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THE PROPERTIES OF THE PREFERENTIAL UPTAKE OF L-LEUCINE BY ISOLATED INTESTINAL EPITHELIAL CELLS

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SUMMARY

1. Epithelial cells isolated from rat intestine have shown the ability to preferentially take up 1 mM L-leucine as compared to the D-isomer. The uptake was found to be concentrative. L-Leucine uptake was inhibited by neutral L-amino acids and basic L-amino acids but was not inhibited by D-valine or D-isoleucine. Galactose and α-methyl-D-glucose were inhibitory; glucose was significantly less inhibitory; and fructose activated uptake. Inhibitors of energy metabolism, sulfhydryl inhibitors, ouabain, and procedures which damaged the morphology of the cell all decreased L-leucine uptake. L-Leucine uptake was decreased in the absence of either Na^+, K^+, Ca^{2+}, or Mg^{2+} and exhibited a broad pH optimum between 4 and 8. L-Leucine uptake was a linear function of time during the first 5 min of incubation. The apparent K_m for L-leucine uptake was 3.2 mM, and L-valine was a competitive inhibitor of L-leucine uptake. Inhibitors of protein biosynthesis did not reduce L-leucine uptake. The efflux of L-leucine from the cells was inhibited by the cold.

2. Brush borders also preferentially bound L-leucine as compared to D-leucine. The binding was quite variable and was not inhibited by L-valine.

3. Preincubation of the cells with Krebs–Tris containing 50 mM L-leucine resulted in a significantly greater subsequent incorporation of iodoacetate into the cells than did preincubation with Krebs–Tris or Krebs–Tris with 50 mM D-leucine. Following homogenization of the cells with 5 mM EDTA and centrifugation, a solubilized protein fraction was found to contain the increased iodoacetate from the L-leucine protected cells.

INTRODUCTION

Current evidence suggests that the binding of an amino acid or sugar to a specific membrane carrier constitutes the initial and structurally specific event in intestinal transport[1,2]. The characteristics of this binding, such as apparent enzyme kinetics and competitive inhibition between structurally similar amino acids or sugars, are similar to the binding of a substrate by an enzyme. More direct evidence that binding may be mediated by a membrane protein is found in studies with bacteria where protein released from osmotically shocked bacteria has been isolated and shown to specifically bind amino acids and sugars[3–5]. One of the major difficulties encountered

in studying the components of membrane transport in intestine in a manner similar to that possible with bacteria or red blood cells has been the unavailability of a relatively purified form of the transporting membrane. One of the more positive approaches to solving this problem was the preparation of purified intestinal brush borders for the study of sugar[6,7] and amino acid[8] binding. However, some of the properties of the brush border binding such as the absence of a Na^+ dependency, the magnitude of the binding K_m, and the lack of inhibition of binding by other sugars or amino acids sharing the same transport pathway, are different from those noted in intact intestine and indicate that a portion of the transport system, perhaps a membrane protein, has been modified or lost during the preparation of the brush borders. Isolated epithelial cells may represent another relatively purified membrane preparation with which to study the properties and components of intestinal transport. Previous work has shown that essentially viable preparations of epithelial cells can be obtained from intact rat[9,10] and rabbit[11] intestine. Except for preliminary reports by STERN AND JENSEN[9] on the active transport of D-glucose and HUANG[11] on the energy-dependent uptake of L-tyrosine and 3-*O*-methylglucose, isolated intestinal epithelial cells have not been employed for transport studies. The purposes of this study were to describe in detail the properties of amino acid uptake by isolated epithelial cells, compare these properties to those of the intact transport system, and to evaluate the applicability of this membrane preparation for the future study of the properties and individual components of amino acid transport.

METHODS

Wistar strain, male rats weighing 180–260 g were used as a source of intestinal tissue. Isolated intestinal epithelial cells were prepared essentially by the method of STERN AND JENSEN[9]. The rats were killed by decapitation; the small intestine was quickly removed and flushed with isotonic saline. The intestine was divided into two equal sections and a polyethylene tubing, 1.5 inches in length, was tied into the end of each segment. Syringes attached to the polyethylene tubing were used to introduce all subsequent media into the intestinal lumen. Each intestinal segment was then filled with an oxygenated, mildly hypertonic (345 mOsm/l) incubation medium (pH 7.2) containing 96 mM NaCl, 8 mM KH_2PO_4, 50 mM sodium citrate·$2H_2O$, 5.6 mM Na_2HPO_4, 1.5 mM KCl, 2.5 mg/ml serum albumin, and 1.5 mg/ml hyaluronidase (Sigma Chemical Co., 370 NF units/mg) and placed in a 50-ml erlenmeyer flask containing 10 ml of gassed saline and incubated 15 min at 33° with agitation. The citrate and hyaluronidase were added to dissociate the epithelial cells from the mucosa[9,10], while the albumin has been reported to stabilize isolated intestinal epithelial cells[10]. The segment was then removed, drained of incubation medium, flushed with cold saline, and partially filled with an oxygenated collection medium containing 137 mM NaCl, 11.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.2 mM KCl, and 5 mg/ml serum albumin (pH 7.2). The partially filled segment was then placed on a towel over an ice block, and the epithelial cells were released from the underlying tissue by patting the serosal side with the fingers. The contents of the lumen were then drained, observed microscopically, and discarded. The manipulation of the tissue was repeated two additional times with fresh collection medium. The luminal contents were collected, observed microscopically, and centrifuged for 5 min at 200 × *g* in a refrigerated

centrifuge. The cell pellet was then dispersed in 20 times its volume of ice cold collection medium without albumin, and centrifuged at 200 × *g* for 5 min in the cold. The viability of the cells in the pellet was determined microscopically after staining with 0.5 % trypan blue in albumin-free collection medium[12]. Smears of the pellet were also stained with Wright's Stain in order to more clearly differentiate and count the cells present in the pellet. Sections of the residual intestine were fixed in 4 % formalin, embedded in paraffin, and stained with haematoxylin and eosin.

