Transport of Dibasic Amino Acids, Cystine, and Tryptophan by Cultured Human Fibroblasts: Absence of a Defect in Cystinuria and Hartnup Disease

ULRICH GROTH and LEON E. ROSENBERG

From the Division of Medical Genetics, Departments of Pediatrics and Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT Transport of lysine, arginine, cystine, and tryptophan was studied in cultured skin fibroblasts from normal controls and from patients with cystinuria and Hartnup disease. Each of these amino acids was accumulated against concentration gradients by energy-dependent, saturable mechanisms. Lysine and arginine were each transported by two distinct processes which they shared with each other and with ornithine. In contrast, cystine was taken up by a different transport system with no demonstrable affinity for the dibasic amino acids. The time course and Michaelis-Menten kinetics of lysine and cystine uptake by cells from three cystinuric patients differed in no way from those found in control cells. Similarly, the characteristics of tryptophan uptake by cells from a child with Hartnup disease were identical to those noted in control cells. These findings indicate that the specific transport defects observed in gut and kidney in cystinuria and Hartnup disease are not expressed in cultured human fibroblasts, thus providing additional evidence of the important role that cellular differentiation plays in the regulation of expression of the human genome.

INTRODUCTION

Cystinuria and Hartnup disease are well recognized, inherited disorders of renal and intestinal amino acid transport. In cystinuria, a specific transport defect for cystine and the dibasic amino acids lysine, arginine, and ornithine has been documented in vivo (1–3) and in vitro (4–6). Hartnup disease is characterized by impaired renal and intestinal absorption of tryptophan and a large group of other monoamino-, monocarboxylic amino acids with neutral, aromatic, and heterocyclic side chains (7). These transport abnormalities, and the autosomal recessive inheritance pattern of both disorders have led to the notion that cystinuria and Hartnup disease are caused by mutations of specific membrane carrier proteins which catalyze group-specific transport of amino acids in gut and kidney. The mutation responsible for cystinuria exhibits tissue as well as substrate specificity. Two groups of workers have shown that peripheral leukocytes from cystinuric patients transported cystine and dibasic amino acids in vitro as well as control leukocytes did (8, 9). To date, such experiments have not been carried out in Hartnup disease, nor have other tissues been examined in either disorder.

Skin fibroblasts, propagated in tissue culture, have become a particularly useful tool for the study of the gene action in man, since they can be obtained without risk and grown in amounts which far exceed those available from other tissue biopsies. More than 35 inherited enzyme defects have been demonstrated in these cells, which have also proven to be uniquely valuable in studying other genetic phenomena in mammalian cells (10). Although skin fibroblasts have been shown to transport amino acids actively in vitro (11), we are aware of no previous attempts to determine if specific, inherited disorders of amino acid transport are expressed in this tissue. In the present study, we have examined the processes by which normal skin fibroblasts transport dibasic amino acids, cystine, and tryptophan, and have compared transport of these compounds in normal cells with that in fibroblasts obtained from patients with cystinuria and Hartnup disease.

METHODS

Cell lines. Three healthy subjects served as control donors for skin biopsies from which diploid fibroblasts were grown

Dr. Groth's present address is the University of Freiburg, Freiburg, Germany.

Received for publication 22 February 1972 and in revised form 29 March 1972.

in tissue culture and subsequently used to establish normal transport parameters for dibasic amino acids, cystine, and tryptophan (cell lines C82, C87, and C120). In addition to these control cell lines, individuals with various genetic diseases which, according to our present knowledge, are not related to defects of transport or metabolism of dibasic amino acids, cystine, or tryptophan were investigated (P1: methylmalonic aciduria; P68: hyperglycinemia; P90: thoracic asphyxiant dystrophy; P96: hyperglycinuria; P117: propionicacidemia.) Cell lines from cystinuric individuals originated from two female (Cy 23 and Cy 24) and one male (Cy 48) adults. Each of these individuals had formed numerous cystine calculi and excreted cystine, lysine, arginine, and ornithine in great excess. Using the classification of Rosenberg (12), their genotypic designations were: Cy 23, I/III; Cy 24, I/I; and Cy 48, I/II. Transport of tryptophan was studied in control cells and in cell line H98, derived from an asymptomatic 10 month old girl who excreted great excesses of alanine, serine, threonine, leucine, isoleucine, valine, tryptophan, phenylalanine, and tyrosine in the urine. This urinary amino acid pattern was typical for homozygous Hartnup disease.

Preparation and incubation of cells. Transport studies in human skin fibroblasts, propagated in tissue culture and harvested as reported by Mahoney and Rosenberg (11), were performed only with nondividing cells at comparable degrees of confluence, conditions which were usually reached three days after serial passage. Cells were incubated at 37°C and pH 7.34 in a phosphate-buffered saline containing the following ion concentrations in mEq/liter: $Na^+ = 156$; $K^+ = 4.2$; $Ca^{2+} = 1.8$; $Mg^{2+} = 1.6$; $Cl^- = 145$; $PO_4^{3+} = 29$; $SO_4^{2-} = 1.6$. Sodium-free buffers were prepared by replacing sodium chloride with equimolar amounts of Tris-HCl (tris-hydromethyl-aminomethane), choline chloride, or potassium chloride. If substrate or inhibitor concentrations greater than 5 mM were used, NaCl was reduced to keep the osmolality of the reaction mixture constant. Incubation duration varied from 1 to 90 min.

In a typical experiment 0.1 ml of cell suspension (1.5–2 $\times 10^5$ cells) were added to 0.4 ml of prewarmed incubation medium in preweighed 3.5 ml polypropylene centrifuge tubes containing substrates, inhibitors, and radioactive compounds to yield the desired concentrations in the total volume of 0.5 ml. Radioactivity in the standard incubation assay varied between 0.1 μCi/ml at low substrate concentrations and 1 μCi/ml at high substrate concentrations. The tube was then stoppered and placed in a 37°C water bath. Thorough and simultaneously gentle mixing of cells and incubation medium was accomplished in a specially designed apparatus, in which the horizontally adjusted tube was moved back and forth along a circular arc at 30 rpm, permitting the cell suspension to move alternatively from one end of the tube to the other. Incubation was terminated by submerging the tube for 5 sec in an isopropyl alcohol-dry ice bath, during which interval the incubation mixture reached a temperature of 4°C. This was followed by centrifugation at 48,000 *g* at 4°C for 10 min in a Sorvall RC2B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). After centrifugation the tubes were prepared for wet weight determination as described previously (13). The cell pellets (usually 3–4 mg wet weight) were disrupted by sonicating for 10 sec at 70 w in 0.5 ml 5% TCA (trichloracetic acid) with the microtip assembly of a Branson cell disruptor, Model W185D (Branson Instruments Co., Stamford, Conn.). Incubation medium was treated with equal volumes of 10% TCA. After removing precipitated components by centrifugation at 48,000 *g* for 5 min, portions of tissue supernatant and incubation medium were counted in a Packard Liquid Scintillation Spectrometer as described previously (14). The protein content of sonicated tissue was assayed by the procedure of Lowry, Rosebrough, Farr, and Randall (15).

Metabolism. The metabolic conversion of L-lysine-U-^{14}C and L-tryptophan-3-^{14}C was investigated in tissue supernatant and incubation medium after extraction of TCA with ether (16) by a single dimension descending paper chromatography in a solvent of butanol: acetic acid: water (4:1:2) and by a single dimension high voltage electrophoresis (13). The fractional recovery of nonmetabolized radioactive substrate was determined as described previously (13).

