UNIVERSITY OF MANITOBA

NEUTRAL AMINO ACID UPTAKE IN CONCANAVALIN A-SENSITIVE AND RESISTANT CHINESE HAMSTER OVARY CELLS GROWN IN TISSUE CULTURE

by

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A Thesis Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of Master of Science.

Department of Microbiology
Winnipeg, Manitoba

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To Orest

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ABSTRACT

Neutral amino acid uptake was studied in two Chinese hamster ovary cell lines (the wild-type, and a concanavalin A-resistant variant, C^R-7). Uptake was studied by time course, sodium ion-sensitivity and inhibition studies. Results indicated the presence of at least three separate systems which operate in the uptake of neutral amino acids in both cell lines. The systems have different amino acid preferences. Two of the systems prefer amino acids with short, polar or linear side chains. The third system prefers the larger amino acids with branched or ringed side groups. No major differences were observed between the wild-type and the C^R-7 cells in relation to the characteristics of the uptake systems. Treatment with concanavalin A inhibited the uptake of certain amino acids in the wild-type but not in the C^R-7 monolayers. The inability of the lectin to affect amino acid uptake in the variant may be related to the resistance of the variant to the cytotoxic effects of concanavalin A.

ABBREVIATIONS

Ala	Alanine
Pro	Proline
Ser	Serine
Gly	Glycine
Met	Methionine
AIB	α-Aminoisobutyric acid
MeAIB	α-Methylaminoisobutyric acid
cLeu	Cycloleucine
Cys	Cysteine
Thr	Threonine
Leu	Leucine
Ile	Isoleucine
Phe	Phenylalanine
Val	Valine
Try	Tryptophan
Tyr	Tyrosine
Na^+	Sodium ion
K^+	Potassium ion
ATPase	Adenosine triphosphatase
αMEM	Alpha-minimal essential medium
FCS	Fetal Calf Serum
DPBS	Dulbecco's Phosphate Buffered Saline

Con A	Concanavalin A
C^R-7	Concanavalin A-resistant cell line
W	Weight
V	Volume

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued) Page

LIST OF FIGURES

LIST OF FIGURES (Continued) Page

Figure

LIST OF TABLES

INTRODUCTION

INTRODUCTION

The differences in the amino acid transport processes between transformed and normal cell lines may be related to the changes in membrane structure that accompany transformation (53, 55, 56). Membrane structural differences relative to amino acid uptake have been investigated with the use of the membrane probe, concanavalin A (54, 56, 57). The C^R-7 variant exhibits many altered membrane properties similar to transformed cells (75). Consequently, it was of interest to compare amino acid uptake in the C^R-7 cells with the uptake in wild-type cells. Amino acid uptake has not previously been described in the C^R-7 cells or any other concanavalin A-resistant variant. Partial characterization of the amino acid uptake systems was necessary in an attempt to uncover any differences between the wild-type and the C^R-7 cells. Further, in view of the studies performed in transformed cells, the effects of concanvalin A on amino acid uptake in both cell lines were examined. This was particularly interesting since any differential effects of concanavalin A on amino acid uptake in the C^R-7 and wild-type cells may be correlated with concanavalin A-resistance.

HISTORY

HISTORY

Neutral amino acid transport

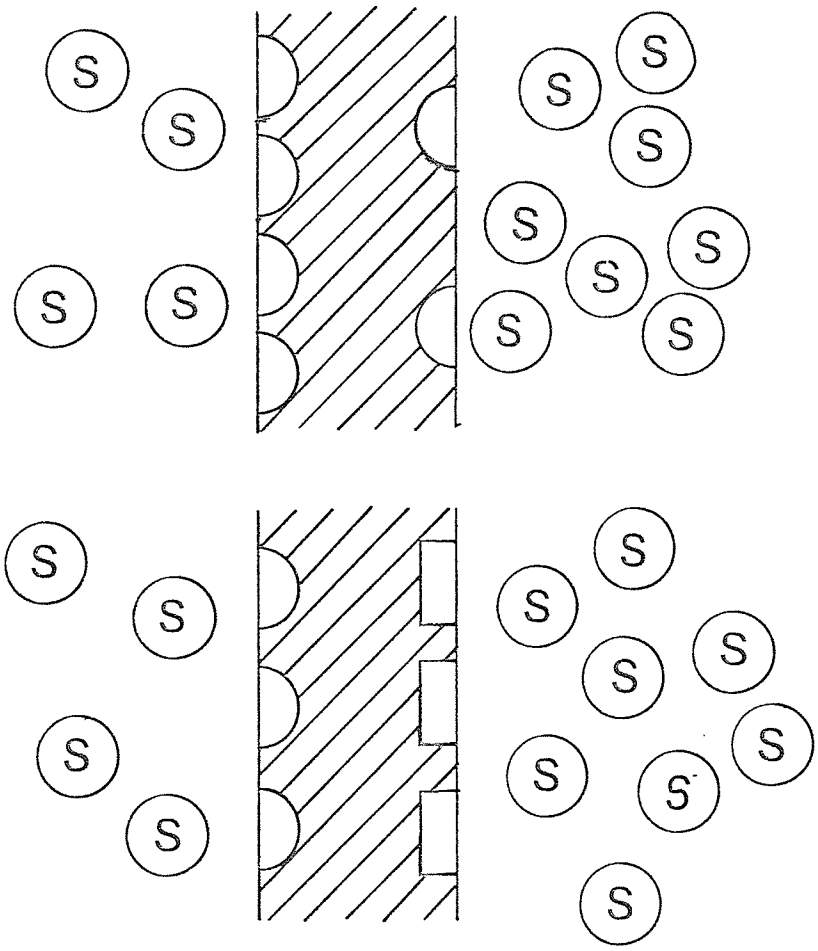
Neutral amino acid transport in animal cells has been extensively studied during the past two decades. Prior to this time, transport was primarily studied in bacterial cells and focused on the problems of ion and sugar transport (1,2). Ironically, "uphill" or "active" transport was first demonstrated using amino acids as substrates (3). In 1913, Van Slyke and Meyer observed that segments of dog intestinal tissue accumulated amino acids from the external medium against a concentration gradient (3).

The term "transport" refers to the mediated movement or transfer of a solute from one compartment to the next across a barrier. The process in animal cells usually includes both uptake and exodus across the plasma membrane. Transport basically proceeds by three steps: the solute is bound at one side of the membrane, transferred across the membrane, and finally released in an unmodified form on the other side of the membrane (1,4). The transfer of a solute which occurs against a concentration gradient at the expense of energy is referred to as "active transport". Energy may either be directly supplied by metabolic reaction (primary active transport) or indirectly derived from another transport process (secondary active transport). The latter may be of major importance to amino acid transport in animal cells. Mediated transport which proceeds without energy expenditure is usually referred to as "facilitated diffusion" (1,4).

The mode of energization of transport is unknown though several theories exist. Two extreme theories are represented by the "pure vectorial force model" (Fig. 1). The former predicts that energy will be introduced to alter the conformation of the transport carrier such that its affinity for the solute will be reduced. This results in asymmetrical distribution of solute across the membrane while equal numbers of receptor sites at each side of the membrane are maintained. Conversely, the carrier of the "pure vectorial force model" remains unaltered but is found in a higher concentration at one side of the membrane. This asymmetrical distribution of carrier results in an asymmetrical distribution of solute. The asymmetry is maintained by vectorial force. The actual mode of energization is probably not represented by either of these two extremes but may be a compromise between the two (1,5).

The amino acids, in particular those which are neutral in reaction at physiological pH are excellent substrates for the investigation of the transport phenomenon. They are important biologically and are present in solution only occasionally as lipid-soluble species. Consequently, the contribution to overall transport by non-specific transport is not a major consideration (1). The neutral amino acids also contain three chemically distinct groups including an amino group, a carboxyl group and a side chain. These groups may be experimentally manipulated in the study of receptor preferences and specifications. Finally, the group of neutral amino acids contains a large number of species which display a wide range of solute structure. This has been indispensible

Figure 1. A diagrammatic representation of the two extreme models (1). The pure vectorial force model (upper diagram) predicts an asymmetrical distribution of receptor sites across the membrane without modification to the sites. Vectorial force maintains the difference. The pure affinity change model (lower diagram) predicts that energy will be supplied to alter the affinity of the receptor sites for the substrate (S). Equal numbers of sites are found on each side of the membrane.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in the discrimination and characterization of the neutral amino acid transport systems in animal cells (1, 4, 6, 7).

The neutral amino acids are transported in many animal cells by three distinct systems. The A, ASC and L systems can be described on the basis of amino acid preference, sodium ion-dependency, sensitivity to both pH and temperature changes, and exchange properties (Table I) (4). The A and L systems were first described in Ehrlich ascites tumor cells by Oxender and Christensen in 1963 (8). The ASC system, which is similar to the A system, was finally characterized in Ehrlich ascites cells four years later (9). Prior to the discovery of the separate systems, the theory that a single agency was responsible for the transport of all neutral amino acids was widely accepted (6). This theory was largely based on competition experiments, in which amino acids present in the external medium were observed to inhibit the uptake of other amino acids. Competition between cationic and neutral or anionic and neutral amino acids was not observed (10,11). The possibility that transport may be mediated by more than one agency was realized following the observation that glycine uptake in the intestine could not be saturated by increasing amounts of glycine (12). Christensen (13) suggested that the entry and the exodus of neutral amino acids may proceed by different routes. Differences observed in the degree of competititon between amino acids for uptake in the Ehrlich ascites cells compounded the problem (14,15). The amino acids DL-leucine, DL-valine, and DL-phenylalanine, were observed to inhibit the uptake of L-methionine but had no marked effect on glycine uptake

TABLE I

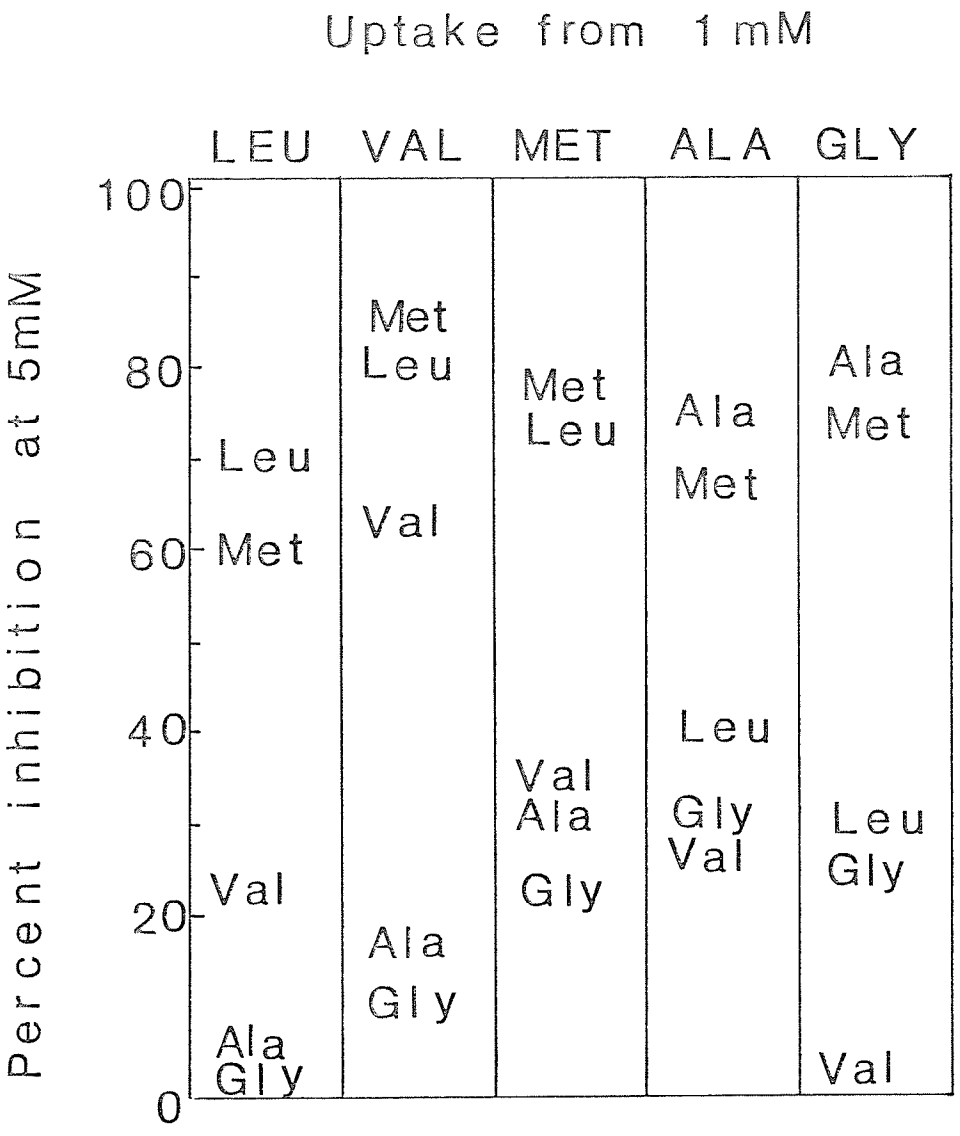
Some properties of the neutral amino acid transport systems found in many animal cells.

System[1]	Some preferred substrates	Na^+-dependence	Sensitivity to pH change	Sensitivity to temperature change	Exchange properties
A	Ala, pro, ser, gly, met, alB, meAlB, cLeu	Yes	Yes	Yes	weak
ASC	Ala, ser, cys, thr	Yes	No	N.D.[2]	variable
L	Leu, ile, phe, met, val, try, tyr, cLeu	No	No	No	strong

[1]The systems are those described by Christensen (1,6,36) for Ehrlich ascites tumor cells but similar systems have been described in many animal tissues and cells. Examples include human fibroblasts (38), human glia and glioma cells (39), hepatocytes (40,41), hepatoma cells (42), diaphragm (43), uterus (44), S37 ascites tumor cells (45), Balb/3T3 cells (22), placenta (46), mouse lymphocytic cells (33), chicken heart cells (35), chick embryo fibroblasts (47,48,49).
[2]Not determined.

(14). Similarly, glycine uptake was found to be inhibited by L-serine, but L-serine and L-leucine did not appreciably compete for uptake (15). These observations were extended to rat intestinal and diaphragm tissue, in which amino acid analogues, α-aminoisobutyric acid and 1-amino-cyclopentane-1-carboxylic acid behaved like glycine and L-valine, respectively (16,17). The detailed experiments of Oxender and Christensen (8) finally resolved the issue. Time course profiles of the uptake of several amino acids in Ehrlich ascites cells suggested the existence of two systems. Amino acids such as glycine, and the amino acid analogue, α-aminoisobutyric acid, reached much higher steady-state levels than were predicted on the basis of their low initial rates of entry. Conversely, the larger amino acids, like leucine and valine, rapidly entered the cell but reached comparatively lower levels of accumulation. This suggested that the rates of exodus of the two "groups" of amino acids were different, with the latter amino acids exciting the cell at rates comparable to their rates of uptake. The simultaneous operation of the two processes would only be possible if two routes of transport existed. Results obtained from competition experiments also suggested the existence of two distinct systems (8). Five amino acids were tested for their ability to inhibit the uptake of one another (Fig. 2). All of the amino acids could inhibit the uptake of one another but not to the same extent. Leucine was observed to greatly inhibit the uptake of valine and methionine, but the uptake of alanine and glycine was not greatly inhibited by leucine. Conversely, alanine effectively competed with glycine for uptake but did not

Figure 2. Inhibition of amino acid uptake in Ehrlich ascites cells by amino acids present in the external medium (8). The uptake of five radiolabelled amino acids (listed at the top of the figure) present at concentrations of 1 mM was studied in the presence of 5 mM non-labelled amino acids (listed in the body of the figure). Uptake proceeded for one minute. Reading across the figure, the trends exhibited for the inhibition by leucine and alanine are shown.


Uptake from 1 mM
LEU
VAL
MET
ALA
GLY
Percent inhibition at 5mM
100
80
60
40
20
0
Leu
Met
Val
Ala
Gly
Met
Leu
Val
Ala
Gly
Met
Leu
Val
Ala
Gly
Ala
Met
Leu
Gly
Val
Ala
Met
Leu
Gly
Val

appreciably inhibit the uptake of either leucine or valine in these cells. The results could not be interpreted to mean that a single agency of transport was eliciting this trend and Oxender and Christensen (8) tentatively identified two systems. These were described as the "alanine-preferring" or A system and the "leucine-preferring" or L system.

The amino acid preferences of the two systems have since been investigated in a number of cell lines (4,5,6). The A system prefers amino acids which contain short, polar or linear side chains (such as alanine and serine). The bulkier amino acids which have branched or ringed side chains (such as leucine or phenylalanine) are the preferred substrates of system L. All of the neutral amino acids show affinity for each of the systems but in general the affinities can be predicted on the basis of these broad structural characteristics. Methionine appears to have a high affinity for both systems A and L. This amino acid may exhibit structural features common to both system A and L requirements (6,7,8).

The ASC system is very similar to the A system in amino acid preference. Amino acids such as alanine, serine, cysteine, and threonine are the preferred substrates (4,5,6). Consequently, the identification of this system was difficult. The existence of the ASC system was first indicated from the results of competition experiments which were designed to minimize the activities of both the A and L systems (18). Ehrlich ascites tumor cells were incubated in excessive amounts of both α-methylaminoisobutyric acid and L-phenylalanine while the uptake of

L-alanine was monitored. A small but significant uptake of L-alanine was still observed which could not be attributed to either the A or L system. Further study led to the characterization of the ASC system which is highly intolerant of N-methylated amino acids; displaying a higher stereospecificity than the A system (9). The intolerance of N-methylated amino acids by the ASC system is a key factor which is often used to differentiate between the A and ASC systems. Consequently, the amino acid analogue, α-methylaminoisobutyric acid is considered a characteristic substrate of system A (1,4,6).

A second characteristic which is usually used to separate systems A and ASC from system L is sodium ion-sensitivity (1,4,19-22). In many animal cells, the amino acid substrates of both the A and ASC systems display a distinct dependence on the presence of sodium ion in the external medium during uptake. Reduction of sodium concentration correspondingly reduces uptake. Conversely, the uptake of L system preferred substrates is relatively insensitive to the presence of sodium ion. Sodium ion is believed to be a cosubstrate which is bound to the carrier along with the amino acid and translocated across the membrane. The energy required for the accumulation of amino acids may be derived from the coupling to the sodium ion flux down its electrochemical gradient (19,20,23-27). This forms the basis of the "gradient-hypothesis" which was first proposed by Crane in the early 1960s as a mechanism for the intestinal transport of sugars (28). The possible importance of the sodium gradient in energizing amino acid uptake was actually implied earlier by Riggs *et al.* (29) though not emphasized.

They suggested that the influx of sodium ion may in some way stimulate the uptake of glycine in Ehrlich cells only as an alternative possibility to the observed efflux of potassium ion which accompanied glycine uptake. The association between the downhill movement of potassium ion and amino acid uptake was widely known at this time, but the role of sodium ion was at best vague (13,30,31). Kromphardt *et al.* (32) finally demonstrated the importance of the extracellular sodium ion concentration to glycine uptake in Ehrlich ascites tumor cells. These results were confirmed by Inui and Christensen (21) with the observation that both K_m and V_{max} values of methionine, α-aminoisobutyric acid, and α-methylamino-isobutyric acid uptake in Ehrlich cells changed as the extracellular concentration of sodium was changed. They also noted that a small part of the uptake of these three amino acids was sodium ion-insensitive. Recent findings in a mouse lymphocytic cell line demonstrated a linear relationship between the reciprocal of the K_m of α-aminoisobutyric acid uptake and the extracellular sodium concentration (33). This observation supports the model which predicts that sodium ion is co-transported with an amino acid by prior complexing to a carrier (19,20, 23,24).