In a typical experiment 0.3 ml of the cells representing an average of 5.25 mg protein was added to 5 ml of oxygenated Krebs–Ringer Tris buffer (pH 7.4) containing 118 mM NaCl, 25 mM Tris–HCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, nonradioactive L- and D-leucine to the desired final concentration, and 10000–20000 counts/min per ml of radioactive L-[^{3}H]leucine and D-[^{14}C]leucine. An aliquot of the incubation medium was taken after the cells were dispersed in the medium for a determination of the initial counts in the reaction mixture. The reaction mixture was then incubated with shaking for the desired time period at 37°. Unless otherwise indicated, the reaction was terminated by pouring the contents of the reaction mixture into a graduated centrifuge tube in an ice bath and centrifuging the cells at 200 × *g* for 5 min in the cold. The cells were then washed and centrifuged 3 additional times with 5 ml cold Krebs–Tris. The final pellet was diluted to 1 ml with cold saline and mixed, and aliquots taken for the determination of radioactivity, protein[13], and for a microscopic examination of viability. All samples were counted in a Tri-Carb liquid scintillation spectrometer (Packard) in a toluene, Triton X-100, 2,5-diphenyloxazole, 1,4-bis-2-(5-phenyloxazolyl)benzene system[14]. The spectrometer was adjusted to permit less than 0.01 % ^{3}H efficiency on the ^{14}C channel and 10 % efficiency of the ^{14}C on the ^{3}H channel[15]. In some experiments the cell pellet was homogenized in 5 % trichloroacetic acid, and aliquots of the supernatant as well as the cell-free reaction medium were subjected to paper chromatographic analysis as described previously[15,16] in order to determine whether leucine metabolism was associated with cell uptake.

In a few experiments, especially those in which the incubation time was a critical factor, a portion of the final reaction mixture was quickly chilled and passed through a previously wetted Millipore filter (SM 5 μ or LS 5 μ, Millipore Filter Corp., Bedford, Mass.) by applying positive pressure to a syringe which fits over the filter holder. The cells on the filter were washed 3 times with cold Krebs–Tris and the filter paper as well as an aliquot of the initial medium counted directly in the scintillation system. The advantage of this procedure was that the cells could be on the filter and washed once in about 10 sec. However, a small (less than 0.4 % of total counts passed through the filter) but variable binding of the isotopes to the filter was observed in the absence of cells even after 3 washings. Therefore, an average filter binding value for each isotope had to be determined for each experiment and subtracted from the total isotope uptake of the cells on the filter. Even with this correction, relatively large standard errors of the mean were obtained for cell uptake on the filters.

Brush borders were prepared from scrapings of rat intestine by disruption of the epithelial cells with a vibra-mixer (Chemapec, Inc.) in ice cold 5 mM EDTA for 20 min, passing the resultant suspension through glass wool to entrap larger particles and remove nuclei[17], and then differentially centrifuging and washing the preparation

as described by EICHHOLZ AND CRANE[18]. The purity of the final brush border preparation was determined by light microscopy. Preferential leucine binding to the brush borders was determined as previously described for the epithelial cells.

In order to calculate the preferential uptake of the L-leucine by the cells or the preferential binding by the brush borders, the counting rate of both isotopes in the original reaction medium was mathematically treated so that 10000 counts/min per ml were equivalent to the initial concentration of the amino acids. With the use of the same proportionality factors employed in converting the medium, the number of counts of each isotope in the cells or bound to the brush borders was converted to a 10000 count equivalent. This manipulation permitted the conversion of counts to nmoles amino acid from identical experiments having somewhat different initial counting rates. The nmoles of the D-leucine taken up by the cell or bound to the brush border, presumably representing diffusion and nonspecific binding, were then subtracted from the nmoles of the L-leucine found in the same preparation. The final uptake or binding was expressed as nmoles L-leucine/mg cell or brush border protein.

The uptake of L- or D-leucine in nmoles/mg protein could be converted into an estimate of the intracellular accumulation of leucine in the cell water by the following formula: mM L- or D-leucine (cellular water) = (μmoles leucine/mg protein) $\times$ (total mg protein in cells)/(cell wet wt.) $\times$ 0.8. The cell wet wt. was calculated as follows: (total mg protein in cells) $\times$ (mg dry wt./mg protein) $\times$ (5 mg wet wt./mg dry wt.). This conversion was based on the assumptions that the dry wt. of a cell is 20 % of its wet wt. and that the density of the cell is about 1 g/ml. The mg dry wt./mg protein in the cells was determined in 32 individual experiments and found to be 1.83 $\pm$ 0.07 (mean and S.E.), and this average value was used in all calculations.

The percent of control values were obtained by determining preferential L-leucine uptake under various experimental conditions and dividing each value by the preferential uptake of L-leucine in the corresponding control system and were expressed as the mean $\pm$ S.E. A paired-difference t test was used to determine if a significant difference existed between control and experimental values. References to statistical significance pertain to the 5 % probability level or lower. In the kinetic studies the regression lines were calculated by the method of least squares. The method used to calculate the confidence limits for the x and y intercepts was that of JERVIS AND SMYTH[19].

The sodium concentration of the incubation media used in sodium replacement studies was determined by direct analysis in a Coleman flame photometer.

The source and specific activity of the radioactive compounds used in this study were: L-[4,5-^{3}H]leucine (58.2 mC/mmole), D-[1-^{14}C]leucine (24.6 mC/mmole), L-[^{3}H]valine (2.52 C/mmole), D-[^{14}C]valine (18.1 mC/mmole), L-[^{3}H]lysine (3.96 C/mmole), D-[1-^{3}H]mannitol (210 mC/mmole), all from New England Nuclear, D-[6-^{14}C]lysine (3.0 mC/mmole) from Calbiochem; and L-[^{14}C]leucine (6.5 mC/mmole), iodo[2-^{14}C]acetic acid (21 mC/mmole) from Amersham/Searle.

Specific modifications of the general methodology which were considered a part of the investigative process (*e.g.* in the sulfhydryl binding experiments) will be described in RESULTS.

RESULTS

Microscopic examination of thin sections of the residual intestine indicated that

only the epithelial cells from the upper portion of the villi had been removed. The lamina propria appeared to be essentially intact. The contents of the first cell collection were discarded on the basis of a microscopic examination which revealed a relatively high content of mucous and bacteria. Fig. 1 shows the morphologic appearance of the cells on a microscope slide under a cover slip following their initial collection, washing, and dilution in the Krebs–Tris–leucine incubation medium. The cells were generally well dispersed, but aggregates of cells were also observed. These

