The Effect of L-Glutamate on Growth of CC9C10 Hybridomas

By

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A Thesis Submitted in Partial Fulfillment of the Requirements of the University of Manitoba for the Degree of

MASTER OF SCIENCE

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Acknowledgments

To any and all, who in some way, be it large or small, assisted me with my research I thank .

I express deep gratitude to **Dr. Michael Butler** for making this project possible and for his help and guidance throughout my stay.

I am also grateful to **Norman Huzel** for his assistance with technical aspects of my research and running the HPLC on my behalf. A true technical wizard if ever there was one.

Norm Barnabe, Andrew Christie, Mike Berry for there assistance and insights into practical aspects of my research.

And to my loving wife **Puala**, to whom I am deeply indebted for her patience and understanding throughout the whole process.

Abstract

In attempts to grow CC9C10 hybridoma cells on less ammoniagenic media by substituting glutamate for glutamine, it was found that elevated glutamate levels were inhibitory to cell growth. Attempts to alleviate inhibition or adapt cells to growth were unsuccessful. It is proposed that elevated glutamate levels impair adequate uptake of cystine, a critical amino acid for the synthesis of glutathione. Glutathione is required by cells to prevent oxidative stress. It was found that CC9C10 hybridomas can take up glutamate from the external medium (k_m 20 mM and V_{max} 5 *n*moles/10^6 cells/min.) in a stereo specific, sodium independent manner. Of the amino acids examined, it was found that cystine and homocysteic acid were the most extensive inhibitors of glutamate uptake and that inhibition was competitive. The uptake of cystine was also found to be (k_m 0.87 mM, V_{max} 0.36 *n*moles/10^6 cells/min.) sodium independent, with elevated glutamate levels causing extensive inhibition. Metabolic profiles of the cells in elevated glutamate levels revealed an overall increase in net production of amino acids, with consumption remaining unchanged. Specifically, production of alanine, serine, asparagine and aspartate increased. Consumption of cystine, arginine, lysine, valine, isoleucine, and phenylalanine decreased, while the consumption of glutamate increased.

The combined kinetic and metabolic results indicate that glutamate and cystine are taken up by uptake system x^-_c. The increasing levels of glutamate in

the medium result in decreased consumption of cystine due to competitive inhibition by glutamate.

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List of Abbreviations

aas	amino acids
ABA	aminobutyric acid
Ac-cys	cysteine derivatized with iodoacetic acid
Ala	alanine
ALS	amyotrophic lateral sclerosis
Arg	arginine
Asp	aspartate
Asn	asparagine
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
BHK	baby hamster kidney cells
CS	Calf Serum
Cys	cystine
1/2 Cys	cysteine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DPBS	Dulbecco's phosphate buffered saline
DTT	dithiothreitol
EAAT	excitatory amino acid transporter
EBM	Eagle's basal medium
EDTA	ethylenediamine-tetraacetic acid
FBS	fetal bovine serum
FCS	fetal calf serum

Gln	glutamine
Glu	glutamate
Gly	glycine
GSH	glutathione, γ-glutamyl cysteinylglycine
HBC	Hepes buffered choline
HBS	Hepes buffered saline
His	histidine
HCA	homocysteic acid
HPLC	high preformance liquid chromatography
IAA	iodoacetic acid
Ile	isoleucine
k_m	Michaelis constant
Leu	leucine
Lys	lysine
2-ME	2-mercaptoethanol
Met	methionine
NADPH	nicotinamide-adenine dinucleotide phosphate
OPA	orthophthaldialdehyde
Phe	phenylalanine
Pro	proline
S.E.M.	Standard error of the mean
Ser	serine
SFM	serum free media (DMEM/F-12 - SFM)
TCA	tricarboxylic acid cycle
Thr	threonine

Tyr	tyrosine
V_{max}	maximum velocity

Chapter 1. Introduction

1. Introduction

1.1 Mammalian Cell Biotechnology Overview

In the past 40 years the use of mammalian cells to manufacture biological products has increased to a point where it now is an important sector of the biotechnology industry. The use of mammalian cells for production purposes dates back to 1949 when Enders and co-workers discovered that the poliomyelitis virus could be grown on mammalian cells in culture (Enders *et al.*, 1949). Five years later in 1954, Salk and colleagues developed a vaccine for polio using poliomyelitis viruses grown on monkey kidney cells (Salk & Salk, 1977). This firmly established the use of mammalian cells in industrial processes and with technological advances, due in large to recent advances in the field of molecular biology, mammalian cell lines are being increasingly used in diverse areas of production. Such areas include production of monoclonal antibodies for therapeutical and diagnostic use (Kohler & Milstein, 1975); production of commercially and therapeutically important proteins and/or enzymes such as interferon (Isaacs and Lindenmann, 1957), plasminogen activator (Kaufman *et al.*, 1985) and erythropoietin (Archer & Wood, 1992); and for medical purposes such as tissue grafts (Langer and Vasanti, 1993) .

Mammalian cells were not, however, universally accepted as the best systems for production of biologicals, primarily due to a lack of fundamental knowledge. Early on it was thought that any and all proteins could be produced

in microbial systems which were easier and cheaper to maintain. However, it quickly became apparent that not all proteins could be produced in microbial cell systems. Fundamental differences in the processing of genes into proteins (most notably splicing of introns), proper folding of the target protein into tertiary or quaternary states, secretion of the target protein into the media for easy recovery, and target protein glycosylation are some of the typical problems associated with microbial cell systems that result in protein inactivity or reduced protein half-life (Adamson & Schmidli, 1986). In order to alleviate these problems it soon became clear that mammalian cells would be required, and once this was acknowledged the field of mammalian cell biotechnology began to develop at a rapid pace.

1.2 Media Development

One reason mammalian cell lines were not readily accepted as vehicles for industrial processes was that initially there were no defined media capable of supporting cell growth. This meant that in the beginning, routine culture of mammalian cells in various biological extracts was akin to an art. Gradually, this began to change with the development of a basal media formulation by Eagle (1955), Eagle *et al.* (1958) for HeLa and mouse L cells. Eagle was the first to define essential media components and relative amounts, these included carbohydrates, amino acids, vitamins, inorganic ions and dissolved gases. However, although this was a step in the right direction, in order to obtain

significant growth and cell yields the addition of animal sera was required. However, it was not known which serum components were required by the cells. In some cases, but increasingly less, animal serum is still in use today. Problems with sera included lot to lot variation, downstream processing and recovery in industrial processes. Other major problems included possible contamination by viruses, mycoplasma and prions all which made for valid reason to become independent of serum addition to basal media (Barnes & Sato, 1980; Butler, 1986; Stoll *et al.*, 1996).

In order to move away from serum based media, much work and development has gone into finding out specifically what the cells require from serum. It is now known that serum contains growth factors, hormones, protection from shear force, buffering capacity and in some cases nutrients (Freshney, 1987; Butler, 1987; Stoll *et al.*, 1996). Knowing this is, it has been possible to formulate serum free media (SFM) for many different cell lines. One of the major problems associated with SFM, is that there is no one universal medium on which all cells will grow. It has been found that almost every cell line requires a different set of growth factors, hormones or attachment factors for optimal cell growth or yield (Murakami, 1989). However defined media allows simple design and interpretation experiments, hormone and drug studies and nutritional studies (Barnes & Sato, 1980).

Although SFM is a step in the right direction it does not entirely alleviate all of the problems associated with serum, it merely reduces them. For instance,

SFM still contains protein additions to the basal media, making downstream recovery of target proteins in industrial processes more difficult. Another problem associated with SFM is the cost of the media itself, pure growth factors, hormones and attachment factors can be extremely expensive in large scale applications (Jayme & Gruber, 1994). This has led to the development of protein free media, but these are still very much in their infancy (Clevland *et al.*, 1983, Stoll *et al.*, 1996). The main benefit of a protein free media, is in the downstream recovery of product.

1.3 Cellular Metabolism and the Importance of Glutamine

The underlying motif for industrial use of mammalian cells, must surely be that of cellular metabolism. Without a fundamental understanding of the metabolic processes which occur within the cell, it is comparatively difficult to adequately design and manipulate a system with an end goal of production (Kromenaker & Srienc, 1994). At any one time there exists, within any heterotrophic aerobic cell, three main type of metabolic processes (Figure 1.1; Atkinson, 1977; Glacken, 1988; Gaertner & Dhurjati, 1993):

(i) *Catabolic metabolism*: Foods are oxidized to carbon dioxide. By way of the glycolytic pathway and citrate cycle energy is produced in the form of ATP, and NADPH regenerated. These same sequences, with the pentose phosphate pathway, also supply the starting materials for all of the cell's biosynthetic processes.

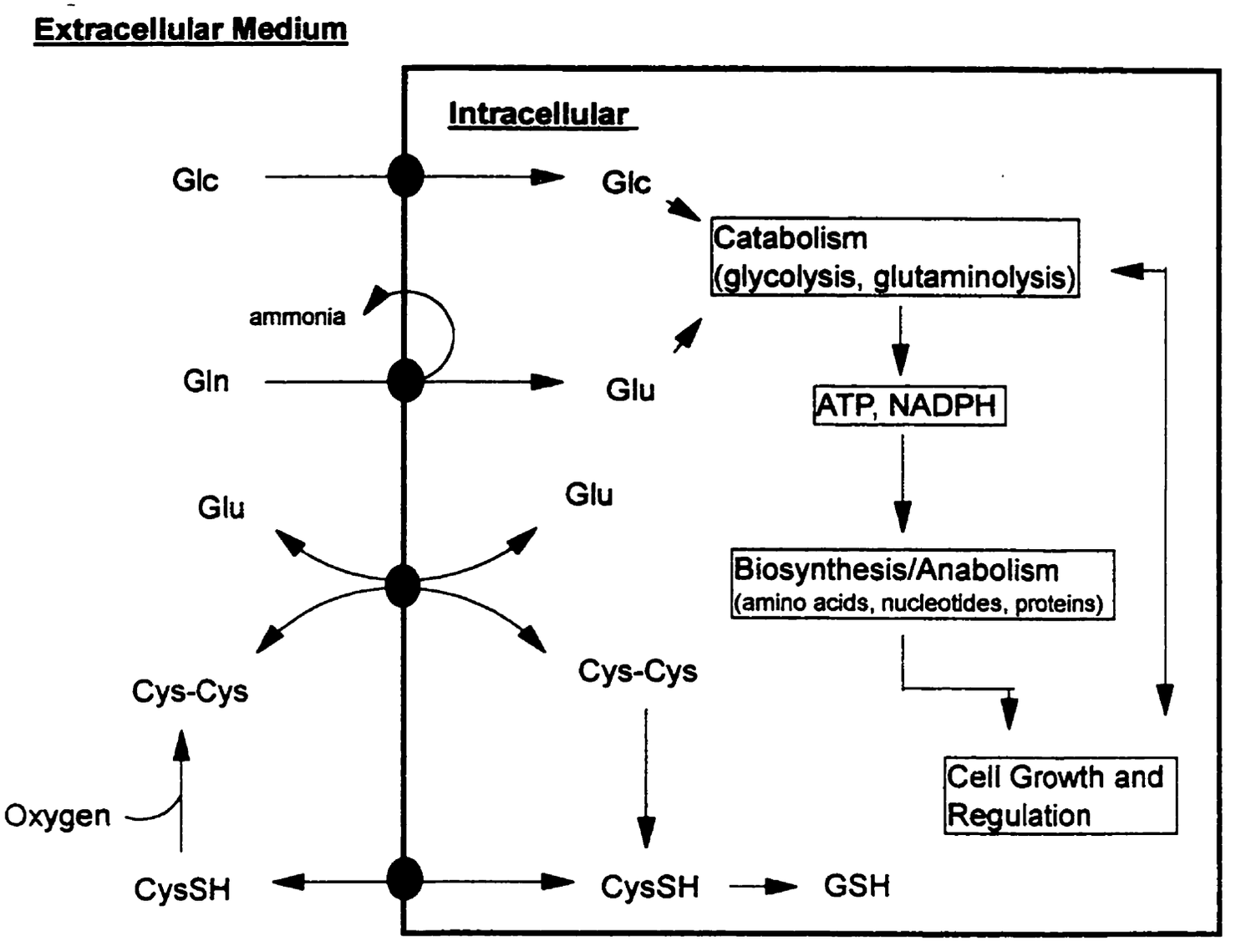
(ii) *Biosynthesis*: Utilization of products from glycolysis and citrate cycle to produce hundreds of cell components. NADPH used as a reducing agent when necessary, and ATP as a coupling or energy producing agent.

(iii) *Growth and Macromolecule Synthesis*: Components made in biosynthesis are utilized for synthesis of complex macromolecules on which biological functionality depends (i.e. proteins, nucleic acids components of membranes etc.). This is where the major portion of a cell's energy is utilized.

These are important concepts that require better understanding, for instance if the expression of an amplified recombinant gene is being studied or produced, it is possible that the energy required to synthesize this protein could constitute a significant proportion of the cell's energy, which in turn could lead to a reduction in cell density (Glacken, 1988).

Accordingly, knowing where and how a cell obtains it's source(s) for catabolic metabolism is influential for the overall cell health and product formation and recovery. Generally mammalian cells derive their energy from two primary sources carbohydrates and amino acids, namely glucose and glutamine respectively (Butler and Jenkins, 1989). The fate of glucose, once transported into the cell can be attributed to two primary pathways: 1) glucose can either be metabolized by the pentose phosphate pathway to produce nucleotides (Zielke *et al.*, 1984; Petch & Butler, 1994) or 2) can undergo glycolysis where it is converted to lactate or acetyl-CoA which can then be used in the production of

Figure 1.1: Primary catabolic pathways associated with glucose and glutamine utilization in mammalian cells and relative fates. (Adapted from Glacken, 1988; Bannai & Ishii, 1988; Bannai, 1984; Kang *et al.*, 1994; Atkinson, 1977)



lipids or are oxidized in the citrate cycle (Butler, 1987; Gaertner & Dhurjati, 1993). The majority of glucose (95%) which enters hybridoma cells is utilized in the glycolytic pathway, evidence has shown that only a small proportion (3.5%) is utilized in the pentose phosphate pathway and an even smaller proportion (0.6%) enters the citrate cycle (Fitzpatrick *et al.*, 1993; Petch & Butler, 1994). These proportions are known to fluctuate depending on the extracellular concentrations of glucose and glutamine (Gaertner & Dhurjati, 1993). For example, it has been shown that if the extracellular glucose concentration is increased there is an observed corresponding increase in the conversion of glucose to lactate, a reduction in the rate of glucose shunted into the pentose phosphate pathway and a reduced rate of glutamine oxidation and oxygen uptake (Glacken, 1988; Miller *et al.*, 1989; Wohlpart *et al.*, 1990; Gaertner & Dhurjati, 1993). It is generally estimated however, that 59% of cellular energy in hybridoma cells is derived from catabolic metabolism of glucose (Petch & Butler, 1994).

The major by-product of glucose utilization is lactate, which is generally believed to exhibit inhibition of cell growth and cell product formation at levels greater than 40 mM (Glacken, 1988; Ozturk *et al.*, 1992; Kromenaker & Srienc, 1994). The amount of lactate produced by glycolysis, has been shown to vary with respect to changes in glucose concentration, cellular activity, and bioreactor activity, and has been reported to be as a high as 25 mM in some hybridoma lines (batch mode; Hassel *et al.*, 1991; Ozturk *et al.*, 1992). Lactate

has been shown to reduce cell growth by lowering the pH of the medium, as mammalian cells only grow well within a narrow pH range (Glacken, 1988).

Amino acids constitute one of the largest groups of compounds actively acquired by mammalian cells. The diverse uses of amino acids within the cell, include direct participation in production of energy, and synthesis of structural proteins and enzymes linked to all aspects of cellular metabolism (Patterson, 1979). The discovery of the ability of mammalian cells to concentrate amino acids dates back to early experiments performed by Van Slyke and Meyer in 1913 (Heinz, 1972). However, characterization of the systems responsible for amino acid transport remained undiscovered until the 1950's when Christensen and his co-workers laid down the basis of amino acid transport systems in Ehlrich ascites carcinoma cells (Christensen *et al.*, 1952). It has been shown that there exists a number of systems which exhibit a large degree of overlap for structurally similar amino acids and possess the characteristics of carrier-mediated transport systems (refer to Table 1.1). Active amino acid uptake in mammalian cells, in general , follows saturation kinetics, and can be expressed by the Michaelis-Menten relation, although the estimates of k_m and maximal flux velocity may not be entirely reliable (Heinz, 1972; Christensen, 1989).

Glutamine is undoubtedly one of the most critical amino acids that cells concentrate. It was not until the early 1970's that glutamine was implicated as an important respiratory fuel (Kovacevic & Morris, 1972; Regan *et al.*, 1973). In

Table 1.1: Summary of mammalian uptake systems known to date, substrate specificity and associated kinetic parameters. (Adapted from Kilberg *et al.*, 1993; Christensen, 1989; Rennie *et al.*, 1995)

System	Substrates	Capacity V_{max} nmoles/10^6 cells/min	Affinity k_m (mM)	Sodium Dependence
Gly	Gly, sarcosine	N.A.	N.A.	Yes
A	Pro, Gly, Ala, Met, Gln	Low (<10)	High (<1)	Yes
ASC	Ala, Ser, 1/2Cys, Thr, Gln	Medium (40)	Medium (3)	Yes
$B^{0,+}$	cationic and zwitterionic aas	High (80)	N.A.	Yes
$b^{0,+}$	cationic and neutral aas	Medium (40)	N.A.	No
N^m	Gln, Asn, His 3-Me His	High (100)	Low (8)	Yes
L	Leu, Phe, 1/2 Cys	Very High (300)	Very low (20)	No
y^+	Arg, Lys	Medium (60)	High (<1)	No
X^-_{AG}	Glu, Asp	Low (8)	High (1)	Yes
x^-_C	Glu, Cys	Low (5)	High (<1)	No

N.A. not available

fact it has been shown that glutamine (present at 5- to 20-fold higher concentration than other amino acids) can provide all the necessary energy required by cells under depleted glucose conditions (Reitzer *et al.*, 1979; Ziekle *et al.*, 1984; Jeong & Wang, 1995). The fate of glutamine, upon entering cells, can be summed up as follows: 56% converted to CO_2; 17% utilized in macromolecules; 14-18% appears in the form of glutamate; and 10-20% is converted into lactate (Reitzer *et al.*, 1979; Zielke *et al.*, 1984; Glacken, 1988). The processes by which glutamine is oxidized to CO_2, catabolized to lactate, or incompletely oxidized to alanine or aspartate was coined glutaminolysis by McKeehan in 1982 due to proposed similarities between it and glycolysis (refer to Figure 1.1; McKeehan, 1982; Jeong & Wang, 1995). Like glucose, glutamine is readily permeable to the cell membrane and both are actively acquired by cells. Metabolites derived in both glycolysis and glutaminolysis are less permeable than glucose and glutamine and are essentially trapped within the cells (McKeehan, 1986).

It is quite apparent then, that mammalian cells derive a significant proportion of their cellular energy from the catabolic metabolism of glutamine. However there exists several problems directly associated with the use of glutamine as an energy source. The first is that glutamine itself is chemically unstable and spontaneously degrades to pyrrolidone carboxylic acid and ammonia (0.2 to 0.6 mM day^{-1}) at 37°C (Bray *et al.*, 1949; Tritsch & Moore, 1962; Butler & Christie, 1994). Significant degradation of glutamine has also

been reported even at 4°C, which limits shelf life of the media and makes sterilization by autoclaving impossible (Tritsch & Moore, 1962). The catabolic utilization of glutamine results in one to two moles of ammonia released by a deamination, and then a transamination step (refer to Figure 1.1). The accumulation of ammonia in the external medium due to enzymatic and chemical degradation of glutamine has been shown to be inhibitory to final cell yields and has also been implicated in reduced product formation (Butler *et al.*, 1983; Butler & Spier, 1984; Glacken, 1988). Much work has been done in attempts to identify and circumvent the problems associated with glutamine usage. Current theories on ammonia toxicity include: disturbance of electrochemical gradients (Glacken, 1988), inhibition of enzymatic reactions (i.e. deamination of glutamate to α-ketoglutarate by glutamate dehydrogenase; Glacken, 1988), intracellular pH shifts (Glacken, 1988; McQueen & Bailey, 1991) which could result in proton gradient disturbances and inhibition of endocytosis and exocytosis (Docherty and Snider, 1991), and an increased demand for maintenance of energy (Martinelle & Hagstrom, 1993). Methods by which the toxic effect of ammonia can be circumvented or alleviated include: enzymatic removal of ammonia by aspartase (Butler, unpublished) or glutamate dehydrogenase (Glacken, 1988), ion exchange strategies (Capiaumont *et al.*, 1995), use of dipeptide in place of glutamine (Christie & Butler, 1994; Butler & Christie, 1994), or adaptation to replacement of glutamine by glutamate (Hassell & Butler, 1990; Hassell *et al.*, 1991). The latter is central to the theme of this thesis. The potential benefits of

replacing glutamine with glutamate are: 1) That it is a relatively simple procedure; 2) It would increase the half life of media significantly; and 3) Has the potential to reduce ammonia production.

1.4 Importance of Glutamate Metabolism and Uptake

Upon entering into the cell glutamine is rapidly converted to glutamate via deamination by glutaminase. In terms of intracellular amino acid pools, glutamate has been shown to be as high as 30 mM or 10- to 20-fold that of other amino acids, depending upon cell line and culture conditions (Reitzer *et al.*, 1979). Glutamate is therefore one of the most important amino acids. It not only plays a central role in cellular catabolic metabolism, but also is one of the major excitatory neurotransmitters (Robinson & Coyle, 1987). In the brain glutamate actively participates in the cytotoxicity of injured brain tissue (Staub *et al.*, 1993). And finally glutamate lends itself to direct use in protein synthesis, and as a precursor for glutathione synthesis (Dall'Asta *et al.*, 1983).

Within mammalian cells, it is generally agreed that there exist at least three alternate catabolic fates for glutamate once it has entered the mitochondria (Moreadith & Lehniger, 1984; Glacken, 1988, Bonarius *et al.*, 1996). All three pathways involve the conversion of glutamate to α-ketoglutarate: The first is a deamination of glutamate by glutamate dehydrogenase to generate α-ketoglutarate and ammonia, the second pathway involves aspartate aminotransferase where glutamate and oxaloacetate are transaminated to

produce α-ketoglutarate and aspartate, and the final pathway involves glutamate, pyruvate and alanine aminotransferase to produce α-ketoglutarate and alanine. Of the three possible fates, it has been shown that production of α-ketoglutarate via glutamate dehydrogenase is either extremely small or that the reaction proceeds in the reverse direction making this an unlikely pathway (Kovacevic & McGivan, 1983; Glacken, 1988). This seems logical, as production of alanine or aspartate as end products in these pathways is less toxic to the cells than ammonia. So, the majority of energy produced within the mitochondrial matrix is derived from aminotransferase transaminations with the subsequent production of aspartate or alanine. α-Ketoglutarate then enters into the citrate cycle where it is used to produce cellular energy.

If glutamate is to be used in place of glutamine, then prior to its fate as a catabolic metabolite, glutamate must be taken up from the external medium into the cell. Much of the work to date on elucidating glutamate transport has been accomplished with medicinal purposes in mind. Early work in non-neuronal cells lines were devised at revealing the causes of cystinuria, a disease associated with the excretion of large quantities of cystine, arginine and lysine, and also cystinosis, a disease associated with cystine storage which can lead to renal failure (Groth & Rosenberg, 1972; DeBrohun-Butler & Spielberg, 1981). In later years much work on glutamate transport has evolved due to interests in nitrogen turn-over within cells and also due to the implication of glutamate as a major catabolic metabolite. The first published existence of anionic amino acid

transporters dates to 1980 when Bannai & Kitamura described the uptake of cystine and glutamate in human diploid fibroblast (Bannai & Kitamura, 1980). This anionic system was initially designated CG, later changed to x^-_C, and was shown to be strictly anionic, specifically for L-glutamate and L-cystine, but not L-aspartate (Bannai & Kitamura, 1980). The system x^-_C is a high affinity, high velocity system and is reported as being sodium ion independent. Bannai hypothesized that the anionic form of glutamate was taken up in exchange for intracellular cystine on a one to one basis (Bannai, 1986). Uptake system x^-_C has also been shown to be up-regulated by starvation as indicated by an increase in protein synthesis, and in terms of glutamate uptake, independent of pH over a range of 6-8 (Bannai and Kitamura, 1982; Forster & Lloyd, 1985; Bannai, 1984).

Since the discovery of system x^-_C, it is now believed that there exists up to three other means by which glutamate uptake can occur (Dall'Asta *et al.*, 1983). The first system, designated X^-_{AG} has been shown to be sodium ion dependent, specific for L-glutamate and L-aspartate, and is a high affinity, low velocity system (Gazzola *et al.*, 1981). In system X^-_{AG} the inward movement of glutamate is coupled to the inward movement of 2 ions of sodium from the external environment (and in some cases an ^-OH or HCO_3^- ion), and the outward movement of one potassium ion from the intracellular space (Sato *et al.*, 1994). The uptake of glutamate was also shown to occur via system ASC at low pH. System ASC is a sodium dependent neutral amino acid uptake system for

alanine, serine and cysteine (Gazzola *et al.*, 1981). The final system by which glutamate can enter the cells was tentatively shown to be via system L, which is a sodium ion independent system specific for leucine. It is thought that uptake of glutamate by this system occurs when glutamate is in its zwitterionic form (Bannai & Kitamura, 1981; Dall'Asta *et al.*, 1983). In culture medium pH ranges from 6.8 to 7.4 under growing conditions, within these ranges a small portion of the glutamate in the medium will exist in the zwitterionic form, however the major proportion will be in its anionic form due to the low pK_a of the carboxyl group and it is believed that this is the form in which glutamate it taken up. Of the four possible uptake systems implicated in glutamate transport, system x^-_C accounts for 20-30% of glutamate uptake and X^-_{AG} accounts for 60% in non-neuronal cell lines, however the relative amount taken up by each system is known to vary with culture conditions (starved vs. non-starved, high density vs. low density; Bannai & Kitamura, 1982).

