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A THESIS

entitled

AMINO ACID TRANSPORT IN CULTURED MAMMALIAN CELLS

by

DOUGLAS MACKENZIE SCOTT

In part fulfilment of the requirements

of the degree of

Doctor of Philosophy

Department of Genetics
University of Glasgow

September 1975

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The Author graduated with a B.Sc. Honours degree in Microbiology from Queen Mary College, University of London in 1971. Subsequent to that, he worked as a research student on the studies described in this thesis from October, 1971 to October, 1974. Since that time he has been employed as a Research Associate in the University of Manchester. The author declares that results presented in this thesis have not been submitted for any other degree.

ACKNOWLEDGMENTS

This thesis is an account of work carried out in the Department of Genetics, from October 1971 to October 1974, under the supervision of Professor J. A. Pateman.

I would like to thank Professor Pateman for his supervision and his encouragement throughout this project. I would further like to thank Dr. R. A. Elton and Mr. A. Gibbs for their help in statistical analysis, and all members of the Department of Genetics, especially Drs. M. Hooper and R. H. Wilson for their helpful discussion.

The receipt of a Postgraduate scholarship for Training in Research Methods from the Science Research Council is also gratefully acknowledged.

ABBREVIATIONS

The following are abbreviations which occur frequently in this thesis:-

EFC_{10}	:-	Glasgow modification of Eagles minimum essential medium, plus 10% foetal calf serum.
EFC_{20}	:-	Glasgow modification of Eagles minimum essential medium, plus 20% foetal calf serum.
EFC_{5}	:-	Glasgow modification of Eagles minimum essential medium, plus 5% foetal calf serum.
$EFC_{0.5}$	:-	Glasgow modification of Eagles minimum essential medium, plus 0.5% foetal calf serum.
$EdFC_{10}$	:-	Glasgow modification of Eagles minimum essential medium, plus 10% dialysed foetal calf serum.
PBS	:-	Phosphate buffered saline (Dulbecco and Vogt 1954).
Cyclic AMP	:-	Adenosine 3' : 5'-cyclic monophosphate.
TCA	:-	Trichloracetic acid.
EMS	:-	Ethyl methane sulphonate.
∝-AIB	:-	∝-Aminoisobutyrate.

SUMMARY

The studies reported in this thesis relate essentially to the transport of the acidic amino acid L-glutamate and the neutral amino acid L-alanine by monolayer cell cultures. The transport of these amino acids was examined in a number of cell lines of different species or tissue origin, but in most detail in the baby syrian hamster kidney cell line BHK21-C13.

Both amino acids were shown to be transported by energy, pH, temperature and Na^+ dependent active transport systems capable of accumulating these amino acids to levels considerably greater than in the surrounding medium. L-glutamate was shown to be transported via a relatively high affinity, low capacity uptake system specific for the acidic amino acids L-glutamate, L-aspartate and certain of their analogues. L-alanine was transported via a separate, relatively low affinity, high capacity transport system of broad specificity. This system appeared capable of transporting all neutral L-amino acids with the exception of L-cysteine and L-cystine. This system however, showed a marked preference for L-alanine and the short polar unbranched neutral amino acids. In addition to this "L-alanine preferring" transport system a second neutral amino acid transport system of preferred substrates L-leucine and the branched chain aliphatic and the aromatic amino acids was indicated. Transport systems of similar specificity and characteristics to the acidic and L-alanine preferring transport systems of BHK21-C13 cells were also observed in a number of diploid or established cell lines of syrian or chinese hamster, mouse, sheep or human embryo. The characteristics of these systems did not appear to be altered following transformation by the DNA tumour virus

SV - 40 or Polyoma.

The level of L-glutamate and L-alanine uptake was also examined in BHK21-C13 cells following different cell or growth conditions, in order to investigate possible factors involved in the transport of their respective transport systems. These transport systems appeared to differ in their control mechanisms. L-alanine uptake was observed to be maximal in cells with low intracellular cyclic AMP levels, but reduced in cells with elevated levels of this cyclic nucleotide. L-glutamate however, appeared to be essentially independent of intracellular cyclic AMP levels. Similar observations were made for a number of transformed or non-transformed cell lines. No evidence for control of the uptake of these amino acids by intracellular amino acid levels was provided by these studies, as the transport of neither of the amino acids was altered following treatments designed to alter cellular amino acid levels. Addition of various hormones or growth in their presence similarly had no effect on L-glutamate or L-alanine uptake, with the exception of insulin, which stimulated L-alanine uptake when added to cells maintained in low serum.

In order to provide further evidence for the postulated acidic and L-alanine preferring transport systems, and possibly supply additional information about the specificity and nature of these uptake systems an attempt was made to isolate mutants defective in the transport of L-glutamate and L-alanine. Two major selection procedures were used, a) involving the selection for cells resistant to toxic amino acid analogues, and b) involving the selection for cells unable to transport radioactive amino acids. Such attempts however, proved unsuccessful.

The results reported in this thesis have thus indicated the presence of active amino acid transport systems in cultured mammalian cells and provided information as to their possible mechanisms of control.

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INTRODUCTION

1.1. Membrane Selectivity: Diffusion & Carrier Transport

The plasma membrane which surrounds all cells is necessary for the maintenance of the cell boundary and the internal environment of the cell. The membrane constitutes a barrier to the entry and exit of solutes but acquires selective permeability by the presence of certain transport systems capable of transporting certain metabolites such as amino acids, sugars, organic acids and certain metal ions, into or out of the cells.

Transfer across biological membranes has been postulated to be of two main types, a) 'Simple or Passive Diffusion', and b) 'Carrier Mediated Transport', which can be further divided into 'Facilitated Diffusion' and 'Active Transport' (Ussing 1952).

1.2. Passive Diffusion

Passive diffusion involves movement entirely under the control of physical forces and without direct interaction with the tissue components. The rate of transfer is directly proportional to the concentration gradient across the membrane and is affected by factors such as temperature, size of the diffusing molecule, solvent viscosity and nature of the membrane.

Diffusion can be defined as the migration of molecules from a region of higher concentration to one of lower concentration as a result of their random motion, and in accordance with the second Law of Thermodynamics. This law describes a system where energy will spontaneously pass from a region of higher energy to a region of lower energy, so that entropy (a measure of 'randomness') in the total system is increasing.

Many metabolic processes controlled by living organisms can produce local decreases in entropy by the use of energy derived from external sources. Furthermore, membrane barriers built and maintained by the use of metabolic energy may retard progress towards increasing entropy by reducing the rate of molecule movement.

The kinetics of diffusion has been adequately described by Riggs (1963) and Hartley and Crank (1949), from which details of this process can be obtained.

1.3. Carrier Transport

The term 'carrier transport' is used to describe metabolite transfer at rates higher than would be obtained merely by diffusion, and such transfer shows many kinetic properties similar to those of enzymes. For example, the rate of entry of a solute may be proportional to the concentration gradient at relatively low concentrations but at higher solute concentrations the rate of penetration reaches a maximum value. The term 'carrier transport' is used to explain such a process and saturation is attributed to the occupation of most of the limited number of sites in the membrane, to which it is believed that the solute must become attached before being transferred across.

Entry of solutes by facilitated (passive) diffusion does not permit movement of solute against a concentration gradient; thus the net flux of solute across the barrier ceases when the concentrations of solute inside and outside the cell are equal. The process of Active Transport, however, permits the transport of solute against a concentration gradient so that an equilibrium can be obtained where the intracellular concentration of the solute may be greater than that in the external environment.

Active transport mechanisms require energy which may be derived

from two general sources: a) an energy-producing chemical reaction coupled to the transport process, or b) an electrochemical gradient of a different solute across the barrier. The first type of system is called a Primary Active Transport System since only one solute is involved, whereas systems involving an electrochemical gradient of one solute which drives the movement of another solute are termed Secondary Active Transport Systems.

1.4. Evidence for specific Amino Acid Active Transport Systems

1.4.1. In Mammalian Systems

Early studies by Van Slyke and Meyer (1913) and Abel et al. (1914) demonstrated that amino acids were removed from the gut to the mesenteric and peripheral blood following administration of certain amino acids or proteins. For many years these and similar observations were interpreted as the absorption of amino acids into the bloodstream by simple diffusion, following their hydrolysis from protein (Verzar and McDougall 1936). However, the work of Gibson and Wiseman (1951) demonstrated that L-stereoisomers of amino acids were absorbed from the small intestine more rapidly than D-isomers, thus indicating that the absorption process was specific and, therefore, not merely passive diffusion. Matthews and Smyth (1954) demonstrated that this difference was also reflected by higher L-amino acid concentrations in the venous blood that drains from the small intestine. In vitro studies using segments of the small intestine also showed the accumulation of L-amino acids but not D-stereoisomers (Agar et al. 1953, 1954; Frindhandler and Quastel 1955).

In addition to in vivo and in vitro work on amino acid transport with the mammalian intestine, considerable work has been carried out by Christensen and his group (Christensen et al. 1952, a,b,c; Christensen and Riggs 1952, Riggs et al. 1952; and Christensen and Henderson 1952).

These workers tested various cell types for their ability to concentrate protein derived amino-acids and their close structural analogues. Ehrlich ascites tumour cells in particular were shown to concentrate most of the naturally occurring amino acids, and maintain higher distribution ratios of these amino acids than most other animal cells (Christensen and Henderson 1952).

Subsequently, studies have been carried out on other systems, showing the active accumulation of a wide variety of amino acids in various tissues. Such tissues include free suspensions of fibroblasts (Wilbrandt 1963), intact diaphragms (Akedo and Christensen 1962), isolated kidney tubules (Christensen and Jones 1962), kidney slices (Rosenberg et al. 1961), liver slices (Hanking and Roberts 1965), brain slices (Smith 1967) and erythrocytes (Christensen et al. 1952a).

1.4.2. In Microorganisms.

The first indications that bacteria could mediate the transfer of amino acids via specific active transport systems came from two main observations. The first was that certain bacteria could accumulate internally high levels of amino acids (Freeland and Gale 1947; Gale 1947, 1954), a process subsequently shown to be energy-dependent (Gale 1954; Chesbro and Evans 1962). The second was that certain cells were unable to utilise an externally applied substrate, although they possessed the relevant enzyme system(s), (Doudoroff 1951, Doudoroff et al. 1956). Such cases were subsequently shown to be due to an inability to transport these substances into the cell (Doudoroff et al. 1956).

The strongest evidence for the presence of membrane-bound specific transport systems is provided from studies on the isolation of macromolecular components of these systems. Penrose et al. (1968), and

Anraku (1968, a,b,c) have examined the properties of purified binding proteins which are specific for a group of amino acids (leucine, isoleucine and valine) and are released from E. coli cells by osmotic shock. Similar studies have been carried out by Ames and Lever (1970, 1972) with the Histidine-Binding Protein from Salmonella typhimurium. The evidence that these binding proteins are involved in the transport of these amino acids is summarised below:

a) The binding protein in unshocked cells is localised in the cell membrane (Penrose et al. 1970); b) Osmotic shock results in a loss of uptake activity and the release of the binding protein into the shock fluid (Piperno and Oxender 1966, Anraku 1968b, Ames and Lever 1970); c) The binding proteins have similar substrate specificities, and dissociation and binding constants to those observed in the unshocked cells; d) There is a repression of leucine uptake and the synthesis of binding proteins when the cells are grown in medium containing leucine (Penrose et al. 1970); e) The uptake of histidine is partially restored following incubation of the osmotically shocked cells in the presence of the binding protein (Anraku 1968c); f) The leucine binding protein has been shown to be capable of reversible conformational changes, although this has not been induced by binding of the amino acid (Penrose et al. 1970); g) Ames and Lever (1970) have isolated mutants defective in the uptake of histidine that have no binding protein and others which increased binding protein and increased histidine transport. Such studies have firmly established the existence of membrane bound transport systems capable of selectively transporting amino acids and other metabolites.

1.5. Transport Models

Transport models have been constructed in order to describe membrane transport at the molecular level. Any such model should be

required to account for the experimental observations and be constructed on the fewest and simplest assumptions.

Cohen and Monod (1957) proposed that the systems for penetration of organic molecules be termed "permeases." They suggested that the cell is bounded by an osmotic barrier which is essentially impermeable to amino acids. However, this impermeability is not absolute and slow leakage tends to slowly equilibriate the intracellular and extracellular concentrations. At intervals along the barriers are proteins (permeases) capable of specifically complexing with an amino acid. This complex associates or dissociates reversibly with the amino acid either inside or outside the barrier, and catalytically equilibriates the internal and external concentrations. When coupled to an energy donor, the internal association is in effect inhibited and the amino acid accumulates within the cells. As the internal concentration rises the non-specific leakage increases until its rate balances that of accumulation at equilibrium. This model assumes that the amino acid is in a free state within the cell. Britten and McClure (1962) discussed the limitations of this model, which although it accounts for a large number of experimental observations in bacteria, is insufficient to explain other phenomena. These include the fact that bacterial pool sizes are not determined by the initial rate of entry of the substrate, and the observation that cells are able to maintain their pools in the absence of external amino acid (Britten and McClure 1962). Subsequently, a number of general Transport System Models have been proposed, based mainly on the cyclical model described by Heinz and Walsh (1958) for Ehrlich ascites cells. These models have been discussed and elaborated by Pardee (1968); generally the process assumes a three step process for transport.

Step 1. Recognition involves the reversible binding for the substrate (A) to a specific macromolecule (X), in the membrane, to form a complex (A-X).

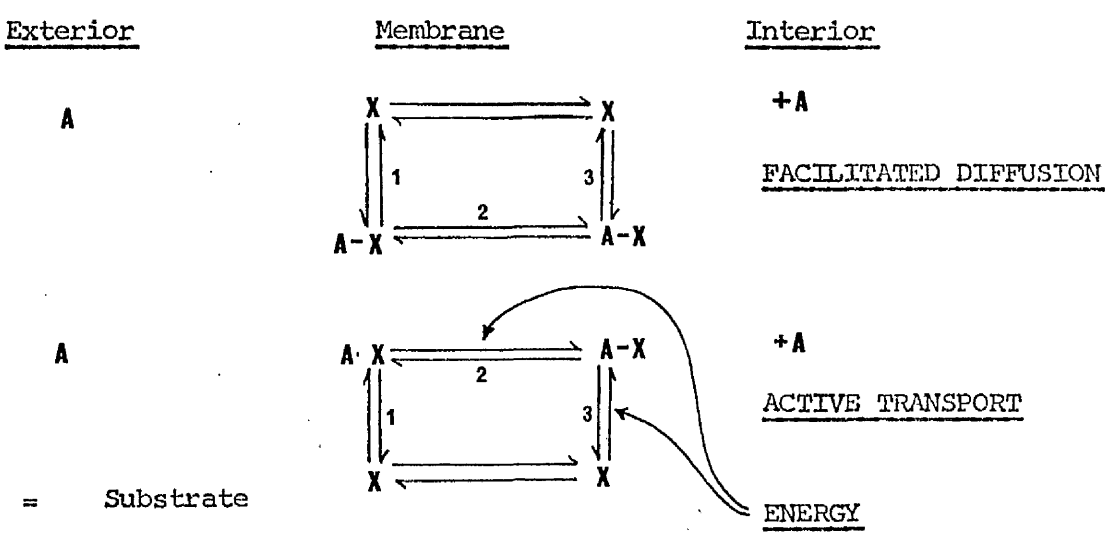
Step 2. Translocation of the substrate across the cell membrane.

Step 3. Recovery, where the substrate is released inside the cell and the macromolecule X is returned to its original state.

These three steps provide an explanation of the phenomenon of facilitated diffusion. The coupling of one of the above steps to metabolic energy makes the process effectively irreversible, and provides a model for Active Transport.

TRANSPORT MODEL - Penrose et al.(1970)

Pardee (1968)



A = Substrate

X = Carrier Mol.

A-X = Substrate Carrier Complex

1 = Recognition Process

2 = Translocation

3 = Recovery Step

This model has been amplified and modified by Oxender (1972) to explain certain phenomena observed in bacteria. Further modification may be necessary to explain the differences observed between different Transport Systems.

1.5. Components of Transport System Models

1.5.1. Recognition Process

Studies with intact cells have provided considerable evidence for a "specific recognition step" in the transport of substrates which takes place according to absorption-saturation kinetics. Evidence for the protein nature of these receptors has been provided from the recent studies described in 1.4.2 with isolation of specific binding proteins.

1.5.2. Translocation

There is really no clear evidence how the central process of transport, Translocation occurs. It has been suggested that the recognition proteins may function as an enzyme and bind the substrate to a low molecular weight carrier. The compound then diffuses across the cell membrane or is transported across the membrane as a result of physical change in the recognition protein. At one time the former hypothesis was favoured and phospholipids suggested as likely carrier molecules. Results on phospholipid turnover make this unlikely (Tarlov and Kennedy 1965). More recently it has been suggested that glutathione may act as a carrier molecule (Orlowski and Meister 1970). Several models such as : a) Rotational Diffusion, whereby small conformational changes may permit passage through the protein itself (Jardetsky 1966); or b) folding, contracting molecules, where one part of the protein may be physically moved through the membrane while the other part remains fixed, have been proposed to account for a protein carrier mediated translocation process. These models require conformational changes during translocation. No alteration of hydrodynamic properties have been observed when proteins bind to the substrate (Pardee 1966; Penrose et al. 1970), however changes in fluorescence of the binding protein, when it combines with its substrate, indicates an alteration

in the environment of the aromatic amino acids of the proteins, (Pardee 1966).

1.5.3. Recovery

So far no evidence is available from biochemical studies regarding the recovery step, where the carrier releases its substrate and returns to its original state.

A novel hypothesis has been proposed by Orlowski and Meister (1970) who suggest that the γ-glutamyl cycle may function in the transport of amino acids. They suggest that such a system has properties which fulfil the requirements of an amino acid transport system. It is proposed that the enzyme γ-glutamyl transpeptidase, (which is probably membrane bound) may combine with free amino acids and carrier (glutathione or possibly γ-glutamyl-cysteine)and could then function in the translocation process. The γ-glutamyl-amino acid formed might then be brought into the cell and the amino acid released, possibly by the hydrolytic activity of the transpeptidase or γ-glutamyl cyclotransferase. Injection of mice with one of several amino acids together with a competitive inhibitor of 5-oxoprolinase (which converts 5-oxoproline to glutamate) results in a greater accumulation of 5-oxoproline than if only inhibitor alone is added (Van der Warf et al. 1974). The authors state that the increased accumulation of 5-oxoproline in the eye is consistent with the involvement of the γ-glutamyl cycle in the transport of amino acid across the cilliary body, into the aqueous humour and then the ocular lens. However, the fact that studies on a human patient with 5-oxoprolinuria, a metabolic disorder characterised by excretion of large quantities of pyroglutamic acid in the urine, show no defect in amino acid absorption is inconsistent with this hypothesis.

1.5.4. Energetics

The step at which energy is required in the process of Active Transport is unclear but in at least two cases the energy input appears to be supplementary to the process of translocation. The facilitated diffusion system for β-thioethyl-glucopyranoside entry into yeast is always present, but this compound is actively transported only under certain inducible growth conditions (Okada and Halvorson, 1963). Similarly facilitated transport of β-galactosides to E. coli is not interfered with when energy inhibitors prevent their entry by Active Transport.

1.6. Coupling of Active Transport to Energy

With reference to the source of energy required to accumulate a substrate against its concentration gradient at least three major mechanisms have been postulated:

a) The Ion Gradient hypothesis was proposed by Riggs et al. (1958) and has been subsequently modified by Mitchell (1967), Morville et al. 1973, and Reid et al. 1974). This hypothesis proposed that amino acid transport is essentially a physiological process where ATP and similar compounds do not participate directly. The amino acid is transported, as a carrier complex, up a concentration gradient at the expense of cations such as Na^+, K^+ or H^+, which move down a concentration gradient. The energy for the former process is obtained by dissipation of the potential energy of the latter. It should be noted that metabolic energy is initially required to set up the counter gradient. The balance of charges would be maintained by either an induced efflux of other cations possibly by an energy dependent reaction or by a stimulated uptake of anions.

b) It has been proposed that the transport process is mediated directly by an energy-rich component built up by the metabolism of the cell.

Quastel (1961) suggested that the energy-rich component may be ATP.

c) It has been suggested that the transport process across cellular membranes may be directly coupled to the redox reaction of the respiratory chain (Conway 1951, 1964).

1.6.1. Energetics in Microorganisms

Studies with bacterial membrane vesicles have shown that the active transport of a wide variety of amino acids appears to be dependent on electron transfer, but is independent of oxidative phosphorylation, or the direct generation or utilisation of ATP (Kaback 1972). These transport systems have been shown to be primarily coupled, in *E. coli*, to D-Lactate oxidation by a membrane bound D-Lactate Dehydrogenase (D-LDH) (Barnes and Kaback 1971, Kaback 1972, Hong and Kaback 1972). Transport of most amino acids in *S. aureus* has been shown to be coupled almost exclusively to α-glycerol phosphate Dehydrogenase (GPDH) (Short *et al.* 1972), whereas in *B. subtilis* it is linked to GPDH, D-LDH and NADH Dehydrogenase (Kaback 1972).

Studies using an artificial electron donor have also shown that amino acid transport in a wide variety of *Pseudomonas*, *Proteus*, *Bacillus* and *Staphylococcus* species, is coupled to Dehydrogenases although specific enzymes have not been identified (Kaback 1972). Little work has been carried out with microbial systems regarding the possibility that solute transport is coupled to the flow of specific cations or protons. Aspartate and glutamate transport in *S. aureus* has been shown to be accompanied by proton movement (Gale and Lewellin 1972) and it is suggested by these authors that the proton gradient might drive transport of these amino acids. Proton transport has also been shown to accompany the transport of certain amino acids in *Saccharomyces* species (Eddy and Nowacki 1971, Seaston *et al.* 1973). Studies on the transport of glutamate, aspartate and alanine in *S. faecalis*, however,

suggest that the transport of these amino acids may be driven by K^+ ion gradients (Harold and Baarda 1967, Harold et al. 1970).

1.6.2. Energetics in mammalian systems

The source of energy for the concentration of amino acids in animal cells is still unclear although considerable work on the energetics of these systems has been carried out. The apparent stoichiometric entry of Na^+ ions and various neutral amino acids, together with the loss of K^+ ions from these systems has led to the hypothesis that energy for amino acid transport may be provided by the energy inherent in Na^+ and K^+ ion gradients (Eddy 1968, 1969). Early experiments by Eddy (1968, 1969) showed that mouse ascites cells, under conditions of complete metabolic inhibition, could concentrate amino acids from the extra-cellular fluid. As this accumulation was approximately one third of that observed under physiological conditions, it was suggested that only part of the energy for driving amino acids is obtained from ionic gradients. The remainder is provided by direct coupling of amino acid transport to cellular metabolism (Kimmich 1970, Schafer and Heinz 1971). Recent work has provided further evidence for all the energy for amino acid transport being supplied by the energy from Na^+ and K^+ ion gradients (Reid et al. 1974). These authors have demonstrated that physiological levels of methionine can be transiently accumulated in metabolically inhibited mouse ascites tumour cells incubated in the presence of valinomycin. Valinomycin, a K^+ selective ionophore, was added to increase the membrane permeability to K^+ and thus alter the membrane potential (which has been shown to affect methionine accumulation) (Gibb and Eddy 1972). The methionine gradient strongly correlated with the magnitude of the ionic gradients, thus suggesting Na^+ and K^+ ions drove the amino acid pump.

1.7. Kinetics of Carrier Transport

1.7.1. Kinetics

The initial rate of entry of a carrier mediated substrate is dependent on its external concentration, and usually obeys saturation kinetics that can be expressed in terms of the Michaelis-Menten equation of enzyme kinetics(Michaelis and Menten 1913). The unidirectional flux, V, is thus given by the expression:-

$$V = \frac{Vmax.\ S}{Km + S}$$

where S is the internal substrate concentration, Km is the external concentration of transported molecules to produce one-half the maximal rate of transport, and Vmax. is the maximum rate of transport.

The similarity between mediated transport systems and enzyme kinetics can be extended to include the action of inhibitors, both competitive and non-competitive. The action of competitive inhibitors is attributed to competition of the inhibitor for the "carrier site" of the permease and has the apparent effect of increasing the Km, while leaving the Vmax. unaltered. In the presence of competitive inhibitor, concentration I, Km is replaced by the expression $(1 + I/Ki)Km$, where Ki is the inhibitor constant-the concentration required to double the apparent value of Km. Non-competitive inhibition effectively reduces the number of carrier sites available to the substrate, thus decreasing the value of Vmax to $\frac{Vmax}{1 + I/Ki}$, where Km is unaltered.

At lower concentration the transport of amino acids shows saturation kinetics that often fit this Michaelis-Menten relationship. However, at high concentrations transport may deviate from this relationship owing to the contribution to total transport of diffusion or an apparently mediated non-saturable component (Christensen and Liang 1966) of transport. As this component is generally small compared

to the total transport it is often only appreciable at very high concentrations. Deviations from the Michaelis-Menten kinetics at moderate concentrations may indicate the operation of a second independent transport system for this amino acid.

1.8. Interactions between Amino Acids

The transport of different amino acid species has been shown to influence the movement of other amino acids across the membrane. This influence may be stimulatory or inhibitory. When an interaction occurs at the same side of the membrane the effect may be considered a cis-effect, and when interaction takes place at the opposite side of the transporting membrane a trans effect. Such phenomena may be described in terms of the hypothetical carrier model.

1.8.1. Cis Inhibition or Competition

The inhibition of amino acid transport by another amino acid is common and often shows the kinetic characteristics for competitive inhibition. This phenomenon is attributed to the competition of these amino acids for the same site on the carrier. Deviations from behaviour predicted solely on the basis of competitive inhibition may be observed, e.g. the Ki of a given amino acid when acting as an inhibitor is different to its Km value when transported. These deviations can often be attributed to the presence of different transport systems with overlapping specificities; for example, the 'A' (alanine-preferring) and the 'L' (leucine-preferring) transport systems in Ehrlich ascites tumour cells, (Oxender and Christensen 1963), the general and neutral systems in Neurospora crassa conidia

(Wolfinbarger and DeBusk 1972); the lysine and the arginine systems in Saccharomyces cerevisiae (Grenson 1966 a & b), and the D-alanine and L-alanine systems in E. coli (Wargel et al. 1970).

1.8.2. Cis Stimulation

In addition to inhibition of amino acid transport by the presence of other amino acids in the extracellular medium, influx may be stimulated by the presence of certain other amino acids or their analogues. This cis stimulation has been observed in Ehrlich ascites cells by Jacquez (1961) who observed a stimulation of tryptophan uptake by the presence of various neutral amino acids and the analogue azaserine. Guroff et al. (1964) have similarly observed the stimulation of the transport of aromatic amino acids by p-Fluoro-phenylalanine in sarcoma cells. Munck (1966) has also demonstrated that transcellular lysine transport in the rat ileum is stimulated by the addition of leucine. The transport of the 'stimulating substrate' was reduced during the stimulation process in these three cases. The actual mechanism of cis-stimulation is at present unclear and it is even disputed that this process is a true cis-effect. Wilbrandt (1961) has suggested that cis-stimulation may be the result of countertransport following entry of some of the stimulating amino acid. Munck (1966) reports that his results were in agreement with this explanation. Guroff et al. (1964), however, reported that stimulation of tryptophan transport in sarcoma cells occurred before sufficient of the stimulating amino acid (phenylalanine) could have entered the cells. Consequently, Guroff proposed that the prior binding of the "stimulating amino acid" to the carrier site, could promote a more rapid attachment of the "stimulated amino acid" by exchange than if the site was previously unoccupied.

Such a phenomenon was observed with substrate binding to enzymes (Heinz 1962). Jacquez (1967) subsequently proposed an explanation, not unrelated to Wilbrandt's hypothesis, which involved two distinct transport systems where rapid uptake of the stimulating amino acid by the first system would enable, on attainment of a certain internal concentration, an exchange counterflow mediated by the other system. Such an explanation is consistent with the stimulation of tryptophan by methionine via the 'A' and 'L' systems in Ehrlich ascites tumour cells (Oxender and Christensen 1963, Jacquez 1967). However, the absence of an increase in influx of lysine following preloading of the intestinal epithelium with leucine would appear inconsistent with this scheme (Munck and Schultz 1969).

1.8.3. Trans Stimulation

The influx and efflux of certain amino acids may be stimulated by solutes on the opposite (trans) side of the membrane. The first observation of this trans-stimulation was reported by Heinz (1954), who observed a stimulation of lysine influx in Ehrlich ascites tumour cells following preloading with glycine. Glycine influx was stimulated by preloading the cells with various other neutral amino acids, particularly those that cis-inhibited glycine transport (Paine and Heinz 1960, Heinz and Walsh 1958).

Two models have been proposed to explain trans-stimulation. The first "accelerated exchange counterflow", suggests that the loaded carrier moves more rapidly than the unloaded carrier, so that at steady state the flow is limited by the return of the empty carrier to its original side. This movement could be accelerated by exchange of substrate at the trans-side, so that the carrier is loaded in both directions. It is generally assumed that the two interacting amino acids are bound at the same site (although this is not necessary) and this mechanism would

allow for different specificities of transport and exchange. The alternative hypothesis, "competitive counterflow", suggests that the stimulating amino acid acts by inhibiting the backward flow of the stimulated amino acid. This mechanism requires the interacting amino acids to have an affinity for the same site, and it is the net flux and not unindirectional flux that is stimulated. Evidence for the former model is provided by the fact that preloading of Ehrlich ascites cells with L-alanine primarily resulted in a stimulation of the unindirectional flux of glycine and not the net accumulation (Heinz and Durbin 1957).

Specificity of trans-stimulation has been claimed (Heinz and Walsh 1958, Paine and Heinz 1960) to be the same as that of cis-inhibition but the observation by Clayman and Scholefield (1968) and the inability of methionine to trans-stimulate glycine influx (Paine and Heinz 1960) shows this is not always the case. Such differences may be interpreted in terms of the transported and stimulating amino acids being bound to different sites in the carrier, or alternatively in terms of two separate transport systems with overlapping affinities. Should this be the case countertransport may effect an energy coupling between these two systems e.g. as in the case of the 'A' and 'L' systems in Ehrlich ascites cells. Thus the 'L' system may accumulate amino acids provided that another substrate is maintained within the cell at a level sufficient to permit effective exchange (Heinz and Walsh 1958, Christensen 1969). Trans stimulation has also been observed in microorganisms, although the mechanism appears to be totally different from that in Ehrlich ascites cells. Gross et al. (1970) demonstrated that preloading of Streptomyces hydrogenans with glutamate, aspartate, or lysine stimulated the uptake of these and all other amino acids tested. Since a lag period of about 20 minutes was observed, it is suggested

that an amino acid derivative is the immediate trans-stimulator and kinetic observations suggest it acts by intensifying the energetic coupling between metabolism and transport.

1.8.4. Trans-Inhibition

Few examples of trans-inhibition, where the influx of an amino acid is inhibited after the cell has been preloaded with the same or a different amino acid, have been reported in animal cells and it is difficult to determine if these phenomena are genuine. In Ehrlich ascites cells the influx of glycine and ∝-AIB is slightly inhibited by preloaded methionine (Paine and Heinz 1960). Similarly trytophan transport has been reported to be inhibited by intracellular glycine (Jacquez 1961). However, it is difficult to determine whether the phenomenon is genuine if the preloaded solute is a good competitive inhibitor of the amino acid under study, since leakage of small quantities of the amino acid could competitively cis-inhibit the test substrate. The trans-inhibition of glycine and ∝-AIB have been interpreted in this way (Paine and Heinz 1960). Trans-inhibition occurs in a number of transport systems of different specificities in microorganisms. Histidine transport has been shown to be specifically inhibited by preloaded histidine in S. cerevisiae (Crabeel and Grenson 1970), and a relatively non specific neutral amino acid transport system in Streptomyces hydrogenans is trans-inhibited by substrates of that system (Ring et al. 1970). Trans-inhibition of the general and acidic transport system in Penicillium chrysogenum has also been shown to occur under conditions where the amino acid is transported more rapidly than it is metabolised. In this respect, L-∝-aminoadipic acid was shown to be a better trans-inhibitor than the preferred substrates of glutamate and aspartate (Hunter and Segel 1971).

The mechanism is unknown but a number of hypotheses have been

suggested. Bradfield et al. (1970) have proposed a simple scheme which assumes that the carrier moves from the outside only when charged with substrate, and from the inside to exterior when unchanged. They suggest that high intracellular concentrations of the substrate, anchors the mobile carrier at the internal site.

1.9. Interaction between Amino Acids and other Solutes

1.9.1. Interaction with Alkali Ions

The interaction between amino acids and various cations such as Na^+ and K^+ has been discussed in sections 1.6.1, 1.6.2, with regard to their possible co-transport and role in the production of energy available for Active Transport.

1.9.2. Interaction with Sugars

A relationship between renal transport of hexoses and amino acids in mammals has been well documented, and amino aciduria is observed to occur in a number of inherited diseases, including diabetes mellitus (Levitin 1974), galactosemia (Segal 1972), and the Fanconi Syndrome (Rosenberg and Scriver 1974). Amino aciduria can also be produced following galactose feeding to rats (Rosenberg et al. 1961), and hexose infusion into humans (Fox et al. 1964).

An interaction between amino acids and sugars in the kidney has been demonstrated in vitro by the observation that glucose, galactose and fructose inhibit the accumulation of certain amino acids by rat kidney cortex slices (Their et al. 1964). This inhibition was shown to be non-competitive. Similar reports of an interaction between hexoses and amino acids have been reported in intestinal transport (Reiser and Christensen 1969).

A number of hypotheses have been postulated to explain these interactions:- a) galactose acts indirectly by competition with amino

acids for available energy supplies (Newey and Smyth 1964); b) galactose gives rise to a toxic metabolite galactose -1-phosphate (Saunders and Isselbacher 1965); or c) the interaction occurs directly at specific binding sites of a polyfunctional carrier, capable of transporting sugars, neutral and basic amino acids, and Na^+ (Alvarado 1966). Chez et al. (1966) have demonstrated that the effect is due to an increase in amino acid efflux; however, the actual mechanism is still unclear. These authors rule out the possibility that galactose-1-phosphate is involved since glucose also inhibits amino acid accumulation. They also rule out the polyfunctional carrier scheme since amino acid influx was uninhibited. Segal et al. (1971) also eliminate the latter hypothesis from their observed differences in the post-natal maturation of the transport systems of sugars and amino acids in these tissues. Chez et al. (1966) suggest that entry of sugars is required for an effect since the inhibitory effect of sugar is reduced by the presence of phlorizin (which is reported to prevent glucose or galactose reabsorption in renal tubules).

1.10. Methods used to study the specificities of Amino Acid Transport Systems

Studies on the kinetics of amino acid transport in microorganisms and mammalian cells or tissues, together with biochemical and kinetic studies with mutants defective in the transport of one or more amino acids have indicated the presence of a number of distinct amino acid transport systems. Some of these systems appear to transport only a single substrate or a group of closely related substrates, whereas others may transport a large class of structurally related or unrelated amino acids.

1.10.1. Kinetic Analysis of Transport

Amino acid transport which exhibits Michaelis-Menten kinetics has been described in section 1.7.1. However, not all amino acid transport conforms to normal Michaelis-Menten kinetics. Biphasic linear plots may indicate that an amino acid may be transported by more than one system e.g. histidine transport in Salmonella typhimurium (Ames 1964). In order to distinguish whether two substrates A and B are transported by the same system Ahmed and Scholefield (1962) devised the ABC test. This test is considered positive if the following criteria are met:-

a) Amino acids A and B competitively inhibit one another;

b) the apparent Km for the transport of A equals the Ki for A (when inhibiting the transport of B) and vice versa;

c) a third amino acid C, if it competes with both A and B must have the same Ki in each case.

If these criteria are met the amino acids are considered to be transported via a single transport system. However, failure to meet criteria b or c does not rule out the possibility of a common transport system but indicates that these amino acids are not exclusively transported by that system. Similarly, should C fail to meet criteria b or c with either of the other amino acids, it should not be concluded that more than two systems are available for the three substrates. For example, if C has an affinity for both systems, the ABC Test would be negative whether an additional system was present or not. Thus this test may indicate the presence of several transport systems for the same amino acid but not the number actually present.

In order to obtain further information as to the number of actual systems involved in the transport of a single amino acid, amino acid analogues with reported affinities for a single transport system have been used. For example, in Ehrlich cells, several amino acids are

normally transported by both the "A" and "L" systems. Christensen et al. (1965) have studied transport limited to one or other system by using a competing analogue specific for the complementry system.

1.10.2. Studies with Mutants defective in Amino Acid Transport

Kinetic analysis of mutants defective in the transport of one or more amino acids has provided considerable information as to the specificities of amino acid transport systems and the number of systems involved in the transport of a single amino acid. Most of the work with transport mutants has been carried out with microorganisms and a large number of such mutants have been isolated and described (see section 1.11.). Detection of amino acid transport defects in whole organisms is more difficult than for microorganisms. However, such defects may have pronounced secondary effects which may be readily observed and which can be traced back to the primary transport defect, e.g. cystinuria, where reabsorption of cystine in the kidneys results in cystine stone formation (Rosenberg and Scriver 1969). Several inheritable diseases of amino acid transport in man have been described (see Table 1.10.2.). Defects in the transport of single amino acids e.g. Tryptophan Malabsorption (Drummond et al. 1964), and groups of amino acids e.g. Hartnup Disease (reviewed by Jepson 1972) indicate the presence in man of amino acid transport systems of narrow and broad specificity. A table of the heritable defects of amino acid transport is listed overleaf. (Table 1.10.2).

1.10.3. Kinetics and Biochemical Studies on Isolated Components of Amino Acid Transport Systems

Recently further information on the specificity of amino acid transport systems has been provided from the kinetic and biochemical studies of Binding-Protein components of microbial amino acid transport systems. For example, the isolation of an Isoleucine/Valine Binding

Table 1.10.2. Inheritable Defects of Amino Acid Transport in Man

Disease	Amino Acids involved in the transport defects	References
Hartnup Disease	Alanine, Serine, Threonine, Valine, Leucine, Isoleucine, Phenylalanine, Tyrosine, Tryptophan, Histidine Lysine, Glutamine, Asparagine	Reviewed by Jepson, (1972)
Methionine Malabsorption Syndrome	Methionine, and to a lesser extent phenylalanine and tryptophan	Jepson et al. (1958) Hooft et al. (1968)
Tryptophan Malabsorption, (Blue Diaper Syndrome)	Tryptophan	Drummond et al. (1965)
Dicarboxylic-amino aciduria	Glutamate, Aspartate	Teijema et al. (1974)
Cystinuria	Arginine, Lysine, Cysteine	Rosenberg et al. (1962) Fox et al. (1964)
Hypercystinuria	Cysteine	Whelan and Scrives (1968) Oyanangi et al. (1970)
Dibasic amino-aciduria	Arginine, Lysine	Holtzapple et al. (1969)
Imino-glycinuria	Glycine, proline, hydroxyproline	Reviewed by Slayman (1973).

Protein in Salmonella typhimurium has indicated the presence of a transport system specific for these amino acids (Rosen 1971a,b). No such system had been clearly indicated from kinetic studies of wild type or mutant cells.

1.11. SPECIFICITY OF AMINO ACID ACTIVE TRANSPORT SYSTEMS

Kinetic and genetic studies of amino acid uptake, in microorganisms and in mammalian cells or tissues, have revealed the presence of a number of distinct active amino acid transport systems. Some of these systems appear to transport only a single substrate, or closely related substrates, whereas others may transport a large class of structurally related or unrelated amino acids.

Most bacteria appear to transport amino acids via amino acid transport systems specific for a single amino acid or structurally related amino acids, e.g. in Escherichia coli arginine and lysine may be separately transported via their own specific transport systems, but also share a common basic amino acid transport system (Brown 1970). Although amino acid specific transport systems have been reported in fungi, e.g. the histidine (Crabeel and Grenson 1970) and lysine (Grenson et al. 1966) transport systems in Saccharomyces cerevisiae; transport systems capable of transporting structurally related amino acids appear to be more common, e.g. the basic amino acid transport systems of S. cerevisiae (Grenson 1966a, b), Penicillium chrysogenium (Hunter and Segal 1969), Neurospora crassa (Pall 1970b; Thwaites and Pendyala 1969; Sanchez et al. 1972). In addition to these specific, or relatively specific transport systems, systems capable of transporting a wide range of neutral, basic (and often acidic) amino acids have been demonstrated in S. cerevisiae (Grenson et al. 1970), P. chrysogenum (Benko et al. 1967, 1969, Hunter and Segal 1971), N. crassa (Pall 1969,

Thwaites and Pendyala 1969) and Arthrobotrys conoides (Gupta and Pramer 1970). A further broad specificity transport system for the neutral L-α-amino acids has also been reported in N. crassa (Pall 1969).

Mammalian amino acid transport systems are less well defined and experiments in vivo and in vitro have indicated possible differences in the transport systems of different tissues (reviewed by Neame 1968). However the presence of broad specificity and relatively narrow specificity transport systems have been demonstrated. Broad specificity transport systems capable of transporting the aliphatic and aromatic neutral amino acids have been demonstrated in a number of systems, including Ehrlich ascites tumour cells (Oxender and Christensen 1963), bone (Finerman and Rosenberg 1966), uterus (Riggs et al. 1968), kidney (Webber 1962; Webber et al. 1962) and intestine (Reiser and Christensen 1971). More specific amino acid transport systems have also been demonstrated, e.g. the glutamate/aspartate active transport system demonstrated in rat spinal cord and brain cortex synaptosomes (Logan and Snyder 1971). At present no mammalian amino acid transport system has been conclusively demonstrated to transport a single amino acid. However, specific systems for methionine and tryptophan have been postulated on the basis of the inheritable diseases Methionine Malabsorption (Hooft et al. 1968, Section 1.10) and Tryptophan Malabsorption (Drummond et al. 1964, Section 1.10) which are thought to be primary defects in the intestinal absorption of these amino acids.

SECTION 2 : MATERIALS AND METHODS

2.0. MATERIALS

2.0.1. Chemicals

All chemicals used were of the highest purity available, and were purchased from Sigma London Chemical Company Limited, Kingston-upon-Thames, Surrey KT2 7BH; Koch-Light Laboratories Ltd., Colnbrook, Bucks; or Calbiochem Ltd., 10 Wyndham Place, London W1 1AS.

2.0.2. Radiochemicals

The following radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks HP7 9LC:

L-[U - ^{14}C] glutamate (specific activity - 225 mCi/mmol)

L-[G - ^{3}H] glutamate (specific activity - 20,000 - 40,000 mCi/mmol)

L-[G - ^{3}H] aspartate (specific activity - 100 to 300 mCi/mmol)

L-[3 - ^{3}H] serine (specific activity - 5000 to 15000 mCi/mmol)

L-[2,3-^{3}H] valine (specific activity - 15,000 to 30,000 mCi/mmol)

L-[2,3-^{3}H] alanine (specific activity - 20,000 to 40,000 mCi/mmol)

L-[4,5-^{3}H] leucine (specific activity 30,000 to 60,000 mCi/mmol)

L-[3,3'-^{3}H] cystine hydrochloride (specific activity 625 mCi/mmol)

G -[^{3}H α]-AIB (specific activity - 200 to 400 mCi/mM) was obtained from NEN Chemicals GmbH, 6072 Dreierchenhain bei Frankfurt /M, Siemenstra SS1, Postfach 71, Germany.

Cyclic AMP assay kit containing [8 - ^{3}H] cyclic AMP (specific activity 25 Ci/mmole) was obtained from the Boehringher Corporation (London) Ltd., Bilton House, 54-58, Uxbridge Road, Ealing, London W5 2TZ.

2.1 MEDIA

2.1.1. Glasgow Modified Eagles Minimum Essential Medium

All cells were maintained in the Glasgow modification of Eagles Minimum Essential Medium (GMEM), described by Macpherson and Stoker (1962), unless specified otherwise in the text. This medium is made up of the following components:

L-Amino Acids	Final Concentration (mM)	Vitamins	Final Concentration (μM)
Histidine	0.1	D-Calcium pantothenate	4.2
Isoleucine	0.4	Folic Acid	4.5
Leucine	0.4	Nicotinamide	16.0
Pheylalanine	0.2	Pyridoxal HCl	10.0
Lysine	0.4	Riboflavin	0.5
Threonine	0.4	Thiamin HCl	6.0
Tryptophan	0.04	i-Inositol	20.0
Valine	0.4	Choline Chloride	14.0
Arginine	0.2		
Tryrosine	0.2		
Cystine	0.2		
Methionine	0.1		
Glutamine	2.0		

Salts	Final Concentration (mM)	Other Components	Final Concentration
NaCl	108.00	Glucose	25 (mM)
KCl	5.00	Penicillin	10^4 units/litre
$MgSO_4$	0.81	Streptomycin	10^4 units/litre
$CaCl_2$	1.80	Antimycotic (n-butyl p-hydroxy benzoate)	0.2 ppm
NaH_2PO_4	0.88		
$Fe(NO_3)3$	0.0025		
$NaHCO_3$	23.00		

This medium was supplemented with foetal calf serum (10%, v/v) unless specified otherwise in the text.

2.1.2 Plating Medium

This medium is comprised of GMEM plus the following non-essential amino acids and nucleotides:

Non-essential L-Amino Acid	Concentration (mM)	Nucleoside	Concentration (mM)
Serine	0.2	Adenosine	.03
Proline	0.2	Guanosine	.03
Aspartic acid	0.1	Cytidine	.03
Glutamic acid	0.1	Thymidine	.03
Alanine	0.1	Uridine	.03
Glycine	0.1		

2.1.3. Storage Medium

GMEM	75% (v/v)
Foetal Calf Serum	20% (v/v)
Glycerol	5% (v/v)

2.1.4. Uptake Salt Solution

All uptake experiments were carried out using the following Hepes buffered salt solution:-

NaCl (114.8mM), KCl (5.4mM), $MgSO_4$ (0.8 mM), $CaCl_2$ (1.8 mM), NaH_2PO_4 (0.9 mM), $Fe(NO_3)_3$ (0.003 mM), Na_2HPO_4 (0.9 mM), Glucose (25 mM) and Hepes (20 mM), pH 7.4. Radioactive substrate amino acid (adjusted to the correct final concentration by cold carrier amino acid) was added prior to transport assays.

2.1.5 Trypsin in Tris-Saline

Trypsin 0.25% (w/v) in Tris-Saline, pH7.3

Tris-Saline:- NaCl (135 mM); KCL (2.6 mM); Na_2HPO_4 (0.63 mM); Glucose (5.6 mM); Tris 10 mM); Penicillin (60 units/litre).

2.1.6. Versene Solution

NaCl (135 mM); KCl (2.6 mM); Na_2HPO_4 (7.2 mM); KH_2PO_4 (2.5 mM), and Versene (10 mM); pH 7.3.

2.1.7. Hank's Balanced Salt Solution

Hank's balanced salt solution (HBSS), pH 7.3, was prepared as described by Hank (1949). This solution is made up of the following components: NaCl (135 mM); KCl (2.7 mM); Na_2HPO_4 (0.38 mM); KH_2PO_4 (0.75 mM); $MgSO_4$ (0.41 mM); $CaCl_2$ (0.25 mM); $MgCl_2$ (0.6 mM); $NaHCO_3$ (2.9 mM); Glucose (5.6 mM).

2.1.8. Phosphate Buffered Saline (PBS)

Phosphate buffered saline was made up of the following components as described by Dulbecco and Vogt (1954):
NaCl (135 mM), KCl (2.7 mM); Na_2HPO_4 (7.2 mM), KH_2PO_4 (2.5 mM); $CaCl_2$ (0.18 mM); $MgCl_2$ (0.6 mM).

2.1.9. Solutions for Protein Determinations

Folins solution C was freshly prepared before use from a 1:50 (v/v) mixture of solutions A and B

Folins Solution	A :	$CuSO_4 \cdot 5H_2O$	5g/litre
	B :	NaK Tartrate	2g/litre
		Na_2CO_3	2g/litre
		NaOH	4g/litre

2.2.1. Normal Culture Conditions

The cell lines were routinely grown as monolayer cultures at 37°C in 8 or 20 oz. medical flat bottles containing 15 or 50 ml.of medium respectively. Larger quantities of cells were grown in 80 oz. flint-glass Winchester bottles, which contained 180 ml. of medium and were rotated on roller culture racks at approximately 3 rev/min. All vessels were gassed with a 95% air:5% CO_2 mixture, in order to initially buffer the medium at about pH 7.4.

Cells were also occasionally maintained in 60 x 15 mm plastic disposable petri dishes containing 4 ml. of medium. These dishes were incubated at 37°C in a humidified atmosphere of 95% air:5% CO_2.

2.2.2. Media

The actual medium used for each cell line is described in Section 3. The basic medium was Glasgow modified Eagle's medium (Macpherson and Stoker 1962), (see Section 2.1.1), which was supplemented with 10 (v/v) Bovine or Foetal Bovine serum. Further additions of non-essential amino acids (see amino acids listed in Section 2.1.2) or Pyruvate ($1x10^{-3}$M final concentration) were made for certain diploid cell lines following isolation.

2.2.3. Harvesting of Cells

At or approaching confluence the cells were passaged as follows: The cells were washed twice with Versene solution (see Section 2.1.6) and detached from the substratum with a solution of one part 0.25% Trypsin in tris saline (see Section 2.1.6) diluted with four parts Versene. On detachment the cells were transferred to an equal volume of the appropriate medium, dispersed by pipetting, further diluted with medium and reseeded into a clean bottle containing fresh medium.

2.2.4. Storage and Recovery of Cells

Cells in log phase of growth were harvested as described in Section 2.1.3, except that an equal volume of calf serum was added to the harvested cells in order to neutralise the trypsin. The cells were then sedimented by centrifugation (x 500 g), the supernatant discarded and storage medium (see Section 2.1.3) added. The cells were redispersed and 2 ml. volumes of cells containing 2 x 10^6 cells/ml. transferred to screw-capped storage vials. The vials were then packed in an expanded polystyrene box with cotton wool and transferred to 70°C in a Revco freezer. On removal from storage the contents of the vial were rapidly thawed at 37°C, the cells seeded into a bottle containing 10 mls. of the appropriate medium and incubated overnight. The medium was then replaced and the cells maintained routinely.

2.2.5. P.P.L.O. Checks

The cell lines were regularly checked at 4 weekly intervals, or following reclamation from storage, for the presence of pleuro-pneumonia like organisms (P.P.L.O. or Mycoplasma). The procedure for detection was a modification of the method of Fogh and Fogh (1964). Cells, inoculated onto coverslips in 30 x 10 mm. petri dishes, were incubated at 37°C for 48 hours in a humid 95% air:5% CO_2 atmosphere. The coverslips were then washed three times with P.B.S. (see Section 2.1.8) and the coverslips transferred to a petri dish containing 4 ml. of 0.9% (w/v) KCl solution. After five minutes 0.2 ml. of 3:1 (v/v) methanol-acetic acid mixture (Carnoy's Fixative) was added to the KCl. This solution was replaced at five and again at ten minutes with 4 ml. of Carnoy's Fixative. The coverslips were then air dried and stained in 2% orcein in 60% acetic acid for ten to fifteen minutes. After washing three times in ethanol the coverslips were transferred to xylol for fifteen minutes, air dried and mounted in De Pex mounting medium.

P.P.L.O. show up as darkly stained granules at the cell periphery, when examined under phase contrast and oil immersion.

The incidence of P.P.L.O. contamination was very low and any contaminated cultures were discarded and fresh stocks recovered from storage.

2.2.6. Cell Counting

Following harvesting and dispersion, the cells were usually counted using a Model D Coulter Counter (Coulter Electronics Ltd.). A 0.1 ml. volume of cells was diluted in 9.9 ml. of millipore filtered 0.9% saline solution. The samples were counted at an Aperture Current setting of 1 and an average threshold setting of 22.5 (which was found to be suitable for all cell lines). The average of two successive readings were taken unless less than 1000 cells were counted, in which case four readings were made. Counts over 10,000 were corrected for coincidence loss by reference to a correction table.

Occasionally cells were counted using a haemocytometer. A single drop of cell suspension was transferred to the haemocytometer chamber and the viable cells counted on opposite grids of the chamber. At least 400 cells were counted. This technique, although less accurate, enables the distinction to be made between viable and non-viable cells. Non-viable cells were identified by their inability to exclude a 1% solution of Trypan Blue, cells that did not take up this dye were considered to be viable.

2.2.7. Cloning Procedure

Isolated colonies were washed twice with 2 ml. of Versene and a drop of Trypsin:Versene solution applied locally with a narrow ended cloning pipette. After 1-2 minutes the whole colony was removed by pipette. The cells were transferred to 2 ml. of medium, dispersed and

grown normally in 1 oz. medical flat bottles until passaging.

2.3. PRODUCTION OF DIPLOID CELL LINES

A number of diploid cell Lines were prepared, from chinese or syrian hamster tissues as described below:
The tissue of the animal from which the cell line was to be obtained was dissected and transferred to a screw top bottle containing 10 ml. of Versene. The tissue was then washed four times in 10 ml. of Versene, removed and minced into small fragments of about 0.5 mm. or less. The fragments were then transferred to a 50 ml. Dimple Flask containing 20 ml. of 0.25% Trypsin in tris-Saline and shaken for twenty minutes at 37°C. After shaking the fragments were allowed to settle, the supernatant containing dispersed cells decanted into 20 ml. of Foetal calf serum and then centrifuged at 500 g. for five minutes. The supernatant was discarded and the cells of the pellet redispersed in Plating Medium (see Section 2.1.2) containing 20% (v/v) Foetal calf serum. The cells were then transferred to a 2 oz. Medical Flat bottle containing 5 ml. of Plating Medium. The bottle was gassed with 95% air:5% CO_2 and the cells incubated at 37°C. Medium was replaced every 48 hours.

When the cells were almost confluent, the cell line was cloned and a cell line started from a single colony of assumed clonal origin. The cells were plated out essentially as described in Section 2.1.1, except that only 100 cells per dish were plated in Plating Medium containing 20% (v/v) non-Dialysed Foetal calf serum. The colonies were removed as described in Section 2.1.7. Karyotype analysis was carried out as described in Section 2.4.

2.4. TRANSFORMATION OF CELLS

Cells were transformed by the DNA virus, SV-40. The method of transformation was based on that described by Black and Rowe (1963).

The cells to be transformed were seeded at 5 x 10^5 in 60 x 15 mm. petri dishes and grown overnight at 37°C in a humidified atmosphere of 95% air:5% CO_2. The cells were washed with P.B.S. (see Section 2.1.8) and 1.5 ml. of Glasgow-modified Eagle's medium, without serum, added to the cells. SV-40 virus (50 pfu/cell) was then added to the medium and the virus allowed to adsorb to the cells for two hours. The unadsorbed virus was removed by several washes with P.B.S. solution. Medium containing 5% (v/v) Foetal Calf serum was added to the petri dish and the cells maintained at 37°C for three weeks. The medium was replaced at three-day intervals. At three weeks foci of 'transformed cells' (approximately 1.5 mm. in diameter) could be seen. Cells were considered transformed by their ability to form rapidly growing, multi-layered, non-contact inhibited colonies. These transformed cells were removed as described in Section 2.1.7.

Control plates, which were not inoculated with virus, failed to show any 'transformed colonies'. The transformed cell lines were capable of high plating efficiency in agar and could be passaged indefinitely without reduction in the growth rate or change in their altered appearance and growth behaviour.

2.5. KARYOLOGICAL PROCEDURES

2.5.1. Cell and Chromosome Preparation

The cells were grown on 22 mm. diameter glass coverslips (Chance No. 1) in EFC 10 for 48 hours. While still in "log phase" growth, colcemid (Ciba) in Hank's Balanced Salt Solution (HBSS) was added at a final concentration of 1 mg/ml. and the cells were incubated for 1.5 hours. The cells were then washed twice with HBSS and incubated for

15 minutes in 60 x 15 mm. petri dishes containing 2 ml. of HBSS. The coverslips were then transferred to a 0.075M solution of potassium chloride. After five minutes at room temperature 0.2 ml. of fresh 3:1 (v/v) methanol:acetic acid mixture was added. The fixative was replaced after five and ten minutes, after which the coverslips were removed and air dried.