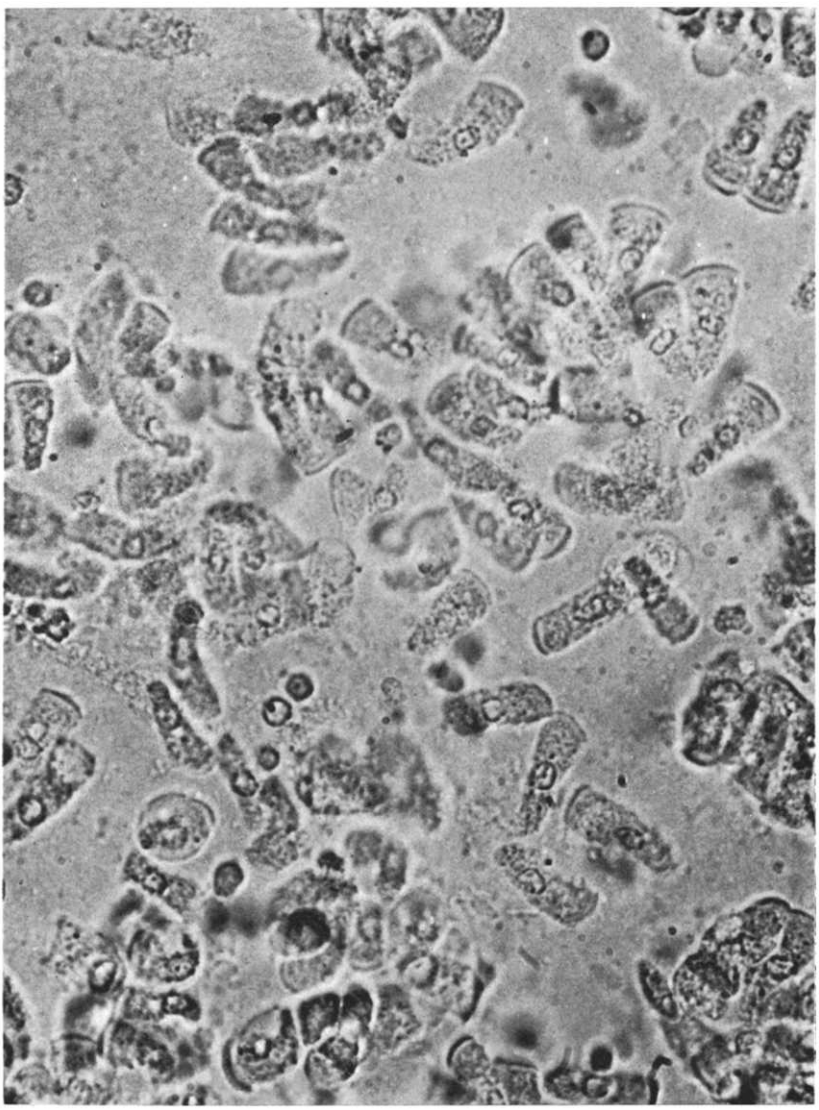

Fig. 1. Intestinal cells were isolated as described in the text and 0.3 ml were dispersed in 5 ml of a Krebs–Tris–1 mM L- and D-leucine incubation medium. An aliquot of the diluted cells was stained with trypan blue and photographed with a Polaroid camera attached to a microscope. Magnification, × 653.

aggregates represented unbroken sheets of epithelial cells, usually 3–7 in number, and cells which had reaggregated (see Fig. 1, lower right hand corner). This reaggregation was apparently the result of a mucous contamination which had become accented by the concentration of the sample under the cover slip. The reaggregated cells tended to disperse when diluted and agitated. In addition, reaggregated cells were only rarely observed in wet preparations of the diluted cells indicating that their presence during incubation was minimal. Addition of the mucolytic compound *N*-acetyl-L-cysteine did not reduce the reaggregation. Small sections of lamina propria as well as a few bacteria were also occasionally observed. The viability of the initial preparation averaged 80 %. This value compares favorably with a cell viability range of 55–90 % reported by STERN AND REILLY[20]. Cell counts of stained thin smears showed an average of 82 % epithelial cells with the rest being mainly lymphocytes. Similar epithelial cell counts have been reported in previous investigations[10,11,20]. After a 15-min incubation at 37° and washing as described in METHODS, the cell viability was reduced to 65 %. The general appearance of the cells was similar to that noted initially. The major differences were that aggregation was less common, the number of round epithelial cells had increased, and there were essentially no bacteria present.

Table I shows the uptake and the intracellular accumulation of 1 mM L- and D-leucine by the isolated epithelial cells collected by the centrifuge or filter method after 15 min incubation. Preferential uptake of the L-leucine was observed using both collection methods with the values obtained from centrifuged cells being about 4 times greater. Microscopic examination of cells eluted from the filters revealed an average viability of 20 % indicating extensive morphologic damage which probably accounts for their comparatively low level of leucine uptake. The morphologic damage is attributed to the relatively large amount of pressure required to pass the fluid through the filter due to the presence of mucous. The uptake by the cells collected by centrifugation was concentrative since the 2.51 mM concentration of L-leucine in the cell water was significantly greater ($P < 0.001$) than that initially present in the incubation medium. In the everted intestinal sac, the intracellular accumulation of 1 mM L-leucine was found to be 2.80 mM after 15 min. An approximation of the intracellular accumulation was also obtained from estimates of the extracellular space

TABLE I

PREFERENTIAL UPTAKE AND INTRACELLULAR ACCUMULATION OF 1 mM L-LEUCINE AS COMPARED TO 1 mM D-LEUCINE BY ISOLATED INTESTINAL EPITHELIAL CELLS AFTER 15 min INCUBATION

Isolated intestinal epithelial cells were incubated for 15 min at 37° in a Krebs–Tris medium containing 1 mM L- and D-leucine. The cells were collected by either centrifugation or on a Millipore filter. Each value represents the mean ± S.E.

Cell collection method	*Protein (mg)*	*1 mM L-leucine*		*1 mM D-leucine*	
		Uptake (nmoles/mg protein)	*Intracellular accumulation (mM)*	*Uptake (nmoles/mg protein)*	*Intracellular accumulation (mM)*
Centrifuge ($n = 133$)	5.25 ± 0.19	18.37 ± 0.60	2.508 ± 0.082	0.63 ± 0.28	0.905 ± 0.036
Filter ($n = 21$)	2.65 ± 0.30	4.41 ± 0.57	0.602 ± 0.147	1.25 ± 0.14	0.171 ± 0.038

of the cell pellet. A mean extracellular space of 81.8 ± 3.7 ($n = 32$) was found from the distribution of tritiated mannitol in the cell pellet[9,21]. STERN AND JENSEN[9] have reported an extracellular space range of from 83 to 95 % for epithelial cell pellets and CRANE AND MANDELSTAM[21] found an extracellular space of 80 % in packed villi. It was also possible to calculate the cell volume of the pellet on the basis of protein content. The protein was converted to cell wet weight and cell volume, as previously described, and the cell volume divided by the pellet volume. On the basis of 32 determinations 83.2 ± 0.7 % of the pellet was extracellular volume. Using an extracellular space of 82.5 % (the average of the values obtained by mannitol space and cell to pellet volume), the intracellular accumulation of L-leucine corresponding to 18.37 nmoles/mg protein was found to be 2.30 mM. In studies using uniformly labeled L-[^{14}C]leucine, the average intracellular accumulation was 2.27 ± 0.17 ($n = 38$) indicating that the magnitude of the L-leucine accumulation was not dependent on the isotope. The lack of a concentrative uptake of the D-leucine is in agreement with the finding that except for D-methionine, D-amino acids are not actively transported[22]. The absence of a concentrative uptake of L-leucine by the filter collection method is again attributed to the extensive morphologic damage of the cells. Unless otherwise indicated, all subsequent studies pertaining to the preferential uptake or binding of L-leucine by isolated cells or brush borders were carried out by the centrifuge collection method.