The other major area of work on glutamate uptake has centered around nervous system cell lines and has focused on the role of glutamate in acute cerebral ischemia, epilepsy, metabolic disorders, brain trauma (Staub *et al.*, 1993). In the brain, glutamate levels are present at 100- to 1000-fold that of the extracellular levels (Robinson *et al.*, 1993). In the central nervous system four isoforms of the Na^+-dependent, high affinity glutamate transporter X^-_{AG} (EAAT1, EAAT2, EAAT3 and EAAT4) have been identified and recently isolated and cloned (Kanai *et al.*, 1995; Nicholson & McGivan, 1996; Rothstein *et al.*, 1996).

The distributions of these isoforms has been worked out by immunochemistry: EAAT3 has been shown to be inducible by increased extracellular osmolarity (Ferrer-Martinez *et al.*, 1995) and is distributed primarily in the brain, but also was found to occur in the kidney and gut; EAAT2 has been pegged as a glial transporter and has been shown to be confined to specific parts of the brain, central nervous system, and placenta; EAAT1 is thought to be confined to the brain, retina, heart and skeletal muscle; and finally EAAT4 has recently been identified in the cerebellum and placenta (Garlin *et al.*, 1995; Nicholson & McGivan, 1996). It has been shown that increases in the extracellular sodium concentration result in increased k_m's and V_{max}'s for system X^-_{AG}, however this effect is most likely a result of induced activity by increased extracellular osmolarity (Robinson *et al.*, 1993; Ferrer-Martinez *et al.*, 1995). Defects in the high affinity sodium uptake system X^-_{AG} , specifically loss of isoform EAAT2, have been associated with amyotrophic lateral sclerosis (ALS), a neurodegenerative disease (Kanai *et al.*, 1994; Rothstein *et al.*, 1996).

Much of the work to date has centered around the sodium dependent system X^-_{AG}, with almost no assessment to the role of system x^-_C. However recently work has begun to assess the role of a second type of glutamate transporter which was Cl^- dependent. This system was shown to exist in synaptic membranes and glial cells (Koyama *et al.*, 1995; Wolosker *et al.*, 1996). The similarities between system x^-_C, and this system in the brain have led to the belief that the function of the Cl^- dependent system is that of a heteroexchange

of glutamate and cystine much the same as system x^-_C (Koyama *et al.*, 1995). As with system x^-_C, up-regulation occurs under ischemic conditions (low O_2 and low glucose), however protein synthesis was not a requirement indicating that post-translational modification could regulate this system (Koyama *et al.*, 1995). This would also seem to indicate the possibility of different isoforms for this uptake system, much the same as system X^-_{AG}.

Although the primary elucidation of glutamate transport has been by the medical profession, the importance of glutamate transport for industrial cell culture purposes should not be overlooked.

1.5 Importance of Cystine Metabolism and Uptake

In the late 1970's, Bannai and colleagues demonstrated that cystine was an essential amino acid, and that without it cells did not survive (Bannai *et al.*, 1977; Bannai & Kitamura, 1982). Since Eagle's early 1950's publication of essential amino acids, including cystine, cystine metabolism and transport have remained largely unstudied owing to the inherent difficulties associated with its chemical properties. Of all the amino acids, cystine is the least soluble and in a pH neutral saturated solution at 25°C it is present at 0.467 mM (Bannai, 1984). Cysteine, the reduced form of cystine, is readily soluble but has been shown to be toxic to certain cell lines, and is rapidly oxidized to cystine under culture conditions (Debrohun-Butler & Spielberg, 1981; Bannai, 1984). Both cystine and cysteine have been shown to undergo sufhydryl exchange with proteins in the

extracellular medium, which has made it difficult to study their metabolism under physiological or serum based culture conditions (Bannai, 1984). Finally, both cystine and cysteine are not readily detectable in amino acid analysis as they fail to form products with ninhydrin or OPA (Walker & Mills, 1995).

In 1977 Bannai and colleagues were the first to examine the physiological importance of cystine. They found that cystine was essential in human embryonic diploid cells and the majority of non-protein thiol was accounted for by glutathione (γ-glutamyl cysteinylglycine, GSH; Bannai *et al.*, 1977). Much work has since been done to elucidate the function and regulation of glutathione and the involvement of cystine and glutamate as essential amino acids in its' biosynthesis. The majority of research to date has primarily centered around fibroblastic cell lines derived from liver or kidney which exhibit a marked affinity for cystine uptake (Meredith & Williams, 1986). On the other hand, blood and lymphoid cell lines remain largely unexamined due to poor uptake of cystine (Bannai, 1984).

Glutathione is a biologically important tripeptide (composed of glutamate, cysteine and glycine), which has been implicated in cellular defense against oxidative damage (Bannai *et al.*, 1977), and as a means of establishing a non-toxic pool of cysteine within the cell (Meredith & Williams, 1986). Glutathione is synthesized (refer to Figure 1.1) in the cell when glutamate and cysteine are coupled through γ-glutamylcysteine synthetase by an amide bond at the γ-carboxyl of glutamate (Creighton, 1993). Then glutathione synthetase adds

glycine by an ordinary peptide bond forming GSH which is then thought to be transported out of the cell by membrane bound γ-glutamyl transpeptidase (Bannai, 1984; Sweiry *et al.*, 1995). The transport of GSH out of the cell is thought to be coupled to the inward transport of γ-glutamyl amino acids which are formed on or near the membrane, and released into the intracellular space (Sweiry *et al.*, 1995). There is a significant amount of controversy surrounding the proposed mechanism of γ-glutamyl transpeptidase as a means of amino acids transport and this has yet to be resolved (Sweiry *et al.*, 1995). The γ-glutamyl amino acid and cysteinyl-glycine are released into the intracellular space and are then recycled back into GSH which is released from the cell completing the cycle.

The uptake of cystine by mammalian cells lines is mediated solely by system x^-_c, which has been shown to have a greater affinity for cystine uptake than for glutamate uptake (Bannai, 1984; Murphy *et al.*, 1989). Glutathione is thought to mediate uptake by this system, as removal of glycine and serine from the extracellular medium promoted glutamate and cystine uptake (Bannai & Kitamura, 1982). Unlike glutamate uptake, cystine uptake was found exhibit a marked dependence on pH with minimal uptake occurring at pH 6 to a 4-fold increase at pH 8 (Bannai & Kitamura, 1982; Forster & Lloyd, 1985). Within the pH range of 6 to 7, cystine exists primarily as zwitterion (electrochemically neutral), at pH 7.4 cystine exists at 70% the zwitterion form and 30% in the electrochemically negative form (net negative charge of -1) which is equivalent

to glutamate over the range of pH 6 to 8 and is the required form of system x_c^- (van Winkle *et al.*, 1992). At pH's near 8, the anionic form of cystine begins to dominate and comprises approximately 90% (Bannai & Kitamura, 1981; Bannai, 1984; Reynolds *et al.*, 1991). Upon entering the cells, cystine is rapidly reduced to cysteine and the mixed disulfide cysteine/GSH (Bannai, 1986). In order for cystine to be taken up from the medium, it has been shown that efflux of one mole of glutamate is required for the influx of one mole of cystine (Bannai, 1986). Accordingly it has been shown that glutamate does accumulate in the medium (Bannai & Kitamura, 1982), but that intracellular levels of glutamate remain significantly higher than the extracellular levels (Bannai, 1986).

In the early 1980's there were several reports on the stimulation, by various thiol compounds, on the uptake of cystine in lymphocytes, bone marrow cells and carcinoma cells (Ohmori & Yamamoto, 1983a; Ohmori & Yamamoto, 1983b; Bannai, 1984). However care should be taken when examining the effects of thiol compounds, as it is not an increase of cystine uptake into the cell which is observed, rather it is an increase in the accumulation of cysteine within the cell. The mechanism of action responsible for the proposed increased cystine uptake is through reduction of cystine in the extracellular medium to mixed thiol containing cysteine which is transported into the cell via an uptake system other than x_c^- (Ohmori & Yamamotob, 1983; Bannai, 1984). Stimulation of system x_c^- was also observed by addition of subtoxic levels of sodium arsenite

however the exact mechanism of induction remains to be defined (Lee *et al.*, 1989; Deneke, 1992).

1.6 Experimental Objectives

The adaptation of mammalian cells to a glutamine reduced or glutamine free media would have potential benefits in industrial process. One of the major concerns of industrial cell culture is the production of ammonia during cellular glutaminolysis. The accumulation of ammonia has been shown to be inhibitory to cell growth and has also been shown to inhibit the production of biologicals. Accumulation of ammonia is generally associated with the use of glutamine as a catabolic metabolite by enzymatic utilization or due to chemical degradation of glutamine. Knowing that within cells glutamine is rapidly converted to glutamate, and that other cell lines can utilize glutamate as a catabolic metabolite in place of glutamine, initial experiments were directed toward adaptation of CC9C10 hybridomas to a glutamate based serum free media with and without glutamine. Finding the cells unadaptable to growth on glutamate, and that in glutamine supplemented media glutamate was inhibitory at higher concentrations, a study was undertaken to assess: 1) The cause of the inhibitory effect of glutamate, i.e. is glutamate inhibiting cellular uptake of cystine which results in oxidative cell death due to lack of glutathione?; 2) Methods by which glutamate and cystine are taken up by the cells in media, i.e. are cystine and glutamate transported by the same system?, is there more than one uptake system responsible for the

uptake of these amino acids?, are they sodium dependent or independent?; 3) Changes in metabolism of glutamate and cystine under normal and inhibited growth conditions, i.e. are there any fluctuations in consumption or production of amino acids under inhibited conditions?; 4) If glutamate is inhibiting cystine uptake and glutathione production is it possible to alleviate or circumvent the inhibition by addition of cystine, addition of glutathione, addition of known reducers?; and finally 5) Is it possible to develop an analytical method by which cystine consumption may be assessed.

Chapter 2. Methods and Materials

Chapter 2. Methods and Materials

2.1 Cell Lines

The hybridoma cell line CC9C10 was acquired from the American Type Culture Collection (ATCC). The cell line was originally derived from the fusion of BALB/c mouse spleen cells with Sp2/O-Ag14 myeloma line. The CC9C10 hybridoma produces monoclonal antibodies against insulin of the IgG_1 class.

The baby hamster kidney (BHK) fibroblast cell line was acquired from the American Type Culture Collection (ATCC). Two cells lines were used, one cultured in basal medium containing 3 mM glutamine (parental) and one adapted to growth on media containing 3 mM glutamate supplemented with 0.25 mM glutamine.

2.2. General Culture Conditions and Cell Line Maintenance

2.2.1 Basal Media Preparation

For CC9C10 hybridomas the basal growth media was DMEM (Gibco, BRL, 4.14 g/L) and Dulbecco Modified Eagle Media / F12 (DMEM/F12; Gibco, BRL, 5.31 g/L) supplemented with sodium bicarbonate (Sigma, 1.85 g/L), glutamine (Sigma, 0.88 g/L), glucose (Sigma, 2.25 g/L), phenol red (Sigma, 15 mg/L), bovine insulin (10 *u*g/mL), bovine transferrin (10 *u*g/mL), pluronic F68 (0.1% w/v), phosphatidlyethanolamine (100 *u*M), ethanolamine (10 *u*M) and NaSe (10 *n*M). This media formulation is commonly referred to as Serum Free Media (DMEM/F12-SFM). After preparation of media in 2 L lots, the pH was

adjusted to 7.2 and the media sterilized by filtration through 5 *u*m filters (Gibco, BRL) into 500 mL sterile media bottles. Media was then stored at 4°C until required.

For BHK fibroblasts the basal growth medium was DMEM (9.2 g/L) supplemented with Hepes (Sigma, 5.3 g/L), sodium bicarbonate (4.11 g/L), L-serine (Sigma, 140.1 mg/L), phenol red (16.8 mg/L), and glucose (5 g/L). The media was then adjusted to pH 7.2 and filter sterilized using 5 *u*m filters into sterile media bottles. At the time of cell passage the basal media was supplemented with filter sterilized Iron Enriched Fetal Calf Serum (FCS, Gibco, BRL, 10%) and L-glutamate or L-glutamine (3 mM, pH 7.2). The L-glutamate containing media was also supplemented with filter sterilized L-glutamine (0.25 mM, pH 7.2). Basal media and FCS were stored at 4°C until required. L-Glutamine and L-glutamate were prepared as filter sterilized stocks of 200 mM and were stored at -8°C until required.

2.1.2 Routine Cell Culture Maintenance

CC9C10 cells were cultured in 75 or 150 cm^2 T-flasks (Corning) at 37°C with a 10% CO_2 overlay. Cells were routinely passaged every 96 hours by diluting cells 10 fold in fresh pre-warmed SFM under aseptic conditions.

BHK fibroblasts were cultured in T-25 cm^2 flasks (Corning) at 37°C with a 10% CO_2 overlay. Routine subculture was performed after cells had reached confluence. Cells were passaged by pipetting off spent media and washing the

confluent monolayer (2X) with pre-warmed Dulbecco's phosphate buffered saline (DPBS, 37°C, without Ca^{+2} and Mg^{+2}) containing ethylenediaminetetraacetic acid (EDTA, Sigma, 0.7 mM, pH 7.2). The cells were then exposed to porcine trypsin (0.25% w/v) in Hanks balanced salt solution containing 1 mM EDTA. The flask was then swirled until the cell monolayer became detached from the flask surface. The cells were then suspended in 10 mL pre-warmed DPBS, and placed in sterile 15 mL centrifuge tubes (Corning) and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and cells resuspended in 5 mL pre-warmed DPBS. A cell count was then taken using a Coulter Counter and cells suspended at 0.7 X 10^5 cells per mL (for cells grown in 3 mM glutamine) or 1 X10^5 cells per mL (for cells grown in 3 mM glutamate).

2.2.3 Culture Storage and Recovery

For long term storage, cells were frozen in liquid nitrogen. CC9C10 or BHK cells were harvested in DPBS to contain approximately 1X10^7 cells per mL. 10% FCS and 10% dimethylsulfoxide (DMSO) were added to the suspension and the suspension then placed in freezing ampules (Corning). The ampules were then sealed, placed in storage boxes and stored at -80°C overnight. The storage boxes were then placed in liquid nitrogen and the contents recorded in the log book.

2.2.4 Cell Enumeration

Viable cell counts were determined using the trypan blue exclusion method (Patterson, 1979). Briefly, an equal volume of culture sample and reagent (0.2% trypan blue in DPBS) were mixed and counted using a haemocytometer under normal conditions, when assessing the viability after cells were concentrated, a 10X dilution was used to achieve accurate counts.

Total cell counts were obtained using a Coulter counter (model Z_F). Briefly, 0.2 mL of sample containing suspended cells was diluted 100 fold in Coulter Buffer (NaCl 7.93 g/L, EDTA 0.33 g/L, KCl 0.4 g/L, Na_2P 1.95 g/L, NaP 0.19 g/L, NaF 0.5 g/L, pH 7.2). The sample was then measured using the Coulter counter.

2.3. Amino Acid Transport and Inhibition Experiments

2.3.1 Glutamine and Glutamate Uptake Protocol for BHK Fibroblasts

BHK cells were harvested from normal culture conditions by the addition of 1.5 mL trypsin (Gibco, 2% in DPBS) for 3-5 minutes. Cells were suspended in 18 mL DPBS and 2 mL iron enriched bovine calf serum added. Cells were suspended as thoroughly as possible in the media by pipetting 15-20 times after which, they were concentrated by centrifugation in a Megafuge (Baxter) at 1500 rpm for 5 minutes. The cells were resuspended in 5 mL pre-warmed DPBS ($37^{O}C$) and total cell counts taken. Cells were suspended in 40 mL of the appropriate media at a density of $0.7X10^5$ cells/mL for cells grown in 3 mM

glutamine or $1X10^5$ cells/mL for cells grown in 3 mM glutamate. Into 24 well plates (Corning), 1 mL of the 40 mL stock was then added and cells were incubated at 37^oC with a 10% CO_2 for 2-3 days for glutamine supplemented media or 3-4 days for glutamate supplemented media. After incubation, media was aspirated off and cells washed twice with pre-warmed uptake buffer (DPBS + 0.01% $CaCl_2$ and 0.01% $MgCl_2$, pH 7.2). The cells were then exposed to 0.5 mL of uptake buffer containing 0.1 uCi U-^{14}C-glutamate or U-^{14}C-glutamine (Amersham) and incubated at 37^oC. Initially concentrations of glutamate or glutamine were held at 3 mM and assay time was varied between 0 and 20 minutes to assess linearity of uptake. Once a linear range was established, concentration was varied between 1 mM and 20 mM and assay time was fixed to determine the rate of glutamate/glutamine uptake. After cells were exposed to the radioactive substrate for the desired period of time, the uptake buffer containing the radioactive substrate was aspirated off. Cells were then washed twice with ice cold DPBS containing 40 mM glutamate or glutamine. After the washes, 1 mL of lysis buffer (0.1 M NaOH + 0.1% w/v SDS) was added to each well. The solution was pipetted into a scintillation vial where 5 mL of Ecolume (ICN) Scintillation fluid was added. Radioactive incorporation was assessed on a Rack Beta Liquid Scintillation Counter (Wallac). Representative cell counts were taken from wells not exposed to radioactivity.

2.3.2 Glutamate, Cystine and Glutamine Uptake Protocol for CC9C10 Hybridomas

Cells were cultured in SFM at 37^{o}C with 10% CO_2 overlay for three days which places cells in mid-log phase (approximately 6X10^{5} cells/mL). From these cultures a viable count was taken (0.2 mL cells + 0.2 mL 0.2% Trypan Blue) after which 100 mL of culture was removed and centrifuged at 1000 rpm in Megafuge for 5 minutes. Spent media was decanted and the cells re-suspended in 5 mL of pre-warmed Hepes Buffered Saline (HBS, NaCl 140 mM, K_2HPO_4 0.7 mM, Hepes 10 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM) or Hepes Buffered Choline (HBC, Chol-Cl 140 mM, K_2HPO_4 0.7 mM, Hepes 10 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM). Cells were then concentrated again and resuspended in 6.2 mL pre-warmed HBS or HBC. A viable cell count was then taken (0.1 mL cells + 0.9 mL 0.2% Trypan Blue) and only cultures with 85% viability or greater were used for experimental purposes. Cells, approximately 1X10^{7} viable cells/mL, were then exposed to 0.1 *u*Ci U-^{14}C-glutamate, U-^{14}C-cystine or U-^{14}C-glutamine with desired concentration of cold amino acid in pre-warmed ependorff tubes with a total volume of 0.5 mL. The cells were then incubated in a G24 Environmental Incubator Shaker (New Brunswick) at 37^{o}C for the desired time. In initial experiments, concentration was fixed at 10 *u*M glutamate, 0.2 mM cystine, and 10 *u*M glutamine while time was varied to assess linearity in HBS and HBC. Once linearity was determined and buffer selected, assay time was fixed and concentration varied to determine rate of uptake. After the desired incubation

period, a the sample was removed and filtered on a HBS or HBC pre-soaked Supor-450 0.45 *u*m 25 mm membrane (Gelman) in a 30-chambered manifold (Millipore). The cells were then washed twice with ice cold uptake buffer. The filters were then removed to scintillation vials where cells were lysed and 5 mL of Eco-Fluor added. Radioactive incorporation was then assessed using a Rack Beta Counter (Wallac).

2.4. Amino Acid Consumption or Production Related Experiments

2.4.1 Reverse Phase C-18 HPLC Amino Acid Analysis

Amino acids were analyzed by the method described by Christie and Butler (1994). Briefly, 100 *u*L amino acid standards (500 mM, Sigma) were mixed with L-α-amino-n-butyric acid, L-glutamine, L-tryptophan, L-asparagine (10 *u*L 25 mM stocks), 360 *u*L water and 500 *u*L 10% trichloracetic acid (TCA). For media samples, 245 *u*L of sample was mixed with 5 *u*L L-α-amino-n-butyric acid (25 mM stock) and 250 *u*L 10% TCA. Standards or samples were then centrifuged (14,000 rpm) for 5 minutes at 4^{O}C. From the supernatant, a 500 *u*L portion was removed and diluted into 1 mL 0.2 M sodium borate buffer (pH 10.4) and centrifuged as above. The supernatant (250 *u*L) was then collected and placed into microfuge tubes (500 *u*L) to which orthophthaldialdehyde (OPA, 250 *u*L) was then added. The tubes were then incubated for 1.7 minutes after which 5 *u*L was autosampled by a SIL-9A autosampler (Shimadzu) and injected into a C-18 reverse phase column (Alltech) on an LKB (LKB-Pharmacia) 2100 series

high pressure liquid chromatography system. Amino acids were eluted from the column using gradients of acetonitrile and methanol and detected by fluorescence. Results were analyzed using Easychrom v.3.2 (Shimadzu) computer software.

2.4.2 Spectrophotometric Determination of Cystine

Cystine utilization rates could not be assessed using pre-column derivatization with OPA protocol for HPLC analysis since it does not form an adduct with OPA. In order to assess cystine utilization in spent media a spectrophotometric assay was employed. The spectrophotometric assay devised by Chrastil (1989) was selected and modified for use with a 96-well plate reader. To 0.3 mL samples of cysteine (1 - 100 mg/mL) in DPBS, 0.1 mL of 6 M HCl was added and mixed. 0.05 mL of 5% $NaHAsO_4$ (Sigma) was added and mixed. After 10 minutes 0.85 mL of 88% HCOOH was added and mixed. Next, 0.1 mL of OsO_4 was added and mixed and mixture left to stand overnight. Optimal wavelength for measurement was stated as 355 nm, however the plate reader did not have this wavelength, so 340 nm was substituted in its place. L-cystine standards were found to be linear over the tested range, however the presence of phenol red in SFM could not be adequately blanked. In order for this to be an effective assay, media used would need to be made without the presence of phenol red or other such pH indicators and pH monitored externally.

2.4.3 Cystine Determination using 3-Bromopropylamine Derivatization

The derivatization of cystine using 3-bromopropylamine was adapted from Hale *et al.*(1994), Jue & Hale (1993) and Jue & Hale (1994). To a sample (100 *u*L) of amino acid standard (cystine 0.125 *u*moles) or a sample containing cysti(e)ne 100 *u*L of 20 mM dithiothreitol (DTT) and 100 *u*L of 50 mM Tris (pH 9) was added and sample allowed to incubate for 30 minutes at 37^{o}C. After the time had elapsed, 3-bromopropylamine (Aldrich) was added to generate a working concentration of approximately 0.1 M. The sample was then incubated for 1 hour at 37^{o}C. Samples were then run on C-18 reverse phase HPLC with pre-column derivatization by OPA. It was found however, that excess bromopropylamine reacted with OPA forming a precipitate that was collected in the HPLC guard column. Therefore in order for this to be an effective method for use in our HPLC system, a method of removing the excess 3-bromopropylamine needed to be devised.

2.4.4 Removal of Excess 3-Bromopropylamine by Anionic Exchange

Dowex-1 anionic resin (Sigma) was prepared by suspending 100 g of resin in 2 L of 0.5 M NaOH, allowing the beads to settle, and removing the layer which remained on the surface. The beads were then collected on a Buchner funnel and then resuspended in 2 L of 0.5 M NaCl and the process repeated. The process was repeated a final time with 2 L of 0.5 M HCl after which the beads were washed on the buchner funnel to near neutrality with water. Beads

were then suspended in 100 mL of water and the slurry stored at $4^{O}C$ until required.

The derivatized sample containing cystine was then diluted in 0.1 M KOH (pH 11) or 0.1 M KOH added to the column prior to sample loading, and the sample layered onto the top of the column. The sample was then allowed to move through the column, after which it was then washed with 10X 5 mL water to remove excess 3-bromopropylamine. The bound amino acids were then eluted using 3X 5 mL 4 M acetic acid. Ninhydrin was used initially to assess whether or not amino acids were bound to the column and number of fractions required to elute all amino acids from the column. Experimental trials (not tested with ninhydrin) were concentrated using a rapid vacuum system. Samples were then reconstituted in their original volumes and were analyzed by HPLC. It was found that excess 3-bromopropylamine was effectively removed from the reaction mixture. However there was also significant loss of basic amino acids, as these amino acids carried a net positive charge (column also carries net positive charge therefore no binding). The protocol was therefore not used, as the loss of basic amino acids and reproducibility in other amino acids could not be alleviated.

2.4.5 Cystine Determination using Iodoacetic Acid Derivatization

The derivatization of cystine in spent media samples was adapted from Birwe and Hesse (1991). To a sample containing cyst(e)ine (150 *u*L standard or sample) 10 *u*L of DTT (96 mM) for a working concentration of 6 mM. This was done to keep cystine in the reduced form cysteine. The reduction was allowed to proceed at room temperature for 10 minutes. After 10 minutes 10 *u*L of iodoacetic acid (680 mM) was added, giving a working concentration of 40 mM for iodoacetic acid. The iodoacetic acid derivatization was then allowed to proceed for 15 minutes at room temperature. The samples were then pre-column derivatized with OPA and loaded onto C-18 reverse phase HPLC.

