidine and leucine were also tested during the initial 24 h of hepatocyte cultures. While the BCH-sensitive uptake of cysteine was found to be somewhat variable, that of both glutamine and leucine decreased steadily over the entire 24-h period (Fig. 1B). In contrast, the Na+-independent uptake of histidine increased by 4- to 5-fold over the 24-h period of the experiment. Other tests have indicated that this increase in activity continues for at least 6 days (data not shown). These results demonstrate the obvious inconsistency of BCH-inhibitable leucine uptake decreasing and, at the same time, BCH-inhibitable histidine uptake increasing. We have recently reported (20) evidence that the Na+-independent transport of both leucine and BCH show kinetic heterogeneity and should, therefore, not be considered System L-specific substrates for isolated hepatocytes. The lag period preceding the increase in histidine uptake varies considerably between experiments and ranges from 12 to 24 h. Despite this variability, the increase in activity is consistently observed in hepatocytes from fed or fasted rats and is not significantly altered by the inclusion of 10% fetal calf serum in the medium or by omission of the 1- to 2-h depletion of intracellular amino acids prior to the transport assays. In fact, incubation for up to 120 min in Na+-free Krebs-Ringer phosphate results in a small, but significant, increase in histidine transport by System N, yet produces no change in the BCH-inhibitable, Na+-independent uptake of histidine (0 min = 23.7 versus 120 min = 24.9 pmol·mg⁻¹ of protein·30 s⁻¹). These results are in agreement with those of Kelley and Potter (8) who showed that incubation of normal hepatocytes in the absence of extracellular amino acids did not produce an increase or decrease in Na+-independent leucine uptake. Nevertheless, as a precaution against possible trans-effects, all of the experiments reported here included a 60-min depletion period in Na+-free buffer prior to the uptake assays. As a further test for possible changes in cellular conditions, it was found that the intracellular water, measured by the 3-O-methylglucose method of Kletzen et al. (21), did not vary significantly from an average of 1.5 μl/mg of protein during the initial 24 h culture period.

To investigate the dichotomy between the changes in the uptake of histidine and leucine shown in Fig. 1B, the kinetics of Na+-independent histidine transport were tested at specific intervals after starting hepatocyte cultures. After 5 h of culture, the Eadie-Hofstee plot of the data deviates from linearity and fits a nonlinear equation (20) describing mediation by two saturable systems (Fig. 2A). If similar measurements are made after 26 h of culture, the results are even more clearly biphasic (Fig. 2B). The kinetic constants indicate that component I, a high affinity low capacity system, increases in activity with time in culture as a result of a change in Vmax (Table II). These results support the data depicted in Fig. 1 showing a time-dependent increase in Na+-independent histidine uptake when assayed at a substrate concentration of 50 μM. In contrast, component II, for which histidine has considerably less affinity, decreases in maximal velocity with respect to time in culture. Although the Km for the second component decreased

| Table I  
| Histidine transport in freshly isolated or cultured hepatocytes  
|  
| 0.1 mM histidine uptake | Cells in suspension | Cells in primary culture |
|--------------------------|---------------------|-------------------------|
| Na+-dependent            | 173                 | 97                      |
| Na+-independent, BCH-inhibitable | 6            | 97                      |

3 Inhibition by excessive amounts of either BCH or the amino acid itself is used to measure the total saturable uptake by Na+-independent systems. The validity of using BCH for this purpose will be documented in Table II.

4 We have found that, under various conditions, histidine may be the preferred System N-specific substrate (19).
FIG. 1. Changes in neutral amino acid transport by rat hepatocytes with time in culture. The Na+-dependent (A) and the Na+-independent (B) uptakes of the indicated amino acid were tested at specific intervals during the first 24 h of hepatocyte primary cultures. The substrate concentration was 50 μM and the cysteine assays included 10 mM dithiothreitol. The data depicted in B represent the saturable uptake only, obtained by assaying transport in the absence or presence of the same amino acid in unlabeled form at 10 mM. The results are the averages of at least 3 determinations with standard deviations of less than 10%. AIB, 2-aminoisobutyric acid.

