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Neutral Amino Acid Transport in Cultivated Human Skin Fibroblasts (38521)

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Most cells are capable of maintaining high intracellular amino acid concentrations even in the presence of low amino acid levels in extracellular fluid. Studies in bacteria (1), erythrocytes (2–5), kidney (6), intestine (7), leukocytes (8, 9), Ehrlich ascites tumor cells (10–12), 3T3 tumor cells (13), and human skin fibroblasts (14, 15) suggest the presence of several amino acid transport systems operating in parallel. These systems can be distinguished by differences in substrate specificities, kinetics of accumulation, sodium dependency, apparent energy requirements, pH and temperature sensitivity, inhibition by other amino acids (16) and stimulation by cations (9). The exact transport systems present in a given cell may vary between and within species.

Christensen and associates (16) have postulated a number of transport systems for the neutral amino acids. Although each system has a high affinity for a specific group of amino acids, it facilitates intracellular accumulation of other amino acids. Alanine, for example, is thought to be accumulated by at least three separate mechanisms, any or all of which may operate in a given cell (16).

Transport of amino acids by separable systems has not been reported in human diploid skin fibroblasts; rather, studies of amino acid transport in fibroblasts have explored gross accumulation of single amino acids or single groups of amino acids (14, 15, 17, 18) and not contributions by or characteristics of separate transport systems. These studies were undertaken to describe the characteristics of neutral amino acid accumulation in growing skin fibroblasts.

Materials and Methods. Skin fibroblast cultures were derived from forearm skin biopsies from six healthy adult and six healthy pediatric controls as previously described (19). Cells were grown in Eagle's Minimal Essential Medium, Earle's base, supplemented with 12% fetal calf serum and penicillin, streptomycin, and fungizone (GIBCO) at 37° in a 5% CO_2–95% air atmosphere. Medium was changed three times weekly. Cells were subcultured at confluency, usually once weekly, by trypsinization with 0.25% trypsin in Puck's Saline A (GIBCO).

During the experiments, cells were studied while attached to glass coverslips. Cells from each (250 ml) 100 mm^2 Falcon flask were suspended in 60 ml of medium and seeded in four to six Petri dishes (Falcon) each containing 20–25 12 mm round coverslips (Kimax). Cells were allowed to settle and attach to the coverslips for approximately 1 hr, and were then returned to the incubator. Medium was changed on alternate days and the day preceding an experiment. Cells reached confluency in 3–6 days. Amino acid accumulation was measured in subconfluent cultures 2–5 days after seeding.

Coverslips from three to six Petri dishes seeded from the same flask on the same day were used in each series of experiments. Medium was aspirated from the Petri dish and the cells were washed twice with phosphate buffered saline (PBS) containing 140 mEq/liter of sodium, 5 mEq/liter of potassium, 5 mEq/liter of calcium, 5 mEq/liter of magnesium, and 0.1% glucose. Ten milliliters of this solution were added to each dish and the cells were returned to the incubator for 60 min to allow depletion of amino acids. Choline was used at an identical concentration to replace sodium as the major extracellular cation when sodium-free phosphate buffered saline was required.

Transport studies were performed using PBS at pH 7.4 with a uniformly labeled ^{14}C-amino acid. Coverslips were transferred to tubes containing 0.5–1.0 ml incubation medium, and incubated with shaking at 37° for varying periods of time up to 90 min. Coverslips were removed and rapidly (in