Chapter 3. Results

Chapter 3. Results

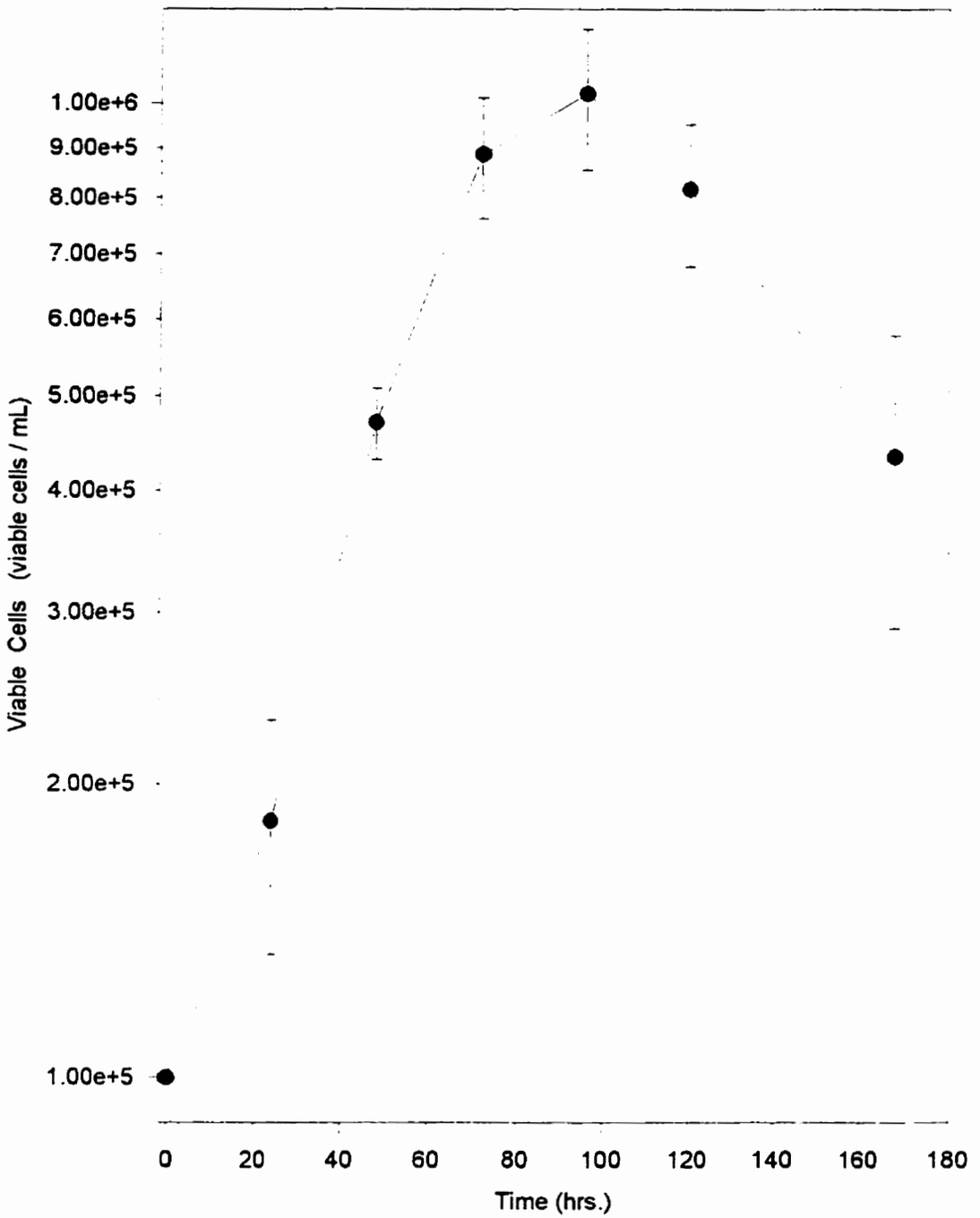
Section 3.1 Effects of Glutamate Supplementation on Cell Growth

3.1.1 Growth Profile of Hybridomas

CC9C10 hybridoma cells were grown in SFM media (Figure 3.1.1) to assess and observe hybridoma growth. CC9C10 hybridomas from routine cell culture (72 hours) were spun down in 30 mL centrifuge tubes. The supernatant was discarded and the cell pellet resuspended in 5 mL pre-warmed DPBS (pH 7.2). A cell count was taken using the Coulter counter to assess cell density. Cells were inoculated at $1X10^5$ cells in 1.5 mL of SFM media in 24 well plates. Cells were incubated at 37°C with 10% CO_2 and samples (0.2 mL) withdrawn every 24 hours to measure cell density. The viable cell density was assessed using trypan blue staining and a haemocytometer.

The growth of CC9C10 hybridomas, as seen in Figure 3.1.1, follows a typical pattern for hybridoma cells. After inoculation cells grow rapidly in the log phase, until they reached a maximum cell density after which followed a rapid cell death. From Figure 3.1.1 it is observed that maximal cell density occurs at roughly 96 hours. This time point was selected as an assay point for future experiments.

Figure 3.1.1 Growth of CC9C10 hybridomas in SFM. Cells were inoculated in 24 well plates at $1X10^5$ cells/mL and plates incubated at 37°C at 10% CO_2 for 168 hours with cell density determined every 24 hours. (n=4, S.E.M.)



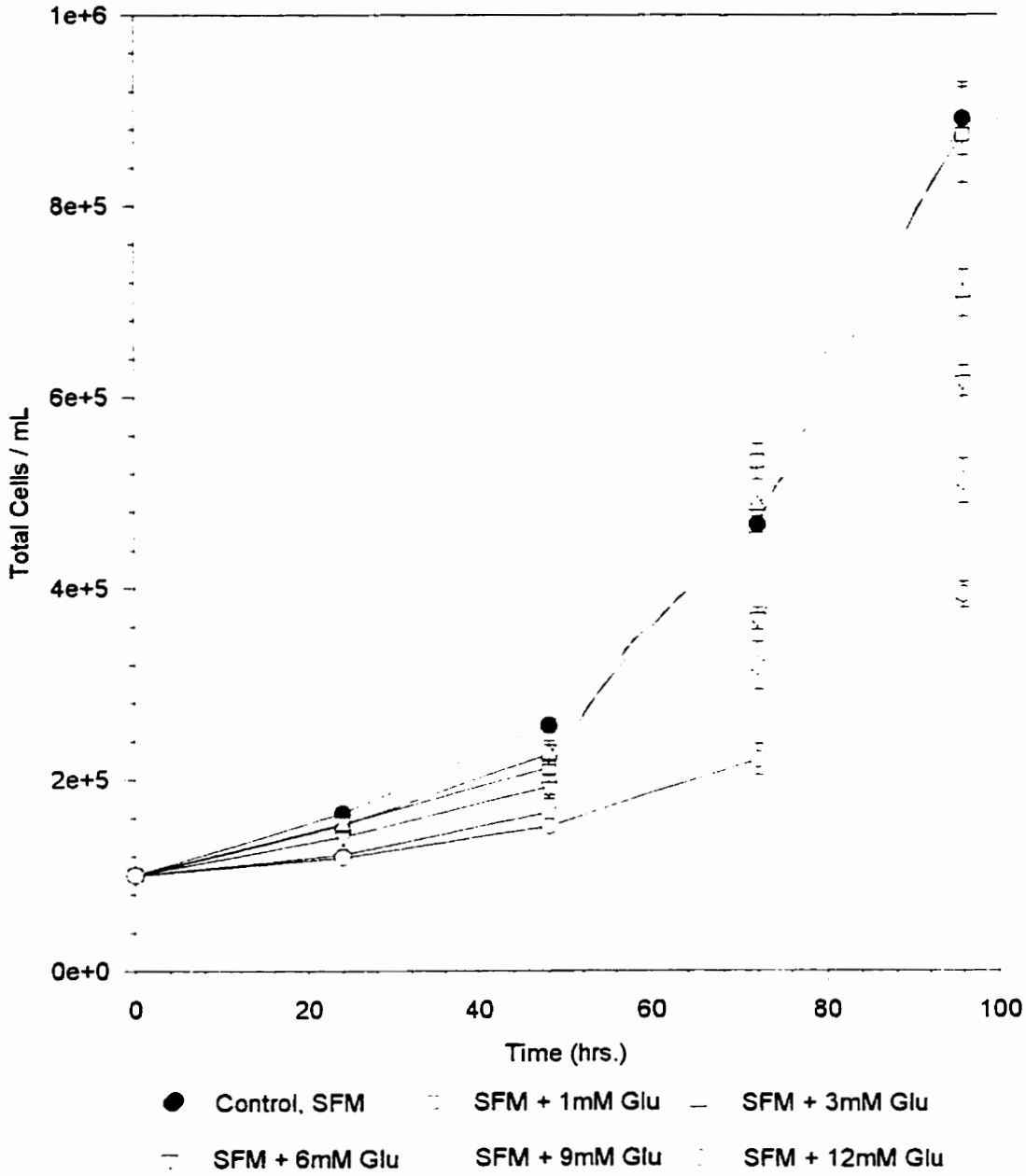
3.1.2 The Effect of Glutamate on Cell Growth

The effect of increasing glutamate concentrations in SFM was assessed on CC9C10 hybridoma cell growth. The ability of the cells to utilize glutamate as a carbon source over glutamine could have the potential to decrease one mole of ammonia produced, for every mole of glutamate during metabolism. The reduction in ammonia is a result of the chemical structure of glutamate which has a carboxyl group in place of one of the amino groups of glutamine. Adaptation to glutamate would also be beneficial in terms of media longevity, as glutamate is not subject to the same chemical decomposition that glutamine is.

In order to assess the ability of CC9C10 hybridoma cells to utilize glutamate, experiments were performed where SFM, containing a fixed glutamine level of 6 mM, was supplemented with increasing amounts of glutamate up to 12 mM (Figure 3.1.2). Cells were harvested from a 72 hour old culture and suspended in fresh SFM at $1X10^5$ cells/mL in 24 well plates containing the experimental level of glutamate (1,3,6,9, and 12 mM). Control cultures consisted of SFM with no additional glutamate. The cell density was determined by Coulter counter from samples (0.2 mL) taken every 24 hours over a 96 hour period.

From Figure 3.1.2 it can be observed that only the addition of 1 mM glutamate to SFM resulted in a similar growth pattern to that of the control (u = 0.024 $hr.^{-1}$ and u= 0.024 $hr.^{-1}$ respectively). Increasing glutamate concentration

Figure 3.1.2 Growth of CC9C10 hybridomas in SFM supplemented with varying amounts of glutamate. CC9C10's were inoculated at $1X10^5$ cells per mL in 24 well plate dishes and incubated at 37°C 10% CO_2 for a period of 96 hours. Total cells were assessed every 24 hours with a Coulter counter. (n=3, S.E.M.)



above 1 mM resulted in decreasing cell densities and specific growth rates, with 12 mM having the lowest cell density after 96 hours of growth. The addition of 3 mM glutamate resulted in a specific growth rate of $u = 0.021$ hr.$^{-1}$ ($p = 0.015$). The addition of 6 mM glutamate also had a specific growth rate of $u = 0.021$ hr.$^{-1}$ ($p = 0.009$), while 9 mM added glutamate had a specific growth rate of 0.020 hr.$^{-1}$ ($p = 0.008$) and 12 mM glutamate a specific rate of 0.017 hr.$^{-1}$ ($p = 0.007$).

A replot of the cell densities at 96 hours (Figure 3.1.3) shows that at concentrations of glutamate where CC9C10 cell growth is inhibited (3 mM to 12 mM), there is a linear relationship between increasing glutamate concentration and decreasing cell densities. The maximal cell density was shown to be $8.8X10^5$ cells per mL giving the half maximal value of $4.4X10^5$ cells per mL. Therefore at the 95% confidence interval, the concentration of glutamate which results in the half maximal cell density is 10.6 ± 0.8 mM.

3.1.3 Adaptation to a Low Glutamine SFM

The ability of CC9C10 hybridoma cells to adapt to a glutamine reduced SFM, supplemented with glutamate was assessed (Figure 3.1.4). Cells were harvested from a 72 hour old culture and resuspended in 6 mL fresh media containing increasing glutamate and decreasing glutamine concentrations. The experimental glutamine levels were reduced by approximately 2 mM for each level of glutamate tested. Glutamate levels selected included non-inhibitory concentrations (4 mM) and inhibitory conditions (>10 mM). The growth of

Figure 3.1.3 Inhibitory effect of glutamate on CC9C10 hybridomas. Cell density was determined after 96 hours of growth in SFM containing increasing amounts of glutamate. Determination of concentration of glutamate which results in half the cell densities achieved under normal growth conditions in SFM. (n=3, S.E.M.)

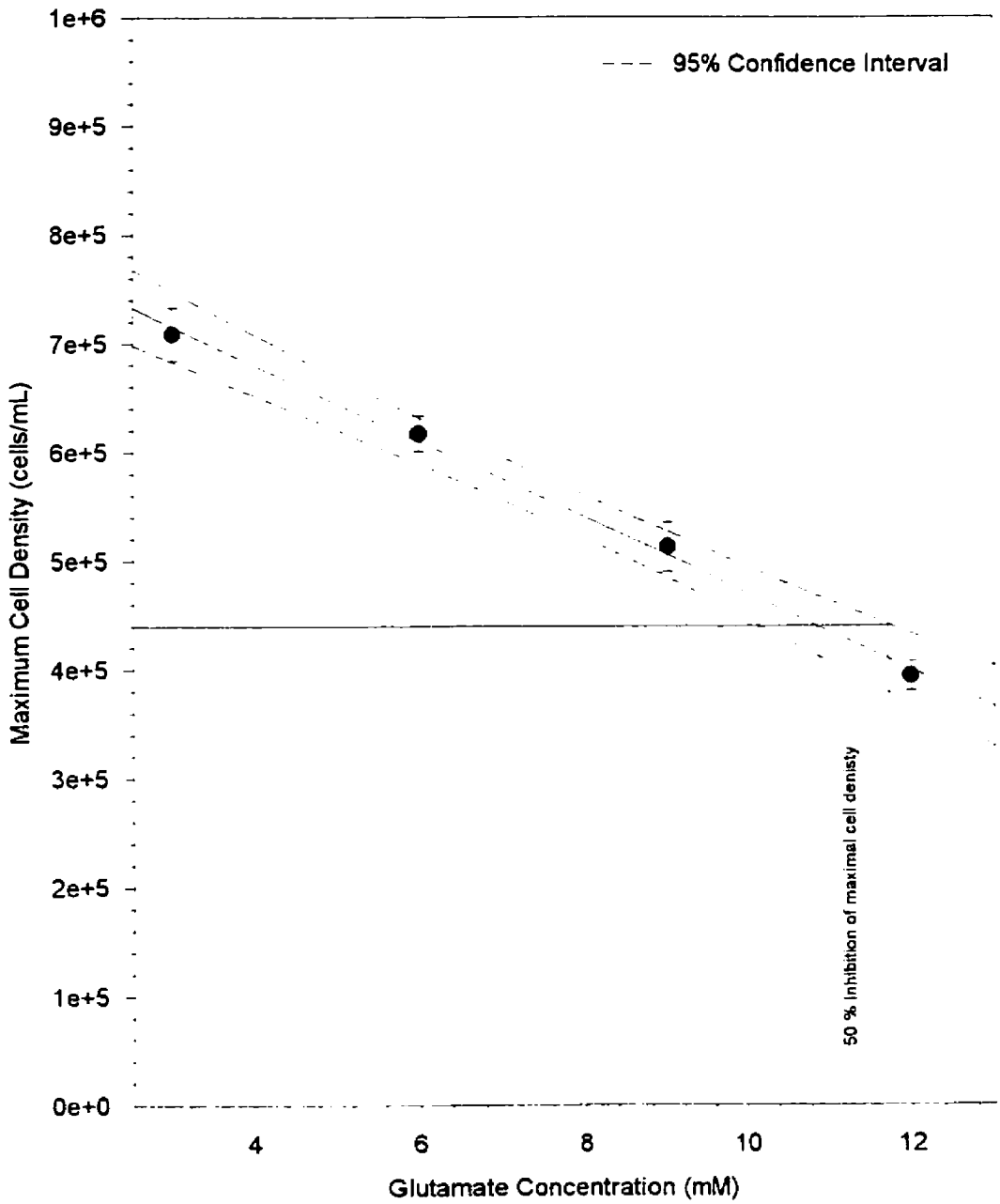
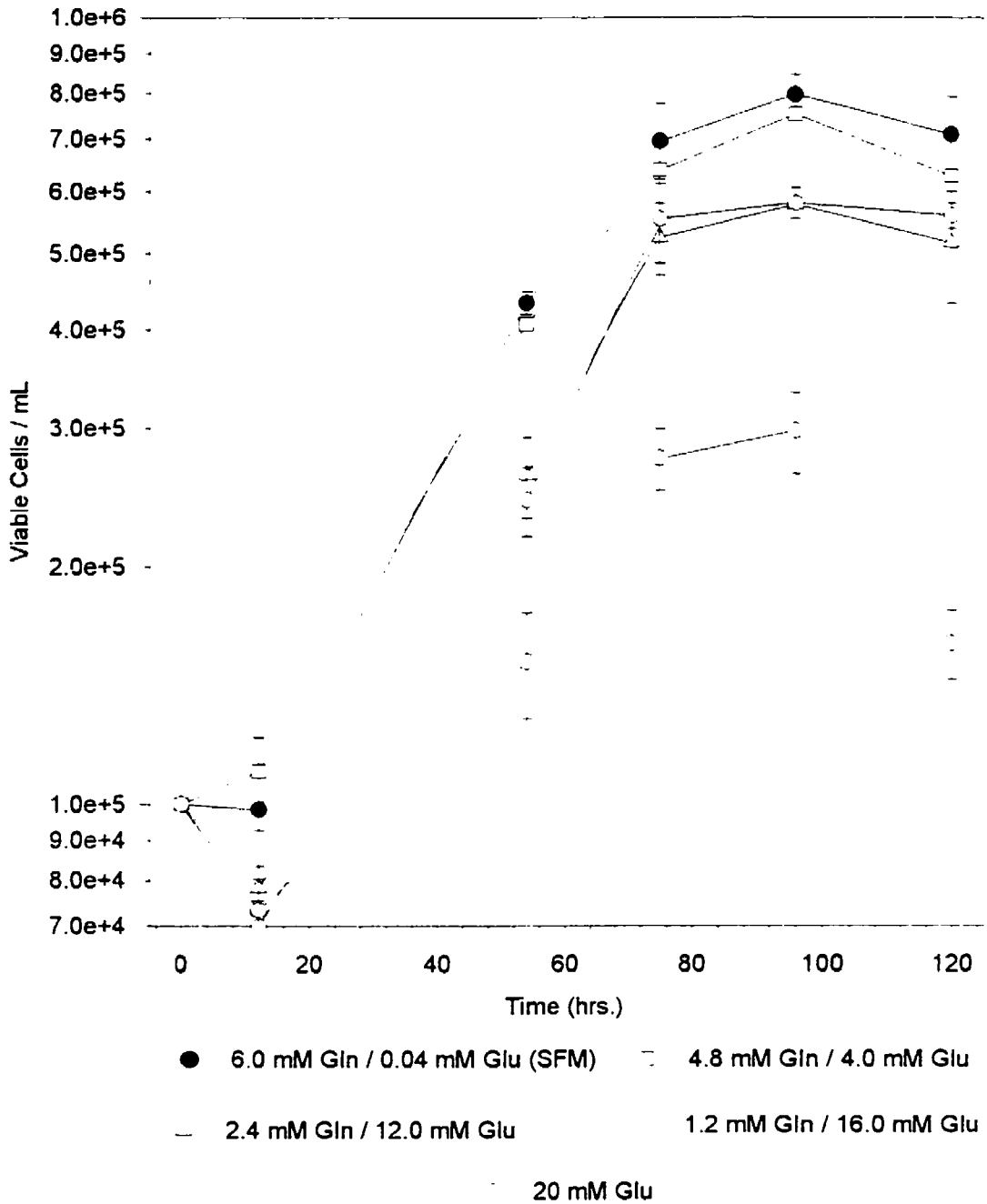


Figure 3.1.4 Effect of increasing glutamate while lowering glutamine concentration on growth of CC9C10 hybridoma cells. Cells were inoculated in 6 well plates (V_T = 6 mL) at 1X10^5 cells per mL. Viable cells were assessed every 24 hours using trypan blue staining and a haemocytometer. (n=3, S.E.M.)



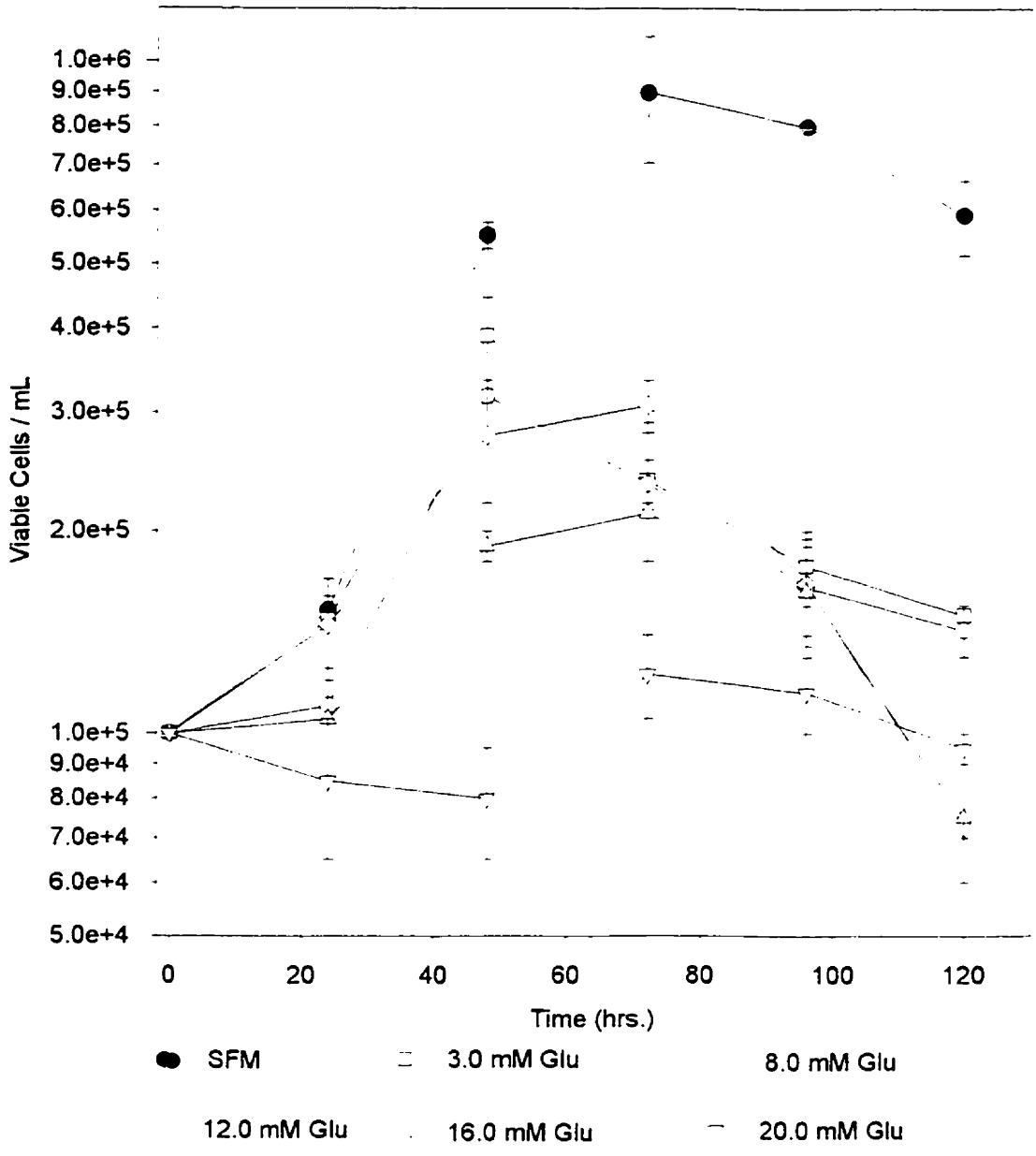
CC9C10 cells in SFM was used as a control. It was found that growth rates were similar between control cells and those grown in 4.8 mM glutamine, 4.0 mM glutamate. Cells grown in 2.4 mL glutamine, 12 mM glutamate were shown to obtain lower final cell densities, with a reduced growth rate. The growth of cells in 1.2 mM glutamine, 16 mM glutamate had a similar growth rate and final cell densities to those grown in 2.4 mM glutamine, 12 mM glutamate. Finally cells grown in 20 mM glutamate had a greatly reduced growth rate and final cell density.

Therefore, CC9C10 hybridoma cells require glutamine at a concentration above 2.4 mM and elevated glutamate levels do not result in growth rates and cell densities similar to those of the control.

3.1.4 Effect of Glutamine Replacement by Glutamate on Cell Growth

In order to assess if supplementation of high levels of glutamate had an effect on cell growth and density, experimentation was done where glutamine was replaced with increasing levels of glutamate in SFM (Figure 3.1.5). Growth of cells in SFM (6 mM glutamine) was used as a control. Glutamine was present in the media at a concentration of 0.5 mM, which is the amount contained in F-12 medium. CC9C10 cells were harvested from a 72 hour old culture. Cells ($1X10^5$ cell per mL) were inoculated in 6 well plates (V_T = 6 mL) containing SFM with the desired concentration of glutamate. From Figure 3.1.5 it can be seen that replacing glutamine with glutamate at any concentration

Figure 3.1.5 Effect of glutamine replacement by glutamate in SFM on cell growth. Cells were inoculated at $1X10^5$ cells per mL in 6 well plates (V_T = 6 mL) and viable counts were assessed every 24 hours by Trypan blue staining using a haemocytometer. (n=2, S.E.M.)



results in decreased growth rate and decreased cell density over the 120 hour period.

3.1.5 Adaptation to Low or Glutamine Free Media by High Density Passage

High density passage of cells, in media known to be sub-optimal for growth, can result in adaptation by cells to normal cell densities and growth rates after several passages (Patterson, 1979). The adaptation process is associated with derepression of enzyme pathways, once inhibitory metabolites or end products from previous growth conditions have been exhausted. Experiments were performed to assess whether high density passage of CC9C10 hybridomas in a SFM containing 6 mM glutamate (glutamine 0.5 mM) in place of 6 mM glutamine would result in growth rates similar to those seen in normal SFM. Cells grown in normal SFM (6 mM glutamine, 72 hour culture) were seeded at $5X10^5$ cells per mL into the SFM containing 6 mM glutamate (V_T = 20 mL). As seen in Figure 3.1.6, growth in initial passage was similar to that seen in normal media with doublings occurring approximately every 24 hours. This is most likely due to residual elevated glutamine levels. However when the cells were split into the 6 mM glutamate based media again, no increase in growth was observed and after 24 hours there was a gradual decrease in viable cell density. After the second passage the media contains glutamine at 0.5 mM and glutamate at 6 mM. Under these conditions cells would be expected to utilize glutamate for growth. As seen in Figure 3.1.6 the cells fail to grow in the

Figure 3.1.6 Adaptation of CC9C10 hybridomas to glutamate based SFM by high density passage. Cells were inoculated at $5X10^5$ cells per mL in two T-125 flasks (V_T = 20 mL) and then passaged at high density. Cell density was assessed every 24 hours using trypan blue and a haemocytometer. (n=2, S.E.M.)