Materials. Uniformly labeled L-lysine-^{14}C (SA: 269 mCi/mmole); L-arginine-^{14}C (SA: 255 mCi/mmole); L-cystine-^{14}C (SA: 271 mCi/mmole); L-tryptophan-3-^{14}C (SA: 29 mCi/mmole); α-aminoisobutyric-1-^{14}C acid (SA: 10.4 mCi/mmole); and glycine-U-^{14}C (SA: 115.8 mCi/mmole) were obtained from New England Nuclear Corp., Boston, Mass. The radiochemical purity of each compound was checked by descending paper chromatography and high voltage electrophoresis in the above mentioned systems and found to be in good agreement with the decomposition rates indicated by the manufacturer. Unlabeled L-lysine monohydrochloride, L-arginine monohydrochloride, L-ornithine monohydrochloride, L-cystine, and L-tryptophan were purchased from ICN Nutritional Biochemical Div., Cleveland, Ohio. Glycine, α-aminoisobutyric acid, and sodium *p*-chloromercuribenzoate were obtained from Schwartz/Mann Research Laboratories, Orangeburg, N. Y. Ouabain was provided by Z. D. Gilman, Inc., Washington, D. C., and sodium cyanide and 2,4-dinitrophenol were purchased from Fisher Scientific Co., Pittsburgh, Pa.

Calculations. The net uptake of substrate by cultured human fibroblasts after a given incubation time is expressed as the distribution ratio, e.g. the ratio of the intracellular substrate concentration, A_c (mmoles/l cell water) to extracellular substrate concentration, A_f (mmoles/l incubation medium). Labeling of substrate with tracer amounts of its radioactive isotope allowed measurement of A_c and A_f in counts per unit time per unit volume. Distribution ratios greater than unity are assumed to represent concentration of substrate against a chemical gradient either by energy dependent active transport mechanisms or exchange diffusion. Distribution ratios less than unity may be achieved by energy-linked uptake, by non-energy-linked facilitated or exchange diffusion, and by physical diffusion. Distribution ratios were calculated according to Rosenberg and Downing (9) using the total tissue water and extracellular fluid space estimates of Mahoney and Rosenberg (11). Unless otherwise stated, the calculated distribution ratios were not corrected for physical diffusion or metabolic conversion of substrate.

The data relating uptake velocity to extracellular substrate concentration were obtained at initial rates of uptake during 1 min incubation intervals. Initial velocities of transport, v (mmoles per liter per minute), were calculated by multiplying the observed distribution ratio by the initial substrate concentration (A_f) in the incubation medium. Physical diffusion was taken into account using the formulation of Akedo and Christensen (17). When the corrected initial velocity, Y, was plotted against substrate concentration by the double reciprocal method of Lineweaver and Burk (18), the data suggested more than one mode of uptake for several substrates. Total transport thus was described as the sum of transport by several transport systems,

using Michaelis-Menten expressions for each of the independent transport systems (equation 1):

$$Y = \frac{V_{max}^1}{1 + \frac{K_t^1}{A_f}} + \frac{V_{max}^2}{1 + \frac{K_t^2}{A_f}} + \cdots \frac{V_{max}^n}{1 + \frac{K_t^n}{A_f}} \quad (1)$$

Statistical analyses were performed with Student's *t*-test.

RESULTS

General properties of fibroblasts

Cell viability. Some properties of human fibroblasts, considered crucial to reliable and reproducible determinations of transport parameters, were studies in different control and cystinuric cell lines (C82, C87, Cy 23, and Cy 24). Cell viability of trypsinized cells, before and after various times of incubation, was judged by trypan blue exclusion. These studies indicated that 90–95% of the cells were viable before incubation, and no significant changes were noted during incubation intervals of 1–60 min. Loss of total cells during the time course of incubation, a problem in our earlier studies, was minimized by using plastic incubation vessels and gentle agitation (see Methods) and averaged about 10% of the initial cell number after incubation periods of 60 min and 15–25% after 90 min. Exposure to 0.25% trypsin for periods as long as 45 min beyond the normal trypsinization period of 10 min required to harvest the cells did not affect cell viability or their ability to transport lysine. In these regards, no differences between control and cystinuric cells were noted.

Relationship between cell protein content and tissue wet weight. A recent study of leucine transport in isolated intestinal cells (19) reported that more reliable estimates of intracellular accumulation were obtained using cell protein determination, than with extracellular space estimates, which involve repeated weighings. We, therefore, examined the relationship between cell protein content and tissue wet weight over a 10-fold range in different experiments with different cell lines. As shown in Fig. 1, tissue wet weight from 0.8 mg to 8 mg correlated closely with the cell protein content. This correlation was independent of individual experiments or the cell lines used. The mean ratio of milligram cell protein to milligram tissue wet weight, determined from 15 experimental points in each of five separate experiments with different cell lines, was 0.13 ±0.01. These findings indicate that the different cell lines used have essentially identical cell protein content and that in our experimental system tissue wet weight determinations provide a reliable index for calculating transport parameters.

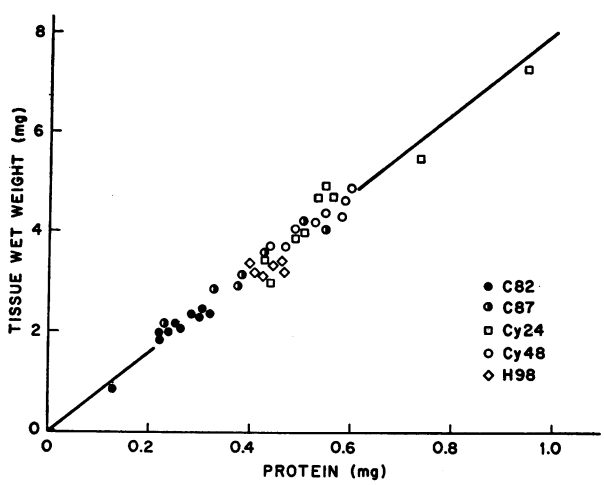

FIGURE 1 Relationship between protein content and tissue wet weight in diploid human fibroblasts. The symbols refer to the different cell lines described in Methods. Each cell line was investigated in a separate experiment.

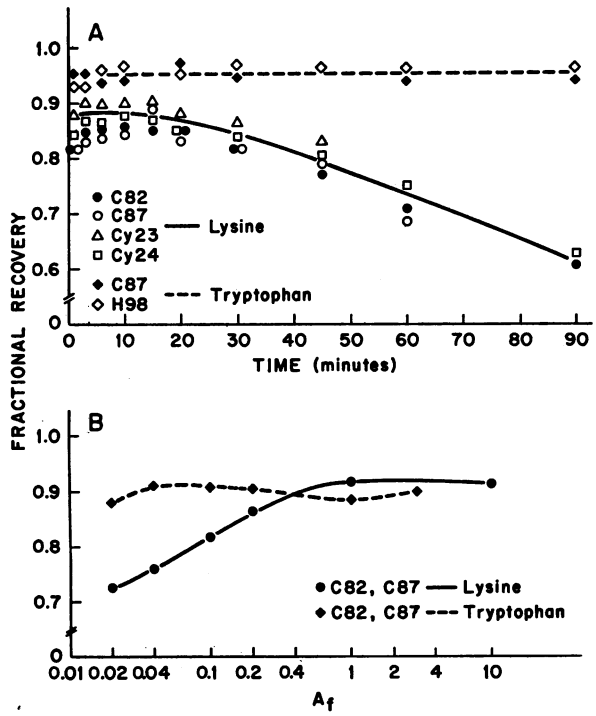

FIGURE 2 Dependence of metabolic conversion of lysine and tryptophan on incubation time (*A*) and on substrate concentration, A_f (*B*). Fractional recovery is defined as the ratio of lysine-^{14}C (or tryptophan) to the total tissue ^{14}C. Each symbol corresponds to a cell line described in Methods and represents the means of single determinations in four sequential cell generations. The substrate concentration for each amino acid reported in *A* is 0.1 mM. The duration of incubation noted in *B* is 1 min.