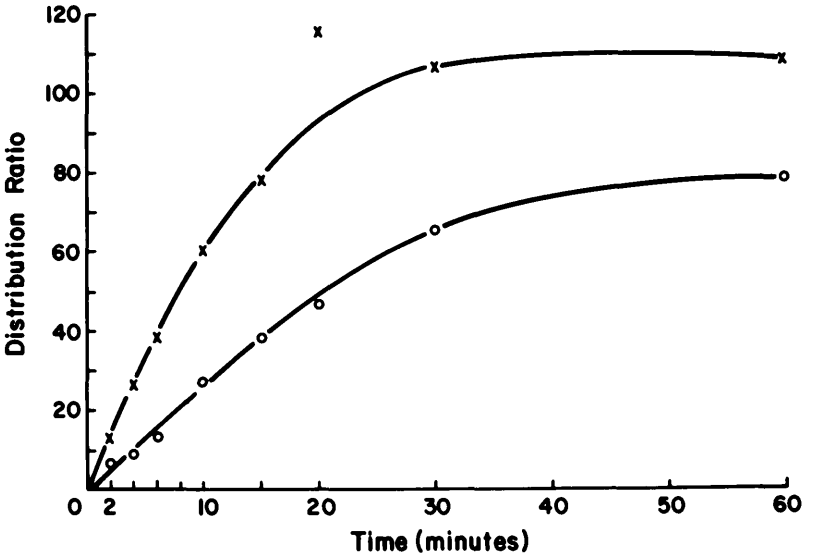

FIG. 1. Distribution ratios achieved by amino acid depleted fibroblasts in a 20 μM solution of amino acid in phosphate buffered saline. Each point represents the mean of determinations in six coverslips in the same experiment. (--X--) represents alanine and (--O--) represents leucine accumulation.

less than 2 sec) dipped through four saline washes at room temperature and placed in liquid scintillation vials. Accumulated radioactivity in cells and in aliquots of the incubation medium was counted in 1:1 toluene: methylcellusolve with 0.4% PPO and 0.005% POPOP in a Nuclear Chicago Mark II scintillation counter. The average number of counts for three to five coverslips was used for calculations. Counts varied from 0 to 5% for replicate coverslips. "Zero time" counts, presumably representing extracellular label not removed by rinsing, were obtained by quickly dipping and removing coverslips from the incubation medium and rinsing as described above.

Four to six coverslips were randomly chosen from each Petri dish and the protein content determined by a modification of the Lowry method (20). Protein concentrations varied by less than 5% between coverslips from the same Petri dish. Intracellular water was measured as space accessible to urea but not mannitol. Replicate coverslips from the same Petri dish were exposed to ^{14}C-urea or ^{14}C-mannitol for 60 min. The coverslips were removed, touched to tissue paper, and counted for radioactivity.

Radiochemicals were obtained from New England Nuclear and were used without further purification. Ouabain, *p*-hydroxymercurobenzoic acid, and unlabeled amino acids were obtained from Sigma. Other chemicals were obtained from Fisher.

Results. Intracellular water space and ^{14}C-amino acid accumulation were both directly proportional to protein content per coverslip as has been reported by others (13). Preincubation with actinomycin D, which inhibits new protein synthesis, did not alter the accumulation of label in the cells, and coverslip radioactivity was considered a measure of free intracellular amino acid content. Distribution ratios for each amino acid cpm/ml of intracellular water/cpm/ml of extracellular water were calculated from a measurement of protein. A distribution ratio of unity indicates identical concentrations of amino acids inside and outside of the cell. Distribution ratios greater than 1 indicate intracellular accumulation of amino acids.

Accumulation of label was time dependent (Fig. 1); the rates of accumulation of the amino acids studied were not identical. Since the original rate of uptake continued to be linear for 15–20 min for each amino acid studied, further experiments were performed using 10-min incubation periods.

The effects of deleting ions from the

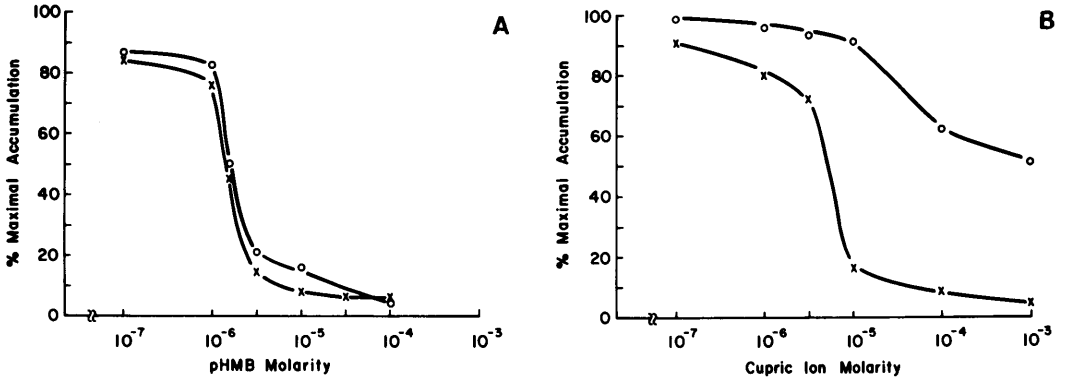

FIG. 2. Inhibition of uptake of ^{14}C-labeled alanine (--X--) and leucine (--O--) by *para*-hydroxymercuro benzoate (2A) and cupric ion (2B).