Paper chromatography of aliquots of the final cell-free incubation medium as well as of supernatants from cell homogenized with 5 % trichloroacetic acid indicated that at least 90 % of the counts was present in the ninhydrin spot corresponding to the R_F of leucine.

The preferential uptake of leucine was found to be directly proportional to the amount of cell protein in the incubation medium over the range 0.67–15.96 mg. This proportionality was ascertained by determining the correlation coefficients between differing amounts of protein and preferential L-leucine uptake in 9 experiments. The average correlation coefficient was 0.915 ± 0.024.

In order to determine whether preferential uptake occurred with other L-amino acids, 1 mM L- and D-valine or lysine was incubated with the cells and uptake measured as described for leucine. On the basis of 12 determinations each, 14.82 ± 0.60 and 14.16 ± 1.14 nmoles/mg protein of L-valine and L-lysine were taken up, respectively.

The properties of the preferential uptake of L-leucine by the cells were next investigated in order to determine whether these properties correspond to those of L-leucine transport by intact intestine. Table II records the effect of 25 mM of the various amino acids and sugars on the preferential uptake of 1 mM L-leucine. The greatest inhibition was observed with the L-isomers of amino acids, such as valine, isoleucine, methionine, phenylalanine, and tryptophane, which are generally accepted as being transported by the neutral amino acid pathway. No additional change in the morphology of the cells was apparent following incubation with 25 mM L-valine. The absence of inhibition with D-valine or D-isoleucine indicates that the uptake system is isomerically specific. There was no decrease in the passive uptake of D-leucine in the presence of the D-valine or D-isoleucine. The smaller inhibition by glycine and proline is probably due to their partial transport by the neutral amino acid pathway as well as by the imino acid–glycine pathway[23]. The basic amino acids

TABLE II

EFFECT OF VARIOUS AMINO ACIDS AND SUGARS ON THE PREFERENTIAL UPTAKE OF 1 mM L-LEUCINE BY INTESTINAL EPITHELIAL CELLS AFTER A 15-min INCUBATION

Isolated intestinal cells were incubated for 15 min at 37° in a Krebs–Tris medium containing 1 mM L- and D-leucine and 25 mM of the various amino acids and sugars indicated. The percent of control values were obtained by determining preferential L-leucine uptake in the presence of the amino acids or sugars and dividing each value by the uptake of L-leucine in the absence of the amino acid or sugar and are expressed as the mean ± S.E. A paired-difference *t* test was used to obtain the probability levels. The numbers in parentheses represent the number of determinations.

Addition to medium (25 mM)	% *Control*	*P*
L-Valine (10)	30.6 ± 3.4	<0.001
D-Valine (10)	97.7 ± 8.3	
L-Isoleucine (8)	36.4 ± 6.0	<0.001
D-Isoleucine (10)	90.7 ± 6.5	
L-Methionine (9)	34.7 ± 2.9	<0.001
L-Phenylalanine (6)	37.2 ± 3.2	<0.001
L-Tryptophane (6)	27.9 ± 5.1	<0.001
Glycine (6)	53.4 ± 5.9	<0.01
L-Proline (6)	53.7 ± 6.1	<0.01
L-Lysine HCl (10)	65.0 ± 6.9	<0.01
L-Arginine HCl (6)	53.8 ± 3.7	<0.01
D-Glucose (12)	80.7 ± 5.4	<0.05
D-Galactose (11)	49.2 ± 4.9	<0.001
α-Methyl-D-glucose (6)	65.3 ± 5.7	<0.01
D-Fructose (12)	141.5 ± 10.6	<0.01

TABLE III

EFFECT OF METABOLIC INHIBITORS AND CONDITIONS WHICH DAMAGE CELL MORPHOLOGY ON THE PREFERENTIAL UPTAKE OF 1 mM L-LEUCINE BY INTESTINAL EPITHELIAL CELLS AFTER 15 min

Unless otherwise indicated, isolated intestinal cells were incubated for 15 min at 37° in a Krebs–Tris medium containing 1 mM L- and D-leucine and the inhibitors at the indicated concentrations. Boiling, freezing and thawing, or osmotic shock of the cells was done prior to leucine uptake. Preferential L-leucine uptake by the boiled cells and the frozen and thawed cells was compared to the leucine uptake of cells kept in the cold for 2 and 10 min, respectively, prior to incubation. The L-leucine uptake by the osmotically shocked cells was compared to the uptake of cells pre-incubated for 10 min in 10 vol. of cold Krebs–Tris. The methods used in obtaining the results shown on this table are the same as for Table II.

Inhibitor	% *Control*	*P*
Anaerobiosis (7) (N_2 gassed)	62.7 ± 6.0	<0.001
$4 \cdot 10^{-4}$ M dinitrophenol (7)	23.3 ± 5.1	<0.001
$2 \cdot 10^{-3}$ M cyanide (10)	44.8 ± 6.1	<0.001
$4 \cdot 10^{-4}$ M *N*-ethylmaleimide (7)	36.2 ± 5.5	<0.001
$5 \cdot 10^{-3}$ M iodoacetate (12)	49.8 ± 4.9	<0.001
$5 \cdot 10^{-4}$ M ouabain (6)	15.1 ± 3.0	<0.001
$5 \cdot 10^{-4}$ M deoxypyridoxine (6)	108.9 ± 9.5	
4° (6)	7.9 ± 0.8	<0.001
Conditions for cell damage		
Boiled for 2 min (10)	6.4 ± 1.7	<0.001
Frozen and thawed × 3 (10)	13.4 ± 3.0	<0.001
10 min osmotic shock in 10 vol. 5 mM EDTA at 4° (16)	13.9 ± 2.5	<0.001

also inhibited leucine uptake. The inhibition of leucine transport by lysine has been reported in rabbit ileum[24] and has been observed in intact rat intestine in our laboratory. The pattern of sugar inhibition of leucine uptake was analogous to that noted for valine, lysine, and glycine transport in intact intestine[16]. The poorly or non-metabolized, actively transported sugars typified by galactose and α-methyl-D-glucose were the most inhibitory. Glucose, which is transported by the same pathway as galactose, produced an inhibition of leucine uptake that was significantly less than that of galactose. Fructose activated leucine uptake 41.5 % indicating that this rapidly metabolized sugar contributes to an energy supply which has become rate limiting. Fructose failed to overcome the inhibition of preferential L-leucine uptake by 10 mM L-valine indicating that energy limitations were not responsible for the valine inhibition.