The process by which energy is derived from the sodium gradient is probably not directly related to the function of the $(Na^+ + K^+)$-ATPase (34,35). Experiments in which cells were treated with ouabain have generally revealed that the uptake of amino acids preferred by both A and ASC systems remained unaffected (33,34). An exception was observed in aggregates of cultured chicken heart cells (35). The uptake of 2-

aminoisobutyric acid in these cells was greatly reduced following a 150 minute preincubation with ouabain. Closer examination revealed that the ionic gradients had been disrupted. Conversely, preincubation with ouabain for only 10 minutes, which was sufficient to inhibit the ATPase but not sufficient to destroy the ionic gradients, had minimal effect on the uptake of 2-aminoisobutyric acid. The Na^+/K^+ pump does not appear to be immediately related to either the sodium gradient or the gradient's influence on amino acid accumulation (35).

The extent to which the A and ASC systems rely on the alkali-metal gradient for energization remains unresolved. Christensen maintains that energy may also be donated from another source (for example, from metabolic or chemical reaction) (19,24,36). Clearly, primary active transport is important in the accumulation of the amino acids preferred by the sodium ion-insensitive L system (19). Evidence also suggests that a small contribution to the uptake of α-aminoisobutyric acid in Ehrlich ascites cells by primary sources may occur (37).

Amino acid accumulation may also result from an exchange phenomenon, in which the uptake of an amino acid is stimulated by the exodus of another amino acid. This phenomenon, also referred to as "accelerative exchange" and "transstimulation" (1,4), was described in Ehrlich ascites tumor cells by Oxender and Christensen during the characterization of systems A and L (8). The exchange properties of system L were observed to be much stronger in comparison to those of system A. Recent studies in Chinese hamster ovary cells have confirmed these observations (22). The uptake of L-phenylalanine was accelerated in Chinese hamster ovary

cells preloaded with either L-phenylalanine or L-leucine. Conversely, cells preloaded with L-alanine, glycine or α-aminoisobutyric acid did not demonstrate increased uptake of either L-alanine or glycine. The operation of these systems in response to existing conditions illustrates the highly complex interaction that occurs between the systems. System A may function primarily in the uptake of neutral amino acids while exodus may be achieved primarily by system L. The interaction may serve to maintain intracellular levels of amino acids. The intracellular amino acid pools may directly regulate cellular growth (1,4,50).

Regulation by the intracellular amino acids may be achieved by feedback mechanisms which act on the transport agencies (51). The endogenous amino acid pools of Balb/3T3 cells have recently been examined, at various cell densities, in relation to neutral amino acid uptake (51,52). The uptake of glycine, L-alanine and L-leucine was monitored in cell monolayers which had attained from 20 to 80 percent confluency. Cells demonstrated changes in amino acid transport as cell density increased. The transport changes correlated to changes in the endogenous amino acid pools. In general, system A activity, as measured by glycine and L-alanine uptake, was observed to decrease as cells approached confluence. Conversely, an increase in system L activity was observed when L-leucine uptake increased. The levels of most intracellular amino acids increased as the cells approached confluence. System A amino acids reached higher levels than system L amino acids. According to Oxender *et al.* (51), as cell populations increase from sparse to near-confluent monolayers, the amino acid pool sizes rise and eventually

feed back to inhibit further increase in amino acid levels. This feedback regulation is achieved by accelerative exchange or trans-stimulation of system L and transinhibition of system A. Trans effects cannot entirely account for the observed decrease in system A activity as experiments have revealed that system A only weakly participates in exchange (8,22). Rather, a number of factors may regulate the activity of system A in response to changes in growth conditions (4).

Amino acid transport changes in transformed cells

Transformed cells exhibit a number of membrane changes which may be related to loss of growth control (53,54). According to Holley (50), malignant growth may result from increases in intracellular concentrations of critical nutrients as a consequence of alterations in transport processes. Changes in amino acid transport have been noted following viral-transformation in a number of cell lines: polyoma virus-transformed 3T3 cells (55), polyoma virus-transformed baby hamster kidney cells (56), SV40-transformed Balb/3T3 cells (56) and murine sarcoma virus-transformed rat liver cells (56). Transformed cells accumulate amino acids at much higher rates in comparison to the non-transformed cell lines. Polyoma virus-transformed 3T3 cells, for example, were observed to concentrate both α-aminoisobutyric acid and cycloleucine between 2-and 2.5-fold more rapidly than observed in the non-transformed 3T3 cells (55). Similarly, polyoma virus-transformed baby hamster kidney cells, SV40-transformed Balb/3T3 cells and murine sarcoma virus-transformed rat liver cells were observed to transport these amino acids at rates which were estimated to be between 2.5- and

3.5-fold higher than the rates determined in their corresponding non-transformed cell lines (56). Though the separate systems of amino acid uptake were not properly discriminated, the increase seemed to be associated with all the amino acids tested. The exceptions were glutamic acid and arginine which were transported at comparable rates in both the polyoma virus-transformed 3T3 cells and the non-transformed cell line (55). These amino acids appeared to be accumulated in polyoma virus-transformed baby hamster kidney cells at increased rates (56).

Kinetic studies of amino acid uptake revealed that the changes observed in transformed cells were always associated with increased V_{max} values (55,56). Changes in K_m values were not observed. According to Isselbacher (56), increased V_{max} estimates reflect an increase in the number of amino acid transport sites in the plasma membrane. The membrane changes which accompany transformation may result in changes to the amino acid transport processes such that the transformed cells exhibit increased transport activity (55).

Concanavalin A and the effects of concanavalin A on amino acid transport in transformed cells

Membrane structural differences, in relation to amino acid uptake between virally-transformed and non-transformed cell lines, have been studied with the use of the membrane probe, concanavalin A (56,57). Concanavalin A is a lectin which is capable of agglutinating a number of different cells (58). The lectin was isolated in 1935 from Jack Bean meal (*Canavalia einsformis*) (59). Concanavalin A can be found as

a monomer, a dimer, a tetramer or a multimer, depending on both pH and ionic conditions (60,61). The molecule dissociates into monomers below pH 4.6 and is found in tetrameric form between pH 5.6 and 7.0 (61). The monomers are identical. Each monomer has a molecular weight of 25,500, and contains one sugar binding site. One calcium and one magnesium binding site is also present per subunit (61,62). Sugar binding is prevented in the absence of calcium and magnesium ions (63). Concanavalin A specifically binds α-D-mannopyranosyl and α-D-glucopyranosyl residues (64,65). The C-3, C-4 and C-6 hydroxyl groups of the D-arabino configuration are involved in concanavalin A interaction (66). The lectin is also capable of binding the non-exposed α-D-mannose residues present in the core region of glycoprotein oligosaccharide chains (66).

The ability of concanavalin A to bind glycoproteins makes it an ideal membrane probe for use in the study of cell surface structure and membrane-associated processes (58). Transformed cells exhibit altered membrane glycoproteins and glycolipids (53). Consequently, concanavalin A interacts differently with transformed cells as opposed to normal cells (58). For example, oncogenically transformed cells are agglutinated by concanavalin A at concentrations that do not cause the agglutination of normal cells (67). The comparative sensitivity of transformed cells to concanavalin A has been exploited to study the membrane changes accompanying transformation (53,54). The effects of concanavalin A on amino acid uptake in transformed cells have been investigated (56,57). Concanavalin A appears to differentially inhibit

amino acid uptake in transformed and non-transformed cell lines. Transformed cells generally exhibit a greater decrease in amino acid uptake than non-transformed cells (56). Inbar *et al.* (57) have reported that the amino acid uptake in normal hamster cells was completely insensitive to concanavalin A notwithstanding the observation that both the normal and transformed cells bound a similar number of lectin molecules at their surfaces. Amino acid uptake in transformed cells incubated with concanavalin A, conversely, was inhibited between 40 and 60% of the controls. Incubation in the presence of both concanavalin A and the hapten, α-methylglucoside, prevented the inhibition of amino acid uptake (57). Further, the effects were reversible upon transfer of transformed cells from concanavalin A containing medium to medium which contained α-methylglucoside in phosphate buffered saline alone (56). According to Inbar *et al.* (57), α-methylglucoside binds to concanavalin A and displaces the lectin from the membrane. The differential inhibition of amino acid transport in the presence of concanavalin A suggests that transformation results in cell surface structural changes which can be related to changes in amino acid transport. Inbar *et al.* (57) have suggested that the amino acid transport sites have been relocated to positions in the membrane which are closer to concanavalin A binding sites. Consequently, the binding of the lectin sterically interferes with amino acid transport activity. Alternatively, since the uptake of many amino acids are effected by concanavalin A following cell transformation, a general alteration in the membrane structure may have occurred which indirectly affects

the efficiency of the amino acid transport carrier (54).

The differential effects of concanavalin A on amino acid uptake in transformed and normal cells may be related to similarly differential cytotoxic effects of this lectin (68). A number of virally transformed cell lines were observed to be more sensitive than their normal counterparts to the cytotoxic effects of concanavalin A. For example, incubation with concanavalin A (50 µg/ml) for 8 hours was sufficient to reduce plated populations of polyoma virus-transformed 3T3 cells by over 60% of controls. The normal 3T3 cell monolayers exhibited no decrease in cell numbers even at concanavalin A concentrations of 1000 µg/ml. The precise mechanisms involved in concanavalin A toxicity are not clear. Kornfeld *et al.* (69) have shown that the initial steps involve binding of the lectin to carbohydrate receptors on the plasma membrane. The lectin is then thought to enter the cell by pinocytosis and cause death by a mechanism which remains to be identified (54). Lectins have been used as selective agents to obtain a variety of resistand variant from established cell lines (70,75).

Properties of the concanavalin A-resistant variant, C^R-7

The cytotoxic effects of concanavalin A were exploited to select several concanavalin A-resistant variants from independent, concanavalin A-sensitive, wild-type Chinese hamster ovary cells (71,73). The variant, C^R-7, is a subclone which was selected from a mixed population of concanavalin A-resistant cells. The mixed population of cells was obtained following ten passages of a wild-type population at 34°C in

growth medium which contained concanavalin A at a concentration of 40 μg/ml (71,74). The C^R-7 cell line was found to be temperature sensitive, with a non-permissive temperature of 39°C (71).

The concanavalin A-resistant variant, C^R-7, exhibited a complex phenotype characterized by altered growth properties and changes in membrane associated properties (Table II) (75). The variant was less sensitive to the cytotoxic effects of concanavalin A with a D_{10} value which was about 2.5-fold higher than that observed with the wild-type. The D_{10} value refers to the concentration of concanavalin A required to reduce cell survival to 10% of controls (72). Increased resistance may be related to the reduced ability of the C^R-7 line to bind concanavalin A. The C^R-7 cells were observed to bind about 2.5- to 3-fold less of the lectin than the wild-type cells. Further, in contrast to parental wild-type cells lectin binding in the C^R-7 cells was non-cooperative. Alterations in C^R-7 cell surface glycoproteins may account for both decreased concanavalin A binding and the decreased ability of the lectin to agglutinate the C^R-7 cells in comparison to the wild-type hamster cells. The latter was particularly pronounced at high concentrations of concanavalin A (500 μg/ml) which were sufficient to completely agglutinate all of the wild-type cells, but only sufficient to agglutinate 50% of the C^R-7 population (71). The C^R-7 cell surface also appears to contain an additional glycoprotein, as determined by surface labelling techniques. This extra glycoprotein has a molecular weight of 155,000 and is conspicuously absent in wild-type cells (76). This unique glycoprotein may contribute to

TABLE II

Modified cellular properties of the concanavalin A-resistant cell line, C^{R}-7, selected in the laboratory of Dr. J.A. Wright.[1]

Obvious temperature-sensitive growth properties.
Altered cellular morphology on culture plates.
Increased sensitivity to some membrane-active agents such as phenethyl alcohol and sodium butyrate.
Altered lectin agglutination properties.
Increased resistance to cytotoxic effects of concanavalin A.
Decreased cellular adhesiveness to substratum.
Defective lectin-receptor mobility characteristics.
Differences in binding mechanism and amount of lectin bound/cell surface area at $4^{o}C$.
Altered surface glycoproteins as detected by lactoperoxidase iodination, galactose oxidase and metabolic labelling procedures.

[1]after Wright (75).

the reduced concanavalin A receptor mobility and the non-cooperative binding mechanism observed with the C^R-7 line. According to Ceri and Wright (76), less than 10% of the C^R-7 cells formed aggregates or caps on incubation with fluorescent labelled concanavalin A. In comparison, 90% of the wild-type cells demonstrated cap formation. Conceivably, the extra component may interfere locally with receptor mobility (76). Clearly, increased resistance to the cytotoxic effects of concanavalin A may be accompanied by membrane structural changes. These membrane changes may result in altered membrane-associated processes. Furthermore, the alterations at the surface membrane appear to be directly due to a defect in glycosyltransferase activities in concanavalin A-resistant cells. Recently, it has been shown (77) that C^R-7 cells contain a lesion in lipid-oligosaccharide biosynthesis which affects the transfer of mannose residues to cell surface glycoproteins. Also, changes in fucosyl transferase activity (77) and glycosidase activity have been described (78,79) for concanavalin A-resistant cells.

MATERIALS AND METHODS

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Cell growth material

The hamster cells were maintained in alpha-minimal essential medium (Flow Laboratories, Rockville, Md.) supplemented with fetal calf serum (Gibco Ltd., Calgary, Alta.) and antibiotics (Gibco Ltd., Calgary, Alta.). The cells were grown either in 16 ounce Brockway bottles (Brockway Glass Co., Brockway, Pa.) or in 35 mm culture plates (Lux Scientific Corporation, Calif.) as described elsewhere (71).

Experimental Material

The radioactive amino acids used in this study were: L-[U- C] proline (10 mCi/mmol and 125 mCi/mmol), L-[U-^{14}C] alanine (10 mCi/mmol and 171 mCi/mmol), L-[U-^{14}C] threonine (10 mCi/mmol), L-[U-^{14}C] leucine (10 mCi/mmol and 150 mCi/mmol), L-[U-^{14}C] isoleucine (10 mCi/mmol), L-[U-^{14}C] phenylalanine (10 mCi/mmol), 2-amino [1-^{14}C] isobutyric acid (60 mCi/mmol), and α-[1-^{14}C] methylaminoisobutyric acid (51.8 mCi/mol). All of these amino acids were purchased from the Radiochemical Centre, Amersham, Ill. The Aqueous Counting Scintillant was also obtained from Amersham. All of the following cold amino acids were obtained from the Sigma Chemical Company, St. Louis, Mo.: L-proline, L-alanine, L-threonine, L-leucine, L-isoleucine, L-phenylalanine, α-aminoisobutyric acid, α-methylaminoisobutyric acid, glycine,

L-serine, L-methionine, L-valine, L-tyrosine, and L-tryptophan. The bacto-trypsin was also supplied by the DIFCO Chemical Company. Concanavalin A was purchased from Calbiochem, San Diego, Calif. The protein was assayed using a kit supplied by Bio-Rad Laboratories, Mississauga, Ont.

METHODS

Cell and culture conditions

Two Chinese hamster ovary cell lines were used: a wild-type cell line and a concanavalin A-resistant variant cell line (C^R-7). These cell lines were kindly provided by Dr. J.A. Wright. The cells were grown in alpha-minimal essential medium (80) which was supplemented with 10% (v/v) fetal calf serum. Penicillin G (100 units/ml) and streptomycin sulfate (100 ug/ml) were also added to the medium. Cells were grown as monolayer cultures either in 16 ounce Brockway bottles or in 35 mm tissue culture dishes. All cells were maintained at 34^oC in a 5% CO_2 atmosphere. Cells used for experiments were grown in 35 mm dishes and the medium was changed every 48 hours. Cell density was measured following removal of the cells with 0.05% (w/v) trypsin in phosphate buffered saline. The trypsinized cells were diluted in isotonic saline (0.18 M NaCl) and counted in a particle counter (Coulter Electronics Ltd.).

Wild-type and C^R-7 growth curves

Growth curves were obtained by seeding exponentially growing cells in 35 mm tissue culture dishes. Wild-type cells were seeded at 4×10^4

cells per plate. C^R-7 cells were seeded at 2 x 10^4 cells per plate. Cell growth was measured on duplicate plates at 24 hour intervals. The doubling time was determined from a plot of log cell number as a function of the culture time.

Protein relationship to cell number

The relationship between protein and cell number was determined for both wild-type and C^R-7 cells. Cell number per 35 mm plate was determined in duplicate plates every 24 hours. Two plates grown in parallel were used for protein determination. The medium was decanted from the latter plates, the cells were washed twice with 1.0 ml phosphate-buffered saline and solubilized with 1.0 ml of 0.2 N NaOH for two hours at room temperature (22, 81, 82). Solubilized samples were neutralized with an equal volume of 0.2 N HCl and assayed for protein by the method of Bradford (83). An alternative method of sample preparation, in which the cells were removed from the plates with trypsin was used to assess the effectiveness of the solubilization technique. Trypsinized cells were centrifuged for 45 seconds at 1000 x g in an International clinical centrifuge to pellet the cells. The pellet was dissolved in 1.0 ml 0.2 N NaOH and assayed for protein as above.

Estimation of cell viability

Cell viability of both the wild-type and the C^R-7 cells was estimated by the trypan blue exclusion test as described by Phillips

(84). Cells were incubated with Dulbecco's phosphate-buffered saline at 34°C for various times ranging from 0 to 120 minutes and were then screened for viability. Cell numbers were also recorded over the experimental period.