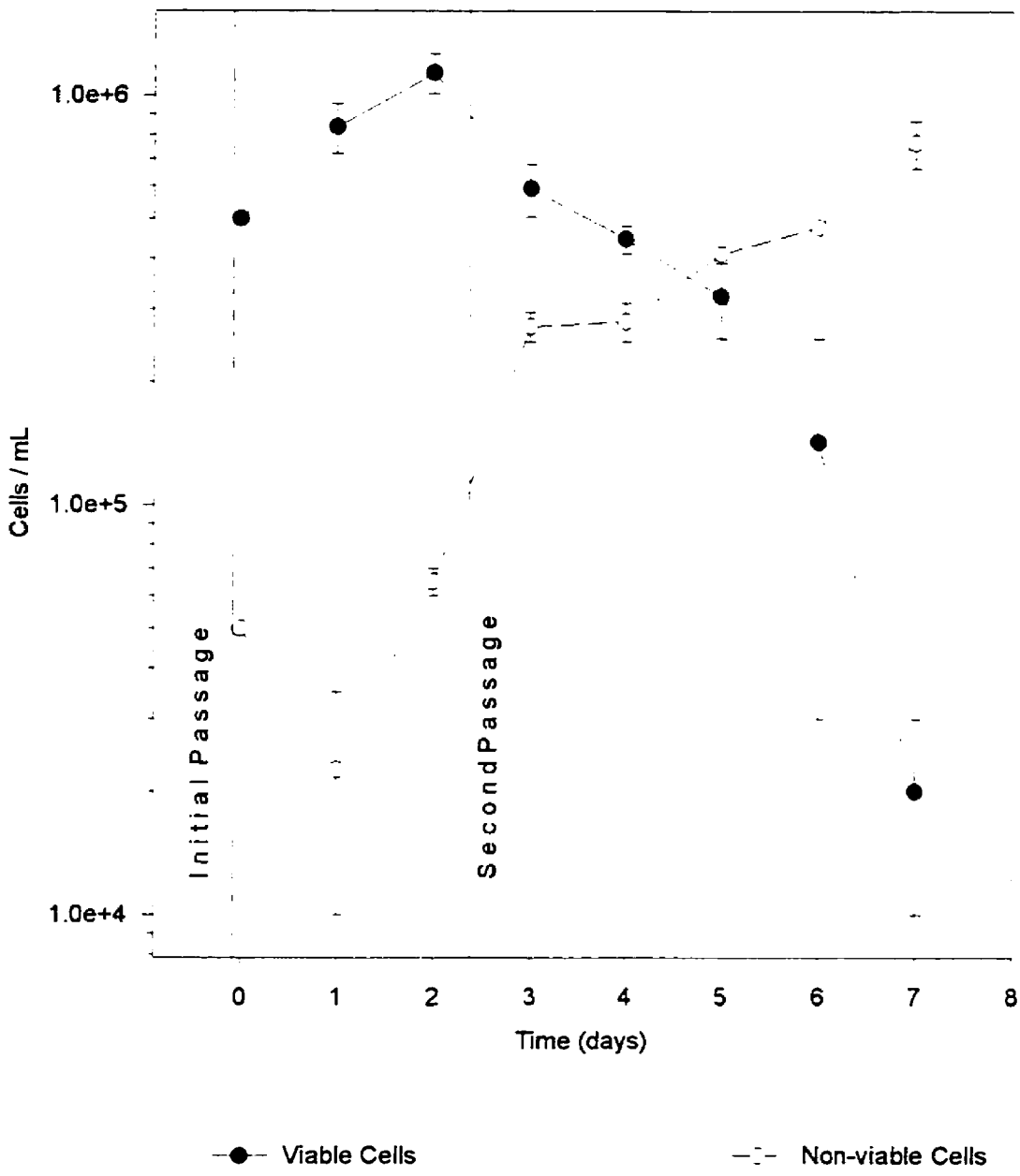
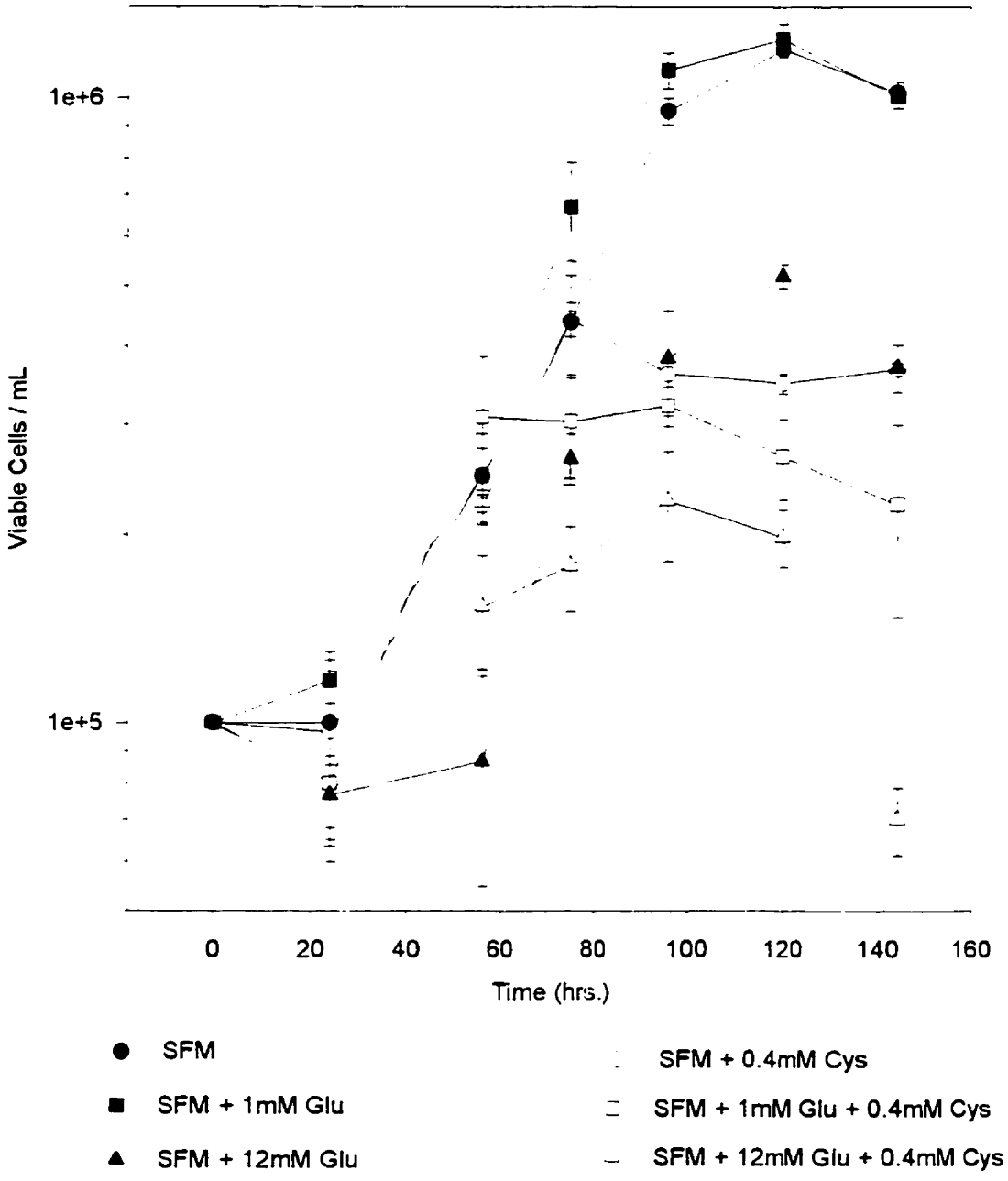


Figure 3.1.7 Effect of addition of 0.4 mM cystine on CC9C10 hybridoma cells grown in SFM (6 mM glutamine) containing varying amounts of glutamate. Cells were inoculated in 6 well plates (V_T = 5 mL) at $1X10^5$ cells per mL and grown at 37°C with 10% CO_2 over a period of 144 hours. Cell growth was assessed every 24 hours using trypan blue stain and a haemocytometer. (n=3, S.E.M.)



a 50% inhibition of maximal cell density. The addition of 0.4 mM cystine to SFM containing 12 mM glutamate resulted in even further inhibition of cell density and cell growth. Therefore the addition of L-cystine could not alleviate the inhibitory effect of glutamate and resulted in reduced cell yields under non-inhibitory conditions.

3.1.7 Effect of Aminoadipate on Cell Growth

Aminoadipate is a known competitive inhibitor of glutamate and cystine uptake into cells and was used to mimic the inhibitory effect caused by glutamate (Bannai & Kitamura, 1981). If the growth inhibitory effect of glutamate was due to competitive inhibition of cystine uptake, it can be argued that aminoadipate should exhibit a similar effect. Cells were inoculated at $1X10^5$ cells per mL and increasing amounts of L-aminoadipate were added to 24 plates containing SFM. The growth rate and maximum cell yield of the control (SFM) and the media containing 1 mM L-aminoadipate were found to be similar (Figure 3.1.8). As in the case of glutamate, increasing L-aminoadipate resulted in reduced maximum cell yields.

A replot of the effect of L-aminoadipate on final cell densities at 96 hours of growth was performed in order to assess the concentration of L-aminoadipate which resulted in 50% of the maximal cell density (Figure 3.1.9). From this it was found that the concentration which resulted in 50% reduction in cell density was 4.4 ± 0.4 mM L-aminoadipate. In comparison to the half-maximal value for

Figure 3.1.8 Effect of L-aminoadipate on growth of CC9C10 hybridoma cells. Cells were grown in 24 well plates, with increasing amounts of L-aminoadipate added to the media, for a period of 96 hours at 37°C with 10% CO_2. Total cells were assessed using a Coulter Counter every 24 hours. (n=2, S.E.M.)

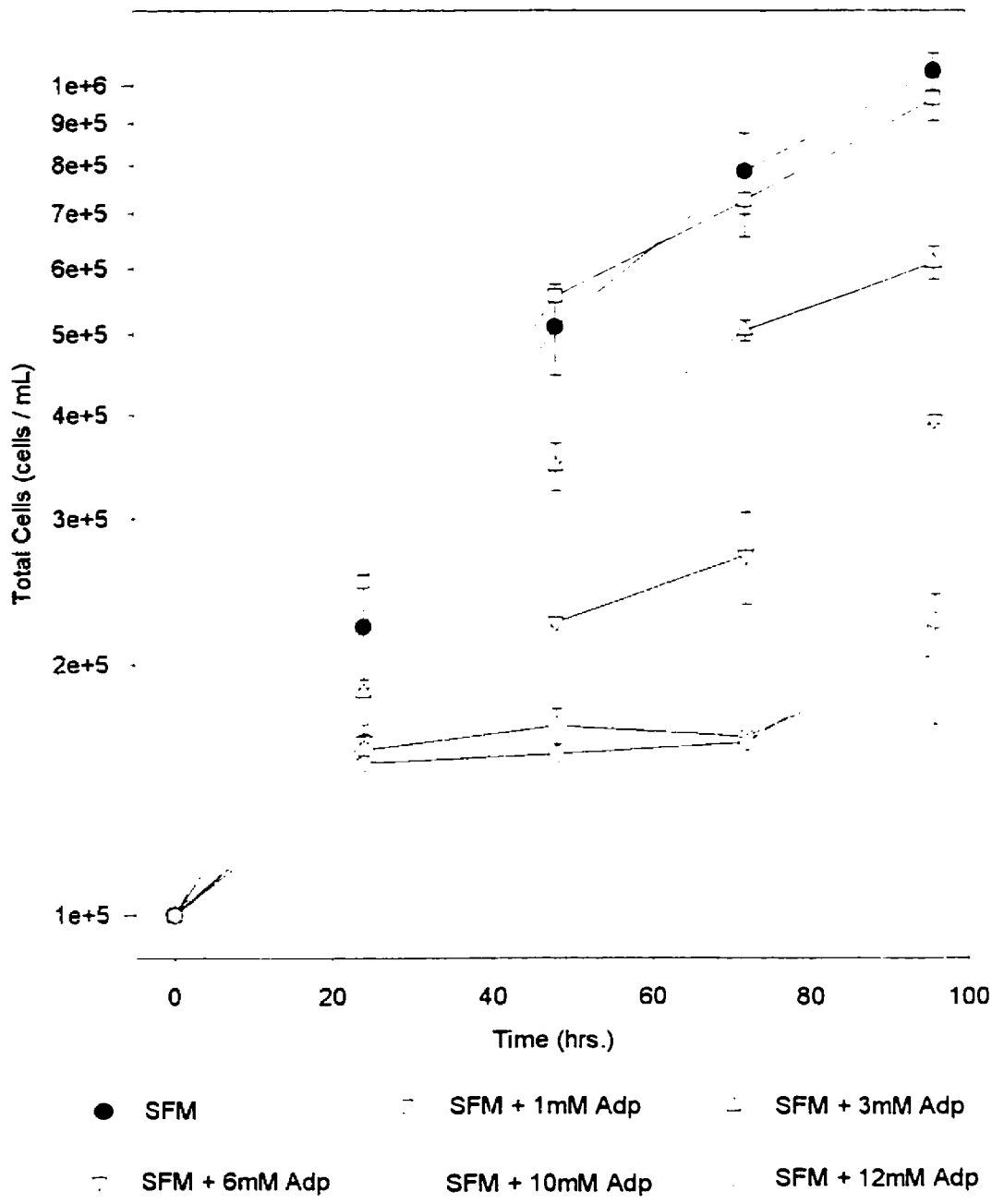
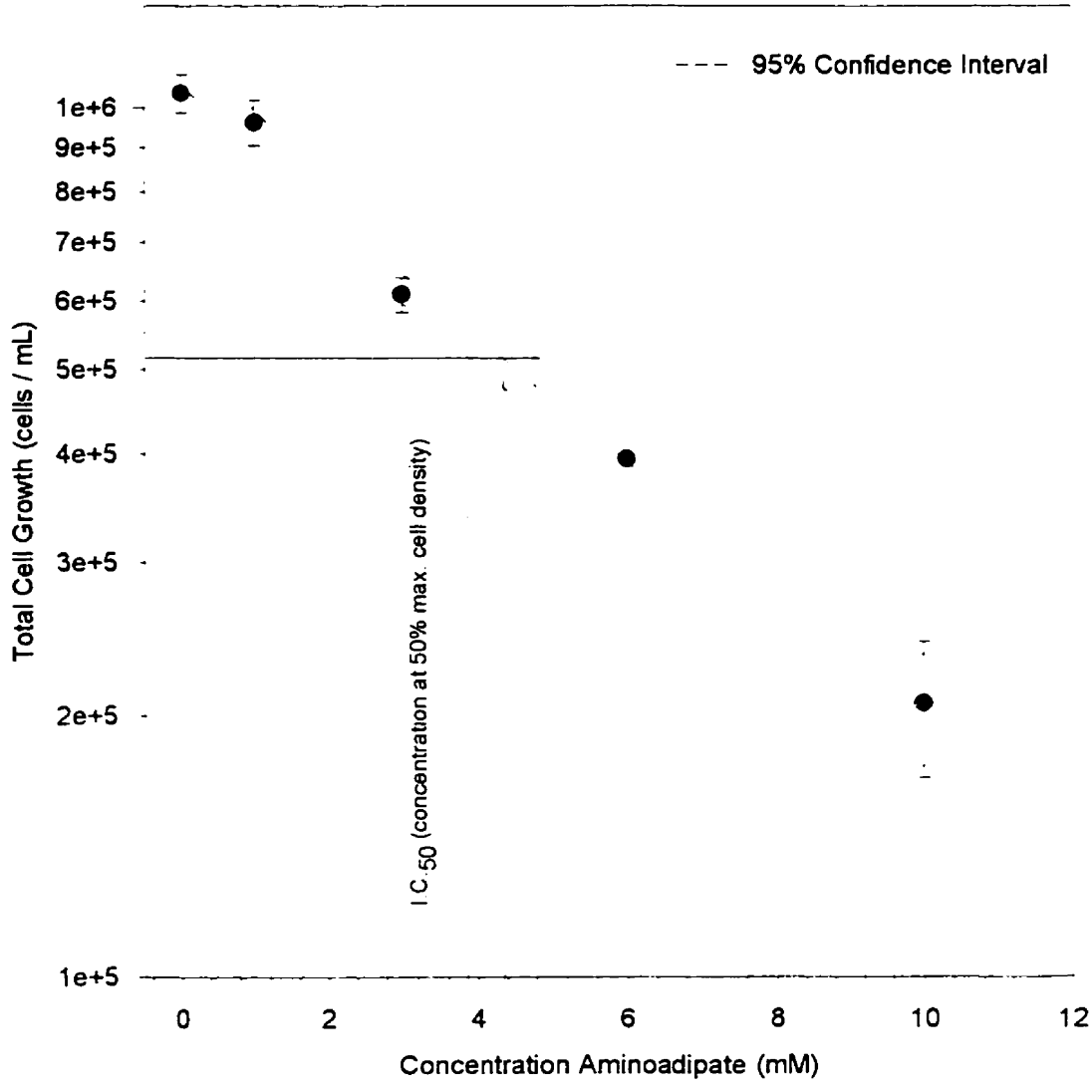


Figure 3.1.9 Inhibitory effect of L-aminoadipate on CC9C10 hybridoma cell density after 96 hours of growth in SFM containing increasing amounts of L-aminoadipate. Determination of concentration of L-aminoadipate which results in half the cell densities achieved under normal growth conditions in SFM. (n=2, S.E.M.)



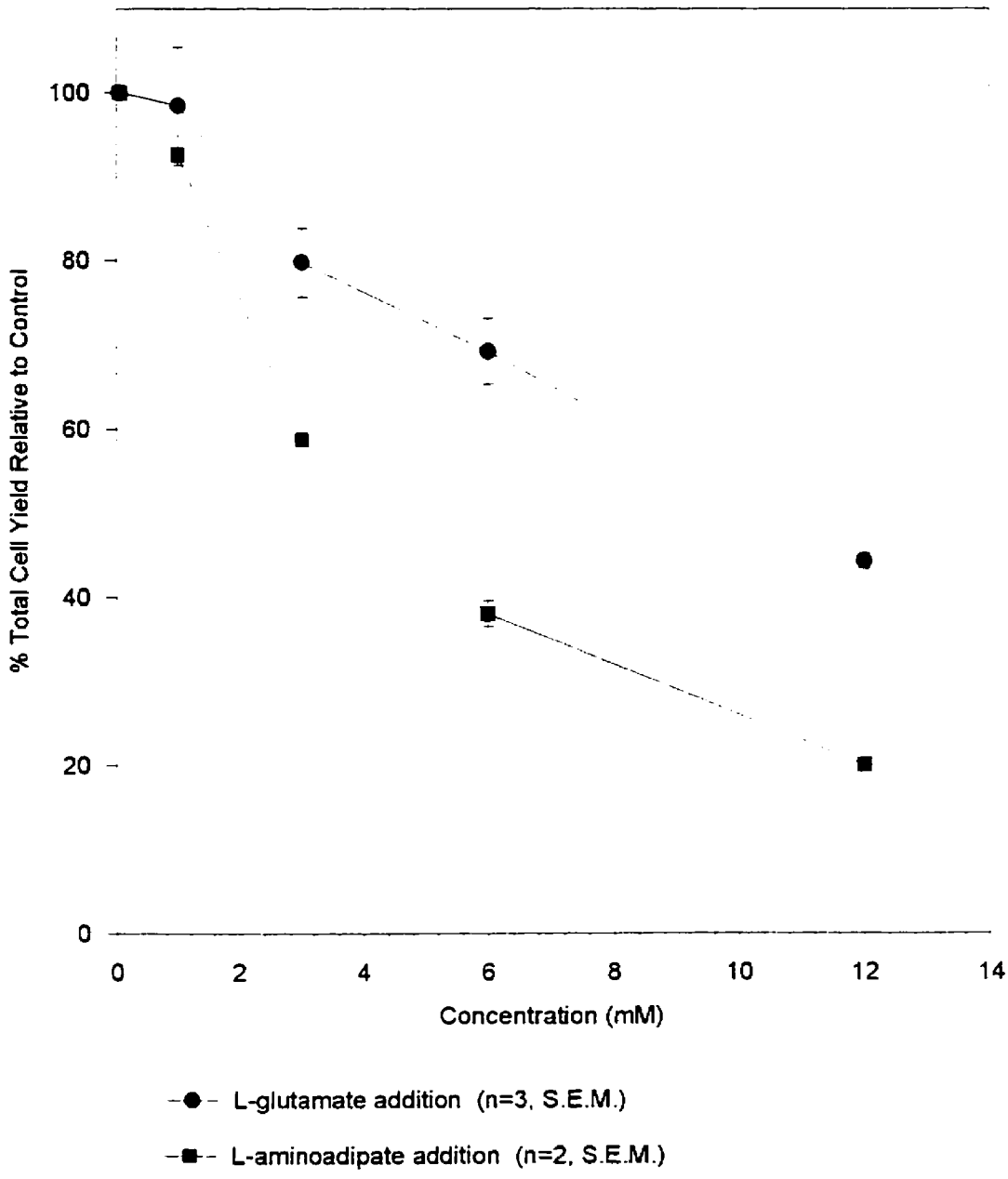
glutamate (10.4±0.4 mM) this represents greater than a two-fold decrease in the concentration required to inhibit cell growth indicating that L-aminoadipate had a much greater effect on final cell density than glutamate did.

Comparing the effects of glutamate and L-aminoadipate (Figure 3.1.10) it can be seen that L-aminoadipate is more inhibitory to cell growth than glutamate. L-Aminoadipate (> 1 mM) consistently resulted in a two-fold lower final cell density.

3.1.8 Alternate Methods of Providing Cystine Under Glutamate Inhibition

Cystine is an essential amino acid for the production of glutathione which is required to prevent cellular oxidative stress. Therefore alternative methods of supplying cystine in the presence of glutamate were investigated. The first method simply involved supplementing cells with 0.4 mM glutathione and examining the effects on cell growth under normal, non-inhibitory and inhibitory levels of glutamate and in SFM where glutamine was replaced by 6 mM glutamate. A 0.4 mM level of glutathione was selected because if 100% of cystine (0.2 mM in SFM) was utilized in the production of glutathione, a theoretical level of 0.4 mM would be expected. The addition of 0.4 mM glutathione to SFM resulted in a growth rate and final cell density that was not significantly different from that of the control growth in SFM. Under non-inhibitive levels of glutamate, 3 mM, there was a slight increase in final cell density when compared to growth in SFM with 3 mM glutamate. However at

Figure 3.1.10 Comparison of inhibitory effects on cell density at 96 hours between L-glutamate and L-aminoadipate relative to control growth of CC9C10 hybridoma cells in SFM.



increasing glutamate concentrations, glutathione was found to have no significant effect at alleviating the inhibitory conditions imposed by glutamate on cell growth and final cell densities. The growth of cells in SFM with 6 mM glutamate replacing glutamine was found to reach cell densities similar to those grown in SFM with 6 mM glutamine, 6 mM glutamate, and 0.4 mM glutathione. The cell densities at 96 hours were then compared between SFM supplemented with glutamate (Figure 3.1.2) and SFM supplemented with glutamate and glutathione (Figure 3.1.11) after normalization to controls (Figure 3.1.12). This plot shows that supplementation of glutathione resulted in no significant difference at the 3 mM glutamate level, and that at increased glutamate levels glutathione supplementation resulted in decreased cell densities at 96 hours.

The second method of cystine supplementation revolved around the oxidative state of cystine in the media. Assuming that competition between cystine and glutamate was responsible for decreased cell densities (due to oxidative stress caused by lack of cystine), then supplying cystine in a reduced or conjugate form may alleviate the observed low cell densities. 2-mercaptoethanol is a known reducer and when placed in media containing oxidized cystine, it rapidly reduces the cystine to cysteine and form a conjugate with the mercaptoethanol having an overall net neutral charge. This provides the cells with a source of cysteine, which is taken up via a neutral uptake system, which the cell can use for glutathione synthesis. The effect of 2-

Figure 3.1.11 Effect of 0.4 mM glutathione (GSH) on CC9C10 hybridoma cells. Cells were grow in SFM with fixed glutamine (6 mM) and varied glutamate (0.04, 3, 6, 12 mM). Cells were also grown in SFM with low glutamine high glutamate (0.8 mM Gln and 6 mM Glu) with addition of 0.4 mM glutathione. Growth in SFM no additives, was used as a control. Total cell growth was assessed every 24 hours for a period of 96 hours using a Coulter Counter. (n=3, S.E.M.)