Metabolic conversion of labeled amino acids. Metabolism of lysine-^{14}C and tryptophan-^{14}C was examined

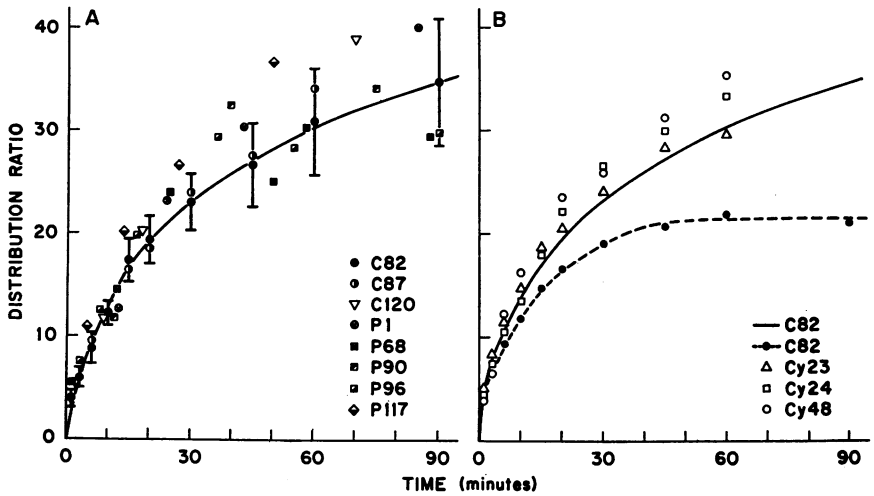

FIGURE 3 Time course of lysine uptake (0.1 mM) by control (*A*) and cystinuric cell lines (*B*). The distribution ratio is defined as the counts/min/ml cell water divided by the counts/min/ml of incubation medium (see Methods). The experimental points for cell lines C82, C87, Cy 23, Cy 24, and Cy 48 represent the means of duplicate observations in each of four separate experiments in sequential cell generations. Values in cell lines C120, P1, P68, P96, and P117 are means of two observations in two cell generations. The solid line in *A* connects the mean values found in cell line C82. The vertical bars indicate one SD for this cell line. The time curve for cell line 82 is reproduced as the solid line in *B*. The dotted line in *B* represents the curve for cell line C82 corrected for metabolism, using the data shown in Fig. 2.

in several fibroblast cell lines. The fractional recovery of nonmetabolized substrate in tissue supernatant and medium was determined for both compounds at medium substrate concentrations of 0.1 mM after different incubation periods, and at various concentrations (0.02–10 mM) after 1 min incubation periods. Time dependent and concentration dependent fractional recovery of both radioactive substances in the incubation medium was found to be 1–2% less than the radiochemical purity of the parent amino acid. The fractional recovery of lysine-^{14}C and tryptophan-^{14}C in the tissue supernatant with increasing duration of incubation is shown in Fig. 2*A*. The recovery of tryptophan remained virtually constant even after 90 min of incubation, whereas an increasing portion of intracellular lysine was metabolized after 30 min. After a 90 min incubation period, nearly 40% of the intracellular radioactivity was found in the form of metabolites of lysine. Fig. 2*B* shows the fractional recovery of lysine-^{14}C and tryptophan-^{14}C over a 50-fold range of substrate concentrations. In all cell lines studied (C82, C87, and H98), tryptophan-^{14}C was not catabolized appreciably over this entire range of substrate concentrations, whereas an increasing fraction of lysine-^{14}C was metabolized at substrate concentrations less than 0.4 mM. Control (C82 and C87) and cystinuric (Cy 23 and Cy 24) cell lines metabolized lysine identically. Unless otherwise stated, no correction for metabolism was applied to the uptake data to be discussed subsequently.

Transport of dibasic amino acids and cystine

Time course of uptake by control cells. Representative curves of uptake of lysine (Fig. 3) and of arginine and cystine (Fig. 4) each at a medium substrate concentration of 0.1 mM, are shown. For each substrate,

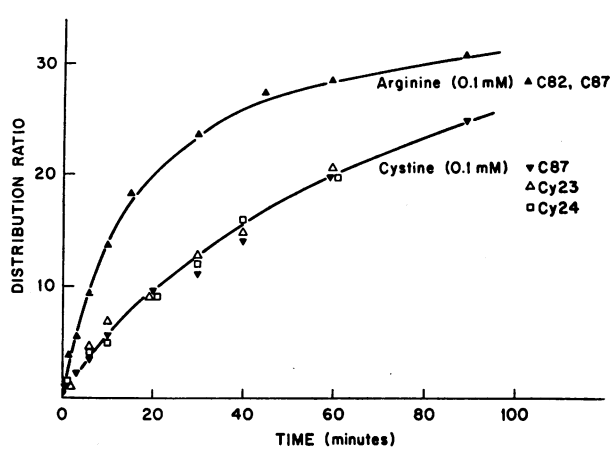

FIGURE 4 Time course of arginine and cystine uptake. Each point represents the mean of at least four observations. See legend of Fig. 3 for details.

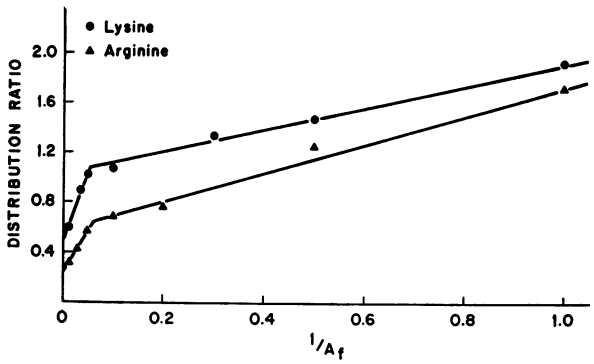

FIGURE 5 Plot of distribution ratio of lysine and arginine uptake against the reciprocal of the substrate concentration, A_f (mM). Incubation duration was 1 min. Apparent diffusion constants were calculated from the ordinate intercepts. Each point represents the means of duplicate observations in each of four different experiments with sequential cell generations. Data were obtained from control cell line C82.

uptake velocity was maximal during the first 1–2 min, after which uptake velocity slowed. Equilibrium conditions (constant distribution ratios) were not achieved, however, even after 90 min of incubation.[1] This apparent failure to reach equilibrium may be explained by the data shown in Fig. 3*B*. The solid line describes the time course of lysine uptake in cell line C82 uncorrected for lysine metabolism, whereas the broken line depicts these same data to which a correction for metabolism was applied by multiplying each isotopic distribution ratio by the value for fractional recovery of true lysine-^{14}C noted in Fig. 2. As can be seen, the accumulation of chemically unaltered lysine does become constant after 45 min of incubation, suggesting that the failure of the uncorrected curve to reach equilibrium reflects intracellular accumulation of labeled-^{14}C metabolites of lysine.

The uptake curve for arginine was almost identical to that for lysine. Accumulation of cystine proceeded initially at a rate significantly slower than that for arginine and lysine but, by 90 min, reached distribution ratios comparable to those for arginine and lysine.

Since diploid fibroblasts have a limited lifespan in culture, it seemed important to determine whether amino acid uptake varied significantly with time in culture. Four separate lysine uptake curves were obtained for control lines C82 and C87 harvested between the 10th and 25th serial generation in culture. No consistent differences were noted with either cell line, and, as seen in Fig. 3*A*, the uptake curves for six other controls lines, harvested between their 8th and 28th generation fell within 1 SD of the mean of the four experiments with line C82. Furthermore, for line C82, the range of experimental error for multiple uptake determinations in a single experiment was comparable to the standard deviation from the mean of values obtained using cells in four different generations. Thus, no significant differences in lysine uptake were observed, either between different generations of a single control cell line, or between different control cell lines harvested after variable durations in culture.