Uptake Experiments

General uptake procedures

Amino acid uptake was measured in 35 mm tissue culture plates by methods similar to those used by Nishino *et al.* (85). Wild-type and C^R-7 cells were allowed to grow in 35 mm plates to dense but not confluent monolayers. These monolayers were used for uptake experiments. Media was removed by aspiration and the cells were washed twice with 2.0 ml of Dulbecco's phosphate-buffered saline, pH 7.4 which was held at room temperature (86). This buffer contained NaCl (8.0 g) KCl (0.2 g), Na_2HPO_4(1.15 g), and KH_2PO_4 (0.2 g) in deionized, bi-distilled water (1000 ml). The buffer also contained 0.01% (w/v) $CaCl_2$ and 0.01% (w/v) $MgCl_2$. In the absence of $CaCl_2$ and $MgCl_2$, the C^R-7 variant dissociated from the surfaces of the plates. $CaCl_2$ and $MgCl_2$ may stabilize the plasma membrane of the C^R-7 in a manner analogous to that observed in erythrocytes (87). Following the buffer wash, 2.0 ml Dulbecco's phosphate-buffered saline which contained 0.1% (w/v) glucose were added to the plates and the plates were incubated for 40 minutes at 34°C. This incubation was necessary in an attempt to reduce the levels of intracellular amino acids which may otherwise influence the uptake of amino acids (38,47). Gazzola *et al.* (38) have

demonstrated in human fibroblasts, preloaded with radiolabelled L-proline, L-alanine, or L-leucine, that a depletion time of 40 minutes was sufficient to reduce intracellular levels of these amino acids by 80%. On this basis, a depletion time of 40 minutes was selected for use in the wild-type and C^R-7 cell experiments. Depletion was terminated by aspiration of the buffer. Uptake was then initiated with the addition of 0.5 ml uptake medium to each plate (85). This volume was sufficient to cover the bottom of the plate. The uptake medium consisted of [^{14}C]-labelled amino acid, corresponding non-radiolabelled amino acid which was added to achieve the desired concentration of amino acid, and 0.1% (w/v) glucose in Dulbecco's phosphate-buffered saline pH 7.4. The uptake medium was held at 34^{o}C. The final specific activity in most experiments was 5 μCi/μmole for amino acid at a 0.1 mM concentration. Uptake was allowed to proceed at 34^{o}C for various times. Trials were also routinely made at 0^{o}C. These plates were adapted for 10 minutes at 0^{o}C prior to the initiation of uptake. Uptake times recorded as 0 minutes of uptake reflect the uptake over the time required to remove the uptake medium immediately following its addition to the monolayers. This operation required 4 seconds to complete. Uptake was terminated by rapid aspiration, to remove the uptake medium, followed by five washes with 2.0 ml of ice-cold Dulbecco's phosphate-buffered saline, pH 7.4 (85,88). The washing buffer did not contain glucose. The entire operation, including the initial aspiration and the five successive washes, required 15 seconds to complete. The cells were then extracted with 1.0 ml of 70% (v/v) ethanol at 80^{o}C for 30 minutes

(82,88). The ethanol-soluble fraction was collected and the plates were washed with an additional 1.0 ml of 70% ethanol. The wash was pooled with the ethanol-soluble fraction. The volume of ethanol-soluble fraction collected varied from plate to plate. Consequently, the ethanol-soluble fraction and pooled wash were evaporated to dryness by a jet of air. The resulting residue was then resuspended in 1.0 ml of 70% ethanol. The addition of the last two steps greatly increased the reproducibility of the results. A sample of the resuspended ethanol-soluble fraction was counted in 5.0 ml of ACS scintillation cocktail in a Beckman LS230 scintillation counter as a quantitative measure of uptake (88). The observed cpm were converted to dpm by reference to the appropriate quench and background curves. The results were finally expressed as nanomoles amino acid taken up per plate as standardized by protein (55,56). All results were corrected for extracellular label by subtracting the values obtained for a zero minute incubation at $0^{o}C$ (90). Protein was estimated, as previously described, from ethanol-insoluble fractions. These fractions were obtained from the plates, following removal of the ethanol-soluble material, by a 2 hour solubilization with 0.2 N NaOH at room temperature (22,81,82). Samples of the ethanol-insoluble fraction were also occasionally monitored for radioactivity to estimate the degree of incorporation of label into macromolecules (81,88).

Time course of amino acid uptake

Each of the amino acids investigated was present in the uptake medium at 0.1 mM and at a final specific activity of 5 μCi/umole.

Uptake times ranged from 0 to 40 minutes with at least four trials per time of incubation. Steady-state levels of uptake were reached within 40 minutes for most of the amino acids studied. Six natural, metabolizable amino acids, and two non-metabolizable amino acid analogues, were examined.

Sodium ion-sensitivity of amino acid uptake

The sensitivity of L-proline, L-alanine, L-leucine and α-methyl-aminoisobutyric acid uptake to the presence of sodium ion was examined over time. This was achieved with the use of sodium ion-containing and sodium ion-free uptake media. The former was prepared as previously described. The sodium ion-free medium was prepared by isoosmotic substitution of NaCl and Na_2HPO_4 in the Dulbecco's phosphate-buffered saline with choline chloride and K_2HPO_4, respectively (82). Choline has been termed the "non-penetrating cation" since its uptake in animal cells is reduced in the absence of sodium ion (91). Consequently, choline cannot replace sodium ions in the generation of a gradient which may energize amino acid uptake (1). Prior to uptake, all monolayers were adapted for 10 minutes in 2.0 ml of either Dulbecco's phosphate-buffered saline or sodium ion-free medium. Uptake was initiated as described previously. The amino acids were present at a concentration of 0.1 mM with a final specific activity of 5 μCi/umole. Two determinations were made for each time and each condition.

Estimation of kinetic parameters of amino acid uptake

The initial rate of uptake was estimated from the one minute uptake of various amino acids at concentrations ranging from .01 mM to 0.5 mM. Initial rates were examined in an attempt to reduce the contribution to the net uptake by exodus processes (1,92). The final specific activity of the amino acids in the uptake medium was 1 μCi/μmole. The relationship between the rate of uptake and amino acid concentration was expressed in a double reciprocal plot. Km and V_{max} values were estimated from the double reciprocal plots (92).

Inhibition of L-proline, L-alanine and L-leucine uptake by various amino acids

Competition between various amino acids for uptake was studied by inhibition experiments. The initial rates of L-proline, L-alanine and L-leucine uptake were examined in the presence of non-radiolabelled amino acids at high concentrations (8,22). The test amino acids were present in the uptake medium at a concentration of 0.1 mM with a 5 μCi/μmole final specific activity. The medium also contained various inhibitor amino acids at a concentration of 5 mM (22). No compensation for the osmotic pressure of added amino acids was made. According to Christensen (1), compensation is required only when the concentration of the added amino acid exceeds 10 mM.

The effects of concanavalin A on the initial rates of L-proline, L-alanine and L-leucine uptake

The concanavalin A used in these experiments was prepared in Dulbecco's phosphate-buffered saline, pH 7.4. Concentrations of 50, 250, and 500 µg/ml were prepared (56,57). Cells were preincubated as described previously, washed twice with 2.0 ml of Dulbecco's phosphate-buffered saline, then 0.5 ml of concanavalin A solution was added to each plate. The plates were incubated for 15 minutes at 34°C. The concanavalin A solution was then rapidly removed by aspiration and the monolayers were washed twice with 2.0 ml of Dulbecco's phosphate-buffered saline to remove the traces of concanavalin A. An additional 15 minute incubation with 0.5 ml of Dulbecco's phosphate-buffered saline followed the incubation with concanavalin A (56). This was removed by aspiration and the uptake of either L-proline, L-alanine or L-leucine was initiated. Amino acids were present in the uptake medium at a concentration of 0.1 mM and at a final specific activity of 5 µCi/µmole. The uptake medium also contained 0.1% (w/v) glucose. According to Inbar *et al.* (57), the presence of glucose, at this concentration, does not interfere with the effects of concanavalin A on amino acid uptake. Uptake was allowed to proceed for one minute in order to study the effects of the lectin on the initial rate of amino acid entry. Control trials were performed by substituting the concanavalin A incubation with a 15 minute incubation in Dulbecco's phosphate-buffered saline. All results are expressed as the mean of duplicate determinations.

RESULTS

RESULTS

Wild-type and C^R-7 growth curves

The growth curves at 34°C of the wild-type and C^R-7 cells were found to be similar (Fig. 3). No lag phase was apparent for the wild-type while a lag of 48 hours was required before exponential growth of the C^R-7 monolayers was initiated. The exponential phase of the wild-type monolayers continued for 144 hours and reached a stationary phase which was equivalent to 3×10^6 cells per plate. The exponential phase of the C^R-7 line continued beyond 192 hours and reached the stationary phase at 2.5×10^6 cells per plate. The doubling time for the wild-type cells was estimated from the curve to be 24 hours. The C^R-7 population doubled every 27 hours. According to Oxender *et al.* (22), amino acid uptake can be compared between two cell lines when the cell lines display similar rates of growth.

Protein relationship to cell number

Standardization of uptake by cellular protein requires prior demonstration of a linear relationship between protein and cell number (55,56). The relationship between protein and cell number for both the wild-type and C^R-7 cells grown in 35 mm tissue culture plates is described in Fig. 4. Both cell lines exhibited a linear relationship over the range of cell numbers used in the experiments. The wild-type cells displayed a linear relationship over the range of cell

Figure 3. Growth curves for the wild-type and C^R-7 Chinese hamster ovary cells grown in 35 mm tissue culture plates at 34°C. The wild-type cells (⊙) were seeded at a density of 4 x 10^4 cells per plate; the C^R-7 cells (△) were seeded at a density of 2 x 10^4 cells per plate. All cells were seeded in 2.0 ml of αMEM containing 10% (v/v) fetal calf serum and maintained at 34°C in a 5% CO_2 atmosphere. Each point represents the mean of two plates.

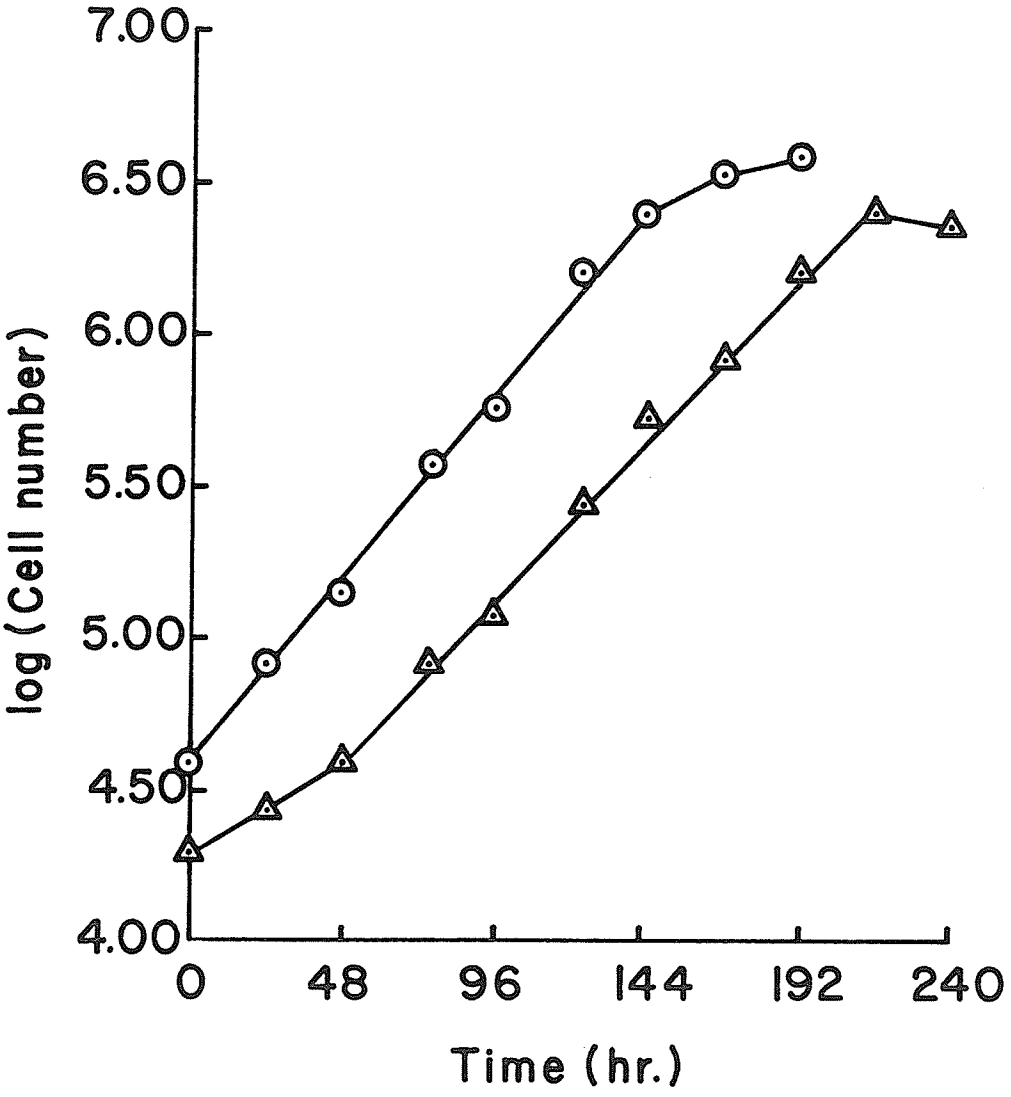
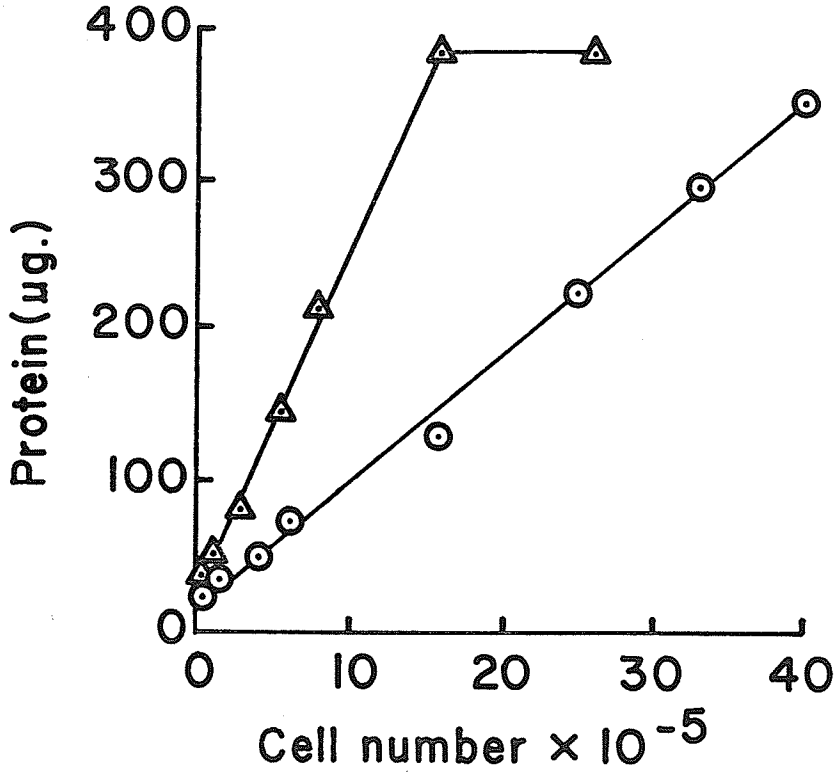

7.00
6.50
6.00
5.50
5.00
4.50
4.00
log (Cell number)
0
48
96
144
192
240
Time (hr.)

Figure 4. Protein relationship to cell number. Wild-type (⊙) and C^R-7 (△) cell numbers per 35 mm plate were determined every 24 hours. Protein was estimated from two plates grown in parallel. Protein was obtained by solubilizing the cells for two hours in 0.2 N NaOH at room temperature. Protein was assayed by the Bradford method (83). Each point represents the mean of two protein estimations and two cell number determinations.


400
300
200
100
0
Protein (µg.)
0
10
20
30
40
Cell number × 10⁻⁵

numbers tested, while the plot for the C^R-7 cells levelled off at approximately 1.6 x 10^6 cells per plate. At the higher cell numbers, the C^R-7 cells had more protein than the wild-type cells at corresponding cell numbers. Uptake experiments were performed with monolayers that corresponded to the linear portions of the curves in terms of cell numbers per plate. Further, attempts to compensate for the greater protein content of the C^R-7 cells were made by selecting monolayers of wild-type and C^R-7 that exhibited similar protein estimations.

The method of obtaining the protein samples used in these experiments was compared to a more conventional method in which the cells are removed from the plates with trypsin (Fig. 5). The method which involved solubilizing the cells with 0.2 N NaOH proved to be as effective as trypsinization in both the wild-type and C^R-7 cells. Microscopic examination following 2 hours of solubilization in 0.2 N NaOH revealed that both the wild-type and the C^R-7 cells were effectively removed from the plates by this method.

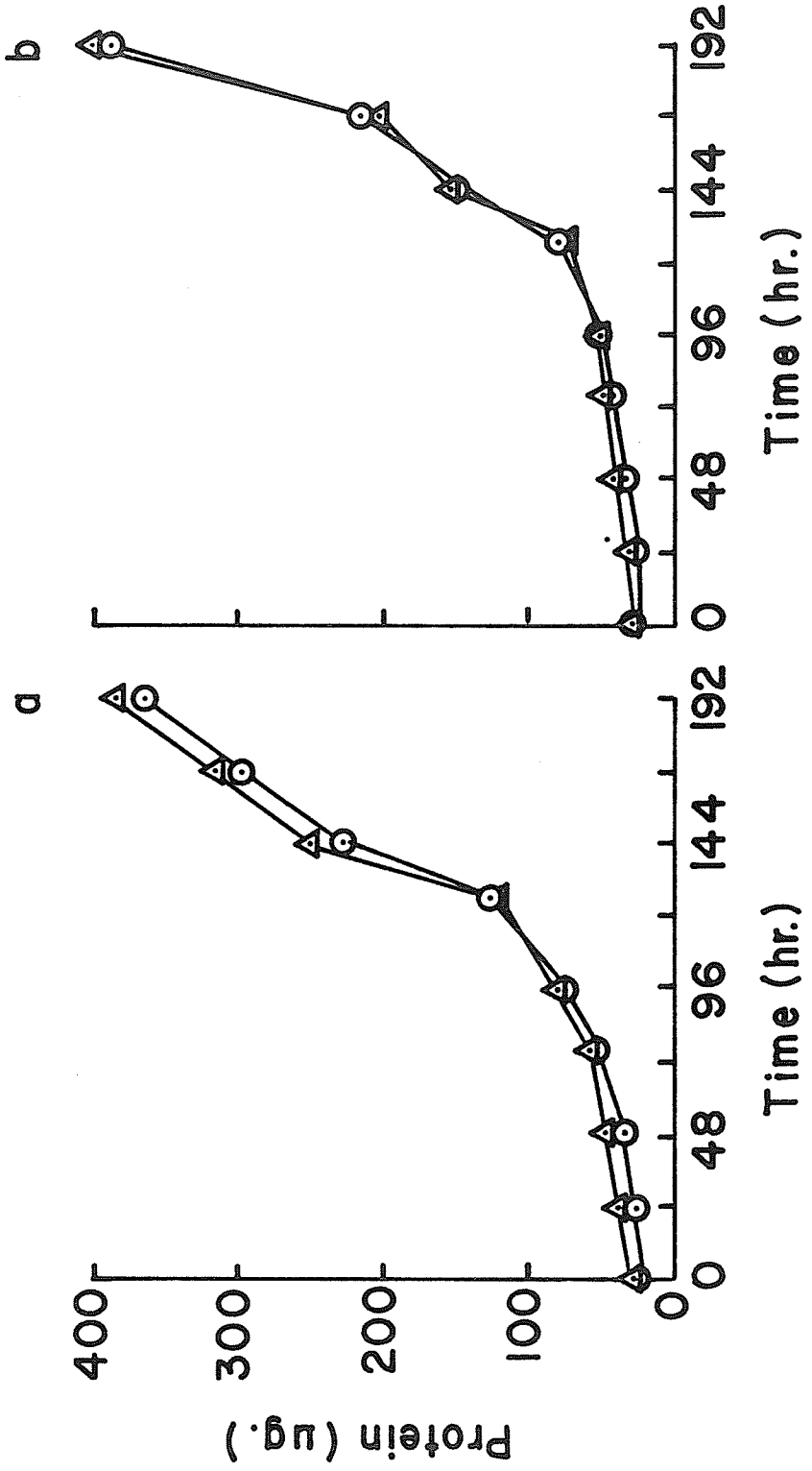
Estimation of cell viability

Trypan blue exclusion tests of both the wild-type and C^R-7 cell viability were made over 2 hours of incubation in Dulbecco's phosphate-buffered saline. Both cell lines remained viable over the entire experimental period. Following 120 minutes of incubation the wild-type and C^R-7 cells were 90 and 92% viable, respectively. Microscopic inspection of the C^R-7 cells at 120 minutes revealed that the

Figure 5. A comparison of two methods for obtaining protein samples. One method involved the two hour solubilization of the cells with 1.0 ml of 0.2 N NaOH at room temperature (⊙). The other method involved trypsinization of the cells with 1.0 ml trypsin, centrifugation of the trypsinized cells at 1000 x g for 45 seconds and resuspension of the pellet in 1.0 ml of 0.2 N NaOH (△). Protein was assayed by the Bradford method (83). Each point represents the mean of two trials. Cells were grown in 35 mm plates in 2.0 ml of αMEM containing 10% (v/v) fetal calf serum at 34°C in a 5% CO_2 atmosphere. Protein samples were obtained every 24 hours.