TABLE I. IONIC DEPENDENCY OF AMINO ACID TRANSPORT.[a]

	Number of samples	Alanine	Leucine
PBS	20	100 ± 6.5	100 ± 6.2
Na^+ free PBS	20	19 ± 6.3	85 ± 8.7
K^+ free PBS	10	87 ± 6.4	55 ± 12.5
Mg^{2+} free PBS	4	45 ± 14.5	78 ± 5.8
Ca^{2+} free PBS	4	103 ± 16.4	80 ± 6.4
Ca^{2+} and Mg^{2+} free PBS	4	46 ± 8.4	55 ± 12.2

[a] These figures represent relative accumulation of radioactive amino acids (± standard deviations) when human skin fibroblasts were exposed for 10 min to 20 μM of ^{14}C-labeled alanine or leucine in phosphate buffered saline with various ions deleted. Mean values were derived from three to six coverslips in "normal" PBS on each day and assigned a value of 100. Relative values in manipulated PBS solutions were derived by comparison to this number.

incubation medium are shown in Table I. Alanine accumulation was almost completely depressed in the absence of sodium, while the uptake of leucine was only slightly diminished. Potassium was required in the extracellular fluid for complete expression of both alanine and leucine accumulation. Divalent ions stimulated accumulation of both amino acids; magnesium depletion diminished alanine accumulation while depletion of calcium, magnesium or both cations was associated with some decrease in leucine accumulation. Preincubation with the fluoride, cyanide, and azide for 60 min and glucose starvation produced no effect on accumulation of radioactive label (Table II). Ouabain, an inhibitor of sodium dependent ATPase, clearly diminished the rates of uptake for both amino acids. *para*-Hydroxymercurobenzoate and cupric ion effectively halted amino acid accumulation (Fig. 2). Kinetic parameters for cellular accumulation of amino acids were ascertained. Alanine accumulation demonstrated a Km of 6.0×10^{-4} M and a V_{max} of 469 μmoles/mg protein/10 min, while leucine accumulation had a Km of 3.3×10^{-4} M and a V_{max} of 150 μmoles/mg protein/10 min.

The effects of unlabeled amino acids in the incubation medium were observed on uptakes of individual radioactive amino acids. The distribution ratio achieved when the incubating medium contained 20 μM radioactive amino acid was compared to that achieved when an unlabeled amino acid was also present in a 1 mM concentration. Maximal "inhibition" was measured using as the inhibitor, a 1 mM solution of the same unlabeled amino acid whose uptake was being studied. This value was thought to indicate identical routes of transport for the test (radioactive) and inhibitor (cold) amino acids. The degree of inhibition by other amino acids can be compared to this

TABLE II. EFFECTS OF METABOLIC INHIBITORS ON AMINO ACID TRANSPORT.[a]

	Number of samples	Alanine	Leucine
Control	20	100 ± 6.5	100 ± 6.4
1 m*M* NaF	8	94 ± 11.5	98 ± 4.2
1 m*M* KCN	8	98 ± 8.4	96 ± 4.4
1 m*M* NaN_3	8	101 ± 6.4	98 ± 6.3
Absent glucose	10	105 ± 11.4	100 ± 4.6
10^{-4} *M* ouabain	10	51 ± 4.5	73 ± 10.3

[a] These figures represent relative accumulation of radioactive amino acid (± standard deviation) when human skin fibroblasts were exposed to 20 μM ^{14}C-labeled alanine or leucine. Cells were preincubated for 60 min in PBS containing no additives or metabolic inhibitors and then studied in PBS containing the same inhibitor plus radioactive amino acid. Mean control distribution ratios obtained on each day were assigned a value of 100 and relative accumulations were derived by comparisons to those values.

maximal inhibition. The presence of methylalanine or a dicarboxylic acid did not diminish uptake of either alanine or leucine. Alanine uptake was inhibited to varying degrees, in order of decreasing inhibition by: (a) Serine, cysteine, and threonine, (b) glycine, (c) the branched chain amino acids and phenylalanine, and (d) lysine. Leucine uptake was inhibited to decreasing extents by: (a) Other branched chain amino acids and phenylalanine, (b) alanine, serine and threonine, (c) lysine, and (d) glycine (Table III).