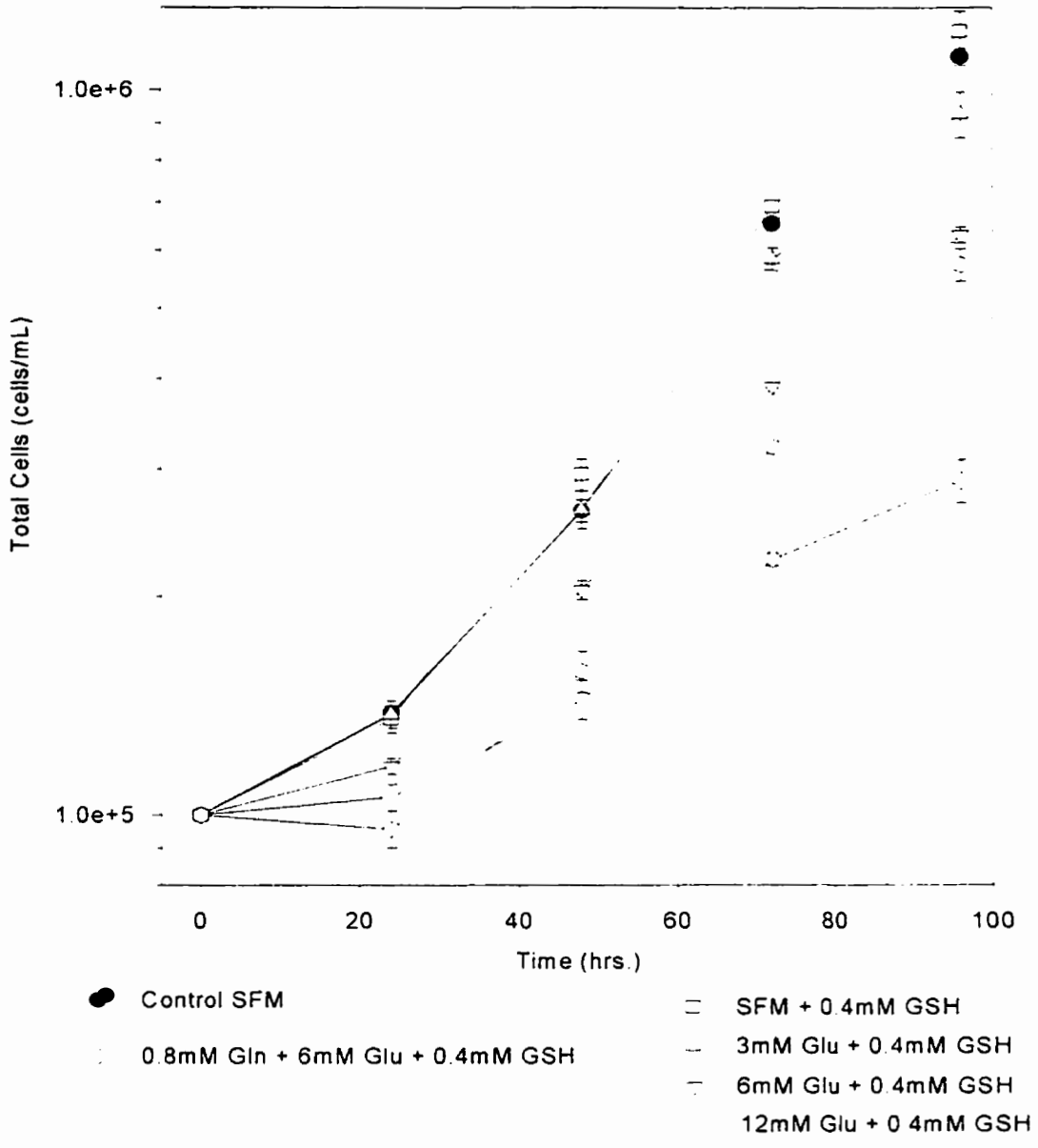
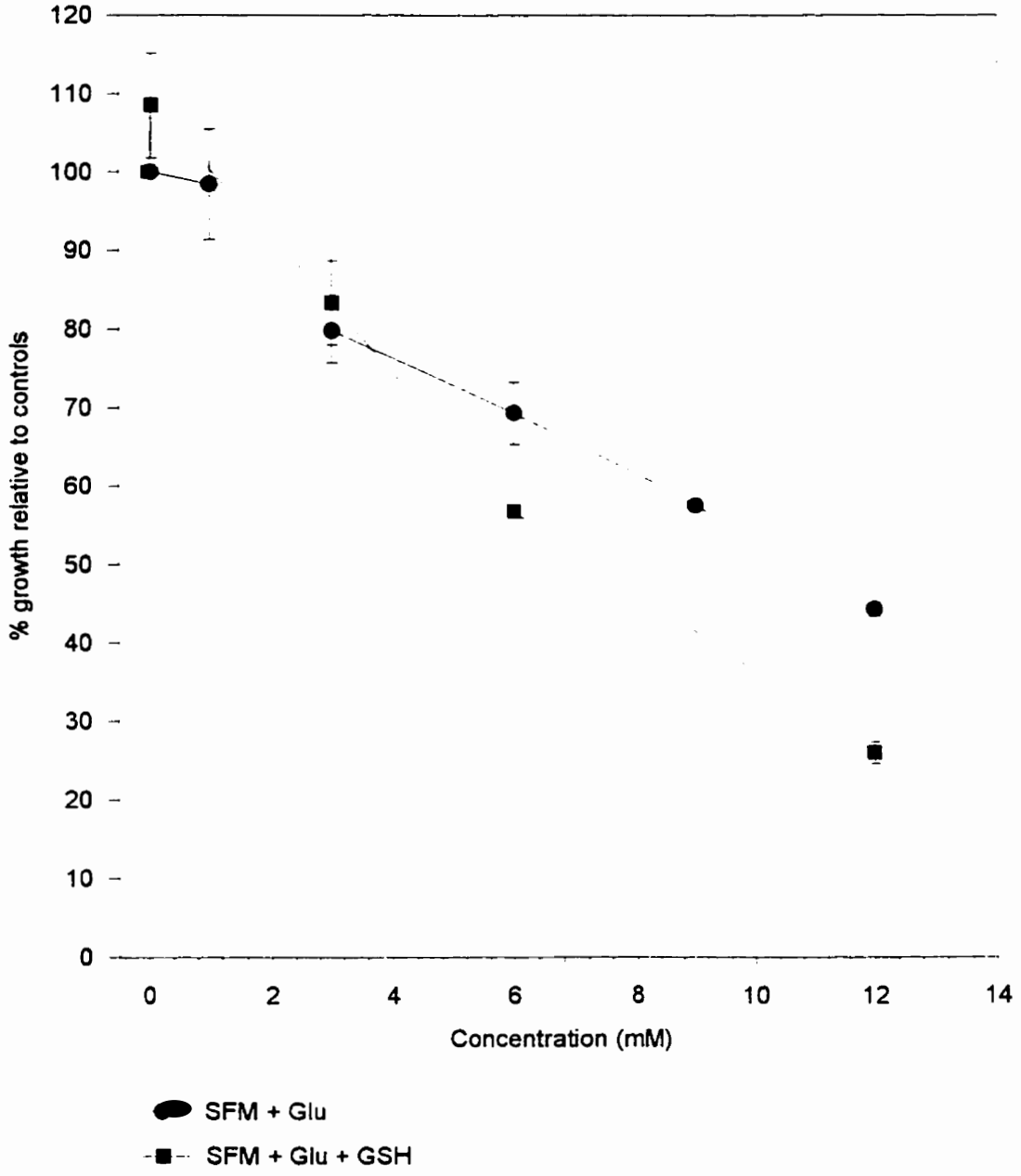
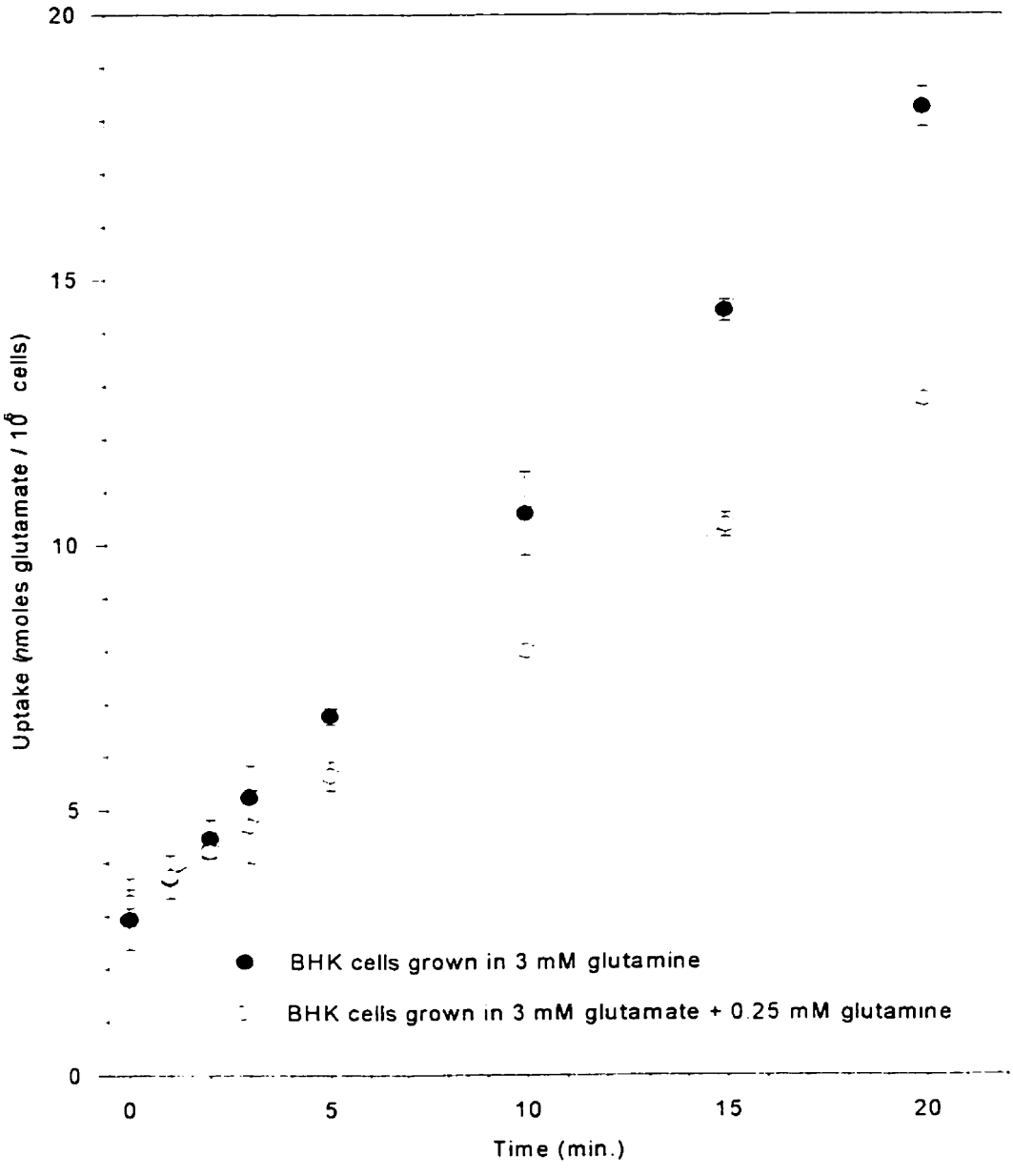


Figure 3.1.12 Effect of glutathione on CC9C10 hybridoma final cell density at 96 hours of growth. Cells were grown in SFM containing varying amounts of glutamate as compared to cells grown in SFM with varying amounts of glutamate, but no glutathione. (n=3, S.E.M.)



mercaptoethanol on growth in SFM and a glutamate based SFM was assessed. It was found that under normal SFM conditions, addition of 2-mercaptoethanol even at 1 *n*M resulted in a decrease of final cell densities and that at levels above 1 *u*M cell growth was significantly reduced (Figure 3.1.13). In SFM where glutamine was replaced by 6 mM glutamate, supplementation with 1 *n*M or 1 *u*M 2-ME had no significant effect on growth. However at concentrations greater than 0.5 mM final cell densities were reduced significantly.

Figure 3.2.1.1 Linearity of glutamate uptake by BHK fibroblastic cells. Cells were grown in 24 well plates until confluence in either DMEM 10% FCS 3 mM glutamine or DMEM 10% FCS 0.25 mM glutamine 3 mM glutamate. Cells were then rinsed with pre-warmed DPBS (2X) and exposed to DPBS containing 3 mM glutamate containing 0.1 *u*Ci U-^{14}C-glutamate for a varying period of time at 37^{o}C. Uptake was terminated by aspiration and radioactive incorporation determined by liquid scintillation counting. (n=2, S.E.M.)



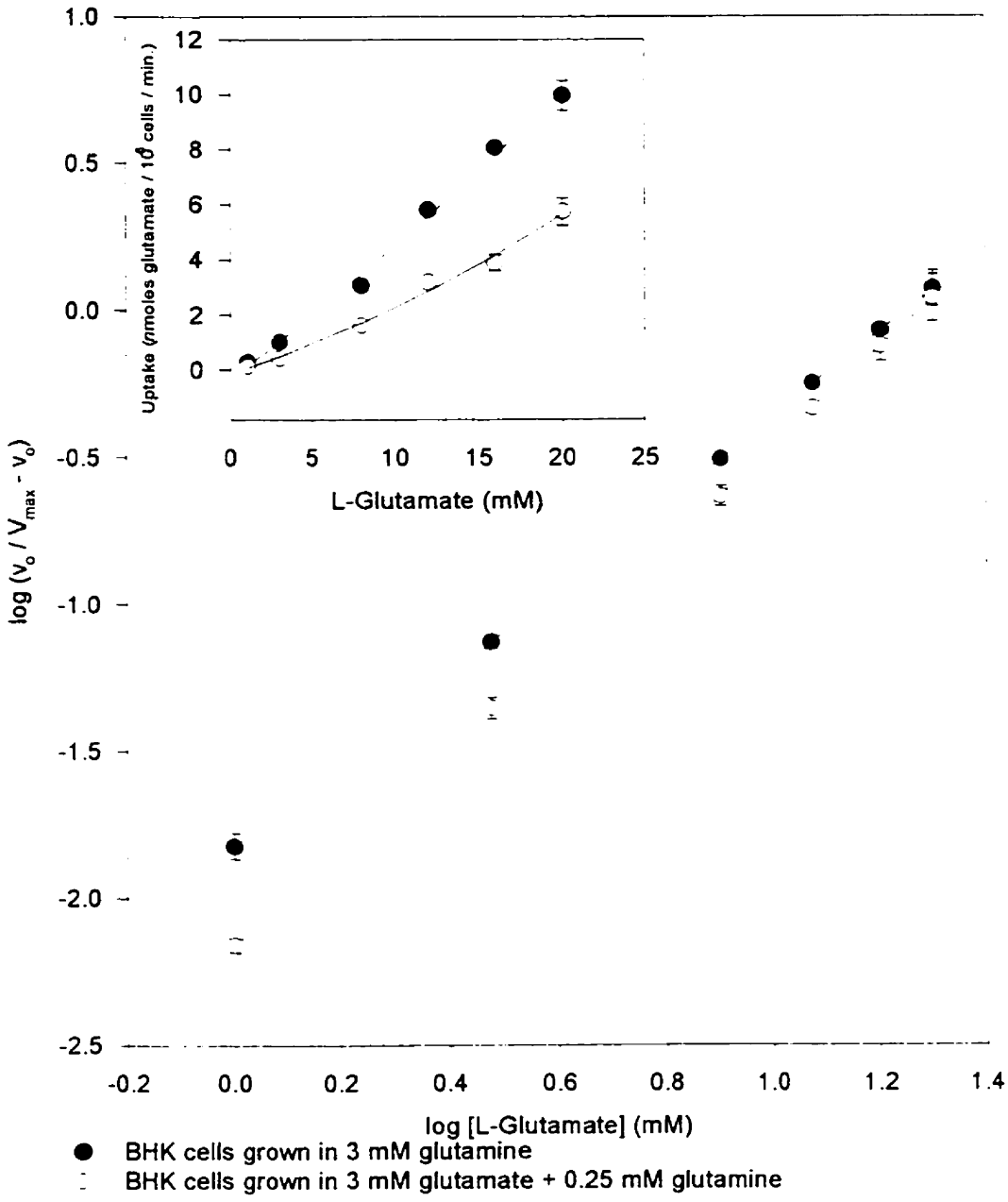
point of 10 minutes was selected as an assay point to provide sufficient radioactive incorporation for accurate measurement.

Having defined a time point at which to assay glutamate uptake, the next step was to vary the concentration of glutamate and assess uptake.. Concentration of glutamate was varied over a range of 1 - 20 mM. This range was selected arbitrarily. As seen in Figure 3.2.1.2 the uptake of glutamate, by both cell lines, does not follow typical Michaelis-Menten saturation curves. When non-linear regression, using the Marquardt-Levenberg algorithm of SigmaPlot, was applied it was found that the uptake of glutamate best fit a second order enzyme curve indicating possible allosteric effects. A Hill plot was then performed and the data fit to an interaction coefficient (*n*) of 1.45±0.09 the parental cell line and 1.46±0.10 for the adapted cell line using a an estimated value for V_{max}. After determining the interaction coefficient, the original data was then fit to the Hill equation:

$$(v = V_{max} / 1+K'/[S]^n).$$

The interaction coefficient was then checked again using the calculated V_{max} and was found to best fit a value of 2 for both parental and adapted cell lines. The maximum velocity (V_{max}) for the cells grown in 3 mM glutamine was found to be 16.70±1.44 *n*moles glutamate/10^6 cells/min. and for cells grown in 3 mM glutamate + 0.25 mM glutamine the maximum velocity was 10.74±2.24 *n*moles glutamate/10^6 cells/min (Table 3.2.2.1). The binding affinity constant K' (in this

Figure 3.2.1.2 Uptake of glutamate by BHK fibroblastic cells in DPBS. Confluent cells ($6X10^5$ cells/cm^2) in 24 well plates were washed twice in pre-warmed DPBS and then exposed to increasing amounts of glutamate containing 1 *u*Ci U-^{14}C-glutamate. Uptake was suspended by aspiration and radioactive incorporation into cells determined by scintillation counting. Inset is *v* v.s. S plot of data depicting non-Michaelis-Menten curves. (n=3, S.E.M.)



case equal to $[S_{1/2}]^2$) was found to be 271.9±45.7 mM for cells grown in 3 mM glutamine and 376.4±100.8 mM for the cells grown in 3 mM glutamate + 0.25 mM glutamine (Table 3.2.2.1). This indicates an overall decrease in V_{max}, with the K' being not significantly different, for the uptake of glutamate by cells which were adapted to growth on a basal media containing 3 mM glutamate + 0.25 mM glutamine.

3.2.2 Characterization of Glutamine Uptake

As glutamine is thought to be one of the primary carbon sources from which cells derive their catabolic energy, an examination of its uptake was performed so that a comparison might be made with glutamate uptake. Initial experiments were designed (in 24 well plates) to assess the linearity of glutamine uptake over a 20 minute period for two BHK cell lines. The BHK cells examined were a normal cell line culture on a basal media supplemented with 3 mM glutamine and 10% FCS, and a second cell line consisting of cells from the first line adapted to growth on a basal media supplemented with 3 mM glutamate, 0.25 mM glutamine and 10% FCS. Cells were grown to 90% confluence. Cells were then exposed to pre-warmed DPBS containing 3 mM glutamine + 0.1 *u*Ci U-^{14}C glutamine. Uptake was terminated by aspiration at the desired time. The cell monolayer was washed twice with ice cold DPBS, exposed to lysis buffer and filters collected in scintillation vials. Radioactive

incorporation was determined using a Rack Beta Scintillation Counter. As seen in Figure 3.2.2.1 the uptake of glutamine was linear over the 20 minute time range. Also the amount of glutamine taken up by the cells supplemented with the 3 mM glutamine was greater than that of the cells supplemented with the 3 mM glutamate over the 20 minute period. As the uptake was linear and due to greater amounts of radioactive incorporation observed, in comparison to glutamate, a time point of 5 minutes was selected for kinetic characterization experiments.

Having found glutamine uptake to be linear, the next step was to characterize the uptake kinetically. For these experiments, the time was held constant at 5 minutes and the concentration varied over an arbitrary range of 1 - 20 mM. As seen in Figure 3.2.2.2 the uptake of glutamine by both BHK cell lines again did not follow typical Michaelis-Menten saturation curves. When non-linear regression, using the Marquardt-Levenberg algorithm of SigmaPlot was applied, it was found that the uptake of glutamine best fit a second order enzyme curve indicating possible allosteric effects. Using a rough estimation of V_{max}, a Hill plot was then graphed and the data best fit to an interaction coefficient (*n*) of 2 for both curves. Having determined the interaction coefficient, the maximum velocity was then calculated using the Hill equation:

$$(v = V_{max} / 1+K'/[S]^n).$$

The maximum velocity (V_{max}) for the cells grown in 3 mM glutamine was found to be 77 ± 11 *n*moles glutamine/10^6 cells/min, and for cells grown in 3 mM glutamate

Figure 3.2.2.1 Linearity of glutamine uptake by BHK fibroblastic cells. Confluent cells, in 24 well plates, were washed with pre-warmed DPBS (2X) and exposed to DPBS containing 3 mM glutamine containing 0.1 *u*Ci U-^{14}C-glutamine for increasing amounts of time. Uptake was terminated by aspiration and radioactive incorporation was determined by liquid scintillation counting. (n=2, S.E.M.)

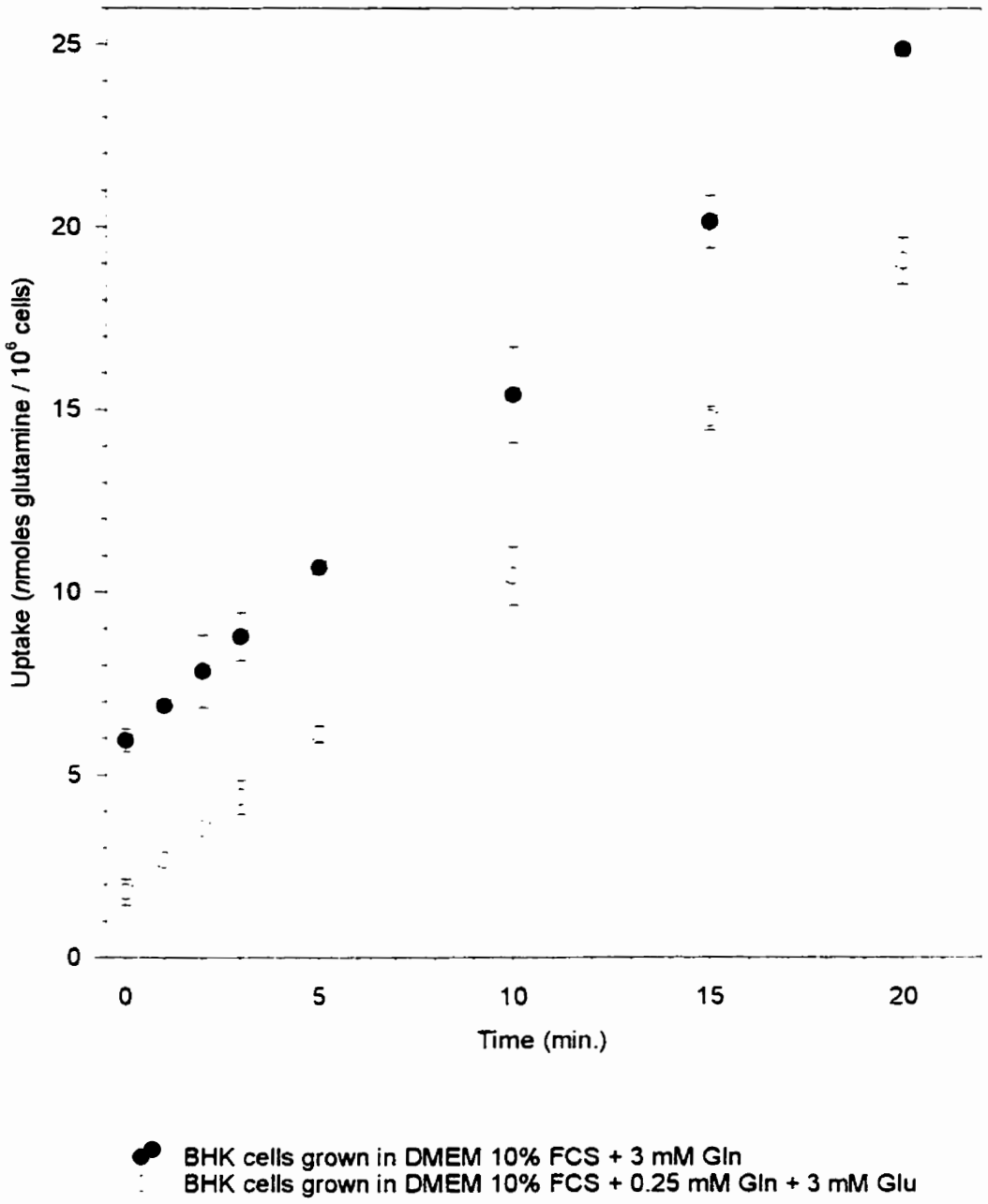
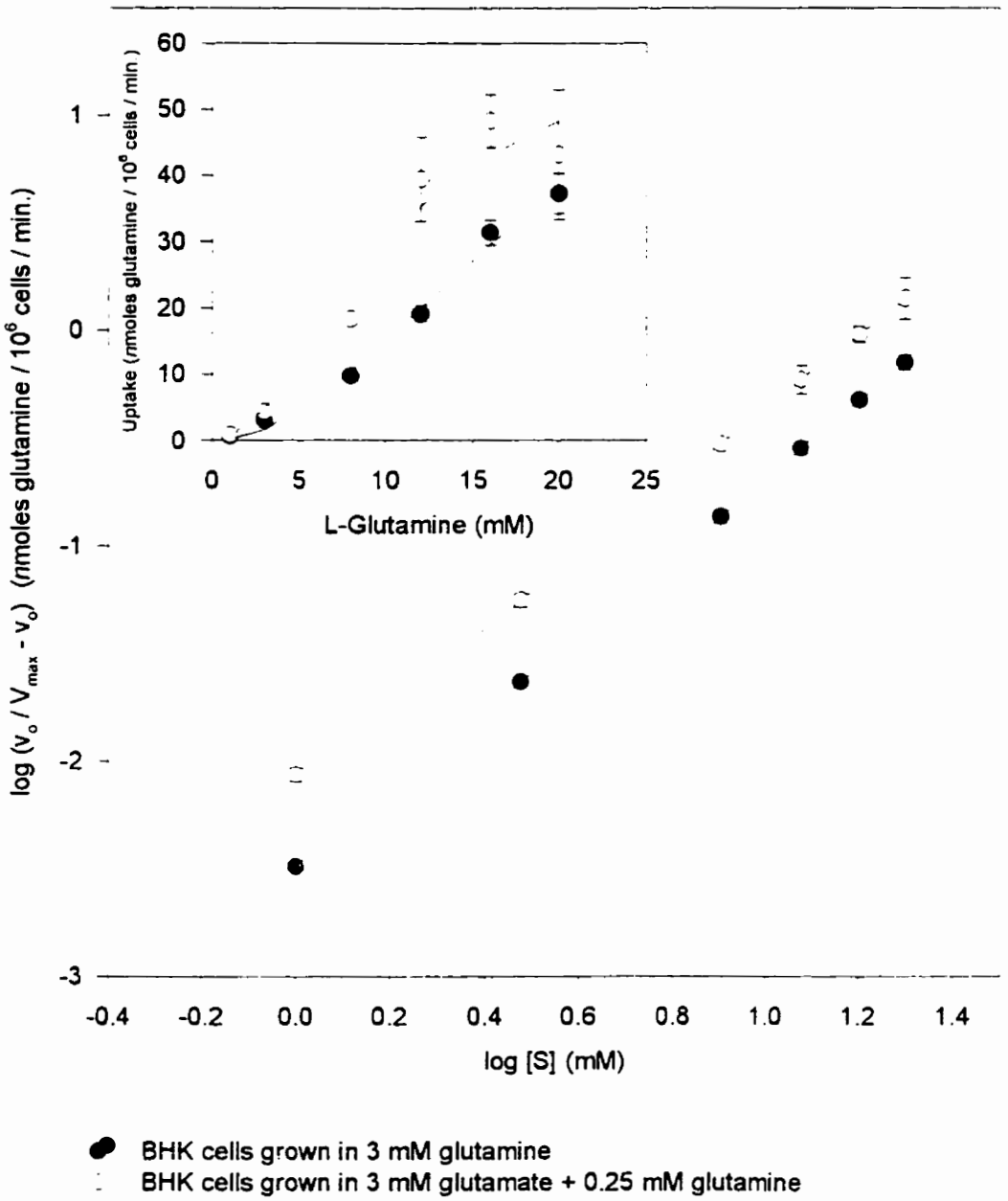


Figure 3.2.2.2 Uptake of glutamine by BHK fibroblastic cells. Confluent cells ($6X10^5/cm^2$), in 24 well plates were washed with pre-warmed DPBS (2X) and exposed to increasing glutamine concentrations containing 0.1 *u*Ci U-^{14}C-glutamine. Uptake was terminated by aspiration and radioactive incorporation was determined by liquid scintillation counting. Inset is *v* v.s. S plot depicting non-Michaelis-Menten curves. (n=3, S.E.M.)