Figure 5a. Wild-type cells.

Figure 5b. C^R-7 cells.


a
b
Protein (ug.)
400
300
200
100
0
0
48
96
144
192
Time (hr.)
Time (hr.)

cells were beginning to "round up". Cell numbers, which were recorded over the incubation period, did not decrease. This indicates that both the wild-type and the C^R-7 cells remained anchored to the plates. Times of uptake incubation in Dulbecco's phosphate-buffered saline never exceeded 120 minutes. The monolayers used in the uptake experiments were viable by extrapolation.

Examination of the ethanol-soluble and insoluble fractions for distribution of label

Examination of the ethanol-soluble and insoluble fractions for distribution of radiolabel revealed that the majority of the label was recovered in the ethanol-soluble fraction (Table III). This was observed at both 1 and 40 minutes of incubation and in both the wild-type and the C^R-7 cells. The ethanol-soluble and insoluble fractions represent uptake and incorporation of amino acids into macromolecules (81,85,89). Results indicated that over time, incorporation increased for all of the amino acids except the two amino acid analogues. Wild-type cell incorporation at one minute amounted to less than 6% of the total activity. Distribution in the C^R-7 cell ethanol-insoluble fraction was less than 4% of the total for all amino acids tested. The results suggest that over the experimental period, the natural amino acids remained largely non-metabolized. Incorporation into macromolecules, following one minute of incubation, comprised only about 5% of the total. These results are in agreement with previous reports (22,81). The apparent incorporation of the amino acid analogues, α-aminoisobutyric

TABLE III

Examination of the ethanol-soluble and insoluble fractions for distribution of radiolabel.[1]

Amino Acid	Dpm of ethanol-soluble fraction[2] (A)		Dpm of ethanol-insoluble fraction[3] (B)		Total Dpm (A + B)		(B/A + B) x 100	
	1 min.	40 min.	1 min.	40 min.	1 min.	40 min.	1 min.	40 min.
Wild-type								
L-proline	1740	23800	101	1470	1840	25300	5.5	5.8
L-alanine	19200	41800	598	3620	19800	45400	3.0	8.0
L-threonine	25700	66500	217	4260	25900	70800	0.84	6.0
L-leucine	14200	29100	403	9470	14600	38600	2.8	25
L-isoleucine	10300	16400	88	2310	10400	18700	0.85	12
L-phenylalanine	12600	22200	644	3340	13200	25500	4.9	13
α-aminoisobutyric acid	2530	43000	134	757	2660	43800	5.0	1.7
α-methylaminoisobutyric acid	1840	34500	106	437	1950	34900	5.4	1.3
C^R-7								
L-proline	5440	39200	109	3830	5550	43000	2.0	8.9
L-alanine	21600	56300	458	5760	22100	62100	2.1	9.3
L-threonine	18900	66600	65	4263	19000	70900	3.4	6.0
L-leucine	17700	19300	413	8310	18100	27600	2.3	30
L-isoleucine	14500	21100	528	4790	15000	25900	3.5	18
L-phenylalanine	10600	19900	142	3430	10700	23300	1.3	15
α-aminoisobutyric acid	4170	55500	122	1023	4290	56500	2.8	1.8
α-methylaminoisobutyric acid	4910	63300	138	1040	5050	64300	2.7	1.6

[1]Results obtained from a typical experiment; the amino acids were present in the uptake medium at 0.1 mM and at a final specific activity of 5 μCi/μmole. Times of incubation ranged over a 40 minute period. Two times are indicated here (1 and 40 mins). Dpm values have been corrected for dpm associated with 0 minute incubations.

[2]Ethanol-soluble fraction was obtained by extraction of the monolayers with 70% ethanol for 30 minutes at 80°C. The soluble fraction and ethanol wash were evaporated to dryness and resuspended in 70% ethanol. A sample was counted in ACS.

[3]Ethanol-insoluble fraction was obtained, following removal of ethanol-soluble material, by solubilization with 0.2 N NaOH for two hours at room temperature. A sample was counted in ACS.

acid and α-methylaminoisobutyric acid, is unexplainable. These two analogues are non-metabolizable which makes them excellent substrates in the study of uptake (1). The present incorporation may represent background though corrections for background were made. The dpm associated with the ethanol-soluble fractions also serve to illustrate typical dpm values obtained in most uptake experiments.

Time courses of amino acid uptake

The time courses of uptake of the various natural amino acids and the two amino acid analogues were studied in both the wild-type and C^R-7 cell lines (Figs. 6-9). Similar results for the wild-type and C^R-7 cells were observed for most of the amino acids tested. In both cell lines, L-proline was taken up slowly, but steadily, over the first five minutes of incubation. A slight shoulder in the curve after this time indicated that the uptake processes were slowed. No steady-state levels were reached even after 40 minutes of incubation. The highest levels of L-proline attained, over the experimental period, were approximately 26 and 19 nmoles per mg protein for the wild-type and C^R-7 cells, respectively (Fig. 6a).

The wild-type and C^R-7 cells, both exhibited rapid initial entry of L-alanine (Fig. 6b). Steady-state levels were reached between 10 and 20 minutes of incubation. The levels reached were high in comparison with the other amino acids. The wild-type reached levels of about 30 nmoles per mg protein, while slightly higher levels of about 34 nmoles per mg protein were attained in the variant. This is the only example in which the steady-state levels reached in the C^R-7 cells are higher than in the wild-type cells.

Figure 6a. Time course of L-proline uptake in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained 0.1 mM L-proline at a final specific activity of 5 μCi/μmole in Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. Uptake was measured in 35 mm plates and estimated from the ethanol-soluble fractions. Each point represents the mean of four trials. The mean cell density of the wild-type population (⊙) was 1.6×10^6 cells per plate and the mean protein content was 0.12 mg. The mean cell density of the C^R-7 population (△) was 9.4×10^5 cells per plate and the mean protein content was 0.18 mg.

Figure 6b. Time course of L-alanine uptake in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained 0.1 mM L-alanine at a final specific activity of 5 μCi/μmole in Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. Uptake was measured in 35 mm plates and estimated from the ethanol-soluble fractions. Each point represents the mean of four trials. The mean cell density of the wild-type population (⊙) was 1.7×10^6 cells per plate and the mean protein content was 0.15 mg. The mean cell density of the C^R-7 population (△) was 9.0×10^5 cells per plate and the mean protein content was 0.17 mg.

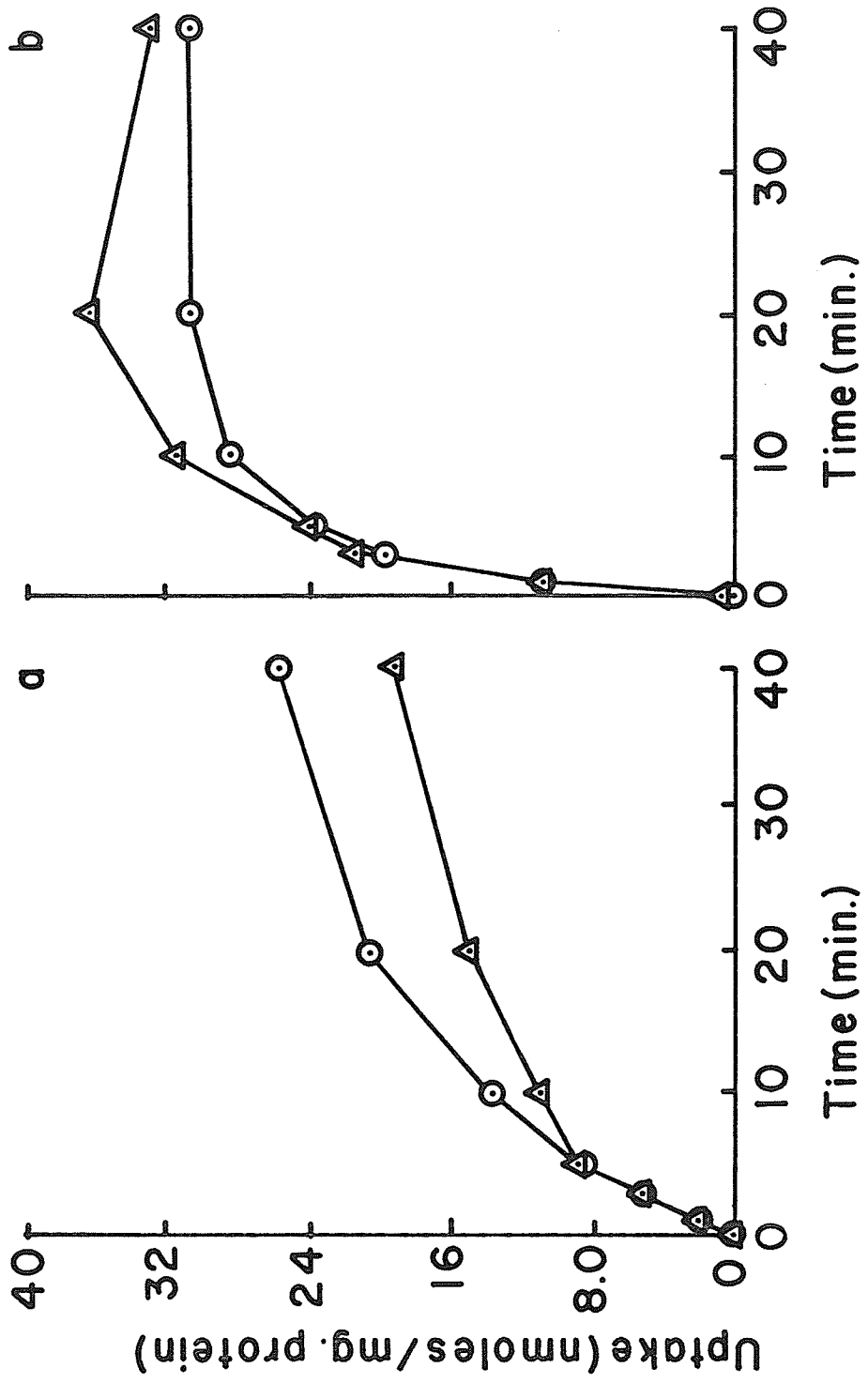

a
b
Uptake (nmoles/mg. protein)
0
8.0
16
24
32
40
0
10
20
30
40
Time (min.)

Figure 7a. Time course of L-threonine uptake in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained 0.1 mM L-threonine at a final specific activity of 5 μCi/μmole in Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. Uptake was performed in 35 mm plates and estimated from the ethanol-soluble fractions. Each point represents the mean of 4 to 6 trials. The mean cell density of the wild-type population (⊙) was 1.6×10^6 cells per plate and the mean protein content was 0.12 mg. The mean cell density of the C^R-7 population (△) was 1.0×10^6 cells per plate and the mean protein content was 0.18 mg.

Figure 7b. Time course of L-leucine uptake in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained 0.1 mM L-leucine at a final specific activity of 5 μCi/μmole in Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. Uptake was performed in 35 mm plates and estimated from the ethanol-soluble fractions. Each point represents the mean of four trials. The mean cell density of the wild-type population (⊙) was 1.7×10^6 cells per plate and the mean protein content was 0.19 mg. The mean cell density of the C^R-7 population (△) was 1.1×10^6 cells per plate and the mean protein content was 0.19 mg.

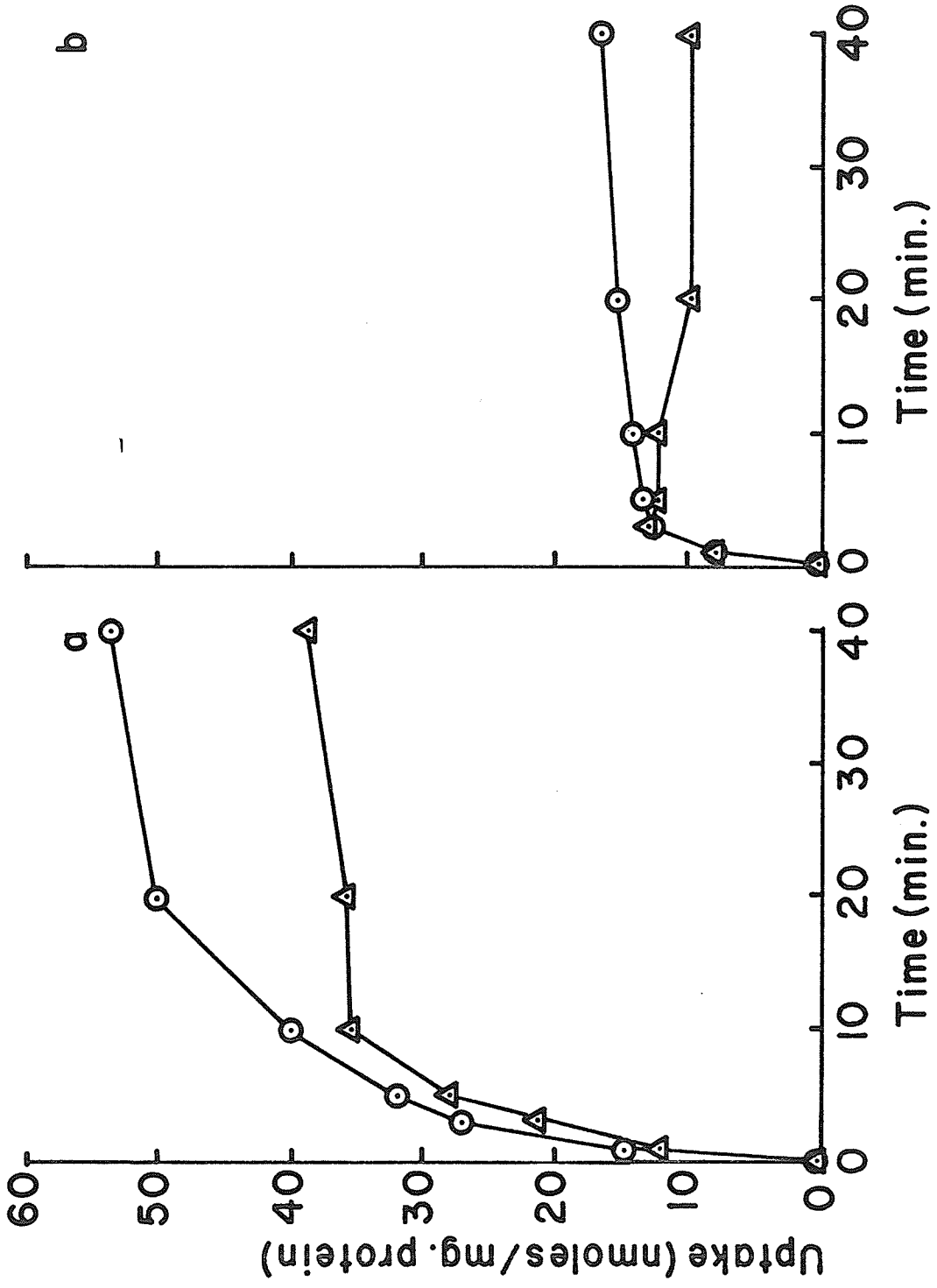

a
b
Uptake (nmoles/mg. protein)
0
10
20
30
40
50
60
Time (min.)
0
10
20
30
40

Figure 8a. Time course of L-isoleucine uptake in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained 0.1 mM L-isoleucine at a final specific activity of 5 μCi/μmole in Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. Uptake was performed in 35 mm plates and estimated from the ethanol-soluble fractions. Each point represents the mean of four trials. The mean cell density of the wild-type population (⊙) was 1.7×10^6 cells per plate and the mean protein content was 0.14 mg. The mean cell density of the C^R-7 population (△) was 9.2×10^5 cells per plate and the mean protein content was 0.18 mg.

Figure 8b. Time course of L-phenylalanine uptake in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained 0.1 mM L-phenylalanine at a final specific activity of 5 μCi/μmole in Dulbecco's phosphate buffered saline with 0.1% (w/v) glucose. Uptake was performed in 35 mm plates and estimated from the ethanol-soluble fractions. Each point represents the mean of four trials. The mean cell density of the wild-type population (⊙) was 1.8×10^6 cells per plate and the mean protein content was 0.16 mg. The mean cell density of the C^R-7 population (△) was 9.7×10^5 cells per plate and the mean protein content was 0.20 mg.