FIG. 2. Kinetics of histidine transport by normal rat hepatocytes. The Na+-independent uptake of histidine was measured early (5 h, A) or late (26 h, B) in culture over the concentration range of 0.002 to 20 mM. The hepatocytes were depleted of amino acids by a 1-h incubation in Na+-free Krebs-Ringer phosphate buffer just prior to the 30-s uptake assays. The kinetic constants, calculated by computer analysis as described in the text, are summarized in Table II. In the particular experiment shown, the more significant measure of rate, \( V_{\text{max}}/K_m \), declined when compared to the earlier time (Table II). These differences become even more apparent if one assays transport after 48 h of culture.

The hepatoma cell line, HTC, has been shown to contain many of the neutral amino acid transport systems present in normal hepatocytes (8, 23, 24). In an attempt to detect the presence of multiple Na+-independent transport systems in HTC cells, the kinetics of histidine, tryptophan, leucine, and BCH were tested in a manner similar to that for normal hepatocytes. The experiments yielded linear Eadie-Hofstee plots for all 4 amino acids as shown for histidine in Fig. 3 (see also Ref. 20). These results suggest the presence of a single saturable system for Na+-independent transport in HTC cells. In support of this conclusion, the uptake of 50 μM tryptophan is inhibited equally as well by leucine, tryptophan, phenylalanine, and BCH. The calculated \( K_i \) values for these inhibitors correspond quite favorably with their apparent \( K_m \) values when tested as substrates (Table III).

Amino acid inhibition of 50 μM histidine or leucine uptake by normal hepatocytes was measured to test for possible differences in substrate specificity between the two Na+-independent systems. Based on the apparent kinetic constants for histidine (Table II) and leucine transport (20), the contribution of components I and II to 50 μM histidine uptake (24 h after initiating primary hepatocyte cultures) would be approximately 75 and 25%, respectively, while their contribution to 50 μM leucine uptake would be about 30 and 70%, respectively. Inhibition of either histidine or leucine uptake was negligible by the charged amino acids, cationic as well as anionic (Table
Na⁺-independent Amino Acid Transport in Rat Hepatocytes

**Summary of the kinetics of Na⁺-independent histidine uptake at 5 and 26 h (Fig. 2) after initiating rat hepatocyte primary cultures.** The nonsaturable rate (Kd) subtracted was 255 ± 7 and 220 ± 7 for 5 and 26 h, respectively. The values of Km, Vmax, and Kd were derived from computer analysis of the uptake data as described in the text. See the legend to Fig. 2 for other details.

**TABLE II**

| Individual Na⁺-independent components | 5-hr cultures | 26-hr cultures |
|--------------------------------------|---------------|---------------|
|                                      | Km (mM)       | Vmax (pmol·mg⁻¹ protein·30 s⁻¹) | Vmax/Km |
| Component I                          | 0.03 ± 0.01   | 6.1 ± 1.4     | 244     |
| Component II                         | 2.95 ± 0.43   | 652 ± 88      | 221     |

*These values are statistically different from the 5-h values at the p < 0.001 level.

**TABLE IV**

**Inhibition of histidine or leucine uptake by cultured hepatocytes**

Hepatocytes were cultured for 24 h in Waymouth's medium. The cells were transferred to Na⁺-free Krebs-Ringer phosphate buffer (pH 7.5) 1 h prior to assaying for inhibition of Na⁺-independent uptake of 50 μM [³H]histidine or [³H]leucine. The uptake of substrate was measured during 30 s and determined in triplicate. The naturally occurring amino acids were in their L-forms. The numbers shown in parentheses are the actual velocities (pmol·milligram⁻¹ of protein·30 seconds⁻¹) for the control samples. The standard deviation from the mean was less than 10%.

| Inhibitor, 10 mM | Control | Inhibition of His/ inhibition of Leu |
|------------------|---------|-------------------------------------|
|                  | Histidine | Leucine |
| None             | 100 (74.0) | 100 (160) | 1.00 |
| Glutamic acid    | 92       | 105     | 0.88 |
| Arginine         | 121      | 129     | 0.94 |
| Homocysteine     | 118      | 136     | 0.87 |
| Lysine           | 101      | 136     | 0.74 |
| Proline          | 127      | 118     | 1.08 |
| Glycine          | 87       | 108     | 0.81 |
| Alanine          | 81       | 105     | 0.77 |
| Serine           | 94       | 112     | 0.84 |
| Threonine        | 58       | 95      | 0.61 |
| Cysteine*        | 29       | 57      | 0.53 |
| Valine           | 29       | 41      | 0.71 |
| Isoleucine       | 29       | 32      | 0.91 |
| Leucine          | 23       | 29      | 0.79 |
| Asparagine       | 71       | 95      | 0.75 |
| Glutamine        | 58       | 85      | 0.68 |
| Methionine       | 28       | 48      | 0.58 |
| Histidine        | 23       | 55      | 0.42 |
| Tryptophan       | 25       | 62      | 0.40 |
| Tyrosine*        | 34       | 75      | 0.45 |
| Phenytoinamide   | 26       | 33      | 0.79 |
| 2-Amino-2-phenylbutyric acid* | 89 | 95 | 0.84 |
| BCH              | 26       | 24      | 1.08 |
| N-Methylphenylalanine | 94 | 103 | 0.91 |