2.5.2. Aceto-Orcein Stain

Air-dried coverslips were stained for 10-15 minutes in 2% orcein in 60% acetic acid. The slides were rinsed with 50% acetic acid, dehydrated in two changes of 95% ethanol for a total of three minutes and absolute ethanol for 30 seconds, and finally cleared with xylol. The slides were permanently mounted in xylol diluted De Pex mounting medium.

2.6. DETERMINATION OF CELL VOLUMES USING A COULTER COUNTER

The mean cell volume of BHK21-C13 cells was determined with a Model D Coulter Counter using the method described by Magath and Berkson (1960). The Counter Counter was set to Aperture 1 and the cells counted at intervals of five threshold units (tu) for the range 10 to 80. The difference between settings 10 and 15 tu (classed as 12.5 tu for purposes of calculation) was the number of cells of mean volume 12.5 tu. By taking the successive differences in this manner the counts for cell diameters of 12.5 tu to 77.5 tu was obtained. The mean cell diameter (expressed in tu) was obtained by dividing the sum of the products of cell diameter and cell number of that diameter by the total number of cells. To convert cell diameter (expressed in tu) to cubic microns the above procedure was repeated using Ragweed pollen spores of known mean diameter, 13.9 μ (Coulter Counter Ltd.). The

actual number of microns equivalent to a threshold unit is:

$$\frac{\text{mean spore diameter (13.9 u)}}{\text{mean spore diameter (tu)}}$$

Thus the mean cell diameter (in cubic microns) is: mean cell diameter (in tu) x number of microns per tu. The mean cell volume is then calculated directly from this value.

2.7. LIQUID SCINTILLATION COUNTING

2.7.1. Liquid Scintillation Spectrometer

All radioactivity assays were carried out using liquid scintillation counting techniques. All samples were counted using a Beckman L.5II Liquid Scintillation System with pre-set and adjustable Plug-In Iso-Set Modules.

2.7.2. Liquid Scintillators

Two commercially prepared scintillation fluids were used, a Dioxan based scintillator, NE250, and a Toluene based scintillator, NE233 (Nuclear Enterprises Ltd.).

NE250 Liquid Scintillator

This scintillator contains purified dioxan and is capable of accommodating considerable amounts of aqueous solutions with relatively little quenching.

NE233 Liquid Scintillator

This scintillator contains highly purified toluene, PPO activator, POPOP spectrum shifter, and naphthalene for reduction of chemical quenching. Although unnecessary for matching response of most photomultipliers, POPOP is useful for reduction of colour quenching.

All liquid scintillation experiments used NE250 liquid scintillator unless specified otherwise.

2.7.3. Quench Correction for [^{3}H] and [^{14}C] Compounds

All radioactive samples after counting were corrected for quenching using a "Channels - Ratio Method". For ^{3}H-Quench Correction two channels were used. Channel A, a pre-set Iso-set Module, which was adjusted to give maximum counting efficiency and Channel B, a variable Iso-set module was adjusted to count approximately 40% of the high energy portion of an unquenched ^{3}H-labelled sample.

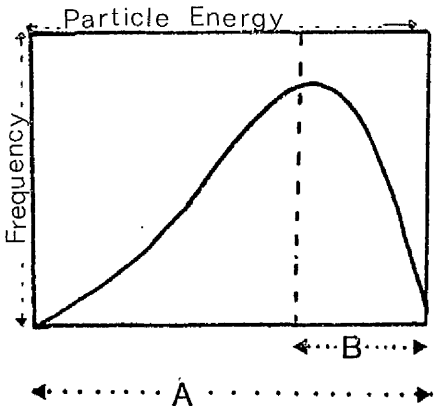


Fig. 2.7.1. Diagram to show sensitivity range of Channels A and B.

A standard curve of counting efficiency versus channels ratio was constructed using a series of increasingly quenched radioactive standards of known activity. (The activity was calculated from that determined by the manufacturer and adjusted for Radioactive decay). A series of scintillation vials was set up in duplicate, using 5 mls. of NE250 liquid scintillator, .005 ml. ^{3}H-Toluene, plus 0 to 0.4 ml. of the quenching agent, chloroform (Analar grade). The total counts per minute (c.p.m.) per channel was determined by using a 10 minute count for each set of vials. Percentage efficiency for each sample was calculated and plotted against Channel Ratio A/B, (Figure 2.7.2a). From this standard curve the counting efficiency and hence the specific activity of a sample may be determined, given the ratio of counts in Channels A and B.

A similar standard curve was constructed for ^{14}C-quenching. The

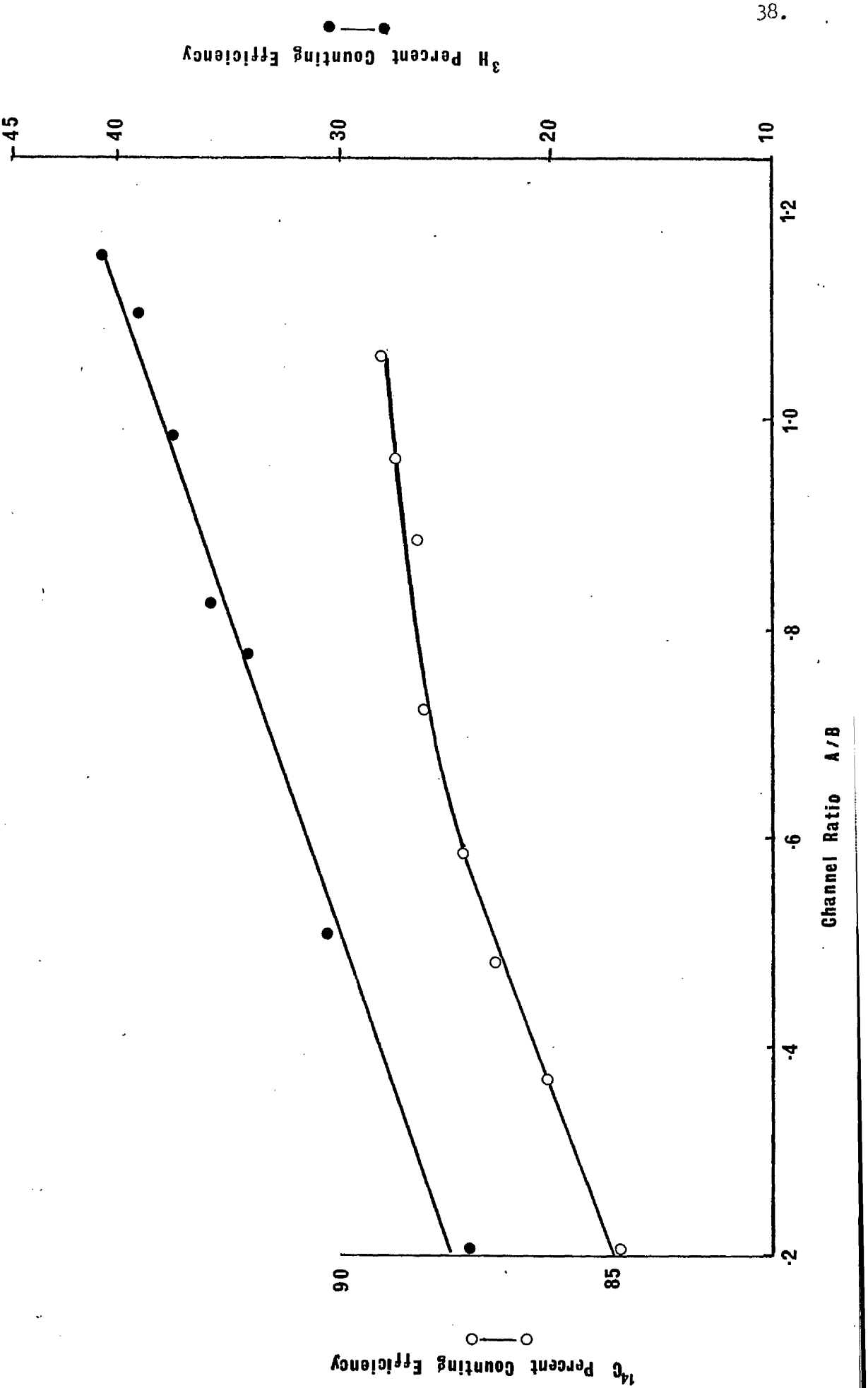
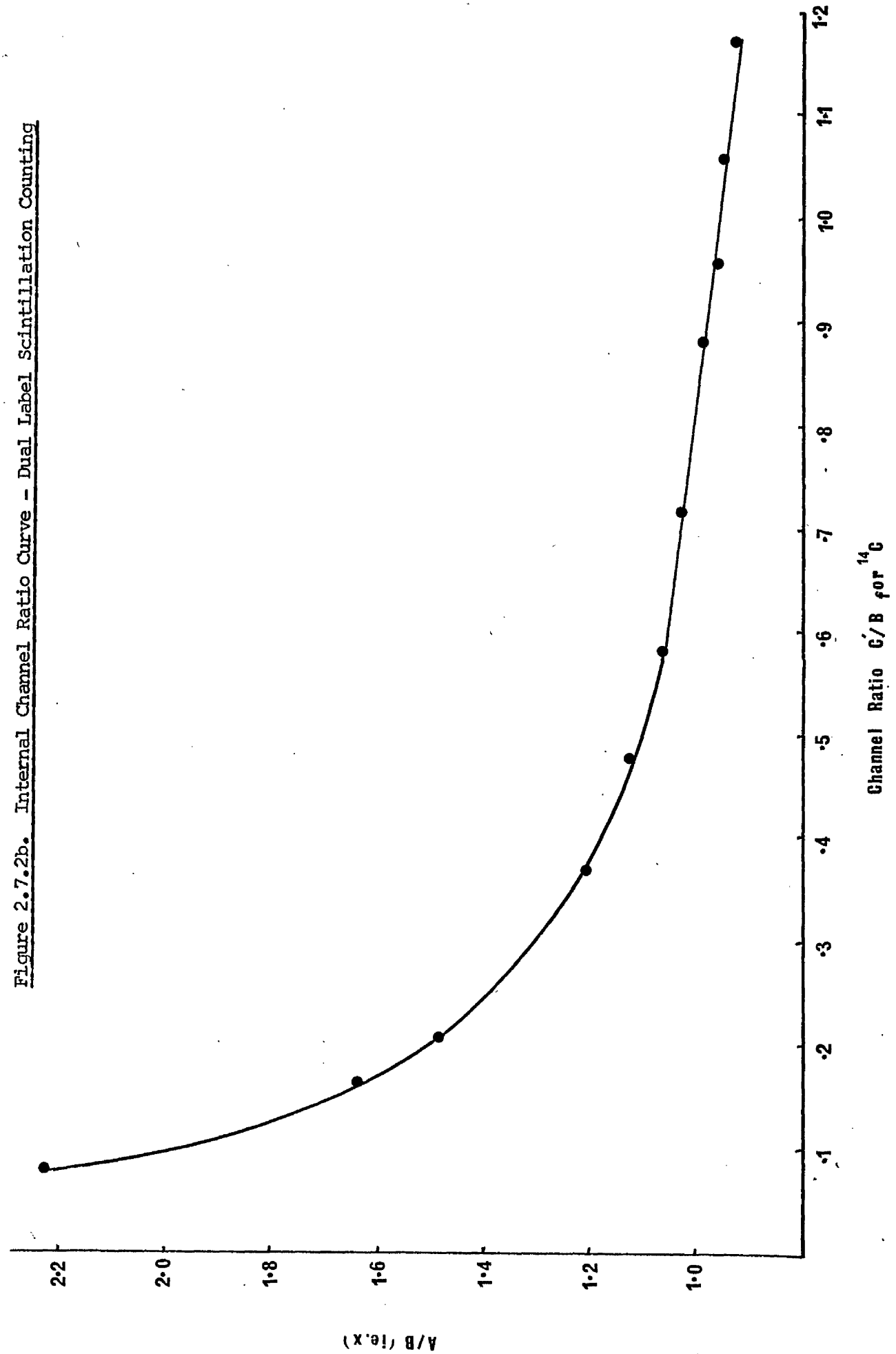


Figure 2.7.2a. ^{3}H and ^{14}C Quench Correction Curves

Figure 2.7.2b. Internal Channel Ratio Curve - Dual Label Scintillation Counting



channels were set as described for the dual label counting (see Section 2.7.4). The standards contained 5 mls. of NE250 scintillation fluid and .005 ml. of ^{14}C-Toluene, plus 0 to 0.4 ml. of chloroform.

2.7.4. Dual Label Scintillation Counting

For dual label scintillation counting, using $[^{3}H]$ and $[^{14}C]$ radioactively labelled amino acids, 3 channels were used. The channels used were as follows:

CHANNEL A - A pre-set Iso-set module, adjusted to give maximum $[^{3}H]$ counting efficiency.

CHANNEL B - A variable Iso-set module, adjusted so that the $[^{14}C]$ c.p.m. in B was approximately equal to the $[^{14}C]$ c.p.m. obtained in C, as shown in Fig. 2.7.3.

CHANNEL C - A pre-set Iso-set module, adjusted for total $[^{14}C]$ c.p.m.

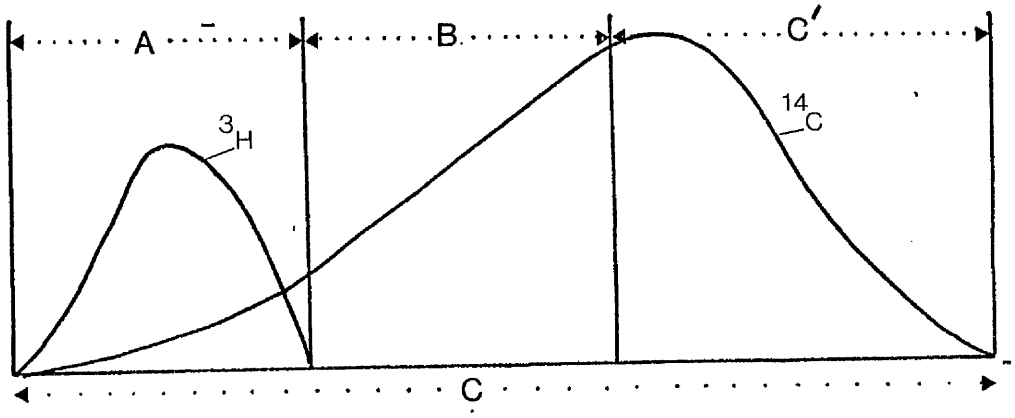


Fig. 2.7.3. Channel Settings for Dual Label Scintillation Counting.

On quenching ^{3}H c.p.m. are lost and a greater proportion of $[^{14}C]$ c.p.m. are counted in Channel B, thus the ratio of C' to B alters and is indicative at the degree of quenching. From this ratio the $[^{3}H]$ c.p.m. in A and the total $[^{14}C]$ c.p.m. can be determined, as indicated in the following steps :

a) $[^{14}C]$ c.p.m. in C' = C - (B+A)

b) Internal Channel Ratio = C'/B

c) Correction factor (x) (to compensate for quenching) is determined from Fig. 2.7.2b a graph of C'/B versus A/B

d) $[^{14}C]$ c.p.m. in A = B.A/B(x)

e) Total $[^{3}H]$ c.p.m. in A = A - (^{14}C in A)

f) Total $[^{14}C]$ c.p.m. = C - (^{3}H in A)

g) $[^{3}H]$ c.p.m. and $[^{14}C]$ c.p.m. are converted to disintegrations per minute (d.p.m.) using Fig. 2.7.2a.

2.8. AMINO ACID TRANSPORT ASSAYS

2.8.1. Growth Conditions Prior to Transport Assays

Cells in "log phase" growth were harvested as described in Section 2.1.3. The cells were then seeded into either 32 mm. diameter wells of 24 hole plastic disposable trays (Biocult-Linbro) containing 22 mm. diameter glass coverslips, or 60 x 15 mm. petri dishes. Medium was added to a total volume of 2 and 4 ml. respectively. The inoculation density used for most cell lines (unless otherwise stated in the text) was 1×10^5 cells per well and 1.5×10^5 per petri dish. The cells were then incubated for 48 hours at 37°C in a humid 95% air:5% CO_2 atmosphere.

The medium for growth and various treatments of the cells prior to transport assays are described in the text.

2.8.2. Uptake Assay - Coverslip Method

This method was based on that described by Foster and Pardee (1969).

Following growth of the cells on coverslips, the growth medium was removed by aspiration and the coverslips rinsed twice by washing

with Uptake Medium (Section 2.1.4) at 37°C. The coverslips were then drained and stacked in a slotted perspex rack. At zero time the rack was immersed into 25 ml. of Uptake Medium which contained radio-actively labelled amino acid and carrier amino acid adjusted to the correct final concentration. At intervals, coverslips were removed with forceps and rinsed for a total of 10 seconds by dipping serially through three containers of warm (37°C) Uptake Medium. The coverslips were then drained, air-dried and transferred to scintillation vials containing 5 ml. of scintillation fluid. A 0.1 ml. sample of radioactive Uptake Medium (for determination of the specific activity of the amino acid) was transferred to a scintillation vial prior to the addition of the coverslips. The radioactivity of this sample and the coverslips was measured by scintillation counting.

Protein measurements were made directly on duplicate coverslip cell cultures by the method of Oyama and Eagle (1956) described in Section 2.8.6.

The rate of transport of amino acids was expressed as pmols/µg Protein/unit time in minutes.

2.8.3. Uptake Assay - Petri Dish Method

This method was a modification of that described by Hatanaka et al. (1969) for sugar transport studies.

After growth of the cells in petri dishes, the growth medium was removed by aspiration and the petri dishes washed twice with Uptake Medium (37°C). The Uptake Medium was removed and 1 ml. of Uptake Medium containing radioactively labelled amino acid added. The petri dish plus contents was then incubated at 37°C for the required time interval after which the Uptake Medium was removed by aspiration and the petri dish washed rapidly four times with warm (37°C) Uptake Medium.

The dishes were then air-dried and 1 ml. of Folin's solution C (see Section 2.8.7) added. Four 0.1 ml. aliquots of this were transferred to scintillation vials containing 5 ml. of scintillation fluid and the radioactivity of the sample measured by scintillation counting. Similarly, four 0.1 ml. aliquots were transferred to 5 ml. of Folin's solution C and the protein content determined as described in Section 2.7.6.

The rate of transport of amino acid was expressed as pmols/µg Protein/min.

2.8.4. Dual Labelled Uptake Procedure

For certain experiments, which will be described in the text, the transport of L-[2,3-^{3}H] alanine and L-[U-^{14}C] glutamate was studied in the same cell sample. The procedure for this method was as described in Section 2.8.2 and 2.8.3; however, the Uptake Medium contained both radioactively labelled amino acids. After scintillation counting of the samples, the respective ^{3}H d.p.m. and ^{14}C d.p.m. were discriminated by the procedure described in Section 2.7.3.

2.8.5. Determination of Km and Vmax for Amino Acid Uptake

Data from which Km and Vmax values for the uptake of a specific amino acid was determined using procedures described in Sections 2.8.2 and 2.8.3. Uptake of amino acids into cells was examined for concentrations ranging from 0.5 or 1 x 10^{-5} to 1 or 4 x 10^{-3}M (see text). Initial rates of uptake were determined from data obtained over the linear phase of transport. This data was initially plotted graphically in order to determine if the data appeared to deviate from linearity, e.g. due to diffusion, inhibition of uptake by an excess of substrate, or transport of the amino acid by more than one major uptake system. For apparently linear plots statistical estimates of

Km and Vmax determinations were made in order that subjective biases from the fitting of data would be avoided. Estimations of Km, Vmax and their standard errors were determined from the weighted least square regression fits of the linear plot $^{s}/_{v}$ versus s, as described by Wilkinson (1961).

2.8.6. Competition Experiments

For amino acid competition studies of the transport of a radioactively labelled amino acid (substrate) was studied in the presence of a second unlabelled amino acid. The procedure was carried out as described in Section 2.7.4, except that the Uptake Medium contained a second non-radioactively labelled amino acid in addition to the substrate. For initial experiments in order to determine whether the competing amino acid was an effective inhibitor of the substrate, a 25 fold molar ratio of competing amino acid was used. In the case of appreciable inhibition of radioactively labelled substrate uptake, further kinetic analysis was carried out to determine if this inhibition was competitive or non-competitive (Dixon and Webb 1964). The uptake of radioactively labelled substrate was examined, in the presence of a single concentration of inhibiting amino acid, over the concentration range studied in the absence of inhibitor. Regression lines for $^{s}/_{v}$ versus s were fitted by the method of weighted least squares, and the Kp value (where the regression line crosses the abscissa) and its standard error determined. Ki values were determined from the relationship $Ki = {}^{I}/_{\frac{Kp}{Km}} - 1$, where I = inhibitor concentration. (See Section 2.14).

2.8.7. Protein Determination Procedure

The method used was based on the Oyama and Eagle (1956) modification of the Lowry method (Lowry et al. 1951) for protein determinations.

The protein determinations were made directly on cells grown on

coverslips or in petri dishes. The coverslips or petri dishes were washed four times with Uptake Medium at 37°C and then air-dried. For protein determinations on coverslips, the coverslips were immersed in 4.8 ml. of Folin's solution C (see Section 2.1.9) and 0.2 ml. of Folin and Ciocalteu's Phenol reagent added. For protein determinations of cells grown in petri dishes 0.5 ml. of Folin's solution C was added and left for 1 hour. A 0.1 ml. aliquot of this protein extract was then added to 4.7 ml. of Folin's solution. The absorbance was determined at 660 nm after 30 minutes. The protein content was obtained from a calibration curve using Bovine Serum Albumin as the standard.

2.9. EXTRACTION AND CHROMATOGRAPHY OF ENDOGENOUS RADIOACTIVE AMINO ACIDS

2.9.1. Extraction of Endogenous Radioactive Amino Acids

Log phase BHK21-C13 cells grown in 60 x 15 mm petri dishes were used. The actual cell numbers were determined with cells trypsinised from duplicate petri dishes. One ml. of Uptake Medium containing 1×10^{-5}M L-[U^{14}C] glutamate or L-[2,3-^{3}H] alanine was added and the cells incubated at 37°C for 10 and 5 minutes respectively. The Uptake Medium of Control Samples also contained 5×10^{-3}M sodium azide (NaN_3) and 2×10^{-3}M sodium cyanide. The Uptake Medium was removed by aspiration and the cells washed four times with warm (37°C) Uptake Medium. Following removal of the Uptake Medium, 0.5 ml. of ice cold 10% TCA solution was added and the cells incubated for 1 hour at 4°C. The cells were removed by scraping, the TCA soluble and insoluble fractions transferred to a 15 ml. centrifuge tube and pooled with two petri dish washings of 0.5 ml. of 10% TCA. The TCA insoluble fraction was sedimented by centrifugation at 10,000g for 14 minutes at 4°C in a M.S.E. Superspeed 65 refrigerated centrifuge. The TCA soluble fraction was pipetted off and the TCA removed by 5 extractions with 2 volumes of

water saturated ether. The ether phase was removed by careful pipetting. A 0.05ml sample was transferred to a scintillation vial containing 5 ml. scintillation fluid and the radioactivity measured.

2.9.2. Ionophoresis

A 0.05 ml. sample of the extract was spotted onto Whatman 3 MM chromatography paper (width 3.0 cm). L-glutamate or L-alanine were separated from other amino acids by low voltage vertical ionophoresis. The method was essentially the same as that described by Evered (1959). After ionophoresis for 3 hours at 200 volts (current,5 m A per strip) in tank buffer pH. 1.9 (58 ml. glacial acetic acid and 26 ml. 25% (w/w) formic acid, diluted to a total volume of 2 litres) the ionophoretogram was air-dried and cut into 1 cm. strips (3 cm. x 1 cm). The strips were transferred to scintillation vials containing 5 ml. of scintillation fluid. The results were expressed as d.p.m. per section of ionophoretogram.

2.10. DETERMINATION OF INTRACELLULAR LEVELS OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE

2.10.1. Preparation of Cells prior to Cyclic AMP Determination

Cells were grown in 60 x 15 mm. petri dishes for 48 hours. The growth medium was then removed, the cells washed three times with warm Uptake Medium, and 0.5 ml. of cold 10% Trichloro-acetic acid (TCA) added. The cells were then incubated at $4^{\circ}C$ for one hour. Fixed cells were scraped from the dish and the complete TCA soluble and insoluble fractions transferred to a cold siliconised certifuge tube, pooled with two 0.5 ml. 5% TCA washes of the plates, and sedimented at 10,000g for 5 minutes at $4^{\circ}C$ in a M.S.E. Medium Refrigerator Centrifuge. All glassware for this procedure was siliconised with triethylchlorosilane

in toluene (Pierce). TCA was removed by 5 extractions with 2 volumes of water saturated ether. The aqueous phase was then dried using a "Speedivac" centrifugal freeze drier, Model 5PS. The dry residue was dissolved in 0.4 ml. of 0.2M Acetate buffer, pH 4.0 and 0.1 ml. samples were assayed for cyclic AMP content, as described in Section 2.10.2.

Cell number was determined from counts made on duplicate petri dishes.

2.10.2. Determination of Cyclic AMP

The samples were assayed for cyclic AMP content by a modification of the Competitive Binding Protein technique described by Gilman (1970).

The standard binding reaction was carried out in a total volume of 0.2 ml. The sample reaction mixture contained 0.1 ml. of sample, 0.05 ml. of 0.2M sodium acetate/acetic acid (pH 4.0), 0.02 ml. of $[^3H]$ - cyclic AMP (0.02 μCi, 0.08 pmols) and 0.01 ml. of redistilled water. The reaction mixture for the standards contained cyclic AMP (1 - 20 pmols) but no sample. A blank, which contained no unlabelled cyclic AMP and to which no binding protein was to be added, and a zero standard containing no unlabelled cyclic AMP were also prepared. These were made up to a total of 0.2 ml. with redistilled water.

The reaction was initiated by the addition of 0.02 ml. of a cyclic AMP Binding Protein (Boehringer-Mannheim). The reaction mixture was mixed by gentle tilting of the tube and then incubated for 100 minutes at $0^{\circ}C$. After the incubation period 1 ml. of ice cold 20 mm. potassium phosphate buffer, pH 6.0, was added. The test mixture was then passed through a 24 mm. cellulose ester (Millipore) filter (0.45 μm), which had been previously rinsed with the buffer. The filter was then washed with 10 ml. of buffer, dried by suction and air-dried. The dry filter

was then placed in a scintillation vial and dissolved in 1 ml. of 2-methoxy-ethanol, with gentle shaking. On dissolution 10 ml. of NE233 scintillation fluid was added, and the radioactivity measured by scintillation counting.

The standard curve was prepared by plotting the ratio of d.p.m. of $\left[^{3}H\right]$-cyclic AMP bound in the absence of unlabelled cyclic AMP (Zero standard blank - Co) and d.p.m. bound in the presence of standard cyclic AMP (Cx) against pmols of unlabelled cyclic AMP per sample. From the Co/Cx ratio for the sample, the number of pmols of unlabelled cyclic AMP was determined from the standard curve.

2.11. DETERMINATION OF CELL VIABILITY USING PLATING EFFICIENCY TESTS

Cells growing in log phase were harvested as described in Section 2.1.3. After dispersal the cells were counted using a haemocytometer, and then inoculated into 60 mm. petri dishes (containing 4 mls of Plating Medium) at a density of 200 cells/dish, unless otherwise stated in the text. Alterations in medium, such as the omission of certain amino acids, or the addition of potentially toxic substances, are stated in the text. Dishes were then placed randomly into sterile plastic sandwich boxes, which contained 10 ml. of sterile distilled water. The box was then briefly gassed with a 5% CO_2 : 95% air mixture, closed and incubated at 37° in a humidified 5% CO_2 : 95% air mixture. After 7 days the medium was poured off, 3 ml. of Leishmann's stain added and left for 10 minutes, diluted with 3 mls. of distilled water and left for a further 10 minutes. The stain was then poured off, the plates rinsed in tap water and allowed to air dry. The colonies were counted under a 10 X magnification.

Results were expressed as the percentage of inoculated cells which gave rise to colonies, relative to the control (which contained

no test substance). The plating efficiency of the control was arbitrarily set as 100%.

2.12. MUTANT SELECTION PROCEDURES

Prior to any mutant selection procedure, cells were treated with the mutagen Ethyl Methane Sulphonate (EMS). The toxicity of this compound was however first determined. The method for this experiment is described below, results of which are described in Section 8.2.1.

2.12.1. Determination of EMS Toxicity

The medium from 10 mm. petri dishes containing BHK21-C13 cells in log phase growth was discarded. The cells were then washed with warm Uptake Medium (37°C), 1 ml. of EFC_{10} containing EMS in the concentration range 1 - 30 mM added and the cells incubated at 37°C for 2 hours. The cells from each dish were then separately harvested (see Section 2.1.3), and four 60 mm. petri dishes containing 500 of the harvested cells established for each EMS concentration. The petri dishes were then incubated for 7 days at 37°C, the medium poured off, and the cells stained and counted as described in Section 2.11.

The procedure for the determination of the toxicity of cells maintained at 34° was carried out as above, except that cells were incubated for up to 12 hours in the presence of a single concentration of EMS (8.5 mM), and subsequently incubated for 12 days at 34°C.

2.12.2. Method for selecting cells resistant to Toxic Amino Acid Analogues

BHK21-C13 cells were grown in 20 oz. medical flat bottles containing 40 ml. of EFC 10 medium, to a final density of approximately 1×10^7 cells. The medium was poured off, the cells washed with warm

Uptake Medium (37°C), and 40 ml. of EFC 10 medium containing a final concentration of 8.5 mM of EMS added. The cells were then incubated at 37°C for 2 hours, after which the medium was removed, the cells washed and harvested as described in Section 2.1.3. After resuspending in Plating Medium, 2 x 10^5 cells were plated into 100 x 20 mm. petri dishes containing a final volume of 15 ml. of the above medium. In later experiments 5 x 10^5 cells were transferred to 75 cm^2 screw cap tissue culture flasks containing 20 ml. of medium. The cells were incubated for 48 hours at 37°C, then washed with warm (37°C) Uptake Medium which was replaced by 20 ml. of modified Plating Medium which contained the analogue. Details of alterations in Plating Medium are given in the text. The cells were again incubated at 37°C and the medium replaced at 4 day intervals. On attainment of a suitable size all colonies were picked by the method described in Section 2.1.7. Following dispersal the cells were cultured in baby bottles containing modified Plating Medium with or without analogue. The cells were harvested while in log phase of growth, the majority frozen and stored (as described in Section 2.2.4) and the remainder tested.

2.12.3. Method for selecting cells unable to incorporate L-[^{3}H]-alanine or L-[^{3}H] glutamate

The procedures for the temperature sensitive (ts) selection of cells unable to transport tritiated L-alanine or L-glutamate, and the non-temperature sensitive (non-ts) selection using L-[^{3}H] glutamate are described in this section. An account of preliminary experiments to determine the toxicity of these radio-active amino acids, under these conditions, is given following a description of the actual selection procedures.

BHK21-C13 cells in log phase growth (approximately 1 x 10^7 cells per 20 oz. medical flat bottle) were exposed to 8.5 mM EMS for 2 hours at 37°C or 6 hours at 34°C for ts and non-ts selection procedures respectively. Cells were then washed with warm Uptake Medium (37°C or 34°C), harvested and 5 x 10^5 cells inoculated into 75 cm^2 screw cap tissue culture flasks or 20 oz. medical flat bottles. Cells were maintained for 40 or 60 hours at 37°C or 34°C (respectively for non-ts and ts selections). Cells for ts selections were then incubated for 24 hours at 39°C. Ts and non-ts cells were then incubated for a further 2 hours at these temperatures in $EdFC_{10}$ containing 2μCi/ml of carrier-free L-[^{3}H]alanine or glutamate. Cells were then transferred to and maintained at 25°C for 5 days. Cells were subsequently incubated at 37°C or 34°C for non-ts and ts selection procedures. Medium was changed at 4 day intervals. On attainment of suitable sizes all colonies were picked by method described in Section 2.1.7, and maintained in baby bottles containing 10 ml. Plating Medium.

Preliminary studies to determine the toxicity of tritiated L-alanine and L-glutamate under these selection procedures were carried out essentially as described above. However, the cells were not treated with mutagen, were inoculated into 25 cm^2 screw cap flasks at a density of 2000 cells per flask, and exposed to 0.5 to 5μCi/ml of radioactive amino acid at either 34° or 37°C (for ts or non-ts selection procedures) for intervals up to 3 hours. Colonies were stained at 16 days and 24 days for non-ts and ts selection procedures. Results were expressed as percentage plating efficiency of control cells, which were not exposed to radioactive amino acids. Experiments were carried out in triplicate.

2.13. SYNTHESIS OF L-[3-^{3}H]- CYSTEIC ACID

L-[3-^{3}H]- cysteic acid was synthesised from L-[3,3-^{3}H] Cystine HCl by the modification of the method of Moore (1963), described by Tabor and Tabor (1971).

L-[3,3-^{3}H] Cystine was spotted onto 3 MM Whatman chromatography paper and allowed to dry. The sample was then moistened with 0.02% Ammonium Molybdate (a catalyst for performic acid oxidation). After drying the sample was moistened with performic acid, formic acid (88%): Hydrogen Peroxide (30%), (9:1, v/v). The wet area of the chromatography paper was then placed over a petri dish (to keep the paper damp and allow the reaction to reach completion) and the sample incubated at 4^{o} for one hour. The strip was dried and the L-cysteic acid separated from impurities by high voltage electrophoresis. Potassium dichromate was used as a marker. After high voltage electrophoresis for 20 minutes at 3,000 volts in tank buffer of Pyridine:Acetic acid:water 250 : 10; 255 (v/v), pH 6.4, the strip was dried. The mobility of L-cysteic acid is 0.5, and the performic acid oxidation product of sulphide is 1.4, relative to dichromate. L-cysteic acid was eluted with water, freeze dried using a "Speedivac" centrifugal freeze drier, Model 5PS, and resuspended in double distilled water. The purity of L-cysteic acid was determined by determining the amount of L-cysteic acid in the proposed L-cysteic acid spot, as compared to that in the total sample applied, following a second electrophoresis of a 5 µl. sample. The L-cysteic acid was found to be greater than 98% pure.

SECTION 2.14. STATISTICAL ANALYSIS OF UPTAKE

The kinetic parameters Km and Vmax of the Michaelis-Menten equation (of linear form $\frac{S}{V} = \frac{Km}{Vmax} + \frac{S}{Vmax}$), and their relevant standard errors, for the uptake of an amino acid were determined by the method of weighted linear regression described by Wilkinson (1961).

Ki values for an amino acid (B) when acting as an inhibitor of the transport of an amino acid (A), were determined from the relationship $Ki = \frac{I}{\frac{Kp}{Km}} - 1$, where I is the inhibitor concentration and where the value Kp (and its standard error) was determined from the transport of amino acid A in the presence of B, as for Km values and their standard errors above.

The standard error (Ki) was determined from the relationship:

$$se\ (Ki) = \frac{Ki^4}{i^2}\left[\frac{Kp^2\ se(Km)^2 + Km^2\ se(Kp)^2}{Km^4}\right]$$

The significance of difference between a) Km values, b) Vmax values was determined from the approximation:

$$z = \frac{X_1 - X_2}{\left(se\ (X_1)^2 + se\ (X_2)^2\right)^{\frac{1}{2}}}$$

where X = Km , or Vmax, and z is distributed according to the standard normal distribution. Values are significantly different if z is lower than p = 0.05.

SECTION 3: CELL LINES

Cell lines of syrian and chinese hamster, mouse, sheep or human embryo origin were used in this study and are listed below. All cells were maintained in Glasgow modified Eagles medium (Macpherson and Stoker 1962) plus 10% (v/v) foetal calf serum, in a humidified atmosphere of 5% CO_2 : 95% air, at 37°C unless specified otherwise in the text.

3.1. Syrian Hamster (Mesocricetus auratus) cell lines

3.1.1. BHK21-C13 cells

History

The BHK21-C13 cell line is an established cell line originally derived from a single cell isolation (Macpherson 1963) of the original BHK21 cell line obtained from the kidneys of day old syrian hamsters (Macpherson and Stoker 1962). Cells of passage number 56 were kindly donated by E. McKay and cells of passage numbers 58 to 75 were used for all experiments.

Morphology: Fibroblast like

Karyology: Chromosome frequency distribution for 50 cells

Chromosome No.	37	39	40	41	42	43	44	45	80+
Cell No.	1	1	2	2	3	8	30	2	1

Protein content/10^6 cells (exponentially growing cells) : 0.50 mg Protein.

Population doubling time: 12 hours.

3.1.2. PyY - BHK21

History

PyY-BHK21, a polyoma transformed clone of BHK21 was isolated by Macpherson and Stoker (1962). The passage number was unknown but cells were not used at passages greater than 12 from the original cell stock obtained from C. Mucci.

Morphology: Bipolar or contracted fibroblasts

Karyology: examined by R.Marshall(1972)

Protein content/10^6cells (exponentially growing cells) : .48 mg Protein

Population doubling time: 10 hours.

3.1.3. Syr K1

History

This diploid cell line was derived from a pair of kidneys from a day old male syrian hamster, as described in Section 2.3. This cell line was recloned and cells of passage numbers between 3 and 10 were used.

Morphology: Fibroblast-like.

Karyology: Chromosome frequency distribution for 50 cells

Chromosome No:	36	42	43	44	45	46	82
Cell No.	1	2	3	41	2	1	1

Modal chromosome number = 44.

Protein content/10^6 cells (exponentially growing cells) : 0.53 mg Protein

Population doubling time: 14 hours.

3.2. Chinese Hamster (Critectulus griseus) cell lines

3.2.1. CHO-KG1

History

The CHO-K1 cell line is a proline requiring derivative (Kao and Puck 1967) of the CHO cell line obtained from the ovary of a chinese hamster (Puck 1958). This cell line was obtained from the American Type Culture Collection, Maryland, recloned and designated CHO-KG1 by R.H. Wilson. Cells were donated by R.H. Wilson and cells of passage 5-15 were used.

Morphology: Epithelial

Karyology: Chromosome frequency distribution for 50 cells

Chromosome No.	17	19	20	21	22	40	42
Chromosomes	1	3	36	5	2	2	1

Modal Chromosome number = 20

Protein content/10^6 cells (exponentially growing cells) : 0.42 mg Protein

Population doubling time: 13 hours.

3.2.2. C4B

History

The C4B cell line was derived from normal peritoneal cells of an adult female chinese hamster with a tetraploid 3-methyl chalenthrene induced fibrosarcoma (Yerganian and Leonard 1961). This cell line was originally designated 16B-FAF-7 but has been repeatedly recloned and redesignated C4B. Cells were donated by R.H. Wilson, and cells of passage 3 to 8 were used.

Morphology: Fibroblast like.

Karyology: Chromosome frequency distribution for 50 cells.

Chromosome No.	19	20	21	22	23	24	34	39	44
Cell No.	1	7	21	10	6	1	1	2	1

Protein content/10^6 cells (exponentially growing cells): 0.39 mg Protein

Population doubling time: 14 hours.

3.2.3. CH1 Series of diploid and SV-40 transformed chinese hamster cell lines

History

Cell lines were prepared from the spleen, liver, lungs and kidneys of a single 10 day old male chinese hamster, as described in Section 2.3. The cell lines were initially grown in plating medium (Section 2.1.2) plus

20% (v/v) foetal calf serum, cloned in this medium and subsequently maintained in Glasgow modified Eagles medium plus 10% (v/v) serum. Cultures of each of these cell lines were transformed by SV-40 virus, as described in Section 2.4. Cells between passage 3 and 12 were used. The normal and transformed cell lines are listed below.

CH1 Series of Cells

Tissue of Origin	Untransformed Cell Line	Corresponding Transformed Cell Line
Lung	CH Lu1	SV-CH Lu1
Kidney	CH Ki1	SV-CH Ki1
Liver	CH Li1	SV-CH Li1
Spleen	CH Sp1	SV-CH Sp1

Morphology:

a) Non-transformed cells: CH Lu1, CH Ki1, CH Sp1 were all fibroblast-like, but CH Li1 was epithelioid.

b) Transformed cells: All transformed cell lines were bipolar, contracted fibroblast-like cells.

Karyology: Chromosome frequency distribution for 50 cells.

	18	19	20	21	22	23	24	25	26	42	44+
CH Lu1			2	3	42		1	1			
SV-CH Lu1		1	7	2	31		4		2		4
CH Ki1			1	1	46	2					
SV-CH Ki1	1		2	5	26		13				1
CH Li1	1		2	1	41	1	3				
SV-CH Li1	1		5		32	5	3		2		2
CH Sp1			1	1	43	3	2				
SV-CH Sp1		1	1	2	36	3	5			1	1

All non-transformed cell lines showed modal chromosome numbers of 22 with at least 80% of the cells possessing this chromosome number. The karyotypes of the transformed cell lines, however, showed more variability.

Protein content and population doubling time

	Protein content/10^6 cells (exponentially growing cells) (mg protein)	Cell doubling time (hours)
CH Lu1	0.47	67
SV-CH Lu1	0.53	22
CH-Ki1	0.40	48
SV-CH Ki1	0.45	29
CH Li1	0.56	36
SV-CH Li1	0.48	16
CH Sp1	0.46	39
SV-CHSp1	0.39	22

3.3. Mouse (Mus Musculus) cell lines

3.3.1. 3T3

History

This "established" cell line was derived from disaggregated albino mouse embryos by Todaro and Green (1962) and evolved under conditions favouring marked contact inhibition (Todaro et al. 1964). Cells were donated by C. Pringle and cells of passage 5 to 10 (from frozen stocks designated passage 1), were used in subsequent experiments.

Morphology: Fibroblast like.

Karyology: Not examined, reported to be hypotetraploid with considerable chromosome breakage and rearrangement (Shannon 1972).

Protein content /10^6 cells: 0.51 mg protein (exponentially growing cells

Population doubling time: 20 hours

3.3.2. Py-3T3 and SV-3T3

History

These Polyoma or SV-40 transformed 3T3 cells were originally isolated by Todaro et al (1964) and donated by H. Otsuka. Cells were stored and cells of passage 3 to 10 (from frozen stocks designated passage 1) were used.

Morphology: Bipolar or contracted fibroblasts.

Karyology: Not examined.

Protein content and population doubling time

Cell Line	Protein content/10^6 cells (exponentially growing cells) (mg Protein)	Cell doubling time (hours)
SV-3T3	0.42	15
Py-3T3	0.48	14

3.4. Sheep (Ovis aries L.) cell lines

History

A number of untransformed cell lines were isolated from embryonic sheep. Cultures of these cells were subsequently transformed by SV-40 virus. The following cell lines were donated by Joan C. M. Macnab.

Tissue of Origin	Untransformed Cell Line	Corresponding Transformed Cell Line
Lung	SH.E.Lu	Py.SH.E.Lu
Kidney	SH.E.Ki	Py.SH.E.Ki
Spleen	SH.E.Sp	Py.SH.E.Sp

All untransformed cell lines were prepared from uncloned material, Polyoma transformed lines were obtained from transformed colonies. Cells of passages 4 to 11 were used.

Morphology:

Untransformed cells : Fibroblast-like

Transformed cells : Bipolar or contracted fibroblasts.

Karyology: Not studied due to the large chromosome numbers in sheep cells.

Protein content and population doubling time

Cell Line	Protein Content/10^6 cells (exponentially growing cells) (mg)	Population doubling time (hours)
SH.E.Lu	0.55	94
Py.SH.E.Lu	0.43	33
SH.E.Ki	0.49	38
Py.SH.E.Ki	0.41	30
SH.E.Sp.	0.52	42
Py.SH.E.Sp.	0.45	34

3.5. Human Cell Lines

3.5.1. HEK and HEL

History

These cell lines were originally derived from male human embryonic kidney and lung by Joan C.M. Macnab. This material was uncloned and cells of passage 4 to 8 were used.

Morphology: Highly elongated fibroblasts.

Karyology: Chromosome frequency distribution for 50 cells

Chromosome No.	42	43	44	45	46	47	48	49	50	51
Cell No. (HEK1	3		2	3	38		2	2		1
(HELu1)		2	3		41		3		1	

Protein content and population doubling time

Cell Line	Protein content/10^6 cells (exponentially growing cells) (mg)	Population doubling time (hours)
HEK	0.46	56
HEL	0.38	42

SECTION 4. L-GLUTAMATE TRANSPORT IN BHK21-C13 CELLS

4.1. INTRODUCTION

The transport of the naturally occurring acidic amino acids glutamate and aspartate has been studied in a wide variety of mammalian and microbial systems (reviewed by Heinz 1972, and Slayman 1973). A number of differences in the relative activity and specificities of transport systems for these amino acids has been observed. Relatively specific transport systems capable of transporting both these amino acids have been reported in mammalian (Schultz et al. 1970, Logan and Snyder 1971, Blasberg 1968, Teijema et al. 1974), and microbial systems (Joiris and Grensen 1969, Hunter and Segal 1971, Pall et al. 1970a, Robinson et al. 1973 a,b, Pateman et al. 1974). Separate aspartate and glutamate transport systems have also been observed in E. coli (Kay 1971, Halpern and Even-Shoshan 1967).

Little work has been carried out involving the transport of glutamate and aspartate in cultured mammalian cells, with the exception of the early work of Christensen and Riggs (1952), Christensen et al. (1952a), Herscovics and Johnstone (1964) and Heinz et al. (1965) with Ehrlichs ascites carcinoma cells. In this chapter the transport of the naturally occurring acidic amino acids, L-glutamate, L-cysteate and L-aspartate, and the properties of a proposed common "Acidic amino acid transport system" are investigated in BHK21-C13 cells. Transport of L-glutamate by cultured cells other than BHK is investigated in Section 7.

4.2. RESULTS

4.2.1. Kinetics of L-Glutamate Uptake into BHK21-C13 Cells

The effect of concentration on the initial rate of uptake of L-[U-^{14}C] glutamate was studied in exponentially growing BHK21-C13 cells. Initial rates of uptake were determined from transport over a ten minute period, during which time L-glutamate uptake was linear for concentrations in the studied range, 0.5×10^{-5} to 1×10^{-3}M. A typical plot for the initial rate of uptake of L-glutamate is shown in Figure 4.2.1a.

A plot of S/v versus S from initial transport rates (Fig. 4.2.1a) for the studied concentration range is shown in Fig. 4.2.1b. This plot is linear indicating that L-glutamate transport appears to follow Michaelis-Menten kinetics, and is consistent with transport via a single transport system over the examined concentration range. Statistically determined estimations of Km and Vmax produced values of $4.6 \pm 0.7 \times 10^{-5}$M and 7.5 ± 0.3 pmol/µg Protein/min.

4.2.2. Effects of Inhibitors on the transport of L-Glutamate in BHK21-C13 cells

The effects produced by a number of inhibitors on the transport of L-glutamate in BHK21-C13 cells are shown in Table 4.2.2. L-glutamate transport was examined in the presence of these inhibitors following preincubation of the cells for 10 minutes in uptake medium containing these compounds. Treatment with azide and cyanide, which both inhibit mitochondrial electron transfer, reduced transport by 92% and 97% respectively. Thus the transport of L-glutamate (1×10^{-5}M) appears to be energy-dependent. Ouabain which inhibits cation transport and certain K^+ dependent ATPases, reduced L-glutamate transport to 30% of the level observed in untreated cells. Such a reduction would be consistent with some of the energy for transport of this amino acid

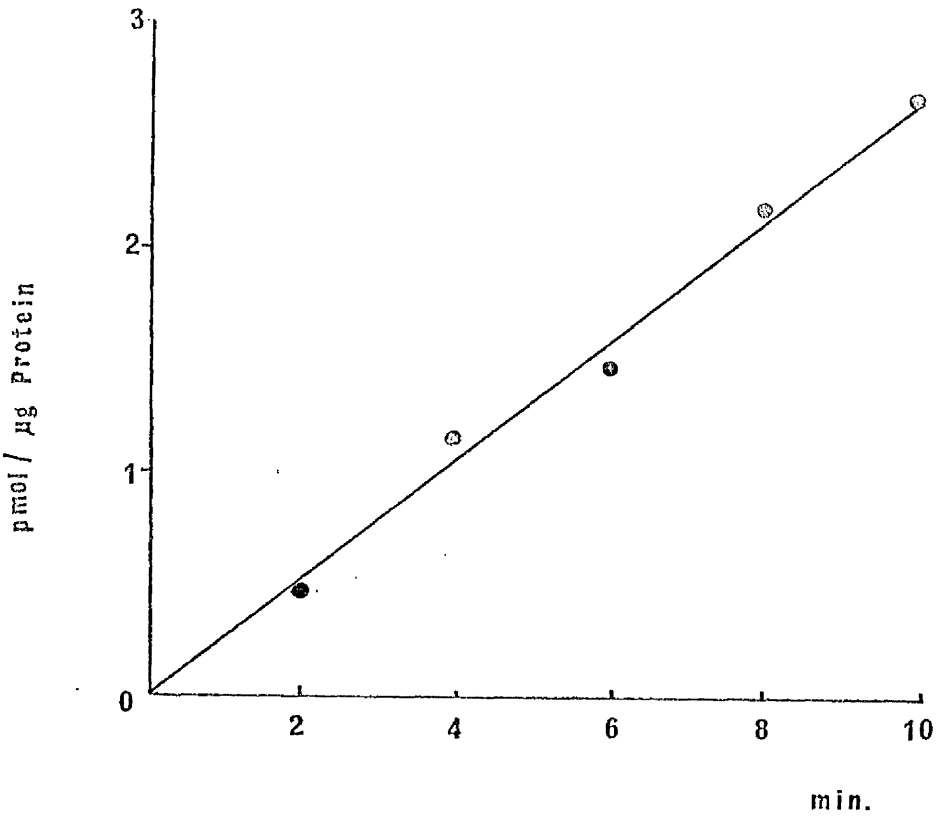


Figure 4.2.1a. Initial rate of L-glutamate uptake into BHK21-C13 cells

Cells were incubated for intervals of up to 10 minutes in Uptake Medium containing L-$\left[U-^{14}C\right]$ glutamate (2×10^{-5}M).

Uptake procedures were carried out as described in Section 2.8.

Figure 4.2.1b. S/v versus S plot of L-glutamate uptake into BHK21-C13 cells

Uptake of L-[U - ^{14}C]glutamate into BHK21-C13 cells was examined for the concentration range 0.5 x 10^{-5}M to 1 x 10^{-3}M. The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations of up to 10 minutes.

S/v values for concentrations of 1 x 10^{-4}M and below are shown on an expanded scale.

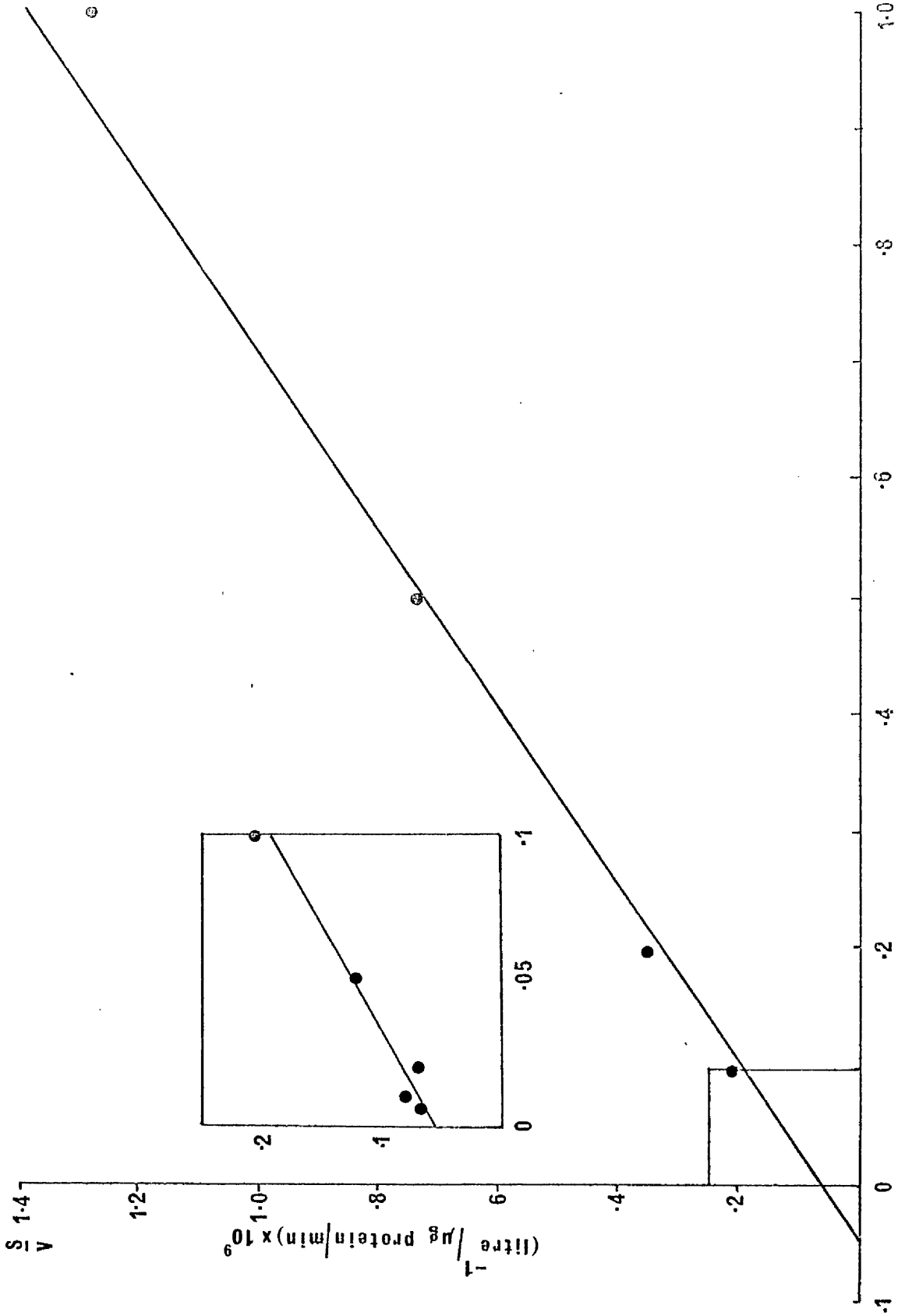


Figure 4.2.1b. S/v versus S plot of L-glutamate uptake into BHK21-C13 cells

Table 4.2.2. Effect of inhibitors on L-glutamate uptake in BHK21-C13 cells.

Inhibitor	Rate of L-glutamate uptake (pmol/μg protein/min)	t	P
None	$2.40 \pm .22$		
Cycloheximide (1×10^{-3}M)	$1.70 \pm .13$	2.67	$0.05 < 0.025$
p-chloro-mercuribenzoate (1×10^{-4}M)	$1.35 \pm .09$	4.46	< 0.005
N-ethyl-maleimide (1×10^{-4}M)	$1.10 \pm .09$	5.45	"
Ouabain (1×10^{-4}M)	$0.72 \pm .10$	7.43	"
Sodium cyanide (1×10^{-3}M)	$0.07 \pm .01$	10.53	"
Sodium azide (1×10^{-3}M)	$0.19 \pm .02$	10.00	"

Uptake of L-[U-^{14}C]glutamate (2×10^{-5}M) was examined in the presence of the above inhibitors, following preincubation of cells in their presence for 10 minutes. Rates of uptake were determined from 5 minute incubations. Values are means of 6 samples, plus SEM.
t values were calculated from comparison with control values, obtained prior to administration of inhibitor. Values were considered significant if P was less than 0.05 (t = 2.20).

being provided by the Sodium Pump. The sulphydryl reagents p-chloromercuribenzoate and N-ethylmaleimide were also seen to inhibit L-glutamate by approximately 43% and 54% respectively. This may indicate that sulphydryl groups are involved in L-glutamate transport, although this inhibition may not be directly at the carrier site.

4.2.3. Accumulation of L-glutamate in BHK21-C13 cells

BHK21-C13 cells were examined to determine if L-glutamate was accumulated to a level greater than that in the surrounding medium. Calculation of the accumulation ratio was based on a comparison of the concentration of extracellular L-[U-^{14}C] glutamate (1×10^{-5}M) with that observed in BHK21-C13 cells after an incubation period of 10 minutes.

A measure of the radioactivity (dpm) of the TCA insoluble fraction indicated that this comprised only 5% of the total sample radioactivity, indicating that only a small proportion of the transported amino acid is incorporated into protein in the period studied.