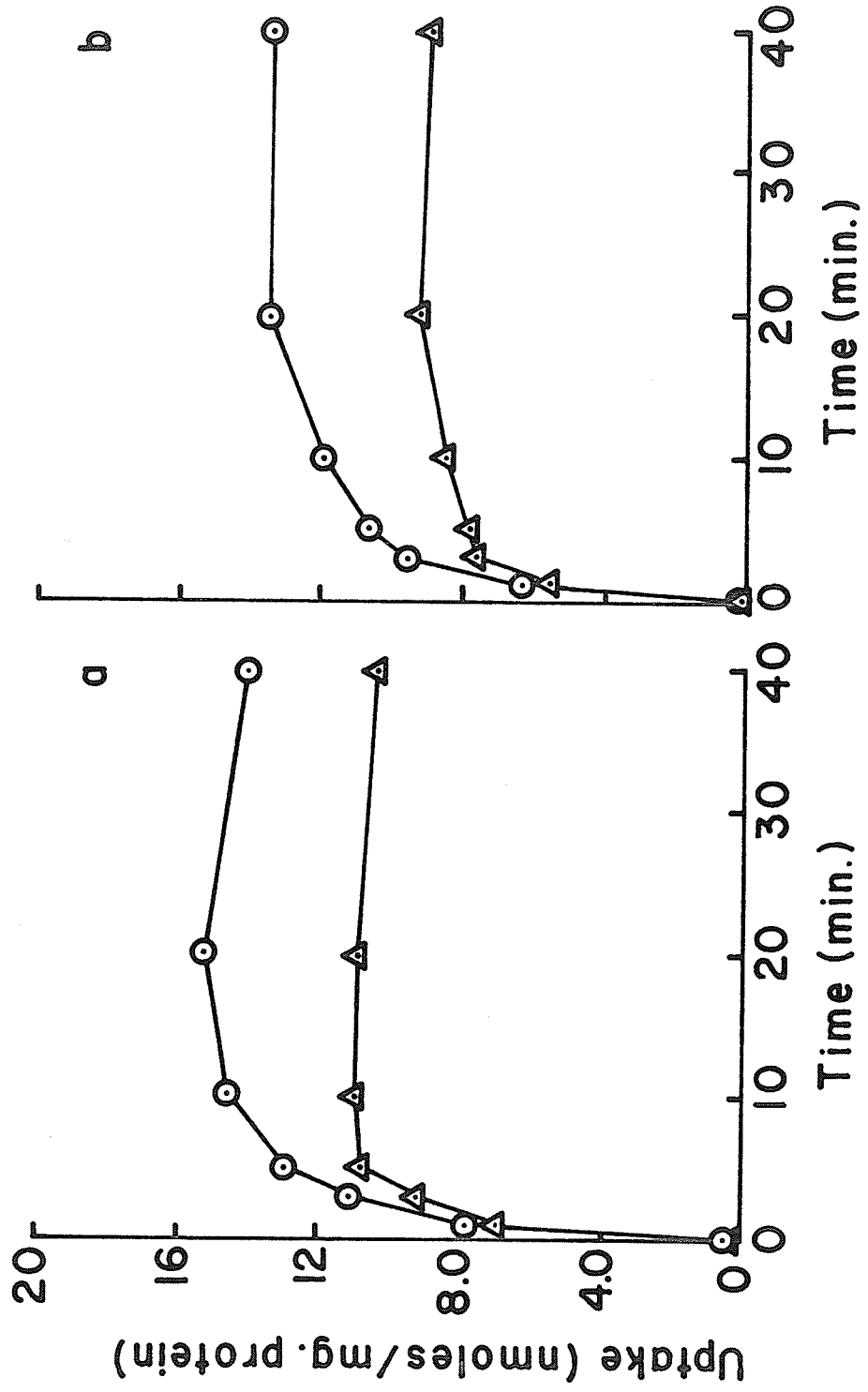
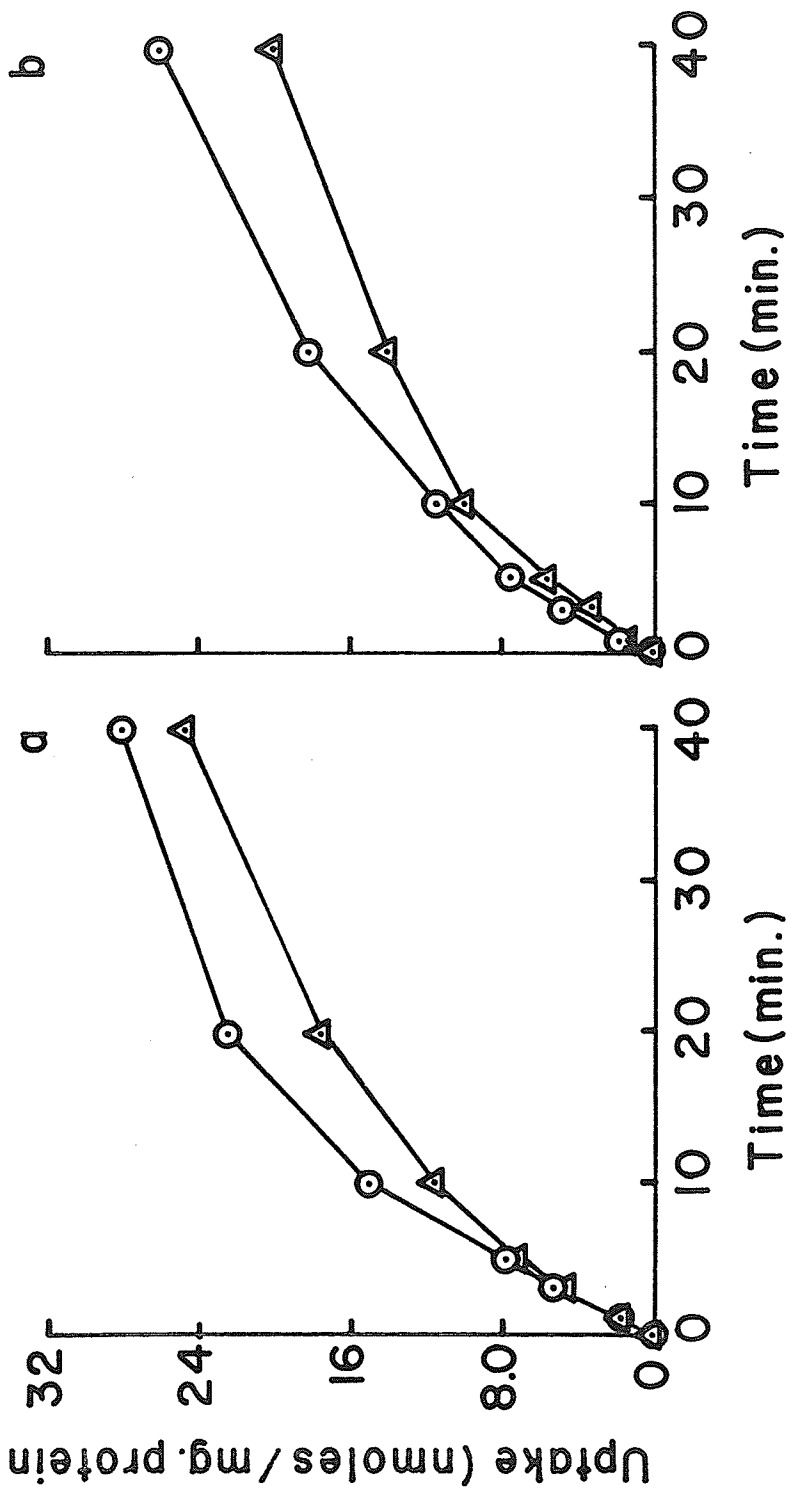

Uptake (nmoles/mg. protein)
20
16
12
8.0
4.0
0
a
b
0
10
20
30
40
Time (min.)

Figure 9a. Time course of α-aminoisobutyric acid uptake in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained 0.1 mM α-aminoisobutyric acid at a final specific activity of 5 μCi/μmole in Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. Uptake was performed in 35 mm plates and estimated from the ethanol-soluble fractions. Each point represents the mean of four trials. The mean cell density of the wild-type population (⊙) was 1.4×10^6 cells per plate and the mean protein content was 0.12 mg. The mean cell density of the C^R-7 population (△) was 8.0×10^5 cells per plate and the mean protein content was 0.19 mg.

Figure 9b. Time course of α-methylaminoisobutyric acid uptake in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained 0.1 mM α-methylaminoisobutyric acid at a final specific activity of 5 μCi/μmole in Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. Uptake was measured in 35 mm plates and estimated from the ethanol-soluble fractions. Each point represents the mean of 2 to 4 trials. The mean cell density of the wild-type population (⊙) was 1.5×10^6 cells per plate and the mean protein content was 0.14 mg. The mean cell density of the C^R-7 population (△) was 1.0×10^6 cells per plate and the mean protein content was 0.18 mg.


Uptake (nmoles/mg. protein)
32
24
16
8.0
0
a
b
0
10
20
30
40
Time (min.)

The uptake profiles of L-threonine were similar to those of L-alanine (Fig. 7a). High steady-state levels of accumulation were obtained following rapid initial entry. Slight differences were observed between the wild-type and C^R-7 cell lines. The steady-state in the wild-type population began at 20 minutes of incubation. The steady-state in the C^R-7 line commenced after 10 minutes. Levels reached in the C^R-7 cells were lower with values of about 36 nmoles per mg protein as compared to 50 nmoles per mg protein in the wild-type cells. The wild-type cells steady-state levels of L-threonine were the highest of all the amino acids tested for the two cell lines.

The amino acids L-leucine, L-isoleucine, and L-phenylalanine displayed similar time courses of uptake (Figs 7b, 8a, and 8b). All three of these amino acids, in both the wild-type and C^R-7 cells were taken up quickly to low steady-state levels between 5 and 10 minutes of incubation. The C^R-7 cells accumulated lower amounts than the wild-type cells. The levels of L-leucine, L-isoleucine, and L-phenylalanine attained in both cell lines were the lowest recorded of the amino acids studied. Levels of L-isoleucine, for example, were only about 14 and 10 nmoles per mg protein in the wild-type and C^R-7 lines, respectively.

The time course profiles of the two amino acid analogues, α-amino-isobutyric acid and α-methylaminoisobutyric acid were similar to the profile of L-proline (Figs 9a and 9b). Uptake was initially slow, but steady over the first 10 minutes of incubation. No definite steady-state was achieved though uptake slowed after 10 minutes. C^R-7 appeared

to accumulate less of these analogues over time than the wild-type.

A comparison between the wild-type and C^R-7 cells initial rates of amino acid uptake indicated that few differences existed (Table IV). The possible exceptions may be the initial rates of entry of L-threonine and α-methylaminoisobutyric acid. Both amino acids were taken up in C^R-7 cells at slightly lower rates than in the wild-type cells. This may partially account for the lower levels reached in the C^R-7 cells as compared to the wild-type cells though the rate of uptake is only one factor which contributes to the steady-state (1). Clearly, from these results, the steady-state level attained cannot always be predicted from the initial rate of entry.

Three natural amino acids were selected for further study on the basis of the results of the time course experiments. These amino acids were L-proline, L-alanine and L-leucine. The amino acid analogue, α-methylaminoisobutyric acid was also studied in an attempt to investigate its similarity to L-proline. This analogue is usually considered to be the characteristic substrate of system A (1,4).

Sodium ion-sensitivity of amino acid uptake

The dependency of amino acid uptake in the wild-type and C^R-7 cells on the presence of sodium ions was studied with the use of Na^+-containing and Na^+-free uptake media. All of the test amino acids displayed Na^+-sensitivity though to different degrees (Figs. 10-13). L-proline and α-methylaminoisobutyric acid uptake demonstrated the most striking Na^+-sensitivity with almost complete loss of uptake in Na^+-free medium.

TABLE IV

Comparison of amino acid uptake in the wild-type and C^R-7 cell lines[1].

Amino acid	Initial rate[2]		
	wild-type (A)	C^R-7 (B)	B/A
L-proline	2.1 ± 0.1	2.1 ± 0.2	1.0
L-alanine	11.0 ± 1.3	11.0 ± 1.3	1.0
L-threonine	15.0 ± 1.3	12.0 ± 0.7	0.80
L-leucine	8.1 ± 1.2	8.3 ± 1.4	1.0
L-isoleucine	7.8 ± 1.0	6.9 ± 0.5	0.88
L-phenylalanine	6.3 ± 1.0	5.5 ± 0.2	0.87
α-aminoisobutyric acid	2.0 ± 0.2	1.8 ± 0.3	0.90
α-methylaminoisobutyric acid	1.8 ± 0.5	1.5 ± 0.2	0.83

[1]Values estimated from Figures 6-9.

[2]nmoles amino acid per mg protein per min.

Figure 10. The effect of sodium ion on L-proline uptake in wild-type and C^R-7 monolayers at 34°C. Uptake of 0.1 mM L-proline at a final specific activity of 5 μCi/μmole was performed in 35 mm plates with either sodium ion-containing (⊙) or sodium ion-free (●) Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. The Na^+-containing medium contained 137 mM NaCl and 8 mM Na_2HPO_4. The Na^+-free medium was prepared by isoosmotic substitution of NaCl and Na_2HPO_4 with choline chloride and K_2HPO_4, respectively. Uptake was estimated from the ethanol-soluble fractions. Each point represents the mean of two determinations. The mean cell density of the wild-type population (a) was 1.6×10^6 cells per plate and the mean protein content was 0.14 mg. The mean cell density of the C^R-7 population (b) was 7.0×10^5 cells per plate and the mean protein content was 0.11 mg.

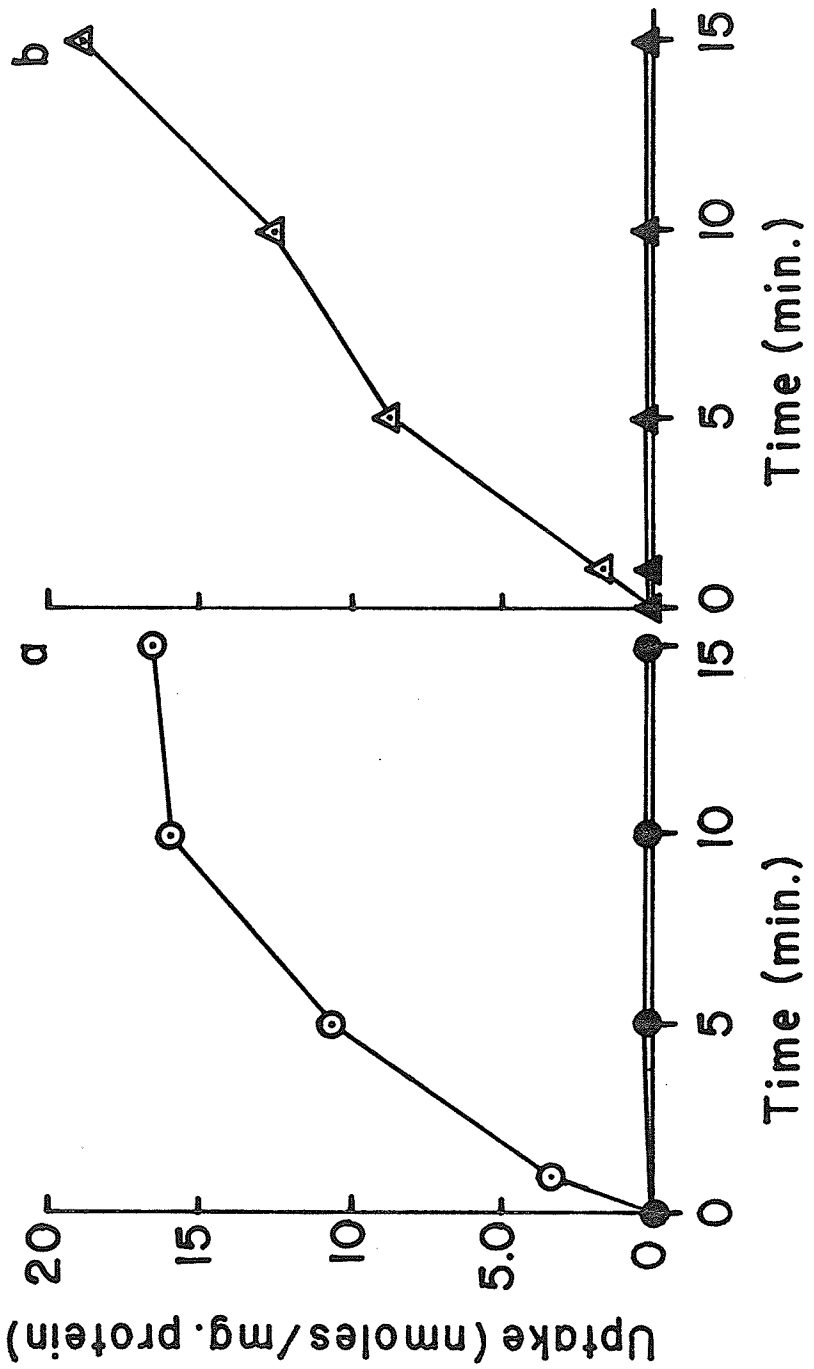

Uptake (nmoles/mg. protein)
20
15
10
5.0
0
0
5
10
15
Time (min.)
a
b
0
5
10
15
Time (min.)

Figure 11. The effect of sodium ion on α-methylaminoisobutyric acid uptake in wild-type and C^R-7 monolayers at 34°C. Uptake of 0.1 mM α-methylaminoisobutyric acid at a final specific activity of 5 μCi/μmole was performed in 35 mm plates with either sodium ion-containing (⊙) or sodium ion-free (●) Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. The Na^+-containing medium contained 137 mM NaCl and 8 mM Na_2HPO_4. The Na^+-free medium was prepared by isoosmotic substitution of NaCl and Na_2HPO_4 with choline chloride and K_2HPO_4, respectively. Uptake was estimated from the ethanol-soluble fractions. Each point represents the mean of two determinations. The mean cell density of the wild-type population (a) was 1.6×10^6 cells per plate and the mean protein content was 0.11 mg protein. The mean cell density of the C^R-7 population (b) was 8.5×10^5 cells per plate and the mean protein content was 0.10 mg.

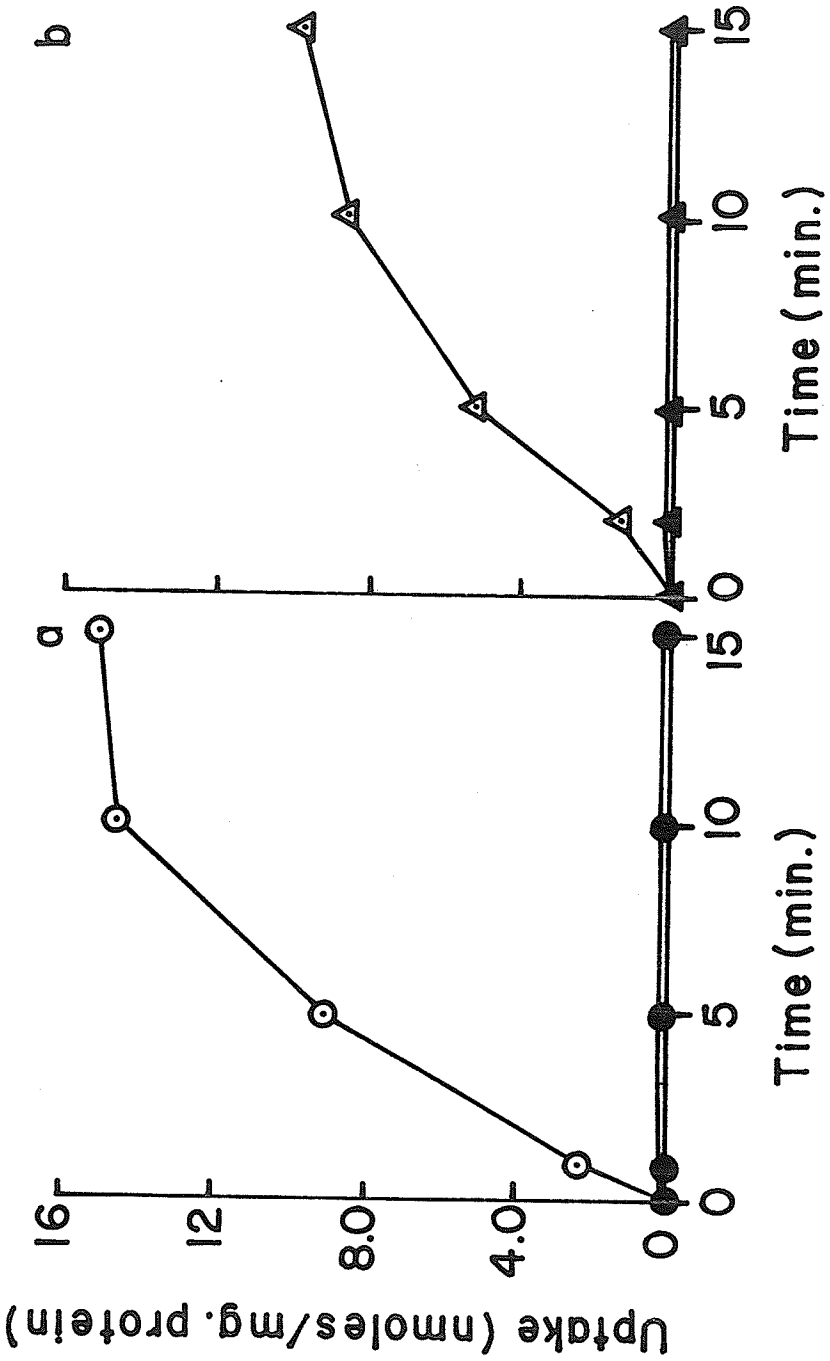

Uptake (nmoles/mg. protein)
16
12
8.0
4.0
0
a
0
5
10
15
Time (min.)
b
0
5
10
15
Time (min.)