Time course of uptake by "cystinuric" cells. As shown in Fig. 3*B*, lysine uptake curves by fibroblasts from three different cystinuric patients (Cy 23, Cy 24, and Cy 48) differed in no way from results noted in controls (Fig. 3*A* and solid line in Fig. 3*B*). Again repeated determinations in each of these "cystinuric" cell lines failed to change with duration in culture. The time course of cystine uptake by two of these cystinuric lines (Cy 23, C24) was also identical to that noted in control cells (Fig. 4).

Initial rate kinetics. The relationship of initial uptake velocity to substrate concentration was investigated for lysine, arginine, and cystine. For each substrate this kinetic analysis revealed saturable transport processes conforming to Michaelis-Menten equations. The distribution ratios for lysine and arginine decreased rapidly in a linear fashion when the substrate concentration exceeded 20 mM (Fig. 5), indicating a nonsaturable portion of the uptake process. According to the method of Akedo and Christensen (17), apparent diffusion constants (K_D) of 0.24 per min for lysine and 0.58 per min for arginine were calculated and subsequently used to correct total uptake velocities (v) to yield initial rates of mediated transport (Y). When the corrected initial rates of lysine and arginine transport were plotted against the initial substrate concentration (A_f) according to the linear transformation of the Michaelis-Menten equation described by Lineweaver and Burk (18), two-limbed curves with a sharp break at an initial substrate concentration of about 1 mM were obtained for lysine (Fig. 6) as well as for arginine (Fig. 7*A*), indicating at least two separate transport mechanisms with widely differing affinities for each of these substrates.

The system preferentially used for substrate accumulation at low substrate concentrations (0.02–1 mM) was arbitrarily called system 1 (high affinity system), whereas the system preferentially mediating translocation of solute at high concentrations (> 1mM), was denoted system 2 (low affinity system). The apparent kinetic constants (Table I) for lysine and arginine are virtually identical for both transport systems.

The initial velocity of cystine uptake was studied over a range of substrate concentrations from 0.25 mM to

[1] Incubations were not usually extended beyond 90 min because increasing cell damage led to variable and erratic results.

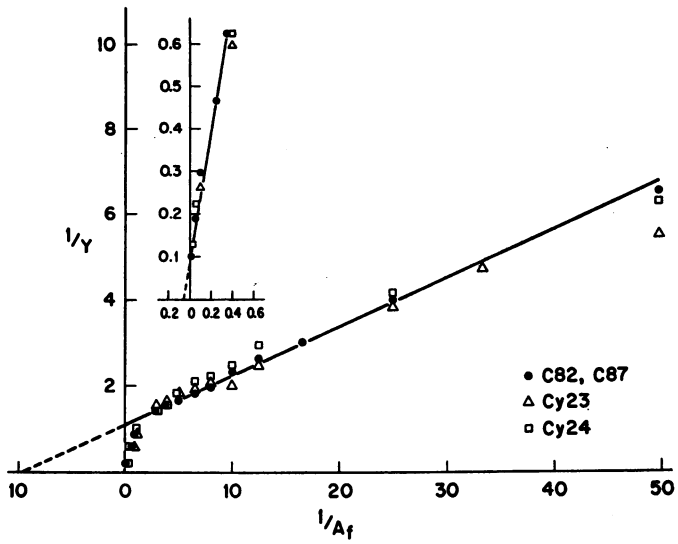

FIGURE 6 Kinetic analysis of lysine uptake in control and "cystinuric" cell lines. Initial rates of uptake, Y (mM/min) are plotted against low and high (inset) substrate concentration A_f (mM), in the incubation medium according to the double reciprocal method of Lineweaver and Burk (18). Each symbol refers to a different cell line identified in Methods. Each point represents the means of eight determinations in four separate experiments with cells from sequential cell generations. Values for control cell lines are means of C82 and C87.

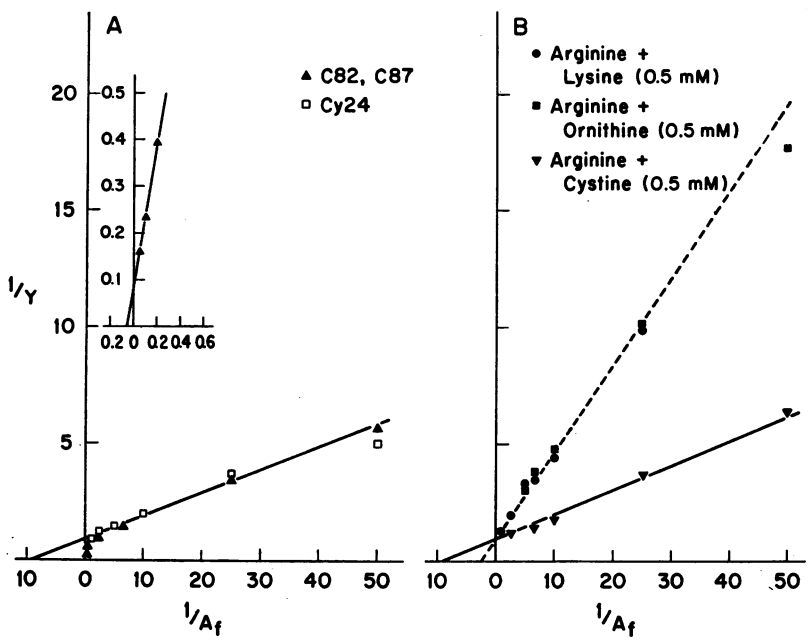

FIGURE 7 Kinetic analysis of arginine uptake (for details see legend of Fig. 6). *A:* Double reciprocal plot of initial rate of uptake versus low and high (inset) arginine concentrations in the incubation medium (A_f). *B:* Double reciprocal plot of initial rate of uptake versus extracellular concentration of arginine in the presence of lysine, ornithine, or cystine. Data are means of at least four observations in control lines C82 and C87.

TABLE I

Kinetic Parameters of Uptake of Dibasic Amino Acids and Cystine by Normal Human Fibroblasts

Substrate or inhibitor	Transport system 1			Transport system 2		
	K_t1	K_i1	$V_{max}1$	K_t2	K_i2	$V_{max}2$
	mM	*mM*	*mmoles/liter/min*	*mM*	*mM*	*mmoles/liter/min*
Lysine	0.10±0.03	0.14‡	0.91±0.09	18.4	—	11.8
Arginine	0.11±0.03	0.15§	1.1 ±0.14	19.0	17.1§	11.7
Ornithine	—	0.14§ 0.15§	—	—	17.1§	—
Cystine	0.74±0.17	—	1.6 ±0.2			

* See text for definition of transport systems 1 and 2 and for definition of K_t, K_i, and V_{max}. All values were derived from initial uptake velocities as described in Methods and Results. Numerical values for transport system 1 represent mean ±SD of at least four separate observations.

‡ Arginine was substrate.

§ Lysine was substrate.

0.5 mM, thus approaching the solubility limit of the compound (Fig. 8*A*). These results also displayed saturability and fitted to Michaelis-Menten kinetics. At initial rates of uptake a nonsaturable component of transport (11) could not be detected and only one mode of uptake was observed. The apparent affinity constant (K_t) was 0.71 mM and the maximal velocity (V_{max}) was 1.67 mmoles/liter per min (Table I).

It should be emphasized that all of these kinetic analyses were carried out in two control cell lines and two cystinuric cell lines. As noted in Figs. 6–8 initial rates of uptake of lysine, arginine, or cystine by cystinuric lines differed in no way from those observed for control cells, either at high or low substrate concentrations.