Ionophoresis of the TCA soluble sample produced a single "spot" which contained approximately 94% of the total label applied, see Figure 4.2.3. The "spot" obtained ran coincidently with control sample runs of L-[U-^{14}C] glutamate.

After 10 minutes incubation in Uptake Medium containing 1×10^{-5}M L-[U-^{14}C] glutamate the intracellular concentration of radioactive L-glutamate was approximately 3.05×10^{-4}M. Sodium azide (5×10^{-3}M) and sodium cyanide (2×10^{-3}M) treated cells showed no radioactive accumulation. This value is

Figure 4.2.3. Ionophoretogram of TCA soluble extract of BHK21-C13 cells following incubation in the presence of L- U-^{14}C glutamate

50 µl of TCA soluble extract of BHK21-C13 cells, obtained following incubation of cells for 10 minutes in Uptake Medium containing L-[U-^{14}C] glutamate (1 x 10^{-5}M), was spotted onto Whatman 3 MM chromatography paper (width 3 cm). The sample was run on low voltage vertical ionophoresis for 3 hours at 200 v (5mA/strip) in tank buffer pH 1.9 (58 ml. glacial acetic acid : 26 ml. 25% (w/v) formic acid, to a final volume of 2 litres). The ionophoretogram was air dried, cut into 1 cm strips, the radioactivity of which was determined by liquid scintillation spectrophotometry. Results are expressed as dpm/cm strip.

Figure 4.2.3. Ionophoretogram of TCA soluble extract of BHK21-C13 cells following incubation in the presence of L-[U-^{14}C] glutamate

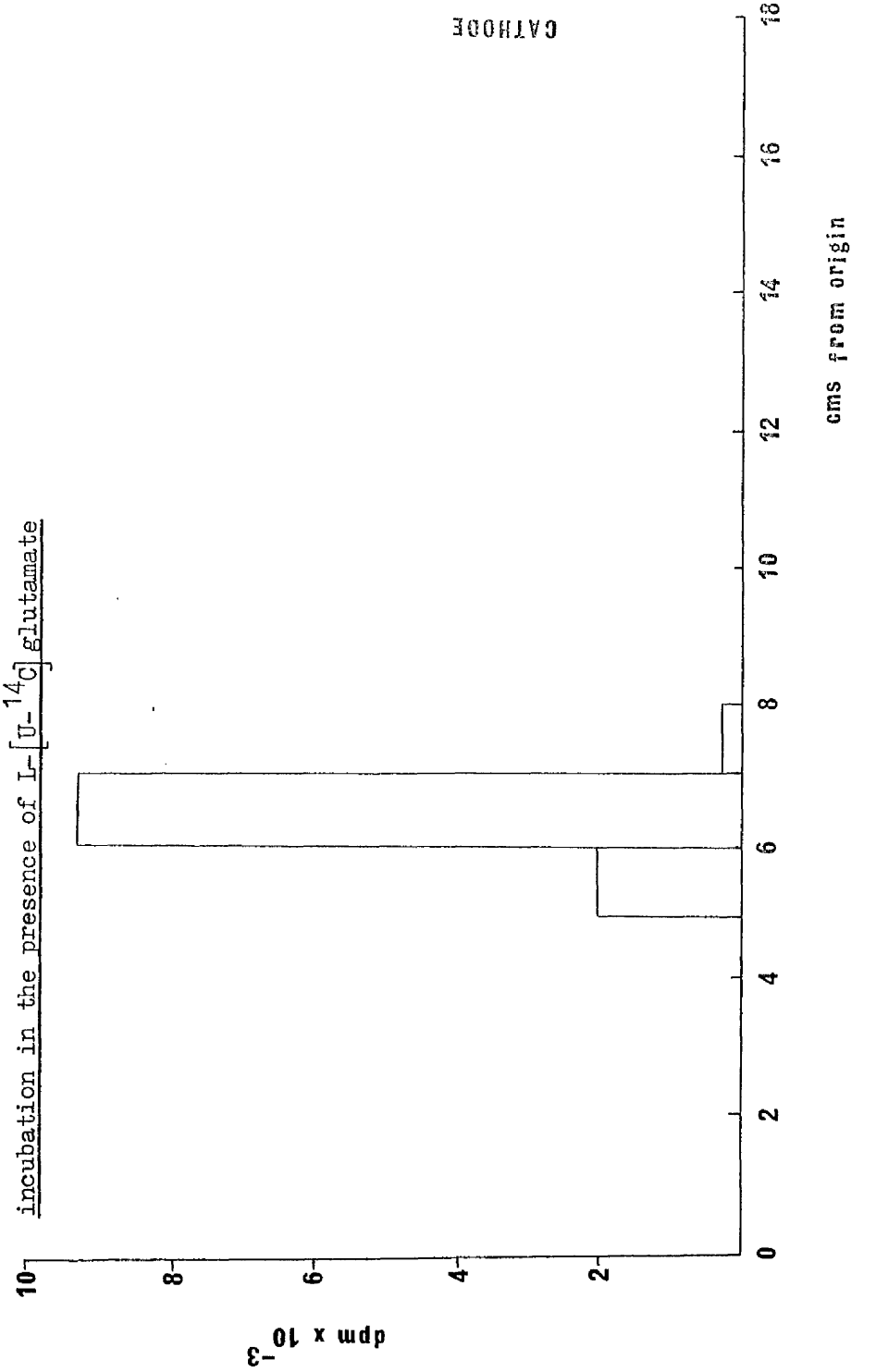


Table 4.2.3. Accumulation of L-glutamate in BHK21-C13 cells.

Total cell volume (μl)	Total dpm in cells	dpm in TCA insoluble fraction	dpm as L-glutamate	dpm in equivalent extracellular volume	Intracellular concentration / Extracellular concentration
2.62 (8.1×10^{5} cells of mean cell volume 3.23×10^{-6} µl)	390,535	19,058	350,786	11,509	30.48
Sodium azide (5×10^{-3}M) and Sodium cyanide (2×10^{-3}M) treated cells.	1,631	107	1,524	11,509	0.13

Cells were incubated for 10 minutes in Uptake Medium containing L-[U-^{14}C] glutamate (1×10^{-5}M). TCA extraction and ionophoresis were subsequently carried out as described in Sections 2.9.1 and 2.9.2. The above values are the means of two experiments.

probably an under-estimate as the total cell volume was considered as the total intracellular space available. These results are summarised in Table 4.2.3.

4.2.4. Effects of Temperature on L-Glutamate uptake in BHK21-C13 cells

Many active transport systems have a high temperature coefficient as energy metabolism is itself temperature dependent. The transport of L-glutamate was measured at various temperatures, see figure 4.2.4. The results given in figure 4.2.4 show that at $1^{o}C$ the observed rate of uptake was only 0.06 p mols/µg protein and remains minimal until above 15^{o} when the rate of transport rapidly increases to an optimum at about 39^{o}, (slightly above the normal growth temperature of 37^{o}.) There is thus an approximate forty-fold increase in uptake between 1^{o} to 39^{o}, with a Q10 value of 2.41 for the increasing rate of transport between 25^{o} and 37^{o}. This large increase in uptake and the non-linear pattern of increase suggests L-glutamate uptake is not a passive process (which has a low temperature coefficient). The decrease in L-glutamate uptake in the cells at higher temperatures is probably due to increasing numbers of dead cells.

4.2.5. Effects of pH on L-glutamate transport in BHK21-C13 cells

The rate of L-glutamate transport in BHK21-C13 cells was measured at various pH values. This transport varied with the pH value of the assay medium, as shown in figure 4.2.5, and showed a pH optimum at approximately pH 7.3. At this value the α-amino group is fully protonated and both the α and the γ-carboxyl groups are in the non-protonated form, $^{-}O_2C.CH_2.CH_2.CH(NH3^{+}).CO_2^{-}$. (pKa values; α-carboxyl, 2.19; γ-carboxyl 4.25; α-amino, 9.67).

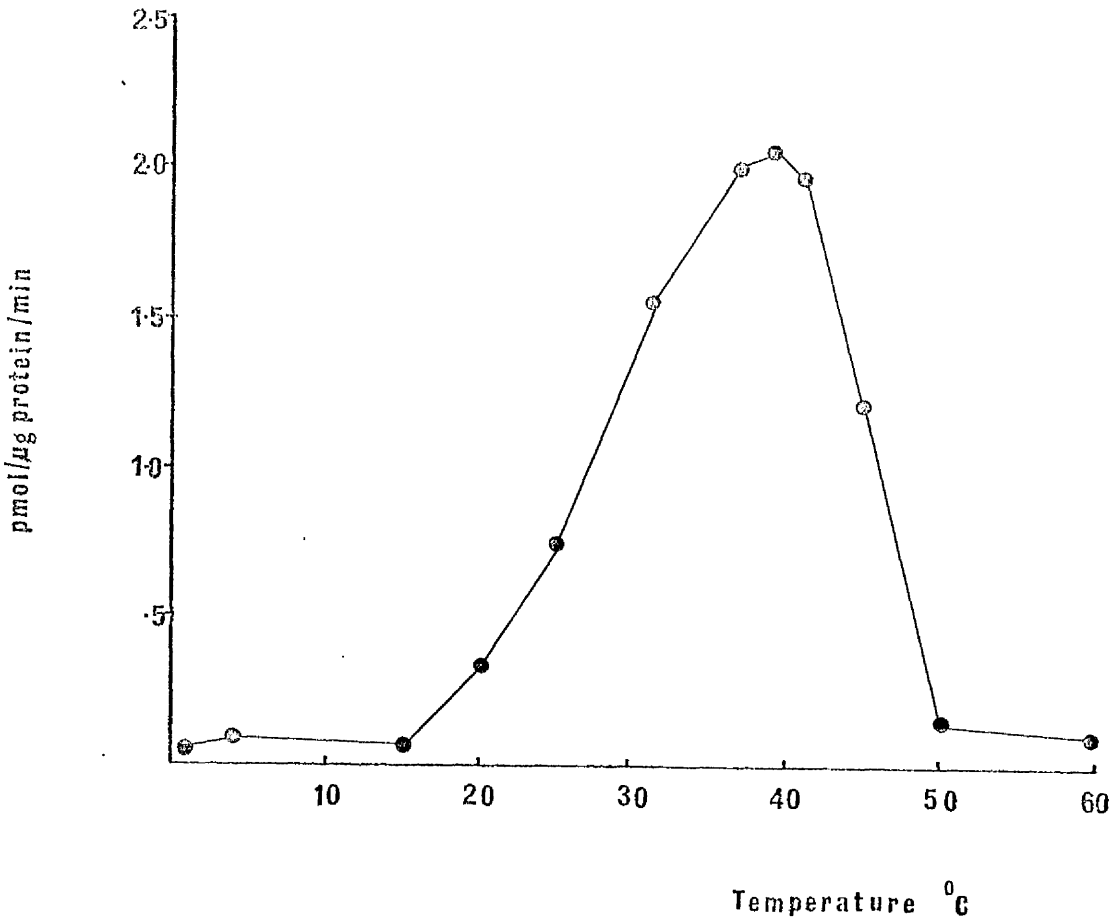


Figure 4.2.4. Effect of temperature on the uptake of L-glutamate into BHK21-C13 cells

The uptake of L-[U-^{14}C]glutamate (2 x 10^{-5}M) was examined at various temperatures following a 10 minute preincubation of the cells at the temperature to be used in the incubation. Initial rates of uptake were based on 10 minute incubations and each point is the mean of six samples.

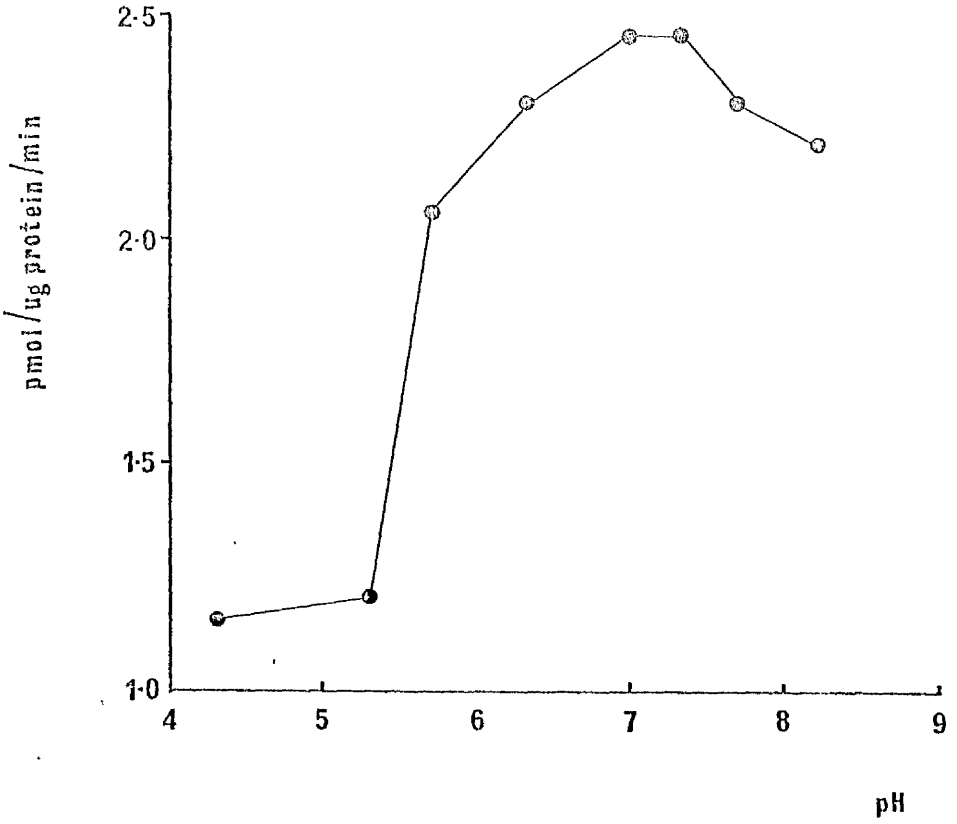


Figure 4.2.5. Effect of pH on L-glutamate uptake into BHK21-C13 cells

The uptake of L-[U-^{14}C]glutamate (2 x 10^{-5}M) was examined at various pH's between 4.3 and 8.3. Initial rates of uptake were based on 10 minute incubations and each point is the mean of six samples.

4.2.6. Effect of Na^+ concentration on L-glutamate uptake into BHK21-C13 cells

A vital role for Na^+ in the active transport of certain amino acids has been demonstrated in a number of mammalian tissues (Schultz et al. 1970, Riggs et al. 1968, Inui and Christensen 1967). Ouabain was demonstrated in section 4.2.2. to severely inhibit L-glutamate uptake, and consequently the possible Na^+ dependence of this transport system was studied. L-glutamate uptake was studied in uptake medium containing different concentrations of Na^+ ions (see figure 4.2.6). NaCl was replaced by ideal equiosmolar concentrations of choline chloride or mannitol to maintain the osmolarity.

The initial rate of L-glutamate uptake can be seen to be reduced as $[Na^+]$ is decreased. When Na^+ is completely replaced by mannitol or choline chloride, minimal uptake of L-glutamate is observed. Transport of L-glutamate would thus appear to show a high degree of dependence on extracellular Na^+.

4.2.7. Specificity of the L-glutamate transport system of BHK21-C13 cells

The specificity of the L-glutamate transport system was investigated by determining the ability of a number of naturally occurring amino acids and certain glutamate and aspartate analogues to inhibit the transport of L-glutamate (2×10^{-5}M). The inhibitory effects of various amino acids and related compounds are shown in Tables 4.2.7(a) and (b). Only L-glutamate, L-aspartate, L-cysteate and DL-4-fluoroglutamate produced considerable inhibition of L-$[U^{-14}C]$ glutamate transport, indicating these amino acids may be transported by the same permease. In order to further investigate this possibility a more detailed kinetic analysis of the transport and inhibition by these compounds was made. These investigations

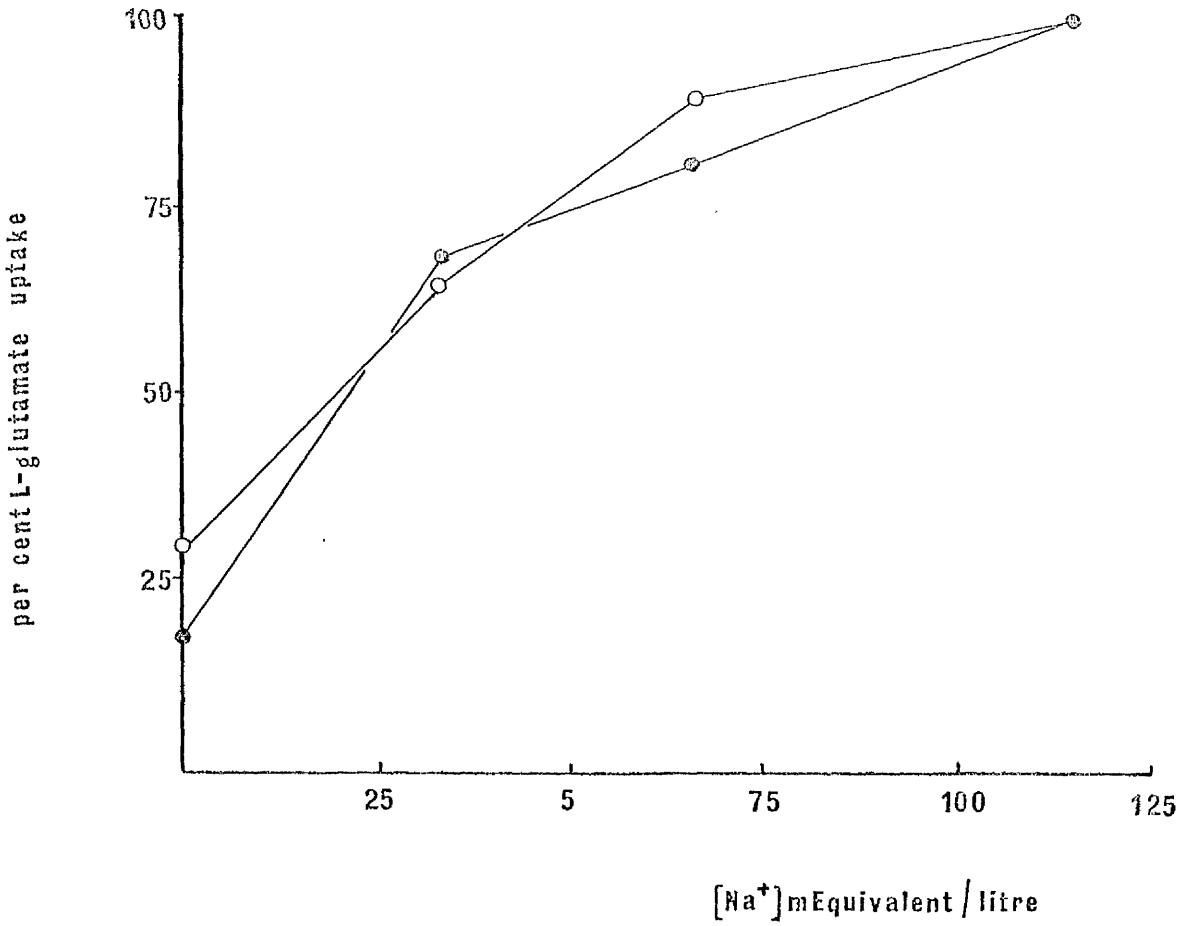


Figure 4.2.6. Effect of Na ion concentration on L-glutamate uptake into BHK21-C13 cells

Uptake of L-[U-^{14}C] glutamate (2 x 10^{-5}M), from Uptake Medium containing varying Na^+ concentrations, was examined for 10 minute incubations. The NaCl component of Uptake Medium was replaced by ideal equiosmolar quantities of D-mannitol or Choline Chloride. Uptake is expressed as percentage uptake from standard Uptake Medium (Section 2.1.4). Each value is the mean of six samples.

Uptake in the presence of D-mannitol (O-O).
Uptake in the presence of Choline Chloride (●-●).

Table 4.2.7a. Inhibition of L-glutamate uptake into BHK21-C13 cells, by different amino acids.

Inhibiting amino acid	Percentage inhibition of L-[U-^{14}C]glutamate uptake.
L-glutamate	89
L-aspartate	94
L-cysteate	79
L-alanine	3
L-serine	5
L-valine	7
L-threonine	6
L-phenylalanine	8
L-tyrosine	6
L-tryptophan	7
L-leucine	10
L-isoleucine	8
L-arginine	3
L-lysine	6
L-cysteine	6
L-cystine	14
L-methionine	15
L-proline	7
Glycine	6
L-glutamine	16
L-asparagine	10
D-glutamate	13

Uptake of L-[U-^{14}C]glutamate (2×10^{-5}M) was examined in the presence and absence of inhibiting amino acid (of concentration 5×10^{-4}M). Incubations were made for 10 minutes. Values are the means of 5 samples and are expressed as percentage inhibition of L-[U-^{14}C]glutamate uptake alone.

Table 4.2.7b. Inhibition of L-glutamate uptake into BHK21-C13 cells, by various amino acid analogues.

Inhibiting amino acid analogue	% inhibition of L-[U-^{14}C] glutamate uptake.
a) ∝-amino group modifications.	
∝-keto glutarate	-8
N-acetyl-L-glutamate	12
N-acetyl-DL-aspartate	6
N-formyl-L-aspartate	10
N-p-nitrobenzoyl-L-glutamate	8
b) ∝-hydrogen group modifications.	
DL-2-methyl-glutamate	6
DL-2-methyl-aspartate	7
c) ∝-carboxylate group modifications.	
∝-amino-butyrate	-3
d) δ-carboxylate group modifications.	
L-glutamic acid-5-methyl-ester	9
L-homocysteate	3
e) Side chain modifications.	
DL-erythro-3-hydroxy aspartate	5
DL-threo-3-hydroxy aspartate	-6
DL-4-fluoroglutamate	71
DL-∝-amino-adipate	14

Uptake of L-[U-^{14}C] glutamate (2 x 10^{-5}M) was examined in the presence and absence of inhibiting amino acid analogue (of concentration 5 x 10^{-4}M, for L-amino acid analogues, or 1 x 10^{-3}M for DL-amino acid analogues). Incubations were made for 10 minutes. Values are the means of 5 samples and are expressed as percentage inhibition of L-[U-^{14}C] glutamate uptake alone.

are described in the following sections, 4.2.7(a) and (b).

4.2.7(a). Kinetics of L-Aspartate and L-Cysteate uptake into BHK21-C13 Cells

The initial rates of L-aspartate and L-cysteate transport, for the concentration range 0.5×10^{-5}M to 1×10^{-3}M were determined from uptake over a 10 minute period, during which time uptake was linear. S/v versus S plots of the initial transport rates are shown in figures 4.2.7(a) and (b). These plots appear to be linear indicating that transport of these amino acids appear to follow Michaelis-Menten kinetics, an observation not inconsistent with each of these amino acids being transported by a single transport system (over the examined concentration range). Statistically determined estimations of Km and Vmax produced values of $3.4 \pm 0.8 \times 10^{-5}$M, and 6.1 ± 0.3 pmols/µg Protein/min for L-aspartate, and $6.9 \pm 0.1 \times 10^{-5}$M and 1.3 ± 0.05 pmols/µg Protein/min for L-cysteate uptake. The kinetics of DL-4-fluoro-glutamate uptake was not studied as radio-actively labelled fluoroglutamate was not commercially available or readily synthesised.

4.2.7(b). Kinetics of Inhibition of L-glutamate and L-aspartate uptake in BHK21-C13 cells

The initial rates of uptake of L-glutamate, for concentrations between 0.5×10^{-5} and 1×10^{-3}M, in the presence of a single concentration of the inhibiting amino acid: L-aspartate (2×10^{-4}M) L-cysteate (5×10^{-4}M) and DL-4-fluoroglutamate (5×10^{-4}M) are shown in figures 4.2.7 c to e. L-aspartate transport (for the concentration range 0.5×10^{-5} to 1×10^{-3}M) in the absence and presence of either L-glutamate (2×10^{-4}M) L-cysteate (5×10^{-4}M) and DL-4-fluoroglutamate (5×10^{-4}M) is shown in figures 4.2.7 f to h. As the Vmax for L-cysteate was extremely low and no radioactive DL-4-fluoro-glutamate was available, kinetic studies for the inhibition of the transport of these compounds were not carried out.

Figure 4.2.7a. S/v versus S plot of L-aspartate uptake into BHK21-C13 cells

L-[G-^{3}H] aspartate uptake into BHK21-C13 cells was examined for the concentration range 0.5 x 10^{-5}M to 1 x 10^{-3}M. The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations of up to 10 minutes.

S/v values for concentrations of 1 x 10^{-4}M and below are shown on an expanded scale.

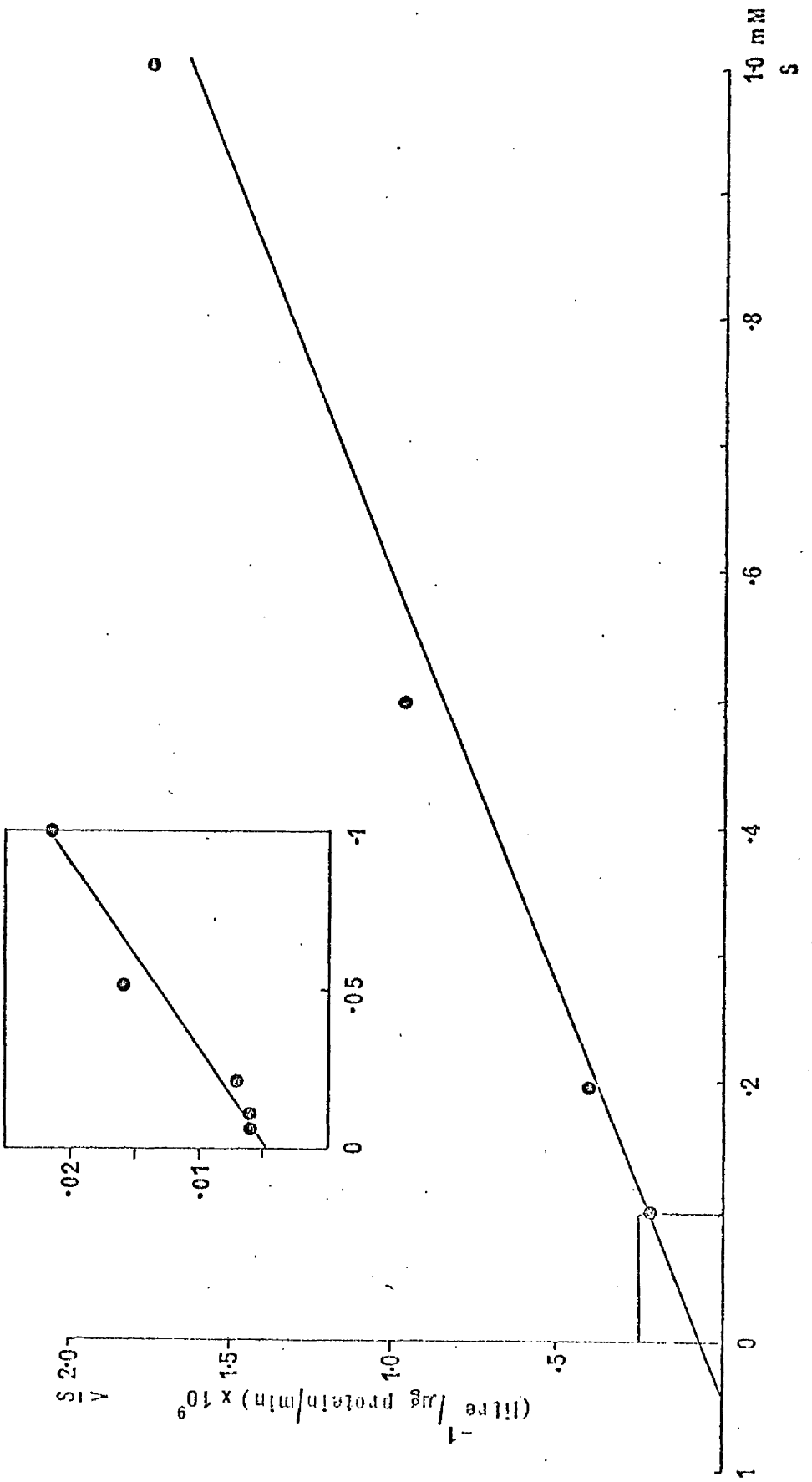


Figure 4.2.7a. S/v versus S plot of L-aspartate uptake into BHK21-C13 cells.

Figure 4.2.7b. S/v versus S plot of L-cysteate uptake into BHK21-C13 cells

L-[U- ^{3}H]-cysteate uptake into BHK21-C13 cells was examined for the concentration range 0.5 x 10^{-5}M to 1 x 10^{-3}M.

The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations of up to 10 minutes.

S/v values for concentrations of 1 x 10^{-4}M and below are shown on an expanded scale.

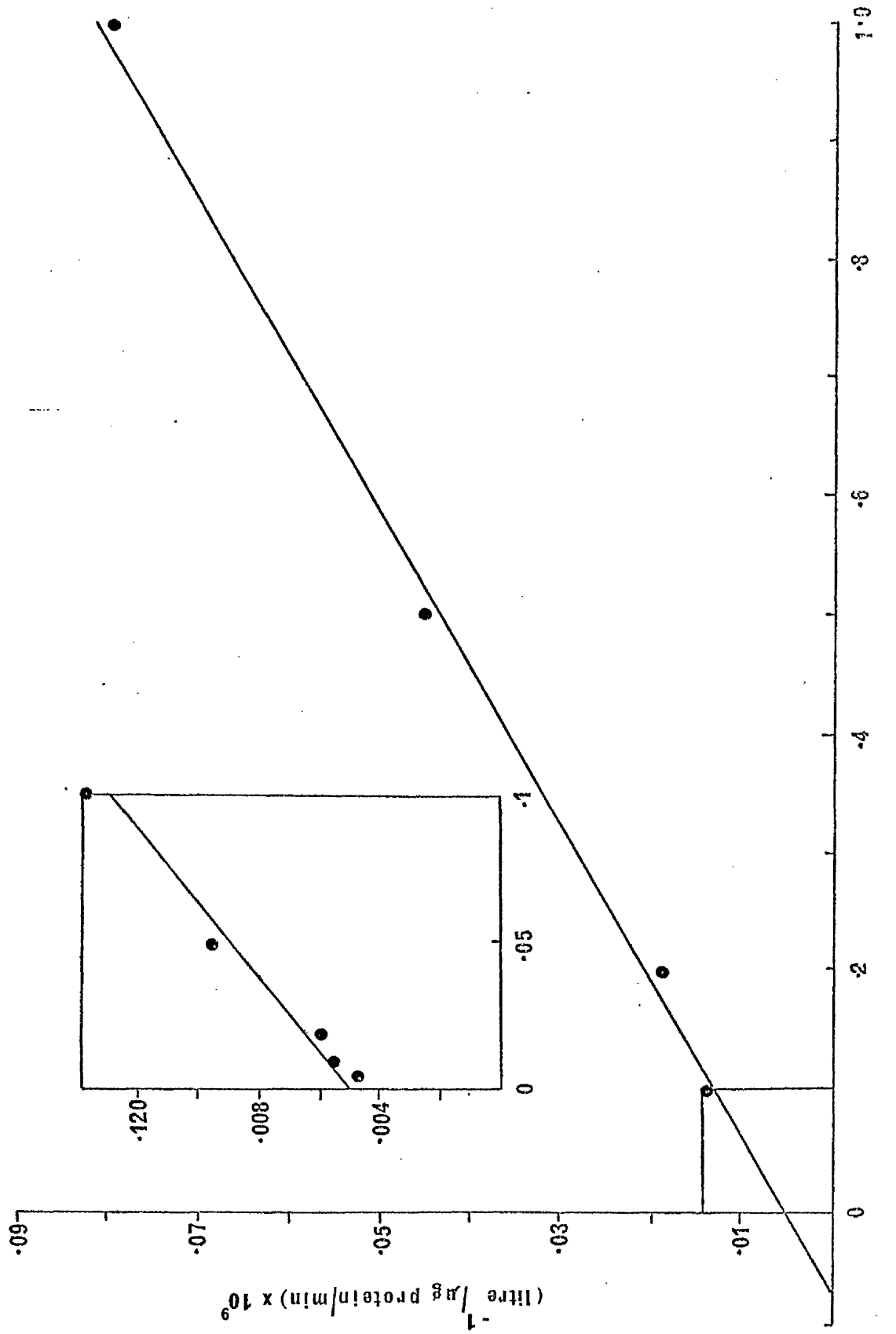


Figure 4.2.7b. S/v versus S plot of L-cysteate uptake into BHK21-C13 cells

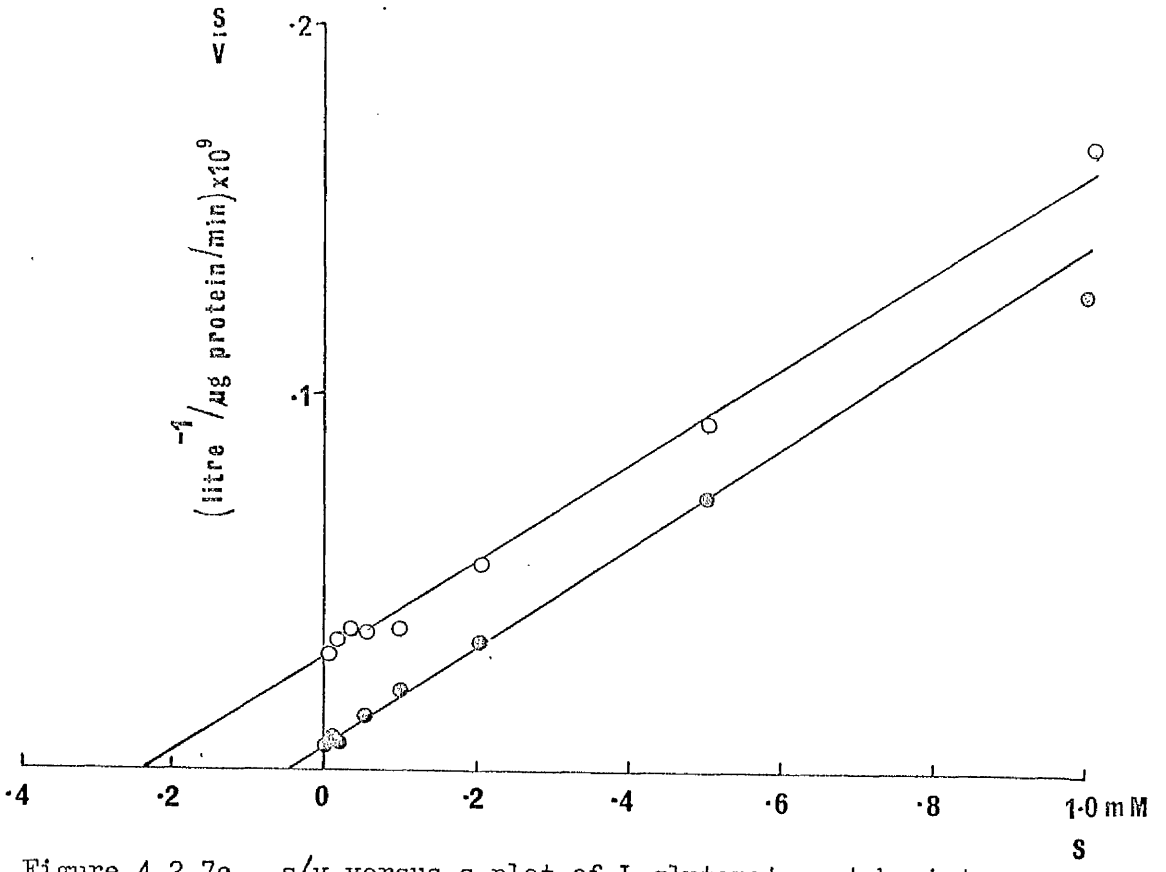


Figure 4.2.7c. s/v versus s plot of L-glutamate uptake into BHK21-C13 cells in the presence and absence of L-aspartate.

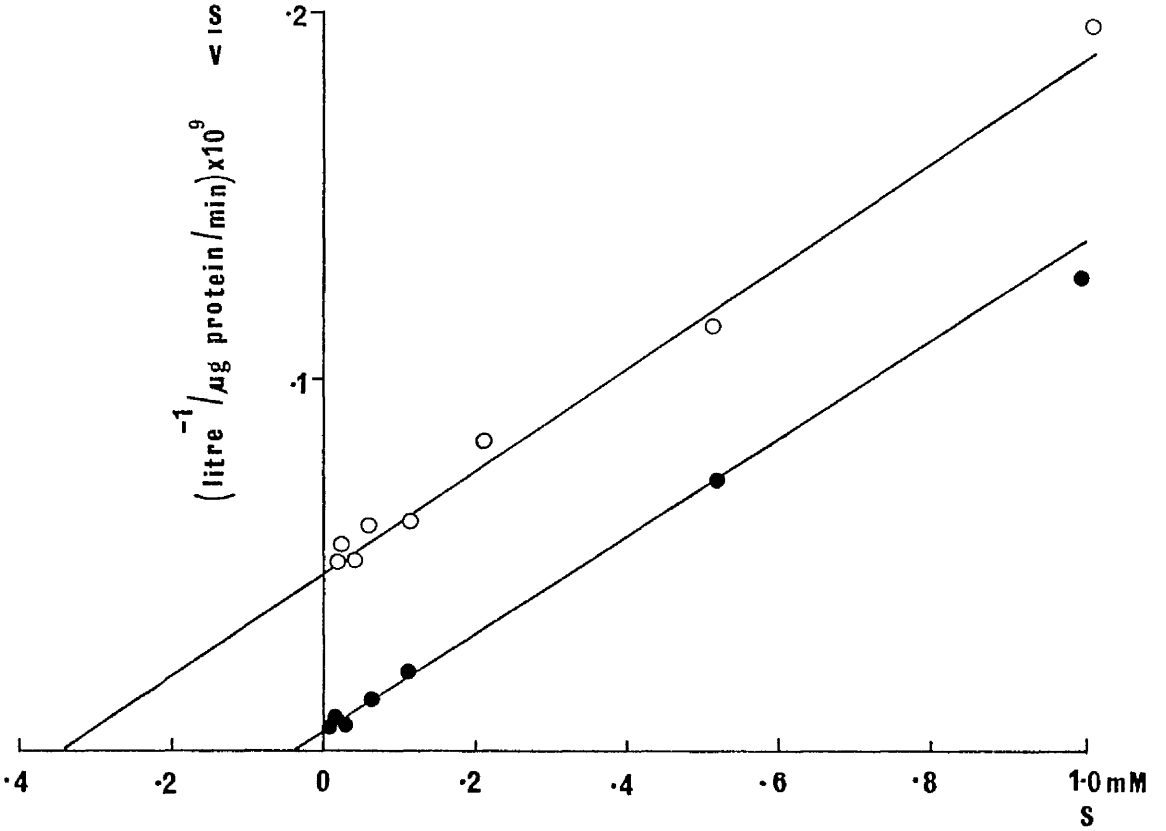


Figure 4.2.7d. s/v versus s plot of L-glutamate uptake into BHK21-C13 cells in the presence and absence of L-cysteate.

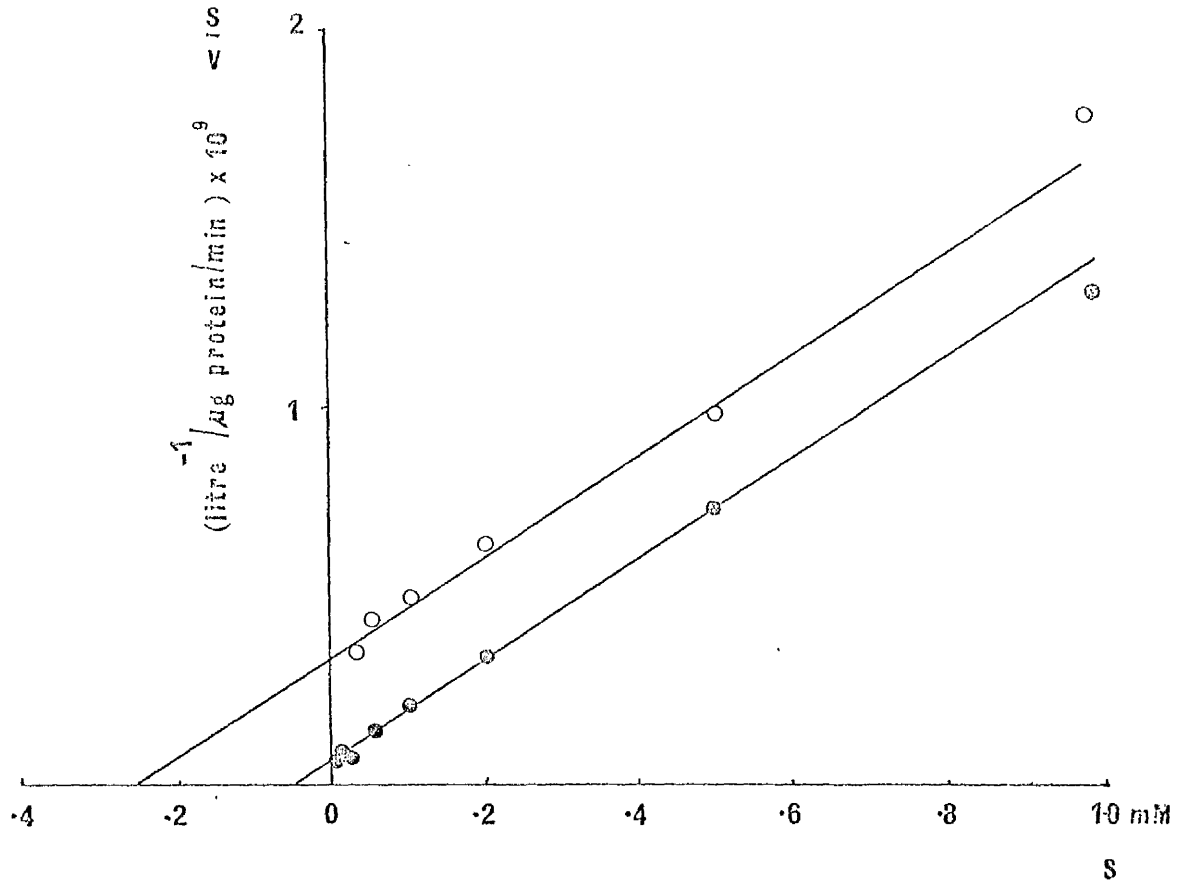


Figure 4.2.7e. S/v versus S plot of L-glutamate into BHK21-C13 cells in the presence and absence of DL-4-fluoroglutamate.

Figures 4.2.7 c to e. S/v versus S plots of L-glutamate transport into BHK21-C13 cells, in the presence and absence of L-aspartate, L-cysteate and DL-4-fluoroglutamate.

Uptake of L-[U- ^{14}C] glutamate into BHK21-C13 cells was examined (for the concentration range 0.5 x 10^{-5}M to 1 x 10^{-3}M L-glutamate) in the presence and absence of:

a) 2 x 10^{-4}M L-aspartate (Figure 4.2.7c)

b) 5 x 10^{-4}M L-cysteate (Figure 4.2.7d)

c) 5 x 10^{-4}M DL-4-fluoroglutamate (Figure 4.2.7e)

The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations of up to 10 minutes.

L-glutamate uptake alone (●-●)

L-glutamate uptake in the presence of competing amino acid (O-O).

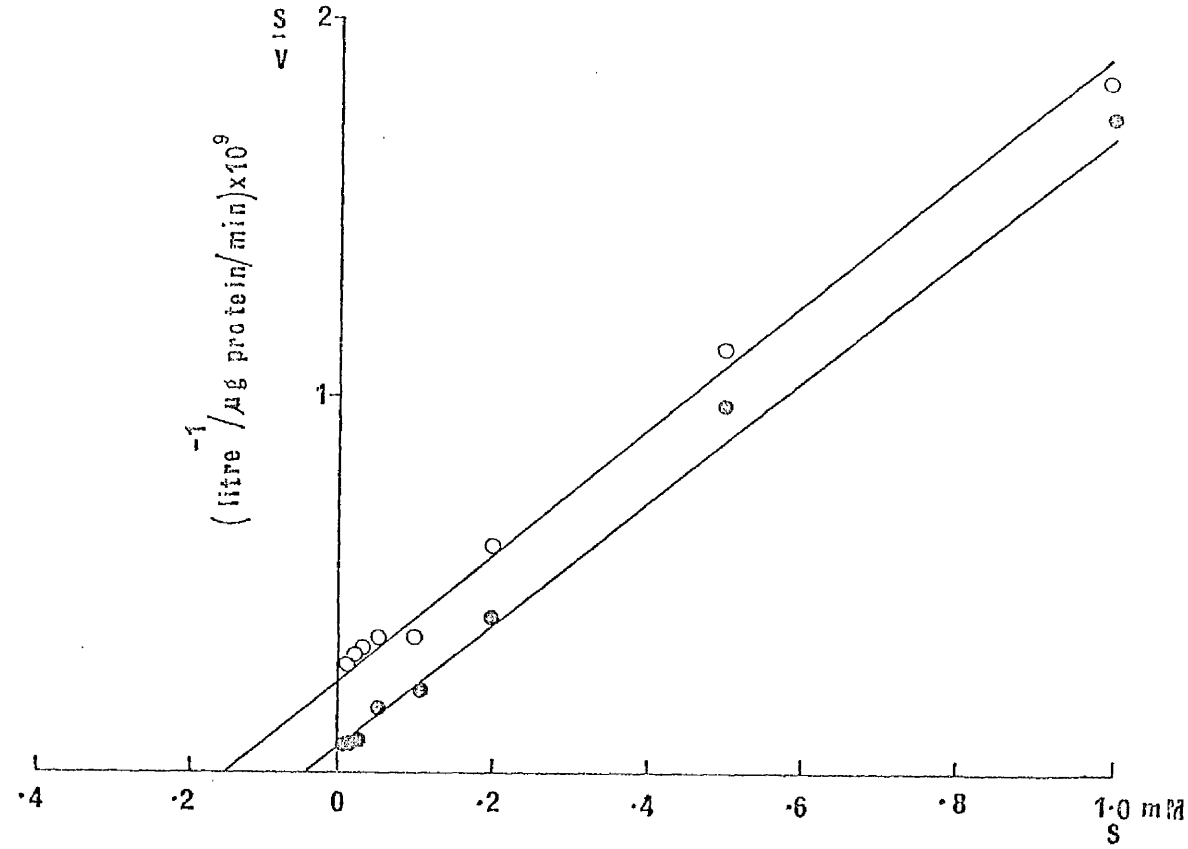


Figure 4.2.7f. S/v versus S plot of L-aspartate uptake into BHK21-C13 cells in the presence and absence of L-glutamate.

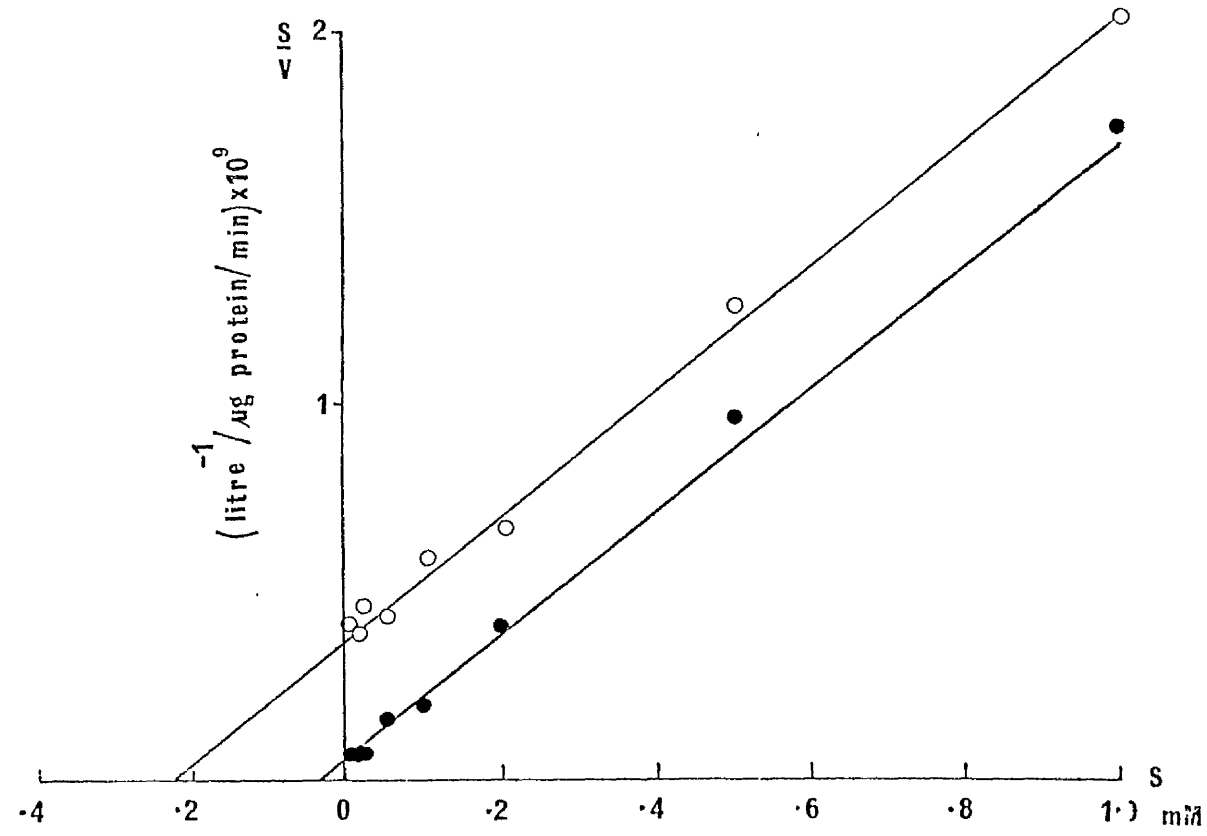


Figure 4.2.7g. S/v versus S plot of L-aspartate uptake into BHK21-C13 cells in the presence and absence of L-cysteate.

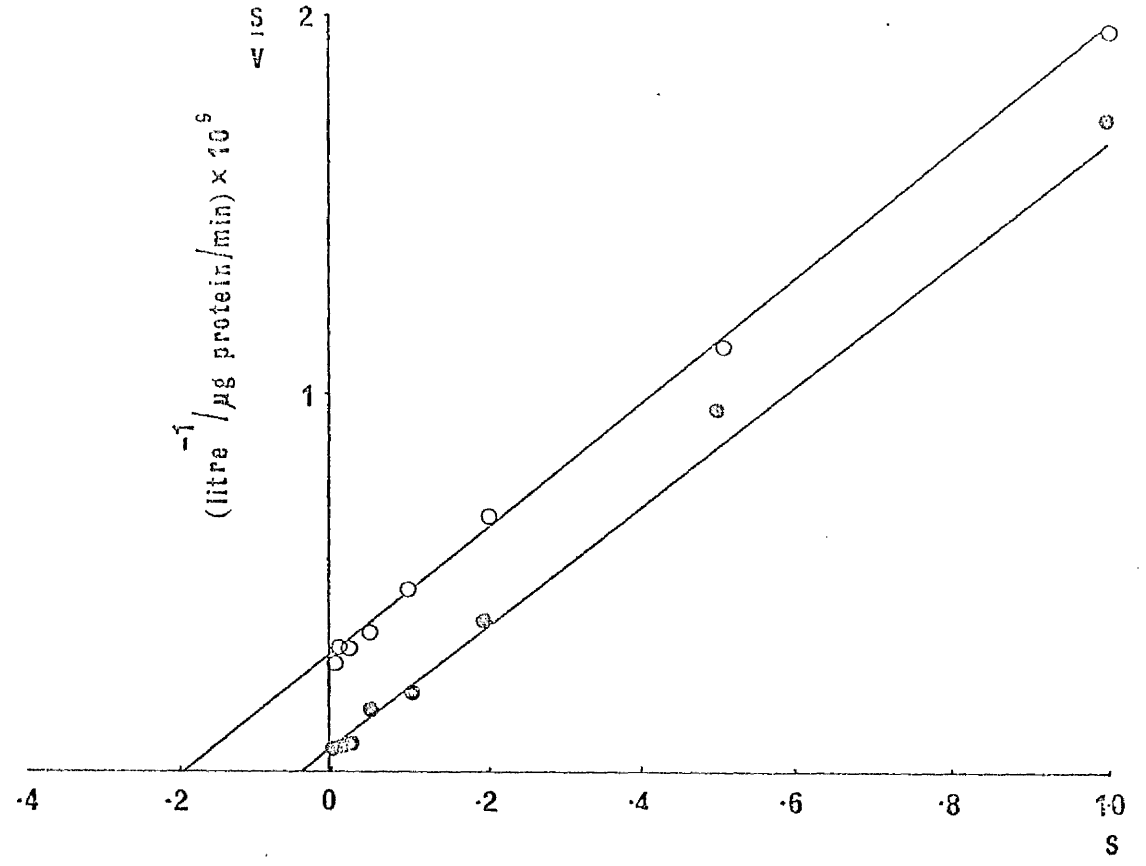


Figure 4.2.7h. S/v versus S plot of L-aspartate uptake into BHK21-C13 cells in the presence and absence of DL-4-fluoroglutamate.

Figure 4.2.7f to h. S/v versus S plots of L-aspartate transport in BHK21-C13 cells, in the presence and absence of L-glutamate, L-cysteate and DL-4-fluoroglutamate.

Uptake of L-$\left[G\ -^{3}H\right]$ aspartate into BHK21-C13 cells was examined for the concentration range 0.5×10^{-5}M to 1×10^{-3}M L-aspartate in the presence and absence of:

a) 2×10^{-4}M L-glutamate (Figure 4.2.7f)

b) 5×10^{-4}M L-cysteate (Figure 4.2.7g)

c) 5×10^{-4}M DL-4-fluoroglutamate (Figure 4.2.7h)

The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations of up to 10 minutes.

L-aspartate uptake alone (●-●)

L-aspartate uptake in the presence of competing amino acid (o-o).

The S/v versus S plots for L-glutamate uptake in the presence of inhibiting amino acids, L-aspartate, L-cysteate, and DL-4-fluoro-glutamate were approximately parallel to that obtained for L-glutamate uptake alone (Figures 4.2.7c to e). Similar results were obtained for L-aspartate uptake in the presence and absence of L-glutamate, L-cysteate and DL-4-fluoroglutamate (Figures 4.2.7 f to h). This data thus indicates that the inhibition, observed for each of these amino acids, was competitive. If the inhibition had been non-competitive the gradient of the regression line for transport in the presence of inhibitor would be greater than in its absence and the line would meet the abscissa at -Km (-Kp would thus equal -Km).

Km values for the transport and estimates of the Ki values for inhibition by these amino acids, are summarised in Table 4.7.2c. Ki values for L-glutamate, L-aspartate and L-cysteate, when acting as inhibitors were similar to their Km values for transport.

This data is consistent with the requirements of the ABC test of Ahmed and Scholefield (1962) for L-glutamate, L-aspartate and L-cysteate being transported via a single system:-

a) L-glutamate and L-aspartate are competitive inhibitors of each other;

b) the Km value for the transport of L-glutamate is equal to its Ki when inhibiting L-aspartate uptake, and vice versa.

c) L-cysteate, a competitive inhibitor of L-aspartate and L-glutamate has a similar Ki in each case.

The similar Kp values obtained for 4-fluoroglutamate when inhibiting the uptake of L-glutamate and L-aspartate are also consistent with this amino acid analogue being transported via this "acidic amino acid transport system".

Table 4.7.2c. Comparison of Km and Ki values for entry of acidic amino acids into BHK21-C13 cells.

Amino Acid	Km (μM)	Ki values when acting as an inhibitor of : L-glutamate (μM)		L-aspartate (μM)	
L-glutamate	46 ± 7	-	-	58 ± 11	(151 ± 19)
L-aspartate	34 ± 8	47 ± 10	(242 ± 18)	-	-
L-cysteate	69 ± 5	77 ± 17	(346 ± 37)	91 ± 27	(221 ± 17)
DL-4-fluoroglutamate	-	114 ± 24	(247 ± 18)	104 ± 31	(197 ± 17)

Km and Ki values for the uptake of these amino acids were determined as described in Sections 2.8.5. and 2.8.6. respectively. Kp values, from which Ki values and their standard errors were calculated, are shown in parenthesis.