+ 0.25 mM glutamine the maximum velocity was 60.7±9.7 *n*moles glutamine/10^6 cells/min (Table 3.2.2.1). The binding coefficient K' was found to be 411±100 mM for cells grown in 3 mM glutamine and 103.3±48.9 mM for cells grown in 3 mM glutamate + 0.25 mM glutamine (Table 3.2.2.1). The maximum velocities for the uptake of glutamine are not significantly different. However, the binding coefficient K' for cells grown in 3 mM glutamate + 0.25 mM glutamine is significantly lower than that of the cells grown in 3 mM glutamine, indicating a greater affinity for glutamine.

Table 3.2.2.1 Summary of kinetic parameters associated with the uptake of glutamate and glutamine for BHK cells grown in 3 mM glutamine and 3 mM glutamate + 0.25 mM glutamine. Values are means (± S.E.M.) for n=3 replicates

	V_{max} (*n*moles/10^6 cells/min.)	$^{\cdot}K'$ (mM)	$^{\cdot\cdot}n$
BHK fibroblasts grown in 3 mM glutamine			
Glutamate	16.70±1.44	271.9±45.7	2
Glutamine	77±11	411±100	2
BHK fibroblasts grown in 3 mM glutamate + 0.25 mM glutamine			
Glutamate	10.74±2.24	376.4±135.8	2
Glutamine	60.7±9.7	103.3±48.9	2

$^{\cdot}$ - K' is equal to $[S]^n$, where *n* the interaction coefficient is 2

$^{\cdot\cdot}$ - *n* is the interaction coefficient determined from Hill plot

Section 3.3. CC9C10 Hybridoma Amino Acid Uptake Kinetic Characterization.

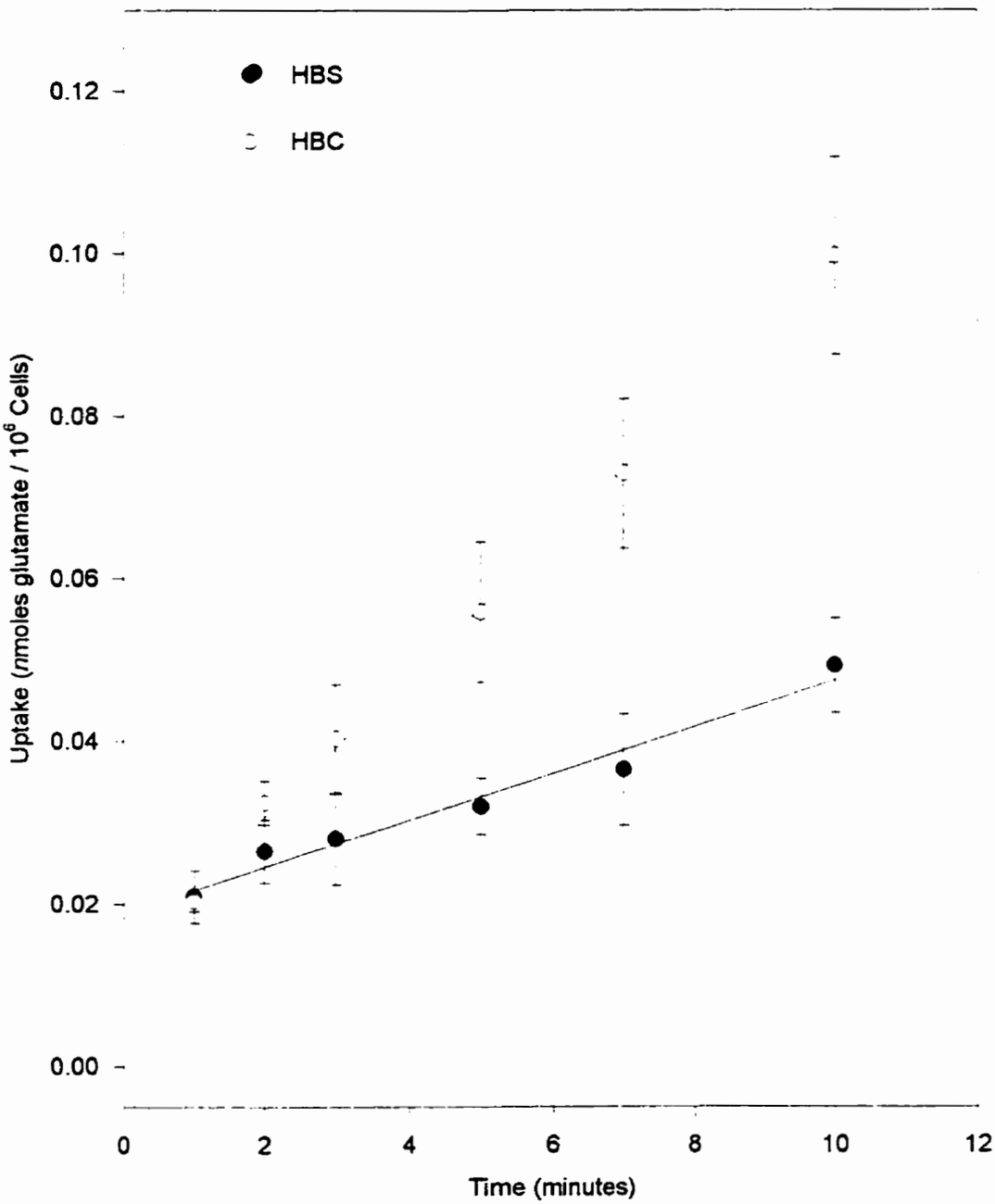
3.3.1. Characterization of L-Glutamate Uptake

3.3.1.1 Uptake Linearity and Kinetic Profile of L-Glutamate

To further investigate how glutamate inhibited cell growth, it was first necessary to assess if glutamate was taken up by the cells from the external medium. The preliminary uptake experiments in BHK fibroblasts had shown that glutamate uptake was linear over a 20 minute period, and a 10 minute time point had been selected for kinetic assays. The 10 minute time point was selected because under inhibitory conditions there would be a measurable amount of glutamate taken up by the cells. Uptake experiments in CC9C10 cells were initially designed to assess linearity of uptake and whether sodium was required for transport. As seen in Figure 3.3.1.1 the uptake of glutamate is sodium independent and is linear over a ten minute period. The presence of sodium in the buffer reduced the uptake by slightly over two-fold. Accordingly, Hepes buffered choline was selected as the uptake medium and a time point of 10 minutes was selected as an assay point to provide sufficient radioactive incorporation for accurate measurement of uptake.

The sodium independent uptake of glutamate was then examined at the 10 minute time point over increasing concentrations of glutamate. This was done in order to estimate kinetic parameters so that the glutamate uptake could be characterized kinetically. The uptake of glutamate was performed over a

Figure 3.3.1.1 Linearity of glutamate uptake by CC9C10 hybridoma cells. Cells were concentrated from mid-log phase growth in SFM and suspended in appropriate buffer containing glutamate (10 *u*M, + 0.1 *u*Ci U-^{14}C glutamate). Assays were performed in either HBS (with sodium) or HBC (sodium replaced by choline), over a period of 10 minutes at 37°C. (n=10, S.E.M.)



range of 0.01 mM to 20 mM. This range was selected as these were the concentrations (1 mM - 20 mM) which had been assessed for growth. The addition of the low concentrations was done in order to further assess the kinetic parameters. The uptake of glutamate depicted a typical Michaelis-Menten saturation curve (Figure 3.3.1.2). The kinetic parameters associated with the curve were found by non-linear curve fitting using the Marquardt-Levenberg algorithm of SigmaPlot to a hyperbolic curve:

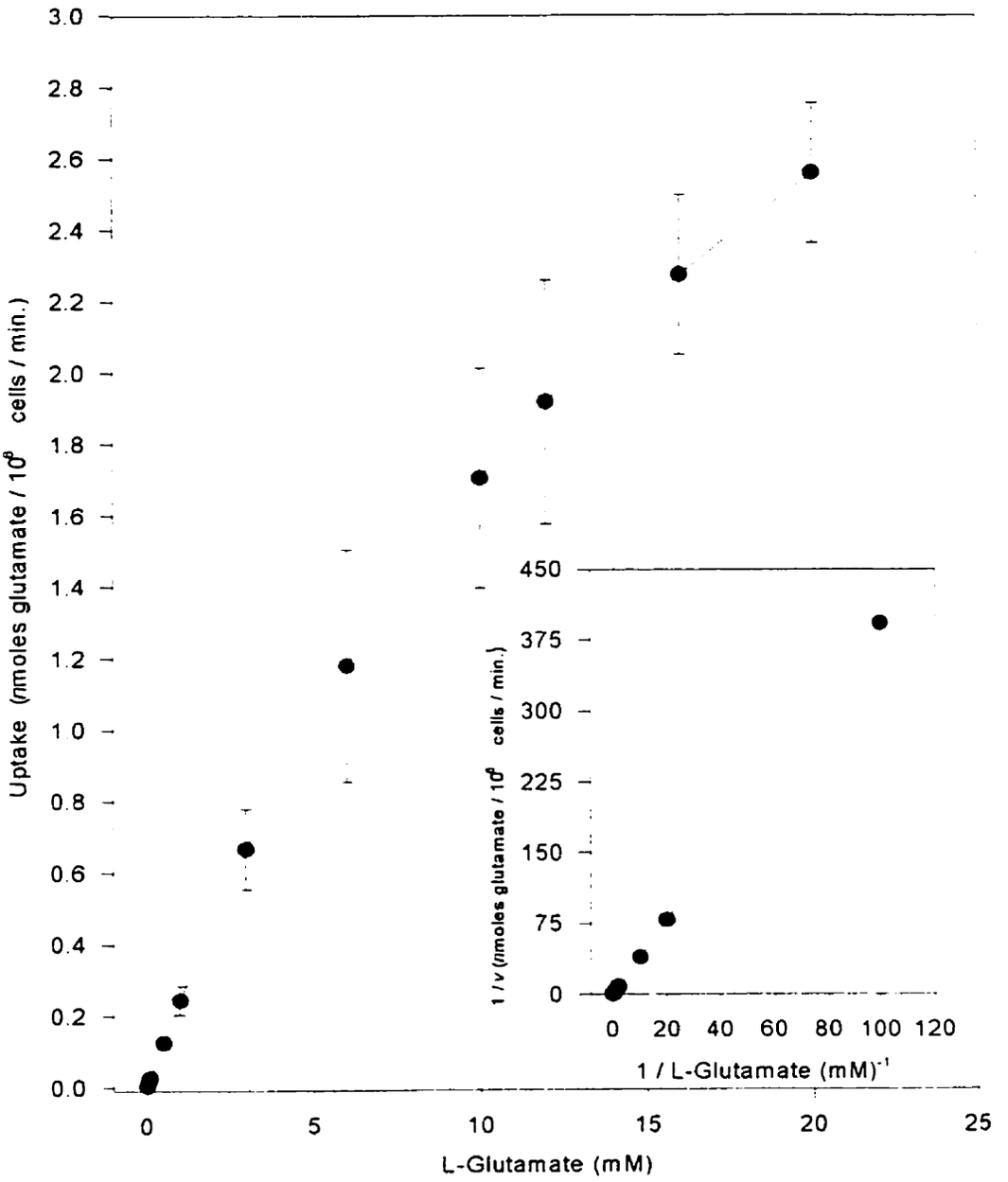
$$(v = V_{max}[S] / (k_m + [S])).$$

The ability of the sodium independent uptake system to bind glutamate was found to be 20±5 mM, while the velocity was found to be 5±1 *n*moles glutamate / 10^6 cells / minute (Table 3.3.1.2).

3.3.1.2 Inhibition of Glutamate Uptake by Amino Acids

Further characterization of the sodium independent uptake system was required to assess the specificity of the system for other amino acids. The ability of the sodium independent uptake system to take up 10 *u*M glutamate in the presence of the test amino acid 1 mM was performed to assess the systems specificity. Cells were harvested from 72 hour old cultures and resuspended in HBC containing radioactive glutamate (0.1 *u*Ci U-^{14}C glutamate), cold glutamate (10 *u*M) and test amino acid (1 mM). Amino acids (Sigma, tissue culture grade) tested included L-glutamine, L-glutamate, D-glutamate, pyroglutamate,

Figure 3.3.1.2 Uptake of glutamate by CC9C10 hybridoma cells in HBC. Cells were concentrated from mid-log phase growth in SFM and suspended in HBC containing increasing concentrations of glutamate. All assays were supplemented with 0.1 *u*Ci U-^{14}C-glutamate. Uptake was terminated at 10 minutes by filtration and radioactive incorporation into cells (trapped on filter pads) was determined by scintillation counting. Inset is Lineweaver-Burke representation of data. (n=13, S.E.M.) k_m = 20±5 mM and V_{max} = 5±1 *n*moles glutamate / 10^6 cells / minute.



L-cysteine, homocysteic acid, L-leucine, L-lysine, L-alanine, L-aspartate, L-asparagine, L-tryptophan, L-phenylalanine, L-valine, L-isoleucine, L-serine, L-threonine, L-methionine, L-proline, L-histidine, and L-arginine. Cells were exposed for the desired period of time and uptake assessed as per Methods and Materials 2.3. Of the amino acids tested it was found that L-valine, L-isoleucine, L-cysteine, L-threonine, L-proline, L-histidine, L-arginine, L-asparagine and homocysteic acid showed a significant difference from control. However, only homocysteic acid extensively inhibited the uptake of L-glutamate, and was selected for further study (Table 3.3.1.1). More notable, is that the system is stereo specific for L-glutamate, as D-glutamate did not extensively inhibit the uptake of L-glutamate. Also pyroglutamate and L-aspartate did not extensively inhibit the uptake of L-glutamate. Cystine was not tested at the point as its' solubility is only 0.4 mM, but was tested later for kinetic analysis. The uptake of 10 *u*M glutamate reported in Table 3.3.1.1 was found not to be significantly different from levels of uptake in Figure 3.3.1.2 (0.0034 ± 0.0006 vs. 0.0035 ± 0.0007 *n*moles/10^6 cells/min.).

3.3.1.3 Kinetic Characterization of Cystine Inhibition of Glutamate Uptake

The sodium independent uptake of glutamate was tested in the presence of 0.2 mM cystine to assess the effect of cystine on glutamate uptake. It was reasoned so far, that because the system is sodium independent, and because

Table 3.3.1.1 Inhibition of amino acids (1 mM) on the uptake of glutamate (10 *u*M, + 0.1 *u*Ci U-^{14}C-glutamate) in HBC. Cells were concentrated from mid-log phase growth in SFM and suspended in buffer containing the appropriate amino acid and glutamate. Control contained 10 uM glutamate (+ 0.1 *u*Ci U-^{14}C-glutamate) in buffer with no supplement. The rate of uptake in the control (designated 100%) was 0.0034±0.0006 *n*moles/10^6 cells/min. (n=3, S.E.M.)

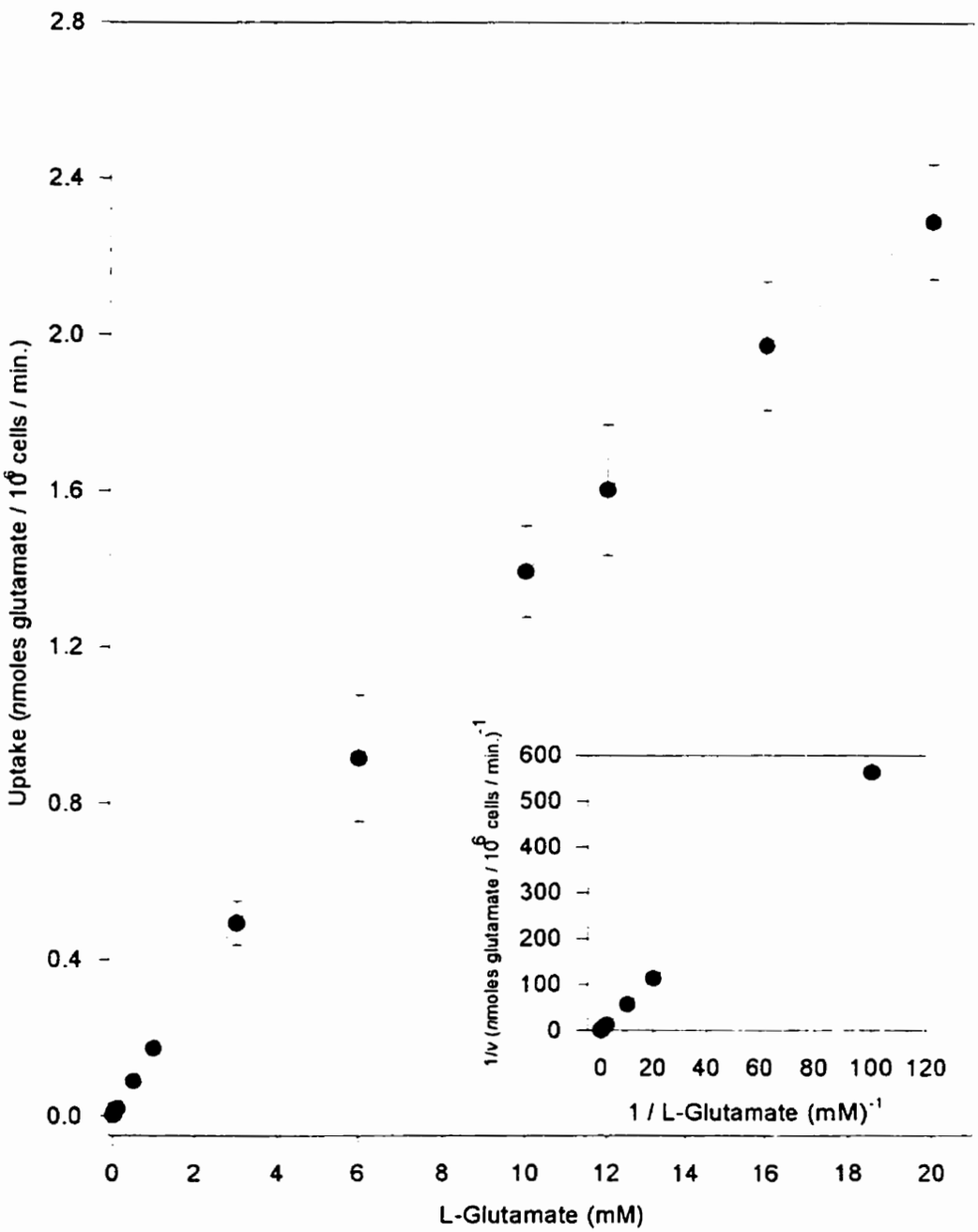
Supplemented Amino Acid	Uptake Rate of L-Glutamate as a % of Control
Control (no supplement)	100±17
L-Alanine	92 ± 15
L-Arginine	75 ± 2
L-Asparagine	56 ± 5
L-Aspartate	73 ± 10
L-Cysteine	66 ± 13
D-Glutamate	84 ± 7
L-Glutamine	119 ± 18
L-Histidine	64 ± 8
Homocysteic Acid	24 ± 4
L-Isoleucine	66 ± 12
L-Leucine	82 ± 14
L-Lysine	109 ± 14
L-Methionine	83 ± 16
L-Phenylalanine	112 ± 25
L-Proline	62 ± 8
pyroglutamate	104 ± 10
L-Serine	103 ± 15
L-Threonine	64 ± 8
L-Tryptophan	97 ± 16
L-Valine	65 ± 4

aspartate showed no significant level of inhibition, that the uptake of glutamate was being mediated by system x^-_c. System x^-_c is a sodium independent uptake system for glutamate and cystine. The level of cystine chosen was the same as level present in SFM. Cells were harvested from a 72 hour old culture and suspended in pre-warmed HBC buffer containing radioactive glutamate (0.1 *u*Ci U-^{14}C glutamate), glutamate (0.01-20 mM) and L-cystine (0.2 mM). Uptake was terminated after desired time by filtration. Filters were removed, placed in lysis buffer and radioactive incorporation assessed using a rack beta scintillation counter. The uptake of glutamate in the presence of 0.2 mM cystine was typical of Michaelis-Menten saturation plots (Figure 3.3.1.3). It was found, using SigmaPlot:

$$v = V_{max}[S] / k_m(1+[I]/k_i)[S]),$$

that the presence of 0.2 mM cystine resulted in an apparent k_m of 36.0±8 mM and a maximum velocity of 6±1 *n*moles glutamate / 10^6 cells / minute (Table 3.3.1.2). In comparison to the V_{max} of glutamate with no inhibitor (Figure 3.3.1.2), it can be seen that there is no significant difference between the maximum velocities. However, in comparison to the k_m of glutamate uptake (20±5 mM), the apparent k_m was found to be higher in the presence of 0.2 mM cystine (36.0±8 mM). This indicates a reduced affinity for glutamate uptake in the presence of cystine, which means that cystine is a competitive inhibitor of glutamate uptake. The sodium independent uptake system of glutamate therefore, also is most likely responsible the uptake of cystine.

Figure 3.3.1.3 Uptake of glutamate by CC9C10 hybridomas in the presence of 0.2 mM L-cystine. CC9C10 cells were concentrated from mid-phase growth in SFM and suspended in HBC buffer containing glutamate (0.1 *u*Ci U-^{14}C-glutamate) and cystine at appropriate levels for a period of 10 minutes. Reactions were terminated by filtration. Inset is Lineweaver-Burke representation of data. (n=12, S.E.M.)
k_m = 36±8 mM and V_{max} = 6±1 *n*moles glutamate / 10^6 cells / minute.