Competitive inhibition. To characterize further the mediated transport systems for lysine, arginine, and cystine, studies of the mutual interaction between dibasic amino acids and cystine were carried out. The data in Fig. 9 describe studies of the initial rate of lysine uptake in the presence and absence of arginine, ornithine, and cystine. In examining the high affinity system (system 1) observed preferentially at low substrate concentration, arginine and ornithine were added at a concentration of 1 mM and cystine was present at 0.5 mM. Arginine and ornithine inhibited lysine uptake markedly and equally, yielding a regression line with an ordinate intercept identical to that observed in the absence of either inhibitor. These results indicate competitive inhibition. The apparent inhibition constant (K_i) for both arginine and ornithine (0.15 mM) was in good agreement with K_t1 for lysine (Table I). In contrast, cystine failed to inhibit lysine uptake throughout this range of substrate concentrations.

Comparable results were found when the low affinity system (system 2) for lysine uptake was examined. Arginine and ornithine, each added at concentrations of 20 mM, inhibited lysine uptake equally and competitively with K_i values very similar to that calculated for K_t2 for lysine. Because of its limited solubility, cystine could not be tested as an inhibitor of system 2 for lysine.

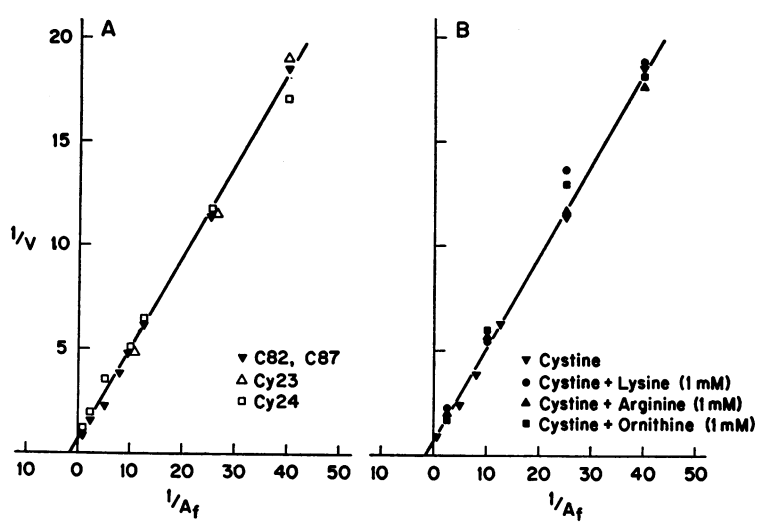

FIGURE 8 Kinetic analysis of cystine transport. (See Fig. 6 for details.) *A:* Double reciprocal plot of initial velocity, V, (mM/min) of cystine uptake in control and "cystinuric" cell lines. Initial velocities are not corrected for diffusion. *B:* Double reciprocal plot of initial velocity of cystine uptake in presence of lysine, arginine, or ornithine, determined in control cell lines C82 and C87. The curve for cystine uptake in control cell lines presented in *A* is reproduced in *B*.

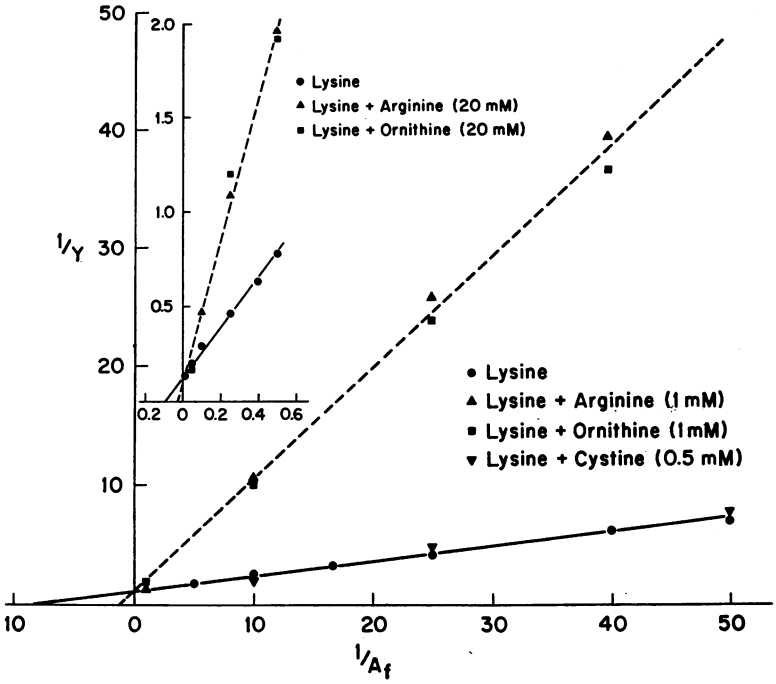

FIGURE 9 Demonstration of competitive inhibition of lysine uptake by arginine and ornithine at low and high (inset) substrate concentrations. The lack of influence of cystine on lysine transport is shown at low lysine concentrations. For details see Fig. 6 and text.

Completely analogous results were obtained when arginine was used as substrate and lysine, ornithine and cystine were used as inhibitors (Fig. 7*B*). Lysine and ornithine inhibited arginine uptake equally and competitively, with K_i values very close to that noted for $K_i 1$ for arginine. Again, cystine failed to alter arginine uptake.

Finally, cystine uptake was examined in the presence of lysine, arginine and ornithine (1 mM). No inhibition of cystine uptake was produced by any of the dibasic amino acids.

The above data indicate that lysine, arginine, and ornithine share a common transport system in cultured human fibroblasts, whereas mediated cystine uptake is accomplished by a completely different transport site. Furthermore, the common K_t and K_i values obtained for lysine, arginine and ornithine demonstrate that these three dibasic amino acids have identical affinity for the shared process.

Effect of extracellular Na^+ and K^+ on initial rates of uptake. The data shown in Fig. 10 demonstrate that the initial rate of lysine uptake by control fibroblasts is not a sodium dependent process. Complete, isosomolar replacement of sodium in the incubation medium by either Tris or choline had no affect on initial uptake velocities over a wide range of substrate concentrations. However, replacement of sodium by potassium led to a distinct reduction in $V_{max}1$. No influence of potassium on system 2 was demonstrated when similar experiments were carried out at high substrate concentrations.

The initial rate of cystine uptake was also shown to be a sodium-independent but potassium-sensitive system. At a substrate concentration of 0.1 mM, substitution of choline for sodium led to no appreciable inhibition of uptake, whereas replacement of sodium by potassium reduced initial uptake of 56% of control values.

Effect of metabolic inhibitors on initial rates of lysine uptake. The influence of several metabolic inhibitors on initial rates of lysine uptake was studied at a low substrate concentration (0.1 mM) where uptake is mediated preferentially by system 1, and at a high substrate concentration (10 mM) where system 2 preferentially brings about solute translocation (Table II). Sodium cyanide and *p*-chloromercuribenzoate inhibited uptake markedly at low and high substrate concentrations, while 2,4-dinitrophenol and ouabain produced only modest inhibitory effects. Each inhibitor except 2,4-dinitrophenol reduced uptake by system 1 more than by system 2.