4.3. DISCUSSION AND CONCLUSION

L-glutamate uptake into BHK21-C13 cells grown in monolayers was studied in detail and appeared to be via a single amino acid transport system over the concentration range studied. This transport system was shown to be energy-linked, highly temperature dependent and exhibited a pH optimum of approximately 7.3. L-glutamate was shown to be accumulated in 10 minutes to a level approximately 30 fold that in the surrounding medium by this transport system.

L-glutamate was shown to have a relatively high affinity for this permease and exhibited a Km of $4.6 \pm 0.7 \times 10^{-5}$M. It should be mentioned here that the values of Km and Vmax were calculated statistically, as graphic plots rarely provide a measure of precision of these values, and in order to avoid possible errors from subjective fitting of the data. S/v plots versus S plots were made in preference to the more commonly used double reciprocal Lineweaver-Burk plot, as statistical considerations discussed by Williamson (1961) and Coloquoun (1970) and studies using simulated data (Dowd and Riggs 1965) suggest that more accurate estimates of these kinetic parameters can be made using the method used in this study.

Detailed kinetic inhibition studies indicated that L-aspartate, L-cysteate and DL-4-fluoroglutamate are also transported by the L-glutamate transport system, which is consequently referred to as the "acidic" amino acid transport system.

This "acidic" amino acid transport system showed similar specificity to the system found in rabbit ileum and jejunum, which was reported to only show an affinity for L-aspartate and L-glutamate (Schultz et al. 1970). L-cysteate transport was not examined in this system. The high affinity transport system of isolated rat synaptosomes of spinal cord is similarly only capable of transporting the acidic amino acids (Logan and Snyder 1971, Roberts 1974). The specificity of the acidic

amino acid transport system in kidney has been reported to show little or no overlap with other amino acid transport systems (Webber 1962, 1963; Neame 1966). However, certain other mammalian acidic amino acid transport systems appear to be less specific in their substrates. Competitive inhibition studies of acidic amino acid transport in brain have indicated that the low affinity system shows some overlap with short chain (but not long chain) basic amino acids, and with some of the neutral amino acid systems (Blasberg and Lajtha 1965, 1966; Neame 1966).

L-glutamate is poorly transported in Ehrlich ascites cells and this system, unlike other mammalian acidic amino acid transport systems, does not appear to transport L-aspartate but is inhibited by certain neutral amino acids (Heinz et al. 1965). No kinetic studies of this inhibitions were carried out so it is unclear whether this inhibition was competitive or non-competitive.

Certain microbial transport systems show very similar specificities to the acidic amino acid transport system of BHK21-C13 cells. Competitive inhibition studies with N. crassa (Pall 1970a) and A. nidulans (Robinson et al. 1973a; Pateman et al. 1974) have demonstrated that these organisms have single transport systems capable of transporting L-glutamate, L-aspartate and L-cysteate. L-cysteate transport does not appear to have been examined in S. faecalis and P. chrysogenum but both these organisms have a single transport system for L-glutamate uptake (Reid et al. 1970, Hunter and Segal 1971). E. coli however, has separate specific glutamate and aspartate transport systems (Halpern and Even-Shoshan 1967, Kay 1971). Of the mammalian acidic amino acid transport systems only that in brain has been shown to accumulate L-glutamate to any appreciable level (Stern et al. 1949). Ehrlich ascites cells have been shown to be capable of accumulating L-glutamate only to levels not more than 1.5 to 1.6 times that in the extracellular medium (Heinz et al. 1964),

although Christensen and Riggs (1952) have demonstrated that these cells are capable of maintaining L-glutamate at levels approximately 16 times that in the surrounding fluid. In vivo and in vitro studies involving acidic amino acid transport in the small intestine have provided no evidence for the accumulation of these amino acids (Wiseman 1953, 1955; Hagihara et al. 1961, 1962; Thier et al. 1964; Neame 1965, 1966). Schultz et al. (1971), however, have demonstrated that these amino acids are absorbed at rates comparable to the neutral amino acids which are capable of accumulation (Wiseman 1953). Transamination of glutamate has been demonstrated in a number of mammalian species, including rat, dog (Neame and Wiseman 1957; Matthews and Wiseman 1953), cat and rabbit (Neame and Wiseman 1958). Similarly aspartate transamination has been observed in rat and dog (Matthews and Wiseman 1953) Neame and Wiseman 1956, 1957). Consequently it has been suggested by these and other authors (including Schultz et al. 1970; Neame 1968) that accumulation of these acidic amino acids is probably prevented by their rapid transamination. Rapid glutamine synthesis from glutamate may also be important in the prevention of glutamate accumulation in certain tissues, e.g. muscle tissue (Marliss et al. 1971) and liver (Lotspeich 1967; Addae and Lotspeich 1968). The activities of glutamine synthetase and glutaminase in liver and kidney have been reported to vary considerably between species (Janicki and Goldstine 1969).

Microbial systems, however, are generally capable of accumulating L-glutamate to much higher levels than any reported mammalian system, eg. E. coli has been demonstrated to accumulate L-glutamate to a level approximately 1700 times that in the surrounding medium (Halpern and Even-Shoshan 1967), and A. nidulans has been reported to accumulate L-glutamate approximately 60 (Pateman et al. 1974) to 230 fold (Robinson et al. 1973a).

The affinity of the BHK21-C13 acidic transport system, as indicated by the Km values for L-glutamate (4.6×10^{-5}M) and L-aspartate (3.4×10^{-5}M), is very similar to the high affinity acidic amino acid transport system found in isolated synaptosomes, obtained from the spinal cord and cerebral cortex of rat (Logan and Snyder 1971). These values, however, are approximately two orders of magnitude less than those of the low affinity system for these amino acids, reported in brain slices (Blasberg 1968), and two to three orders of magnitude (depending on Na^+ concentrations) less than values reported for rabbit ileum and jejunum (Schultz et al.1970). L-glutamate is transported with an unusually high Km of 2×10^{-2}M in Ehrlich ascites cells, (Heinz 1965). It should be noted however, that this value was calculated from concentrations considerably lower than this concentration, and saturation was not observed over the concentration range studied. In view of the exceptionally high Km and the incomplete saturability of this system it is possible that this L-glutamate system is comparable to the "non-saturable component" reported in Ehrlich ascites cells for the transport of B-alanine, taurine and betaine (Christensen and Liang 1966). The Km's for specific L-glutamate and L-aspartate transport systems in micro-organisms vary considerably with the organism. These values range from 3.7×10^{-6}M for L-aspartate transport in E. coli (Kay 1971) to 1.8×10^{-4}M for L-glutamate uptake in A. nidulans (Robinson et al. 1973). L-cysteate transport has been examined in detail in N. crassa (Pall 1970) and to a lesser extent in A. nidulans (Robinson et al. 1973a) and Km values of 7×10^{-6}M and 1.9×10^{-4}M respectively have been obtained.

Details of the acidic amino acid transport system of BHK21-C13 cells and a number of transport systems capable of transporting those amino acids is summarised in the following table, Table 4.3.1.

Table 4.3.1. ACIDIC AMINO ACID TRANSPORT SYSTEMS.

A. MAMMALIAN SYSTEMS.

System	Substrates (Km values in parenthesis, x 10^{-5}M)	Ki values when acting as inhibitors of: L-glutamate (x 10^{-5}M)	L-aspartate (x 10^{-5}M)	References
BHK21-C13 cells				
	L-glutamate (4.6)		5.8.	Section 4, this
	L-aspartate (3.4)	4.7		thesis.
	L-cysteate (6.9)	7.7	7.5	
Brain				
	L-glutamate (48)		20	
	D-glutamate (300)		180	Blasberg (1968)
Ileum & Jejenum (Rabbit)				
Values at 140mM Na^+	L-glutamate (710)			Schultz et al (1970
Synaptosomes (Rat)				
a) Spinal cord				
Low affinity	L-glutamate (170)		300	Logan and Snyder
	L-aspartate (370)	350		(1971).
High affinity	L-glutamate (36)			
	L-aspartate (17)			

System	Substrates (Km values in parenthesis x 10^{-5}M)	Ki values when acting as inhibitors of : L-glutamate (x 10^{-5}M)	L-aspartate	References
b) Cerebral Cortex				
Low affinity	L-glutamate (150)			Roberts (1974)
	L-aspartate (368)			
High affinity	L-glutamate (3.6)		4.0	
	L-aspartate (1.7)			
	D-glutamate			
	D-homocysteate			
	L-cysteate			
Ehrlich ascites cells				
	L-glutamate (0.2M)			Heinz et al (1965)
	Neutral amino acids?			

B. MICROBIAL SYSTEMS

System	Main substrates (Km values in parenthesis, x 10^{-6}M)	Additional Inhibitors of substrates : (Ki values in parenthesis, x 10^{-4}M)	References
E.coli K12			
a) Glutamate	L-glutamate (7.7)	Competitive : D-glutamate (24.5), L-glutamine (33.5) L-glutamate-α-ethyl ester (1.75), α-methyl-DL-glutamate (16.3) L-glutamate-α-methyl ester (21.0), β-hydroxy-DL-glutamate (12.5) Non-Competitive : DL-aspartate, α-keto-glutarate	Even-Shoshan (1967)
b) Aspartate	L-aspartate (3.7)	Competitive : D-aspartate (2.0), L-glutamine (8.4) L-glutamate (6.4), β-methyl-DL-aspartate (5.7) DL-threo-β-hydroxy aspartate (1.8) DL-erythro-β-hydroxy aspartate (5.7)	Kay (1971)
c) C_4 dicarboxylic acids	aspartate, fumarate, malate, maleate, succinate (10-30)	Competitive : D-aspartate, C_4 dicarboxylic acids, DL-erythro-β-hydroxy aspartate	Kay and Kornberg (1971)

System	Main substrates (Km values in parenthesis, x 10^{-6}M)	Additional inhibitors of substrates (Ki values in parenthesis, x 10^{-4}M)	References
N. crassa			
	L-glutamate (16) D-glutamate (90) L-aspartate (13) D-aspartate (5.4) L-cysteate (7)	DL-∝-methylaspartate, DL-erythro-hydroxy-aspartate, L-aspartic acid ∝-methyl ester, DL-∝-methyl-glutamate, L-glutamic acid -∝-methyl ester.	Pall (1970b)
S.faecalis			
	L-glutamate (30) L-aspartate (10)	L-aspartate (7)	Reid et al (1970)
	L-glutamate (12 x 10^{-3}M) L-aspartate (0.8 x 10^{-3}M)	L-aspartate (0.8 x 10^{-3}M)	

System	Main Substrates (Km values in parenthesis, x 10^{-6}M)	Additional Inhibitors of substrates (Ki values in parenthesis, x 10^{-4}M)	References
P.chrysogenum			
	L-glutamate (20 to 100, depending on pH).	DL-glutamate, L-aspartate, L-glutamine, ∝- amino adipate (Inhibition not determined if competitive or non-competive).	Hunter and Segal (1971)
S.cerevisiae	Possible substrates (Km values not determined)	glutamate, aspartate and ∝-amino adipate	Joiris and Grenson (1969)
A.nidulans			
a)	L-glutamate (110) L-aspartate (130)	Competitive inhibitors : L-glutamate (95) L-aspartate (140), L-cysteate (55 and 500 for inhibition of L-glutamate and L-aspartate respectively).	Pateman et al (1974)
b)	L-glutamate (180) L-aspartate (100) L-cysteate (190)	Competitive inhibitors : L-glutamate (150), L-aspartate (120), L-cysteate (240 for inhibition of L-glutamate uptake	Robinson et al (1973a)

System	Main Substrates (Km values in parenthesis, x 10^{-6}M)	Additional Inhibitors of substrates (Ki values in parenthesis, x 10^{-4}M)	References
N.Crassa			
	L-glutamate (16) D-glutamate (90) L-aspartate (13) D-aspartate (5.4) L-cysteate (7)	DL-β-methylaspartate, DL-erythro-hydroxy-aspartate, L-aspartic acid β-methyl ester, DL-α-methyl-glutamate, L-glutamic acid-α-methyl ester.	Pall (1971)
S.faecalis			
a) High affinity	L-glutamate (30) L-aspartate (10)	L-aspartate (7)	Reid et al (1970)
b) Low affinity	L-glutamate (12 x 10^{-3}M) L-aspartate (0.8 x 10^{-3}M)	L-aspartate (0.8 x 10^{-3}M)	

The inhibition studies with naturally occuring amino acids or analogues of glutamate or aspartate provide information as to the molecular configuration required for transport via the "acidic" amino acid transport system of BHK21-C13 cells. As previously mentioned only the L-stereoisomers of glutamate and aspartate appear to be transported. Removal or substitution of the α-amino group (e.g. in α-keto glutarate, or N-formyl DL-glutamic acid), and α-carboxyl group (e.g. α-amino butyrate) completely abolished the ability of the compound to be transported by the acidic amino acid transport system. Substitution of the α-hydrogen by an aliphatic group (e.g. in α-methyl-DL-glutamic acid), increase of the glutamic acid "side chain" length by a single methylene group (as in α-amino-adipic acid, or substitution of a β-hydrogen atom by a different group (e.g. as in DL-3-hydroxy-aspartic acid or DL-3-Methyl-Aspartic acid also completely abolished the affinity of the compound for the permease. However, substitution of a γ-hydrogen atom by a fluoro group in the glutamate analogue 4-fluoro-glutamic acid reduced, but did not completely remove, affinity for the permease. A second anionic group is also necessary for transport via this system, as esterification (e.g. as in DL-glutamic acid γ-methyl ester) or amidation (e.g. as in L-glutamine or L-asparagine) resulted in complete loss of affinity for the "acidic" transport system. Substitution of the β-carboxyl group by a sulphonate group (L-cysteic acid), however, apparently satisfies the general specificity requirement of the "acidic" amino acid permease (R^{-} $CH.(NH_3^{+})$ CO_2^{-}). R contains no more than 3 carbon atoms, and substitution of a hydrogen at the γ-carbon position does not completely abolish transport of the analogue by the "acidic" transport system, apparently unlike substitutions of a hydrogen at the β-carbon position.

Very few studies into the structural requirements for transport

via the mammalian acidic amino acid transport systems have been carried out. The high affinity acidic amino acid transport system of brain, which shows marked similarities to the BHK21-C13 system, is an exception. The L-stereoisomers of glutamate and aspartate appear to be transported in preference to the D-stereoisomers by the rat small intestine (Gibson and Wiseman 1951), and by the low affinity acidic amino acid transport system in brain (Blasberg 1968). However, in vivo renal reabsorption studies in dog showed little difference in the absorption of L-glutamate or D- or L-aspartate (Webber 1963). The high affinity system in brain is similar to the BHK21-C13 system as it showed no affinity for glutamate analogues modified from glutamate as follows:
substitution of the α-hydrogen by a methyl group (McLennan et al.1971), esterification of the α- and γ-carboxyl groups (Curtis et al. 1972; Halderman and McLennan 1972) and N-methylation of the α-amino group (Roberts 1974). This system is also similar to the BHK21-C13 system, as replacement of the β-carboxyl group of L-aspartate by a sulphonate group (L-cysteate) does not abolish affinity for the system. However, DL-homoscysteate (where the γ-carboxyl group of glutamate is replaced by a sulphonate group) is transported in brain but not by BHK21-C13 cells. None of the tested analogues of glutamate or aspartate (L-glutamic acid α-methyl ester, L-asparagine, N-acetyl L-glutamic acid or β-alanine) inhibited L-glutamate, or D- or L-aspartate reabsorption in dog kidney (Webber 1963).

More extensive studies of the structural requirements for transport by systems capable of transporting acidic amino acids have been carried out in microorganisms. These systems show a number of similarities and differences from the described BHK21-C13 system. These systems are generally less stereo-specific than BHK21-C13 cells, and the separate aspartate and glutamate systems of E. coli (Kay 1971;

Halpern and Even-Shoshen 1967), as well as the common acidic amino acid transport systems of P. chrysogenum (Hunter and Segal 1971) and A. nidulans (Robinson et al. 1973a) exhibit a relatively high affinity for D- as well as L-stereoisomers. The acidic system of N. crassa shows an even higher affinity for D- than L-aspartate, but prefers the L- to the D-stereoisomer of glutamate (Pall 1970b). These transport systems show some heterogeneity with respect to their requirement for an intact α-amino group. The single acidic amino acid transport system of N. crassa (Pall 1971) and A. nidulans (Robinson et al. 1973a) are similar to the BHK21-C13 system as modifications of this group completely remove the affinity of the compound for the transport system. The affinity is merely reduced in the glutamate transport system of E. coli (Halpern and Even-Shoshan 1967). Removal of the α-amino group however, results in the complete loss of affinity for the transport system in each of the above cases. Similarly, removal of the α-carboxyl group (e.g. as in α-amino butyrate) also results in the loss of ability to be transported via the glutamate transport system of E. coli (Halpern and Even-Shoshan 1967) and the acidic amino acid transport system of A. nidulans (Robinson et al. 1973a). The α-hydrogen, shown to be essential in BHK21-C13 cells, was also necessary for transport by the A. nidulans system (Robinson et al. 1973a), but could be substituted by an α-methyl group in the E. coli systems (Kay 1971; Halpern and Even-Shoshan 1967), and the N. crassa system (Pall 1970b). The importance of chain length also appeared to vary with the organism. In E. coli a decrease or increase of one methylene groups in the side chain results in almost complete abolition of affinity for the transport system (Kay 1971; Halpern and Even-Shoshan 1967). The acidic transport system of N. crassa (Pall 1971), P. chrysogenum (Hunter and Segal 1971), A. nidulans (Robinson et al. 1973a) and possibly Yeast (Joiris and Grenson 1969)

appears to be less specific and show some affinity for α-amino adipate (which contains 3 side chain methylene groups). BHK21-C13 cells show no affinity for this compound. Substitutions of the aspartate or glutamate side chains by groups such as hydroxyl or methyl groups (e.g. as in β-hydroxy DL-glutamate or aspartate, or β-methyl DL-aspartate), merely results in a reduced affinity for transport by the E. coli systems (Kay 1971; Halpern and Even-Shoshan 1967), and the N.crassa (Pall 1970b) acidic amino acid transport system. The absolute requirement for a second anionic group for transport by the acidic amino acid transport system of BHK21-C13 cells does not appear to hold for certain microbial systems. Esterification of glutamate or aspartate (e.g. L-glutamate- α -ethyl ester, or L-glutamate- γ ethyl ester) has been shown to reduce but not completely abolish transport by the acidic amino acid transport systems of N.crassa (Pall 1970b) and A.nidulans (Robinson et al. 1973a), and the glutamate transport system of E.Coli (Halpern and Even-Shoshan 1967). It should be noted that Robinson et al. question the validity of their results and suggest the esters may be metabolised and the inhibition observed is due to the formation of the parent amino acid. Similarly the amination of aspartate and glutamate results in a marked reduction but not a complete loss of affinity for the transport systems in E.coli (Kay 1971; Halpern and Even-Shoshan 1967). Glutamine also reduces glutamate transport in P.chrysogenum (Hunter and Segal 1971), however these authors did not determine if this inhibition was competitive or non-competitive.

Absolute confirmation of the existence of an "acidic" amino acid transport system of BHK21-C13 cells would have been provided by isolation of mutants defective in the structural protein, or regulation of this permease. Efforts to isolate such a mutant were unsuccessful. The methods used in the attempted isolation of amino acid transport mutants and the possible reasons for the failure to isolate these mutants are described in Section 7.

SECTION 5. L-ALANINE TRANSPORT IN BHK21-C13 CELLS

5.1. INTRODUCTION

The transport of L-alanine has been examined in a number of mammalian and microbial systems. This amino acid appears to be transported in most mammalian tissues, e.g. kidney (Webber et al. 1961; Webber 1962; Neame 1965, 1966), small intestine (Wiseman 1955; Hagihara et al. 1962), by a broad specificity neutral amino acid transport system(s). Detailed studies of neutral amino acid transport in Ehrlich cells have indicated that L-alanine may be transported via three transport systems of completely or partially overlapping specificities (Oxender and Christenson 1963; Christensen et al. 1967; Inui and Christensen 1967). In micro-organisms the number of, and specificities of, transport systems capable of L-alanine transport varies considerably with the organism studied. This amino acid may be transported via broad specificity transport systems, e.g. in S. cerevisiae (Grenson et al. 1970) and a number of filamentous fungi (Benko et al. 1967, 1969; Pall 1969; Gupta and Pramer 1970); or as is generally the case in bacteria (Wargel et al. 1970; Hechtman and Scriver 1970a and b; Reitz et al. 1967) via systems specific, or relatively specific, for this amino acid.

5.2. RESULTS

5.2.1. Kinetics of L-alanine uptake into BHK21-C13 cells

L-alanine uptake into BHK21-C13 cells was examined for the concentration range 1×10^{-5}M to 4×10^{-3}M. Initial rates of transport were based on uptake over 4 minutes, over which period L-alanine uptake was linear. (Figure 5.2.1a shows a typical plot of uptake from 2×10^{-5}M L-alanine over this period). An S/v versus S plot was linear (Figure 5.2.1b) indicating that the transport of L-alanine appears to follow Michaelis-Menten kinetics, over the concentration

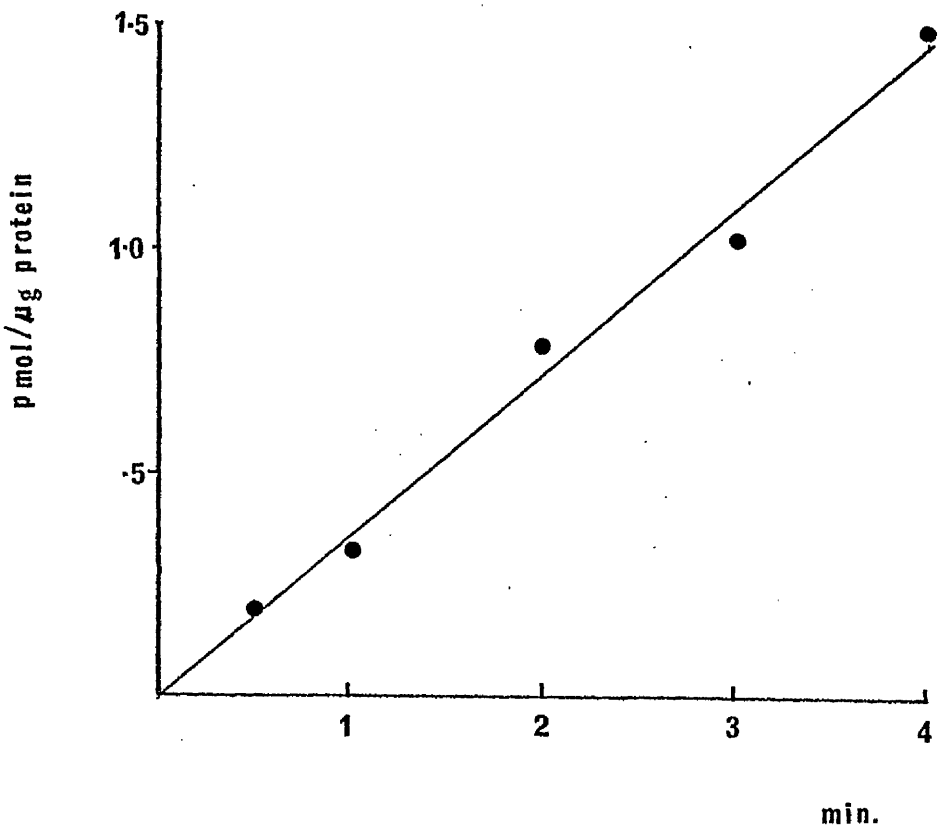


Figure 5.2.1a. Initial rate of L-alanine uptake into BHK21-C13 cells

Cells were incubated for intervals of up to 4 minutes in Uptake Medium containing L-[2,3-^{3}H] alanine (2×10^{-5}M). Uptake procedures were carried out as described in Section 2.8.

Figure 5.2.1b. S/v versus S plot of L-alanine uptake into BHK21-C13 cells

Uptake of L-[2,3 - ^{3}H] alanine into BHK21-C13 cells was examined for the concentration range 1 x 10^{-5}M to 4 x 10^{-3}M. The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations of up to 10 minutes.

S/v values for concentrations of 1 x 10^{-3}M and below are shown on an expanded scale.

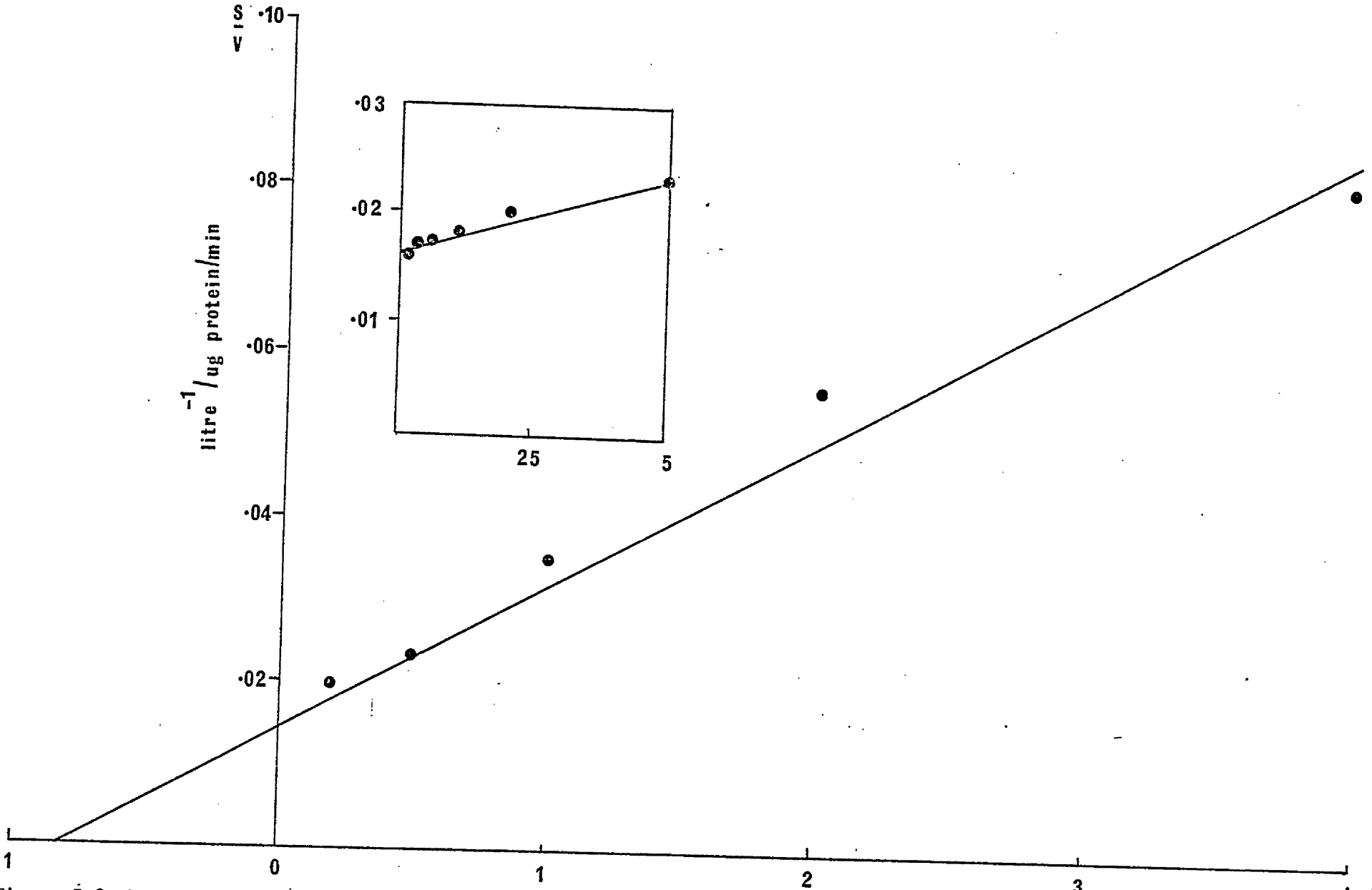


Figure 5.2.1b. S/v versus S plot of L-alanine uptake into BHK21-C13 cells

range studied. Statistically determined estimates of the Km and Vmax values for L-alanine transport gave values of $0.81 \pm .16 \times 10^{-3}$M and 57.7 ± 4.1 p mol/µg protein/min.

5.2.2. Effect of inhibitors on the uptake of L-alanine in BHK21-C13 cells

Inhibition of L-alanine uptake in BHK21-C13 cells following 10 minutes preincubation and transport (over a 4 minute period) in the presence of various inhibitors is summarised in Table 5.2.2. Sodium azide and sodium cyanide, inhibitors of energy metabolism, reduced L-alanine uptake by approximately 96 and 88% respectively, indicating that this uptake is energy dependent. N-ethyl-maleimide (NEM) and p-chloromercuribenzoate inhibited L-alanine by approximately 66 and 77% respectively indicating that sulphydryl groups may be involved in the transport of this amino acid, although this inhibition may not be acting directly at the carrier site. Ouabain also produced a large inhibition of L-alanine uptake (approximately 78%) suggesting that L-alanine uptake may be dependent on cation transport.

5.2.3. Accumulation of L-alanine in BHK21-C13 cells

The ability of BHK21-C13 cells to accumulate L-alanine was examined. After incubation for 4 minutes in uptake medium containing L-[2,3-^{3}H]-alanine approximately 98% of the total radioactivity remained in the TCA soluble fraction. Results of the ionophoresis of these samples are represented in Figure 5.2.3. Approximately 93% of the radioactivity applied co-chromatographed with control L-alanine samples. A comparison of the intracellular and extracellular concentrations of L-[2,3,-^{3}H] alanine indicated that L-alanine (1×10^{-5}M) was accumulated intracellularly to a level approximately 5.03×10^{-4}M within 4 minutes. No accumulation of L-alanine uptake was observed in medium containing

Table 5.2.2. Effect of inhibitors on L-alanine uptake in BHK21-C13 cells.

Inhibitor	Rate of L-alanine uptake (pmol/μg protein/min)	t	P
None	11.0 ± 1.0		
Cycloheximide (1×10^{-3}M)	9.6 ± 0.8	0.91	<0.05
p-chloro-mercuribenzoate (1×10^{-4}M)	4.1 ± 0.8	4.46	<0.001
N-ethyl-maleimide (1×10^{-4}M)	3.7 ± 0.5	5.61	"
Ouabain (1×10^{-4}M)	2.5 ± 0.4	6.69	"
Sodium cyanide (2×10^{-3}M)	1.4 ± 0.3	7.94	"
Sodium azide (5×10^{-3}M)	0.7 ± 0.2	8.60	"

Uptake of L-[2,3-^{3}H]alanine (2×10^{-4}M) was examined in the presence of the above inhibitors, following preincubation of cells in their presence for 10 minutes. Rates of uptake were determined from 4 minute incubations. Values are means of 6 samples plus SEM.
t values were calculated from comparison with control values, obtained prior to administration of inhibitor. Values were considered significant if P was less than 0.05 ($t = 2.20$).

Figure 5.2.3. Ionophoretogram of TCA soluble extract of BHK21-C13 cells following incubation in the presence of L-[2,3 - ^{3}H] alanine

50 µl of TCA soluble extract of BHK21-C13 cells, obtained following incubation of cells for 10 minutes in Uptake Medium containing L-[2,3 - ^{3}H] alanine (1 x 10^{-5}M), was spotted onto Whatman 3 MM chromatography paper (width 3 cm). The sample was run on low voltage vertical ionophoresis for 3 hours at 200 v (5mA/strip) in tank buffer pH 1.9 (58 ml. glacial acetic acid : 26 ml. 25% (w/v) formic acid, to a final volume of 2 litres). The ionophoretogram was air dried, cut into 1 cm strips, the radioactivity of which was determined by liquid scintillation spectrophotometry. Results are expressed as dpm/cm strip.

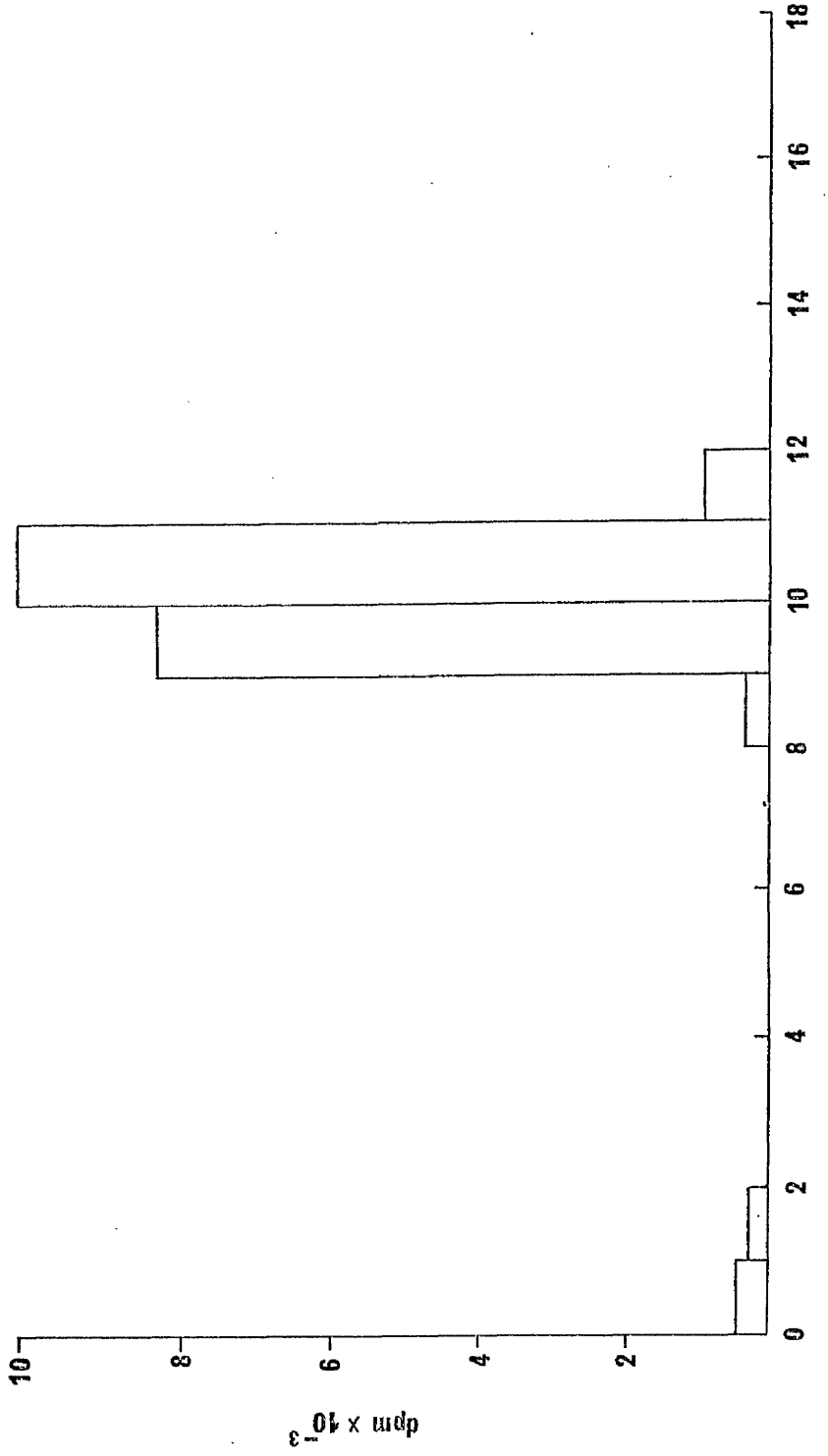


Figure 5.2.3. Ionophoretogram of TCA soluble extract of BHK21-C13 cells following L-[2,3,-^{3}H] alanine

5 x 10^{-3}M sodium azide and 2 x 10^{-3}M sodium cyanide. These results are summarised in Table 5.2.3.

5.2.4. Effect of temperature on the uptake of L-alanine in BHK21-C13 cells

The effect of temperature on the uptake of L-alanine is shown in Figure 5.2.4. Uptake of L-alanine from a 2 x 10^{-4}M solution was increased approximately 75 fold over the temperature range 1°C to a temperature optimum of 39°. Above this temperature optimum the rate of uptake fell off rapidly probably due to cell death. The Q_{10} value for L-alanine uptake between 25° and 37°C was 2.57.

5.2.5. Effect of pH on L-alanine uptake into BHK21-C13 cells

Figure 5.2.5 shows the effect of varying the pH of the uptake medium (for the range 4.3 to 8.3) on the uptake of L-alanine. It can be seen that the uptake is markedly pH sensitive. L-alanine transport at pH 4.3 was approximately 16% of the level observed at the approximate pH optimum of 7.6, although it increased rapidly with increasing pH between pH 5 and the optimum (after which the rate appeared to decrease). Over the pH range studied L-alanine carries no net charge, the α-carboxyl group being fully charged and the α-amino group fully protonated (except for very small levels of uncharged amino groups at high pH values). The pKa values for the α-carboxyl and α-amino groups are 2.35 and 9.62 respectively. The fact that this amino acid bears no net charge would indicate that the observed pH dependence is a characteristic of the transport system itself and is not due to changes in the charge of the amino acid.

5.2.6. Effect of sodium ion concentration on L-alanine uptake into BHK21-C13 cells

Transport of certain neutral amino acids in intact intestine

Table 5.2.3. Accumulation of L-alanine in BHK21-C13 cells.

Total Cell Volume (µl)	Total dpm in cells	dpm in TCA soluble fraction	dpm as L-alanine	dpm in equivalent volume	Intracellular Concentration / Extracellular Concentration
2.07 (7.9×10^{5} cells of mean cell volume 2.62×10^{-6} µl)	352,024	343,223	317,825	6,320	50.3
Sodium Azide (5×10^{-3}M) and Sodium Cyanide (2×10^{-3}M) treated cells	2,865	12	1,367	6,320	0.22

Cells were incubated for 4 minutes in uptake medium containing, L-[2,3,-^{3}H] alanine (1×10^{-5}M). TCA extraction and ionophoresis were subsequently carried out as described in Section 2.9.1. and 2.9.2. The above values are the means of two experiments.

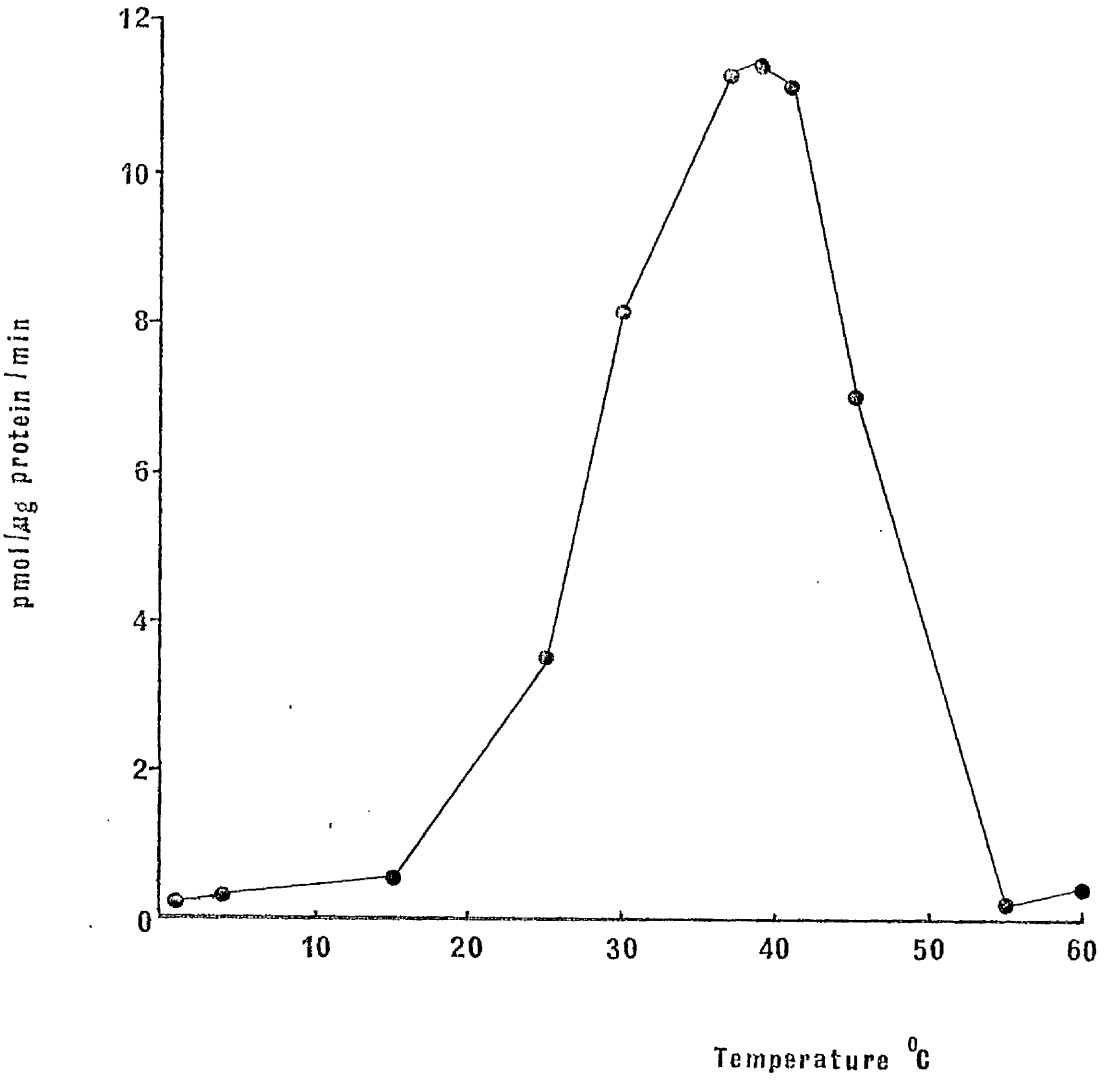


Figure 5.2.4. Effect of temperature on the uptake of L-alanine into BHK21-C13 cells

The uptake of L-[2,3-^{3}H] alanine (2 x 10^{-4}M) was examined at various temperatures following a 10 minute preincubation of the cells at the temperature to be used in the incubation. Initial rates of uptake were based on 4 minute incubations and each point is the mean of 6 samples.

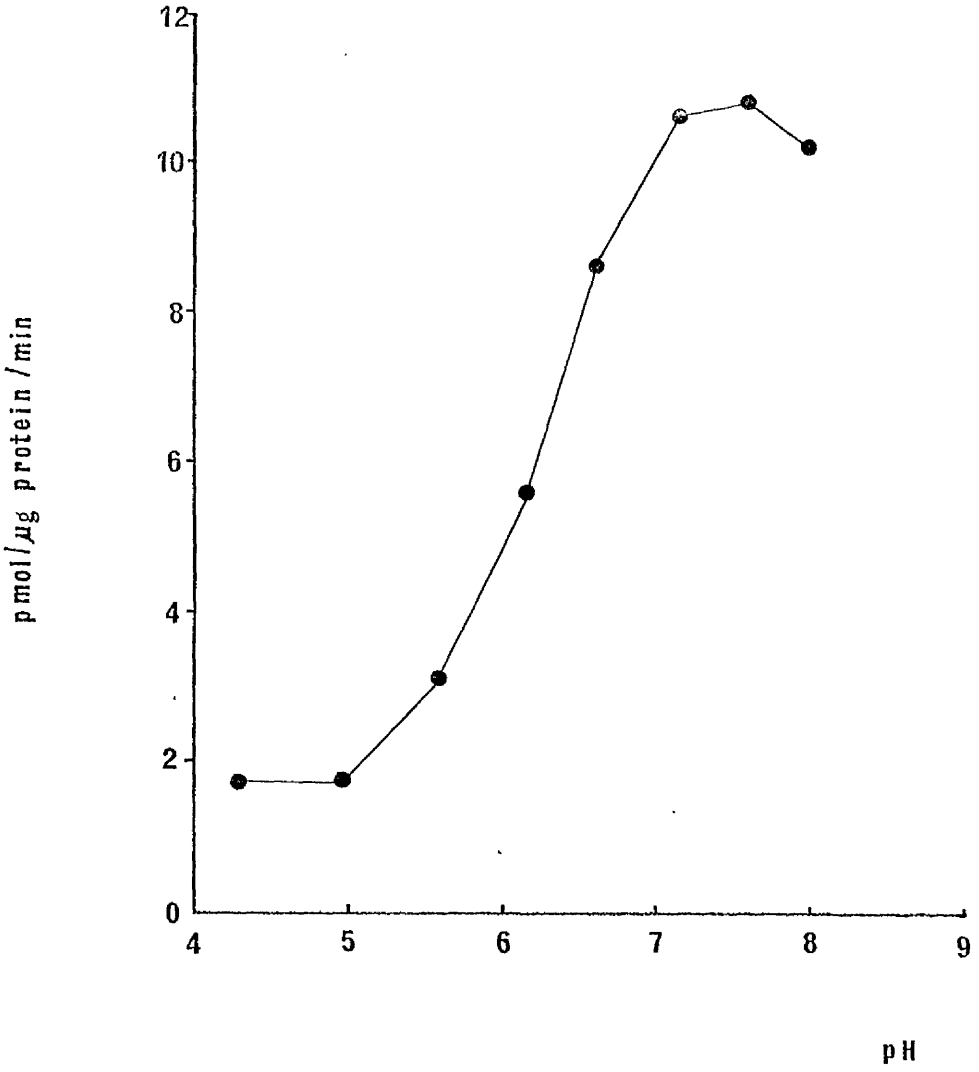


Figure 5.2.5. Effect of pH on L-alanine uptake into BHK21-C13 cells

The uptake of L-[2,3-^{3}H] alanine (2 x 10^{-4}M) was examined at various pH's between 4.3 and 8.3.

Initial rates of uptake were based on 4 minute incubations and each point is the mean of 6 samples.

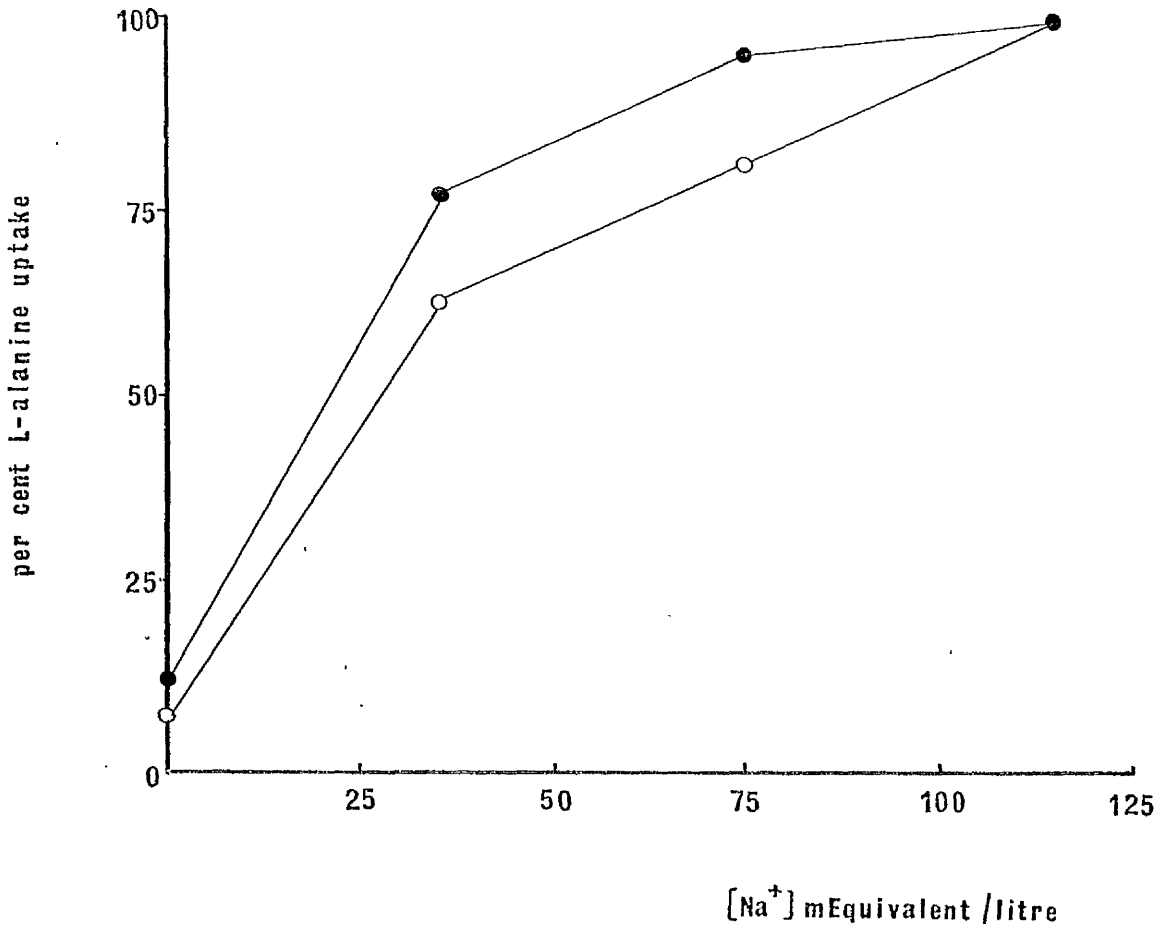


Figure 5.2.6. Effect of Na^+ ion concentration on L-alanine uptake into BHK21-C13 cells

Uptake of L-[2,3-^{3}H]alanine (2×10^{-4}M) from Uptake Medium containing varying Na^+ concentrations, was examined for 4 minute incubations. The NaCl component of Uptake Medium was replaced by ideal equiosmolar quantities of D-mannitol or Choline Chloride. Uptake is expressed as percentage uptake from standard Uptake Medium (Section 2.1.4). Each value is the mean of 6 samples.

Uptake in the presence of D-mannitol (O-O).

Uptake in the presence of Choline Chloride (●-●).

(Schultz et al. 1966), isolated epithelial cells (Reiser and Christiansen 1971) and Ehrlich cells (Inui and Christensen 1967) has been demonstrated to require the presence of Na^+ in the incubation medium. Ouabain which inhibits cation transport, has been demonstrated in Section 5.2.3 to inhibit L-alanine uptake. This section consequently examines the effect of sodium ion concentration on L-alanine uptake. Uptake of L-[2,3-^{3}H] alanine from a 2 x 10^{-4}M solution was examined at different Na^+ concentrations. Na^+Cl^- was replaced by either choline chloride or D-mannitol. L-alanine uptake was seen to be dependent on Na^+ concentration as transport was decreased as the Na^+ component of the medium was reduced; complete removal of the Na^+ component of Na^+Cl^- resulting in an approximately 90% reduction in L-alanine transport, (Figure 5.2.6).

5.2.7. Specificity of the L-alanine uptake system in BHK21-C13 cells

The specificity of the L-alanine system in BHK21-C13 cells was examined by investigating the ability of other amino acids to inhibit L-alanine uptake. L-[2,3-^{3}H]alanine (1 x 10^{-4}M) was examined in the presence of a 25 fold molar concentration of inhibiting amino acid (Table 5.2.7a) or amino acid analogue (Table 5.2.7b). All naturally occurring neutral α-amino acids, with the exception of L-cysteine and L-cystine inhibited L-alanine uptake by at least 20%. The neutral amino acids can be divided into three main groups depending on their ability to inhibit L-alanine uptake:

a) most effective (80% inhibition):- L-alanine, L-serine, L-threonine,

b) intermediate (50 to 79% inhibition):- L-glutamine, L-asparagine, L-methionine, L-proline and L-glycine,

c) least effective (20 to 49% inhibition):- L-leucine, L-isoleucine, L-valine, L-phenylalanine, L-tyrosine and the basic aromatic amino acid L-histidine.

Little inhibition was produced by the acidic or basic L-α-amino acids.

Table 5.2.7. Inhibition of L-alanine uptake by different amino acids in BHK21-C13 cells.

Inhibiting amino acid	Percentage inhibition of L-[2,3,-^{3}H] alanine uptake
L-alanine	92
L-serine	87
L-threonine	84
L-valine	31
L-leucine	35
L-isoleucine	34
L-phenylalanine	25
Glycine	56
L-tryptophan	26
L-tyrosine	21
L-histidine	26
L-aspartate	7
L-glutamate	9
L-arginine	7
L-lysine	11
L-cysteine	12
L-cystine	7
L-methionine	68
L-proline	74
L-glutamine	68
L-asparagine	60
D-alanine	21
D-serine	24

Uptake of L-[2,3-^{3}H]alanine (1 x 10^{-4}M) was examined in the absence and presence of inhibiting amino acid (of concentration 2.5 x 10^{-3}M). Rates of uptake were determined from 4 minute incubations. Values are the means of 5 samples and are expressed as percentage inhibition of L-[2,3-^{3}H]alanine uptake alone.

Table 5.2.7b. Effect of amino acid analogues on the uptake of L-alanine in BHK21-C13 cells.

Inhibiting amino acid analogue	% inhibition of L-[2,3,-^{3}H] alanine uptake
a) ∝-amino group modifications	
N-methyl-DL-alanine	86
N-acetyl-DL-alanine	7
DL-alanine hydroxamate	15
b) ∝-hydrogen group modifications	
∝-AIB	81
∝-Methyl serine	82
c) ∝-carboxylate group modifications	
DL-alanine-methyl-ester	10
1-amino-ethyl-ester	4
d) Other analogues	
Cycloleucine	9
DL-Cycloserine	14

Uptake of L-[2,3,-^{3}H] alanine (1 x 10^{-4}M) was examined in the absence and presence of inhibiting amino acid (of concentration 2.5 x 10^{-3}M for L-amino acids, or 5 x 10^{-3}M for DL-amino acid analogues). Rates of uptake were determined from 4 minute incubations. Values are means of 6 estimates and are expressed as percentage inhibition of L-[2,3,-^{3}H] alanine alone.

The D-stereoisomers of alanine and serine produced considerably less inhibition than the corresponding L-stereoisomer. In order to determine if the observed inhibition was competitive or non-competitive, L-alanine transport was examined in the presence of selected amino acids from each of the above groups. L-alanine uptake was examined in the presence of L-serine, L-methionine, L-glycine, L-leucine and L-phenylalanine, see Figures 5.2.7 a to e. S/V versus S plots of L-alanine uptake in the presence of these amino acids (and L-alanine alone) were constructed. As the regression lines obtained for L-alanine uptake in the absence and presence of these amino acids were approximately parallel it can be concluded that the inhibition of L-alanine observed, at least for the representative amino acids, was competitive.

As the neutral amino acids exhibit a wide variation in their abilities to inhibit L-alanine uptake, and more than one neutral amino acid transport system has been reported in Ehrlich cells (Oxender and Christensen 1963; Inui and Christensen 1967; Christensen et al. 1965), the possibility of additional neutral transport systems in BHK21-C13 cells was investigated. If amino acids are transported via a single transport system Ki values for amino acid when acting as an inhibitor should equal its Km for uptake. Evidence for more than one neutral transport system is provided from a comparison of Km and Ki values for effective (L-alanine and L-serine) and relatively ineffective inhibitors (L-leucine and L-valine) of L-$[2,3,-^{3}H]$ alanine uptake (Table 5.2.7c). Initial rates of uptake of L-serine were based on 4 minute incubations, during which time transport was linear for the examined concentration range (1×10^{-5} to 4×10^{-3}M). Initial rates of L-leucine and L-valine, however, were based on 1 minute incubations, as for most concentrations transport was not linear for incubation times greater than this. S/V versus S plots for L-serine uptake alone and in the presence of L-alanine

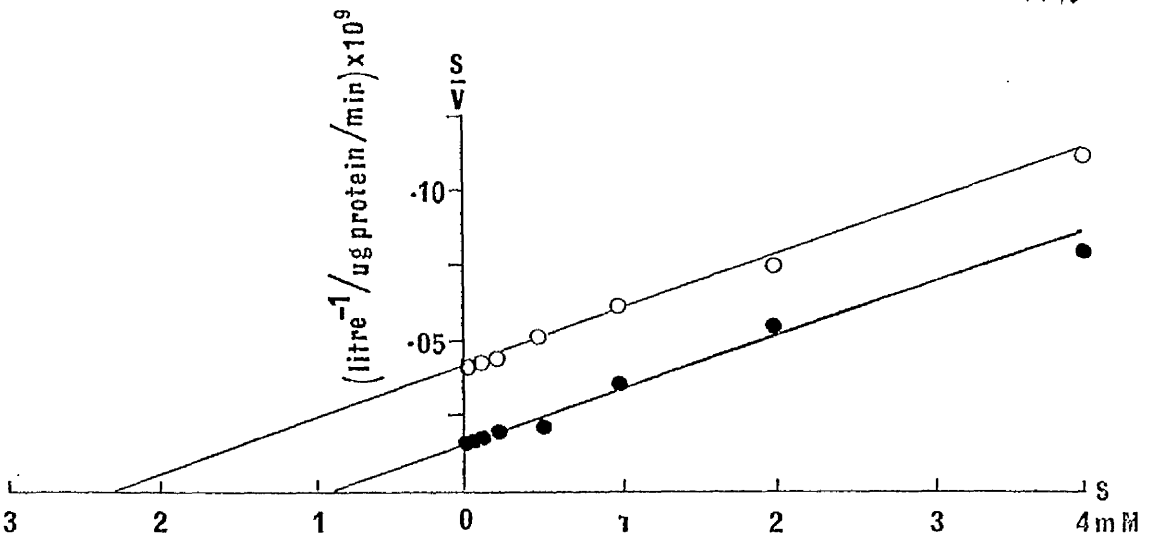


Figure 5.2.7a. S/v versus S plots of L-alanine uptake into BHK21-C13 cells in the presence and absence of L-methionine.