Inhibitors of energy metabolism produced large decreases in the preferential uptake of L-leucine by the cells (Table III) supporting the contention that uptake is an active process (Table I). Sulfhydryl inhibitors, such as *N*-ethylmaleimide and iodoacetate, also inhibited uptake. Valine transport in intact intestine has previously been found to be inhibited 75–80 % by sulfhydryl inhibitors[25]. Ouabain produced a very large inhibition indicating that leucine uptake is dependent on cation transport. The lack of inhibition with deoxypyridoxine shows that transamination and decarboxylation reactions are not involved in leucine uptake and provides further evidence against vitamin B_6 action as an amino acid carrier[26]. Leucine uptake exhibited a marked temperature dependence with values 8 % of control levels being obtained at 4°. Boiling the cells for 2 min prior to incubation virtually abolished leucine uptake. Other procedures which had a less drastic effect on cell morphology, such as freezing and thawing 3 times and a form of osmotic shock (preincubation of the cells for 10 min in cold 5 mM EDTA, pH 7.4), reduced the subsequent uptake of L-leucine to about 14 % of the leucine uptake by comparably treated control cells (see legend, Table III). Subsequent studies have shown that osmotic shock from cold 5 mM Tris–HCl (pH 7.4) for times as short as 1 min resulted in essentially the same loss of leucine uptake capacity.

Since active amino acid transport in intact intestine requires the presence of Na^+ in the incubation medium, it was of importance to determine whether preferential L-leucine uptake by the cells was also Na^+-dependent. In these experiments the cells were washed with either 10 vol. of 4° isotonic choline chloride or isotonic KCl prior to incubation. The ability of these cells to preferentially take up L-leucine when incubated in the Krebs–Tris medium was not significantly different from the uptake of L-leucine by cells prepared by the usual procedure. The Na^+ concentration in the cell water of the choline chloride and KCl washed cells was 74.2 ± 5.9 mM ($n = 6$) and 81.6 ± 4.9 mM ($n = 6$), respectively. The effect of the Na^+ concentration of the incubation medium over the range 0–118 mM on preferential L-leucine uptake by the choline chloride or KCl washed cells was investigated using either choline chloride or KCl as isotonic replacements for NaCl (Fig. 2). Replacement of the NaCl by choline chloride (Na^+ concentration of medium after incubation 3.7 mM) and KCl (Na^+ concentration of medium after incubation 2.4 mM) resulted in a decrease of leucine uptake of 64 % and 75 %, respectively. The final concentration of L-leucine in the cell water was not greater than the L-leucine concentration in the Na^+-free media. In agreement with the findings in intact tissue[27], 59 mM Na^+ maintained

optimum amino acid uptake when choline was the replacement cation, but produced significant inhibition when K^+ was the replacement cation.

Because ouabain (Table III) produced a much larger inhibition of leucine uptake than could be accounted for by an effect on Na^+ transport, the results of the isotonic replacement of the other cations of the incubation medium by NaCl were next investigated (Table IV). The replacement of K^+ resulted in a decrease of leucine uptake of at least as great a magnitude as that produced by the replacement of Na^+. The replacement of Ca^{2+} and/or Mg^{2+} resulted in much smaller decreases in uptake.

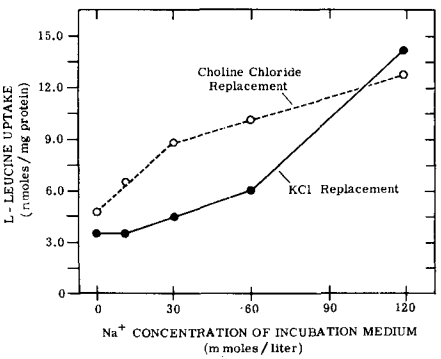

Fig. 2. Effect of the Na^+ concentration of the incubation medium on the preferential uptake of L-leucine. The desired Na^+ concentration was attained by isotonic substitution of the NaCl of the Krebs–Tris medium with either choline chloride or KCl. Each point represents the mean of 6 determinations.

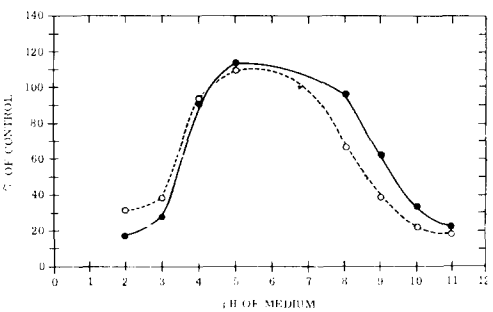

Fig. 3. Effect of the pH of the Krebs–Tris incubation medium (●—●) and of the Krebs–Tris incubation medium with NaCl replacing all the Ca^{2+} and Mg^{2+} (○---○) on the preferential uptake of L-leucine. Each point represents the mean of at least 6 determinations.

Fig. 3 shows the effect of varying the pH of the incubation medium over the range 2–11 on the preferential uptake of L-leucine. The solid line represents the uptake of leucine in the Krebs–Tris medium. A broad pH optimum range was observed between 4 and 8 with an apparent maximum between 5 and 7.4. However, at pH values above 8 the Ca^{2+} and Mg^{2+} precipitated from the medium and uptake values at these pH's were corrected for the percent decrease that resulted from the absence of Ca^{2+} and Mg^{2+} from the incubation medium (Table IV). In addition, the effect of pH on leucine uptake was determined in a Krebs–Tris medium in which the Ca^{2+} and Mg^{2+} had been replaced by NaCl (Fig. 3, dashed line). The pH optimum using this medium showed a marked shift away from the alkaline side with a pH value as low as 8 now producing a significant decrease of leucine uptake.