Transport of tryptophan

As mentioned earlier, tryptophan transport has been shown to be defective in the gut and kidney of patients with a second inborn error of transport, Hartnup dis-

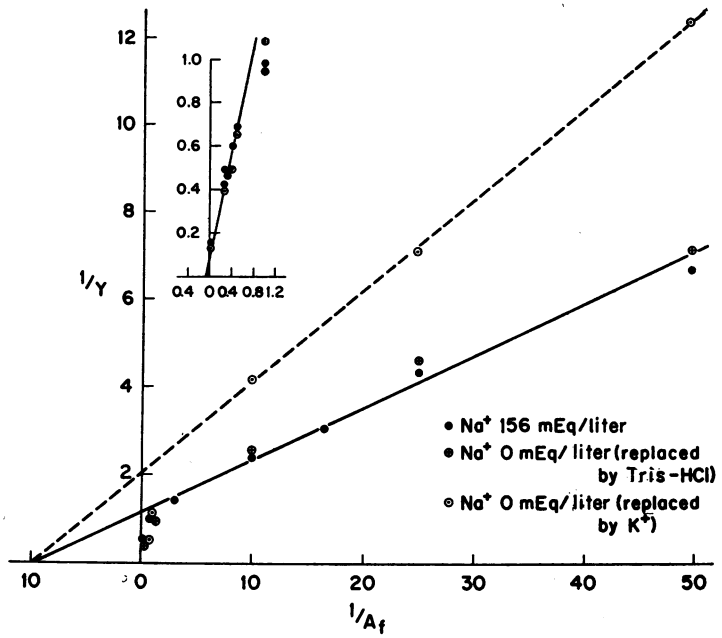

FIGURE 10 Effect of sodium replacement in the incubation media by Tris and choline on initial rates of lysine uptake at low and high substrate concentrations. Data from control cells are plotted according to the double reciprocal method. (For details see Fig. 6.)

ease. In the following experiments we characterized tryptophan transport in fibroblasts from controls (C82 and C87) and from a child with documented Hartnup disease (H98). These results are summarized in Fig. 11.

The accumulation of tryptophan (0.1 mM) proceeded rapidly during the first 2 min to reach a plateau after 60 min of incubation and (Fig. 11*A*) remained constant up to 90 min. No differences in time dependent uptake were observed between control cell lines and the Hartnup cell line.

Tryptophan uptake was saturable. A diffusion constant ($K_D = 0.42$) was calculated as described above and used for correction of initial uptake velocities (Fig. 11*B*). Initial rates of transport conformed to Michaelis-Menten equations, and double reciprocal analysis (Fig. 11*C*) revealed the presence of two transport systems (Fig. 11*C*). System 1 (high affinity system) had a K_t1 of 0.02 mM and a $V_{max}1$ of 2.0 mM/min. The kinetic parameters of system 2 (low affinity system) were: $K_t2 = 12.5$ mM; $V_{max}2 = 10$ mM/min. No differences in initial rates of uptake were found between control cell lines and the Hartnup cell line at substrate concentrations ranging

TABLE II

Effect of Metabolic Inhibitors on Initial Uptake Velocity of Lysine at Low and High Substrate Concentrations

Inhibitor*	Inhibition		Significance of difference between substrate concentrations
	0.1 mM L-lysine	10 mM L-lysine	
	%	%	
Sodium cyanide (5×10^{-3} M)	81.7±4.6	51.5±5.1	$P < 0.01$
Parachloromercuribenzoate (1×10^{-4} M)	71.0±5.0	46.1±4.0	$P < 0.01$
2, 4-Dinitrophenol (1×10^{-4} M)	21.2±6.4	15.3±5.5	$P > 0.1$
Ouabain (5×10^{-4} M)	31.5±2.3	9.0±6.2‡	$P < 0.01$

* Human diploid fibroblasts were preincubated with inhibitor for 5 min before the addition of the substrate. Initial rates of uptake were determined after 1 min incubation intervals. Results are expressed as per cent inhibition of uptake in the presence of inhibitor compared to controls studied in the same experiment. Values represent mean ±1 SD of at least four observations.

‡ Not significantly different from zero ($P > 0.05$).

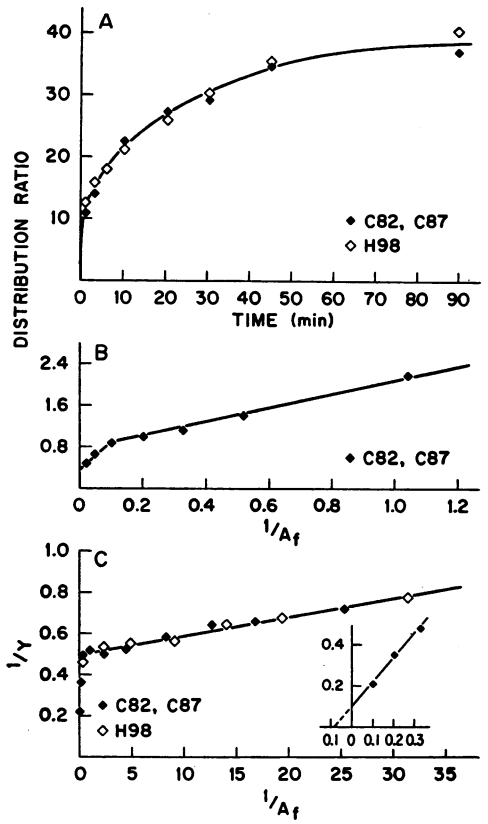

FIGURE 11 Parameters of tryptophan transport. *A:* Time course of tryptophan uptake in control and "Hartnup" cell lines; *B:* Plot used to determine the apparent diffusion constant for initial rates of tryptophan uptake (for details see legend of Fig. 5); *C:* Double reciprocal plot of the initial rates of tryptophan uptake vs. low and high (inset) tryptophan concentrations in the incubation media (for details see legend of Fig. 6). All values represent the mean of at least four observations in each cell line used.

from 0.03 to 1 mM. Tryptophan uptake in cell line H98 was not determined for substrate concentrations higher than 1 mM.

The initial rate of tryptophan uptake was sodium independent at low and high substrate concentrations. Replacement of sodium in the medium by potassium inhibited tryptophan transport at low but not at high substrate concentrations (Table III). In contrast, transport of two other neutral amino acids, α-aminoisobutyric acid and glycine, was shown to be sodium-dependent at both low and high substrate concentrations (Table III). Thus, the sodium independent nature of the transport processes for lysine, cystine, and tryptophan was shown not to be a technical artifact.

The effect of metabolic inhibitors on initial rate of tryptophan uptake is shown in Table IV. 2,4-dinitrophenol and ouabain failed to inhibit tryptophan transport significantly either at low or high substrate concentrations. Cyanide inhibited uptake at low but not at high concentrations, whereas *p*-chloromercuribenzoate inhibited uptake at both substrate concentrations, with significantly greater inhibition observed at the low substrate concentration.

DISCUSSION

This study has two related aims: characterization of the transport processes for several amino acids in diploid human fibroblasts; and assessment of the expression of the mutant phenotype in cultured diploid fibroblasts from patients with cystinuria and Hartnup disease. Although the negative data referable to the latter aim has the broader clinical significance, the former topic raises equally significant biological questions which will be discussed first.

Transport properties of dibasic amino acids, cystine,

TABLE III

Effect of Sodium Replacement by Choline and Potassium on Initial Uptake Velocities of Various Amino Acids at Low and High Substrate Concentrations

Amino acid*	Per cent of control					
	Amino acid concentration (0.1 mM)			Amino acid concentration (10 mM)		
	Extracellular sodium replaced by:			Extracellular sodium replaced by:		
	Choline	Potassium	S‡	Choline	Potassium	S‡
Tryptophan	98.7±5.9§	51.8±8.7	$P < 0.01$	103.5±8.3§	95±11.8§	$P > 0.1$
Glycine	32.2±2.8	19.9±4.3	$P < 0.01$	43.3±6.7	43± 3.1	$P > 0.1$
α-aminoisobutyric acid	48.7±4.5	29.8±3.6	$P < 0.01$	53.2±3.5	54± 4.6	$P > 0.1$

* Experimental conditions: cells were harvested in sodium containing buffer, divided, and washed twice in sodium free buffers containing isoosmolar amounts of choline or potassium. Cells were resuspended and incubated in the appropriate buffer for 1 min. Results are expressed as per cent uptake in sodium free incubation medium compared with controls studied in the same experiment. All data indicate means ±SD of four determinations.