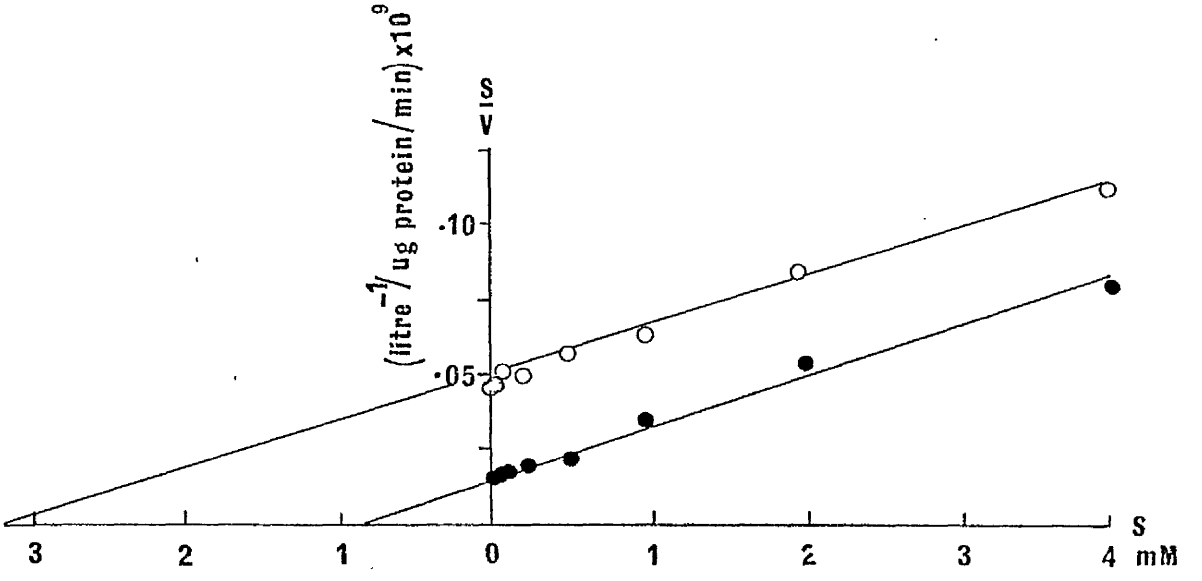


Figure 5.2.7b. S/v versus S plots of L-alanine uptake into BHK21-C13 cells, in the presence and absence of L-leucine.

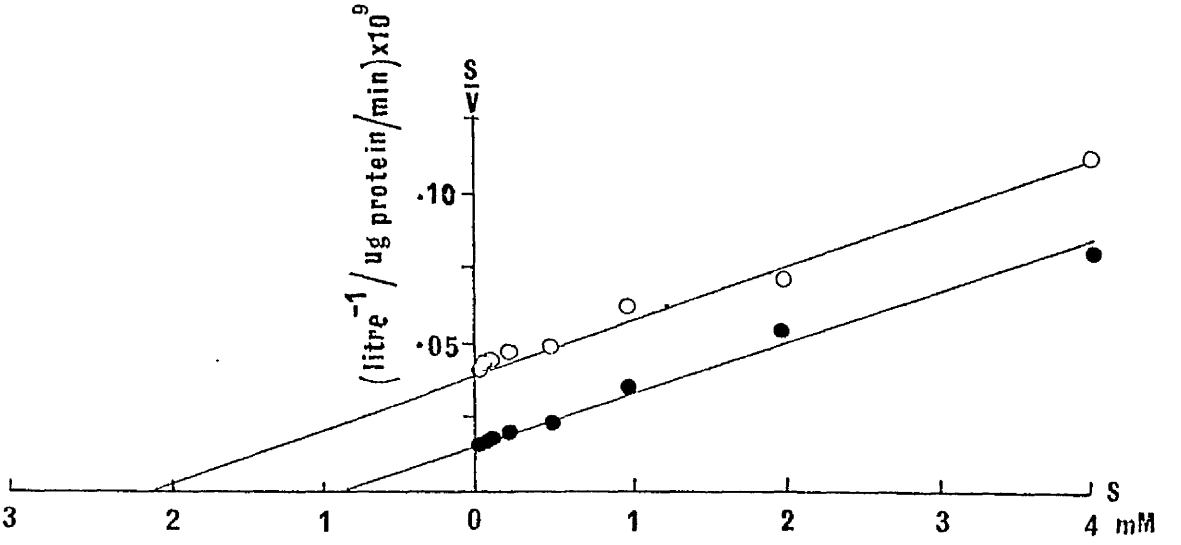


Figure 5.2.7c. S/v versus S plots of L-alanine uptake, into BHK21-C13 cells in the presence and absence of L-phenylalanine.

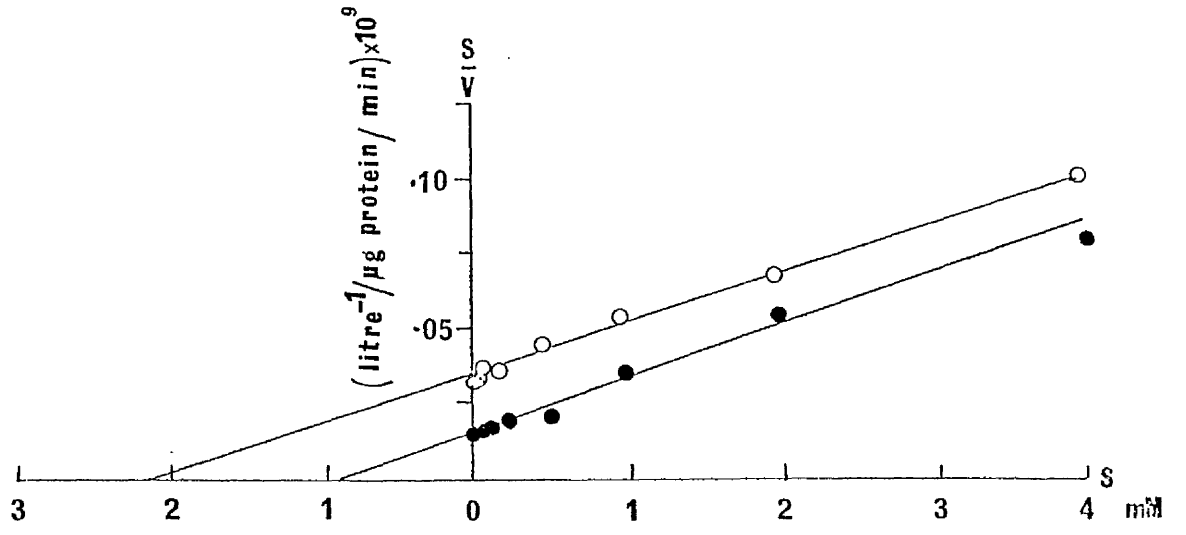


Figure 5.2.7d. S/v versus S plots of L-alanine uptake into BHK21-C13 cells, in the presence and absence of Glycine.

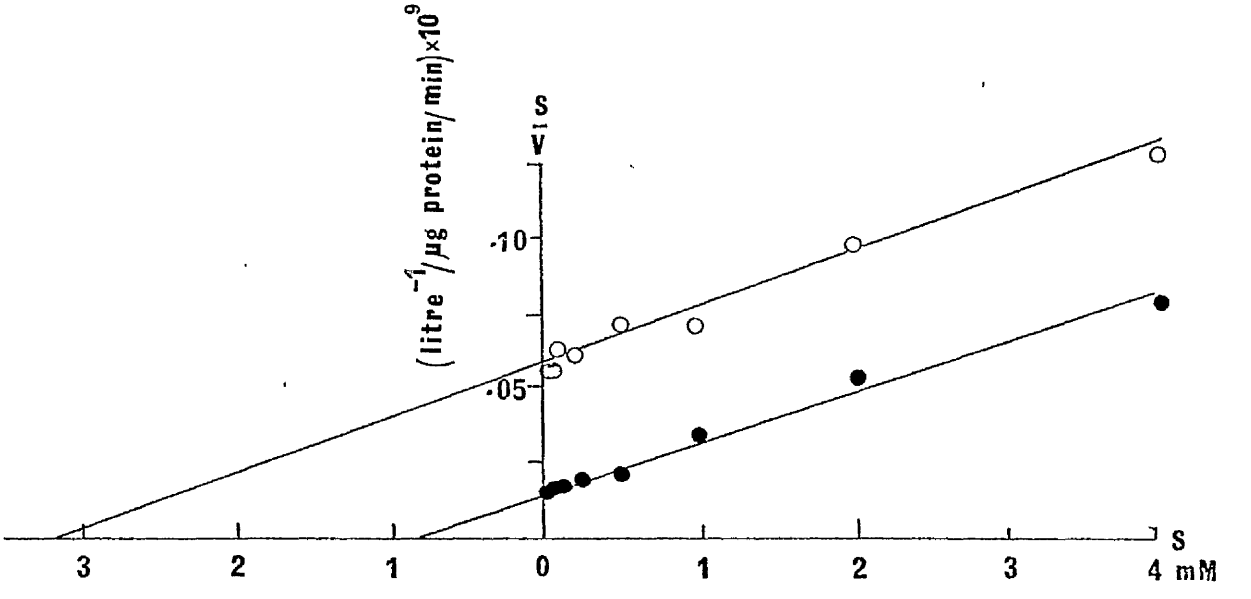


Figure 5.2.7e. S/v versus S plots of L-alanine uptake into BHK21-C13 cells, in the presence and absence of L-serine.

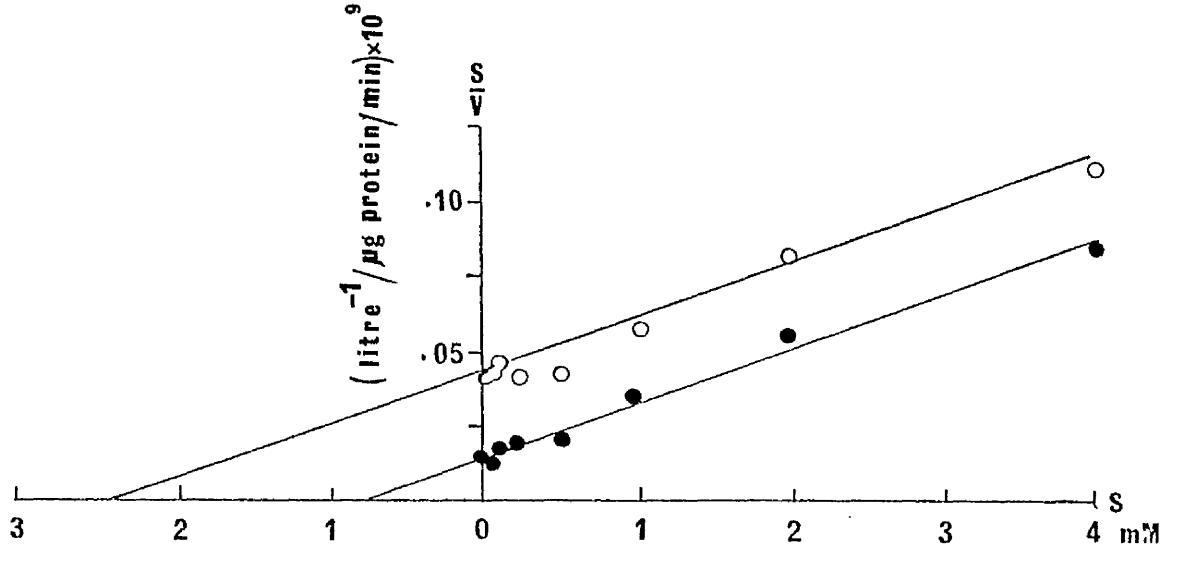


Figure 5.2.7f. S/v versus S plots of L-serine uptake into BHK21-C13 cells, in the presence and absence of L-alanine.

Figures 5.2.7a to e. S/v versus S plots of L-alanine uptake in L-alanine uptake in BHK21-C13 cells in the presence and absence of L-methionine, L-leucine, L-phenylalanine, glycine and L-serine

Uptake of L-[2,3 - ^{3}H] alanine into BHK21-C13 cells was examined (for the concentration range 1 x 10^{-5}M to 1 x 10^{-3}M L-alanine) in the presence and absence of

a) 2 x 10^{-3}M L-methionine (Figure 5.2.7a)

b) 1 x 10^{-2}M L-leucine (Figure 5.2.7b)

c) 1 x 10^{-2}M L-phenylalanine (Figure 5.2.7c)

d) 2 x 10^{-3}M Glycine (Figure 5.2.7d)

e) 2 x 10^{-3}M L-alanine (Figure 5.2.7e)

The assay procedure was carried out as described in Section 2.8, and the rates of transport were based on incubations of up to 4 minutes.

L-alanine uptake alone (●-●)

L-alanine uptake in the presence of competing amino acid (O-O)

Figure 5.2.7f. S/v versus S plot of L-serine uptake in BHK21-C13 cells in the presence and absence of L-alanine

Uptake of L-[3-^{3}H] serine into BHK21-C13 cells was examined (for the concentration range 1 x 10^{-5}M to 1 x 10^{-3}M L-serine in the presence (O-O) and absence (●-●) of L-alanine (2 x 10^{-3}M). The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations up to 4 minutes.

Figure 5.2.7g. S/v versus S plot of L-leucine uptake in BHK21-C13 cells in the presence and absence of L-valine and L-alanine

Uptake of L-[4,5 - ^{3}H] leucine into BHK21-C13 cells was examined (for the concentration range 1 x 10^{-5}M to 1 x 10^{-3}M L-leucine) alone (●-●) and in the presence of 1 x 10^{-2}M L-alanine (O-O) or 2 x 10^{-3}M L-valine (Δ-Δ). The assay procedure was carried out as described in Section 2.8, and rates of transport were based on one minute incubations.

Figure 5.2.7h. S/v versus S plot of L-valine uptake in BHK21-C13 cells in the presence and absence of L-leucine

Uptake of L-[2,3 - ^{3}H] valine into BHK21-C13 cells was examined (for the concentration range 1 x 10^{-5}M to 1 x 10^{-3}M L-valine) alone (●-●) and in the presence of 2 x 10^{-3}M L-leucine (O-O). The assay procedure was carried out as described in Section 2.8, and rates of transport were based on one minute incubations.

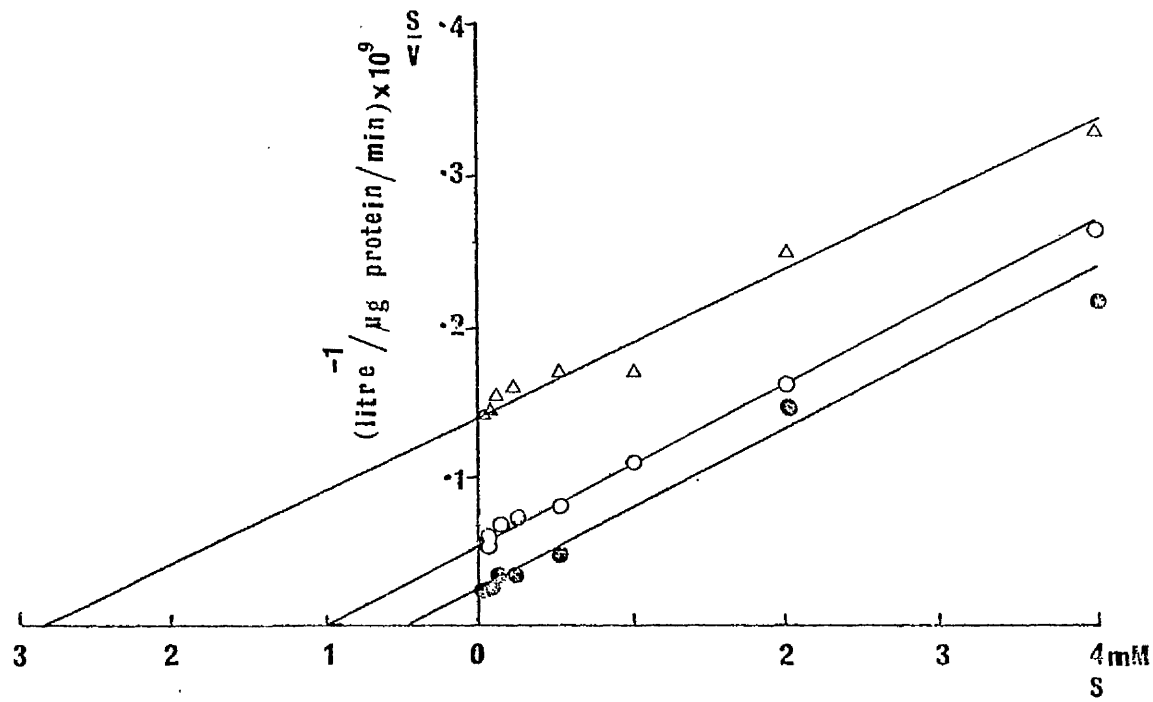


Figure 5.2.7g. S/v versus S plots of L-leucine uptake into BHK21-C13 cells, in the presence and absence of L-alanine and L-valine.

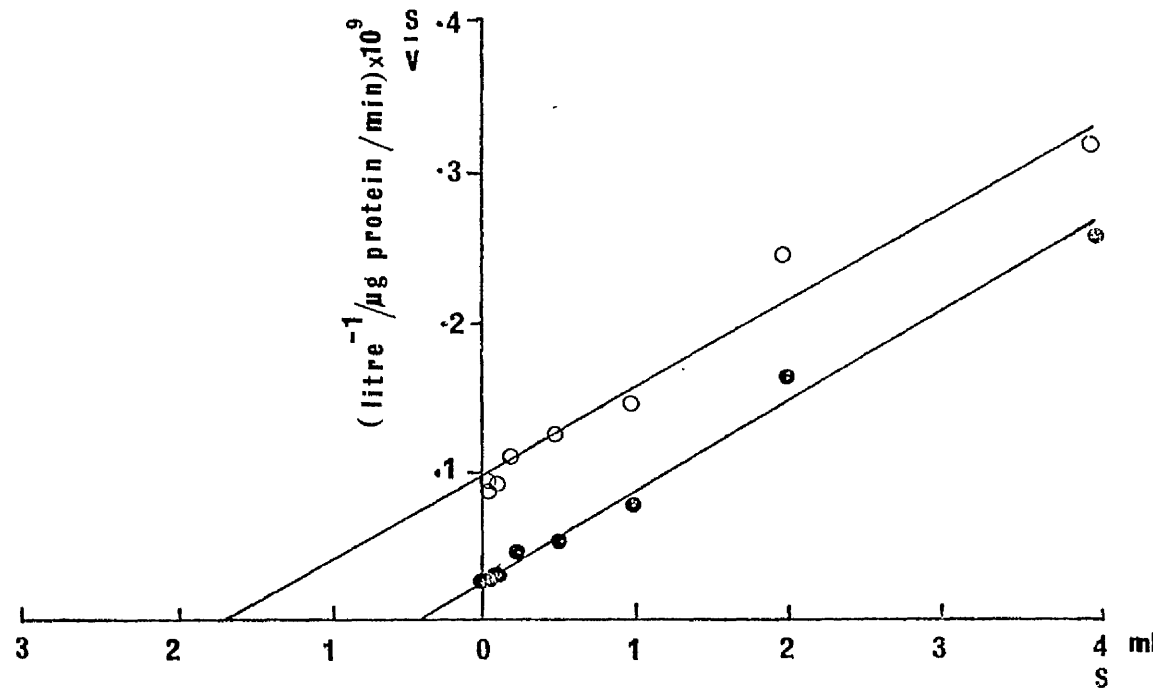


Figure 5.2.7h. S/v versus S plots of L-valine uptake into BHK21-C13 cells, in the presence and absence of L-leucine.

Table 5.2.7c. Comparison between Km and Ki values for entry of amino acids into BHK21-C13 cells.

Amino Acid	Km (mM)	Ki values when acting as inhibitors of :			
		L-serine	L-alanine	L-leucine	L-valine
L-alanine	.81 ± .16	.99 ± .28 (2.27 ± .24)	-	10.63 ± 1.58 (0.99 ± .10)	-
L-serine	.75 ± .12	-	.68 ± .18 (3.18 ± .29)	-	-
L-valine	.39 ± .06	-	-	.42 ± .15 (2.92 ± .48)	-
L-leucine	.51 ± .10	-	1.92 ± .47 (3.17 ± .21)	-	.61 ± .13 (1.67 ± .1

Km and Ki values for the uptake of these amino acids were determined as described in Sections 2.8.5. and 2.8.6. Kp values from which Ki values were calculated are shown in parenthesis.

Table 5.2.7d. Inhibition of L-leucine uptake into BHK21-C13 cells, by different amino acids.

Inhibiting Amino Acids	Percentage inhibition of L-[4,5-^{3}H]leucine uptake
L-leucine	84
L-phenylalanine	87
L-valine	81
L-glutamine	55
L-alanine	21
L-arginine	14

Uptake of L-[4,5-^{3}H]leucine (1 x 10^{-4}M) was examined in the presence and absence of inhibiting amino acid (of concentration 2.5 x 10^{-3}M). Rates of uptake were determined from 1 minute incubations. Values are the means of 5 samples and are expressed as percentage inhibition of L-[4,5-^{3}H]leucine uptake alone.

and L-leucine uptake in the presence of L-alanine and L-valine are shown in Figures 5.2.7 f and g respectively. L-valine uptake alone and in the presence of L-leucine is shown in Figure 5.2.7h.

Km values for the uptake of L-alanine and L-serine were similar to their Ki values, when acting as inhibitors of the transport of each other. Similar observations were made for L-leucine and L-valine uptake and reciprocal inhibition (Table 5.2.7c).

However, the Km values for L-alanine and L-leucine uptake differed considerably from the Ki values obtained for these amino acids, when acting as inhibitors of the transport of the other amino acid.

These data are consistent with transport of L-alanine and L-serine via a single transport agency; similarly L-leucine and L-valine would appear to be transported via a separate single transport system. However, as L-alanine and L-leucine each appear to be a competitive inhibitor of the other's transport, it would appear that each of these amino acids shows some affinity for the other system. The limited affinity of L-alanine for transport via the "L-leucine preferring transport system" is indicated by its limited inhibition of L-[4,5-^{3}H] leucine uptake, as compared to L-leucine itself, L-valine and L-phenylalanine (Table 5.2.7d). Thus it would appear that there are at least two transport systems capable of transporting the α-amino neutral amino acids.

5.3. DISCUSSION AND CONCLUSIONS

The uptake of L-alanine was examined in detail in monolayer cultures of exponentially growing BHK21-C13 cells. This amino acid was strongly accumulated against a concentration gradient and its transport was energy-, Na^{+}-, temperature- and pH-dependent. L-alanine uptake appeared to follow Michaelis-Menten kinetics for the concentrations studied and statistically determined estimates of the Km and Vmax values for L-alanine gave values of $0.81 \pm .16 \times 10^{-3}$M and 57.7 ± 4.1 pmol/μg

protein/min respectively. L-[2,3-^{3}H] alanine uptake was inhibited by a large number of neutral L-α amino acids although their effectiveness in inhibiting its uptake varied considerably. This inhibition was studied in more detail in certain cases and shown to be competitive. The inhibition was of similar specificity to that observed with the "Alanine preferring" ("A-") transport system of Ehrlich cells (Oxender and Christensen 1963). Like the latter system, the major system mediating L-alanine uptake in BHK21-C13 cells showed preference for the short chain aliphatic or hydroxy substituted amino acids and showed a marked preference for the L-stereoisomers of the substrate amino acids. L-cysteine, although an effective inhibitor of L-alanine uptake in Ehrlich cells, did not appreciably inhibit L-alanine uptake in BHK21-C13 cells.

Inhibition of L-alanine uptake by structural analogues of substrates of the "L-alanine preferring transport system" provided additional information about its structural requirements in BHK21-C13 cells. These studies indicated that the α-carboxyl group is essential for transport via the "L-alanine preferring transport system" as esterification or replacement of the α-carboxyl group completely abolished affinity for this system. Replacement of the α-hydrogen by an α-methyl group however, appeared to have little effect on ability to be transported by the "L-alanine preferring transport system", as α-AIB was almost as effective as L-alanine in inhibiting L-[^{3}H] alanine uptake. The L-alanine preferring transport system however, appears to tolerate only certain modifications of the α-amino group of substrate amino acids, as replacement of this group by a N-acetyl or a hydroxamate group almost completely abolished affinity for transport via the 'L-alanine preferring transport system'. N-methylation (as exhibited by N-methyl-DL-alanine) produced little alteration of affinity and

appeared to inhibit L-[2,3-^{3}H]alanine uptake as effectively as L-alanine. N-methylation of most substrates of the 'A' system of Ehrlich cells was shown to produce little reduction in ability to be transported via the 'A' system, and N-methylation of α-AIB actually increased its affinity for transport via this system (Christensen et al. 1965). In addition to the 'A' system in Ehrlich cells two additional neutral amino acid transport systems have been demonstrated: a) a Na^+ sensitive ASC transport system (preferred substrates alanine, serine, cysteine) which was demonstrated by studies utilising the fact that it apparently does not transport N-methyl amino acids (Christensen et al. 1967); and b) a Na^+- and pH- independent "leucine preferring" ('L') transport system. As L-alanine uptake was reduced by approximately 90% by N-methyl-DL-alanine, it is unlikely that a system of similar specificity to the 'ASC' system of Ehrlich cells is important in the transport of L-alanine in BHK21-C13 cells. Systems of similar specificity to the 'ASC' system of Ehrlich cells have, however, been demonstrated in rabbit reticulocyte (Winter and Christensen 1965) and pigeon erythrocyte (Eavensen and Christensen 1967).

Inhibition studies have indicated the presence of a second amino acid transport system capable of transporting neutral amino acids in BHK21-C13 cells. This system showed similar specificity to the 'L'-system of Ehrlich cells as L-[^{3}H] leucine uptake was most effectively inhibited by short branched chain aliphatic neutral amino acids (as indicated by L-leucine and L-valine), aromatic amino acids (as indicated by L-phenylalanine), or L-methionine. L-alanine and the basic amino acid arginine were relatively ineffective inhibitors of L-leucine uptake, although this inhibition was shown to be competitive at least for L-alanine. As the Ki value for L-alanine when acting as an inhibitor of L-leucine uptake is very large compared to its Km for uptake, and

L-alanine transport is reduced by approximately 90% by Na^+ removal, it is unlikely that this L-leucine preferring system is important in the uptake of L-alanine.

Broad specificity neutral α-amino acid transport systems have also been reported in a number of mammalian or avian tissues. However, these systems generally have not been studied in great detail (reviewed by Neame 1968). Christensen (1969) has proposed that a system of similar specificity (and possibly other characteristics) to the L-system of Ehrlich cells may be present in all cells, and that A-like systems may also occur in most cell types. These systems, when both are present, are considered not to be exclusive but are of overlapping specificities and capable of transporting both aliphatic and aromatic amino acids (Oxender and Christensen 1963; Christensen et al. 1965; Christensen et al. 1967). Systems of similar specificity to the L-system have been reported or indicated in a number of tissues including: isolated intestinal epithelial cells (Reiser and Christiansen 1971), syrian hamster embryo cells in culture (Hare 1967), and human (Winter and Christensen 1965) and pigeon erythrocytes (Eavensen and Christensen 1967). 'A'-like transport systems have been reported or indicated in rat intestine (Munck 1966), rat calvarium (Finerman and Rosenberg 1966), kidney (Webber et al. 1961; Webber 1962) and chick embryo heart cells (Gazzola et.al. 1971; Franchi-Gazzola et al. 1972). No 'A'-transport system however is present in human (Winter and Christensen 1965) or pigeon erythrocytes (Eavensen and Christensen 1967). Confirmation of the existence in human intestine and kidney of at least one broad specificity system, capable of transporting aliphatic and aromatic neutral α-amino acids, is indicated by the apparent deficiency in the transport of these amino acids in Hartnup disease (Baron et al. 1956; Jepson 1972). In addition to these broad specificity neutral α-amino acid transport systems, more specific systems capable of transporting

only a single amino acid are indicated in man by the occurrence of the apparent defects of transport of methionine (Hooft et al. 1965) and tryptophan (Drummond et al. 1964). A transport system shared by proline, hydroxyproline and glycine,and separate glycine and amino acid transport systems have also been indicated by studies on patients with iminoglycinuria (Goodman et al. 1967; Scriver 1968; Rosenberg et al. 1968), and direct in vitro studies on a number of mammalian tissues e.g. rat kidney slices (Wilson and Scriver 1967), hamster intestine (Lin et al. 1962), foetal rat bone (Finerman and Rosenberg 1966), and rabbit renal tubules (Hillman and Rosenberg 1969). The existence of seperate proline and glycine transport systems are also indicated by the development of ability to transport proline and glycine at different times following birth (Baerlocher et al. 1970, 1971a and b). Broad specificity amino acid transport systems capable of transporting the neutral amino acids have also been demonstrated in microorganisms. General systems capable of transporting neutral, basic and acidic amino acids (but not the imino acid proline) have been reported in Saccharomyces cerevisiae (Grenson et al. 1970), Penicillium chrysogenum (Benko et al. 1967, 1969; Hunter and Segal 1971), Neurospora crassa (Pall 1969; Thwaites and Pendyala 1969) and Arthrobotrys conoides (Gupta and Pramer 1970). An additional neutral L-α-amino acid transport system, designated System I by Pall (1969), has also been demonstrated in N. crassa (Pall 1969; Wolfinbarger and De Busk 1971 ; Wiley and Matchett 1966). Gits and Grenson (1969) have also reported a system in S. cerevisiae specific for methionine, leucine, valine, isoleucine, alanine and threonine. The neutral amino acids however, in bacteria appear to be transported via a number of amino acid-specific, or group-specific systems. The characteristics of these bacterial, fungal and mammalian transport systems are briefly summarised in Table 5.3.1. It can be seen from this table that the observed Km values for L-alanine

TABLE 5.3.1. NEUTRAL AMINO ACID TRANSPORT SYSTEMS

I) MAMMALIAN SYSTEMS

i) "L"-System	Specificity	Km's ($\times 10^{-3}$ M)	References
(formally described for Ehrlich cells)	Transports most neutral amino acids (not α-AIB), or N-methyl amino acids)	0.3 to 4 L-leu (0.3)	Oxender and Christensen (1963), Christensen et al (1965)
Possibly similar systems in:			
Isolated intestinal epithelial cells		L-leu 3.2	Reiser and Christiansen (1971)
Cultured syrian hamster embryo cells		L-phe .15 (estimated from data)	Hare (1967)
Human erythrocyte		L-leu 1.8 L-phe 4.3 L-met 5.2	Winter and Christensen (1964)
BHK21 - C13 cells		L-leu 0.5 L-val 0.4	Section 5, this thesis

ii) "A-" System	Specificity	Km's (x 10^{-3}M)	References
(formally described for Ehrlich cells)	Transports all neutral amino acids, preferred substrates L-ala and thpse with short polar side chains.	0.5 - 4 L-ala (0.5).	Oxender and Christensen (1963) Christensen et al (1965, 1967)
Possibly similar systems in :			
rat intestine			Munck (1966)
rat Calvarium			Finerman and Rosenberg (1966)
Chick embryo heart cells		∝-AIB 0.72	Gazzola et al (1972) Franchi-Gazzola et al
BHK21 -C13 cells		L-ala 0.8 L-ser 0.6	Section 5, this thesis.

iii) "ASC" - System	Specificity	Km's (x 10^{-3})	References
(described for Ehrlich cells)	L-Ala, L-ser, L-thr, L-pro, L-cysteine not methyl-substituted amino acids.	0.2 (L-cysteine) 1.0 (L-ser) 1.4 (L-ala)	Christensen et al (1965, 1967)
Similar systems described for :			
rabbit reticulocyte			Winter and Christensen (1965)
pigeon erythrocyte			Eavenson and Christensen (1967)
iv) Glycine specific			
as described for avian erythrocytes and rabbit reticulocytes	gly, sarcosine		Vidavar et al (1964) Winter and Christensen (1965)
possibly kidney			De Vries et al (1957)

v) Transport defect in man indicate in addition to a braod specificity, neutralα-amino acid transport system, possible specific methionine and tryptophan, and additional imino acid/glycine transport systems. Reviewed by Slayman (1973)

II) MICROBIAL SYSTEMS

i) Broad Specificity Systems

System and Specificity	Microorganism	Km's ($\times 10^{-6}$ M)	References
a) General System			
Neutral, basic and acidic amino acids.	S.cerevisiae	3 - 10	Grenson et al (1970)
	P.chrysogenum		Benko et al (1969)
	N.crassa	.2-1200	Hunter and Segal (1971) Pall (1969)
	A.conoides	15 - 75	Gupta and Pramer (1970)
b) Neutral System			
Neutral amino acids	N.crassa	100 - 500	Pall (1969), Wolfinbarger and DeBusk (1971), Wiley and Matchett (1966)
Met,leu,val,ileu,ala	S.cerevisiae		Gits and Grenson (1969)

ii) Specific or relatively specific systems, as demonstrated by the systems for Ecoli

Amino acid or Group of amino acids	Transport Systems and Specificity	Km's (x 10^{-6}M)	References
a) Gln	Gln and certain Gln analogues	0.008	Weiner and Heppel (1971)
b) Leu, Ileu, Val.	Number of systems is unclear, possibly		
	a) share system plus	2	Reviewed by: Slayman (1973)
	b) specific system for each amino acid.	50	
c) Gly, ala, ser.	2 or possibly 3 separate systems.		
	a) L-ala and probably L-ser.	57	Wargel et al (1970)
	b) and possibly c) for gly, D-L-ala and D-ser		Oxender (1972a)
d) Aromatic amino acids :	At least 5 systems :		
	a) General aromatic system	.4 - .6	Brown (1970)
trp, Tyr, Phe	b) tyr,phe c) Phe d) 2 Trp systems	approximately 2	

and L-leucine transport in BHK21-C13 cells are similar to those reported for Ehrlich cells and certain other mammalian systems, and the neutral L- amino acids of N. crassa, but 2-5 orders of magnitude higher than most bacterial neutral amino acid transport systems.

Valuable information as to the complete specificity of the L-alanine transport system in BHK21-C13 cells, and its relative importance in the transport of its substrates could be provided by the isolation of L-alanine transport defective mutants. Section 9 describes attempts to isolate such mutants and discusses possible reasons for lack of success in their isolation.

SECTION 6. CONTROL OF THE ACIDIC AMINO ACID AND L-ALANINE PREFERRING TRANSPORT SYSTEMS OF BHK21-C13 CELLS

6.1. INTRODUCTION

Although considerable information is available regarding the nature and specificity of amino acid transport systems, relatively little is known about their control mechanisms. Intracellular concentrations of naturally occurring amino acids or their analogues have been shown in microorganisms to influence the inward transport of these molecules, either by transinhibition (Ring and Heinz 1966, Pall 1971; Benko et al. 1967) and/or by control mechanisms effective on protein synthesis (Pall 1971). The actual control mechanisms for the regulation of amino acid transport systems in mammalian cells are unclear, although there is some evidence that these systems may also be controlled by their substrate molecules (Phang et al. 1971). Mammalian amino acid transport systems have also been commonly shown to be altered in vivo and in certain cases in vitro by various peptide, amino acid derivative or steroid hormones, including insulin (Kipnis and Noall 1958; Wool et al. 1965), growth hormone (Riggs and Walker 1960; Hjalmarsen and Ahren 1967), 3,3',5-triodothyronine (T3) (Adamson and Ingbar 1967), cortisol (Baril et al. 1969; Mohri 1967) and epinephrine (Sanders and Riggs 1967). Recent studies with cultured mammalian cells have indicated that a number of additional factors or conditions may be important in the transport of low molecular weight metabolites. These include cell density (Sefton and Rubin 1971; Bose and Zlotnick 1973; Foster and Pardee 1969), serum concentration (Foster and Pardee 1969; Shodell and Isselbacher 1973; Herschko et al. 1971), and intracellular cyclic AMP levels (Kram et al. 1973; Kram and Tomkins 1973).

In this chapter the effects of medium modification, cell density,

various serum treatments, hormones and dibutyryl cyclic AMP, on the transport of L-alanine and L-glutamate in BHK21-C13 cells are examined, in order to investigate possible regulatory mechanisms for the acidic and L-alanine preferring transport systems. The intracellular cyclic AMP levels of these cells following certain of the above conditions or treatments are also reported in this chapter, in view of a possible correlation between L-alanine transport and intracellular cyclic AMP levels.

6.2. RESULTS

6.2.1. Effects of medium modifications on L-glutamate and L-alanine uptake in BHK21-C13 cells

L-alanine and L-glutamate transport was examined in BHK21-C13 cells following growth or incubation of these cells in conditions designed to alter intracellular amino acid levels. The transport of these amino acids was examined following:

a) incubation or growth of cells in EdFC plus relatively high concentrations of certain amino acids (shown in Sections 5.2.7 and 4.2.7 to be substrates of the L-alanine preferring and acidic amino acid transport systems), see Tables 6.2.1a and b; and

b) growth in the presence of $EdFC_{10}$ supplemented with non-essential amino acids (see Section 2.1.2) and the subsequent removal of substrate or other amino acids, see Tables 6.2.1c and d.

Incubation or growth of cells in the presence of high concentrations of acidic or neutral amino acids ($2x10^{-3}M$) did not appreciably alter the respective rates of transport of L-glutamate or L-alanine. The lack of effect of these relatively long or short term treatments on the transport of these amino acids would indicate that these transport

Table 6.2.1a. L-alanine uptake following preloading of BHK21-C13 cells with amino acids transported by the L-alanine preferring transport system.

Incubation period in the presence of selected amino acids (hours)	Added amino acids: L-ala, L-ser, L-thr (preferred substrates)	Added amino acids: All neutral ∝-amino acids
	L-alanine uptake (pmol/μg protein/min)	
0	50.9 ± 5.0	53.1 ± 5.5
0.25	53.9 ± 4.6	47.4 ± 4.6
1	55.5 ± 3.8	56.8 ± 4.9
2	44.7 ± 2.3	46.1 ± 3.5
4	48.2 ± 4.2	44.4 ± 4.7
8	42.1 ± 3.7	54.8 ± 4.2
48	47.6 ± 4.1	47.6 ± 3.2

Cells were grown for 48 hours in $EdFC_{10}$ (or for the 48 hour sample in $EdFC_{10}$ plus the above amino acids, of concentration 2×10^{-3}M). Uptake of L-[2,3,-^{3}H]alanine (2×10^{-3}M) was then examined following these growth conditions or at intervals following the addition of the above amino acids (2×10^{-3}M). Rates of L-alanine uptake were determined from 4 minute incubations. All values are the means of 8 estimations plus SEM.

Table 6.2.1b. L-glutamate uptake following preloading of BHK21-C13 cells with amino acids transported by the acidic amino acid transport system.

Incubation period in the presence of selected amino acid (hrs.)	Added Amino Acids: L-glutamate, L-aspartate	Added Amino Acids: All neutral amino acids, plus histidine
	L-glutamate uptake pmol/μg protein/min	L-glutamate uptake pmol/μg protein/min
0	6.5 ± .5	6.3 ± .5
0.25	7.0 ± .6	5.4 ± .7
0.5	6.3 ± .5	5.7 ± .4
1.0	6.9 ± .5	6.9 ± .7
2.0	7.5 ± .8	6.1 ± .6
4.0	8.1 ± .7	7.1 ± .7
8.0	7.4 ± .4	5.9 ± .7
48.0	6.5 ± .7	5.4 ± .7

Cells were grown for 48 hours in $EdFC_{10}$ (or for the 48 hour sample in $EdFC_{10}$ plus the above amino acids, of concentration 2×10^{-3}M). Uptake of L-[U-^{14}C] glutamate (2×10^{-3}M) was then examined following these growth conditions or at intervals following the addition of the above amino acids (2×10^{-3}M). Rates of L-glutamate uptake were determined from 10 minute incubations. All values are the means of 8 estimations plus SEM.

Table 6.2.1c. L-alanine uptake following removal of amino acids from the growth medium.

Interval following amino acid removal	Amino acids removed from Growth Medium		
	L-alanine, L-serine, L-threonine.	All neutral amino acids.	All amino acids
	L-alanine uptake (pmol/μg protein/min)		
0	45.0 ± 4.9	45.0 ± 4.9	40.9 ± 3.1
1	47.4 ± 2.7	48.6 ± 3.3	47.6 ± 1.9
2	43.0 ± 3.6	44.9 ± 2.8	39.4 ± 3.3
4	37.6 ± 3.4	35.3 ± 2.1 (t = 3.57)*	36.4 ± 1.6
8	41.6 ± 1.8	33.8 ± 2.1 (t = 5.91)*	25.2 ± 2.4(t = 4.00)*

Cells were grown for 48 hours in the presence of $EdFC_{10}$ supplemented with non-essential amino acids (Section 2.1.2.). Uptake of L-[2,3,-^{3}H] alanine (2×10^{-3}M) was susequently examined in these samples, and in samples incubated in medium less the above amino acids. Rates of uptake were determined from 4 minute incubations . All values are means of 6 samples plus SEM.

* Indicates value significantly different from control value (Sample 0) obtained prior to amino acid removal, (p 0.05, t = 2.20).

Table 6.2.1d. L-glutamate uptake following removal of amino acids from the growth medium.

Interval following amino acid removal	Amino acids removed from Growth Medium			
	L-glutamate, L-aspartate	Neutral amino acids	Basic amino acids	All amino acids
	L-glutamate uptake (pmol/ug protein/min)			
0	6.9 ± .9	6.9 ± .9	7.2 ± .8	7.2 ± .8
1	7.4 ± .7	5.5 ± .2	6.5 ± .5	6.4 ± .5
2	6.8 ± .4	8.1 ± .4	5.9 ± .5	6.9 ± .5
4	5.4 ± .2	7.4 ± .4	7.5 ± .5	5.8 ± .6
8	6.5 ± .7	5.2 ± .4	5.4 ± .4	4.6 ± .5(t = 3.29)*

Cells were grown for 48 hours in the presence of $EdFC_{10}$, supplemented with non-essential amino acids (Section 2.1.2.). Uptake L-[U-^{14}C] glutamate (final concentration, 2 x 10^{-5}M) was subsequently examined in these samples and in samples incubated in medium less the amino acids. Rates of uptake were determined from 10 minute incubations. All values are means of 6 samples plus SEM.

* Indicates value significantly different from control value (Sample 0) obtained prior to amino acid removal, (p 0.05, t = 2.20).

systems are not transinhibited, transtimulated or repressed by their respective substrate amino acids. The absence of increased transport of L-alanine and L-glutamate following removal of substrate amino acids (or amino acids from which they may be synthesised) is also inconsistent with these transport systems being subject to repression by the presence of substrate amino acids. The observed time-dependent reductions in transport are probably due to a reduction in the synthesis or replacement of specific permease protein(s) and/or proteins involved in general cell metabolism.

6.2.2. Transport of L-glutamate and L-alanine at different cell densities of BHK21-C13 cells

The rates of L-alanine and L-glutamate uptake were examined in BHK21-C13 cells at different cell densities (see Figure 6.2.2a). Similar rates of L-glutamate transport were observed at all cell densities examined. However, L-alanine uptake was reduced in cells which exhibited marked cell to cell contact (confluent cultures). L-alanine uptake in confluent cells was reduced to a level approximately 57% of that observed in exponentially growing cultures. The basis for this reduced rate of uptake in confluent cultures was investigated by examining the kinetics of L-alanine uptake in these cells. L-alanine uptake was examined for concentrations between 1×10^{-4}M and 4×10^{-3}M for 4 minute incubations of cells and a plot of the S/v versus S values constructed, Figure 6.2.2b. This plot was linear over the concentration range studied. The statistically determined estimates for Km and Vmax for L-alanine transport in confluent BHK21-C13 cells were $0.95 \pm 0.17 \times 10^{-3}$M and 35.1 ± 2.5 p mol/ug protein/min respectively. The Vmax observed for these confluent cells was thus approximately 40% lower than that observed in exponentially growing cells ($Z = 2.47$, $P < 0.05$), although the Km value was not significantly different ($Z = 0.54$, $P > 0.05$).

Figure 6.2.2a. L-alanine and L-glutamate uptake during growth of BHK21-C13 cells

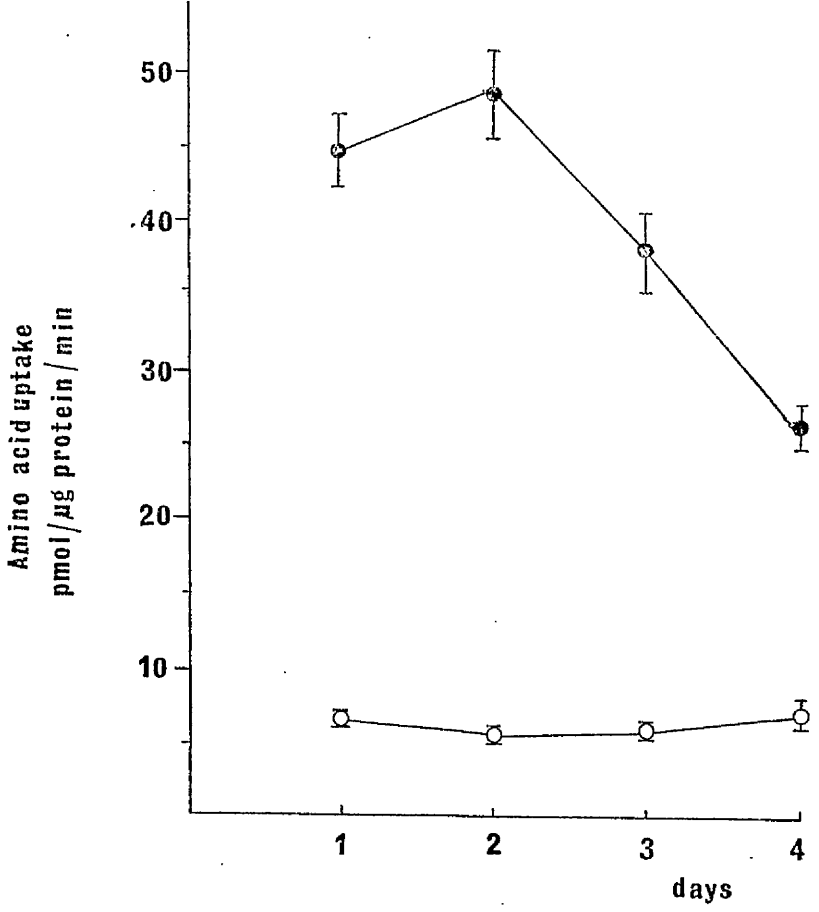
1×10^5 cells per 5cm dish were seeded on day 0. The uptake of L-[U-^{14}C]glutamate (2×10^{-4}M) and L-[2,3-^{3}H]alanine (2×10^{-3}M) were simultaneously examined, by dual labelling techniques, at 24 hour intervals. Rates were determined for 4 minute incubations. Values are the mean of 6 samples.

L-glutamate uptake (○-○)

L-alanine uptake (●-●)

Cell protein (▲-▲)

Cells per dish (□-□)



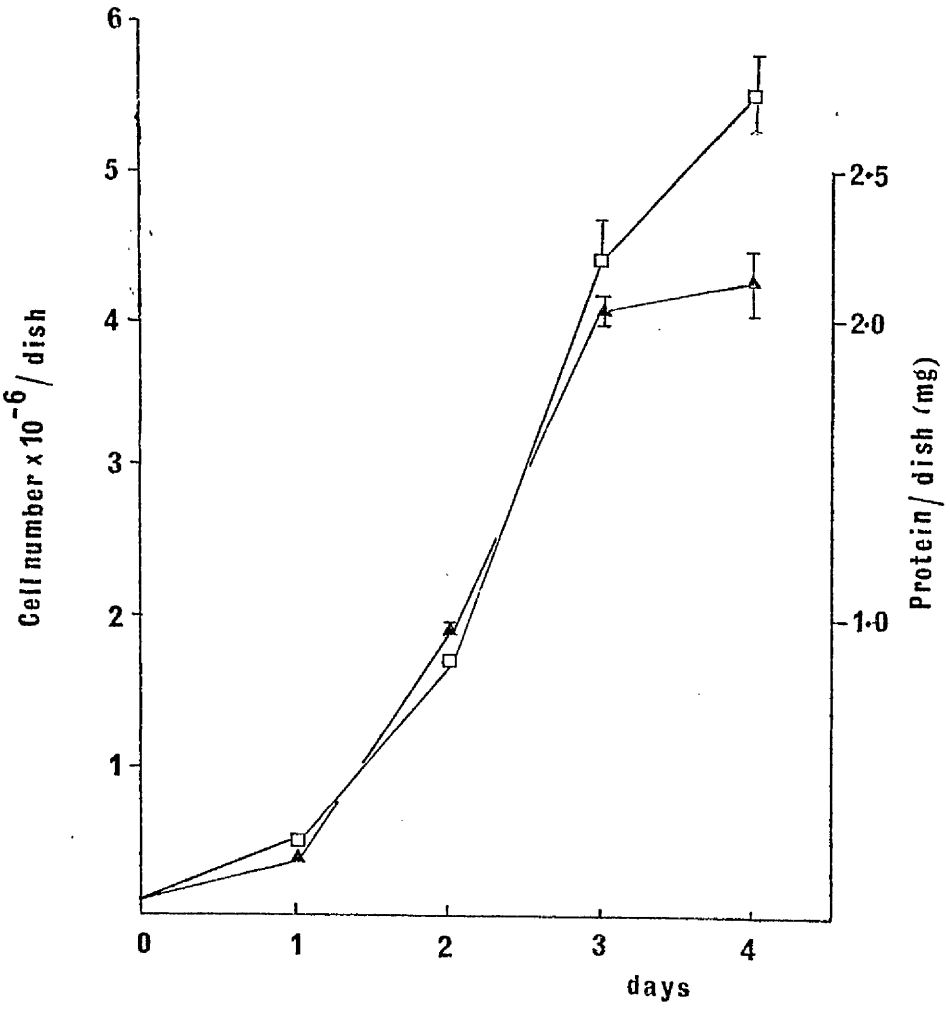


Figure 6.2.2a. L-alanine and L-glutamate uptake during growth of BHK21-C13 cells.

Figure 6.2.2b. S/v versus S plot of L-alanine uptake in confluent BHK21-C13 cells

Uptake of L-[2,3 - ^{3}H] alanine into confluent BHK21-C13 cells was examined for the concentration range 1 x 10^{-5}M to 4 x 10^{-3}M. The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations of up to 4 minutes. S/v values for concentrations 0.5 x 10^{-3}M and below are shown on an expanded scale.

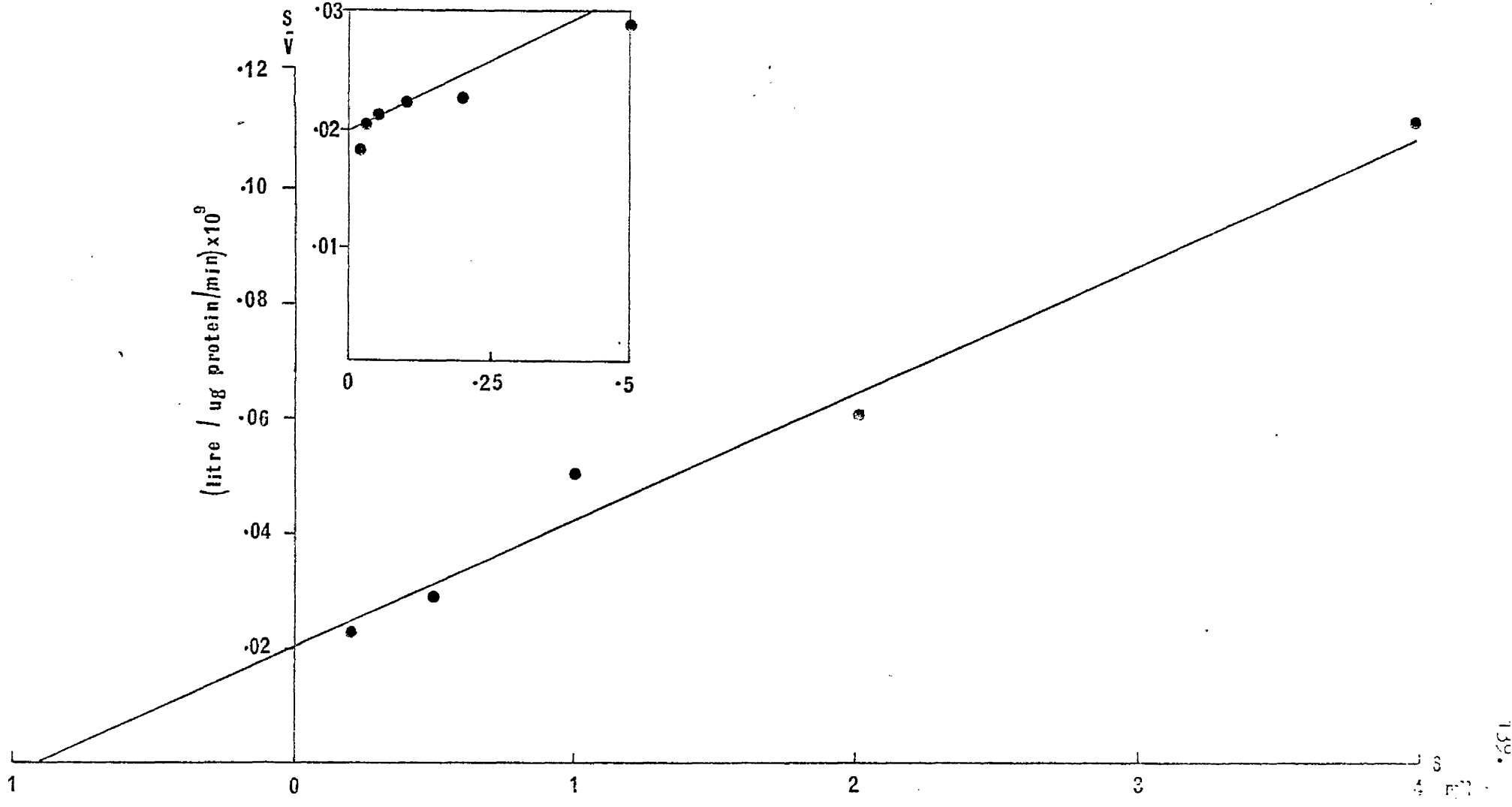


Figure 6.2.2b. S/v versus S plot of L-alanine uptake in confluent BHK21-C13 cells.

Thus it would appear that there is an apparent reduction in the activity, or number of L-alanine preferring transport sites available to the substrate amino acids.

6.2.3. Effects of various hormones on the rates of uptake of L-glutamate and L-alanine uptake in BHK21-C13 cells

The effect of various hormones on the uptake of L-alanine and L-glutamate in BHK21-C13 cells was examined. The rates of transport of these amino acids were examined at intervals following the addition of these hormones to cells grown in GMEM plus 10% or .5% (v/v) serum.