Figure 12. The effect of sodium ion on L-alanine uptake in wild-type and C^R-7 monolayers at 34°C. Uptake of 0.1 mM L-alanine at a final specific activity of 5 μCi/μmole was performed in 35 mm plates with either sodium ion-containing (⊙) or sodium ion-free (●) Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. The Na^+-containing medium contained 137 mM NaCl and 8 mM Na_2HPO_4. The Na^+-free medium was prepared by isoosmotic substitution of NaCl and Na_2HPO_4 with choline chloride and K_2HPO_4, respectively. Uptake was estimated from the ethanol-soluble fractions. Each point represents the mean of two determinations. The mean cell density of the wild-type population (a) was 1.6×10^6 cells per plate and the mean protein content was 0.12 mg. The mean cell density of the C^R-7 population (b) was 9.3×10^5 cells per plate and the mean protein content was 0.09 mg.

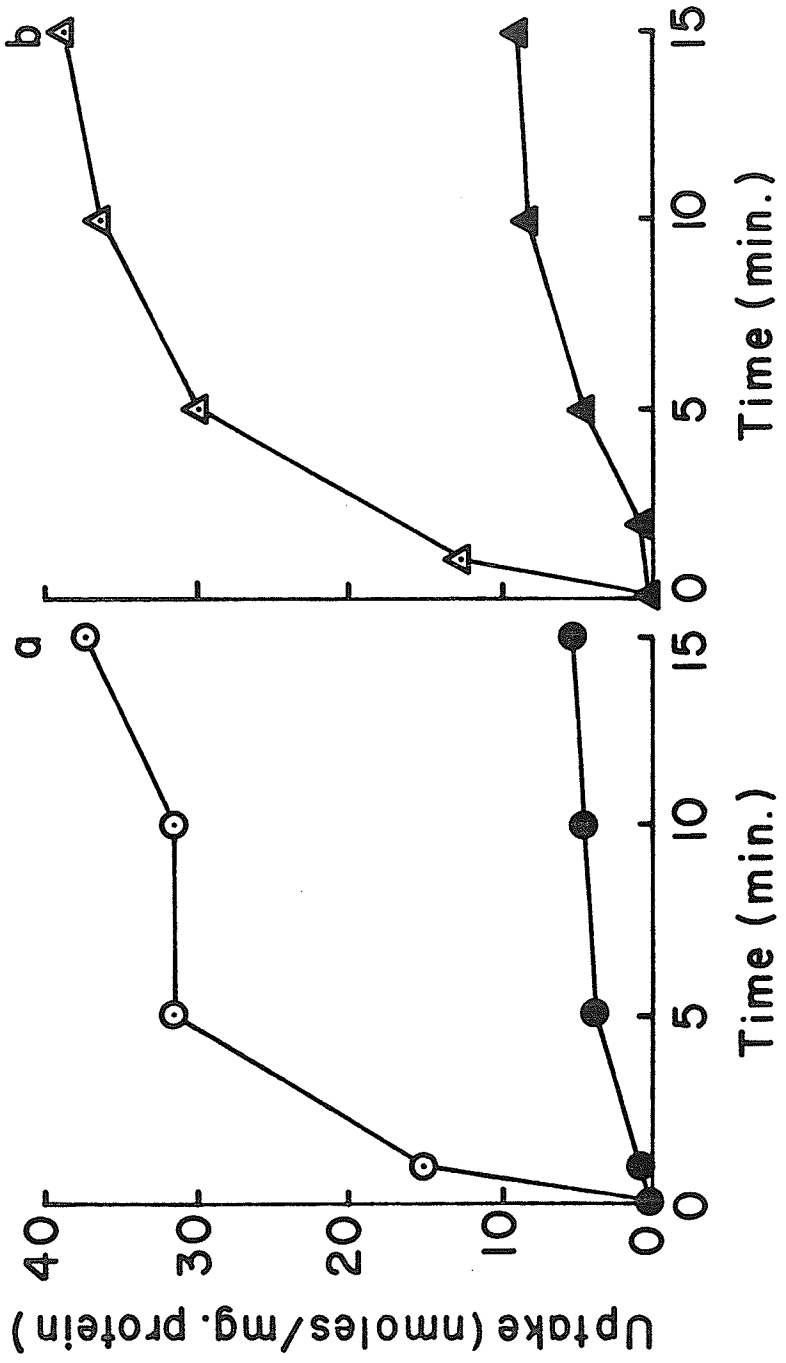
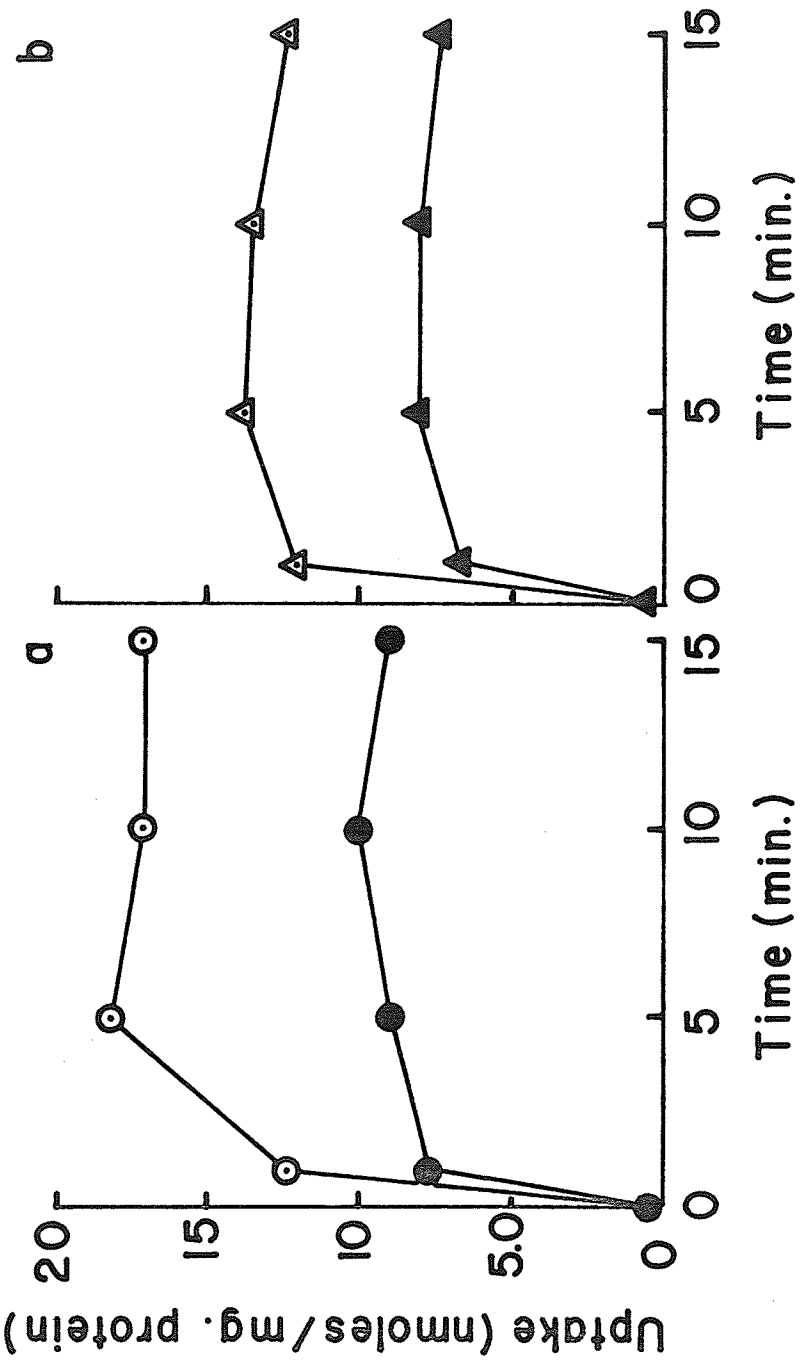

Uptake (nmoles/mg. protein)
40
30
20
10
0
a
b
0
5
10
15
Time (min.)

Figure 13. The effect of sodium ion on L-leucine uptake in wild-type and C^R-7 monolayers at 34°C. Uptake of 0.1 mM L-leucine at a final specific activity of 5 μCi/μmoles was performed in 35 mm plates with either sodium ion-containing (⊙) or sodium ion-free (●) Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. The Na^+-containing medium contained 137 mM NaCl and 8 mM Na_2HPO_4. The Na^+-free medium was prepared by isoosmotic substitution of NaCl and Na_2HPO_4 with choline chloride and K_2HPO_4, respectively. Uptake was estimated from the ethanol-soluble fractions. Each point represents the mean of two determinations. The mean cell density of the wild-type population (a) was 1.4×10^6 cells per plate and the mean protein content was 0.11 mg. The mean cell density of the C^R-7 population (b) was 9.4×10^5 cells per plate and the mean protein content was 0.11 mg.


Uptake (nmoles/mg. protein)
20
15
10
5.0
0
a
b
0
5
10
15
Time (min.)

Na^+-insensitive uptake of L-proline comprised only about 2% and 5% of the total uptake in wild-type and C^R-7 cells, respectively (Figs. 10a and 10b). Na^+-insensitive uptake of the analogue was negligible in both cell lines (Figs. 11a and 11b). The total uptake of α-methylaminoisobutyric acid was in general lower in the variant as compared to the wild-type line. The uptake of L-alanine in both the wild-type and C^R-7 lines appeared to be less dependent on the presence of sodium ions (Figs. 12a and 12b). At least 15% and 23% of the total uptake of this amino acid in the wild-type and C^R-7 cells, respectively, was contributed by Na^+-independent agencies. The uptake of L-leucine was the least dependent on the presence of sodium ions (Figs. 13a and 13b). Uptake in Na^+-free medium reached 50% of the uptake levels attained in Na^+-containing medium in wild-type cells. The C^R-7 cells displayed even less Na^+-sensitivity with 60% of the total uptake contributed by Na^+-independent routes. The results obtained for C^R-7 cells in both the presence and absence of the cation are correspondingly lower than for the wild-type.

Estimation of the kinetic parameters of amino acid uptake

The estimated K_m and V_{max} values for the uptake of L-proline, α-methylaminoisobutyric acid, L-alanine, and L-leucine, in both the wild-type and C^R-7 cells, are indicated in Table V. These values were obtained from the double reciprocal plots of velocity as a function of amino acid concentration (Figs. 14-17). The wild-type and C^R-7 cells exhibit similar kinetic parameters for the uptake of L-proline,

TABLE V

Kinetic parameters for amino acid uptake in the wild-type and C^R-7 cell lines.

Amino acid	wild-type		C^R-7	
	K_m[1]	Vmax	K_m	Vmax
L-proline	0.46	17.0	0.61	20.0
α-methylaminoisobutyric acid	0.11	2.6	0.10	2.9
L-alanine	0.13	35.0	0.14	29.0
L-leucine	0.09	16.0	0.05	9.9

[1]Estimated K_m and V_{max} values were determined from Figures 14 to 17 and are expressed in millimolar concentrations and nanomoles amino acid per mg protein per minute, respectively. Final specific activity was 1 μCi/μmole.

Figure 14. Double-reciprocal plot of L-proline uptake in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained L-proline at various concentrations and at a final specific activity of 1 μCi/μmole in Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. Uptake was performed in 35 mm plates for 1.0 minute and was estimated from the ethanol-soluble fractions. Each point represents the mean of two determinations. The mean cell density of the wild-type population (⊙) was 1.4×10^6 cells per plate and the mean protein content was 0.15 mg. The mean cell density of the C^R-7 population (△) was 7.0×10^5 cells per plate and the mean protein content was 0.10 mg.

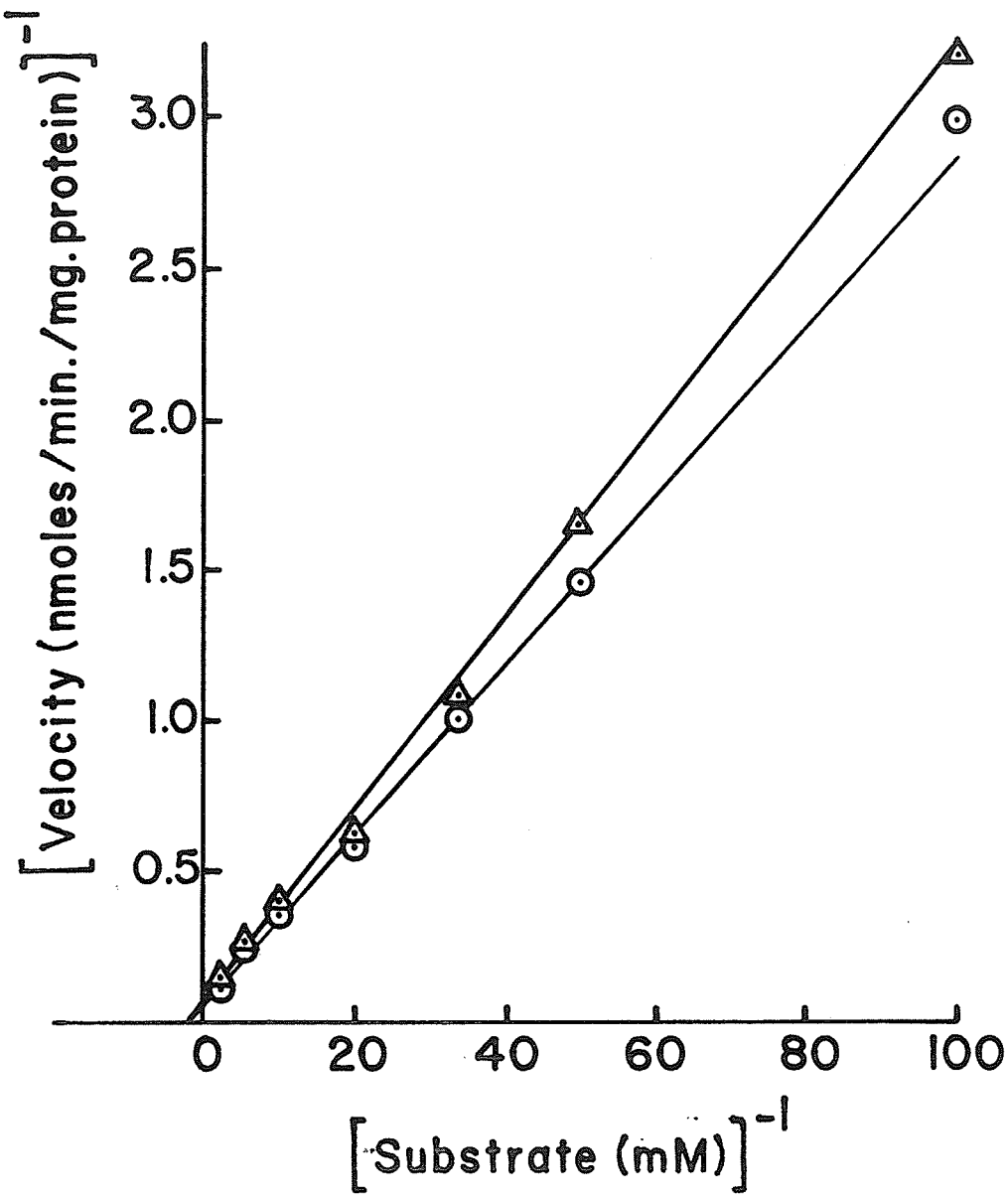

[Velocity (nmoles/min./mg. protein)]$^{-1}$
3.0
2.5
2.0
1.5
1.0
0.5
0
20
40
60
80
100
[Substrate (mM)]$^{-1}$

Figure 15. Double-reciprocal plot of α-methylaminoisobutyric acid in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained α-methylaminoisobutyric acid uptake at various concentrations and at a final specific activity of 1μCi/μmole in Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. Uptake was performed in 35 mm plates for 1.0 minute and was estimated from the ethanol-soluble fractions. Each point represents the mean of two determinations. The mean cell density of the wild-type population (⊙) was 1.8×10^6 cells per plate and the mean protein content was 0.18 mg. The mean cell density of the C^R-7 population (△) was 7.7×10^5 cells per plate and the mean protein content was 0.16 mg.

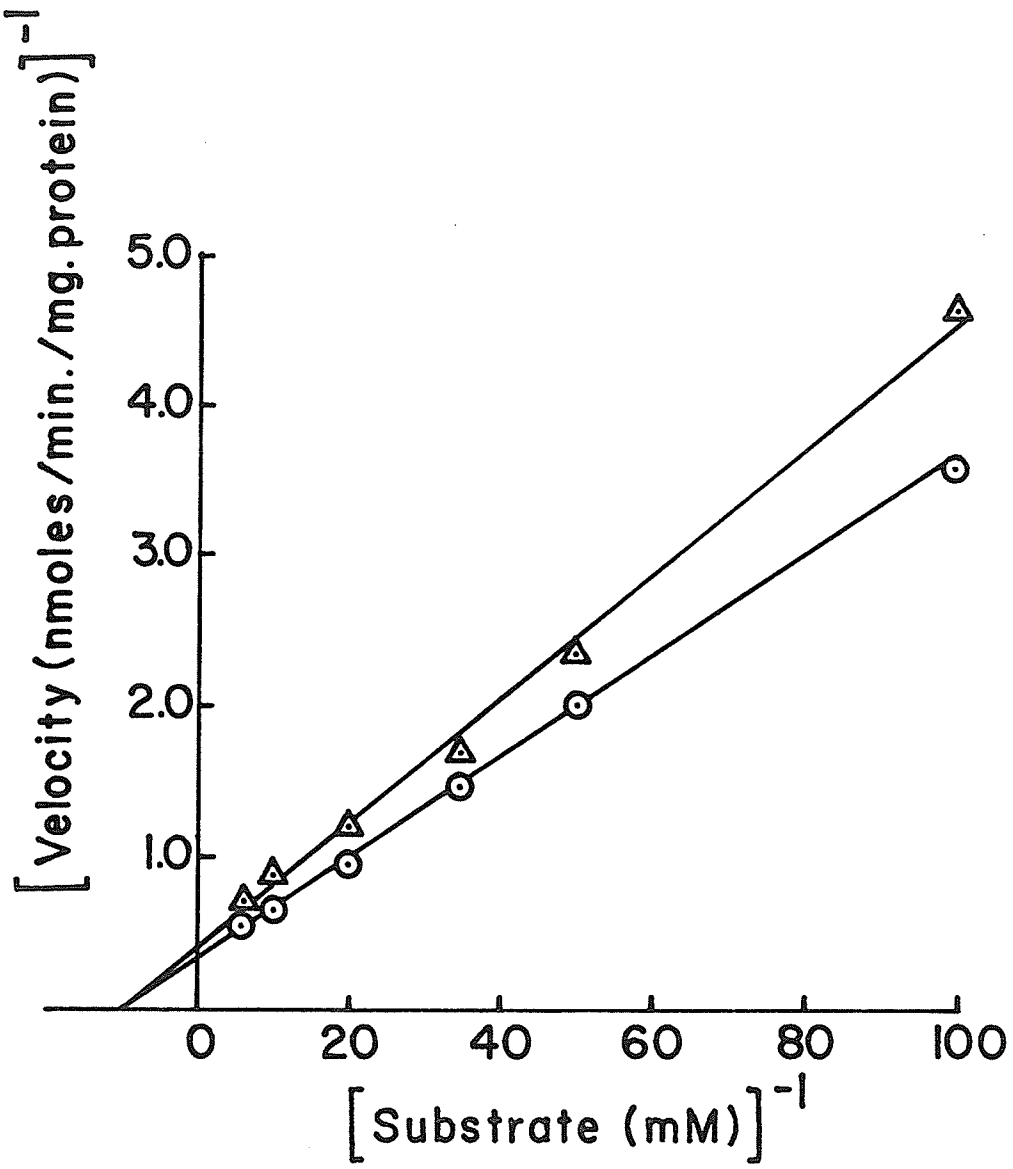

[Velocity (nmoles/min./mg. protein)]⁻¹
5.0
4.0
3.0
2.0
1.0
0
20
40
60
80
100
[Substrate (mM)]⁻¹

Figure 16. Double-reciprocal plot of L-alanine uptake in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained L-alanine at various concentrations and at a final specific activity of 1 μCi/μmole in Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. Uptake was performed in 35 mm plates for 1.0 minute and was estimated from the ethanol-soluble fractions. Each point represents the mean of two determinations. The mean cell density of the wild-type population (⊙) was 1.7×10^6 cells per plate and the mean protein content was 0.16 mg. The mean cell density of the C^R-7 population (△) was 7.2×10^5 cells per plate and the mean protein content was 0.18 mg.