The preferential uptake of 1 mM L-leucine in cells that had been washed 6 times with ice cold Krebs–Tris was not significantly different from the uptake of the cells after 3 washings ($n = 9$). This finding indicates that there is very little release of leucine by the cells at 4°. However, when considered together with the peak in leucine uptake shown around pH 5, this resistance of intracellular leucine to washout raised the possibility that leucine uptake represents, at least in part, an incorporation into intracellular protein. In order to investigate this possibility, cells from 8 individual experiments were homogenized with 2 vol. of cold 5 % trichloroacetic acid following 1 mM L-leucine uptake. The mixture was centrifuged in the cold, the supernatant

collected, and the residue washed once with the trichloroacetic acid. All the counts originally present in the cells were recoverable in the supernatant indicating that leucine was not incorporated into protein. In addition, the effect on leucine uptake produced by compounds influencing protein biosynthesis was also investigated (Table V). Neither puromycin, chloramphenicol, or tetracycline, at concentrations reported to be inhibitory to protein biosynthesis, lowered the uptake of leucine by the cells. ATP, which was added to stimulate amino acid activation, produced an inhibition of leucine uptake. Finally, when the cells from 14 experiments having an average preferential uptake of 10.12 nmoles L-leucine/mg protein were incubated for 5 min at 37° in either a Krebs–Tris medium or a Krebs–Tris medium containing 5 mM cold L-leucine, the leucine content of the cells was decreased 78 % and 89 %, respectively. This rapid loss of intracellular leucine further indicates that leucine is not incorporated into protein but rather is in a free form as predicted by the reversibility of leucine uptake by the cells (see Fig. 5 for apparent K_m value).

TABLE IV

EFFECT OF CHANGES IN THE IONIC COMPOSITION OF THE INCUBATION MEDIUM ON THE PREFERENTIAL UPTAKE OF 1 mM L-LEUCINE BY INTESTINAL EPITHELIAL CELLS AFTER 15 min

The isolated cells were incubated for 15 min at 37° in a Krebs–Tris–1 mM L- and D-leucine medium modified by isotonic replacement of K^+, Mg^{2+} and/or Ca^{2+} by NaCl. Each value represents the mean ± S.E. from at least 7 determinations. % of control and probability values were obtained as described in Table II.

Medium	*L-Leucine uptake (nmoles/mg protein)*	*P*	*% Control*
Control	8.76 ± 0.75		
No K^+	2.06 ± 0.57	<0.001	23.5
No Ca^{2+}	6.88 ± 0.68	<0.05	78.5
No Mg^{2+}	5.98 ± 0.59	<0.05	68.3
No K^+, Ca^{2+}	2.51 ± 0.53	<0.001	28.7
No K^+, Mg^{2+}	2.77 ± 0.40	<0.001	31.6
No Ca^{2+}, Mg^{2+}	5.01 ± 0.56	<0.01	57.2
No K^+, Ca^{2+}, Mg^{2+}	1.32 ± 0.15	<0.001	15.1

TABLE V

EFFECT OF COMPOUNDS INFLUENCING PROTEIN BIOSYNTHESIS ON THE PREFERENTIAL UPTAKE OF 1 mM L-LEUCINE BY INTESTINAL EPITHELIAL CELLS AFTER 15 min

The isolated cells were incubated for 15 min at 37° in a Krebs–Tris–1 mM L- and D-leucine medium containing the inhibitors of protein biosynthesis or ATP at the indicated final concentration. Each value is the mean of 6 determinations ± S.E. % of control and probability values were obtained as described in Table II.

	L-Leucine uptake (nmoles/mg protein)	*% Control*
None	9.58 ± 0.79	
$2 \cdot 10^{-4}$ M puromycin	9.53 ± 0.85	99.6
$2 \cdot 10^{-5}$ M chloramphenicol	9.06 ± 0.32	94.6
$2 \cdot 10^{-5}$ M tetracycline	10.04 ± 1.02	104.8
$2 \cdot 10^{-3}$ M ATP	5.68 ± 0.24	59.3
	P <0.001	

Fig. 4 shows the effect of time on the preferential uptake of 1 mM L-leucine. Uptake was a linear function of time through the first 5 min of incubation (correlation coefficient of 0.985), reached a maximum at about 15 min, and declined at 30 min.

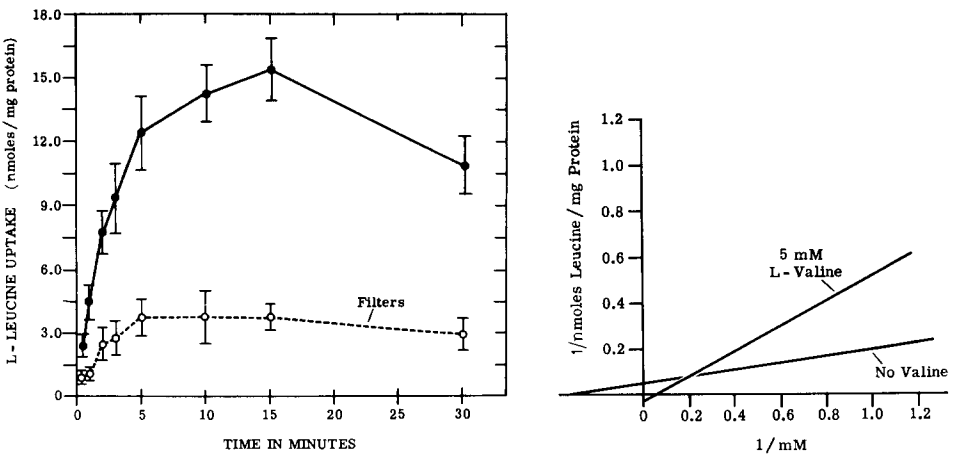

Fig. 4. Effect of time on the preferential uptake of L-leucine by isolated intestinal epithelial cells. Each point represents the mean of at least 12 determinations.

Fig. 5. Lineweaver–Burk plot of the data shown in Table VI. Equations of these regression lines with the 95% confidence limits of their intercepts are as follows: leucine uptake in the absence of valine-equation to line, $y = 0.0313 + 0.0996x$; intercept on y axis $(1/v_{max}) = 0.0313 \pm 0.0161$; intercept on x axis $(-1/K_M) = -0.3140 \pm 0.1507$. Leucine uptake with 5 mM L-valine-equation to line, $y = -0.0192 + 0.3680x$; intercept on y axis $= -0.0192 \pm 0.0688$; intercept on x axis $= 0.0523 \pm 0.1327$.

Since the filter method of cell collection permitted a much more precise control of incubation time, a similar time study was carried out using the filters. The results, though based on uptakes of much lower magnitude, again showed that initial velocity was maintained through the first 5 min of incubation (correlation coefficient of 0.965), that activity was optimum after 15 min, and that activity declined after 30 min. HUANG[11] has previously reported that L-tyrosine uptake by isolated epithelial cells was at initial velocity through the first 10 min of incubation.