Cells were grown in low serum containing medium as well as at normal serum levels as it is possible that hormonal effects may be masked by high serum concentration. No alterations in the rates of transport of L-alanine or L-glutamate were observed for insulin (0.013, 0.13 and 1.3mM), epinephrine (0.01 and 1mM), growth hormone (0.01 and 1mM), cortisol (0.01 and 1mM) or 3', 3'5' - triiodothyronine (T3) (1mM) treated cells which were maintained in 10% serum. Representative results, as exhibited by cortisol (1mM) are shown in Table 6.2.3a. Insulin (1.3mM), however, stimulated growth and increased L-alanine uptake in cells maintained in 0.5% serum, Table 6.2.3b. No effect was produced by the lower concentrations of this hormone. (It should be noted that cells grown in low serum levels show reduced rates of L-alanine transport, see Section 6.2.4b.).

6.2.4. The effect of serum treatments on the rate of uptake of L-alanine and L-glutamate in BHK21-C13 cells

6.2.4a. Effects of dialysed and non-dialysed bovine and foetal bovine sera on L-alanine and L-glutamate uptake

L-alanine and L-glutamate uptake was examined in BHK21-C13 cells following growth of these cells in medium containing either 10% (v/v) dialysed or undialysed bovine or foetal bovine sera, see Table 6.2.4a.

Table 6.2.3a. L-alanine and L-glutamate uptake following addition of Cortisol to BHK21-C13 cells.

Interval following hormone administration	L-alanine uptake (pmol/μg protein/min) following growth in:		L-glutamate uptake (pmol/μg protein/min) following growth in:	
	EFC_{10}	$EFC_{0.5}$	EFC_{10}	$EFC_{0.5}$
0	45.9 ± 2.7	13.7 ± 1.9	7.2 ± .5	8.3 ± .9
0.5	41.6 ± 2.3	11.3 ± 1.0	8.4 ± .5	8.8 ± .9
1	40.2 ± 3.9	17.0 ± 1.1	7.4 ± .9	9.7 ± .7
2	48.8 ± 2.4	12.9 ± 1.6	9.0 ± .7	7.7 ± .5
6	40.0 ± 1.7	11.2 ± 1.9	6.7 ± 1.9	6.4 ± .4
12	48.2 ± 3.1	13.2 ± 1.3	8.9 ± 1.3	8.9 ± .7
48	41.4 ± 2.9	14.4 ± .9	8.0 ± .7	7.1 ± 1.0

Cells were grown for 48 hours under normal growth conditions (Section 2.2.1.), or in the presence of Cortisol (1×10^{-3}M). Measurements of the uptake of L-[2,3-^{3}H]alanine (2×10^{-3}M) and L-[U-^{14}C]glutamate, (2×10^{-4}M) were simultaneously determined subsequent to growth, or at intervals following Cortisol addition. Rates of uptake were determined from 4 minute incubations. Values are means of 8 values plus SEM.

Table 6.2.3b. L-alanine and L-glutamate uptake following addition of insulin to BHK21-C13 cells.

Interval following hormone administration	L-alanine uptake (pmol/μg protein/min) following growth in		L-glutamate uptake (pmol/μg protein/min) following growth in	
	EFC_{10}	$EFC_{0.5}$	EFC_{10}	$EFC_{0.5}$
0	40.7 ± 3.2	12.7 ± .8	6.6 ± .4	7.3 ± .7
0.5	36.9 ± 2.7	16.0 ± 1.0	6.1 ± .3	6.4 ± .6
1	45.1 ± 1.5	12.6 ± 1.0	7.6 ± .4	7.5 ± .7
2	43.2 ± 3.1	14.8 ± 1.4	6.5 ± .3	5.9 ± .5
6	36.6 ± 2.5	17.7 ± 1.1 (t = 3.20)*	7.4 ± .4	7.2 ± .7
12	34.2 ± 2.1	24.0 ± 1.9 (t = 6.59)*	5.8 ± .5	7.6 ± .2
48	45.0 ± 1.8	23.4 ± 1.9 (t = 6.13)*	6.4 ± .7	6.4 ± .6

Cells were grown for 48 hours under normal growth conditions (Section 2.2.1.), or in the presence of Insulin (1.3mM). Measurements of the uptake of L-[2,3-^{3}H] alanine (2×10^{-3}M) and L-[U-^{14}C]glutamate, (2×10^{-4}M) were simultaneously determined subsequent to growth, or at intervals following Insulin addition. Rates of uptake were determined from 4 minute incubations. Values are means of 8 values plus SEM.

* Indicates value significantly different from control value (Sample 0) obtained prior to hormone administration, (p 0.05, t = 2.20).

Table 6.2.4a. Effect of Dialysed or Undialysed Bovine or Foetal Bovine Sera on L-alanine and L-glutamate uptake.

Serum	L-alanine uptake (pmol/μg protein/min)	L-glutamate uptake (pmol/μg protein/min)
Foetal Bovine	41.5 ± 1.8	7.0 ± 0.5
Dialysed Foetal Bovine	37.5 ± 2.0	6.4 ± 0.4
Bovine	39.8 ± 2.3	6.6 ± 0.4
Dialysed Foetal Bovine	44.1 ± 1.9	7.2 ± 0.2

Uptake of L-[2,3-^{3}H] alanine (2×10^{-3}M) and L-[U-^{14}C] glutamate (2×10^{-4}M) was examined following growth of cells in medium containing 10% (v/v) of the above sera. Rates of uptake were determined from 4 minute incubations. Values are means plus SEM of 10 estimations.

L-glutamate uptake $F_{3,36} = 0.89$, $p > 0.05$ ($F = 2.86$).

L-alanine uptake $F_{3,36} = 2.13$. $p > 0.05$ ($F = 2.86$).

However, no significant differences in the transport rates of these amino acids were observed following growth in medium containing these different sera. The lack of effect on L-alanine or L-glutamate uptake of dialysis of serum would indicate that low molecular weight dialysable factors are probably unimportant in the regulation of these transport systems. The lack of any difference in the transport of these amino acids between cells grown in media containing foetal bovine serum and cells grown in media containing adult bovine serum may be due to the presence of similar high molecular weight serum transport factors in these sera, or the independence of L-glutamate and L-alanine transport from serum growth factors.

6.2.4b. Effect of serum concentration on the growth of BHK21-C13 cells and the uptake of L-alanine and L-glutamate in these cells

The growth characteristics of BHK21-C13 cells grown in different serum concentrations are shown in Figure 6.2.4a. BHK21-C13 cells grown in medium containing 10% (v/v) serum on reaching confluence divide more slowly and attain a maximum cell density of approximately 2.4×10^5 cells/cm^2. Cells grown in medium containing 5% (v/v) serum exhibit a slightly reduced rate of growth and saturation density. However, in medium containing 25% (v/v) serum these cells show a reduced density dependent inhibition of growth and continue to divide until they reach a saturation density of approximately 3.5×10^5 cells/cm^2. In the presence of 0.5% (v/v) serum, cells initially divide very slowly and within 48 hours cease to divide and produce apparently quiescent cultures (a condition which will subsequently be referred to as 'Serum Arrest'). A number of differences in the rates of transport of L-alanine were observed between cells maintained in these different serum concentrations, although similar rates of L-glutamate uptake were obtained in confluent

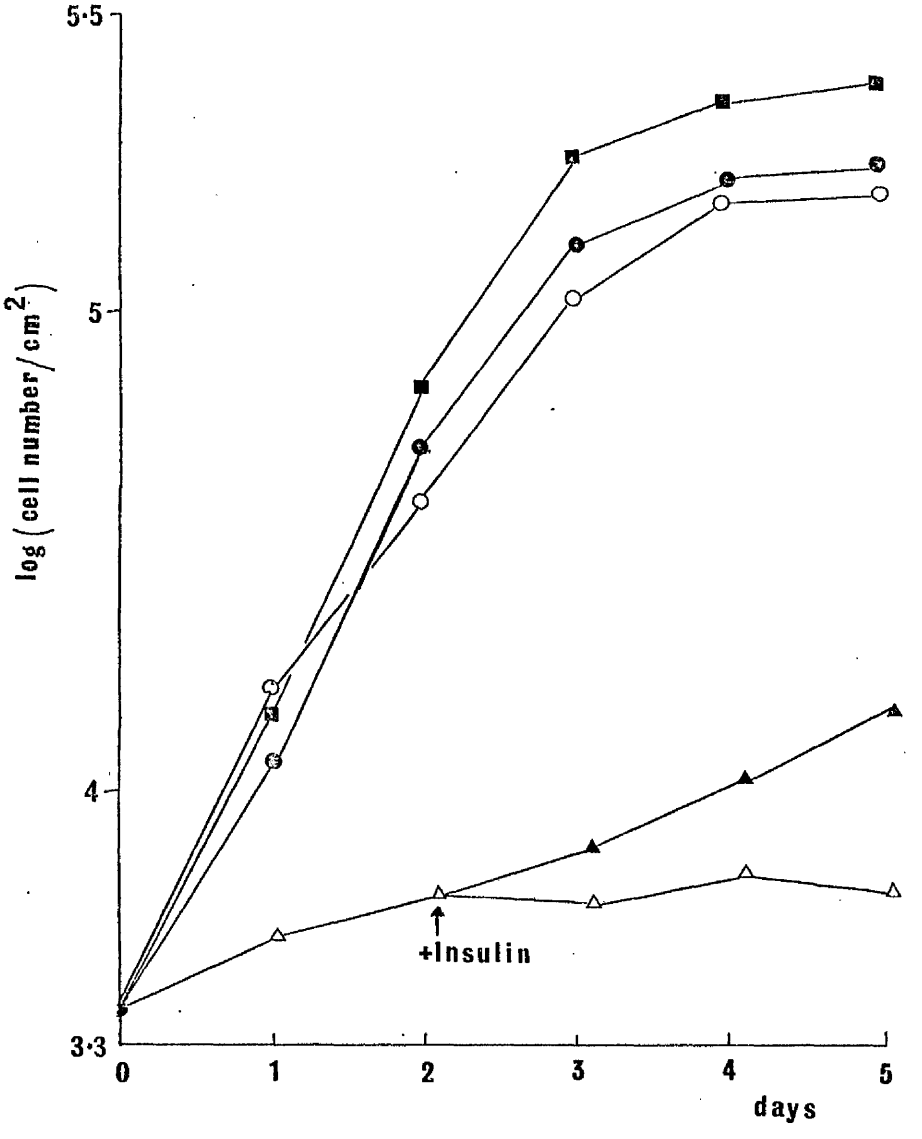


Figure 6.2.4a. Growth characteristics of BHK21-C13 cells in different serum concentrations.

Cells were inoculated at a density of 3.5×10^3 cells/cm^2 in 6.0 cm petri dishes, and grown under standard conditions (Section 2.2.), except for the medium modifications indicated below. Medium was changed every 48 hours. Cells were harvested and counted at intervals. Each value is the mean of duplicate cultures.

Cells maintained in EFC_{25} (■-■)
Cells maintained in EFC_{10} (●-●)
Cells maintained in EFC_{5} (○-○)
Cells maintained in $EFC_{0.5}$ (△-△)
Cells following addition of Insulin (1.3 nM) to $EFC_{0.5}$ (▲-▲)

or non-confluent cultures irrespective of serum concentrations, Table 6.2.4b. The rates of L-alanine uptake obtained for exponentially growing cultures of BHK21-C13 cells maintained in medium containing 5 to 25% (v/v) serum, did not differ significantly. However, L-alanine uptake was reduced in confluent cells grown in 5 or 10% (v/v) serum but not those in 25% (v/v) serum. Serum arrested cells (cells grown for 48 hours in 0.5%, v/v serum) showed rates of L-alanine uptake approximately 34% of those observed in exponentially growing cells. In order to determine whether the L-alanine uptake observed in serum arrested cells was mainly due to transport via the L-alanine preferring transport system, the kinetics of L-alanine uptake (Figure 6.2.4b) and the ability of selected amino acids to inhibit the uptake of this amino acid (Table 6.2.4c) were examined in these cells. Statistically determined estimates for the Km and Vmax values for L-alanine uptake in these cells were $0.70 \pm 0.9 \times 10^{-3}$M and 18.6 ± 0.8 pmol/min/μg protein. The maximum rate of transport was thus approximately 39% of the value obtained in normal serum levels ($z = 9.42$, $P < 0.05$) although the Km value was not significantly altered ($z = 0.61$, $P > 0.05$).

Although the maximum rate of L-alanine transport was considerably reduced in serum arrested cells the observations that: a) the Km value for transport was not significantly altered, and b) the inhibition of L-alanine uptake was similar to that observed in exponentially growing cells; would indicate that the observed transport was via the L-alanine preferring transport system.

Analysis of variance for L-alanine and L-glutamate uptake in BHK21-C13 cells grown in medium containing different serum concentrations.

L-glutamate uptake : $F_{6,80} = 1.76$, $P \Rightarrow 0.05$ ($F = 2.33$).
L-alanine uptake : $F_{6,80} = 23.80$, $P \Leftarrow 0.05$ ($F = 2.33$).

Difference between means of L-alanine uptake.

a) Cells grown in different serum concentrations.

Growth conditions			L.S.D.	Difference between Means
EFC_{10}	versus	EFC_{25}	6.08	2.6
EFC_{10}	versus	EFC_{5}	6.24	5.8
EFC_{5}	versus	EFC_{25}	6.24	3.2
EFC_{10}	versus	$EFC_{0.5}$	6.08	27.6*
EFC_{25}	versus	$EFC_{0.5}$	6.08	25.0*
EFC_{5}	versus	$EFC_{0.5}$	6.24	21.8*

b) Confluent versus non-confluent cells grown in different serum concentrations.

Non-confluent cells		Confluent cells	L.S.D.	Difference between Means
EFC_{10}	versus	EFC_{10}	6.08	16.0*
EFC_{25}	versus	EFC_{25}	5.82	2.9
EFC_{5}	versus	EFC_{5}	6.00	13.6*
EFC_{5}	versus	EFC_{25}	6.00	0.3
EFC_{10}	versus	EFC_{5}	5.82	19.4*
EFC_{25}	versus	EFC_{5}	5.82	16.8*
EFC_{10}	versus	EFC_{25}	5.82	5.5

* Indicates difference between the means is significant at the $P = 0.05$ level of significance.

L.S.D. is the least significant difference.

Table 6.2.4b. Effect of serum concentration on L-alanine and L-glutamate uptake in confluent and non-confluent BHK21-C13 cells.

Serum concentration (% v/v)	L-alanine uptake in pmol/ug protein/min		L-glutamate uptake in pmol/ug protein/min	
	Non-confluent	Confluent cells	Non-confluent	Confluent cells
25	38.9 ± 2.8 (12)	36.0 ± 1.4 (14)	5.8 ± .5 (12)	7.4 ± .5 (14)
10.	41.5 ± 3.4 (12)	25.5 ± 1.5 (12)	6.5 ± .6 (12)	5.9 ± .8 (12)
5	35.7 ± 2.9 (11)	22.1 ± 1.4 (14)	6.8 ± .4 (11)	6.8 ± .6 (14)
0.5	13.9 ± 1.4 (12)	Not determined	5.4 ± .6 (12)	Not determined

Cells were grown for 48 hours in Glasgow modified Eagles medium containing the above levels of serum. Confluent cultures were obtained within this incubation period by seeding 1.5 times the normal cell inoculum. Rates of uptake of L-[2,3,-^{3}H] alanine (2×10^{-3}M) and L-[U-^{14}C] glutamate (2×10^{-4}M) were determined (simultaneously by dual labelling methods) from 4 minute incubations. The mean values and SEM of the uptake rates are given with the number of independent estimations in parenthesis.

Figure 6.2.4b. S/v versus S plot of L-alanine uptake in BHK21-C13 cells following growth in low serum

Uptake of L-[2,3, - ^{3}H] alanine uptake into BHK21-C13 cells was examined, following growth of cells for 48 hours in $EFC_{0.5}$, for the concentration range 1 x 10^{-5}M to 4 x 10^{-3}M. The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations of up to 4 minutes.

S/v values for concentrations 0.5 x 10^{-3}M and below are shown on an expanded scale.

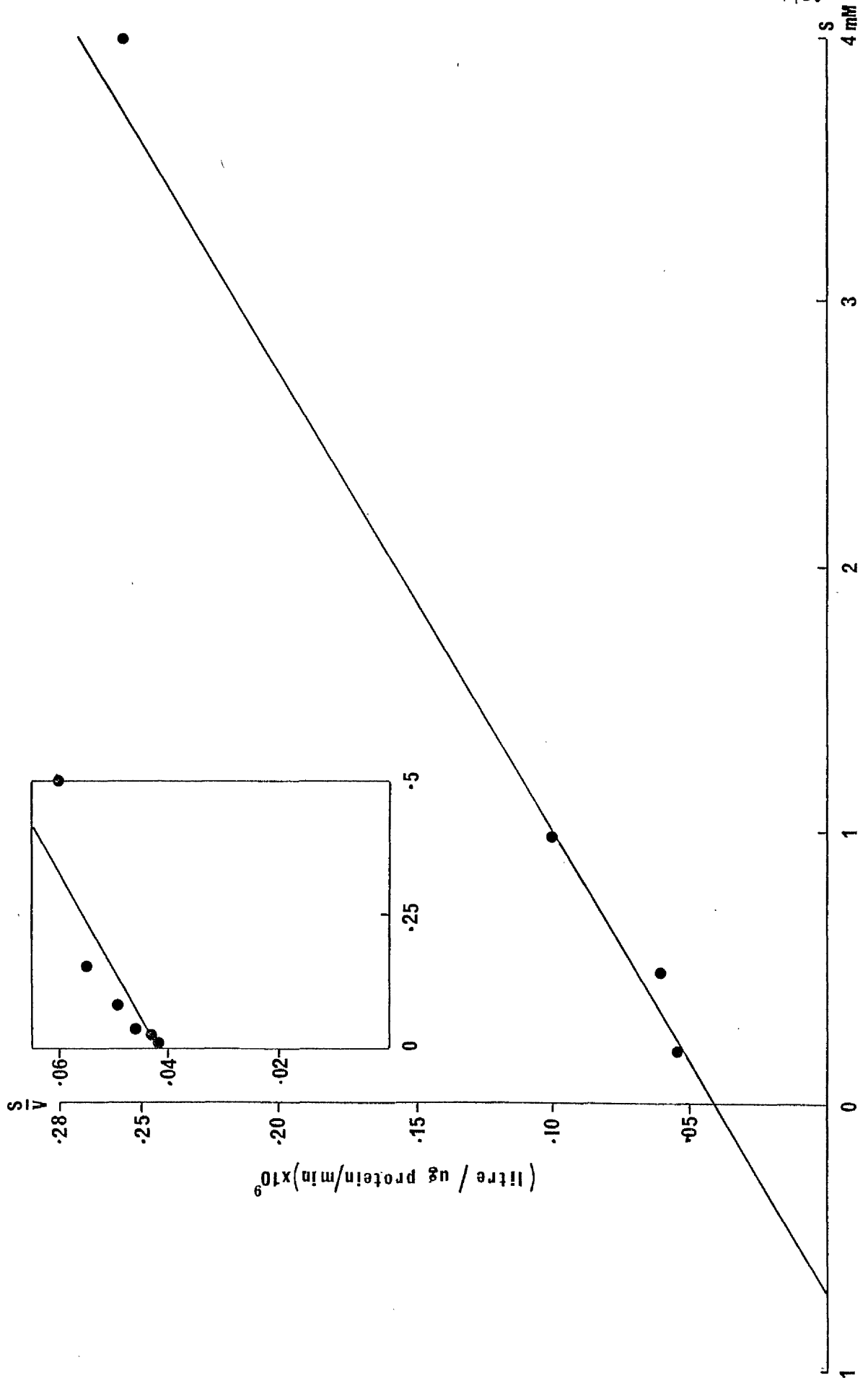


Figure 6.2.4b. s/v versus s plot of L-alanine uptake in cells grown in low serum

Table 6.2.4c. Inhibition of L-alanine uptake in BHK21-C13 cells following growth in medium containing low (0.5%, v/v) serum concentration

Inhibiting Amino Acid	Percentage Inhibition of L-[2,3,-^{3}H] alanine uptake
L-alanine	84
L-serine	79
L-leucine	38
L-valine	36
L-methionine	58
L-proline	53
L-phenylalanine	27

Uptake of L-[2,3,-^{3}H] alanine (final concentration 1 x 10^{-4}M) was examined in the presence and absence of inhibiting amino acid (2.5 x 10^{-3}M). Rates of uptake were determined from 4 minute incubations. Values are the means of 6 samples and results are expressed as percentage inhibition of L-[2,3-^{3}H] alanine uptake alone.

6.2.4c. L-glutamate and L-alanine uptake following the addition of serum containing medium to confluent or serum arrested BHK21-C13 cells

The effect on L-alanine and L-glutamate uptake of the addition of fresh foetal calf serum to exponentially growing, confluent or serum arrested BHK21-C13 cells is shown in Figures 6.2.4c,d and e. The uptake of these amino acids was examined simultaneously by means of dual-labelling experiments, L-alanine and L-glutamate data was discriminated as described in Section 2.7.4. The addition of serum to these cells did not alter the uptake of L-glutamate. L-alanine transport was similarly unaltered by the addition of serum (10%) to exponentially growing cells, or addition of serum of final concentration less than 5%, to exponentially growing, serum arrested or confluent cells. Addition of 5% (v/v) serum to serum arrested or confluent cells, produced a slight stimulation of L-alanine uptake, although 10 and 25% (v/v) serum increased L-alanine uptake to a rate comparable to that observed in exponentially growing cultures. Maximum rates of uptake were observed within 8 and 16-24 hours respectively for confluent and serum arrested cells. Similar results were obtained for the addition of fresh serum containing medium.

Subsequently to these experiments Shodell and Isselbacher (1973) reported a more marked stimulation of α-AIB uptake in serum arrested cells. The experiments were therefore repeated using α-AIB and L-alanine. The cells were pretreated as described by these authors. After serum arrest the cells were washed twice with phosphate buffered saline (PBS) and incubated in PBS for 45 minutes. This medium was then replaced with fresh medium containing 10% (v/v) serum, and α-AIB uptake examined at 4, 8, 16 and 24 hours. However, similar increases were obtained for these amino acids whether the cells were preincubated in PBS or not, and maximum transport was observed by about 16 hours after serum addition (Figure 6.2.4f).

Figure 6.2.4c. L-alanine and L-glutamate uptake in exponentially growing BHK21-C13 cells following serum addition

L-|2,3 - ^{3}H| alanine ($2 \times 10^{-3}M$) and L-|U ^{14}C|glutamate ($2 \times 10^{-4}M$) uptake into exponentially growing BHK21-C13 cells was examined at intervals following the addition of various concentrations of fresh foetal calf serum to these cells. The uptake of these amino acids was examined simultaneously by dual labelled uptake procedures (Section 2.8.4). Rates of transport were determined from 4 minute incubations and are the means of 6 samples, plus SEM.

L-alanine uptake (●-●) and L-glutamate uptake (■-■) following addition of 25% (v/v) serum.
L-alanine uptake (○-○) and L-glutamate uptake (□-□) following addition of 10% (v/v) serum.
L-alanine uptake (▲-▲) and L-glutamate uptake (△-△) following addition of 5% (v/v) serum.

Figure 6.2.4d. L-alanine and L-glutamate uptake in confluent BHK21-C13 cells following serum addition

L-[2,3 - ^{3}H] alanine ($2 \times 10^{-3}M$) and L-[U ^{14}C] glutamate ($2 \times 10^{-4}M$) confluent BHK21-C13 cells was examined at intervals following the addition of various concentrations of fresh foetal calf serum to these cells. The uptake of these amino acids was examined simultaneously by dual labelled uptake procedures (Section 2.8.4). Rates of transport were determined from 4 minute incubations and are the means of 6 samples, plus SEM.
L-alanine uptake (●-●) and L-glutamate uptake (■-■) following addition of 25% (v/v) s rum.
L-alanine uptake (○-○) and L-glutamate uptake (□-□) following addition of 10% (v/v) serum.
L-alanine uptake (▲-▲) and L-glutamate uptake (△-△) following addition of 5% (v/v) serum.

L-alanine uptake (▼-▼) and L-glutamate uptake (▽-▽) following addition of 2% (v/v) serum.

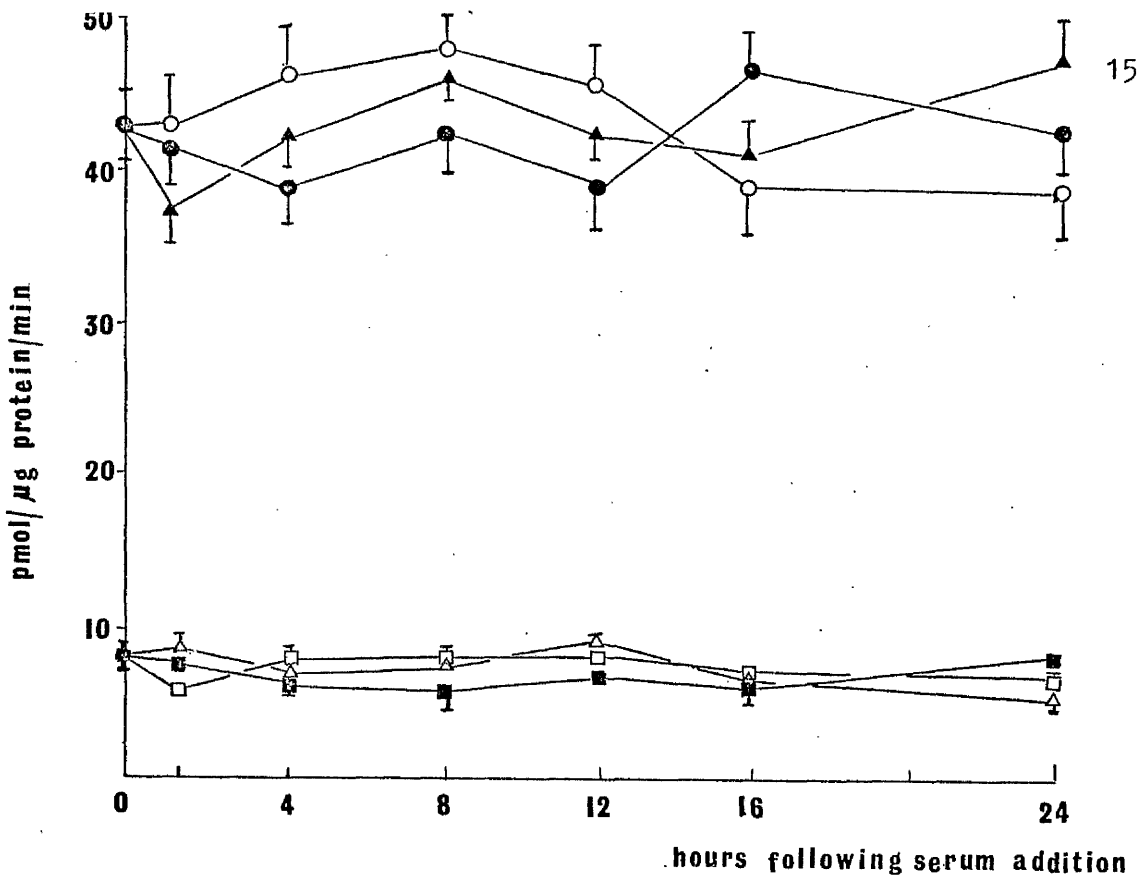


Figure 6.2.4c. L-alanine and L-glutamate uptake in exponentially growing BHK21-C13 cells following serum addition.

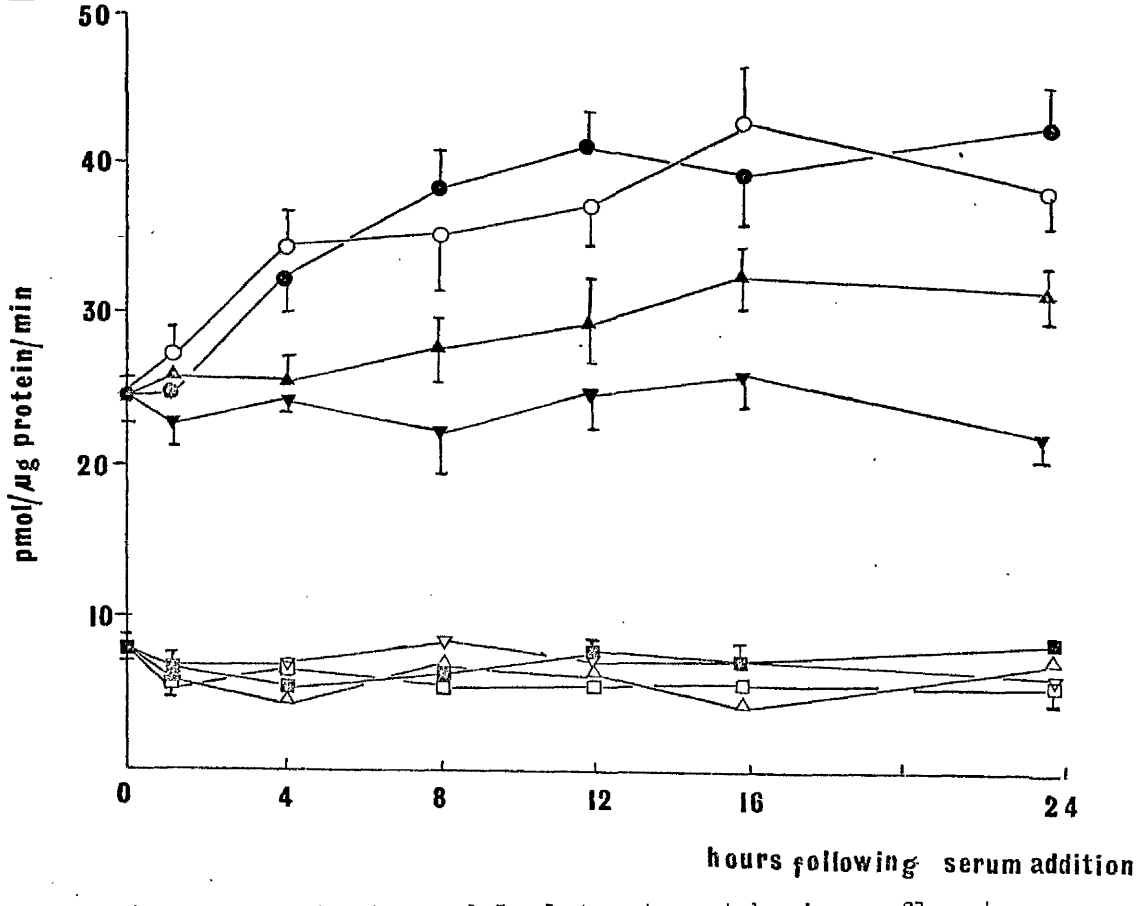


Figure 6.2.4d. L-alanine and L-glutamate uptake in confluent BHK21-C13 cells following serum addition.

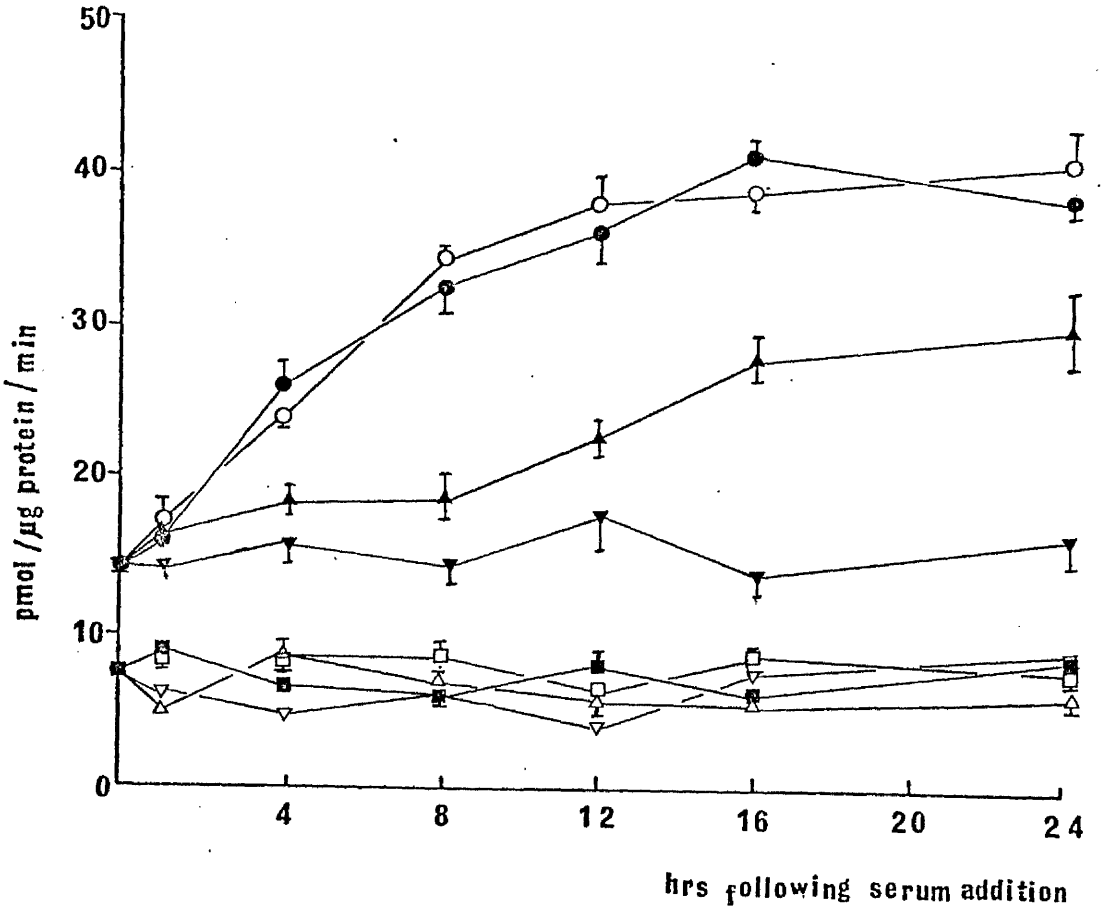


Figure 6.2.4e. L-alanine and L-glutamate uptake following serum addition to BHK21-C13 cells grown in low serum containing medium.

BHK21-C13 cells were grown for 48 hours in $EFC_{0.5}$.
L-[2,3 - ^{3}H]alanine ($2 \times 10^{-3}M$) and L-[U - ^{14}C] glutamate ($2 \times 10^{-4}M$) uptake was then examined at intervals following the addition of fresh foetal calf serum to these cells. The uptake of these amino acids was simultaneously examined by dual labelled uptake procedures (Section 2.8.4.). Rates of transport were determined from 4 minute incubations and are the means of 6 samples, plus SEM.

L-alanine uptake (●-●) and L-glutamate uptake (■-■) following addition of 25% (v/v) serum.
L-alanine uptake (○-○) and L-glutamate uptake (□-□) following addition of 10% (v/v) serum.
L-alanine uptake (▲-▲) and L-glutamate uptake (△-△) following addition of 5% (v/v) serum.
L-alanine uptake (▼-▼) and L-glutamate uptake (▽-▽) following addition of 2% (v/v) serum.

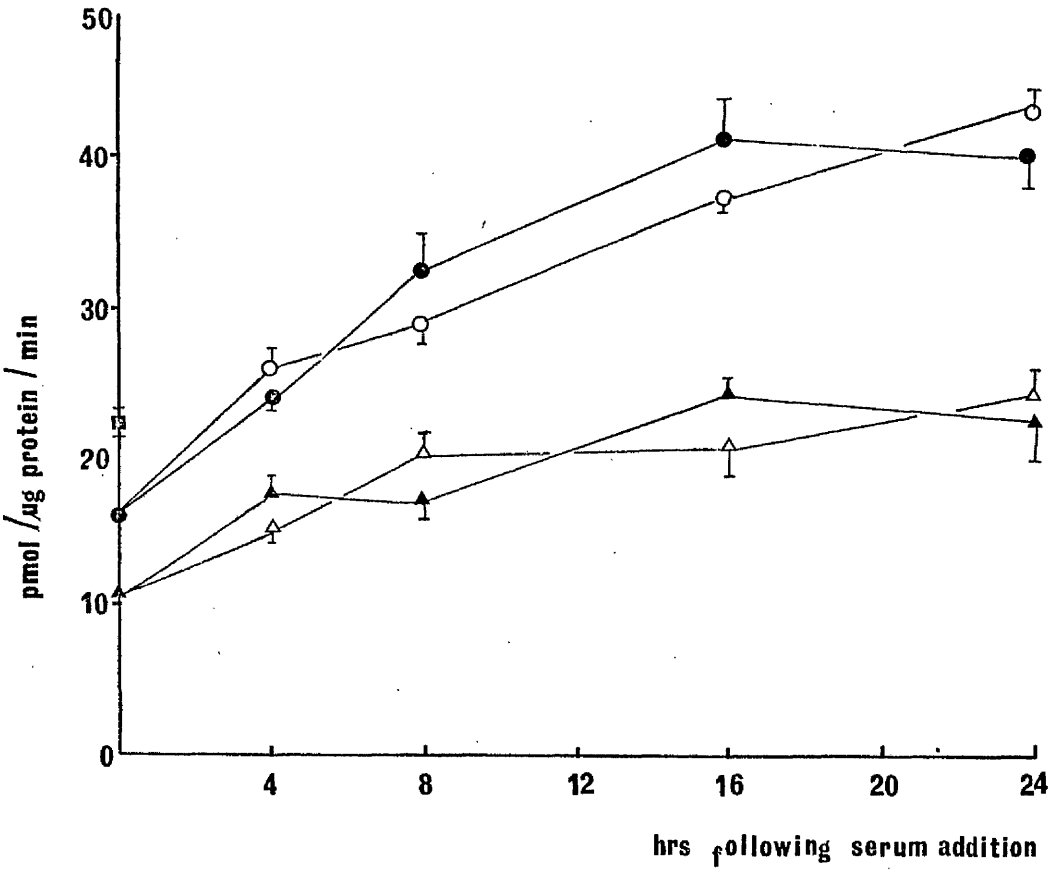


Figure 6.2.4f. L-alanine and ∝-AIB uptake following serum addition to BHK21-C13 cells grown in low serum containing medium

BHK21-C13 cells were grown for 72 hours in $EFC_{0.5}$. L-[2,3,-^{3}H] alanine and [G -^{3}H]∝-AIB uptake was examined at various intervals following the addition of 10% (v/v) foetal calf serum. Serum was added a) immediately following the above growth conditions, or b) following 45 minutes preincubation of cells in PBS medium (Section 2.1.8). Rates of transport were determined for 4 minute incubations and are the means of 8 samples, plus SEM.

L-alanine uptake in PBS pretreated cells (○-○)

L-alanine uptake in non-pretreated cells (●-●)

∝-AIB uptake in PBS pretreated cells (△-△)

∝-AIB uptake in PBS pretreated cells (▲-▲)

∝-AIB uptake in exponentially growing cells (■-■)

6.2.5. Effects of dibutyryl cyclic AMP on the growth of BHK21-C13 cells, and on the transport of L-alanine and L-glutamate in these cells

Dibutyryl cyclic AMP plus or minus theophylline was shown to alter the morphology and growth characteristics of BHK21-C13 cells. BHK21-C13 cells maintained in the presence of dibutyryl cyclic AMP became narrower and more elongated than untreated cultures (see Plates 6.2.5a and b). These cells initially showed a very reduced rate of growth and produced quiescent cultures within 48 hours (Figure 6.2.5a). Addition of theophylline alone similarly reduced the rate of growth and saturation density of these cells. All treated cells showed approximately 94 to 98% viability, as indicated by trypan blue staining. Following removal of dibutyryl cyclic AMP, cells regained their normal growth rate (Figure 6.2.5a) and morphological appearance.

L-alanine uptake was significantly reduced in cells grown in the presence of dibutyryl cyclic AMP (0.5 to 2 x 10^{-3}M), dibutyryl cyclic AMP (2 x 10^{-4}M) plus theophylline (1 x 10^{-3}M) or theophylline (1mM) alone. The reduced rate of transport observed following growth in the presence of dibutyryl cyclic AMP (1 to 2 mM) alone and in the presence of theophylline was similar to that observed for cells grown in low serum (0.5% v/v), Section 6.2.4b. L-alanine uptake was not significantly reduced by butyric acid (1 x 10^{-3}M) or 5'-AMP (1 x 10^{-3}M), indicating that the observed effect produced by dibutyryl cyclic AMP was not due to butyric acid release, or degradation to 5'-AMP. L-glutamate uptake was not significantly reduced by these treatments. This data is summarised in Table 6.2.5a.

L-alanine uptake examined in the presence of dibutyryl cyclic AMP (0.2 x 10^{-4}M) and theophylline (1 x 10^{-4}M) is likely to still be essentially via the L-alanine preferring transport system as, a) inhibition of L-alanine uptake by other amino acids was similar to that observed in

Plate 6.2.5a. BHK21-C13 cells grown in EFC_{10}

Photograph of BHK21-C13 cells following growth for 48 hours at $37^{o}C$ in EFC_{10} medium.

Plate 6.2.5b. BHK21-C13 cells grown in EFC_{10} plus dibutyryl cyclic AMP and theophylline

Photograph of BHK21-C13 cells following growth for 48 hours at $37^{o}C$ in EFC_{10} medium plus dibutyryl cyclic AMP ($.2 \times 10^{-3}M$) and theophylline ($1 \times 10^{-3}M$).

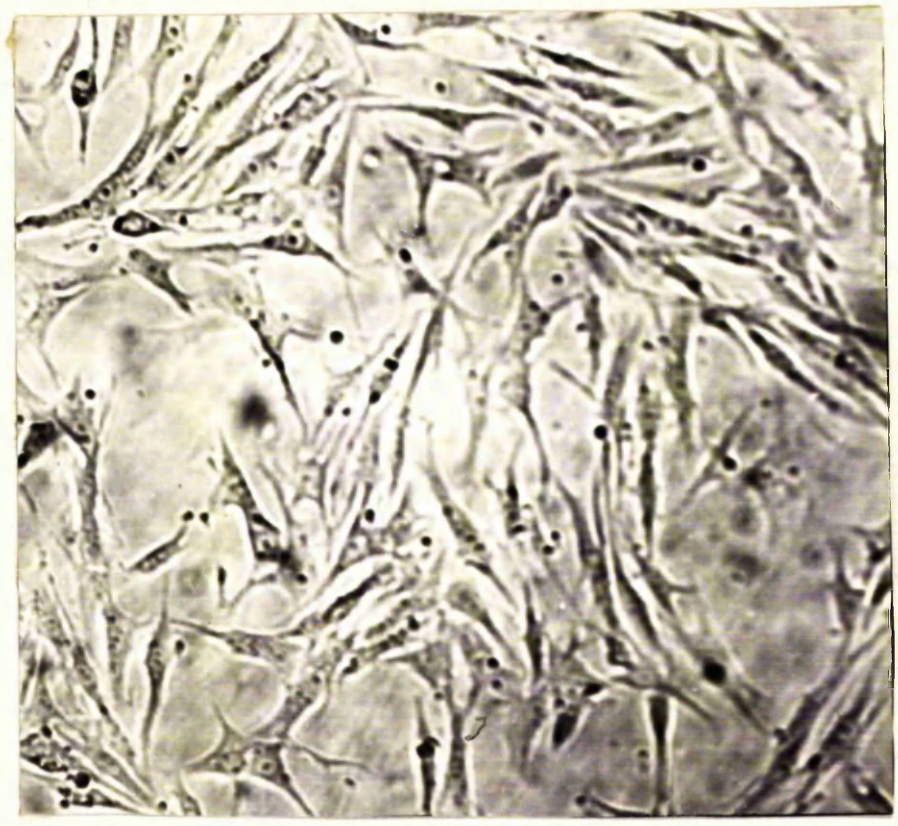


Plate 6.2.5a. BHK21-C13 cells grown in EFC_{10}

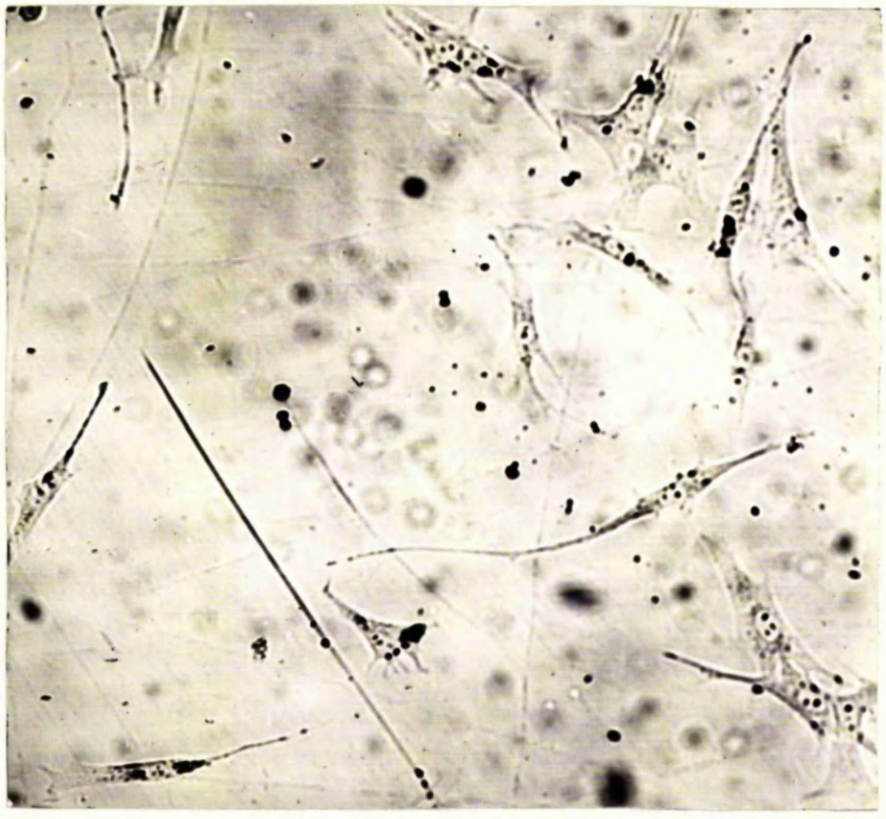


Plate 6.2.5b. BHK21-C13 cells grown in EFC_{10} plus dibutyryl cyclic AMP and theophylline

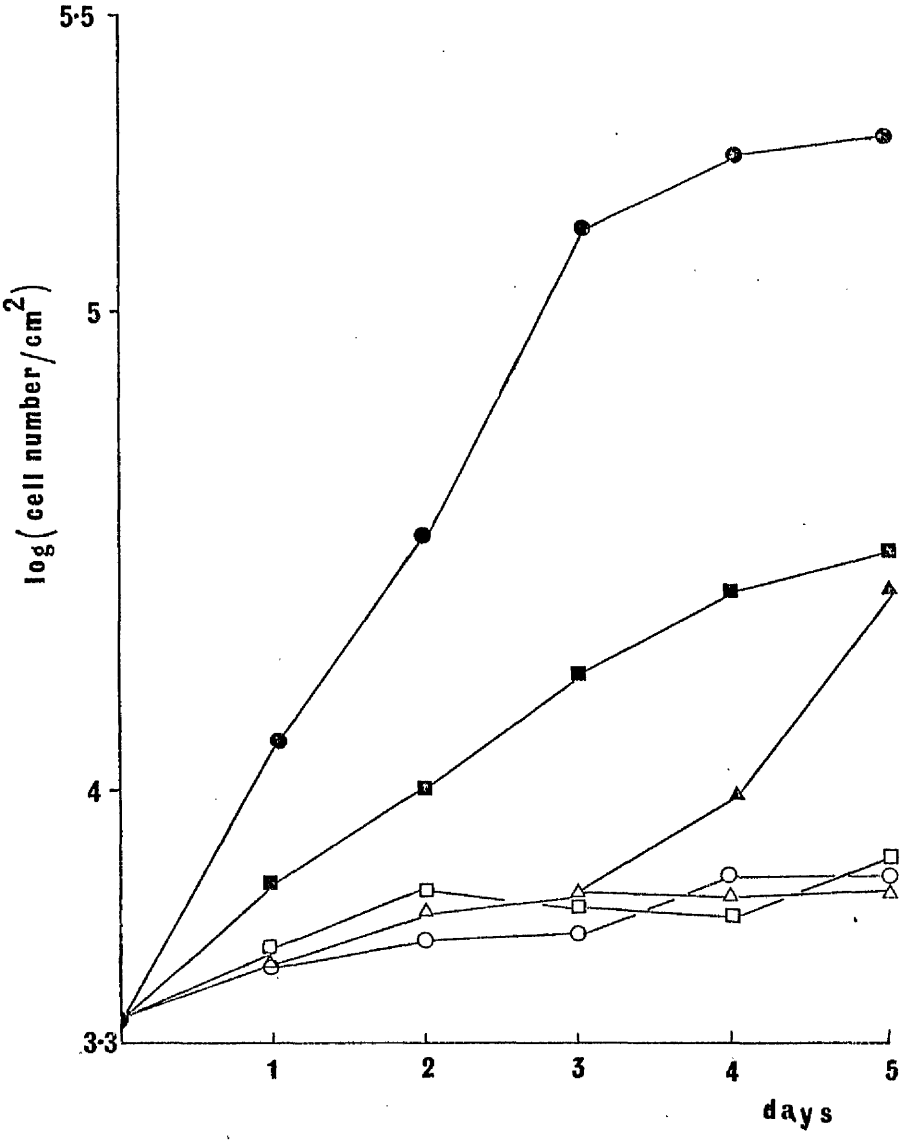


Figure 6.2.5a. Effect of dibutyryl cyclic AMP, plus or minus theophylline, and theophylline alone on the growth of BHK21-C13 cells.

Cells were inoculated at a density of 3.5×10^3 cells/cm^2 in 6.0 cm petri dishes, and grown under standard conditions (Section 2.2.), except for the medium modifications indicated below. Medium was changed every 48 hours. Cells were harvested and counted at intervals. Each value is the mean of duplicate cultures.

Cells maintained in EFC_{10} (●-●)

Cells maintained in EFC_{10} + theophylline (1mM) (■-■)

Cells maintained in EFC_{10} + dibutyryl cyclic AMP (0.2mM) and theophylline (1mM) (○-○)

Cells maintained in EFC_{10} + dibutyryl cyclic AMP (1mM) (△-△)

Cells maintained in EFC_{10} + dibutyryl cyclic AMP (2mM) (□-□)

Cells following removal of dibutyryl cyclic AMP (1mM) (▲-▲)

Analysis of variance of data for L-alanine and L-glutamate uptake in BHK21-C13 cells following treatment with dibutyryl cyclic AMP and related compounds.

L-glutamate uptake : $F_{8,120} = 1.57$, $P > 0.05$ ($F = 2.01$)

L-alanine uptake : $F_{8,120} = 14.96$, $P < 0.05$ ($F = 2.01$)

Difference between means of L-alanine uptake

Growth conditions			L.S.D.	Difference between Means
EFC_{10} versus :				
EFC_{10} plus dibutyryl cyclic AMP	(0.2mM)		11.66	1.2
" " " "	(0.5mM)		10.12	16.8*
" " " "	(1.0mM)		8.43	16.0*
" " " "	(2.0mM)		11.20	28.3*
" " " "	(0.2mM)			
plus theophylline	(1.0mM)		8.55	27.6*
" theophylline	(1.0mM)		10.86	12.2*
" 5'-AMP	(1.0mM)		10.86	6.0
" Butyric acid	(1.0mM)		10.86	2.3
EFC_{10} plus dibutyryl cyclic AMP	(0.2mM)	versus:		
EFC_{10} " " " "	(1.0mM)		11.97	16.8*
" " " "	(2.0mM)		14.49	27.1*
" " " "	(0.2mM)			
plus theophylline	(1.0mM)		12.07	26.5*
EFC_{10} plus dibutyryl cyclic AMP	(0.5mM)	versus:		
EFC_{10} " " " "	(1.0mM)		10.56	9.2
" " " "	(2.0mM)		13.10	4.4
" " " "	(0.2mM)			
plus theophylline	(1.0mM)		10.65	10.8*
EFC_{10} plus dibutyryl cyclic AMP	(1.0mM)	versus:		
EFC_{10} " " " "	(2.0mM)		11.55	2.3
" " " "	(0.2mM)			
plus theophylline	(1.0mM)		8.98	1.6
EFC_{10} plus dibutyryl cyclic AMP	(2.0mM)	versus:		
EFC_{10} " " " "	(0.2mM)		11.52	0.7

* Indicates difference between the means is significant at the P = 0.05 level of significance.

L.S.D. is the least significant difference.

Table 6.2.5a. Effect of dibutyryl cyclic AMP, plus or minus theophylline, and related compounds on L-alanine and L-glutamate uptake in BHK21-C13 cells.

Growth Conditions	L-alanine uptake (pmol/μg protein/min)	L-glutamate uptake (pmol/μg protein/min)
EFC_{10}	41.2 ± 1.8 (28)	7.5 ± 0.4 (28)
EFC_{10} + dibutyryl cyclic AMP (0.2mM)	40.0 - 3.0 (9)	7.0 - 0.6 (9)
EFC_{10} + dibutyryl cyclic AMP (0.5mM)	24.4 ± 1.3 (13)	5.9 ± 0.3 (13)
EFC_{10} + dibutyryl cyclic AMP (1.0mM)	15.2 ± 1.3 (23)	6.5 ± 0.4 (23)
EFC_{10} + dibutyryl cyclic AMP (2.0mM)	12.9 ± 0.7 (10)	5.5 ± 0.4 (10)
EFC_{10} + dibutyryl cyclic AMP (0.2mM) + theophylline (1.0mM)	13.6 ± 0.7 (22)	6.2 ± 0.4 (22)
EFC_{10} + theophylline (1.0mM)	29.0 ± 1.9 (8)	6.6 ± 0.6 (8)
5'-AMP (1.0mM)	35.2 ± 3.1 (8)	6.9 ± 0.8 (8)
Butyric acid (1.0mM)	43.5 ± 2.4 (8)	7.2 ± 0.8 (8)

BHK21-C13 cells were grown for 48 hours in the above media and the uptake of L-[2,3,-^{3}H] alanine (2 x 10^{-3}M) and L-[U-^{14}C] glutamate susequently examined. Rates of uptake were determined from 4 minute incubations. The mean values and their SEM are given with the number of independent estimations in parenthesis.

Table 6.2.5b. Inhibition of L-alanine uptake in BHK21-C13 cells following growth in medium containing dibutyryl cyclic AMP and theophylline

Inhibiting Amino Acid	Percentage Inhibition of L-[2,3,-^{3}H] alanine uptake
L-alanine	75
L-serine	77
L-leucine	31
L-valine	39
L-methionine	64
L-proline	54
L-phenylalanine	23

Uptake of L-[2,3,-^{3}H] alanine (1×10^{-4}M) was examined in the presence and absence of inhibiting amino acid (2.5×10^{-3}M). Rates of uptake were determined from 4 minute incubations. Values are the means of 6 samples and results are expressed as percentage inhibition of L-[2,3,-^{3}H] alanine uptake alone.

Figure 6.2.5b. S/v versus S plot of L-alanine uptake into BHK21-C13 cells following growth in medium containing dibutyryl cyclic AMP plus theophylline

BHK21-C13 cells were grown for 48 hours in EFC_{10} containing dibutyryl cyclic AMP (0.2×10^{-3}M) plus theophylline (1×10^{-3}M). The uptake of L-[2,3 - ^{3}H] alanine uptake into these cells was then examined for the concentration range 1×10^{-5}M to 4×10^{-3}M. The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations of up to 4 minutes.

S/v values for concentrations 0.5×10^{-3}M and below are shown on an expanded scale.