*10 mM dithiothreitol was included.
* Final concentration was 2 mM.
* 10 mM L-mixture.
all medium changes, i.e. allowing the cells to remain in the original culture medium, enhances the increase in System L1 activity.

To study further the possible mechanism for the stimulation of Na\(^{+}\)-independent histidine uptake, the sensitivity of this increase to inhibitors of both RNA and protein synthesis was determined. In a representative experiment, there was a 47% increase in activity between 24 and 30 h of culture; this increase in transport was totally blocked by the inclusion of cycloheximide in the medium. For individual cell preparations, the inhibitor of RNA synthesis, actinomycin D, ranged from 50 to 100% in its effectiveness in blocking the stimulation of uptake.

The data presented in Table V show the uptake of cysteine, 2-aminoisobutyric acid, BCH, and histidine by primary cultures of hepatocytes from near term fetuses. The velocities for all 4 amino acids were increased (3 to 30 times) in the fetal cells when compared to those of the maternal parent which were isolated at the same time. While the activity of Systems A and N declined with time in culture, System ASC remained unchanged (Table V). In contrast, the sodium-independent uptake of both histidine and BCH increased between 6 and 24 h in a manner similar to that seen for the adult cells. This response occurred despite the fact that the Na\(^{+}\)-independent uptake of both amino acids was already considerably higher in the fetal cells. Histidine transport was 767 and 47 pmol·mg\(^{-1}\)·h\(^{-1}\) for fetal and adult hepatocytes, respectively, whereas the rates for BCH uptake were 680 (fetal) and 235 (adult) pmol·mg\(^{-1}\)·h\(^{-1}\) of protein·30 s\(^{-1}\).

discussion

Isolated rat hepatocytes in primary culture appear to contain two unique agencies for the Na\(^{+}\)-independent transport of neutral amino acids. One of these processes, component II, is characterized as a low affinity (K_m values between 1 and 3 mM) high capacity system which transports leucine and other amino acids at relatively high rates in freshly isolated cells, but rapidly declines in activity during the initial 24 h of culture. If one calculates, using the appropriate kinetic constants (20), the contribution of this system, to 50 pmol leucine uptake, it represents about 94 and 10% at 4 and 24 h of culture, respectively. The inhibition data of Table IV indicate that this system is strongly inhibited by valine, leucine, phenylala-

Table V

| Substrate, 50 \(\mu\)M | Ion dependence (system tested) | 6 h cultures | 24 h cultures | 48 h cultures |
|------------------------|---------------------------------|--------------|--------------|--------------|
| Cysteine               | Na\(^{+}\)-dependent (ASC)      | 627          | 618          | 0.99         |
| 2-Aminoisobutyric acid | Na\(^{+}\)-dependent (A)        | 195          | 20           | 0.10         |
| BCH                    | Na\(^{+}\)-independent (L1 and/or L2) | 680         | 1676         | 2.47         |
| Histidine              | Na\(^{+}\)-dependent (N)        | 767          | 83           | 0.11         |
|                        | Na\(^{+}\)-independent (L1 and/or L2) | 767         | 904          | 1.18         |

Conversely, component I is minimally expressed in freshly isolated cells of either fed or fasted rats (data not shown) and develops with time in primary hepatocyte cultures. We do not understand the regulatory processes necessary for the expression of this activity, but we do recognize that they may be quite complex and related to composition of the culturing medium and not merely to the length of time in culture. In this regard, one does not seem to alter the response significantly by including 10% fetal calf serum in the medium or changing the medium at different schedules over the first 48 h. As indicated above, the stimulation of System L1 activity does appear to be larger and more consistent if no medium changes are made after the initial plating of the cells. One aspect which has not been investigated in detail is the possible effects of differences in the preparations of collagenase used to isolate the cells. Regardless, once component I has been expressed by the cells, it can be shown to be completely inhibited by cysteine, valine, leucine, isoleucine, leucine, methionine, histidine, tryptophan, tyrosine, phenylalanine, and BCH. The differences in the specificity of inhibition along with the biphasic kinetics of common substrates serve to distinguish these two transport systems.