The age of the person from whom a skin fibroblast culture was derived, the age of the culture in passage number, the degree of confluency of cells in culture and the use of Hank's F-10 medium in place of Minimal Essential Medium as a growth medium did not alter the characteristics of amino acid accumulation described.

Discussion. These data indicate that neutral amino acid transport in the human skin fibroblast has both sodium dependent and sodium independent components. Accumulation of short chain neutral amino acids is largely sodium dependent, although it also occurs to a small degree in the absence of sodium. Branched chain amino acids are accumulated primarily by mechanisms which operate in the absence of sodium; however, they also compete for sodium dependent sites. Binding constants for short chain and branched chain amino acids are similar but not identical. These data are compatible with either of two explanations.

Christensen (21) has postulated several transport systems for neutral amino acids. These include a sodium dependent system which accepts *N*-methylated amino acids (A), a sodium dependent system with specificity for short chain amino acids but not accepting *N*-methylated amino acids (ASC), and a sodium independent system which transports branched chain amino acids (L). If his postulates are correct, these data support the hypothesis that the ASC and L systems but not the A system operate in the cultivated human skin fibroblast. This is an unusual complement of transport systems previously reported only as occurring transiently in reticulated red blood cells (5). Fibroblasts would then be an excellent

TABLE III. INHIBITION OF AMINO ACID UPTAKE BY AMINO ACIDS IN THE INCUBATION MEDIUM.

Inhibition by:	Alanine	Leucine
Alanine	98	81
Leucine	65	96
Lysine	20	50
N-methylalanine	−2	−10
Serine	98	80
Threonine	97	82
Glycine	92	45
Phenylalanine	63	94
Isoleucine	60	97
Glutamic acid	−2	3

This table demonstrates the degree of inhibition of ^{14}C-alanine or ^{14}C-leucine uptake in the presence of unlabeled amino acids. Each figure represents the mean of three to six determinations on different cell lines. Radioactive amino acid was present at 20 μM; unlabeled amino acid as 1 m*M* solution.

tissue for studying the ASC system without A inhibitors.

On the other hand, these data do not preclude the possibility that the same system transports both short chain and branched chain neutral amino acids in the human skin fibroblast. Sodium has been thought to assist in the binding of a variety of amino acids (22). Our data are compatible with the hypothesis that sodium enhances binding of short chain amino acids to a system transporting a variety of neutral amino acids. In the absence of sodium, binding to longer, aliphatic amino acids might occur preferentially.

Inhibition by *N*-methylated amino acids of amino acid uptake in kidney and intestine (13) but not in red blood cells (3) or undifferentiated skin fibroblasts suggests that a system accepting *N*-methylated amino acids (Christensen's "A" system) may be limited to cells with specialized transport functions.

In the fibroblast, classic metabolic blocking agents are poor inhibitors of amino acid accumulation while ouabain, an inhibitor of sodium–potassium dependent ATPase, diminishes uptake of both alanine and leucine to some degree. These findings suggest that amino acid transport is linked to the maintainence of the transmembrane sodium gradient.

Inhibition of uptake by *para*-hydroxymercurobenzoate and cupric ion suggest that free sulfhydryl groups at the surface of the membrane, bindable by these agents, are important in transport processes.

Variations in culture technique, growth medium, and donor or culture age have been noted to alter many biochemical parameters in human skin fibroblasts (23). Alterations in each of these parameters induced no change in the characteristics of amino acid accumulation in cultures of human skin fibroblasts.

These studies clearly indicate that both sodium dependent and sodium independent components are important in transport of neutral amino acids in the cultivated human skin fibroblasts. Both of these components serve to allow accumulation of short chain and branched chain amino acids simultaneously but to varying degrees.

Summary. The accumulation of neutral amino acids was studied in cultivated human skin fibroblasts. Transport was blocked by sulfhydryl binding agents, but proceeded without inhibition in the presence of metabolic blocking agents. Both sodium dependent and sodium independent components of neutral amino acid accumulation were found. The sodium dependent component preferentially transports short chain amino acids, while the sodium independent system transports long chain amino acids. These components operate simultaneously and with overlap of substrate specificity. Both may function as parts of a single neutral amino acid transport system.

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