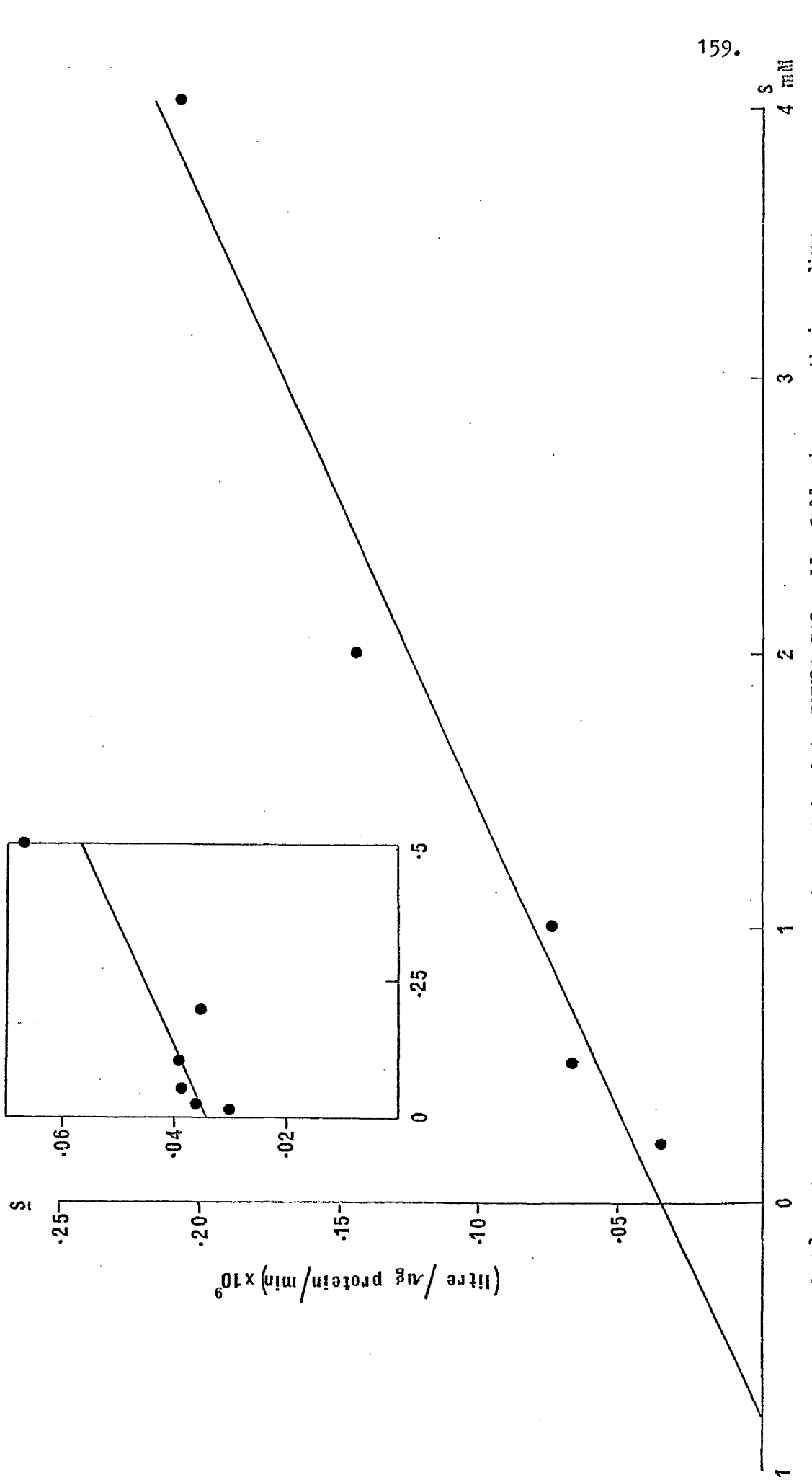


Figure 6.2.5 b. S/v versus S plot of L-alanine uptake into BHK21-C13 cells following growth in medium containing dibutyryl cyclic AMP plus theophylline.

exponentially growing cells (Table 6.2.5b); and b) the Km $1.05 \pm 0.15 \times 10^{-3}$M) for its uptake was not significantly different ($z = 1.11$, $P > 0.05$) from cells maintained under normal growth conditions. The Vmax for L-alanine uptake, however was reduced by approximately 59% to 23.0 ± 2.1 pmol/μg protein/min ($z = 7.53$, $P < 0.05$). The s/v versus s plot for L-alanine uptake following growth of cells in the presence of dibutyryl cyclic AMP (0.2 mM) and theophylline (1.0 mM) is shown in Figure 6.2.5b.

6.2.6. Cyclic AMP levels in BHK21-C13 cells

Cyclic AMP levels were determined in BHK21-C13 cells at different densities, or following growth conditions or treatments shown to alter L-alanine transport. The values for these cyclic AMP levels are shown in Table 6.2.6. Cyclic AMP levels in BHK21-C13 cells were lowest during exponential growth and were increased 1.9 and 2.8 times respectively in confluent and serum arrested cells. Treatment of cells with theophylline, an inhibitor of the phospho-diesterase which converts cyclic AMP to 5'-AMP, also increased cyclic AMP levels. High cyclic AMP levels observed in serum arrested cells were reduced by the addition of insulin (1.3 mM) or fresh serum containing medium to these cells. Cyclic AMP levels were also reduced in confluent cells to which fresh serum containing medium had been added. L-alanine transport activity was thus high in BHK21-C13 cells with low intracellular cyclic AMP levels and reduced in cells with elevated levels of this cyclic nucleotide. This relationship between intracellular cyclic AMP levels and L-alanine transport suggests that cyclic AMP may be involved in the regulation of the L-alanine preferring transport system. As little difference between the rates of L-glutamate uptake are observed in cells with high or low cyclic AMP levels, it is unlikely this cyclic nucleotide is important in the regulation of the acidic amino acid transport system.

Analysis of variance of data for cyclic AMP levels in BHK21-C13 cells.

$F_{5,24} = 14.78$, $P < 0.05$, ($F = 2.62$).

Difference between means of cyclic AMP levels for different cell or growth conditions.

Samples		L.S.D.	Difference between Means
Non-confluent versus	confluent	2.56	4.6*
" " "	serum arrested	2.56	8.9*
" " "	non-confluent + theophylline	2.96	5.5*
" " "	serum arrested + serum	2.96	0.8
" " "	serum arrested + insulin	2.96	3.2*
Confluent "	serum arrested	2.56	4.3*
" "	non-confluent + theophylline	2.96	0.9
" "	serum arrested + serum	2.96	3.8*
" "	serum arrested + insulin	2.96	0.6
Serum arrested "	non-confluent + theophylline	3.45	3.4
" " "	serum arrested + serum	3.45	8.1*
" " "	serum arrested + insulin	3.45	5.7*
Non-confluent + theophylline "	serum arrested + serum	3.45	4.7*
" "	serum arrested + insulin	3.45	2.3
Serum arrested + serum "	serum arrested + insulin	3.45	2.4

* Indicates difference between the means is significant at the P = 0.05 level of significance.

L.S.D. is the least significant difference.

Table 6.2.6. Cyclic AMP levels in BHK21-C13 cells

Cell Condition or treatment	Cyclic AMP Level (pmol/mg protein)
Non-confluent (Exponential phase)	5.1 ± 0.5 (6)
Confluent	9.7 ± 0.7 (6)
Serum arrested	14.0 ± 0.9 (6)
Non-confluent + theophylline (1×10^{-3}M)	10.6 ± 1.8 (4)
Serum arrested, 4 hours following serum addition (10%, v/v)	5.9 ± 1.3 (4)
Serum arrested, 4 hours following addition of Insulin (1.3mM)	8.3 ± 1.8 (4)

Cyclic AMP levels were determined in the above treated cells following TCA extraction, see Section 2.10. The mean values and SEM are given with the number of independent estimations in parenthesis.

6.3. DISCUSSION AND CONCLUSIONS

One of the most important groups of compounds transported by active transport systems are amino acids, but although considerable progress has been made in determining the specificity of amino acid transport systems, very little is known about the mechanisms involved in their regulation.

A number of reports have shown that microbial amino acid transport systems may be inhibited by high intracellular levels of substrate (Crabeel and Grenson 1970; Pall 1971) or non-substrate amino acids (Ring et al. 1970). Relatively few reports of reductions in amino acid transport have been made following preloading of mammalian cells with amino acids (Paine and Heinz 1960; Jacquez 1961). It has been suggested however, that these reductions in transport are not due to transinhibition but due to competitive inhibition of uptake by preloaded amino acid, which had leaked out of the cells (Paine and Heinz 1960; Heinz 1972). No evidence for transinhibition of the acidic or L-alanine transport systems of BHK21-C13 cells was provided from this study (Section 6.2.1). Furthermore the absence of stimulation of transport following preincubation of BHK21-C13 cells in medium containing high concentrations of substrate amino acids would indicate that exchange (counterflow) is unimportant in the transport of the major substrates of these amino acid transport systems. Oxender and Christensen (1963) have similarly observed that the 'A'-system of Ehrlich ascites tumour cells operates minimally, if at all by exchange, although the 'L'-system operates freely by exchange.

Amino acid transport systems have also been reported to be regulated by control mechanisms effective on protein synthesis (Pall 1971; Pateman et al. 1974; Robinson et al. 1973a and b, Gazzola et al. 1972 Franchi-Gazzola et al. 1973). Removal of substrate or other amino acids did not produce any increase in activity of either the acidic or L-alanine

preferring transport systems of BHK21-C13. These results differed from similar studies with the 'A'-system of chick embryo heart cells (a system of similar specificity to the alanine preferring transport systems of BHK21-C13 and Ehrlich ascites tumour cells), which showed a protein and RNA synthesis dependent increase in activity up to eight hours following amino acid removal (Gazzola et al. 1972; Franchi-Gazzola et al. 1973).

Insulin has been shown to increase the transport of ∝-AIB and certain neutral amino acids in a wide variety of tissues including diaphragm muscle (Wool et al. 1965), foetal membranous bone (Hahn et al. 1969), rat liver (Tews et al. 1970) and monolayer cultures of Reuber hepatoma cells (Krawitt et al. 1970). Although insulin did not alter L-glutamate or L-alanine uptake in BHK21-C13 cells grown in medium containing 10% (v/v) serum, L-alanine transport was increased in cells maintained in 0.5% (v/v) serum. The observed decrease in intracellular cyclic AMP levels obtained following addition of insulin to serum arrested cells would indicate that this increased uptake may have been mediated by reduced cyclic AMP levels. Insulin has also been shown to mediate other effects via decreased cyclic AMP levels, e.g. the antilipolytic action on adipose tissue, although activities such as increased glucose transport have been shown to be independent of altered cyclic AMP levels (Butcher et al. 1972). As insulin treatment also produced some increase in growth of serum arrested cells, it is possible that the increased L-alanine transport is not a primary response to the hormone but may be secondary to the ability of insulin partially to replace serum as a cell growth requirement (De Asua et al. 1973).

Growth hormone (Riggs and Walker 1960) and epinephrine (Sanders and Riggs 1967) have been shown to increase ∝-AIB transport in a number of tissues, whereas T_3 and cortisol have been shown to increase uptake

in some tissues but reduce it in others (reviewed by Riggs, 1970). However, none of these hormones affected the uptake of L-glutamic acid or L-alanine by BHK21-C13 cells, even at levels greatly above physiological concentrations. This may be due to the presence in serum of unusually high levels of specific binding proteins, which are thought to be involved in hormone distribution and function *in vivo* (reviewed by Westphal, 1970). Alternatively it may reflect a) a lack of hormonal control over L-glutamate or L-alanine transport at the organism level; b) the absence of hormone-specific receptor sites from the tissue, from which the BHK21-C13 cell line is derived; or c) the loss of such receptor sites in the process of adaptation of cells to growth in culture.

The effects of serum concentration on the growth of BHK21-C13 cells and L-alanine transport were similar to those observed in other cell lines. Cells grown in medium containing high concentrations of serum have been shown to grow to far greater densities than when grown in medium containing the normal 10% (v/v) serum concentration (Todaro *et al.* 1965; Holley and Kiernan 1968; Foster and Pardee 1969). However, when maintained in the presence of low levels of serum the cells rapidly cease growth and produce "quiescent" cultures. (Burk 1970, Shodell and Isselbacher 1973; Todaro *et al.* 1965, Oey *et al.* 1974). L-alanine uptake in BHK21-C13 cells (Section 6.2.4b) was demonstrated to be lower in confluent cultures maintained in 10% serum than in exponentially growing cultures, but showed no reduction in confluent cells grown in higher serum concentrations. A similar observation has been made for α-AIB uptake in 3T3 cells (Foster and Pardee 1969). The transport of L-alanine (Section 6.2.4b) and α-AIB (Section 6.2.4c) in BHK21-C13 cells grown in low serum exhibited reduced transport rates compared to exponentially growing cells. Complete serum deprivation (18 hours) has also been shown to reduce the quantity of leucine, thymidine and uridine taken into 3T3 cells (Herschko *et al.* 1971). As the cells were pulsed with labelled metabolite for 60 minutes,

these results reflect the quantity of compound taken into the cells, but not initial rates of transport. Isselbacher (1972), however, reported no reduction in α-AIB uptake in BHK21-C13 cells grown in 0.5% serum. Possible reasons for the difference in response of these cells and the BHK21-C13 cells used in the studies in this thesis are discussed later.

The addition of fresh serum, or serum containing medium to confluent, or serum arrested cells has been shown rapidly to stimulate the uptake of various low molecular weight metabolites, such as nucleotides (Cunningham and Pardee 1969), sugars (Cunningham and Pardee 1969; Sefton and Rubin 1971) and phosphate ions (Cunningham and Pardee 1969; Blat et al. 1973). Although amino acid uptake in confluent or quiescent cell cultures has been reported to be stimulated by serum addition, these increases are less rapid than for the above compounds and the responses are more varied. In this study (Section 6.2.4c) L-alanine but not L-glutamate uptake was shown to be increased by serum addition to confluent or serum arrested BHK21-C13 cells. The observed increase in L-alanine uptake following serum addition to confluent BHK21-C13 cells was similar to that obtained for cycloleucine in confluent 2RA and 3T6 cells but different to that for WI-38 cells, during the 6 hour period over which this stimulation was examined (Costlow and Baserga 1974). It should be noted that little difference in the transport rates of cycloleucine were observed between confluent or non-confluent cells, although this may be due to cycloleucine being mainly transported by a system other than the L-alanine preferring transport system, as this amino acid analogue appeared to have little affinity for this system in BHK21-C13 cells (Section 5.2.7). Shodell and Isselbacher (1973) have examined the effect of serum addition on uptake of α-AIB (shown in Section 5.2.7 to be a substrate of the L-alanine

preferring transport system) and cycloleucine in serum arrested BHK21-C13 cells. The observed stimulation of transport however, differed from that described for L-alanine in Section 6.2.4c. of this thesis. Shodell and Isselbacher (1973) reported that the increase in uptake was proportional to the added serum concentration (1-10%) and reached a 'plateau' level, 5.5 to 6 times higher than that of unstimulated cells by about 4 hours. The results described in Section 6.2.4c, however, show no stimulation of L-alanine uptake by serum at concentrations of less than 5% and no difference in stimulation between 10% and 25% serum. Maximum levels of L-alanine uptake, similar to those observed in exponentially growing BHK21-C13 cells and equivalent to an approximately three-fold increase, were not attained until about 16 to 24 hours following serum addition. Similar responses were obtained for α-AIB uptake (Section 6.2.4c). The reason for the differences in the response of BHK21-C13 cells in these studies and those of Shodell and Isselbacher is uncertain. It is unlikely, however, that these differences are merely due to differences in experimental procedures as preincubation of cells in PBS (as per Shodell and Isselbacher) did not alter the transport responses of the studied amino acids. As Isselbacher (1972) observed no reduction in α-AIB uptake in BHK21-C13 cells grown in 0.5% (v/v) serum it is possible that the differences in response may be a reflection of physiological differences in the BHK21-C13 cell lines used. (Pitts 1970). Comparisons with the Shodell and Isselbacher (1973) paper are, however, difficult to make as considerable differences in α-AIB transport rates (approximately 500 fold) are observed between this paper and a previous study by Isselbacher (1972).

Dibutyryl cyclic AMP has been demonstrated to produce alterations in the morphology (Hsie and Puck 1971; Johnson et al. 1971; Gazdar

et al. 1972; Johnson and Pastan 1972), and reduce population doubling times and saturation densities (Johnson and Pastan 1972; Sheppard 1971, Blat et al. 1973) of a number of normal and transformed cell lines and BHK21-C13 cells. Treatment of cells with cyclic AMP or its dibutyryl derivative has also been reported to alter the transport of certain low molecular weight metabolites. Increased transport of sugars (Gazdar et al. 1972) and nucleotides (Roller et al. 1974; Hare 1972) have been reported in some cells following addition of cyclic AMP or dibutyryl cyclic AMP, but decreased uptake of these metabolites have been reported in others (Grimes and Schroeder 1973; Hauschka et al. 1972; Kram and Tomkins 1973). BHK21-C13 cells grown in the presence of dibutyryl cyclic AMP alone or plus theophylline were shown in this thesis to exhibit reduced rates of L-alanine uptake (Section 6.2.6), although L-glutamate uptake was apparently unaltered. Uptake of α-AIB and L-glutamine (both apparently substrates of the BHK21-C13 cells) have also been shown to be reduced in CHO cells following growth in the presence of dibutyryl cyclic AMP and theophylline. As dibutyryl cyclic AMP has been used to demonstrate the regulatory functions of cyclic AMP in a number of systems (Robinson et al. 1970; Paston and Perlman 1971) it would indicate that cyclic AMP may be involved in the regulation of the L-alanine preferring transport system of BHK21-C13 cells (and possibly that of CHO cells). As L-glutamate is essentially unaltered by dibutyryl cyclic AMP treatment, it would seem unlikely that this transport system is similarly controlled.

Cyclic AMP levels in BHK21-C13 cells, reported in Section 6.2.6, showed similar alterations to those observed in other cell lines. Cyclic AMP levels are at their lowest levels in exponentially growing

cells, are increased in confluent (Otten et al. 1972b, Oey et al. 1974; Heindrick and Ryan 1974) or serum arrested cultures (Oey et al. 1974; Otten et al. 1972a) but may be reduced by the addition of serum (Burger et al. 1972; Otten et al. 1972b; Sheppard 1971; Froehlich and Rachmelar 1972) or insulin (Otten et al. 1972a).

The activity of the L-alanine preferring transport system (as indicated by L-alanine uptake, thus strongly correlates with intracellular cyclic AMP levels of BHK21-C13 cells:

a) maximal activity is observed in cells with low cyclic AMP levels (exponentially growing cultures);

b) minimal activity is observed in cells with high cyclic AMP levels (confluent or serum arrested cells);

c) reduced activity is observed in cells with intermediate cyclic AMP levels (confluent or theophylline treated cells)

d) the activity is increased following treatments shown to decrease cyclic AMP levels (the addition of serum to serum arrested or confluent cells, or insulin to serum arrested cells).

This correlation between cyclic AMP levels and L-alanine preferring transport system activity in BHK21-C13 cells provides strong evidence for the hypothesis that this transport may be regulated by cyclic AMP levels.

SECTION 7. L-GLUTAMATE AND L-ALANINE UPTAKE IN VARIOUS CELL LINES OF DIFFERENT TISSUE AND ANIMAL ORIGIN

7.1. INTRODUCTION

Early studies have shown quantitative differences in the ability of certain mammalian tissues to accumulate amino acids *in vivo* (Kamin and Handler 1951; Lajtha 1958; Chirigos et al. 1960) and *in vitro* (Neame 1962, Vidavar *et al.* 1964). Of the tissues examined those containing rapidly dividing cells or those specialised to transport or conserve amino acids generally showed a greater capacity for accumulation.

It has been suggested (Holley 1972) that growth of mammalian cells may be directly regulated by intracellular concentrations of "low molecular weight nutrients" which in turn would be regulated by their transport systems. Alterations in intracellular levels of these nutrients, caused by changes in uptake mechanisms, are postulated as the primary cause of malignancy. Support for such a hypothesis is provided by alterations in the transport abilities of certain hexose sugars in transformed cells (Bose and Zlotnick 1973; Isselbacher 1972; Hatanaka et al. 1969, 1970, a,b).

In this chapter possible quantitative differences in the uptake of L-alanine and L-glutamate are investigated in a number of cell lines derived from different tissues of mouse, sheep, hamster or human origin. The transport of these amino acids is also examined in certain of these cell lines following transformation by DNA tumour viruses, in an attempt to clarify the conflicting reports regarding possible alterations in amino acid uptake in transformed cells (Eagle, Piez and Levy 1972; Hare 1967; Foster and Pardee 1969; Isselbacher 1972).

The effects on L-alanine and L-glutamate uptake of growth of cells in the presence of dibutyryl cyclic AMP, or conditions shown to

increase cyclic AMP levels in BHK21-C13 cells, were also investigated in a number of "normal" and virus transformed cell lines.

7.2. RESULTS

7.2.1. Kinetics of L-glutamate and L-alanine uptake in "normal" and virus transformed cell lines

The effect of concentration on the initial rates of uptake of L-alanine and L-glutamate were examined in a number of "normal" (i.e. non-transformed diploid or established) cell lines derived from different tissues from human embryo, sheep, mouse, and syrian or chinese hamster origins. The transport of these amino acids were also examined in a number of Polyoma or Simian Virus-40 (SV-40) transformed cell lines, derived from certain of the above "normal" lines.

Initial rates of uptake for L-[U-^{14}C] glutamate (for concentrations between .5 x 10^{-5}M and 1 x 10^{-3}M) and L-[2,3-^{3}H] alanine (for concentrations between 1 x 10^{-5}M and 4 x 10^{-3}M) uptake were determined over 10 and 4 minute period respectively, during which time uptake was linear for the concentrations of amino acids studied. Plots of S/v versus S were constructed from the data obtained for the transport of these amino acids in each of the cell lines examined. These plots were linear, indicating that the transport of these amino acids appeared to follow Michaelis-Menten kinetics, an observation not inconsistent with the hypothesis that each of these amino acids are transported by single transport systems. Representative plots for L-glutamate and L-alanine uptake respectively, are shown in Figures 7.2.1a and b, and 7.2.1c and d. The statistically determined estimates of Km and Vmax for the uptake of these amino acids in all examined cell lines are shown in Tables 7.2.1a and 7.2.1b. Kinetic constants plus

Figures 7.2.1a and b. S/v versus S plots of L-glutamate uptake in CH Ki1 and SV-CH Ki1 cells, respectively

Uptake of L-[U - ^{14}C] glutamate into CH Ki1 and SV-CH Ki1 cells was examined for the concentration range 0.5 x 10^{-5}M to 1 x 10^{-3}M. The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations of up to 10 minutes.

S/v values for concentrations 0.1 x 10^{-3}M and below are shown on an expanded scale.

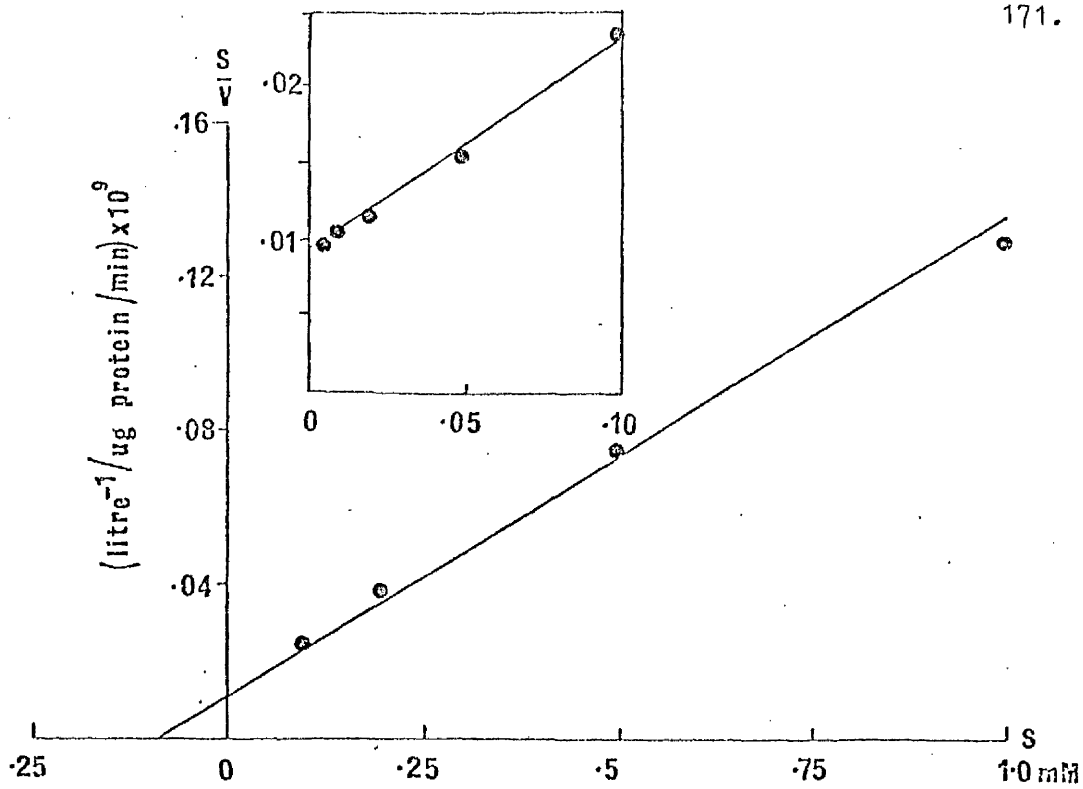


Figure 7.2.1a. S/v versus S plot for L-glutamate uptake into CH Ki1 cells.

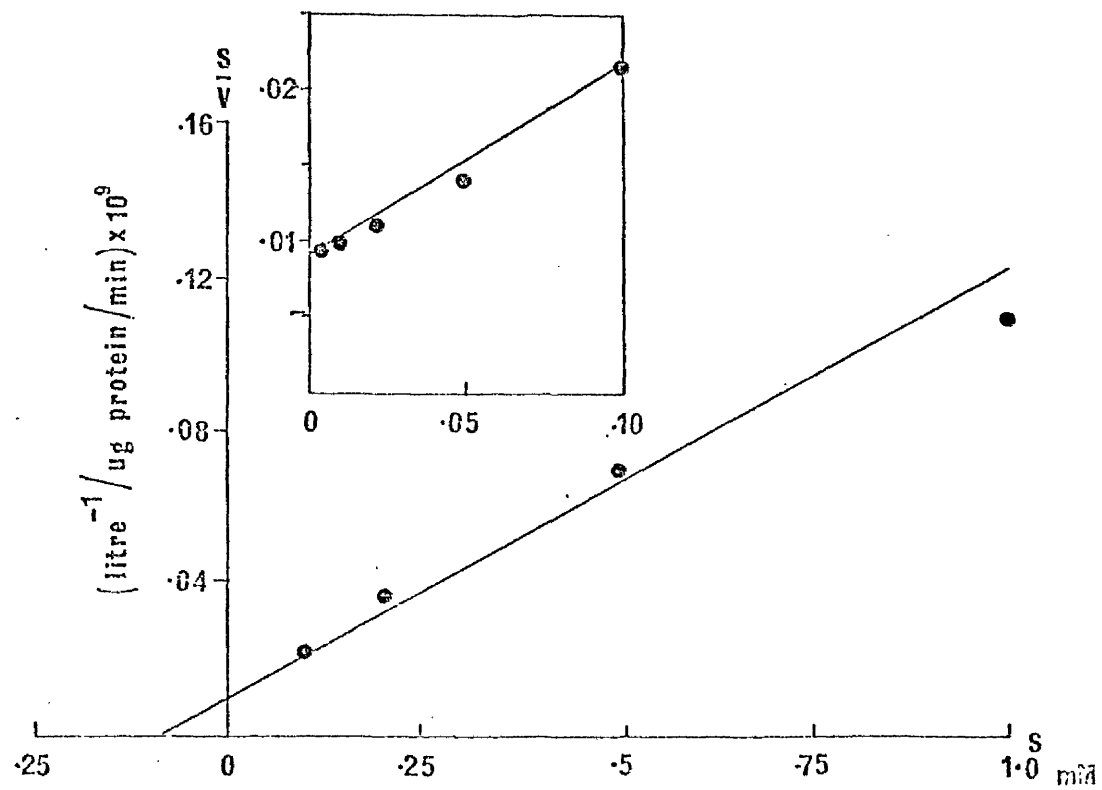


Figure 7.2.1b. S/v versus S plot for L-glutamate uptake into SV-CH Ki1 cells.

Figures 7.2.1c and d. S/v versus S plots of L-alanine uptake in CH Ki1 and SV-CH Ki1 cells, respectively

Uptake of L-[2,3 - ^{3}H] alanine into CH Ki1 and SV-CH K1 cells was examined for the concentration range 1 x 10^{-5}M to 4 x 10^{-3}M. The assay procedure was carried out as described in Section 2.8, and rates of transport were based on incubations of up to 4 minutes. S/v values for concentrations 0.5 x 10^{-3}M and below are shown on an expanded scale.

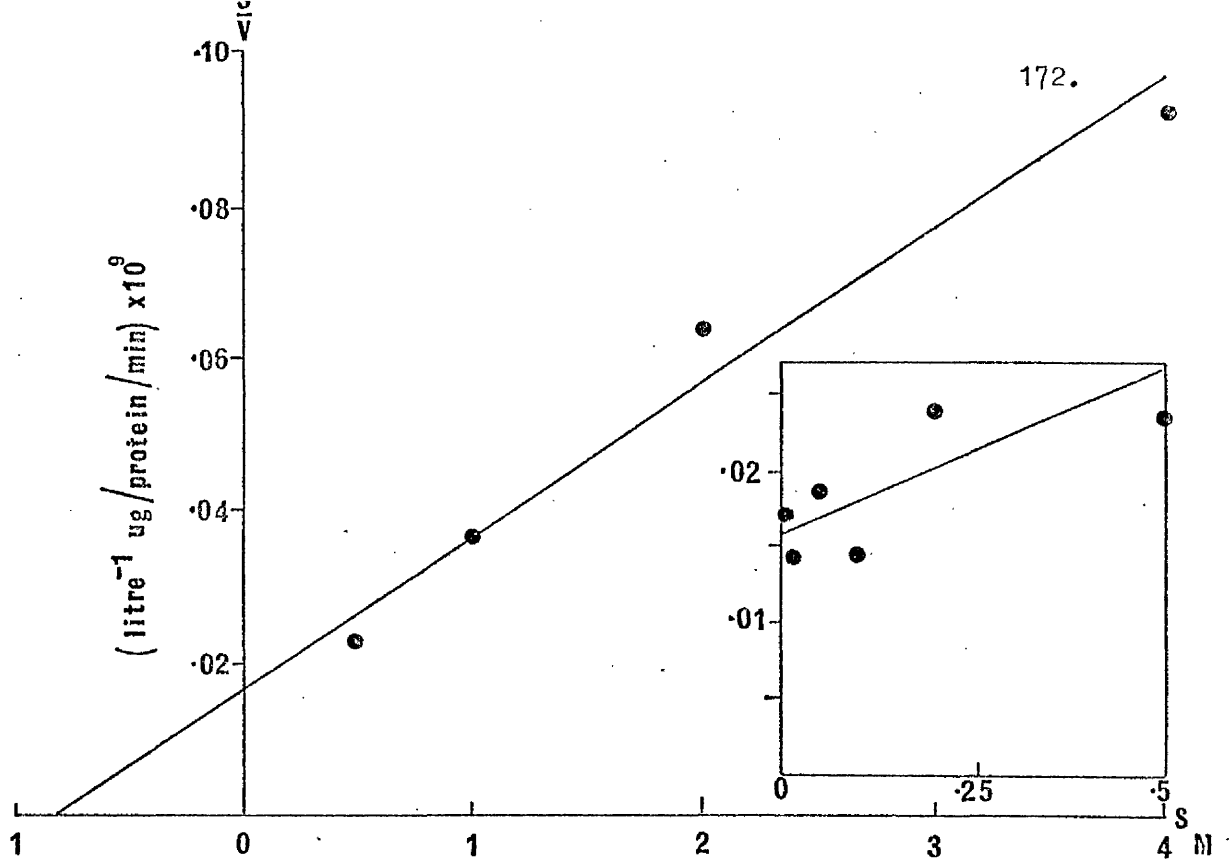


Figure 7.2.1c. S/v versus S plot of L-alanine uptake in CH Ki1 cells

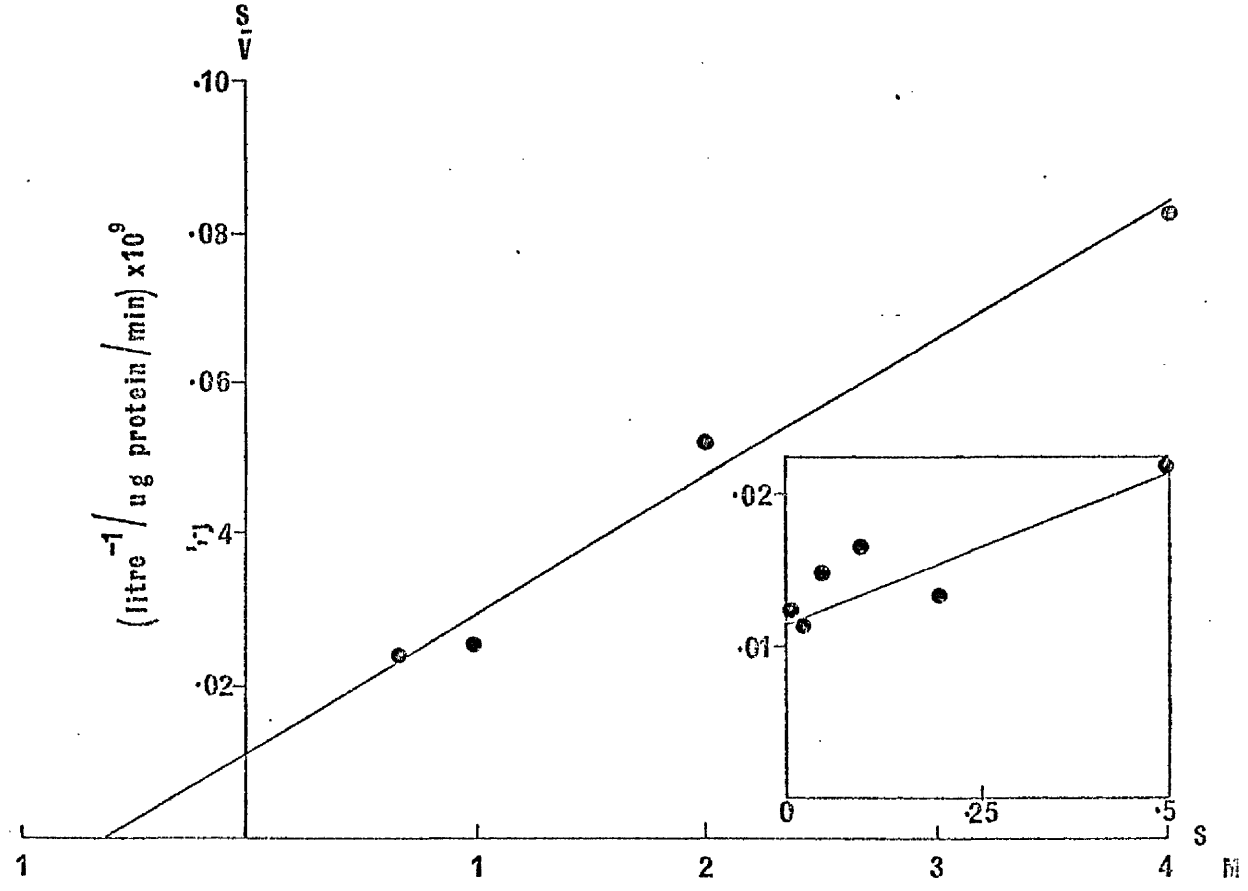


Figure 7.2.1d. S/v versus S plot of L-alanine uptake in SV-CH Ki1 cells

Table 7.2.1a. Km and Vmax values for the uptake of L-alanine by various 'normal' and transformed cell lines of hamster, sheep and mouse origin.

Cell Line	Km ($\times 10^{-4}$M)		Vmax pmol /µg protein/min	
BHK21-Cl3	8.1 ± 1.6	(7.9 ± 1.2)	57.7 ± 4.1	(61.2 ± 3.7)
PyY	7.6 ± .9	(6.7 ± .8)	54.2 ± 2.1	(52.1 ± 2.8)
Syr 1	9.9 ± 1.6		56.0 ± 3.8	
CH-Sp1	7.4 ± 1.3		53.4 ± 4.0	
SV-CH Sp1	7.0 ± 1.0		44.3 ± 2.2	
CH-Li1	7.3 ± 1.0		51.9 ± 2.5	
SV-CH Li1	6.7 ± .6		47.7 ± 1.5	
CH-Lu1	8.1 ± 1.7		49.1 ± 3.6	
SV-CH Lu1	7.7 ± 1.4		45.9 ± 3.2	
CH-Ki1	6.2 ± 1.4		54.5 ± 4.4	
SV-CH Ki1	7.9 ± 1.4		48.1 ± 3.2	
CH-Ki2	5.3 ± 0.8		59.4 ± 3.4	
3T3	7.6 ± 1.2	(6.9 ± 1.4)	34.6 ± 2.0	(37.7 ± 2.6)
Py-3T3	7.4 ± 1.3	(5.9 ± 1.0)	39.2 ± 2.4	(37.6 ± 2.3)
Sv-3T3	7.2 ± 1.0		31.2 ± 1.6	
SH.E.Ki	6.7 ± 1.0	(5.4 ± 1.0)	41.3 ± 2.4	(46.8 ± 2.6)
Py.SH.E.Ki	5.2 ± 0.7	(5.1 ± .7)	44.6 ± 2.0	(40.5 ± 1.7)

The Uptake of L-[2,3 - ^{3}H] alanine (for the concentration range 1 x 10^{-5}M to 4 x 10^{-3}M L-alanine) was examined under standard conditions (Section 2.8.) for each of the above cell lines. Kinetic constants plus standard errors were determined from initial rates of transport, by the method of Wilkinson (1961). Values in parenthesis indicate those values obtained from transport, following preincubation of cells in amino acid free medium (see text).

Table 7.2.1b. Km and Vmax values for the uptake of L-glutamate by various 'normal' and transformed cell lines of hamster, sheep, mouse and human embryonic origin.

Cell Line	Origin of Cell line	Km ($\times 10^{-5}$M)		Vmax pmol/μg protein/min.	
	Syrian Hamster				
BHK-21-C13	Kidney	4.6 ± .7	(4.8 ± .8)	7.5 ± .3	(7.0 ± .3)
PyY	"	4.5 ± 1.0	(4.7 ± .4)	6.3 ± .4	(6.0 ± .1)
Syr1	"	4.1 ± .9		6.5 ± .4	
	Chinese Hamster				
CH-Sp1	Spleen	6.7 ± .8		8.4 ± .3	
SV-CH-Sp1	"	5.4 ± .3		6.6 ± .1	
CH-Li1	Liver	5.6 ± 1.0		7.9 ± .4	
SV-CH-Li1	"	7.6 ± .8		8.7 ± .3	
CH-Lu1	Lung	5.4 ± .7		8.5 ± .3	
SV-CH-Lu1	"	6.1 ± .7		8.1 ± .3	
CH-Ki1	Kidney	8.1 ± .7		7.9 ± .2	
SV-CH-Ki1	"	8.7 ± 1.3		8.7 ± .4	
C4B	Peritoneal effusate	4.1 ± .6		4.1 ± .2	
CHO-KG1	Ovary	7.2 ± 1.1		4.5 ± .2	
	Sheep				
SH.E.Lu	Lung	7.7 ± 1.4		2.5 ± .1	
Py.SH.E.Lu	"	7.2 ± .8		2.6 ± .1	
SH.E.Ki	Kidney	7.9 ± .8	(8.0 ± .9)	2.6 ± .1	(2.1 ± .1)
Py.SH.E.Ki	"	8.9 ± 1.2	(7.4 ± 1.3)	2.6 ± .1	(2.0 ± .1)
SH.E.Sp	Spleen	8.9 ± 1.5		2.6 ± .1	
Py.SH.E.Sp	"	10.8 ± 2.3		2.6 ± .2	
	Mouse				
3T3	disaggregated embryo	6.1 ± 1.1	(4.9 ± .6)	2.3 ± .1	(2.5 ± .1)
Py-3T3	"	7.4 ± 1.5	(6.5 ± .9)	2.9 ± .2	(2.3 ± .1)
SV-3T3	"	7.2 ± 0.7		2.9 ± .1	

Cell Line	Origin of Cell Line	Km ($\times 10^{-5}$M)	Vmax pmol/µg protein/ min.
	Human Embryo		
HEK	Kidney	8.9 ± .7	1.9 ± .04
HEL	Lung	8.9 ± .7	2.1 ± .05

The uptake of L-[U ^{14}C]glutamate (for the concentration range, 0.5 x 10^{-5}M to 1 x 10^{-3}M L-glutamate) was examined under standard conditions (Section 2.8) for each of the above cell lines. Kinetic constants plus standard errors were determined from initial rates of transport, by the method of Wilkinson (1961). Values in parenthesis indicate those values obtained for transport, following preincubation of cells in amino acid free medium (See text).

standard errors were determined from initial rates of transport, by the method of Wilkinson (1961). Values in parenthesis indicate those values obtained for transport following preincubation of cells in amino acid free medium (see text).

It has been reported (Isselbacher 1972) that preincubation of cells in medium without serum or amino acids is necessary to demonstrate differences in amino acid transport between transformed and non-transformed cell lines. Consequently L-glutamate and L-alanine uptake was examined following preincubation (45 minutes) in medium without serum and amino acids (Uptake Medium) and immediately following normal growth conditions. However, little difference in the kinetic constants for the uptake of either of these amino acids was observed between preincubated or non-preincubated non-transformed cells and their transformed derivatives. Consequently in other cases amino acid uptake was examined immediately following growth. Similar Km and Vmax values were observed for either L-alanine or L-glutamate uptake in the 'normal' cell lines and their virus transformed derivatives.

Km values of between approximately .4 and 1 x 10^{-5}M were observed for L-glutamate uptake in all cell lines examined, whereas the values for L-alanine were approximately an order of magnitude greater (approximately 5 x 10^{-4}M). Maximum rates of L-alanine uptake were also greater (between 30 and 60 pmol/µg protein/min) than those obtained for L-glutamate (approximately 2 to 9 pmol/µg protein/min).

7.2.2. Possible relationship between L-alanine and L-glutamate uptake rates and rates of cell growth

It has been suggested (Neame 1962) that amino acid transport may reflect the rate of cell division within a tissue. In order to determine if the Vmax values for L-alanine or L-glutamate were related to the rate of cell division in cultured cells the population doubling

Table 7.2.2a. Population doubling times of a number of "normal"and virus transformed cell lines.

Cell Line	Population Doubling time (hours)
Syrian Hamster	
BHK21 - C13	12
PyY	11
SyrK1	14
Chinese Hamster	
CHK1	48
SV-CHK1	29
CH-Li1	36
SV-CH Li1	16
CH-Sp1	39
SV-CH Sp1	22
CH-Lu1	67
SV-CH Lu	51
CHO-KG1	13
C4B	14
Sheep	
SH.E.Ki	39
Py.SH.E.Ki	30
SH.E.Sp	34
SH.E.Lu	94
Py.SH.E.Lu	33

Cells were seeded at a population density of $1x10^{-5}$ cells/60mm. dish and cells harvested and counted at 24 or 48 hour intervals. Population doubling times were calculated for cells in "exponential" growth phase.

times were determined for a number of cell lines (Table 7.2.2a). No relationship between the rates of L-glutamate or L-alanine uptake and population doubling times was observed.

7.2.3. Specificity of L-glutamate and L-alanine transport systems in various cell lines

The actual specificities of amino acid transport systems have been demonstrated to vary between tissues (Neame 1964, 1966, 1968; Neame and Ghadially 1967). In order to determine whether the specificities of the L-glutamate and L-alanine transport system(s) differed in cell lines derived from different tissues, the ability of certain amino acids to inhibit the uptake of L-glutamate and L-alanine were examined in selected cell lines. The results of these inhibition studies are shown in Tables 7.2.3a and b, respectively. Similar inhibition of L-alanine and L-glutamate uptake was observed for each of the cell lines examined, indicating that the L-glutamate and L-alanine transport systems in these (and BHK21-C13) cell lines have similar specificities. As similar specificities were observed for the 'normal' and their respective virus transformed lines it would appear that the specificities of these transport systems are unaltered following cell transformation.

7.2.4. Effect of cell density, and growth of cells in the presence of low serum or dibutyryl cyclic AMP on the rate of L-alanine and L-glutamate uptake in various cell lines

The L-alanine preferring transport system of BHK21-C13 cells was shown in Section 6 to exhibit reduced activity in cells with elevated cyclic AMP levels or following growth in the presence of dibutyryl cyclic AMP plus or minus theophylline, whereas the acidic amino acid transport system was unaffected. The possibility that L-alanine and

Table 7.2.3a. Inhibition of L-glutmate uptake by certain amino acids in various "normal" and virus transformed cell lines from tissues of hamster and and sheep.

Cell line	% inhibition of L-[U - ^{14}C]glutamate uptake by:				
	L-glu	L-asp	L-ala	L-phe	L-arg
BHK21 - C13*	97	94	6	7	5
PyY	95	98	8	7	8
CH Lu1	92	87	5	8	14
CH Li1	94	91	7	6	8
SV-CH Li1	89	96	12	13	7
CH Sp1	95	95	8	-6	11
SH.E.Ki	85	97	10	15	12
Py.SH.E.Ki	94	88	4	7	8

Uptake of L-[U - ^{14}C] glutamate (2×10^{-5}M) was examined in the presence and absence of inhibiting amino acid (5×10^{-4}M). Rates of uptake were determined from 10 minute incubations. Values are means of 5 estimates and are expressed as percentage inhibition of L-[U - ^{14}C] glutamate uptake alone.

* Value from Table 4.2.7a.

Table 7.2.3b. Inhibition of L-alanine uptake by certain amino acids in various "normal" and virus transformed cell lines from tissues of hamster and sheep..

Cell line	% inhibition of L-[2,3 - ^{3}H] alanine uptake by :				
	L-ala	L-leu	L-trp	L-glu	L-arg
BHK21 - C13*	92	35	26	9	7
PyY	87	39	34	4	12
CH Lu1	84	30	20	-6	4
CH Li1	81	44	37	7	8
SV-CH Li1	89	37	28	-2	7
CH Sp1	94	26	37	11	11
SH.E.Ki	84	41	25	6	9
Py.SH.E.Ki	86	46	17	4	6

Uptake of L-[2,3 - ^{3}H] alanine ($1x10^{-4}$M) was examined in the presence and absence of inhibiting amino acid($2.5x.10^{-3}$M). Rates of uptake were determined from 4 minute incubations. Values are means of 5 estimates and are expressed as percentage inhibition of L-[2,3 - ^{3}H] alanine uptake alone.

* Values from Table 5.2.7a.

Table 7.2.4a. Effect of cell density and growth of cells in the presence of low serum or dibutyryl cyclic AMP plus theophlline on the rates of uptake of L-glutamate in various transformed or non-transformed cells.

Cell line	Exponentially growing cells	Confluent cells	Cells grown in low serum	Cells grown in EFC_{10} + dibutyryl cyclic AMP plus theophylline
PyY	5.1 ± .6	6.0 ± .5	6.2 ± .6	5.5 ± .5
CH-Sp 1	6.0 ± .5	5.4 ± .3	6.7 ± .4	6.1 ± .6
SV-CHSp 1	5.4 ± .3	7.1 ± .7	6.3 ± .7	5.9 ± .5
CH-Li 1	6.4 ± .2	5.4 ± .3	6.9 ± .6	5.9 ± .6
SV-CHLi 1	6.6 ± .3	5.8 ± .3	6.4 ± .6	5.3 ± .3

Cells were grown for 48 hours in the presence or absence of dibutyryl cyclic AMP (2×10^{-4}M) plus theophylline (1×10^{-3}M). or on $EFC_{0.5}$. Confluent cell cultures were obtained by seeding 1.5 times the normal cell inoculumn in EFC_{10}. Rates of L-[U- ^{14}C] glutamate (2×10^{-4}M) were determined for 10 minute incubations. Values are expressed as means of 8 samples plus SEM.

Analysis of variance for above data:

PyY	$F_{3,28} = 1.59$, $P>0.05$ ($F = 2.96$)
CH-Sp 1	$F_{3,28} = 1.34$, $P>0.05$ ($F = 2.96$)
SV-CHSp 1	$F_{3,28} = 1.24$, $P>0.05$ ($F = 2.96$)
CH-Li 1	$F_{3,28} = 1.91$, $P>0.05$ ($F = 2.96$)
SV-CH-Li 1	$F_{3,28} = 2.41$, $P>0.05$ ($F = 2.96$)

Analysis of variance for data obtained for L-alanine uptake in various transformed and non-transformed cell lines of different cell densities, or maintained in the presence of low serum or dibutyryl cyclic AMP plus theophylline.

PyY :- $F_{3,28} = 10.10$, $P < 0.05$ ($F = 2.96$)

CH-Sp1 :- $F_{3,28} = 20.77$, $P < 0.05$ ($F = 2.96$)

CH-Li1 :- $F_{3,28} = 23.83$, $P < 0.05$ ($F = 2.96$)

SV CH-Sp1 :- $F_{3,28} = 4.86$, $P < 0.05$ ($F = 2.96$)

SV CH-Li1 :- $F_{3,28} = 9.24$, $P < 0.05$ ($F = 2.96$)

		PyY		CH-Sp1		SV CH-Sp1		CH-Li1		SV CHLi1	
		L.S.D.	Diff.in Means	L.S.D.	Diff. in Means	L.S.D.	Diff.in Means	L.S.D.	Diff.in Means	L.S.D.	Diff.in Means
Exponentially growing cells	versus confluent cells	6.04	5.3	6.02	15.5*	10.72	2.0	7.14	17.8*	8.53	3.8
"	" cells grown in low serum	"	12.0*	"	16.5*	"	15.2*	"	14.0*	"	8.2
"	" cells grown in medium + dibutyryl cyclic AMP and theophylline	"	13.7*	"	20.6*	"	8.8	"	17.8*	"	14.9*
Confluent cells	" cells grown in low serum	"	6.7	"	1.0	"	13.2	"	6.2	"	12.0*
"	" cells grown in medium + dibutyryl cyclic AMP and theophylline	"	8.4*	"	5.1	"	6.8	"	6.9	"	18.7*
Cells grown in medium + dibutyryl cyclic AMP and theophylline	" cells grown in low serum	"	1.7	"	4.1	"	6.4	"	5.7	"	6.7

* Indicates difference between the means is significant at the P = 0.05 level of significance.

L.S.D. is the least significant difference.

Table 7.2.4b. Effect of cell density and growth of cells in the presence of low serum or dibutyryl cyclic AMP plus theophylline on the rates of uptake of L-alanine in various transformed or non-transformed cells.

Cell line	Exponentially growing cells	Confluent Cells	Cells maintained in low serum	Cells in EFC10 + dibutyryl cyclic AMP and theophylline
PyY	33.1 ± 2.3	27.8 ± 1.8	21.1 ± 2.0	19.4 ± 1.9
CH Sp1	36.5 ± 2.5	21.0 ± 1.7	20.0 ± 2.1	15.9 - 1.5
SV-CH Sp1	42.6 ± 2.9	40.6 ± 3.6	27.4 ± 1.8	33.8 ± 2.3
CH Li1	45.3 ± 2.8	27.5 ± 2.5	21.3 ± 1.7	20.6 ± 1.5
SV-CHLi1	40.0 ± 2.6	43.8 ± 2.9	31.8 ± 3.0	25.1 ± 2.3

Cells were grown for 48 hours in the presence or absence of dibutyryl cyclic AMP ($2x10^{-4}M$) plus theophylline ($1x10^{-3}M$), or in EFC0.5. Confluent cell cultures were obtained by seeding 1.5 times the normal cell inoculum in EFC10. Rate of L-[2,3 $-^{3}H$] alanine ($2x10^{-3}M$) uptake were determined for 4 minute incubations. Values are expressed as means of 8 samples plus SEM.

Figures 7.2.4a to e. Effects of low serum and dibutyryl cyclic AMP plus theophylline on the growth of various cell lines.

Cells were inoculated at 3.5 or 7.0 x 10^3 cells/cm^2 in 60 cm. petri dishes, and grown under standard conditions (Section 2.2). Medium was changed every 2 days. Cells were harvested and counted at intervals. Each value is the mean of duplicate cultures.

Cells maintained in EFC_{10} (●—●)

Cells maintained in $EFC_{0.5}$ (o—o)

Cells maintained in EFC_{10} and dibutyryl cyclic AMP (2 x 10^{-4}M) and theophylline (1 x 10^{-3}M). (■—■)

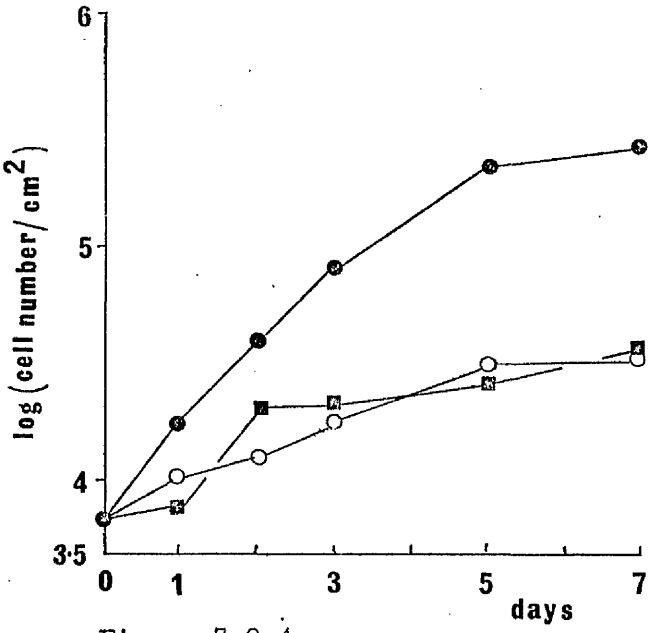


Figure 7.2.4a.
Effect of low serum and dibutyryl cyclic AMP and theophylline on the growth of SV-CH Li1.

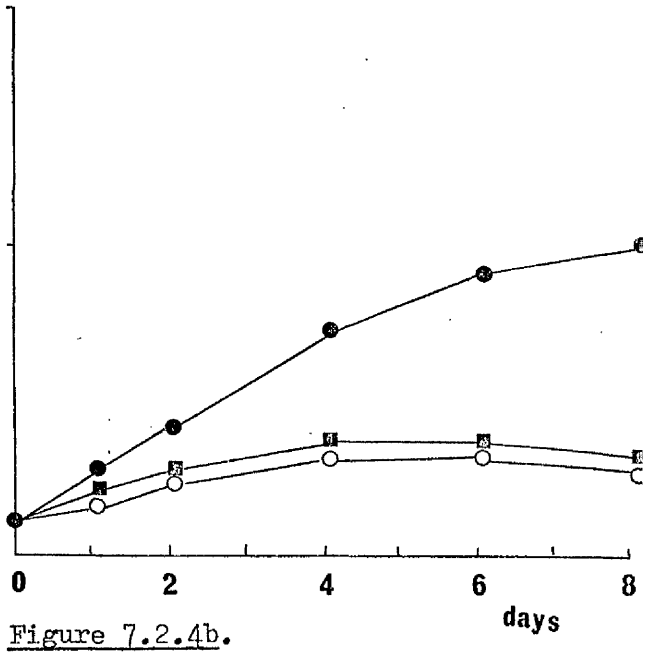


Figure 7.2.4b.
Effect of low serum and dibutyryl cyclic AMP and theophylline on the growth of CH Li1.

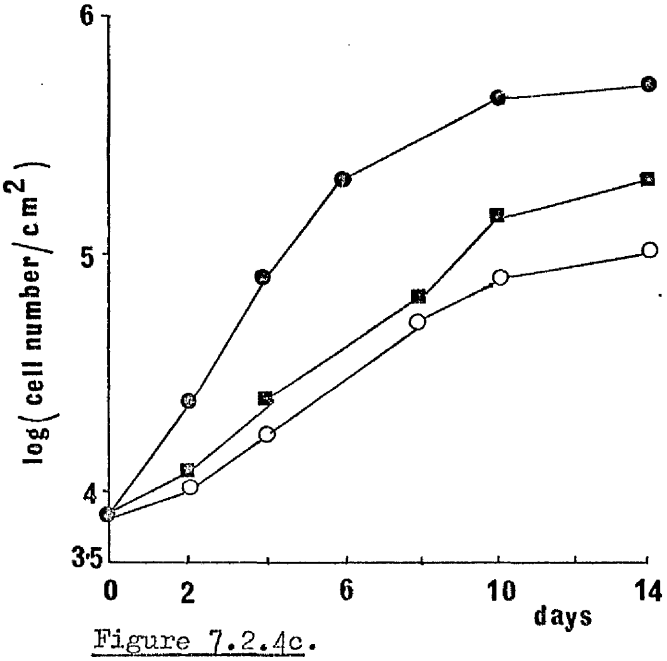


Figure 7.2.4c.
Effect of low serum and dibutyryl cyclic AMP plus theophylline on the growth of SV-CHSp1.