Table VI shows the effect of the concentration of L- and D-leucine over the range 0.5–10 mM on the preferential uptake of L-leucine after 5 min in the presence and absence of 5 mM L-valine. As the substrate concentration increased, the efficiency of the uptake decreased, and at 10 mM the average intracellular accumulation of L-leucine was 10.31 mM, a value not significantly different from the concentration in the incubation medium. Similar decreases in the efficiency of amino acid accumulation as a result of increases in the medium concentration have been observed with intact intestine[15,16]. As the leucine concentration increased, the magnitude of the inhibition produced by the valine decreased indicating a competitive inhibition. Fig. 5 shows the data of Table VI plotted according to the method of Lineweaver and Burk. The equation of the line representing L-leucine uptake in the absence of valine was $y = 0.0313 \pm 0.0996x$. The intercept on the y axis, $1/v_{max}$, was 0.03128 with 95% confidence limits of $\pm$ 0.01612. The intercept on the x axis, $-1/K_m$, was -0.3140 with 95% confidence limits of $\pm$ 0.1507. The apparent K_m for preferential L-leucine

TABLE VI

EFFECT OF LEUCINE CONCENTRATION ON THE PREFERENTIAL UPTAKE OF LEUCINE BY ISOLATED INTESTINAL EPITHELIAL CELLS IN THE PRESENCE AND ABSENCE OF 5 mM L-VALINE AT 5 min

The cells were incubated 5 min at 37° in a Krebs–Tris medium containing L- and D-leucine at the initial concentrations indicated in the presence and absence of 5 mM L-valine. Each value represents the mean of 18 determinations ± S.E. % inhibition was obtained by subtracting the % of control from 100%. % of control and probability values were obtained as described in Table II.

Leucine concn. (mM)	*L-Leucine uptake (nmoles/mg protein)*		*P*	*% Inhibition*
	5 mM L-valine	*No valine*		
0.5	1.77 ± 0.22	5.14 ± 0.62	<0.001	65.6
1.0	4.35 ± 0.43	9.27 ± 1.08	<0.01	53.1
5.0	18.24 ± 1.90	23.77 ± 2.44	<0.05	23.3
10.0	24.28 ± 2.33	28.96 ± 2.72		16.2

TABLE VII

EFFECT OF 10 mM L-VALINE ON THE PREFERENTIAL BINDING OF 1 mM L-LEUCINE BY INTESTINAL BRUSH BORDERS AFTER 15 min

Brush borders were prepared as described in the methodology and incubated for 15 min at 37° in a Krebs–Tris medium containing 1 mM L- and D-leucine and, where indicated, 10 mM L-valine. Each value represents the mean of 20 determinations ± S.E.

Intestinal preparation	*Leucine binding (nmoles/mg protein)*		*Preferential L-leucine binding (nmoles/mg protein)*
	L-Leucine	*D-Leucine*	
Brush borders	8.07 ± 2.21	1.99 ± 0.49	6.12 ± 2.05
Brush borders + 10 mM L-valine	9.66 ± 2.22	2.89 ± 0.59	6.77 ± 2.07

uptake of 3.2 mM is in excellent agreement with an apparent K_m of 2.8 mM for leucine accumulation in intact intestine[16]. The equation of the line and the x and y intercepts ± the 95% confidence limits for leucine uptake in the presence of 5 mM L-valine were $y = -0.0192 + 0.3680x$, $y = -0.0192 \pm 0.0688$ and $x = 0.0523 \pm 0.1327$, respectively. These lines with overlapping y intercepts and different x intercepts are characteristic of competitive inhibition.

On the basis of 33 determinations, purified preparations of intestinal brush borders were able to preferentially bind 5.72 ± 1.68 nmoles/mg protein of L-leucine. The results with the brush borders were quite variable with binding values ranging between 0 and 37.23 nmoles/mg protein. In addition, brush border binding of L-leucine was not inhibited by the presence of 10 mM L-valine (Table VII) indicating that the properties of leucine binding to the brush borders were different from those of leucine uptake by the cells.

Using the isolated epithelial cell as a source of the transport proteins which are believed to participate in membrane transport[5], experiments have been initiated with the aim of isolating and characterizing a leucine binding protein[3]. The method used is basically the same as that employed in the laboratory of KENNEDY and co-workers[28,29] for the study of the β-galactoside transporting protein of *Escherichia coli*.

TABLE VIII

IODOACETATE BINDING TO ISOLATED INTESTINAL EPITHELIAL CELLS AFTER PREINCUBATION WITH AND WITHOUT 50 mM L-LEUCINE FOLLOWED BY INCUBATION WITH 5 mM IODOACETATE

Isolated intestinal cells were preincubated 15 min at 37° in one of the indicated preincubation media. Iodoacetate was then added to each media to give a concentration of 5 mM and the incubation continued for an additional 5 min. The cells were then collected by centrifugation, washed twice with saline and incubated at 37° for 5 min in a Krebs–Tris medium containing radioactive and nonradioactive 5 mM iodoacetate. The reaction was stopped by addition of 2-mercaptoethanol and the cells washed twice in saline. Aliquots of the cells were then analyzed for radioactivity and protein.

Preincubation medium	*Iodoacetate incorporation (nmoles/mg protein)*
Krebs–Tris (6)	21.16 ± 3.21
	$P < 0.01$
Krebs–Tris + 50 mM L-leucine (6)	42.42 ± 6.36
Krebs–Tris + 50 mM D-leucine (4)	20.43 ± 3.55

The cells were first preincubated for 15 min at 37° in a Krebs–Tris medium containing 50 mM L-leucine in order to saturate the leucine uptake sites. Cells incubated in Krebs–Tris or in Krebs–Tris containing 50 mM D-leucine served as controls. Iodoacetate was then added to the medium to a final concentration of 5 mM in order to irreversibly combine with available sulfhydryl groups and the incubation allowed to proceed 5 min longer. The cells were then centrifuged and washed twice with saline to remove the leucine and excess iodoacetate, and dispersed in a Krebs–Tris medium containing 5 mM radioactive and nonradioactive iodoacetate. After a 5-min incubation at 37°, the iodoacetate was neutralized with 2-mercaptoethanol, and the cells collected and washed twice with saline. Table VIII shows that the cells protected with L-leucine had a significantly greater incorporation of iodoacetate than did cells incubated in Krebs–Tris with or without D-leucine. In one experiment a portion of the cells was homogenized in cold 5 mM EDTA and centrifuged in the cold for 120 min at 136000 × *g*. Analysis of the residue revealed that the amount of iodoacetate (nmoles/mg protein) was the same after preincubation with either Krebs–Tris alone or Krebs–Tris containing 50 mM L- or D-leucine. However, the iodoacetate in the supernatant of the cells protected with L-leucine was twice as great as in the cells preincubated in Krebs–Tris or Krebs–Tris with D-leucine. 90% of the iodoacetate present in the supernatant of the cells protected with L-leucine was precipitated along with added albumin (5 mg/ml) by 5% trichloroacetic acid.