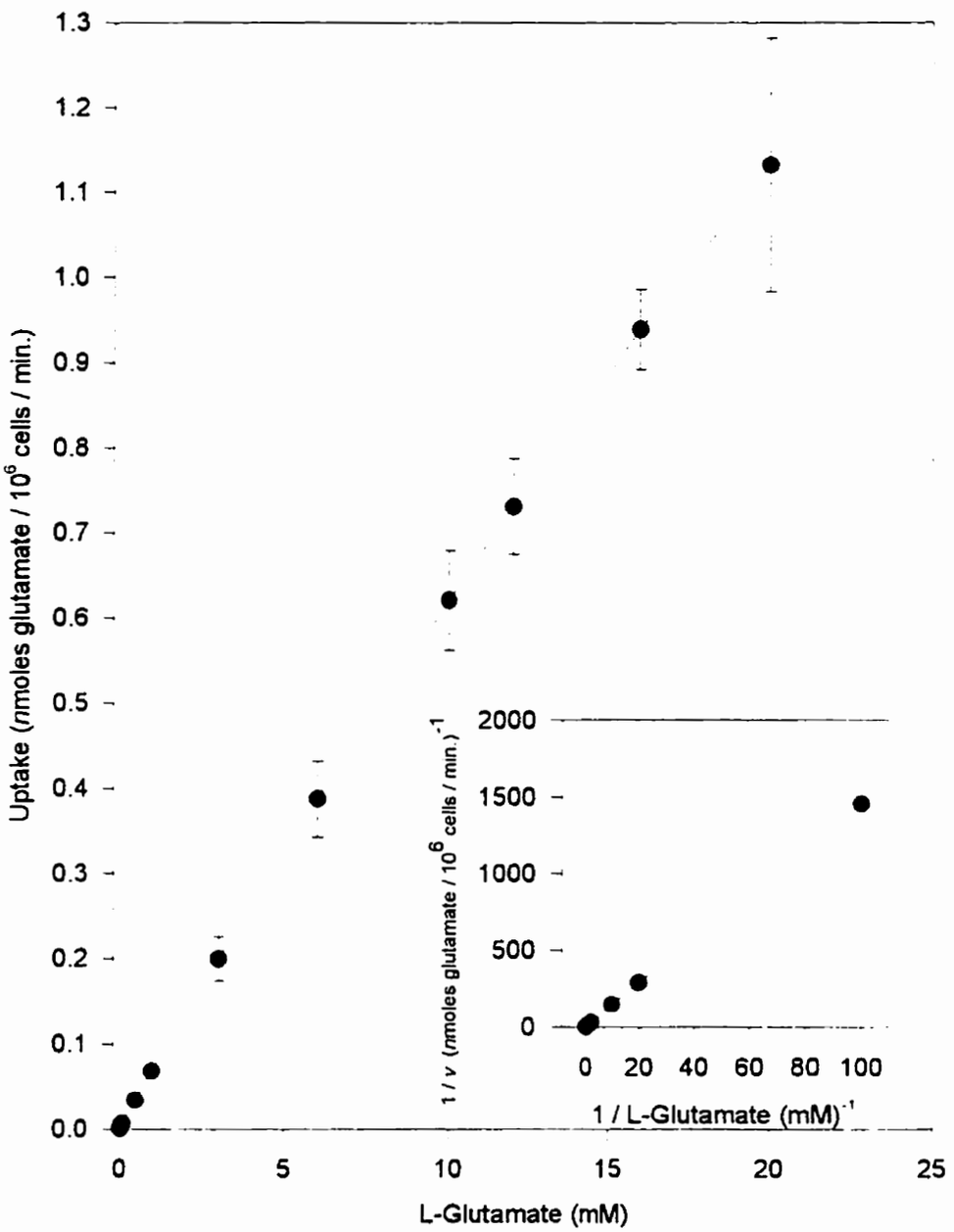
3.3.1.4 Kinetic Characterization of Homocysteic Acid Inhibition of Glutamate Uptake

As homocysteic acid was the only one of the amino acids tested (for inhibition of 10 *u*M glutamate) which was found to extensively inhibit the uptake of L-glutamate. Further kinetic assessment was performed to determine the type of inhibition it exerted, and to further define the substrate specificity of the glutamate uptake system. A level of 0.4 mM homocysteic acid (structurally similar to glutamate carrying a net negative charge of 2) was selected as this is approximately 10X the amount of glutamate normally present in SFM. The cells were prepared and experiment performed as per Methods and Materials 2.3.2 except for the additional presence of the inhibitor (0.4 mM homocysteic acid). In the presence of 0.4 mM homocysteic acid it was found that glutamate uptake was a typical Michaelis-Menten saturation curve (Figure 3.3.1.4). The kinetic parameters were determined by SigmaPlot:

$$(v = V_{max}[S] / k_m(1+[I]/k_i)[S]).$$

The apparent k_m of glutamate in the presence of 0.4 mM homocysteic acid was found to be 93$\pm$2 mM and the maximum velocity was 6.1$\pm$0.5 *n*moles glutamate / 10^6 cells / minute (Table 3.3.1.2). In comparison to the uptake of glutamate with no inhibitors (Figure 3.3.1.2), it can be seen that the maximum velocity is not significantly different, but the apparent k_m of the uptake system for glutamate is greatly increased in the presence of homocysteic acid. This indicates that

Figure 3.3.1.4 Uptake of glutamate by CC9C10 hybridomas in presence of homocysteic acid. Cells were concentrated from mid-log phase growth in SFM and suspended in HBC buffer containing HCA (0.4 mM) and increasing glutamate (0.1 *u*Ci U-^{14}C glutamate). Assay was terminated by filtration. Inset is Lineweaver-Burke representation of data. (n=2, S.E.M.).
k_m = 93±2 mM and V_{max} = 6.1±0.5 *n*moles glutamate / 10^6 cells / minute

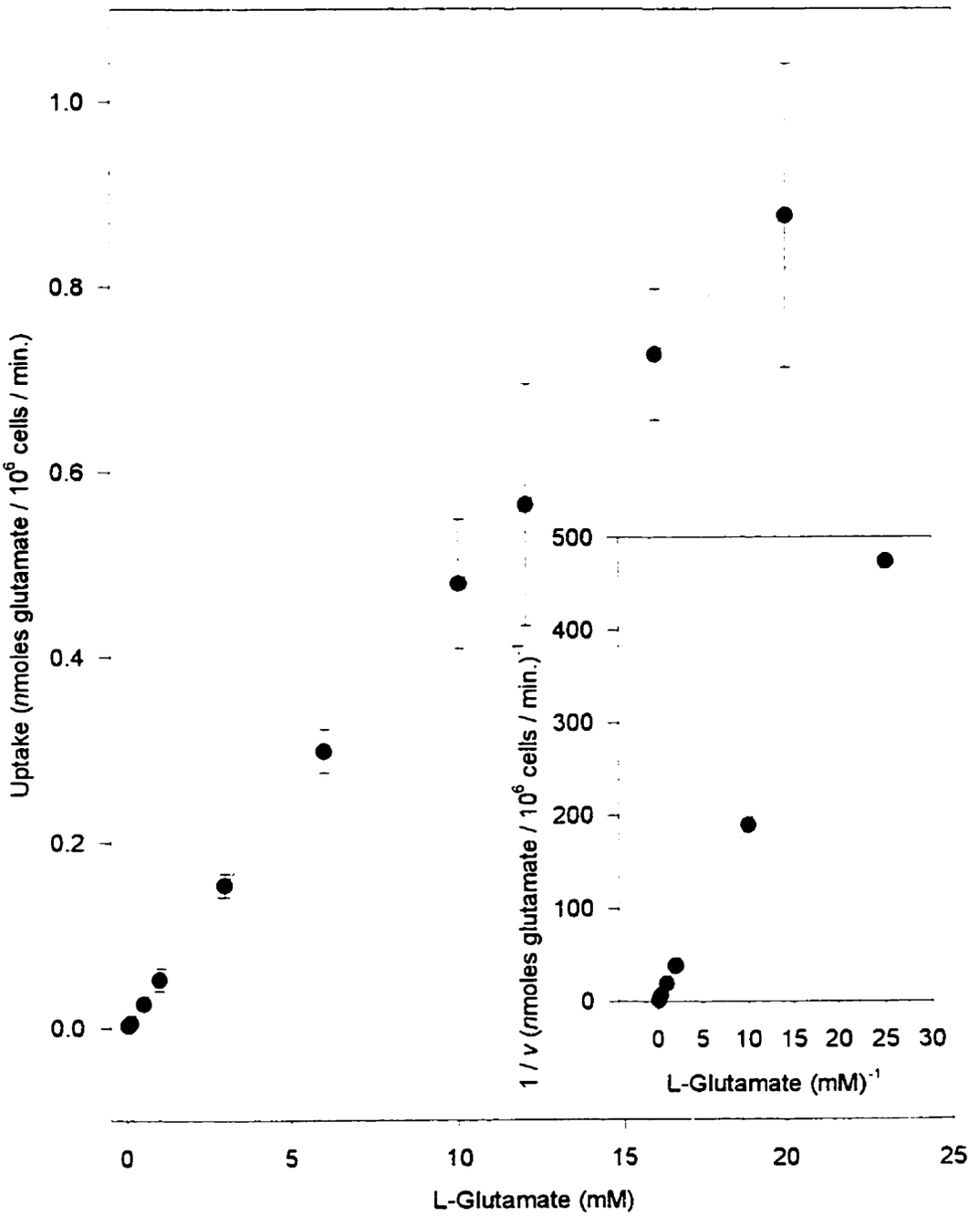


homocysteic acid is a competitive inhibitor of glutamate uptake. In comparison to uptake of glutamate in the presence of cystine, uptake of glutamate in the presence of homocysteic acid was found to have a higher apparent k_m (93±2 in comparison to 36±0.5 mM respectively) of glutamate. This greatly reduced binding affinity of glutamate in the presence of homocysteic acid indicates a stronger affinity of the system for homocysteic acid over cystine.

3.3.1.5 Kinetic Characterization of Glutamate Uptake in SFM

To assess the effectiveness of CC9C10 cells to sequester glutamate from the external medium, it was necessary to assess kinetic parameters under normal growth conditions. The uptake of glutamate was therefore assessed in culture medium (DMEM/F-12 - SFM), so that determined kinetic parameters could be compared to glutamate uptake in a single buffer system (no inhibitors). Cells were harvested from a 72 hour old culture and resuspended in pre-warmed SFM containing radioactive glutamate (0.1 *u*Ci U-^{14}C glutamate) and glutamate (0.1-20 mM). Uptake was terminated by filtration. Filters were collected, placed in lysis buffer and radioactive incorporation determined by rack beta scintillation counting. The uptake of glutamate was found to exhibit a typical Michaelis-Menten saturation curve (Figure 3.3.1.5). The kinetic parameters were

Figure 3.3.1.5 Uptake of glutamate by CC9C10 hybridomas in SFM. Cells were concentrated from mid-log phase growth and resuspended in SFM containing glutamate (0.1 *u*Ci U-^{14}C glutamate) for a period of 10 minutes at 37°C. Assay was terminated by filtration. Inset is Lineweaver-Burke representation of data. (n=6, S.E.M.)
k_m = 97±5 mM and V_{max} = 5±1 *n*moles glutamate / 10^6 cells / minute



determined using SigmaPlot. The apparent k_m of the uptake system for glutamate in SFM was found to be 97±5 mM and the maximum velocity was found to be 5±1 *n*moles glutamate/10^6 cells/min. (Table 3.3.1.2). In comparison to glutamate uptake in a single buffer system, it can be seen that the velocity was the not statistically significantly different, but the apparent affinity of the system (k_m) for glutamate was greatly reduced (20 mM vs. 97 mM).

3.3.1.6 Summary of Glutamate Uptake by CC9C10 Hybridomas under all Experimental Conditions Examined

In order to better visualize the effects that inhibitors have on glutamate uptake, Lineweaver-Burke representations of all conditions examined were plotted (Figure 3.3.1.6). A summary table of all kinetic parameters associated with glutamate uptake was also compiled (Table 3.3.1.2). As seen in Figure 3.3.1.6, the addition of inhibitors or uptake in SFM resulted in steeper slopes than that of glutamate uptake in HBC. The uptake of glutamate in SFM was found to cause the largest reduction in glutamate uptake. HCA was found to be the next largest inhibitor (due to structural similarities shared with glutamate), while cystine was found to cause the least amount of reduction in glutamate uptake, but was still significantly different from the uptake of glutamate. The uptake velocities (Table 3.3.1.2) are not significantly different from one another, while all the k_m's are. This indicates that HCA, cystine and compounds in SFM are all competitive inhibitors of the uptake of glutamate.

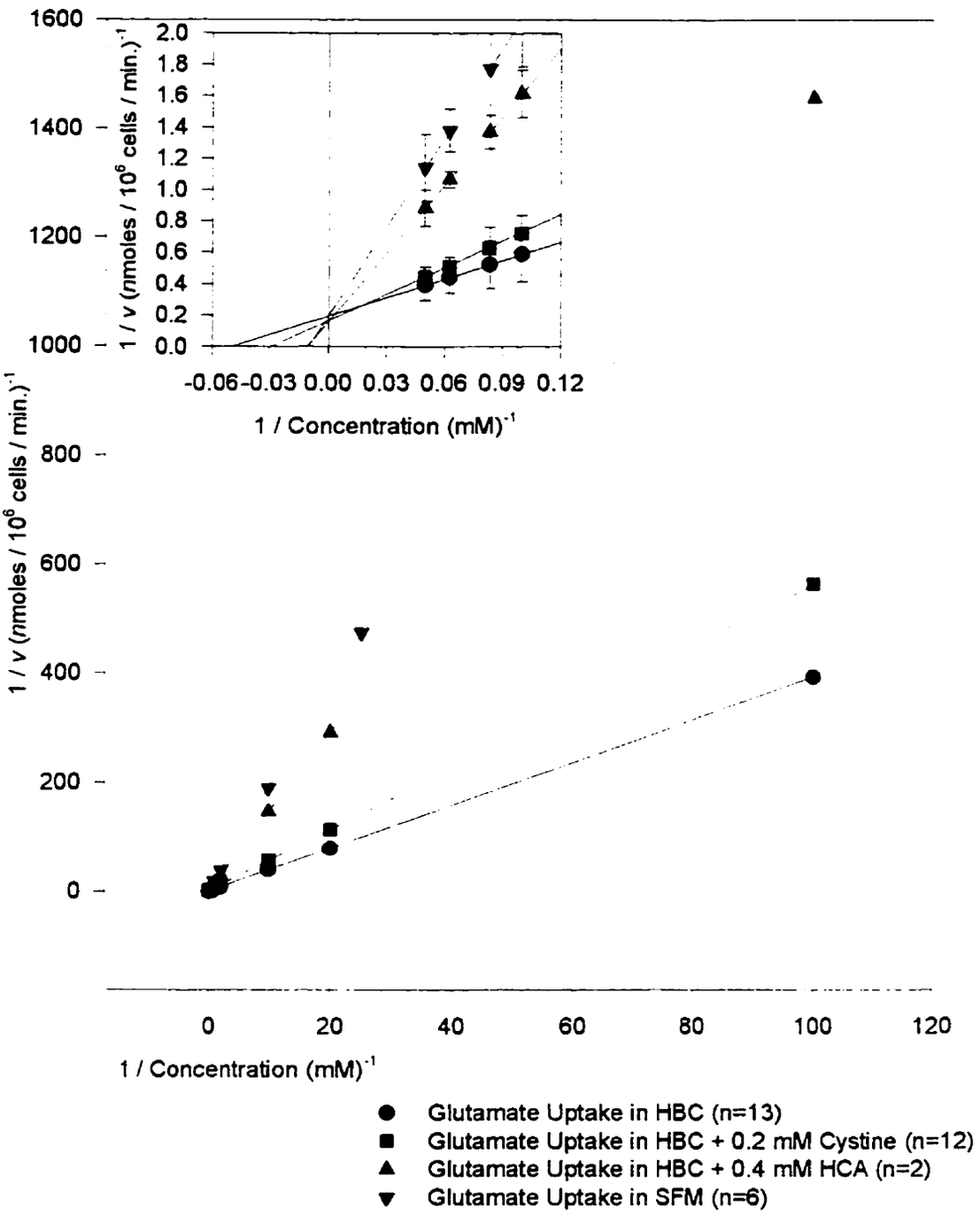
Table 3.3.1.2 Summary of kinetic parameters associated with the uptake of glutamate in CC9C10 hybridomas in HBC, in SFM and in HBC with the inhibitors cystine (0.2 mM) or homocysteic acid (0.4 mM).

Conditions	$^{*}k_m$ (mM)	V_{max} *n*moles / 10^6 cells /min	K_i (mM)
Glutamate (Control)	20±6	5±1	*N.A.
Glutamate + 0.2 mM Cystine	36±8	6±1	4±1
Glutamate + 0.4 mM HCA	93±2	6.1±0.5	9±2
Glutamate in SFM	98±5	5±1	N.A.

*k_m is the apparent k_m under all conditions examined, except for the uptake of glutamate

N.A. not applicable

Figure 3.3.1.6 Lineweaver-Burke representation of uptake of glutamate by CC9C10 hybridoma cells in presence or absence of inhibitors examined. Inset depicts where lines intersect axes. Points are means (± S.E.M.).

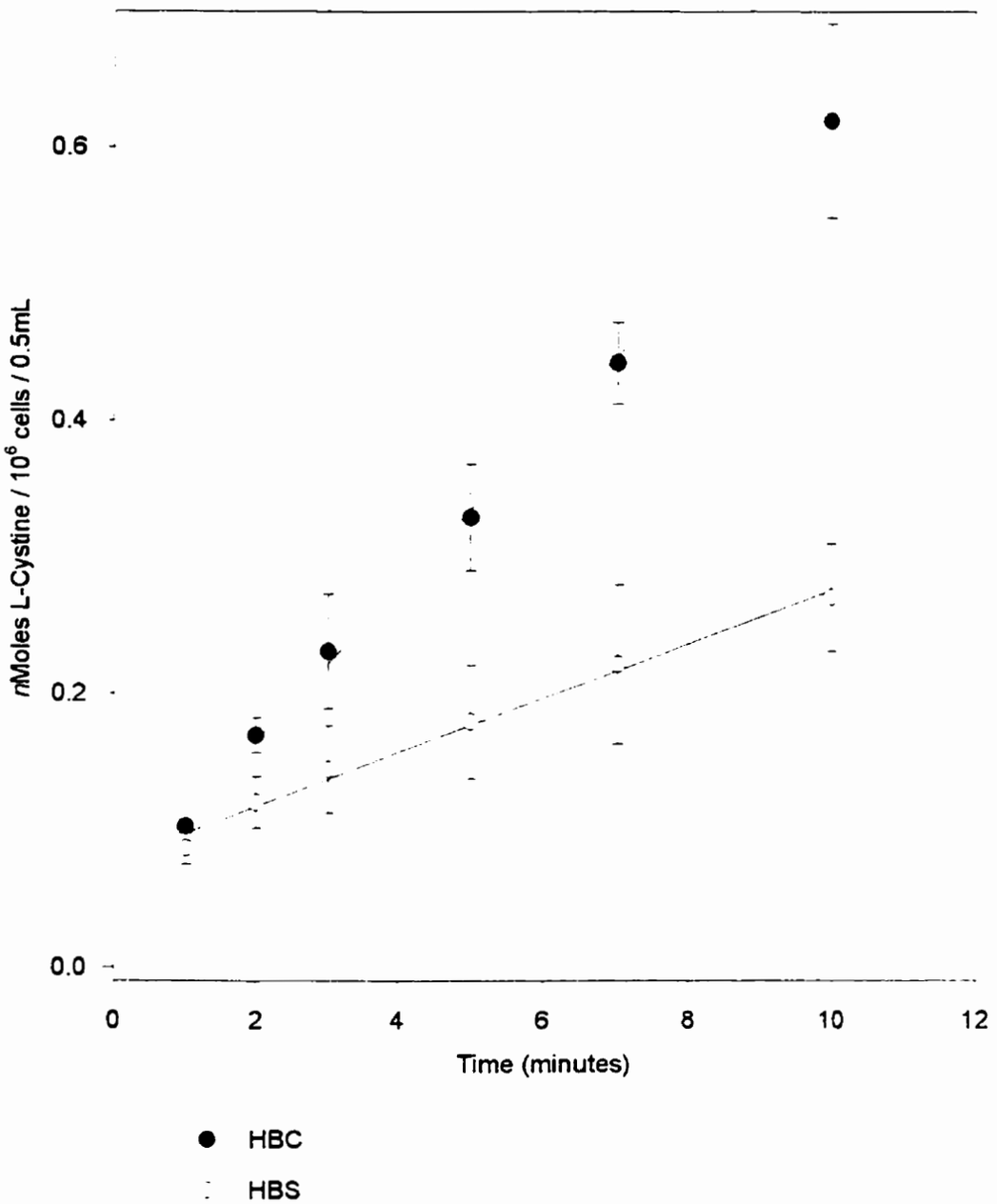


3.3.2. Characterization of L-Cystine Uptake

3.3.2.1 Uptake Linearity and Kinetic Profile of L-Cystine

The uptake of L-cystine by CC9C10 hybridoma cells was performed in order to assess whether or not cells were able to take up cystine from the external medium. It was also necessary to assess whether or not glutamate was inhibitory to cystine uptake. Initial experiments were performed to assess whether the presence of sodium was required for uptake and to determine linearity of uptake over time. A concentration of 0.2 mM cystine was used to duplicate that which is normally found in SFM. Cells were harvested from a 72 hour old culture and resuspended in pre-warmed HBS or HBC containing radioactive cystine (0.1 *u*Ci U-^{14}C cystine) and cystine (0.2 mM). After desired time, uptake was terminated by filtration. Filters were collected, placed in lysis buffer and radioactive incorporation measured using a rack beta scintillation counter. The uptake of L-cystine in HBS and HBC was found to be linear over a 10 minute period (Figure 3.3.2.1). The presence of sodium in the buffer resulted in one third of the cystine being taken up over the 10 minute period, in comparison to the uptake in buffer where sodium was replace by choline. This demonstrates that cystine, as glutamate, is also taken up from the external medium by a sodium independent means. The HBC buffer (sodium replaced) and a time point of 3 minutes were selected for kinetic uptake studies, as it was determined that these were sufficient for measurement of cystine uptake.

Figure 3.3.2.1 Linearity of L-cystine uptake by CC9C10 hybridoma cells. Cells were concentrated from mid log-phase growth in SFM. Cells were assayed for linearity of uptake of cystine (0.2 mM, + 0.1 *u*Ci U-^{14}C cystine) in HBS and HBC over a 10 minute period at 37°C. Assays were terminated by filtration. (n=5, S.E.M.)



The assay of cystine was performed in HBC in order to characterize kinetically the uptake system responsible for cystine uptake and to assess whether this was the same system responsible for glutamate uptake. Due to the limited solubility of cystine, a narrow concentration range (0.01 - 0.4 mM) was chosen. Cells were harvested from a 72 hour old culture and resuspended in pre-warmed HBC containing radioactive cystine (0.1 μCi L-^{14}C cystine) and cystine (0.01-0.4 mM). Uptake was terminated by filtration. Filters were collected, placed in lysis buffer and radioactive incorporation measured using a rack beta scintillation counter. The uptake of cystine was found to exhibit a typical Michaelis-Menten saturation curve (Figure 3.3.2.2). The kinetic parameters associated with the curve were determined by SigmaPlot analysis:

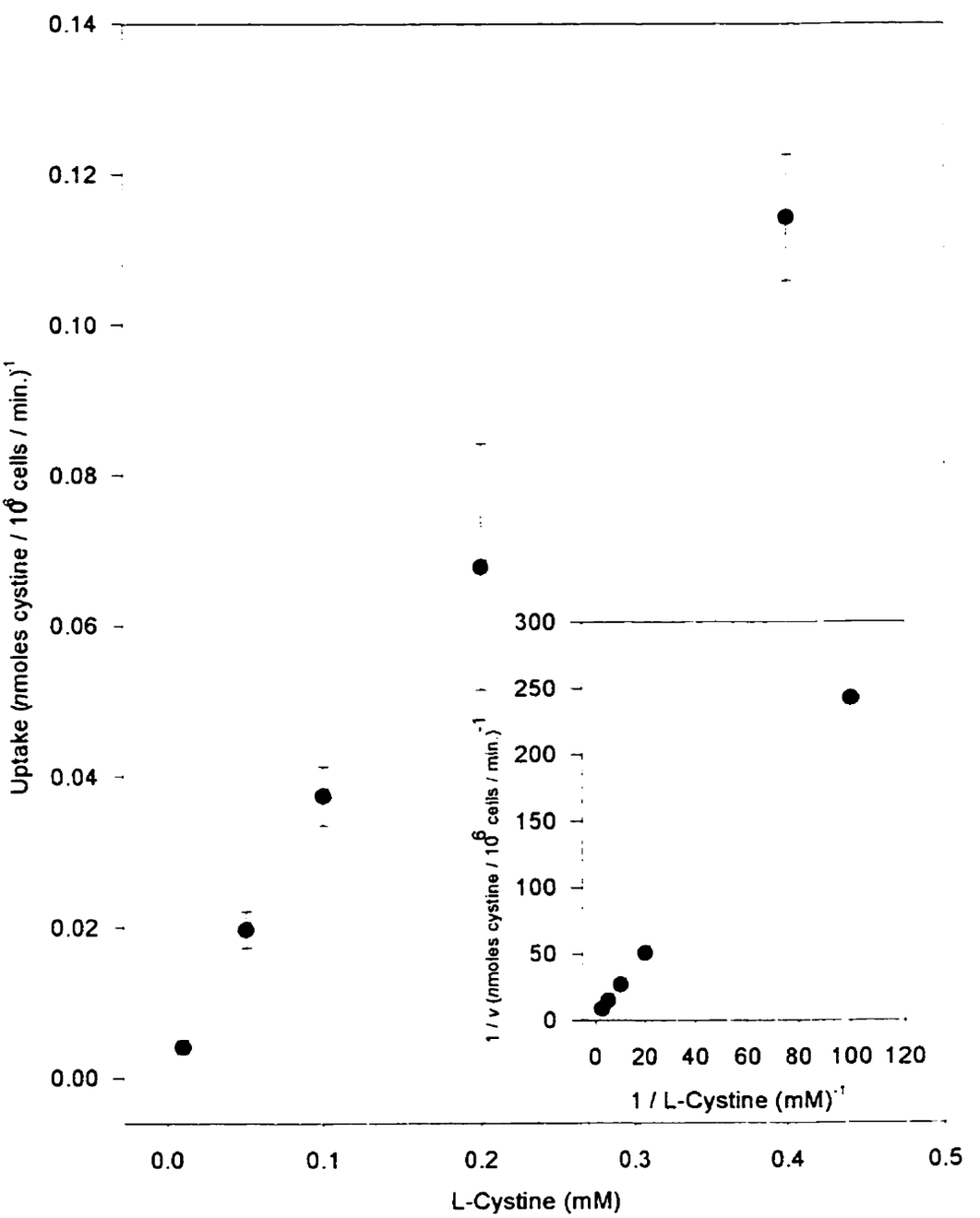
$$v = V_{max}[S] / k_m + [S]$$

The k_m of the uptake system for cystine was found to be 0.9$\pm$0.1 mM and had a maximum velocity of 0.36$\pm$0.03 *n*moles cystine / 10^6 cells / minute (Table 3.3.2.1). In comparison to the uptake of glutamate in the presence of cystine (Figure 3.3.1.2) it can be seen that cystine has a much lower k_m (and therefore greater affinity) for the uptake system, but a significantly lower maximum velocity (0.9 vs. 20 mM, and 5 vs. 0.36 *n*moles / 10^6 cells / minute).