‡ S, significance of difference between choline and potassium containing buffer.

§ Not significantly different from control ($P > 0.1$).

TABLE IV

Effect of Metabolic Inhibitors on Initial Rates of Tryptophan Uptake at Low and High Substrate Concentration

Inhibitors*	Inhibition of uptake at concentrations of		Significance of difference between substrate concentrations
	0.1 mM	10 mM	
	%	%	
Sodium cyanide (5×10^{-3} M)	49.2±7.1	8.4±4.4	$P < 0.01$
p-chloromercuribenzoate (1×10^{-4} M)	91.4±2.8	43.9±4.5	$P < 0.01$
2, 4-Dinitrophenol (1×10^{-4} M)	3.9±4.0‡	4.2±5.8‡	$P > 0.1$
Ouabain (5×10^{-4} M)	23.4±3.9	14.2±6.6	$P = 0.05$

* See Table II for details.

‡ Not significantly different from zero ($P > 0.1$).

and tryptophan. Our data relating metabolic conversion of lysine and tryptophan (Fig. 1) to the transport of these substances by intact fibroblasts provide a basis for the theoretical interpretation of the observed time curves of uptake. Equilibrium conditions were not achieved for lysine uptake even after 90 min of incubation (Fig. 3). Instead, the total intracellular radioactivity increased approximately linearly after 45 min of incubation, indicating a "stationary state" with a constant net inflow of substrate. According to Curran and Schultz (20), this condition can be described as a time independent state by the derivative expression

$$\frac{dA'_c}{dt} = \text{constant} \qquad (2)$$

in which A'_c denotes total radioactivity inside the cell. If the time curve for lysine uptake is corrected for that portion of lysine-^{14}C metabolized by the cell at any given time (Fig. 3*B*), the course of uptake after 45 min of incubation shows another time independent state which reflects the condition of equilibrium and may be described by the expression

$$\frac{dA_c}{dt} = 0 \qquad (3)$$

in which A_c is the fraction of chemically unaltered lysine. The steady-state condition of a metabolizable substrate, assuming unit area and unit volume is given by

$$J_i - \nu_i J_r = 0 \qquad (4)$$

in which J_i is the net flow, ν_i the stoichiometric coefficient of the substance in the reaction and J_r the rate of chemical reaction (20). Applied to our data, this equation states that during the portion of the time curve when a stationary state has been achieved, the rate of consumption of lysine within the cell is exactly balanced by an inflow of lysine, so that its intracellular concentration remains constant. The validity of this interpretation is strengthened by the contrasting properties of the uptake curve for tryptophan (Fig. 11*A*), which was shown not to be metabolized under our experimental conditions. In this instance, equilibrium conditions were reached after 50 min of incubation, satisfying equation 2.

Since $^{14}CO_2$ evolution from lysine or tryptophan was not measured in our experiments and since efflux of trace amounts of labeled metabolites into the incubation medium may have escaped our detection, our estimates of substrate conversion may be low. This discrepancy does not affect the validity of our interpretation of the time curves as long as all flows of matter not explicitly determined in our system are time independent themselves. Such conditions might be expected in our experiments since the system was left for a sufficiently long time without external constraint.

Estimates of the kinetic parameters of the saturable transport systems for dibasic amino acids, cystine and tryptophan were made after very brief exposure of cells to external amino acids, assuming that the intracellular concentration found after such short incubation intervals represented the initial rate of uptake. Lineweaver-Burk plots (18) of the initial rates of uptake, corrected for an uptake portion due to diffusion (17), indicated two transport systems with widely differing affinities and capacities for each of the substrates investigated except cystine. The transition from one mode of uptake to the other occurred at substrate concentrations in the range of 1 mM, a concentration roughly one order of magnitude above physiological plasma amino acid values (21). If we extrapolate from cultured human fibroblasts to human fibroblasts in vivo, these data suggest that translocation of these solutes at physiological plasma amino acid concentrations is preferentially brought about by the high affinity system (system 1). The biological significance of the low affinity systems remains unexplained. More than one transport system for a given amino acid (one acting at low, the other at high substrate concentrations) have been found in a variety of microorganism (22–24)

and mammalian cells (13, 25–27) but a molecular understanding of these systems is lacking. In this context the observation of Boos, Gordon, and Hall (28) that the galactose-binding protein from *Escherichia coli* undergoes a conformational change at high substrate concentrations leading to an increase in the K_m of about two orders of magnitude should be mentioned, since similar changes could affect the postulated membrane carrier proteins for amino acids in human fibroblasts.

In agreement with previous observations using rat kidney (29) or human leukocytes (8), competitive inhibition studies at low substrate concentrations revealed that cystine uptake by diploid fibroblasts is not mediated by the common transport system shared by the dibasic amino acids. In rat kidney and in leukocytes, arginine had an affinity for this shared system about twice that estimated for lysine. Our data with fibroblasts, however, indicate that lysine, arginine, and ornithine have identical affinities, not only for transport system 1 (high affinity) but also for system 2 (low affinity). The affinity of lysine for system 1 in fibroblasts ($K_t = 0.1$ mM) is virtually identical with that observed in human leukocytes ($K_t = 0.08$ mM) (8). Similar identity was found for the affinity constants of cystine transport in the gut (5) ($K_t = 0.71$ mM) and in fibroblasts ($K_t = 0.74$ mM). In contrast, the K_t of lysine transport in human gut mucosa (5) for the transport system shared by dibasic amino acids and lysine is about 25 times higher than that in human leukocytes (8) and fibroblasts. All these observations support the notion that some properties of amino acid transport systems in man are tissue-specific while others may be identical in several tissues. More information about the properties of transport systems in different tissues is needed before we can begin to understand the role of differentiation in the regulation of transport processes.

The transport systems in human fibrobasts for dibasic amino acids, cystine, and tryptophan are not sodium-dependent, in conformance with observations in human leukocytes (8, 30) and in Ehrlich ascites cells (25, 31). Since lysine transport in intestine has been shown to be sodium-dependent (32, 33) we may conclude, again, that the process of differentiation changes certain important properties of transport systems.

The inhibitory effect of high extracellular potassium concentrations on lysine, cystine, and tryptophan uptake by their high affinity systems (system 1) and the absence of this effect on their low affinity systems (system 2) (see Fig. 10 and Table II) is consistent with previous observations (34, 35). If we assume that system 1 is mediated and energy-dependent, and that system 2 is mediated but energy-independent (see Table IV) our findings are consistent with an inhibitory action of potassium on the energy link for system 1, but this represents only one of several possible interpretations.

Absence of transport defect in fibroblasts from patients with cystinuria and Hartnup disease. Diploid human fibroblasts grown in tissue culture are presumed to contain the complete genome. The failure to demonstrate transport defects for dibasic amino acids and cystine in "cystinuric" cell strains as well as for tryptophan in a cell line from a patient with Hartnup disease indicates that the gene loci mutant in these diseases are not expressed in cultured human fibroblasts under the conditions of our study. This observation has some important consequences for further attempts to investigate the biochemical nature of transport defects. Movement of solutes across biological membranes has long been described in phenomenologic terms, assuming the existence of hypothetical carrier proteins in the translocation process. In recent years, proteins have been isolated from microbial species which appear to be integral parts of the transport system (36). Since such analyses depend on the availability of large amounts of pure membrane components, the ability to grow human cells in culture and to compare membrane structure with transport functions in normal vs mutant cells would appear to be uniquely advantageous. It is disappointing that cultured human fibroblasts, a readily available tissue source, do not express the same genetic information as such differentiated tissues as kidney and gut do. This experimental handicap may be overcome successfully in two ways. It may be possible to induce or activate previously unexpressed gene loci in fibroblasts. Preliminary attempts in our laboratory to grow fibroblasts in minimal medium for dibasic amino acids failed to alter the transport characteristics of these cells. Nonetheless, further attempts along these lines seem important since we know little about the nature of stimuli required to induce differentiated functions. Alternatively, technical progress in the ability to grow and maintain differentiated cells (e.g., gut or kidney) in tissue culture may enable workers to use both biochemical and genetic approaches to the study of transport regulation in human tissue.