DISCUSSION

The use of isolated epithelial cells in studying the properties of intestinal transport has many obvious advantages[11]. Intact intestine is comprised mainly of nonabsorptive tissue which can contribute to the properties of *in vitro* transport and which presents a nonphysiological diffusion barrier to the transport of absorbed substances. Previous attempts to obtain an intestinal preparation composed primarily of absorptive units have involved stripping the mucosa from the underlying tissue with a glass slide[30]. These mucosal strips have been shown to consist of the epithelial lining

and the underlying lamina propria[31]. The major advantages of the single cells over mucosal scrapings in studying transport are as follows: (a) there are only two transport compartments, cells and medium; (b) there is a greater proportion of absorptive to nonabsorptive material; (c) there is less variability in the composition of the preparation thus permitting a better quantitation of transport; and (d) the lamina propria constitutes enough of a diffusion barrier so that it may serve as a secondary accumulating compartment[32]. In studies aimed at isolation of discrete binding sites or individual transport proteins, the advantages of starting with single cells as compared to mucosal strips are even more apparent. The relative stability of the cells to incubation conditions is another factor in favor of their use for transport studies. It has been reported that as many as 60 % of the cells incubated for 60 min at 30° retain their viability[20]. In view of the very large activation of leucine uptake produced by fructose, the viability of the cells may be maintained or even improved by the addition of an energy producing metabolite to the incubation medium.

Previous reports on the uptake of sugars or amino acids by isolated epithelial cells have been based on determinations of the extracellular space of the residual cell pellets[9,11]. In our studies variations of considerable magnitude were found in the extracellular space of cell pellets treated in the same fashion. Using our data as an example, an error of 5 % in the extracellular space could result in a 33 % error in the magnitude of the intracellular accumulation. Since the precision of the protein analysis was greater than 97 %, we believe that a more reliable estimate of the intracellular accumulation can be obtained on the basis of cell protein.

In order to determine whether isolated epithelial cells could serve as a purified membrane preparation with which to study the properties of intestinal transport, it was first necessary to ascertain whether the cells still contained the entire system required for physiological amino acid transport. To do this, we compared the properties of leucine uptake by the cell to the transport properties of leucine and similarly transported neutral amino acids in intact intestine. On the basis of energy dependence, inhibition by sulfhydryl binders, sodium dependence, general pH optimum, effect of substrate, apparent K_m, and the selective inhibitions produced by sugars and other amino acids, the properties of leucine uptake by the cells were generally the same as those of leucine transport in intact intestine. It has recently been reported that L-leucine transport in intact rat jejunum is significantly greater at pH 6.3 than at pH 7.3 (ref. 33). The general peak of L-leucine uptake observed between pH 5 and 7.4 may be a reflection of the contribution of epithelial cells from the jejunum to the overall activity. The neutral amino acids utilizing the imino acid–glycine preferring pathway exhibited a greater increase in transport at the lowered pH than did neutral amino acids utilizing the methionine preferring pathway[33,34]. Since leucine has been reported to have affinities for both of these pathways[23], the shift of the pH optimum of leucine uptake to the acid side in the absence of Ca^{2+} and Mg^{2+} may represent a specific requirement of these cations for the methionine preferring pathway. The lack of total inhibition of L-leucine uptake by neutral amino acids, such as L-valine and L-methionine which apparently have little affinity for the glycine pathway[35], can also be explained by a partial uptake of leucine by the glycine pathway. The one major area of divergence was in the action of K^+, Ca^{2+}, and Mg^{2+}, all of which were required for the optimal uptake of leucine by the cells but were not required for optimal transport of valine in intact intestine[27]. One of the initial consequences of

a limited energy supply in intestinal tissue is a decrease in intracellular K^+ (ref. 36). Direct measurements of the K^+ or Na^+ concentration in the cell water is difficult because of the small volume of cell water and the presence of these cations in the media used in obtaining a cell pellet which is 82.5 % extracellular volume. However, analysis of cells washed an additional time with 4° isotonic choline chloride resulted in a K^+ concentration in the cell water of 34.1 ± 3.7 mM ($n = 6$) which is considerably lower than the K^+ concentration reported in mucosal strips from rabbit intestine[37]. It can therefore be concluded that the epithelial cells lose K^+, and probably Ca^{2+} and Mg^{2+} during incubation and these cations must be supplied in the incubation medium. In intact intestine these cations are presumed to be present intracellularly in amounts sufficient to satisfy the requirements for ATPase or other metabolic reactions involved in amino acid transport. It appears then that single cells represent a system suitable for the study of various aspects of intestinal transport and especially appropriate for those problems complicated by the presence of the serosal membrane[24].

The same principles involved in the concentration of amino acids in the epithelial cells of intact intestinal mucosa appear to be operative in the isolated cells. The distribution of the leucine in the cells is probably not uniform. Autoradiographic studies have shown that 1 mM L-valine and 1 mM L-methionine are concentrated 5–50 times in the epithelial cells, but that the concentration was much greater near the brush border than at the basal pole of the cell[38]. The concentration of leucine by the cells does not appear to be mediated by an intimate association of leucine with a macromolecular component found near the brush border since leucine can be readily washed out of the cell at 37°.

Having established that the cells contain the components of the amino acid transport system, we were then particularly interested in attempting to isolate a specific leucine binding protein from the cell membrane. On the basis of work done with bacteria such transport proteins appear to be relatively labile[3–5]. Binding studies using intestinal brush borders may be interpreted as confirming the presence of such proteins in intestinal membranes. Brush borders are prepared by disrupting the epithelial cells in 5 mM EDTA, a procedure which is more drastic than the osmotic shock treatment used to release bacterial proteins. The failure of valine to inhibit leucine binding to brush borders in the present study and the nature of the changes in the properties of glucose binding to brush borders[6] could be explained by the loss of a protein which gives the carrier its specificity and kinetic characteristics. The presence of a specific L-leucine-binding protein in the isolated cells has been suggested by the iodoacetate binding studies. The increase in iodoacetate binding due to L-leucine protection could be traced to a protein fraction which had been solubilized by homogenization of the cells in 5 mM EDTA. Assuming a relationship between the increase in iodoacetate binding and L-leucine protection, these studies indicate that a leucine-binding protein can possibly be identified on the basis of iodoacetate incorporation. It is also noteworthy that conditions used to prepare brush borders result in a release of the preferential iodoacetate binding of the cells. Since iodoacetate was found to enter the epithelial cells after a 5-min incubation, it cannot be excluded that a portion of the increase in iodoacetate binding found in L-leucine protected cells represents events occurring in the cell cytoplasm. One of the more important properties of leucine uptake by the cells was its essential irreversibility in the cold. It is hoped that the binding of leucine to lyophilized cells, various fragments of the

cell membrane, or protein solubilized from cell membrane preparation may similarly be stabilized by the cold, thus permitting identification of active uptake sites on the basis of substrate binding.

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