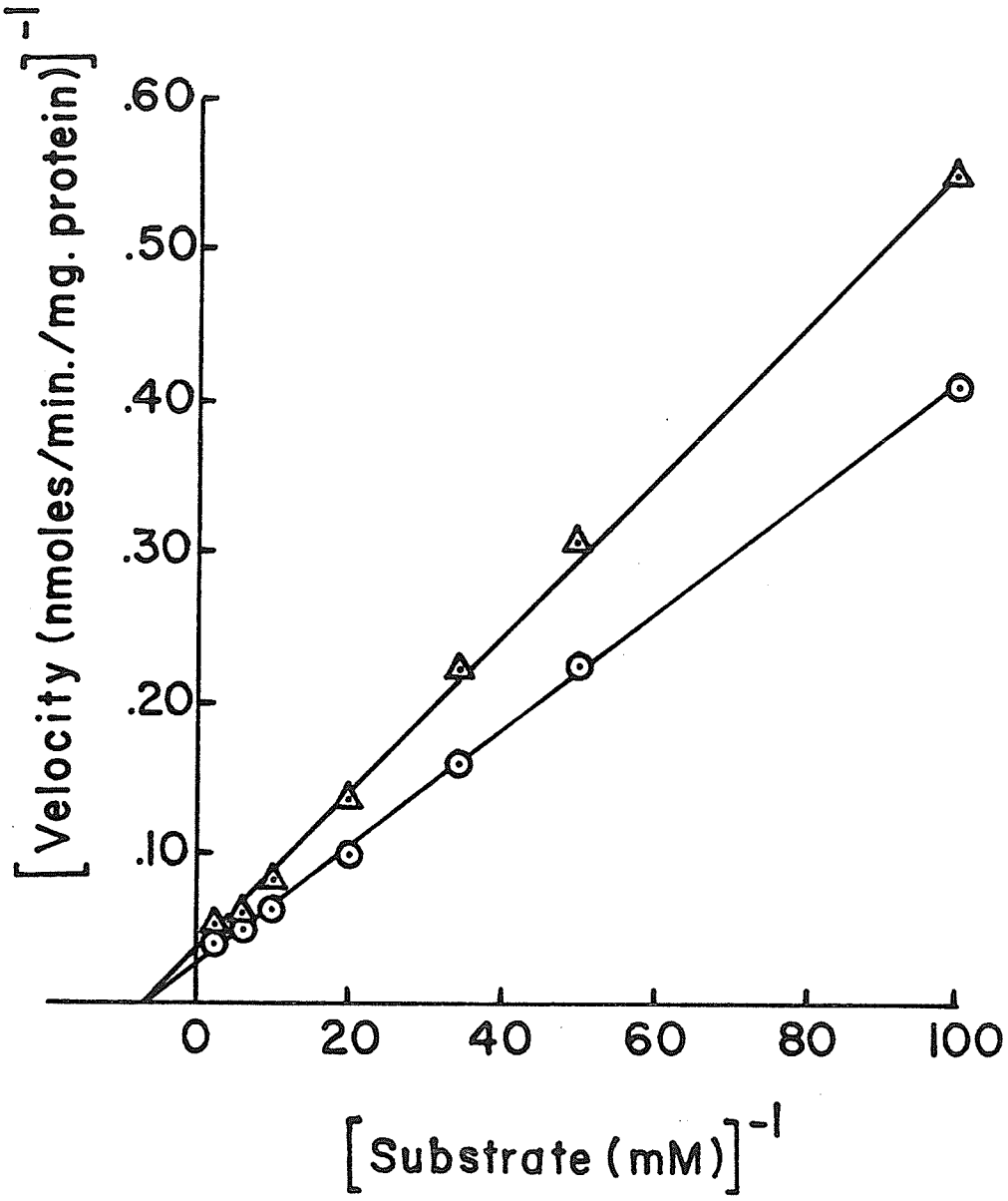
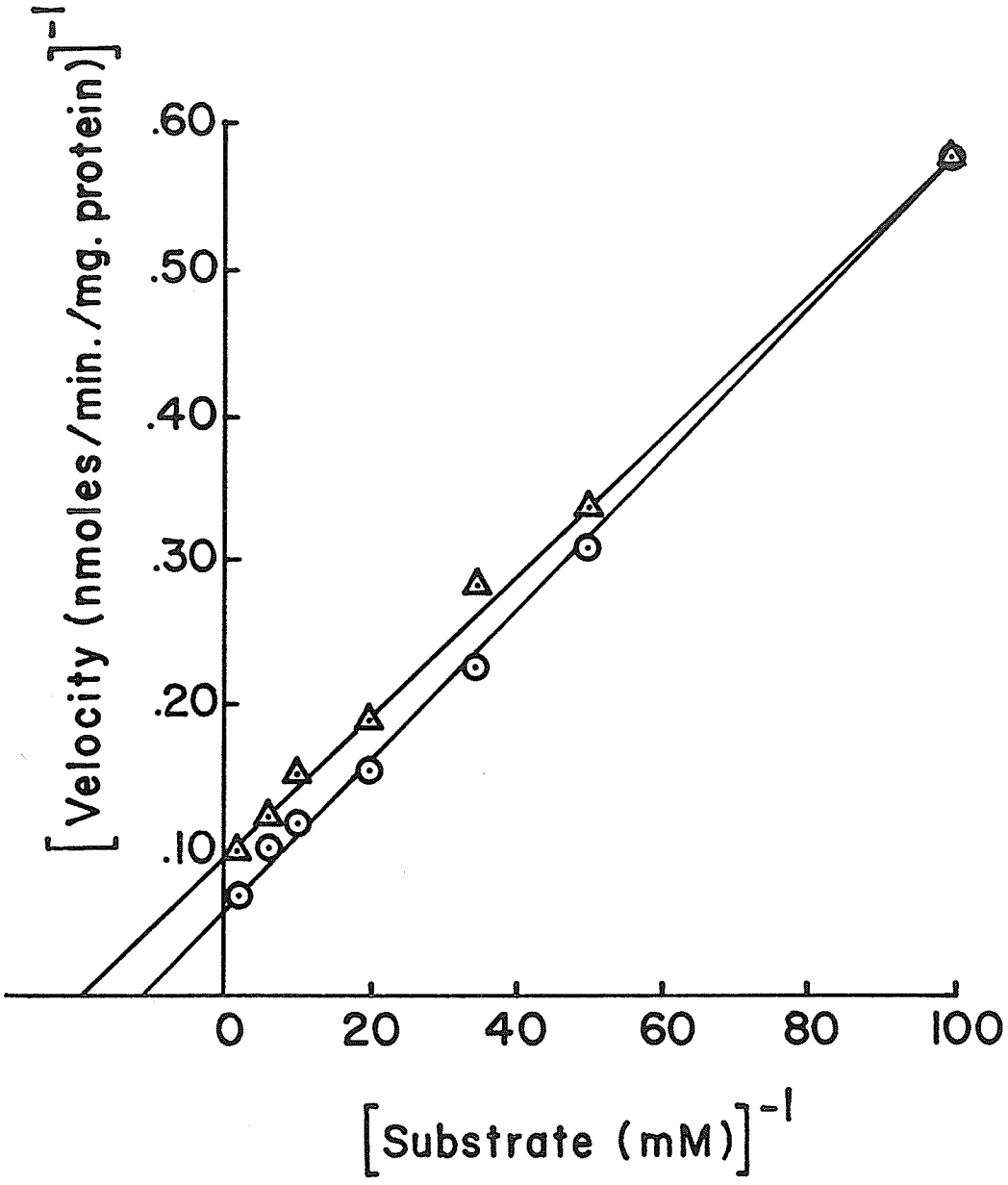

[Velocity (nmoles/min./mg. protein)]⁻¹
.60
.50
.40
.30
.20
.10
0
20
40
60
80
100
[Substrate (mM)]⁻¹

Figure 17. Double reciprocal plot of L-leucine uptake in wild-type and C^R-7 monolayers at 34°C. The uptake medium contained L-leucine at various concentrations and at a final specific activity of 1 μCi/μmole in Dulbecco's phosphate-buffered saline with 0.1% (w/v) glucose. Uptake was performed in 35 mm plates for 1.0 minute and was estimated from the ethanol-soluble fractions. Each point represents the mean of two determinations. The mean cell density of the wild-type population (⊙) was 1.2×10^6 cells per plate and the mean protein content was 0.10 mg. The mean cell density of the C^R-7 population (△) was 7.0×10^5 cells per plate and the mean protein content was 0.14 mg.


[Velocity (nmoles/min./mg. protein)]$^{-1}$
.60
.50
.40
.30
.20
.10
0
20
40
60
80
100
[Substrate (mM)]$^{-1}$

α-methylaminoisobutyric acid, and L-alanine. The K_m and V_{max} values of L-leucine entry are lower in the C^R-7 cells than in the wild-type cells. The variant appears to have a K_m which is half of the wild-type value. The V_{max} is similarly reduced in the C^R-7 line.

Inhibition of L-proline, L-alanine and L-leucine uptake by various amino acids

Inhibition studies were conducted in an attempt to further characterize the amino acid uptake systems in both the wild-type and C^R-7 cell lines. The test amino acids used were L-proline, L-alanine, and L-leucine. Several amino acids were tested for their ability to compete with these radiolabelled amino acids for uptake. Wild-type and C^R-7 cells exhibited no major differences (Table VI). All of the inhibitor amino acids were observed to compete with the test amino acids for uptake, but the degree of inhibition varied with the test amino acid. The inhibition of L-proline uptake in both the wild-type and C^R-7 lines was the greatest with the short, linear amino acids such as L-alanine and L-serine. The one exception was with L-threonine, which did not greatly inhibit the uptake of L-proline. Glycine, which is the simplest in structure, was not as effective an inhibitor as the larger, linear amino acids. The amino acid analogue, α-methylaminoisobutyric acid, was also a strong inhibitor of L-proline uptake. The bulkier amino acids, which have either branched or ringed side groups, did not appreciably inhibit the uptake of L-proline. This group included L-leucine through to L-tryptophan. Similar results

TABLE VI

Inhibition of amino acid uptake in wild-type and C^R-7 monolayers at 34°C by various amino acids[1].

Inhibitor (5 mM)	Percent inhibition of uptake in wild-type			Percent inhibition of uptake in the C^R-7		
	L-proline	L-alanine	L-leucine	L-proline	L-alanine	L-leucine
None	0	0	0	0	0	0
Glycine	50	49	33	53	32	17
L-alanine	91	91	66	86	89	57
L-serine	80	90	66	78	90	59
L-threonine	22	78	84	17	63	87
L-proline	85	48	43	81	42	26
L-methionine	94	85	91	91	74	91
α-methylaminoisobutyric acid	78	20	9	81	14	13
L-leucine	37	63	94	39	51	93
L-isoleucine	7	38	95	14	15	95
L-valine	25	46	92	9	25	91
L-phenylalanine	26	34	93	22	8	92
L-tyrosine	24	26	94	30	6	93
L-tryptophan	19	43	96	23	6	96

[1]The uptake medium contained either L-proline, L-alanine or L-leucine at 0.1 mM and a final specific activity of 5 μCi/μmole, 5 mM inhibitor amino acid and 0.1% (w/v) glucose in Dulbecco's phosphate-buffered saline. Uptake was performed in 35 mm plates for 1.0 minute and was estimated from the ethanol-soluble fractions. Each value is expressed as percent inhibition of the control value, which was obtained in the absence of 5 mM inhibitor amino acid, and is an average of duplicate trials. Wild-type cell numbers ranged from 1.5×10^6 to 1.7×10^6 cells per plate. Wild-type protein ranged from 0.15 to 0.16 mg. C^R-7 cell numbers ranged from 9.6×10^5 to 1.1×10^6 cells per plate. C^R-7 protein ranged from 0.18 to 0.25 mg.

were observed for the inhibition of L-alanine uptake. The linear amino acids were more effective inhibitors in comparison to the bulkier amino acids. Unlike L-proline uptake, L-alanine uptake was inhibited by L-threonine. Conversely, L-proline which effectively inhibited its own uptake, was not similarly effective in competing with L-alanine for uptake. Further, α-methylaminoisobutyric acid was less effective as an inhibitor of L-alanine uptake than of L-proline uptake. A significant degree of inhibition was observed in the presence of L-leucine in both the wild-type and C^R-7 cell lines. This inhibition was not as pronounced in the C^R-7 cells. The remaining amino acids with branched or ringed side groups, including L-isoleucine through to L-tryptophan, did not inhibit L-alanine uptake to the same extent as did L-leucine. The C^R-7 cells exhibited less competition for uptake by these amino acids than did the wild-type cells. L-phenylalanine, for example, inhibited the uptake of L-alanine in the wild-type by 34% while in the C^R-7 line an inhibition of 8% was observed. The uptake of L-leucine in both the wild-type and C^R-7 cell lines was greatly inhibited by all of the bulkier amino acids. Inhibition by the linear amino acids was not as pronounced. L-threonine, which inhibited L-alanine uptake, was also an effective competitor of L-leucine uptake in both cell lines. L-alanine and L-serine both significantly inhibited L-leucine uptake. The analogue, α-methylaminoisobutyric acid, had a negligible effect. L-methionine, as observed for the previous test amino acids, was also an effective inhibitor of L-leucine uptake.

The effects of concanavalin A on the initial rates of L-proline, L-alanine, and L-leucine uptake

Concanavalin A at three concentrations was tested for its effects on the uptake of L-proline, L-alanine, and L-leucine in wild-type and C^R-7 cells (Table VII). At all concentrations tested, no effects were observed on the initial rates of uptake of either L-proline, L-alanine, or L-leucine in the C^R-7 cells. Conversely, in the wild-type, inhibition of the initial rate of L-alanine and L-leucine uptake was observed. The initial rates of L-alanine and L-leucine uptake gradually decreased as the concentration of concanavalin A increased from 50 to 500 µg/ml. Maximum inhibition was about 30% of the control for the initial rates of uptake of both L-alanine and L-leucine. This was observed at a concanavalin A concentration of 500 µg/ml. L-proline uptake in the wild-type line was essentially unaffected by concanavalin A. At 500 µg/ml, a slight inhibition of the initial rate of uptake was observed, but this was not as pronounced as the inhibitions of the other test amino acids. The C^R-7 line exhibited no inhibitions of initial rates in the presence of the lectin.

TABLE VII

The effects of concanavalin A on the initial rates of L-proline, L-alanine, and L-leucine uptake in wild-type and C^R-7 monolayers at 34°C[1].

Concanavalin A concentration (μg/ml)	L-proline		L-alanine		L-leucine	
	Initial rate[2]	% of control	Initial rate[2]	% of control	Initial rate[2]	% of control
Wild-type[3]						
0 (Control)	2.5 ± 0.1	100	12.0 ± 0.5	100	8.4 ± 0.1	100
50	2.4 ± 0.2	96	11.0 ± 1.3	92	7.8 ± 1.0	93
250	2.6 ± 0.2	104	9.6 ± 1.2	80	6.6 ± 0.2	79
500	2.2 ± 0.1	88	8.3 ± 0.9	69	6.0 ± 1.1	71
C^R-7[4]						
0 (Control)	1.6 ± 0.2	100	9.6 ± 0.7	100	7.5 ± 0.4	100
50	1.7 ± 0.3	106	9.2 ± 0.8	96	7.4 ± 0.2	99
250	1.7 ± 0.1	106	9.2 ± 1.0	96	7.8 ± 0.4	104
500	1.7 ± 0.1	106	8.8 ± 0.8	92	7.6 ± 0.1	101

[1]Concanavalin A was prepared in DPBS. Cells were incubated with the Con A for 15 minutes followed by a 15 minute incubation with DPBS only. The amino acids were present in the uptake medium at 0.1 mM at a final specific activity of 5 μCi/μmole.
[2]Initial rate was estimated from one minute of uptake and is expressed as nmoles per mg protein per minute. Each value represents the mean of two determinations. All values were corrected for non-specific binding as estimated from 0 minutes of uptake at 0°C. The control values were obtained from two 15 minute incubations with DPBS only.
[3]Wild-type averaged at 1.4×10^6 cells per plate and 0.12 mg protein.
[4]C^R-7 averaged at 1.1×10^6 cells per plate and 0.18 mg protein.

DISCUSSION

DISCUSSION

The uptake of neutral amino acids in many animal cells occurs by three distinct systems which have been designated A, ASC and L (1,4). These three systems can be distinguished on the basis of a number of criteria including amino acid preferences and sensitivity to sodium ion (Table I) (4). Oxender and Christensen first described the A and L systems from results of time course and inhibition studies (8). The comparison of the time courses of a number of amino acids revealed two categories of uptake. An amino acid, such as α-aminoisobutyric acid, was taken up slowly to high steady-state levels. Amino acids, such as leucine and valine, reached low steady-state levels, but initial rates of entry were high (8). Similar patterns of uptake were observed in the wild-type and C^R-7 Chinese hamster ovary cells (Figs. 6-9). An additional category, in which amino acids were rapidly taken up to high steady-state levels, was also observed. This category included the amino acids L-alanine and L-threonine. In both the wild-type and C^R-7 cells, these two amino acids were taken up rapidly over the first 10 minutes of incubation and attained high steady-states between 10 and 20 minutes (Figs. 6b and 7a). Edmondson *et al.* (41) have also reported the rapid accumulation of L-alanine in freshly isolated rat hepatocytes, with steady-state levels observed in 15 minutes of incubation. Similar findings were also reported for Balb/3T3 cells (22). The uptakes of L-proline, α-aminoisobutyric acid, and α-methylamino-

isobutyric acid in the wild-type and C^R-7 cells are similar to those previously reported (38, 41, 55, 56). Uptake of these three amino acids in the wild-type and C^R-7 cells was slow and fairly linear over time and no steady-state was obvious by 40 minutes of incubation (Figs. 6a, 9a, and 9b). The levels attained by 40 minutes of incubation suggested that a very high steady-state would eventually be reached. Steady-state levels of α-aminoisobutyric acid were also not observed in either baby hamster kidney cells (56) or Chinese hamster ovary cells (55) for over 60 minutes of incubation. Edmondson *et al.* (41) have reported that α-aminoisobutyric acid uptake in hepatocytes finally attained steady-state levels, comparable to those of L-alanine, in 90 minutes of incubation. The uptake profiles of L-leucine, L-isoleucine, and L-phenylalanine in both the wild-type and C^R-7 cells agree with the profiles described in Ehrlich ascites tumor cells (Figs. 7b, 8a and 8b) (8). Uptake of these amino acids was fairly rapid initially, but only low levels of accumulation were observed. The steady-states commenced between 5 and 10 minutes of uptake. Balb/3T3 cells similarly accumulated L-leucine and L-phenylalanine, but the steady-state levels reached appeared to be much lower than those observed in the wild-type and C^R-7 cells used in this study. Further, steady-state levels were reached in two minutes (22, 51, 52). The Balb/3T3 monolayers were less than 50% confluent in comparison to both the wild-type and C^R-7 cells which were near confluent. System L uptake appears to be stimulated as cells approach confluence (51). According to Gazzola *et al.* (38), the

uptake of system L reactive amino acids, such as L-leucine, increases as cells approach confluence due to increased intracellular concentrations of amino acids. The trans-stimulation of L-leucine and L-phenylalanine was also described in Balb/3T3 cells to be the result of increased levels of endogenous L-system amino acids (51). A similar phenomenon may be in operation in the wild-type and C^R-7 cell lines though attempts were made to deplete the cells of intracellular amino acids prior to amino acid uptake. Conceivably, higher levels of L-leucine, L-isoleucine, and L-phenylalanine may be a characteristic of the wild-type and C^R-7 cells, and may be subject to other, as yet unidentified, control mechanisms.