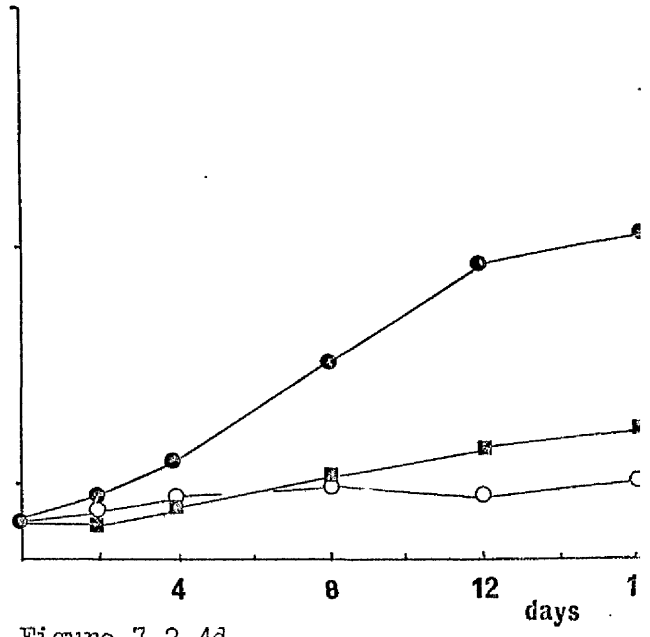


Figure 7.2.4d.
Effect of low serum and dibutyryl cyclic AMP plus theophylline on the growth of CH Sp1.

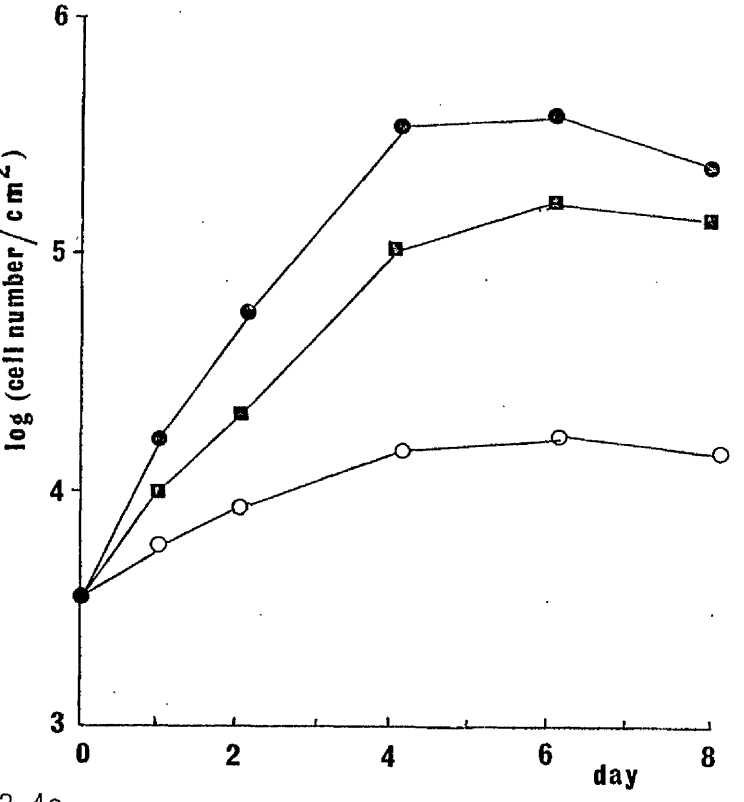


Figure 7.2.4e.

Effect of low serum and dibutyryl cyclic AMP plus theophylline on the growth of PyY.

Figures 7.2.4a to e. Effect of low serum, and dibutyryl cyclic AMP plus theophylline on the growth of various cell lines.

Cells were inoculated at 3.5 or 7.0 x 10^3 cells/cm^2 in 60mm. petri dishes, and grown under standard conditions (Section 2.2.). Medium was changed every 3 days. Cells were harvested and counted at intervals. Each value is the mean of duplicate cultures.

Cells grown in EFC_{10} (●-●)
Cells grown in $EFC_{0.5}$ (○-○)
Cells grown in EFC_{10} plus dibutyryl cyclic AMP (2 x 10^{-4}M) and theophylline (1 x 10^{-3}M) (■-■)

L-glutamate transport systems of other "normal" or virus transformed cell lines may be similarly regulated was investigated. L-alanine and L-glutamate uptake was examined in confluent cultures and following growth in the presence of dibutyryl cyclic AMP plus theophylline or in medium containing low serum concentrations. These results for L-glutamate and L-alanine uptake are shown in Tables 7.2.4.a and b respectively. Similar rates of L-glutamate uptake were observed for transformed and "normal" cells examined following growth in these conditions. L-alanine uptake, however, was reduced in these cells following growth in medium containing low serum or dibutyryl cyclic AMP plus theophylline. No significant reduction in L-alanine uptake was observed in confluent transformed cell lines, whereas L-alanine transport was reduced in confluent non-transformed cells.

The effects of low serum and dibutyryl cyclic AMP plus theophylline on the growth of selected transformed and non-transformed cell lines was examined (Figures 7.2.4a to e). Non-transformed cells rapidly produced quiescent cultures in medium containing low serum or dibutyryl cyclic AMP plus theophylline. Transformed cells, however, were capable of maintaining growth at a reduced rate under these conditions, although the final density attained was apparently less than for cells grown in EFC10.

7.3. DISCUSSION AND CONCLUSIONS

L-glutamate and L-alanine uptake were examined in a variety of cell lines derived from human embryonic, mouse, sheep, syrian or chinese hamster origin. The transport of these amino acids was similar to that observed in BHK21-C13 cells as L-glutamate appeared to be transported by a high affinity, low capacity amino acid transport system and L-alanine by a relatively low affinity, high capacity system(s).

Although it has been suggested (Neame 1962) that the relative ability of a tissue to transport amino acids may be greater in tissues containing rapidly dividing cells, or tissues specialised to conserve amino acids, L-alanine and L-glutamate uptake appeared to be independent of the tissue of origin or the rate of growth of these cells. Similar Vmax values were generally observed for L-glutamate or L-alanine uptake in cell lines derived from the same species whether these lines were derived from the same (e.g. CH1 series of cell lines) or different animals (e.g. the sheep cell lines). However, the quasi-diploid C4B, and hypodiploid CHO-KG1 cell lines showed lower Vmax values for L-glutamate uptake than the other chinese hamster cell lines. This reduced L-glutamate uptake may reflect differences in transport in the tissues from which these cell lines were derived, cell line peculiarities, or possibly these cells may have been derived from a different cell type to the CH1 series of cells. The affinity of the examined cell lines for the L-alanine and L-glutamate transport systems, as indicated by their Km values, were similar to those observed in BHK21-C13 cells the Km values for L-alanine uptake being at least an order of magnitude higher than those obtained for L-glutamate.

Certain differences in the specificities of amino acid transport systems in various tissues have been indicated from inter-tissue differences in the ability of certain amino acids to inhibit the uptake of other amino acids (Neame 1964, 1966, 1968; Neame and Ghadially 1967). However, the L-alanine and L-glutamate transport systems in cell lines derived from different species or tissues showed (apparently) similar specificities. The lack of variation between these different systems may reflect the presence of similar transport systems in the parent tissue, the expression of only certain transport systems when cells are maintained in culture, or alternatively, the cell lines may have been

derived from a similar cell type present in these tissues. Transformation was not demonstrated to alter the specificities of these transport systems.

Holley (1972) has proposed that the "malignant state" is the consequence of increased intracellular concentrations of nutrients (normally present at growth limiting levels), produced by changes in uptake mechanisms. Support for this hypothesis is provided from increased transport of certain sugars in cells following the transformation by RNA or DNA tumour virus (Hatanaka et al. 1969; Hatanaka and Gilden 1970; Hatanaka et al. 1971; Hatanaka and Hanafusa 1970; Hatanaka et al. 1970a; Isselbacher 1972). Amino acid transport has been little studied in either transformed or non-transformed cell lines and it is unclear whether amino acid uptake is generally increased following transformation. No significant differences in the rates of uptake of the examined amino acids were observed between "normal" cell lines or cell lines derived from malignant tissue (Eagle et al. 1961) or between normal and virus transformed cell lines (Hare 1967; Hatanaka et al. 1969). However, Foster and Pardee (1969) have noted increased uptake (approximately two fold) of two non-metabolisable amino acids and L-glutamine in Polyoma transformed as compared with untransformed 3T3 cells. However, no change in arginine or glutamate uptake was observed in these cells. Isselbacher (1972) similarly observed increased transport of the non-metabolisable amino acids in Polyoma transformed BHK21-C13 cells, and other virus transformed cell lines, compared to their non-transformed parent cell lines. However, unlike Foster and Pardee, this author reported increased uptake of L-arginine and L-glutamate in these cells. This increase in L-glutamate uptake in Polyoma transformed BHK21-C13 cells was not confirmed in this study (Section 7.2.1). Similarly no significant alterations in the Km or Vmax values were observed between a number of "normal"

mouse, chinese hamster or sheep derived cell lines and their respective Polyoma or SV-40 transformed lines. The observation that preincubation of cells in amino acid free medium is required to demonstrate differences between transformed and non-transformed cells (Isselbacher 1972) was also not confirmed in this study (Section 7.2.1).

Treatment of BHK21-C13 cells with dibutyryl cyclic AMP plus or minus theophylline, or growth in low serum, was demonstrated in Section 6.2.5 to initially increase the population doubling time and rapidly produce quiescent cells. Similar observations were made for the non-transformed cell lines examined in this section. Transformed cell lines also showed reduced growth rates however, these cells continued growth for several days. Similar observations have been made for dibutyryl cyclic AMP treated transformed cells (plus or minus theophylline) by other authors (Sheppard 1971; Johnson and Pastan 1972; Blat et al. 1973).

Results presented in Section 6.2.5 demonstrated that L-alanine uptake was considerably reduced in BHK21-C13 cultures grown in the presence of dibutyryl cyclic AMP and theophylline, or cells with elevated cyclic AMP levels (confluent cells, or cells grown in low serum). L-glutamate uptake was essentially unaltered under these conditions. Similar observations were made for the transport of these amino acids in the examined non-transformed cell lines. L-alanine uptake was also reduced in transformed cell lines grown in the presence of dibutyryl cyclic AMP plus theophylline, or in medium containing 0.5% serum. No significant effect on L-alanine uptake was observed between confluent or non-confluent cultures. As the "normal" cell lines showed reduced L-alanine transport in the presence of dibutyryl cyclic AMP, or conditions shown to increase intracellular concentrations of cyclic AMP in BHK21-C13 (Section 6.2.6) or other non-transformed cell lines (Kram et al. 1973; Otten et al. 1971a, b; Heindrick and Ryan 1970) it is possible that

cyclic AMP is also involved in the regulation of the L-alanine transport system(s) in these cell lines. The L-alanine transport system(s) in transformed cells would also appear to be under cyclic AMP regulation as its activity was reduced by dibutyryl cyclic AMP or growth in low serum conditions. The lack of significant reduction of L-alanine uptake in confluent transformed cell cultures is not inconsistent with the involvement of cyclic AMP in the control of L-alanine transport, as transformed cell lines have been shown to maintain low cyclic AMP levels on confluence (Otten et al. 1971a and b).

SECTION 8. THE ATTEMPTED ISOLATION OF MUTANT BHK21-C13 CELLS DEFECTIVE IN THE TRANSPORT OF L-GLUTAMATE OR L-ALANINE

8.1. INTRODUCTION

The isolation of mutants altered in the transport of a particular amino acid substrate(s), have provided considerable information with regard to the number and specificity of amino acid transport systems in microorganisms (Kay 1971; Ames and Lever 1970; Pall 1969, 1970a; Grenson 1966a, b; Grenson et al. 1970; Crabeel and Grenson 1970). These mutations have also provided information as to the mechanism of permease regulation (Pateman et al. 1974), and the probable nature and identity of their component proteins (Ames and Lever 1970). The production and study of amino acid transport mutants in cultured mammalian cells should provide similar information regarding the specificity of the transport systems in these cells and enable the biochemical and genetical study of these systems. This chapter describes attempts to isolate mutants defective in the "acidic" and "L-alanine preferring" transport systems of BHK21-C13 cells. Two major selection procedures were employed. The first involved the selection for cells resistant to the toxic effects of amino acid analogues transported by the transport system under investigation. The second method, involving " ^{3}H -suicide" selects for cells which are unable to transport radioactive amino acids into the cell under normal growth conditions, or alternatively for cells capable of transporting amino acids under certain ("permissive") but not other ("non-permissive") conditions. The latter technique should be particularly useful for the isolation of cells carrying mutations which affect transport systems essential for growth.

8.2. RESULTS

8.2.1. Determination of EMS toxicity to BHK21-C13

The mutagen EMS has been demonstrated to increase significantly the frequency of forward or reverse mutations in cultured mammalian cells (Chu and Malling 1968; and Meiss and Basilico 1972), and consequently all selection procedures were carried out with EMS treated cells. As Chu and Malling (1968) demonstrated that this mutagen appreciably reduced cell survival (as indicated by reduced plating efficiency), the relationship between the EMS concentration applied and cell survival was determined (see Figure 8.2.1a).

A 2-hour exposure of 8.5×10^{-3}M EMS was found to give a 50% survival of BHK21-C13 cells; this concentration was used for all subsequent selection procedures. A 6-hour exposure of cells to this compound was required to produce a similar effect at $34^{\circ}C$ (Fig. 8.2.1b).

8.2.2. Evaluation of L-glutamate and L-aspartate analogues as potential selective agents for acidic amino acid transport mutants of BHK21-C13 cells, and results of selection procedures

To be suitable for use as an agent for the selection of mutants defective in the studied transport system, an analogue should be transported by this transport system and be toxic to the cell at useful concentrations. Cells defective in the transport of the analogue (and hopefully the normal substrates that share the transport system) should thus acquire resistance to this compound and be capable of growth under conditions unfavourable to "wild-type" cells.

A number of L-glutamate and L-aspartate analogues were tested for their toxicity (as indicated by reduced plating efficiency of BHK21-C13 cells in the presence of these analogues) and ability to be transported via the acidic amino acid transport system (as shown by their ability to inhibit L-glutamate uptake), see Table 8.2.2a. Of

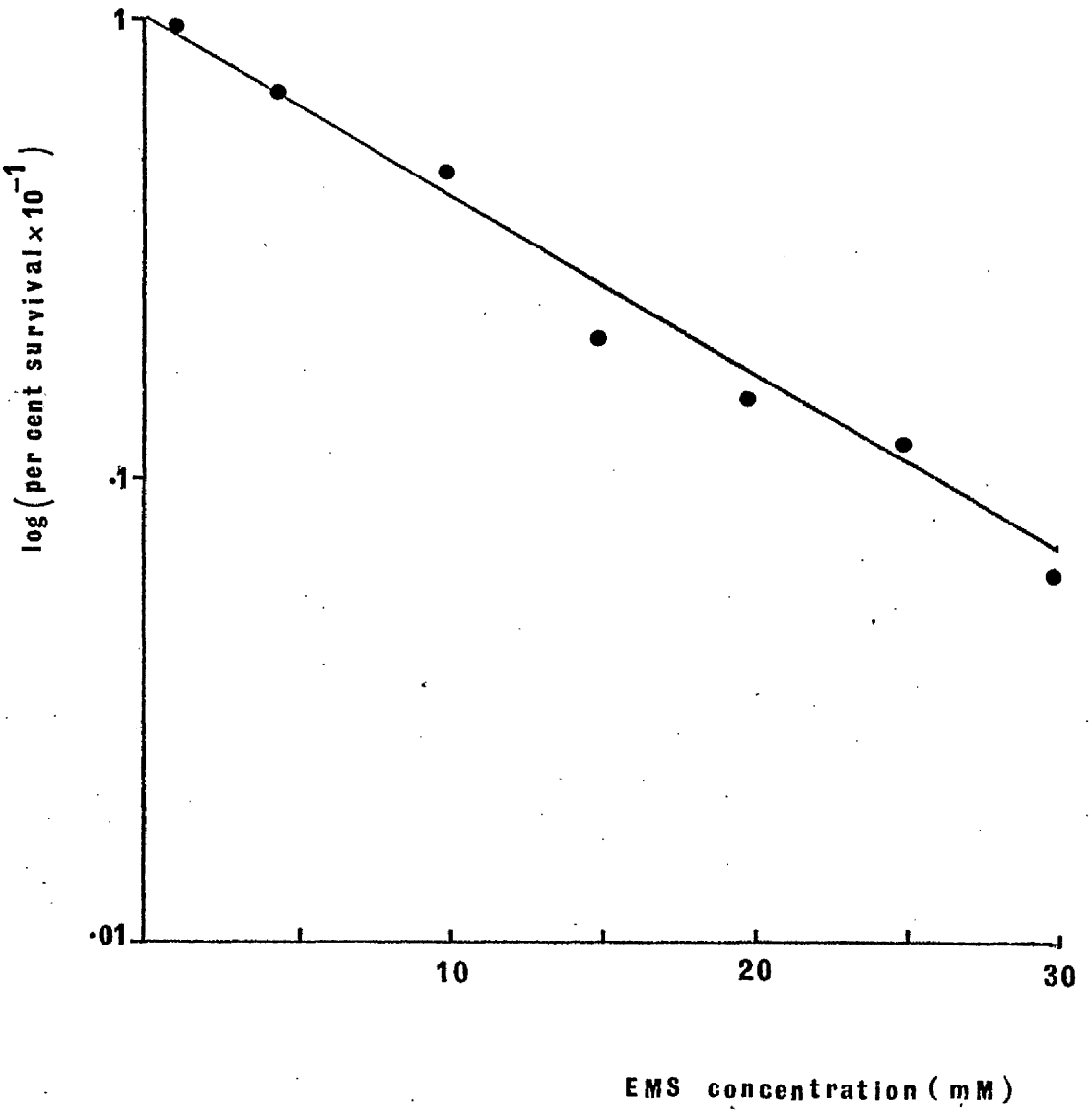


Figure 8.2.1a. Toxicity of EMS to BHK21-C13 cells

Cells in log phase growth were exposed to various concentrations of EMS for 2 hours at 37°C, harvested and inoculated in 60 mm petri dishes at a density of 500 cells per dish. Cells were then maintained under normal conditions for 7 days, examined and stained as described in Section 2.11. Results are expressed as a percentage of non-EMS treated cells which gave rise to colonies. The experiment was carried out in triplicate.

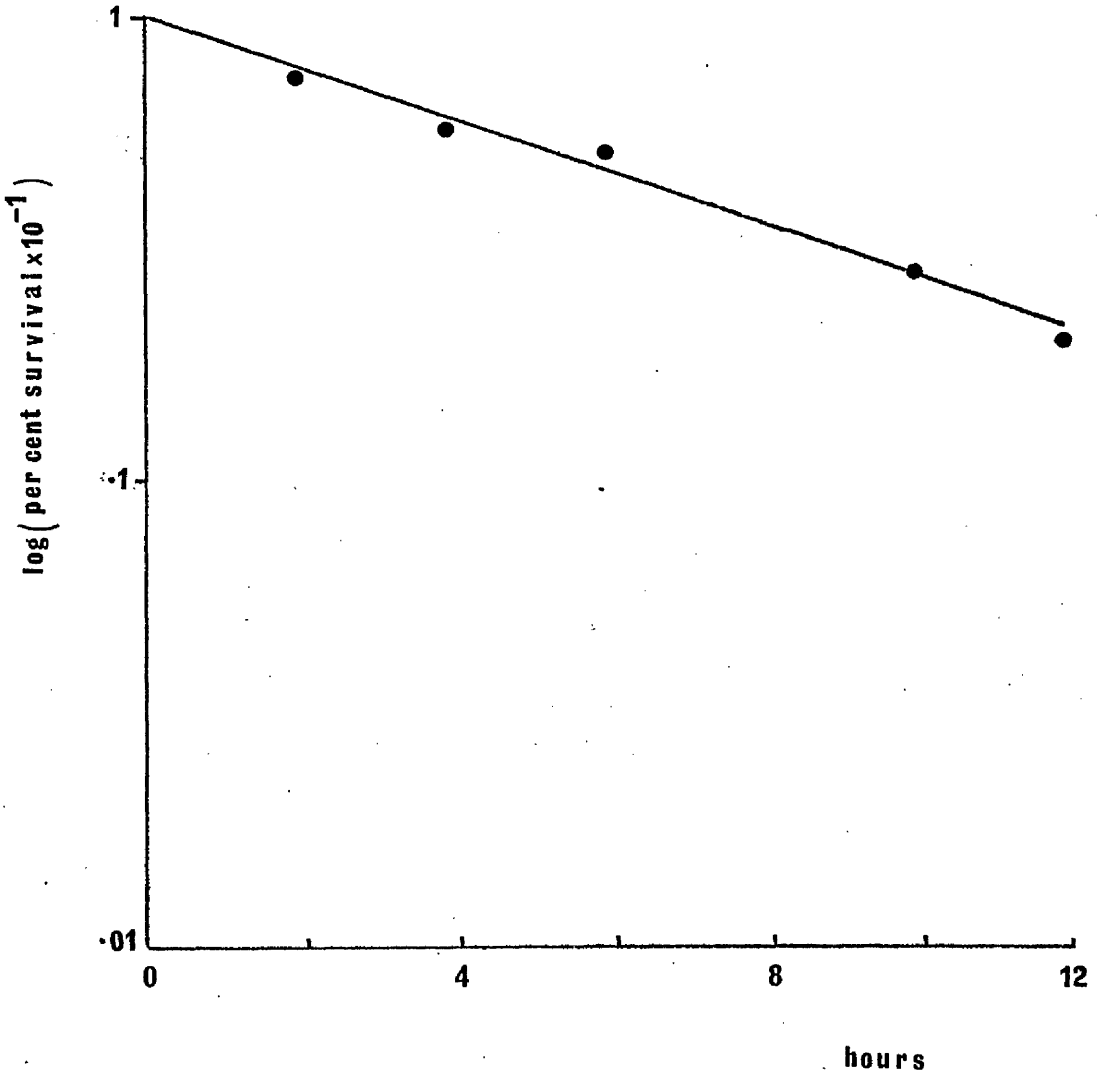


Figure 8.2.1b. Toxicity of EMS to BHK21-C13 cells when incubated at 34°C

Cells in log phase growth were exposed for various intervals to EMS (8.5 mM) at 34°C, harvested and inoculated in 60 mm. petri dishes at a density of 500 cells per dish. Cells were then maintained at 34°C for 12 days, examined and stained as described in Section 2.11. Results are expressed as a percentage of non-EMS treated cells, which gave rise to colonies. The experiment was carried out in quadruplicate.

these analogues only 4-fluoroglutamate was both toxic to BHK21-C13 cells (the survival curve for these cells in the presence of this analogue is shown in Figure 8.2.2.) and an effective inhibitor of L-glutamate uptake. L-cysteate, although a more effective inhibitor of L-glutamate uptake than 4-fluoroglutamate was not suitable as a selective agent as it was not sufficiently toxic to BHK21-C13 cells at low concentrations.

Cells were inoculated as relatively sparse cultures (2×10^5 cells/100mm dish, a density sufficiently sparse for no cell to cell contact) and the selective agent added at approximately 40 hours following mutagen removal (during which time approximately 3 cell divisions had occurred), conditions shown to be optimal for mutant selection (Chu and Malling 1968). These authors have also shown that at lower concentrations of selective agent the survival frequency is greater than at higher concentrations, although the resistance of the majority of these cells was lower than that of mutants obtained at higher concentrations. In order to select for more resistant cells, selections were carried out at the relatively high concentration of 4-fluoroglutamate concentration of 40 μM. However, as no colonies were initially obtained at this concentration, additional selections were subsequently carried out at 30 μM. A number of "potentially resistant" colonies were obtained under these selective conditions, and subsequently tested for resistance to 4-fluoroglutamate and ability to transport L-glutamate. Only one cell line BHK21-FGR301 showed any resistance to 4-fluoroglutamate, see Figure 8.2.2 ; however this cell line showed no defect in L-glutamate transport. In an attempt to obtain more resistant cells derived from this colony, a second selective procedure was carried out at 40 μM. Four colonies were obtained, but none of these showed altered L-glutamate uptake, or increased resistance compared to FGR301. The results of these selection procedures are summarised in Table 8.2.2b.

Table 8.2.2a. Toxicity of Glutamate and Aspartate analogues to BHK21-C13 cells, and their effectiveness as inhibitors of L-glutamate uptake.

Amino Acid Analogue	% Inhibition of L-glutamate uptake *	Concentration to produce 50% inhibition of colony formation (mM)	Lowest concentration to produce toxic effects on dense cultures (mM)
N-formyl-L-aspartate	10	2	10
N-acetyl-L-glutamate	12	8	.0
N-acetyl-DL-aspartate	6	10	NT
DL-4-fluoroglutamate	71	.008	.06
L-cysteate	79	10	10
L- -amino adipate	14	6	10

*Data taken from Table 4.2.7b.

Uptake of L-[U -^{14}C] glutamate (2×10^{-5}M) was examined in the presence of L-amino acid (5×10^{-4}M) or DL-amino acid (1×10^{-3}M) analogues. Determination of the toxicity of amino acid analogues by plating procedures was carried out as described in Section 2.12. Toxicity of analogues to dense cell cultures was indicated by marked cell detachment.

NT indicates not tested.

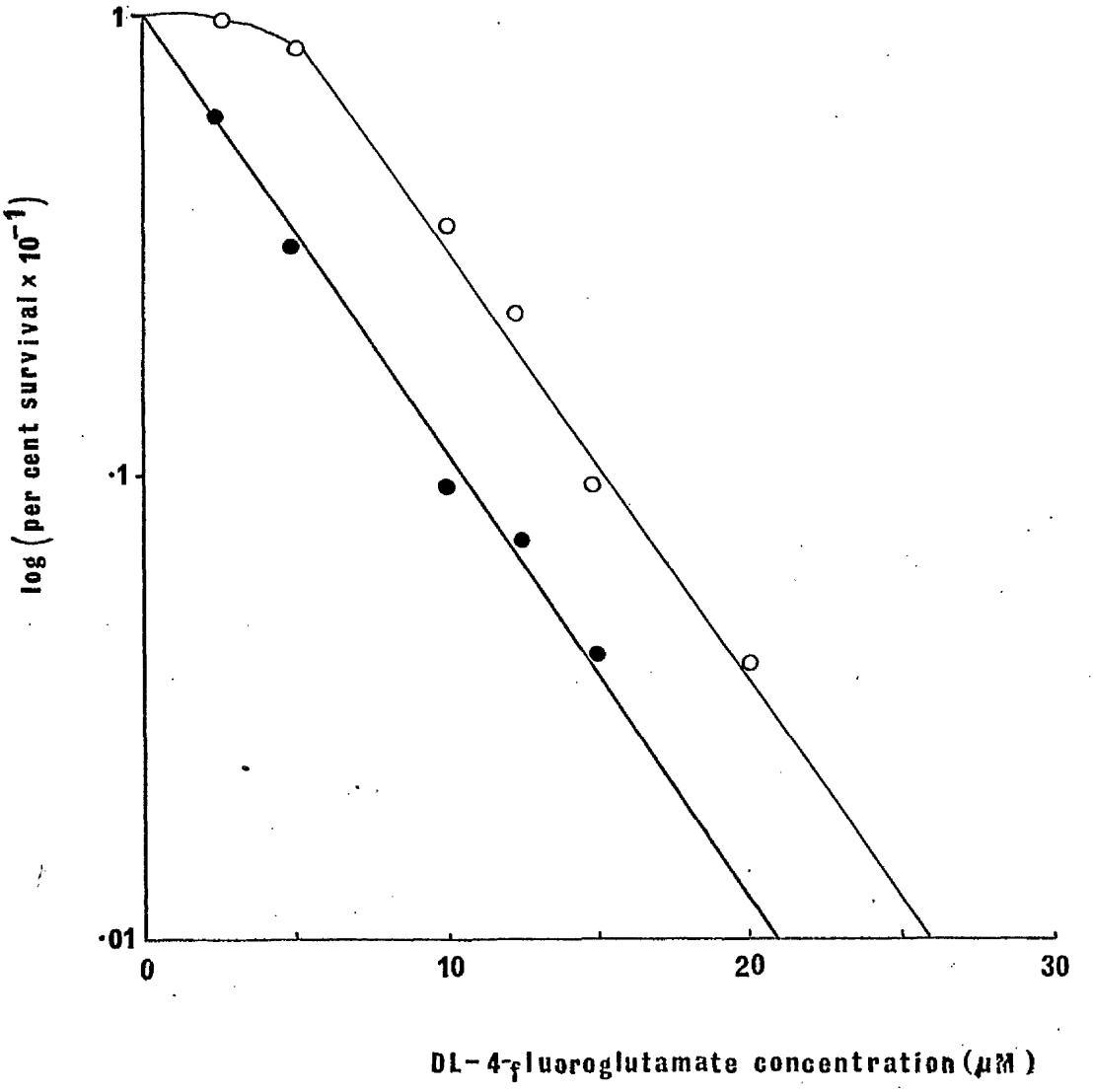


Figure 8.2.2. Toxicity of DL-4-fluoroglutamate to BHK21-C13 cells

Cells in log phase were harvested, dispersed and inoculated in 60 mm. petri dishes (containing 4 mls. Plating Medium less L-glutamate and L-aspartate) at a density of 200 cells/dish. DL-4-fluoroglutamate (concentrations 2.5 to 30 uM) was added, and the cells maintained under normal conditions for 7 days (Section 2.11). Colonies were examined and stained (Section 2.11) after this interval.

BHK21-C13 cells grown in the presence of DL-4-fluoroglutamate (●-●).

Clone FGR-301 grown in the presence of DL-4-fluoroglutamate (O-O).

Table 8.2.2b. Summary of selections for cells resistant to 4-fluoroglutamate.

No. of experiments.	Selection concentration of 4-fluoro-glutamate (μM)	No. of cells seeded after EMS treatment (x 10^{-7})	Estimated viable cell No. (x 10^{-7})	No. of colonies produced	4-fluoroglutamate resistance	No. defective in L-glutamate uptake
11	30	8.8	3.3	41	1 (FGR 301)	-
5	40	4.5	1.7	19*	-	-

* This figure includes 4 lines derived from a second round selection procedure of FGR 301 cells.

Selection procedures and L-glutamate uptake experiments were carried out as described in Sections 2.13.2. and 2.8. respectively. 4-fluoroglutamate resistance was examined by the standard procedures, described in Section 2.12., for concentration between 1 x 10^{-5}M and 5 x 10^{-5}M.

8.2.3. Evaluation of a number of neutral amino acid analogues as agents for the selection of mutants defective in the L-alanine preferring transport system

The potential of a number of neutral amino acid analogues as agents for the selection of mutants defective in the "L-alanine preferring" transport system was examined. The relative toxicities of these analogues to BHK21-C13 cells, and their respective abilities to be transported via this system (as indicated by inhibition of L-alanine uptake) are shown in Table 8.2.3. None of these analogues fulfilled the requirements of such a selective agent, as although N-methyl-DL-alanine, α-AIB and α-methyl-serine were shown to be effective inhibitors of L-alanine uptake, these compounds were not considered sufficiently toxic for the required selection procedures. As no amino acid analogue was found to be suitable as a selective agent attempts to isolate mutants defective in L-alanine transport were not possible by the analogue resistance technique.

8.2.4. L-[^{3}H] alanine and L-[^{3}H] glutamate toxicity to BHK21-C13 cells, and results of selection procedures using these radioactive amino acids as selective agents

The toxicities of L-[^{3}H]glutamate and L-[^{3}H]alanine were determined, prior to their use as selective agents for the isolation of L-glutamate and L-alanine transport defective mutants in BHK21-C13 cells. The toxicity of L-[^{3}H] alanine, for the concentration range 0.5 to 5μCi/ml, is shown in Figure 8.2.4a. Figure 8.2.4b shows the results obtained for temperature sensitive (ts) and non-temperature sensitive (non-ts) selections using L-[^{3}H] glutamate. Similar "kill" curves were obtained for both radioactive amino acids, and little difference was observed between the ts and non-ts selections for L-[^{3}H]glutamate toxicity.

Table 8.2.3. Toxicity of D-neutral amino acids or amino acid analogues to BHK21 - C13 cells and their effectiveness as inhibitors of L-alanine transport.

Amino Acid Analogue	% Inhibition of L-glutamate uptake*	Concentration to produce 50% inhibition of colony formation (mM)	First concentration to produce toxic effects on dense cultures (mM)
N-methyl-DL-alanine	86	10	NT
N-acetyl-DL-alanine	7	9	NT
∝-alanine hydroxamate	15	3	10
∝-AIB	81	8	NT
∝-methyl-serine	87	9	NT
DL-alanine-methyl-ester	10	10	
D-alanine	21	10	NT
D-serine	24	7	10

*Data from Table 5.2.7b.

Uptake of L-[2,3 -^{3}H] alanine (1 x 10^{-4}M) was examined in the presence of D-amino acids (2.5 x 10^{-3}M) or DL-amino acid analogues (5 x 10^{-3}M). Determination of the toxicity of amino acid analogues by plating procedures was carried out as described in Section 2.12. Toxicity of analogues to dense cell cultures was indicated by marked cell detachment.

NT indicates not tested.

Figure 8.2.4a. Survival curve for BHK21-C13 cells exposed to L-[^{3}H] alanine (ts selection procedure)

Cells in log phase growth were harvested and 2000 cells inoculated into 25 cm^2 flasks containing 2 mls EFC_{10}. Cells were allowed 4 hours for attachment and L-[2,3 - ^{3}H] alanine added to a final concentration of 0.5 to 5 uCi/ml. Cells were incubated at 39°C, in the presence of radioactive amino acid for intervals up to 3 hours, after which the cells were washed and the medium replaced by Plating Medium. Cells were then incubated at 25°C for 5 days, transferred to 34°C and then colonies stained and counted (see Section 2.11) at 24 days. The experiment was carried out in triplicate.

L-[2,3 - ^{3}H] alanine concentration:
- 0.5 µCi/ml (△–△)
- 1.0 µCi/ml (□–□)
- 2.0 µCi/ml (○–○)
- 3.0 µCi/ml (▽–▽)
- 5.0 µCi/ml (●–●)

Figure 8.2.4a. Survival curve for BHK21-C13 cells exposed to L-[^{3}H] glutamate (ts and non-ts selections).

Cells in log phase growth were harvested and 2000 cells inoculated into 25 cm^2 flasks containing 2 mls EFC_{10}. Cells were allowed 4 hours for attachment and L-[^{3}H] glutamate added to a final concentration of 0.5 to 5 uCi/ml. Ts selection procedures were carried out as described in the legend to Figure 8.2.3a. For non-ts selections all procedures were carried out at 37°C, except for incubation at 25°C as above. Cells for non-ts selections were counted at 24 days.

L-[^{3}H] glutamate concentrations:

	ts selection	non-ts selection
0.5 µCi/ml	(▲–▲)	(△–△)
2.0 µCi/ml	(■–■)	(□–□)
5.0 µCi/ml	(●–●)	(○–○)

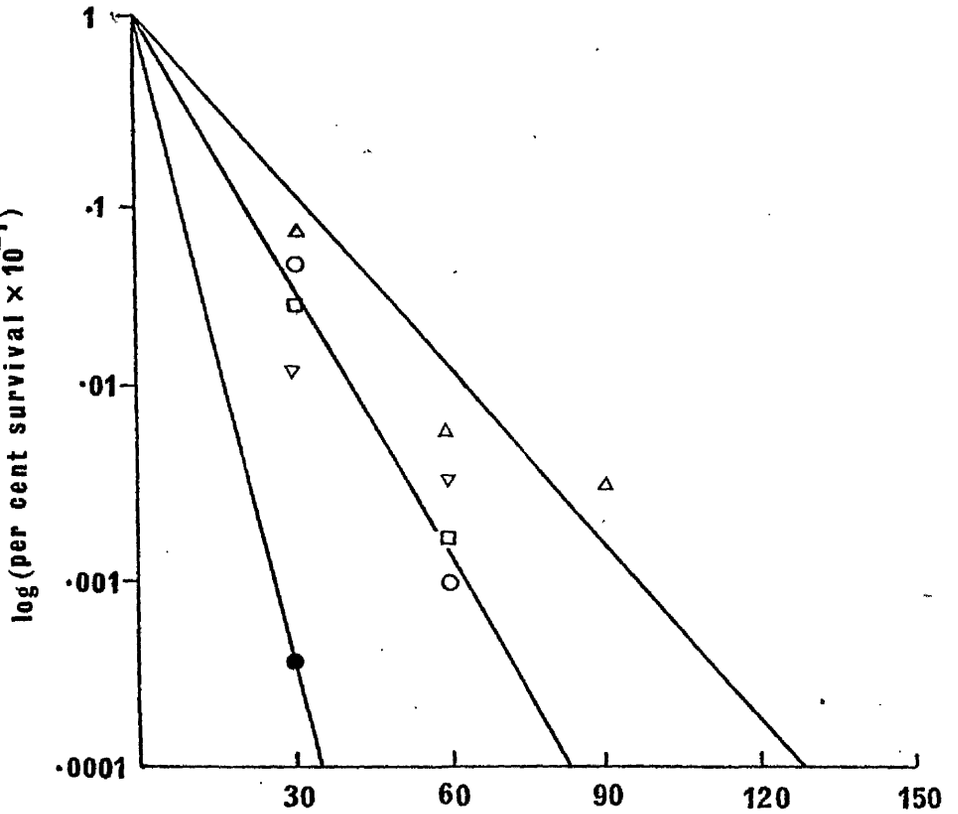


Figure 8.2.4a. Survival curve for BHK21-C13 cells exposed to L-[^{3}H] alanine

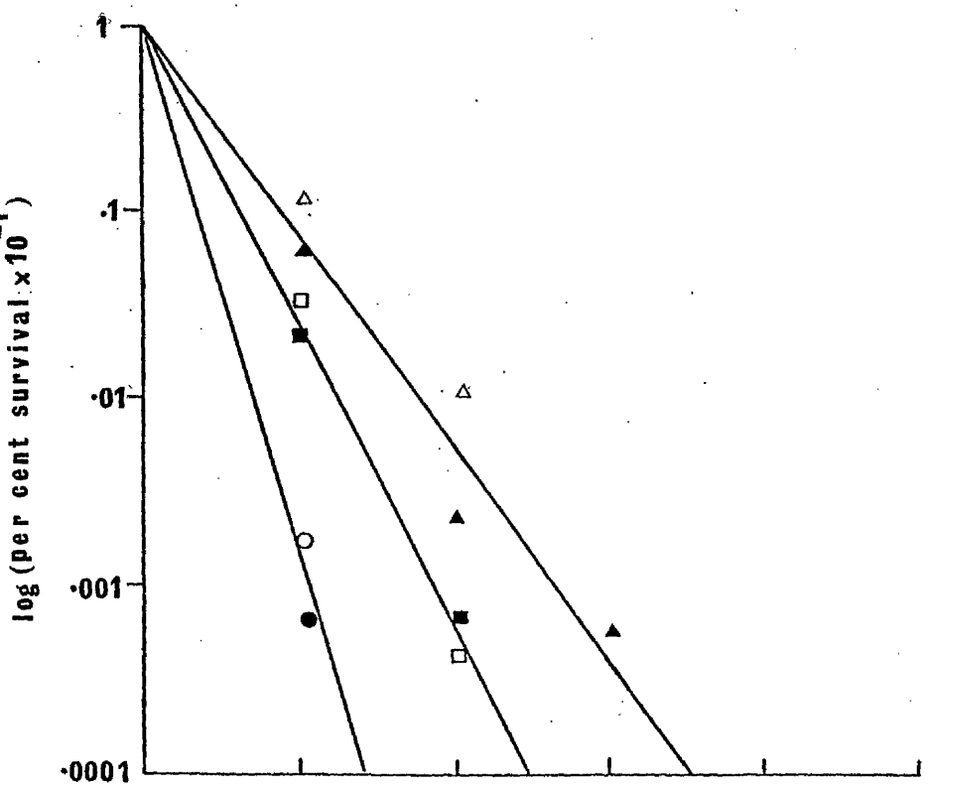


Figure 8.2.4b. Survival curve for BHK21-C13 cells exposed to L-[^{3}H] glutamate

Use of 5μCi/ml radioactive L-glutamate and L-alanine produced almost complete "kill" of these cells with a 30 minute exposure, compared with 60 to 90 minutes for 1 to 3 μCi/ml for L-[^{3}H]alanine and 2μCi/ml for L-[^{3}H]glutamate, and between 90 and 130 minutes for 0.5 μCi/ml. A 2 hour exposure interval was chosen for facility of operation, using 2μCi/ml of radioactive amino acid. Prior to the actual selection procedure cells were exposed to 8.5 mM EMS for 2 hours at 37° for the non-ts selection procedure, and 6 hours at 34°C (the permissive temperature) for ts selections (time intervals shown to produce approximately 50% "kill" at these temperatures, see Figures 8.2.1a and b). The cells were then maintained for 40 or 60 hours at 37° or 34°C, for non-ts and ts selections respectively, in order to allow approximately 3 cell divisions, a condition shown to be optimal for full expression of induced mutant phenotype (Chu and Malling 1968; Orkin and Littlefield 1971). Cells for the ts selection were then transferred to the non-permissive temperature (39°) for 24 hours in order to allow expression of the mutant phenotype. Following a 2 hour exposure to the selective agent the cells were incubated at 25°C, in order to prevent rapid cell division, the consequent overgrowth of cells, and dilution of incorporated radioactive amino acids into new cells. The cells were maintained at this temperature for 5 days to allow cells that had incorporated radioactive amino acid to be killed by irradiation. Cells unable to incorporate the radioactive amino acid should not have been killed. The cells surviving the ts selection were then transferred to the permissive temperature (34°), at which the mutation should not be expressed. Prior to examination of the cells at the non-permissive temperature (39°) cells were incubated at 39°C in order to allow phenotypic expression of the transport defect. Cells from non-temperature sensitive selections, following incubation at 25°C, were transferred and maintained at 37°C. A number of colonies were obtained following the above ts selection procedures. However, none of the colonies selected with either L-[^{3}H]glutamate or L-[^{3}H]alanine showed

Table 8.2.4. [^{3}H]- Amino acid selections

a) [^{3}H]- Glutamate suicide selections

No. of Experiments	No. of cells seeded after EMS treatment	Estimated viable cell number	No. of Colonies	No. defective in L-glu uptake.
4 (non ts selection)	6×10^7	3×10^7	12	0
2 (ts selection)	2×10^7	1×10^7	4	0

b) [^{3}H]- L-Alanine suicide selections

				No. defective in L-ala uptake.
4 (ts selection)	8×10^7	6×10^7	28	0
4 (2nd round ts selection)	4×10^7 *	3×10^7	6	0

* Indicates cells derived from colonies of 1st round ts selection procedure.

Selection procedures and L-[U-^{14}C]glutamate uptake experiments were carried out as described in the text and Sections 2.13.3. and 2.8. respectively.

amino acid transport different from wild type cells at the non-permissive ($39^{o}C$) or permissive temperature ($34^{o}C$). Cells put through a second L-[^{3}H]alanine selective procedure, similarly showed no altered L-alanine uptake. Results of these "^{3}H-suicide" selections are shown in Table 8.2.4.

8.3. DISCUSSION AND CONCLUSIONS

One of the most powerful techniques for obtaining amino acid transport mutants in bacteria (Kay 1971; Halpern and Umbarger 1961; Rosen 1971a; Kessel and Lubin 1965), fungi (Sinha 1969; Hochberg *et al.* 1972; Lester 1966; Stadler 1966; Wolfinbarger and De Busk 1971), and yeast (Grenson 1966a,b;Gits and Grenson 1967; Grenson and Hennaut 1971), has been the selection of mutants resistant to toxic amino acid analogues due to the inability of these cells to transport these analogues or the normal substrate. A number of mammalian cell lines resistant to the toxic effects of various compounds have been isolated that have been subsequently shown to owe their resistance to transport defects, for example: Actinomycin D in HeLa cells (Goldstein *et al.* 1966), amethopterin in a mouse leukaemia cell line (Fischer 1962), ^{3}H thymidine in chinese hamster DON cells (Breslow and Goldsby 1969) and colchicine in CHO cells (Till *et al.* 1973). In addition to the inadvertant isolation of these transport mutations Till *et al.* (1973) have specifically attempted to isolate mutants altered in transport abilities. These authors selected for ouabain resistant cells and obtained two mutant cell lines which showed altered K^{+} transport. The first showed some ouabain insensitivity with regard to K^{+} transport, the transport of this ion being less severely inhibited in the presence of ouabain than in "wild type" cells, and the second mutant showed reduced K^{+} transport even in the absence of ouabain.

Most of the amino acid analogues examined in this chapter have

been demonstrated to be toxic to a variety of microorganisms (Shive and Skinner 1963), and certain of these have been used to isolate amino acid transport mutants in bacteria, e.g. DL-(threo)-β-hydroxy-aspartate (Kay 1971), 2-methyl-DL-glutamate (Halpern and Umbarger 1961) and D-cycloserine (Kessel and Lubin 1965) in E. coli; and D-serine in E. coli (Oxender 1972), Streptococcus st. Challis (Reitz et al. 1967) and A. nidulans (Kinghorn, personal communication, 1973). These analogues, however, with the marked exception of 4-fluoroglutamate were relatively non-toxic to BHK21-C13 cells. This may be due to the inability of most of these analogues to be effectively transported by the acidic or "L-alanine preferring" transport systems or, alternatively, due to their detoxification, protection by serum,or autolysis. Although 4-fluoro glutamate would appear to be ideal for the selection of mutants defective in acidic amino acid transport (as this analogue was shown to be both extremely toxic and transported by the acidic amino acid transport system) no cell lines defective in L-glutamate uptake were obtained, although one cell line, BHK21-FGR301, showed slight 4-fluoroglutamate resistance but normal L-glutamate uptake. In addition to this slightly resistant cell line a number of apparently "wild type" cells were obtained under the selective conditions. A number of explanations are possible for the apparent "escape" from killing by the selecting agent: a) the selection procedure may have been insufficiently severe to kill all "wild type" BHK21-C13 cells; b) the genotype may have been altered subsequent to the initial selection procedure between the time of selection and testing, or c) alternatively the cells may be capable of some form of short term "adapted" phenotypic resistance, which may be lost on removal of 4-fluoroglutamate. This latter suggestion would appear unlikely as maintenance of cells in the presence of low levels of 4-fluoroglutamate did not prevent "loss" of resistance.

Radioactive "suicide" techniques have also been used to isolate transport mutants in microorganisms. Harold et al. (1965), for example, using ^{32}P as a selective agent have isolated S. faecalis mutants which were defective in phosphate uptake. Till et al. (1973) have suggested that modification of such a technique may provide a method suitable for the selection of conditional-lethal mutations defective in metabolite transport. A number of cell lines showing temperature sensitive growth properties have been isolated from monkey BS-C-1 cells (Naha 1969), mouse L-cells (Thompson et al. 1970), chinese hamster CHO cells (Thompson et al. 1971) and BHK21-C13 cells (Meiss and Basilico 1972), although none of these mutations have been definitively shown to be altered in metabolite transport. However, no cell lines defective in amino acid transport were obtained from the ts or non-ts selection procedures involving the use of radioactive amino acids, described in this section. Similar explanations for the "escape" of apparently "wild-type" cells obtained following these procedures can be postulated as for the analogue resistance selection procedure, with the exception of adaptation. It is however, possible that at least some of these cell lines obtained following the ts selections may have been ts for a characteristic other than amino acid transport, as this was the only characteristic the cells were examined for.

The lack of success in the isolation of mutants defective in the acidic or "L-alanine preferring" transport systems, even following mutagenesis, would indicate mutation frequencies of less than 9×10^{-7} and 4.5×10^{-7} (for viable EMS treated cells) respectively for these systems. Much higher frequencies have been reported for other mutant characteristics in mammalian cells. For example: Thompson et al. (1971) have isolated ts mutants at a rate of 6×10^{-3} for mutagenised cells and

Chu and Malling (1968) report a mutation frequency for 8-azaguanine resistance of 2.6×10^{-5} for non-mutagenised cells and 1.5×10^{3} for mutagenised cells. The high mutation frequency for $HGPRT^{-}$ (azaguanine resistance) is probably due to the fact that this locus is X-linked and it may be that other ts mutations isolated at high frequencies may be similarly X-linked. High mutation frequencies may also be due to chromosome rearrangements leading to homozygosity for the gene affected, or partial or total deletions of the chromosome loci causing localised haploidy for the mutated genes. The apparently low frequency for transport mutant isolation may be due to homozygosity of BHK21-C13 cells for the normal permease gene and should the mutations be recessive, two mutations at this locus will be required before the altered phenotype can be detected. A further explanation for the lack of success in the isolation of acidic amino acid transport mutants may be that such a mutation is normally lethal, in which case no such mutants should be detectable using 4-fluoroglutamate resistance or non-temperature sensitive selection procedures. However, as this transport system has been demonstrated normally to transport only the non-essential amino acids, L-glutamate and L-aspartate, it would seem unlikely that the loss or alteration in the activity of this transport system would be lethal, unless such a mutation resulted in unfavourable alterations in the membrane surrounding this altered permease.

SECTION 9: CONCLUDING DISCUSSION

Amino acids play diverse roles in cellular metabolism as they provide the basic structural components of proteins and enzymes required for all aspects of cell metabolism. The regulation of their transport and metabolism consequently provides numerous potential sites for the regulation of cell metabolism or growth. Until recently amino acid transport studies in mammalian systems have been carried out using a number of *in vivo*, e.g. oral loading (Milne *et al.* 1960) and renal clearance (Crawhall et al. 1969), or *in vitro* techniques including the use of tissue slices or explants (Rosenberg *et al.* 1967) or Ehrlich ascites tumour cells (reviewed by Christensen 1969). The use of homogeneous populations of cultured mammalian cells for transport studies should provide a powerful additional tool for the characterisation of these systems both at the cellular and molecular level. Cells in culture provide a system which has certain advantages over "non-isolated" cell systems which have been used for transport studies. These advantages include a) a more rapid and complete contact between the cell population and compounds under study; b) a less complex two compartment system comprising incubation medium and intracellular space (extracellular space having been eliminated); and c) a more uniform composition of cells (Guidiotti *et al.* 1969). However, probably the greatest advantage of cultured cells is that they provide a system comparable to micro-organisms, whereby a genetic approach to transport studies is possible.

Studies reported in this thesis indicate the possible kinds of information that may be readily obtained about the amino acid transport systems in these cells: a) Classical kinetic studies of amino acid uptake may provide information as to the affinity, capacity and specificity of the transport system(s) involved in the uptake of amino

acids in these cells. These kinetic studies can be extended to include a comparison of amino acid transport in cell lines of different tissue or species origin, or in "normal" or transformed cells;
b) The examination of amino acid transport in cells following a variety of pre-treatments, e.g. growth or incubation in medium containing hormones or altered amino acid or serum levels; may provide information about the possible modes of regulation of these transport systems. Studies of this nature, which are reported in this thesis, have indicated in BHK21-C13 cells the existence of a relatively high affinity low capacity transport system specific for acidic amino acids, and a relatively low affinity, high capacity L-alanine preferring transport system which is apparently capable of transporting all neutral α-amino acids (Section 4.2 and 5.2). The presence of a second broad specificity neutral amino acid transport system was also indicated (Section 5.2.7). Similar acidic and L-alanine preferring transport systems were also observed in a number of transformed or untransformed cell lines of different species or tissue origin. Similar kinetic constants for amino acid uptake were obtained for each of the normal cell lines and their corresponding virus transformed cell lines (Section 7.2). "Regulatory studies" described in Section 6 have indicated that the L-alanine preferring transport system may be regulated by intracellular cyclic AMP levels, whereas the activity of the acidic amino acid transport system is apparently unaltered by changes in levels of this cyclic nucleotide.

These observations suggest possible future experiments which may provide additional information about the transport systems examined in this thesis, and possibly other amino acid transport systems present

in BHK21-C13 cells. The apparent difference in the regulation of the acidic amino acid and L-alanine preferring transport systems indicates some heterogeneity of control of amino acid transport systems. Examination of the transport of other amino acids following growth in the presence of dibutyryl cyclic AMP, or conditions shown to elevate intracellular cyclic AMP levels should indicate whether other amino acid transport systems are also controlled by cyclic AMP levels, or whether this regulatory mechanism is unique to the L-alanine preferring system. Cyclic GMP has been shown to antagonise the effects produced by cyclic AMP, both in vivo (Sutherland 1972) and in cells in culture (Kram and Tomkins 1973). Examination of the effect on L-alanine uptake of addition of cyclic GMP (or its dibutyryl derivative) to dibutyryl cyclic AMP treated cells or cells with elevated cyclic AMP levels, may indicate whether this second cyclic nucleotide is also involved in the regulation of the transport of this amino acid. Although the majority of the literature (Hare 1967; Eagle et al. 1961; Hatanaka et al. 1969; Hatanaka and Hanafusa 1970) and the studies reported in this thesis (Section 7.2.1) indicate that amino acid transport is not generally altered following virus transformation, increased transport of certain amino acids (or their analogues) has been reported (Foster and Pardee 1969; Isselbacher 1972). It is possible that the transport of only certain amino acids is increased following transformation. In order to determine whether this is indeed the situation the transport of other amino acids requires to be examined in a number of normal and virus-transformed cell lines.

The examination of amino acid uptake in cultured cells, purely by kinetic studies, does however have certain limitations, as although

amino acid uptake may appear to follow Michaelis-Menten kinetics, transport of an amino acid by more than one transport system generally cannot be excluded. Determination of the actual number of transport systems capable of transporting an amino acid has been possible in microorganisms by the kinetic analysis of both wild type cells and cells defective in the transport of the amino acid, e.g. the basic amino acids in S. cerevisiae (Grenson et al. 1966). Such an approach with cultured mammalian cells may yield similar information. Section 8 reports the attempted isolation of mutants, defective in the proposed 'acidic' or'L-alanine preferring' transport systems of BHK21-C13 cells, by "radioactive suicide" techniques or by selection for cells resistant to toxic amino acid analogues. The isolation of such mutants would have provided confirmation of these proposed systems and possibly indicated the presence and specificity of additional systems available for the transport of neutral amino acids. It is possible that the use of alternative toxic amino acid analogues, or use of combinations of analogues (apparently transported by the same system) may prove more successful for mutant selection. An approach alternative to the selection for amino acid transport mutants could be the setting up of cell lines from individuals with inborn errors of amino acid transport, such as Cystinuria, Dicarboxylic-aminoaciduria (see Section 1.10.2). It would be hoped that these cell lines would express the mutant phenotype in culture. Kinetic studies of amino acid uptake in transport defective and "wild-type" cells could provide information which may eventually enable the complete characterisation of the mammalian amino acid transport systems. Genetic analysis of these mutants by cell hybridisation techniques may also provide information as to the chromosomal location of the genes coding for these transport systems and the number of subunits in each system. Transport mutants may also provide valuable chromosomal markers and the isolation and genetic

analysis of mutants comparable to the inherited transport defects of man may provide a means for the early diagnosis of these defects. Transport defective mutants should also provide a means whereby mammalian transport proteins may be identified and isolated, thus enabling the study of amino acid transport systems at the molecular level. Identification and isolation of transport proteins by procedures similar to those described for bacteria e.g. by "differential labelling" or "substrate recognition" techniques (reviewed by Pardee 1969) should be possible.

Cell culture techniques should thus enable information to be obtained about mammalian amino acid transport systems that would be difficult, if not impossible, to obtain by other procedures.

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