On the basis of preliminary results (20), we originally thought component I to be somewhat analogous to the aromatic amino acid transport system in human red blood cells, System T (22). The results of Table IV show quite clearly, however, that component I is not restricted to mediation of aromatic amino acid uptake. Similarly, the data accumulated thus far do not allow us to assign either one of the systems to a System L-like activity. Consequently, we choose to term the two components as Systems L1 and L2, at least until further identifying information can be obtained. It should be noted that most of the previous studies involving hepatic Na\(^{+}\)-independent transport were designed such that System L2 would be the predominant agency observed.

The apparent regulation of System L1 in normal adult hepatocytes represents one of a few reports of such control for a Na\(^{+}\)-independent amino acid transport system. In general, System L has been shown to be unresponsive to either hormonal stimulation or adaptive control (9). Two apparent exceptions to the inertness of System L for regulation have been reported. Adamson and Inghar (25, 26) reported that uptake by cartilage tissue of those amino acids which have historically been thought of as System L substrates was increased by thyroid hormones to a greater extent than the uptake of typical System A substrates. It should be noted, however, that the uptake of some substrates previously associated with System L, namely isoleucine and valine, was not affected by the hormone treatment. Furthermore, the authors pointed out that the aromatic amino acids tyrosine, trypto-
phospholipids, and phenylalanine, all of which were affected by the thyroid hormone, formed a "distinct class of amino acids" because their transport was not strongly inhibited by either alanine or leucine (25). In fact, the group of amino acids that Adamson and Ingbar showed to be responsive to hormonal stimulation resembles the list of the best inhibitors of System L1 in hepatocytes (compare Table III and Fig. 2 of Ref. 26).

Moore et al. (27) and Shotwell et al. (28) have described a temperature-sensitive, leucyl-tRNA mutant Chinese hamster ovary cell line in which the Na+-independent uptake of the branched chain and aromatic amino acid is increased when the cells are grown at the marginally permissive temperature. They conclude, based on substrate specificity of the enhanced system, that System L is responsible for the phenomenon. It appears pertinent, however, that both leucine and phenylalanine yielded biphasic kinetics for transport in these cells (28).

In contrast to the normal hepatocyte, the hepatoma cell line, HTC, does not yield biphasic kinetics for leucine, BCH, tryptophan, or histidine (20) (Fig. 3), and reciprocal inhibition analysis (Table III) supports the conclusion that the transformed cell contains only one Na+-independent transport system for neutral amino acids. These preliminary experiments suggest that the Na+-independent transport in the HTC cell appears to be homogeneous. The significance of the lack of one of these systems to be expressed in the transformed cell is unclear. With respect to the proposal that "oncology is blocked ontogeny" (29), we have found that System L1 activity appears to be expressed in fetal hepatocytes. In fact, the basal Na+-independent rates of both histidine and BCH uptake are much higher in the fetal cells when compared to those of the mother, yet the uptake of these amino acids increased with time in culture in a manner similar to that seen in the adult cells. The differences in Na+-independent transport between the fetal and adult hepatocytes should be included in a series of changes that occur during maturation of these cells. Such changes, including the lack of a system for the cationic amino acids (30) and another showing affinity for glutamate and cystine (31), are different from those seen in this report in that the changes in Na+-independent uptake occur rapidly and without any recognized evidence for cellular differentiation.

The physiological importance of System L1 with respect to hepatic metabolism remains unresolved. Of considerable interest is the apparent inverse relation between the time-dependent changes in System L1 activity with those found for Systems ASC, N, and L2 in cultured hepatocytes. If this relationship corresponds to changes occurring in vivo, it could provide the hepatocyte with a mechanism to significantly alter the net flux of many, if not most, of the neutral amino acids. Studies are currently in progress to characterize System L1 fully and to determine what factors control its regulation.

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