The time courses of amino acid uptake in the wild-type and C^R-7 cells revealed three patterns of uptake. Amino acids, such as L-alanine or L-threonine, rapidly entered the cells and reached high steady-state levels. Conversely, amino acids like L-leucine, L-isoleucine, and L-phenylalanine, reached only low levels of steady-state, regardless of the high initial rates of entry. Finally, amino acids such as L-proline, α-aminoisobutyric acid, and α-methylaminoisobutyric acid, were slowly accumulated to high levels which did not plateau in 40 minutes of incubation. Clearly, the steady-state level attained can not always be predicted by the initial rate of uptake. The steady-state relies on the net operation of a number of factors including both uptake and exodus (1,4), Amino acids which demonstrate only low steady-states while their initial rates of uptake are high are probably more strongly involved in exodus than the amino acids of the other two categories (1,8). These results suggest the existence of two systems

(and possibly a third) for neutral amino acid uptake in the wild-type and C^R-7 cells.

The differences observed between the amino acid uptake profiles of wild-type and C^R-7 cells are slight in comparison to those reported for transformed cells and their corresponding normal cell lines (55, 56). The wild-type and C^R-7 profiles are comparable up to the steady-states, after which lower levels were consistently observed in the variant (Figs. 6-9). The only exception was observed with L-alanine uptake, which displayed slightly higher sustained levels in the C^R-7 (Fig. 6b). The initial rates of uptake (Table IV) were similar in the wild-type and C^R-7 lines indicating that few differences in actual uptake of these amino acids exists. L-threonine and α-methylaminoisobutyric acid may be taken up by the C^R-7 cells at slightly lower initial rates but the differences observed are not as great as those observed between transformed and non-transformed cells (55,56). Polyoma virus-transformed baby hamster kidney cells consistently displayed a 2.5- to 3.5-fold higher uptake of both α-aminoisobutyric acid and cycloleucine at all times, including initial uptake, in comparison to the non-transformed cells (56). The differences reported between the wild-type and C^R-7 cells are not as dramatic and are only obvious during steady-state accumulation. The lower steady-states attained by the C^R-7 cells may reflect increased rates of exodus of these amino acids or an altered response to endogenous amino acids. Conceivably, the depletion of endogenous amino acids may not be as complete in the wild-type as in the C^R-7 population. Alternatively, since steady-state levels varied from experiment to experiment with the same amino acid, differences

between the wild-type and C^R-7 lines may merely reflect experimental variation. This is difficult to justify in view of the observation that 7 of the 8 amino acids tested revealed lower levels in the variant as compared to the wild-type population.

The Na^+-sensitivity of amino acid uptake has been studied extensively in a number of cell lines (1,4,19-22). This property has often been used to differentiate the A and ASC systems from the L-system. The latter is relatively Na^+-insensitive in comparison to both systems A and ASC, which display marked Na^+-dependency (Table I) (4). Energization of the A and ASC systems may be partly achieved by a coupling to the sodium ion-flux down its concentration gradient. Consequently, in the absence of Na^+, the gradient is dissolved and the uptake of A and ASC reactive amino acids is reduced (23-27). The Na^+-sensitivity of L-proline, α-methylaminoisobutyric acid, L-alanine, and L-leucine uptake has been described in the wild-type and the C^R-7 cells (Figs. 10-13). All of the amino acids displayed Na^+- sensitivity, but to different degrees. Both L-proline and α-methylaminoisobutyric acid uptake displayed the most striking dependence on sodium ion with almost complete loss of uptake in the absence of sodium (Figs. 10 and 11). The uptake of L-alanine was also greatly dependent on sodium ion, but to a lesser extent (Fig. 12). At least 15% and 23% of the total uptake of this amino acid in the wild-type and C^R-7 cells, respectively, was contributed by Na^+-independent agencies. Similar findings have recently been described in Chinese hamster ovary cells grown on glass coverslips (93). The uptake of L-leucine was the least Na^+-sensitive

in both the wild-type and C^R-7 cells (Fig. 13). Previous reports with Balb/3T3 cells described the uptake of L-leucine as Na^+-insensitive (22). The present study revealed that in both the wild-type and C^R-7 populations, a significant portion of the total uptake was contributed by Na^+-sensitive agencies. The recent report with cultured human fibroblasts supports this data (38). At least 25% of L-leucine uptake in the fibroblasts was attributed to Na^+-sensitive routes. The Na^+-sensitivity of L-leucine uptake in the wild-type and C^R-7 cells is more pronounced, with an estimated 50% and 40% of the total contributed by Na^+-sensitive routes, respectively (Fig. 13). The reasons for the enhanced Na^+-sensitivity are unknown, but may be related to the higher steady-state levels of L-leucine attained in these cells in comparison to other cell lines. Regardless, neutral amino acid uptake, in both the wild-type and C^R-7 cell lines proceeds by at least two systems which can be discriminated on the basis of Na^+-sensitivity. At least one of these systems is Na^+-insensitive and largely responsible for the uptake of L-leucine. This system may be similar to system L described in Ehrlich ascites tumor cells (8). No major differences, in relation to sodium ion-sensitivity, were observed between the wild-type and C^R-7 cells (Figs. 10-13).

Estimations of the kinetic parameters of amino acid uptake from double-reciprocal plots have been useful in comparing uptake in transformed and non-transformed cells (55,56). Isselbacher (56) has indicated that uptake is not likely to conform to classic Michaelis-Menton kinetics. Consequently, two cell lines should not be

compared solely on the basis of kinetic parameters. The kinetic parameters of L-proline, α-methylaminoisobutyric, L-alanine, and L-leucine uptake in the wild-type and C^R-7 lines were estimated from their respective double-reciprocal plots (Table V, Figs. 14-17). The K_m estimates were similar to those previously reported for L-alanine and L-leucine uptake in Chinese hamster ovary cells (22). The latter report also described a biphasic double-reciprocal plot for L-leucine uptake. This was not observed in the current study for either the wild-type or the variant populations. Possibly, the concentration range used was too narrow to detect this phenomenon. Similarly, Ehrlich ascites tumor cells did not display a biphasic double-reciprocal plot for L-leucine uptake (8). The wild-type and C^R-7 cells exhibited similar kinetic parameters with the exception of the L-leucine uptake parameters (Table V). The K_m and V_{max} values for L-leucine uptake in the C^R-7 line were considerably lower than the wild-type values. Considerable variation in the kinetic analysis of L-leucine entry into Chinese hamster ovary cells has been reported (22,51). A similar variation may be reflected in the K_m and V_{max} differences observed between the wild-type and C^R-7 cells in this study. Differences reported between transformed and normal cell lines are much greater than those observed between the wild-type and C^R-7 cells (55,56). The V_{max} values for both α-aminoisobutyric acid and cycloleucine uptake in SV40-transformed Balb/3T3 cells were estimated to be 3-fold higher than the values in the corresponding normal cells (56). Further, no changes in K_m were reported. The differences observed between the wild-type and C^R-7 cell lines for L-leucine uptake

were probably the result of experimental variation. Alternatively, alterations in the C^R-7 membrane structure may indirectly affect L-leucine uptake in a general and non-specific manner.

Inhibition studies have been indispensible in the differentiation of the A, ASC and L systems. The systems exhibit characteristic amino acid preferences which may be described by inhibition experiments designed to study the competition between various amino acids for uptake (1,4). The existence of the A and L systems was first described in Ehrlich ascites tumor cells partly from the results of inhibition studies (Fig. 2) (8). Generally, the A system displays reactivity with the amino acids which have either short, linear, or polar side groups. The L-system prefers the bulkier amino acids which have either branched or ringed side groups. The ASC system is similar to the A system in amino acid preference, but it is intolerant of N-methylated amino acids. The amino acid analogue, α-methylaminoisobutyric acid, is consequently a characteristic substrate of system A (1,4,6,13,20,36). Similar divisions of amino acid preference were observed in both the wild-type and C^R-7 cell lines (Table VI). The uptake of radiolabelled L-proline and L-alanine was generally inhibited by the amino acids which have short, linear or polar side chains, such as L-serine. The bulkier amino acids such as L-isoleucine and L-tryptophan had minimal effect. The converse was observed with L-leucine. These results suggest the existence of at least two systems, as previously reported, which display different amino acid preferences. One of these systems may be similar to system L, which is largely responsible for the uptake of

amino acids, such as L-leucine (4). Closer examination of the results revealed that two distinct systems were responsible for the uptake of amino acids with short, linear or polar side groups (Table VI). The uptake of L-proline was greatly inhibited by α-methylaminoisobutyric acid. The uptake of L-alanine and L-leucine, conversely, was not greatly inhibited by this analogue (Table VI). As previously mentioned, N-methylated amino acids are not tolerated by system ASC. This allows α-methylaminoisobutyric acid to be used as a characteristic substrate of system A (4). The ability of this analogue to greatly inhibit L-proline, but not L-alanine, uptake suggests that two different systems are in operation. These may be analogous to the A and ASC systems, respectively. Further, L-proline has been described as a model substrate of system A in cultured human fibroblasts (38). According to Christensen (6), the imino acid structure of proline is analogous to the N-methyl group of α-methylaminoisobutyric acid. Consequently, only limited uptake of L-proline by system ASC will occur. This amino acid, in both the wild-type and the C^R-7 lines, did not greatly compete with L-alanine for uptake (Table VI). The system which was responsible for the majority of L-alanine uptake is similar to system ASC. This is further supported by the results of inhibition by L-threonine. This amino acid, which has been described as a preferred substrate of system ASC (4), was largely incapable of inhibiting L-proline uptake. Conversely, the inhibition of L-alanine uptake in the presence of L-threonine, was marked. L-threonine was also a potent inhibitor of L-leucine uptake in both the wild-type and C^R-7 cell lines

(Table VI). L-threonine was not an effective inhibitor of L-leucine uptake in either Ehrlich ascites tumor cells (8) or Balb/3T3 cells (22). A number of other anomalies concerning the inhibition of L-leucine uptake was also observed in this study. The amino acids, L-alanine and L-serine, which do not inhibit L-leucine uptake in Balb/3T3 cells (22), significantly competed with L-leucine uptake in both the wild-type and C^R-7. Since these are all substrates of the system which interacts with L-alanine, a significant portion of L-leucine uptake may also be mediated by this system. Further, L-leucine was capable of competing with L-alanine for uptake (Table VI). Results, previously discussed, indicated that 50% and 40% of the total leucine uptake in wild-type and C^R-7 cells, respectively, was contributed by Na^+-sensitive agencies (Fig. 13). Since L-alanine uptake was highly Na^+-sensitive (Fig. 12), the Na^+-sensitive component of L-leucine uptake was possibly mediated by the system responsible for alanine uptake. The uptake of L-proline was also Na^+-sensitive (Fig. 10), but in the present study, the system responsible for L-proline uptake probably did not contribute to L-leucine uptake, as the analogue, α-aminoisobutyric acid, did not inhibit L-leucine uptake (Table VI). Further, L-proline exhibited less inhibition of L-leucine uptake than did L-alanine. The significance of the enhanced Na^+-sensitive uptake of L-leucine by this system is unknown. Gazzola *et al.* (38) have also reported a significant contribution by Na^+-sensitive agencies to the total uptake of L-leucine in fibroblasts. Contrary to the results of this study, the uptake of L-leucine was inhibited by α-methylaminoisobutyric acid (38).

Three separate systems appeared to function in the uptake of neutral amino acids in the wild-type and C^R-7 cell lines. The system which was largely responsible for L-proline uptake differed from that which mediated L-alanine uptake. In addition to having affinity for both L-alanine and L-serine, the former system demonstrated high affinity for the amino acid analogue, α-methylaminoisobutyric acid. Further, this system did not appreciably interact with L-threonine or any of the bulkier amino acids with branched or ringed side groups (Table VI). Based on the extreme Na^+-sensitivity of both L-proline and α-methylaminoisobutyric acid uptake (Figs. 10,11), this system is probably also dependent on the sodium ion. These characteristics have also been associated with system A (1,4,6,13,20,36). Consequently, the system responsible for most L-proline uptake in both the wild-type and C^R-7 cell lines, may be analogous to system A. The system which was studied with L-alanine was also highly reactive with L-serine and L-threonine, but did not tolerate α-methylaminoisobutyric acid. This system demonstrated only low affinity for all of the bulkier amino acids but L-leucine. The uptake of L-leucine may be partly mediated by this route. Based on the Na^+-sensitivity of L-alanine uptake (Fig. 12), the system which is responsible for L-alanine uptake may also be Na^+-sensitive. This system may be similar to the ASC system (1,4). A Na^+-insensitive system which mediated a considerable portion of L-leucine uptake (Fig. 13) may also be reactive with other structurally similar amino acids, such as isoleucine and phenylalanine. This system may be similar to system L. As supported by previous studies (8,22,38),

inhibition of L-proline, L-alanine, and L-leucine uptake was achieved to a certain extent by all of the inhibitor amino acids (Table VI). The three systems are all likely to have an affinity, if only a limited affinity, for all of the neutral amino acids. Methionine, which appeared to greatly inhibit the uptakes of all of the test amino acids, may display structural features which are recognized by all three systems (8).

No major differences between the wild-type and the C^R-7 cell lines were disclosed by the inhibition studies (Table VI). The inhibition of L-alanine uptake by the bulkier amino acids was generally less in the variant than in the wild-type cells. This may indicate that these amino acids may not utilize the system, which was responsible for the uptake of alanine, to the same extent in the variant as in the parental cells. Generally, similar patterns of inhibition were observed with the C^R-7 cells as compared to the wild-type cells.

Differences between the wild-type and C^R-7 uptakes of neutral amino acids were observed following incubation with concanavalin A (Table VII). Concanavalin A is a lectin which can be used as a membrane probe to study membrane structural differences between cell lines (58). The lectin has been used to study the membrane changes which accompany transformation, relative to amino acid uptake (56,57). The uptake of L-alanine and L-leucine was significantly reduced in the wild-type cells following incubation with concanavalin A. Maximum inhibition was about 30% and was observed at the highest concentration of lectin tested (500 µg/ml). Conversely, no effect on neutral amino acid

uptake in the C^R-7 cells was observed following treatment with the lectin. Further, L-proline uptake did not decrease in the wild-type cells (Table VII). This indicates that the effects of concanavalin A may involve specific amino acid uptake systems. Possibly, only the system which is shared by L-alanine and L-leucine is affected. SV40-transformed hamster cells behaved similarly to the wild-type Chinese hamster ovary cells of this study (57). The uptake of several amino acids was inhibited, in the transformed cells, following treatment with concanavalin A. The normal cell line was not affected (57). Another study, with polyoma virus-transformed and non-transformed baby hamster kidney cells, revealed that neutral amino acid uptake in both cell lines was reduced following treatment with concanavalin A. The transformed cell line exhibited a more pronounced inhibition than the normal cell line (56). The differential effects of concanavalin A on amino acid uptake in transformed and non-transformed cells may be similar to the effects observed with the wild-type and C^R-7 cells. The different responses to the lectin, detected by differences in neutral amino acid uptake, suggest that the wild-type and C^R-7 cells have membrane structural differences which may be related to amino acid uptake. Inbar *et al.* (57) have suggested that the amino acid transport sites of the transformed cell membrane may have been re-located to positions closer to the concanavalin A binding sites. Consequently, steric hinderance of amino acid uptake may occur following binding of concanavalin A. A similar explanation may suffice to explain the differential effects of the lectin on the wild-type and C^R-7

cell uptake of neutral amino acids. Conceivably, amino acid uptake sites in the C^R-7 cells may have been relocated to positions away from concanavalin A binding sites. The binding of the lectin to the C^R-7 cell surface would consequently not interfere with neutral amino acid uptake. Alternatively, the differential effects of concanavalin A on the wild-type and C^R-7 cell amino acid uptake may be related to the different abilities of these cell lines to bind concanavalin A (Table II). The SV40-transformed and non-transformed hamster cells were estimated to bind similar numbers of concanvalin A molecules (57). Conversely, the C^R-7 cells used in this study were reported to bind from 2.5- to 3-fold less lectin than the wild-type (72). Consequently, a higher concentration of concanavalin A may be required to elicit an effect on the uptake of amino acids by the C^R-7 cells. The highest concentration used in this study (500 µg/ml) has previously been shown to be sufficient to agglutinate only 50% of the C^R-7 cells. In comparison, all of the wild-type cells are agglutinated at this concentration (71). The distribution of concanavalin A on the wild-type and C^R-7 cell surfaces may also be important, particularly in terms of the possible steric hinderance of amino acid uptake. The C^R-7 cell line has a reduced concanavalin A receptor mobility. Consequently, cap formation is prevented in this cell line (76). Cap formation in the wild-type may be all that is required to cause the steric interference of neutral amino acid uptake. The inability of concanavalin A to inhibit L-proline uptake in the wild-type (Table VII) suggests that the effects of this lectin on amino acid uptake may be mediated by more

specific membrane interactions. Regardless, the position of the neutral amino acid uptake sites, relative to concanavalin A binding sites, may be an important feature in the resistance of the cells to the cytotoxic effects of concanavalin A (57). The possibility that concanavalin A may exert its effects on amino acid uptake as a secondary consequence of some other interaction can not be excluded from the results of this study.

CONCLUSION

The wild-type and C^R-7 Chinese hamster ovary cell lines contain at least three separate systems which operate in the uptake of neutral amino acids. The systems expressed several characteristics in common with the A, ASC, and L systems which have been previously described in a variety of cell lines (1,4,6,13,20,36). Few major differences between the wild-type and C^R-7 cells were observed in relation to the characteristics of the uptake systems.

Concanavalin A was observed to have a differential effect on the neutral amino acid uptake in the wild-type and C^R-7 cell lines (Table VII). This effect may be related to a similar phenomenon which was observed in transformed and non-transformed cell lines (56,57). The insensitivity of the C^R-7 cell line to the cytotoxic effects of concanavalin A may be related, at least in part, to the failure of the lectin to influence neutral amino acid uptake in the C^R-7 cells.

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