ACKNOWLEDGMENTS

We wish to thank Mrs. Jane Hunt and Mrs. Gail Marchant for invaluable technical assistance, and Dr. Maurice J. Mahoney for much valuable advice. Dr. Groth was supported by a James Hudson Brown Fellowship from the Yale University School of Medicine. This work was supported by research grants from the John A. Hartford Foundation and the National Institute of Arthritis and Metabolic Diseases (AM 12579).

REFERENCES

1. Robson, E. B., and G. A. Rose. 1957. The effect of intravenous lysine on the renal clearance of cystine, arginine, and ornithine in normal subjects, in patients with

cystinuria and Fanconi syndrome and in their relatives. *Clin. Sci.* **16**: 75.
2. Arrow, V. K., and R. G. Westall. 1958. Amino acid clearances in cystinuria. *J. Physiol.* **142**: 141.
3. Milne, M. D., A. M. Asatoor, K. D. G. Edwards, and L. W. Loughridge. 1961. The intestinal absorption defect in cystinuria. *Gut.* **2**: 323.
4. Fox, M., S. Thier, L. E. Rosenberg, W. Kiser, and S. Segal. 1964. Evidence against a single renal transport defect in cystinuria. *N. Engl. J. Med.* **270**: 556.
5. Thier, S., M. Fox, S. Segal, and L. E. Rosenberg. 1964. Cystinuria: in vitro demonstration of an intestinal transport defect. *Science (Wash., D. C.)*. **143**: 482.
6. Thier, S. O., S. Segal, M. Fox, A. Blair, and L. E. Rosenberg. 1965. Cystinuria: defective intestinal transport of dibasic amino acids and cystine. *J. Clin. Invest.* **44**: 442.
7. Jepson, J. B. 1972. Hartnup disease. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Frederickson, editors. McGraw-Hill, Inc., New York. 3rd edition. 1486.
8. Becker, F. F., and H. Green. 1958. Incorporation of cystine and lysine by normal and "cystinuric" leukocytes. *Proc. Soc. Exp. Biol. Med.* **99**: 694.
9. Rosenberg, L. E., and S. J. Downing. 1965. Transport of neutral and dibasic amino acids by human leukocytes: absence of defect in cystinuria. *J. Clin. Invest.* **44**: 1382.
10. Mellman, W. J. 1971. A biochemical genetics view of human cell culture. *In* Advances in Human Genetics. H. Harris and K. Hirschhorn, editors. Plenum Press, New York. **2**: 259.
11. Mahoney, M. J., and L. E. Rosenberg. 1970. Uptake of α-aminoisobutyric acid by cultured human fibroblasts. *Biochim. Biophys. Acta.* **219**: 500.
12. Rosenberg, L. E. 1966. Cystinuria: genetic heterogeneity and allelism. *Science (Wash., D. C.)*. **154**: 1341.
13. Hillman, R. E., I. Albrecht, and L. E. Rosenberg. 1968. Identification and analysis of multiple glycine transport systems in isolated mammalian renal tubules. *J. Biol. Chem.* **243**: 5566.
14. Hillman, R. E., and L. E. Rosenberg. 1969. Amino acid transport by isolated mammalian tubules. II. Transport systems for L-proline. *J. Biol. Chem.* **244**: 4494.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265.
16. Rosenberg, L. E., J. C. Crawhall, and S. Segal. 1967. Intestinal transport of cystine and cysteine in man: evidence for separate mechanisms. *J. Clin. Invest.* **46**: 30.
17. Akedo, H., and H. N. Christensen. 1962. Nature of insulin action on amino acid uptake by the isolated diaphragm. *J. Biol. Chem.* **237**: 118.
18. Lineweaver, H., and D. Burk. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**: 658.
19. Reiser, S., and P. A. Christiansen. 1971. The properties of the preferential uptake of L-leucine by isolated intestinal epithelial cells. *Biochim. Biophys. Acta.* **225**: 123.
20. Curran, P. F., and S. G. Schultz. 1968. Transport across membranes: general principles. *Handb. Physiol. Sect. 6.* **3**: 1217.
21. Rosenberg, L. E., and C. R. Scriver. 1969. Orders of amino acid metabolism. *In* Diseases of Metabolism. P. K. Bondy, editor. W. B. Saunders Company, Philadelphia, Pa. 6th edition. 366.
22. Ames, G. F. 1964. Uptake of amino acids by *Salmonella typhimurium*. *Arch. Biochem. Biophys.* **104**: 1.
23. Ring, K. 1969. Die Induktion des aktiven Transportes neutraler Amino-Säuren bei Streptomyces hydrogenans. *Biochem. Biophys. Acta.* **183**: 375.
24. Grenson, M., M. Mousset, J. M. Wiame, and J. Brechet. 1966. Multiplicity of the amino acid permeases in Saccharomyces cerevisiae. I. Evidence for a specific arginine-transporting system. *Biochim. Biophys. Acta.* **127**: 325.
25. Christensen, H. N. 1964. A transport system serving for mono- and diamino acids. *Proc. Natl. Acad. Sci. U. S. A.* **51**: 337.
26. Rosenberg, L. E., I. Albrecht, and S. Segal. 1967. Lysine transport in human kidney: evidence for two systems. *Science (Wash., D. C.)*. **155**: 1426.
27. Matthews, R. H., C. A. Leslie, and P. G. Scholefield. 1970. Histidine uptake and exchange in S_{37} ascites tumor cells. *Biochim. Biophys. Acta.* **203**: 457.
28. Boos, W., A. Gordon, and R. E. Hall. 1971. Substrate induced conformational change of the galactose-binding protein of E. coli. *Fed. Proc.* **30**: 1062. (Abstr. 58)
29. Rosenberg, L. E., S. J. Downing, and S. Segal. 1962. Competitive inhibition of dibasic amino acid transport in rat kidney. *J. Biol. Chem.* **237**: 2265.
30. Yunis, A. A., G. K. Arimura, and D. M. Kipnis. 1963. Amino acid transport in blood cells. I. Effect of cations on amino acid transport in human leukocytes. *J. Lab. Clin. Med.* **62**: 465.
31. Oxender, D. L., and H. N. Christensen. 1963. District mediating systems for the transport of neutral amino acids by the Ehrlich cell. *J. Biol. Chem.* **238**: 3686.
32. Munck, B. G. 1970. Interactions between lysine, Na^+ and Cl^- transport in rat jejunum. *Biochim. Biophys. Acta.* **203**: 424.
33. Rosenberg, I. H., A. L. Coleman, and L. E. Rosenberg. 1965. The role of sodium ion in the transport of amino acids by the intestine. *Biochim. Biophys. Acta.* **102**: 161.
34. Schultz, S. G., R. E. Fuisz, and P. F. Curran. 1966. Amino acid and sugar transport in rabbit ileum. *J. Gen. Physiol.* **49**: 849.
35. Curran, P. F., S. G. Schultz, R. A. Chez, and R. E. Fuisz. 1967. Kinetic relations of the Na-amino acid interaction at the mucosal border of intestine. *J. Gen. Physiol.* **50**: 1261.
36. Pardee, A. B. 1968. Membrane transport proteins. *Science (Wash., D. C.)*. **162**: 632.