3.3.2.2 Inhibition of Cystine Uptake by Glutamate

As the kinetic parameters associated with cystine uptake were not yet readily comparable to those of glutamate uptake. It was therefore necessary to

Figure 3.3.2.2 Uptake of L-cystine by CC9C10 hybridoma cells in HBC. Cells were grown to mid-log phase in SFM, concentrated and then suspended in the appropriate level of L-cystine (0.1 *u*Ci U-^{14}C cystine). Uptake was assayed for 3 minutes with increasing L-cystine concentrations at 37°C. Assays were terminated by filtration. Inset is Lineweaver-Burke representation of data. (n=3, S.E.M.)
k_m = 0.87±0.1 mM and V_{max} = 0.36±0.03 *n*moles cystine / 10^6 Cells / minute

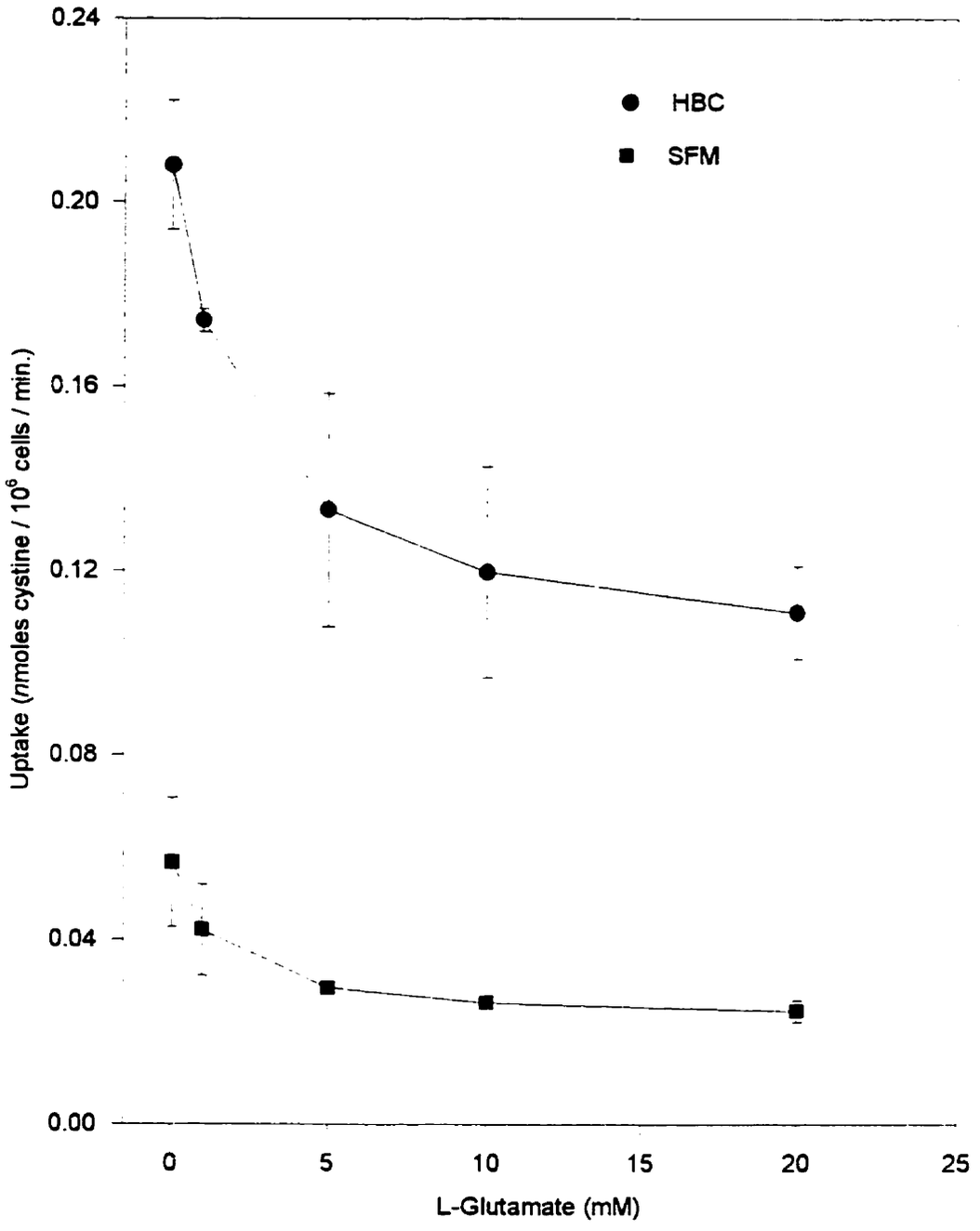


assess if the presence of glutamate had any effect on cystine uptake. Because the solubility of cystine is limited, it was decided that cystine would be fixed at 0.2 mM (normal media levels) and glutamate concentration would be increased to assess its inhibitory ability. It was also decided to assess the effects of increasing glutamate concentration on cystine uptake in SFM. Cells were harvested from a 72 hour old culture and resuspended in pre-warmed HBC containing radioactive cystine (0.1 *u*Ci U-^{14}C cystine), cystine (0.2 mM) and glutamate (1 - 20 mM). The uptake of cystine without any supplementary glutamate was used as a control. Uptake was terminated by filtration. Filters were collected, placed in lysis buffer and radioactive incorporation assessed using a rack beta scintillation counter. The uptake of cystine in HBC with increasing glutamate concentrations show an inverse relationship between increasing glutamate concentration and cystine uptake. As the glutamate concentration increased, the amount of cystine taken up from the external buffer decreased (Figure 3.3.2.3). The kinetic parameters associated with the curve were found by non-linear curve fitting using the Marquardt-Levenberg algorithm of SigmaPlot to a competitively inhibited system

$$(v = v_o - (I_{max}[S] / I_{0.5}+[S]).$$

The maximal inhibition (I_{max}) was found to be 0.1077$\pm$0.02712 *n*moles cystine/10^6 cells/min. and the inhibitor concentration required for half-maximal inhibition ($I_{0.5}$) was 2.2$\pm$0.8 mM. A similar relationship was observed in SFM, however the

Figure 3.3.2.3 Effect of increasing glutamate concentration on the uptake of L-cystine in CC9C10 hybridomas. Uptake was assessed in HBC and SFM at a 3 minute time point over a varied glutamate concentrations with a constant cystine concentration (0.1 mM, 0.1 *u*Ci U-^{14}C cystine). (n=3, S.E.M.)



uptake of cystine in SFM was greatly reduced in comparison to the HBC buffer. The associated parameters were found to be I_{max} = 0.03404±0.0004 *n*moles cystine/10^6 cells/min. and $I_{0.5}$ = 1.39±0.07 mM. This demonstrates that glutamate is an inhibitor of cystine uptake and that cystine and glutamate are both likely to be taken up by the same sodium independent uptake system x^-_C.

Table 3.3.2.1 Summary of kinetic parameters associated with the uptake of cystine by CC9C10 hybridomas in HBC and in the presence of increasing inhibitor (glutamate in HBC and SFM).

Conditions	k_m (mM)	$^{\cdot}V_{max}$ or I_{max} *n*moles / 10^6 cells /min	$^{\cdot\cdot}I_{0.5}$ (mM)
Cystine	0.9±0.1	0.36±0.03	N.A.
0.2 mM Cystine + Glutamate in in HBC	N.A.	0.1077±0.02712	2.2±0.8
0.2 mM Cystine + Glutamate in SFM	N.A.	0.0340±0.0004	1.39±0.07

$^{\cdot}$ V_{max} is the maximum velocity of cystine uptake in HBC, where as I_{max} is the maximal inhibition of glutamate on cystine uptake in HBC and SFM.

$^{\cdot\cdot}$ $I_{0.5}$ is the glutamate concentration that cause half maximal inhibition of cystine uptake

3.3.3. Characterization of L-Glutamine Uptake

3.3.3.1 Uptake Linearity and Kinetic Profile of L-Glutamine

The uptake of glutamine was also examined in CC9C10 hybridoma cells. Initially the uptake of glutamine was assessed in HBS and HBC to assess where glutamine uptake was sodium dependent or independent. As shown in Figure 3.3.3.1 the uptake of glutamine is sodium dependent and is linear over a seven minute period. The absence of sodium in the uptake buffer reduced the uptake by approximately one fourth of that when sodium was present. Therefore HBS buffer was selected as the buffer for further uptake studies. A time point of 5 minutes was selected as an assay point as sufficient radioactive incorporation into the cells provided accurate measurement.

Initial experiments were designed to assess the sodium dependent uptake of glutamine at the 5 minute time point while the concentration of glutamine was increased over a range of 0.1 to 8 mM. This was performed to estimate the kinetic parameters of glutamine uptake in CC9C10 hybridomas. The range of glutamine selected for experimentation covered levels above and below that present in SFM under normal growing conditions. The addition of lower (< 1 mM) concentrations was added to better assess kinetic parameters. The uptake of glutamine depicted a typical Michaelis-Menten saturation curve (Figure 3.3.3.2). The kinetic parameters associated with the curve were found by non-linear curve fitting using the Marquardt-Levenberg algorithm of SigmaPlot to a

Figure 3.3.3.1 Linearity of glutamine uptake by CC9C10 hybridoma cells. Cells were concentrated from mid-log phase growth in SFM and suspended in appropriate buffer containing 10 *u*M glutamine with 0.1 *u*Ci U-^{14}C-glutamine. Assays were performed in HBS or HBC over a period of 7 minutes at 37°C. Uptake was terminated by filtration and radioactive incorporation determined by liquid scintillation counting. (n=3, S.E.M.)

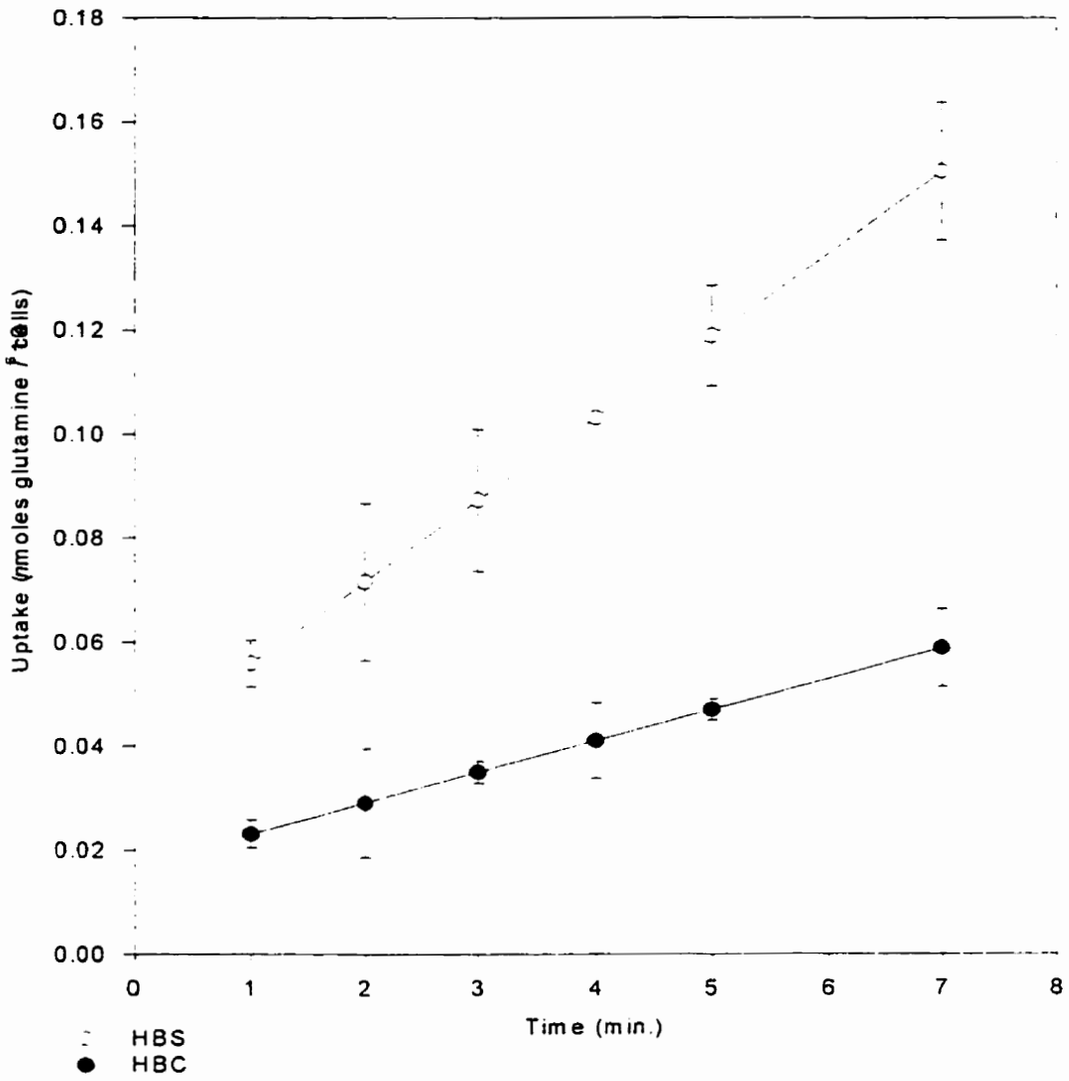
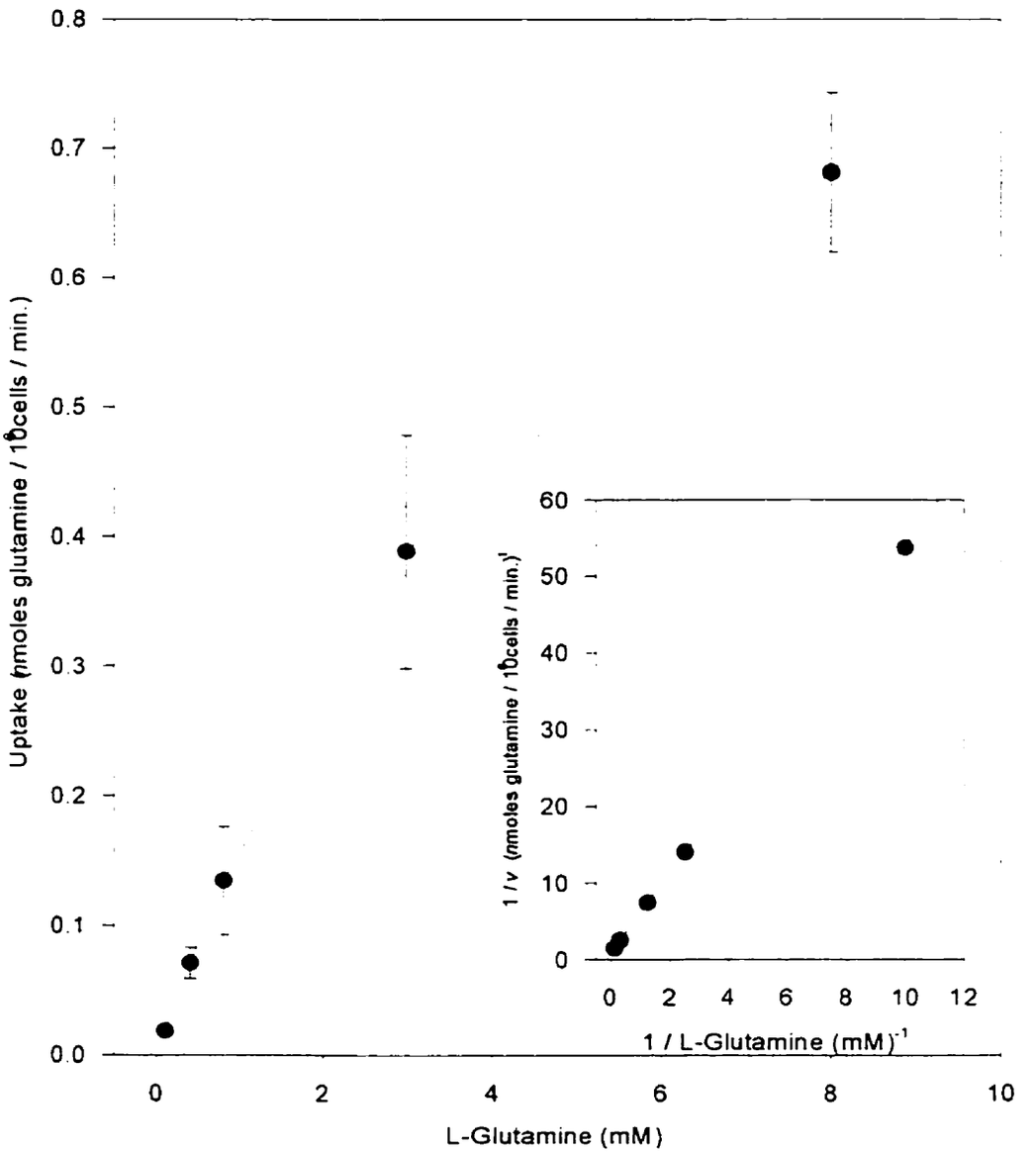


Figure 3.3.3.2 Uptake of glutamine by CC9C10 hybridoma cells in DPBS. Cells were concentrated from mid-log phase growth in SFM and suspended in DPBS containing increasing concentrations of glutamine. All assays were supplemented with 0.1 *u*Ci U-^{14}C-glutamine. After 5 minutes at 37°C, uptake was terminated by filtration and radioactive incorporation determined by liquid scintillation counting. Inset is Lineweaver-Burke representation of data. (n=3, S.E.M.)
k_m = 6.58±0.03 mM and V_{max} = 1.2±0.1 *n*moles glutamine/10^6 cells/min.



hyperbolic curve:

$$v = V_{max}[S] / k_m + [S]$$

The ability of the sodium dependent uptake system to bind glutamine was found to be 6.58 ± 0.03 mM, while the velocity was found to be 1.2 ± 0.1 *n*moles glutamine/10^6 cells/minute.

3.3.3.2 Inhibition of Glutamine Uptake by Amino Acids

Further characterization of the sodium dependent uptake system was required to assess the specificity of the system for other amino acids. The ability of the sodium dependent uptake system to take up 10 *u*M glutamine containing 0.1 *u*Ci U-^{14}C glutamine in the presence of 1 mM test amino acid was performed. Testing was performed in DPBS as this was done prior to use of HBS as an uptake buffer. It was found that uptake of glutamine in the presence of L-alanine, L-leucine, L-isoleucine, L-serine, L-cysteine, L-threonine, L-methionine, and L-arginine were significantly different from the control. However, L-serine and L-cysteine had the most extensive effect on glutamine uptake (Table 3.3.3.1). Arginine was also observed to cause a greater reduction of glutamine uptake.

Table 3.3.3.1 Inhibition of amino acids (1 mM) on the uptake of glutamine (10 uM, 0.1 *u*Ci U-^{14}C-glutamine) in DPBS. Cells were concentrated from mid-log phase growth in SFM and suspended in buffer containing the appropriate amino acid and glutamine. Control contained 10 *u*M (+ 0.1 0.1 *u*Ci U-^{14}C-glutamine) in buffer with no supplement. The rate of uptake of glutamine in the control (designated 100%) was 0.0082±0.0007 *n*moles glutamine/10^6 cells/min. (n=3, S.E.M.)

Supplemented Amino Acid	Uptake Rate of L-Glutamine as a % of Control
Control (no supplement)	100±9
L-Alanine	64 ± 11
L-Arginine	48 ± 1
L-Asparagine	N.D.
L-Aspartate	81 ± 21
L-Cysteine	36 ± 7
L-Glutamate	87 ± 12
D-Glutamate	105 ± 13
L-Histidine	82 ± 25
homocysteic acid	N.D.
L-Isoleucine	75 ± 7
L-Leucine	66 ± 9
L-Lysine	107 ± 16
L-Methionine	53 ± 4
L-Phenylalanine	N.D.
L-Proline	N.D.
pyroglutamate	N.D.
L-Serine	39 ± 4
L-Threonine	65 ± 11
L-Tryptophan	N.D.
L-Valine	N.D.

N.D. = Not Determined

Section 3.4. Metabolic Analysis of CC9C10 Hybridoma Cells

3.4.1 Standardization of L-Cystine Labeling for HPLC Detection

In order to assess whether cystine consumption was being affected by elevated glutamate levels it was necessary to develop an HPLC method by which cystine could be readily detected.

The detection of amino acids from biological fluids is important in a variety of areas such as optimizing cellular growth condition or diagnosing metabolic disorders. Use of high performance liquid chromatography (HPLC) is a very sensitive method for detecting and quantitating amino acids from biological fluids. In HPLC analysis, perhaps the most popular pre-column derivatization reagent is *o*-phthaldialdehyde (OPA). In alkaline media in the presence of a thiol compound (such as 2-ME), OPA reacts with primary amines to form the intensely fluorescent adduct *N*-alkyl-2-alkylthio-substitued isoindole (Fujwara *et al.*, 1987).

However there are a number of problems associated with the use of OPA (Walker & Mills, 1995). Firstly, derivatives are known to be unstable. This problem has been alleviated by automating injecting procedures. Secondly, some amino acids do not adequately resolve, i.e. glycine and threonine. Thirdly, OPA does not form adducts with proline or hydroxyproline. Fourthly, OPA reacts extremely slowly with cysteine, so that under normal conditions little, if any cysteine is resolved (cystine is reduced to cysteine in OPA derivatizing protocol).

A number of pre-column derivatization techniques (prior to OPA labeling) were attempted, however it was found that reducing cystine to cysteine via DTT and labeling with iodoacetic acid (IAA; see below) at room temperature was the most quantitative and efficient.

Cystine + Cysteine + DTT → 3 Cysteine + IAA → 3 Ac-Cysteine

Initial experimentation centered on detection limits of the HPLC method. Levels of L-cystine (25-400 *u*M) were chosen to represent decreasing amounts from the theoretical maximum present in SFM (0.4 mM). The decreasing amounts were used to represent consumption of the amino acid by cells. The response of the derivatized cystine relative to the internal standard aminobutyric acid (ABA) was found to be linear over the concentration range (Figure 3.4.1). This indicated that the protocol would be acceptable for detecting cystine at or below levels present in the media.

The next problem was to assess whether labeling with iodoacetic acid would effect any other amino acids in SFM extracts. A standardization of HPLC relative response to ABA was done on cystine spiked media. SFM, and SFM with spiked cystine (0.1 - 0.4 mM) were reduced using DTT (6 mM), labeled with iodoacetic acid, subjected to pre-column labeling with OPA and run on the HPLC. The response relative to ABA was found to be linear of over the concentration range (0-0.4 mM) tested (Figure 3.4.2). Also it was observed that the derivatized cystine, acetylcysteine (Ac-Cys) eluted as a unique peak with

Figure 3.4.1 HPLC relative response standardization for iodoacetic acid labeling of L-cystine. L-Cystine (25-400 uM) was reduced (6 mM DTT) and then derivatized with iodoacetic acid (680 mM). Samples were then loaded onto reverse phase C-18 HPLC to assess range of linearity. (n=2, S.E.M.)

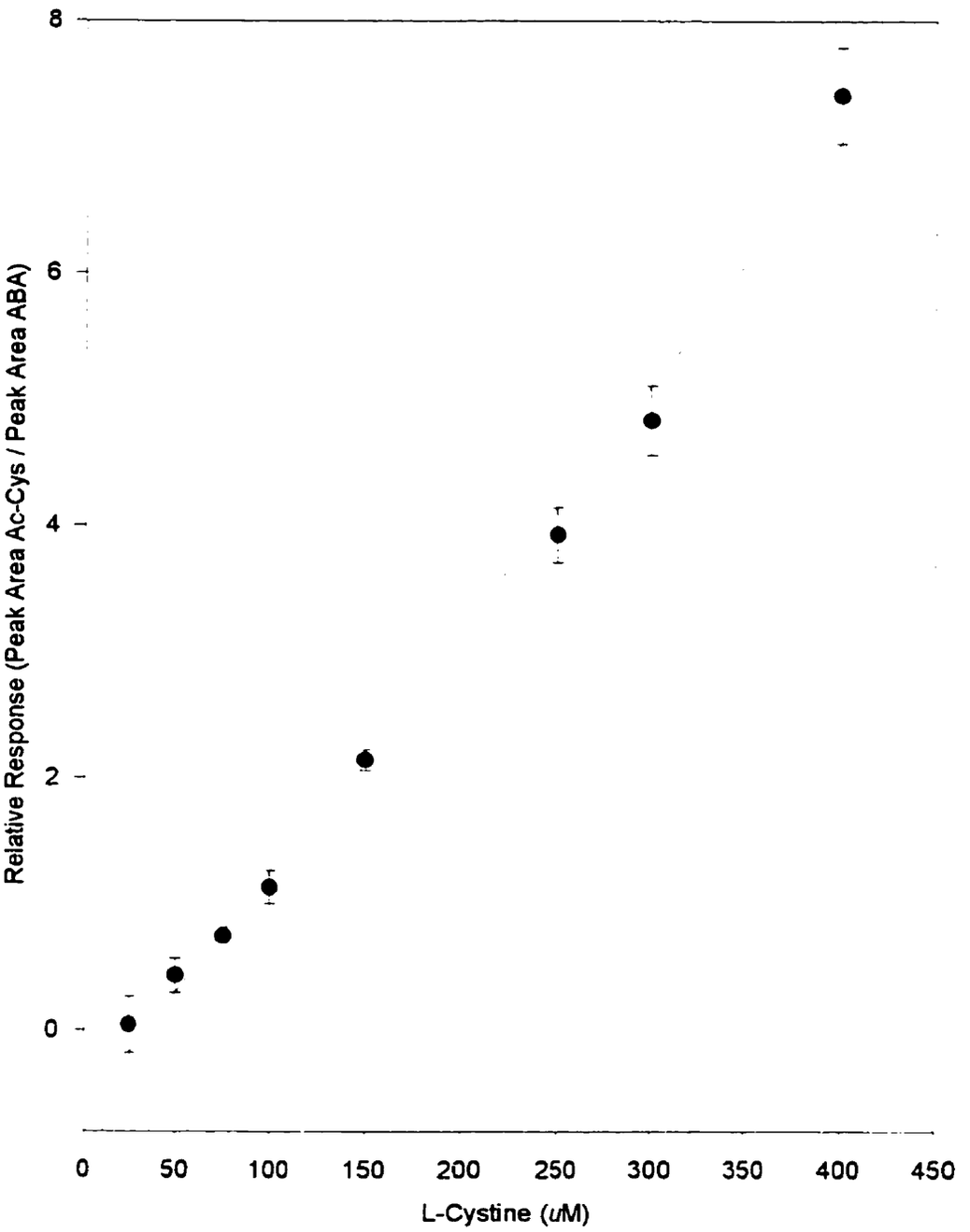
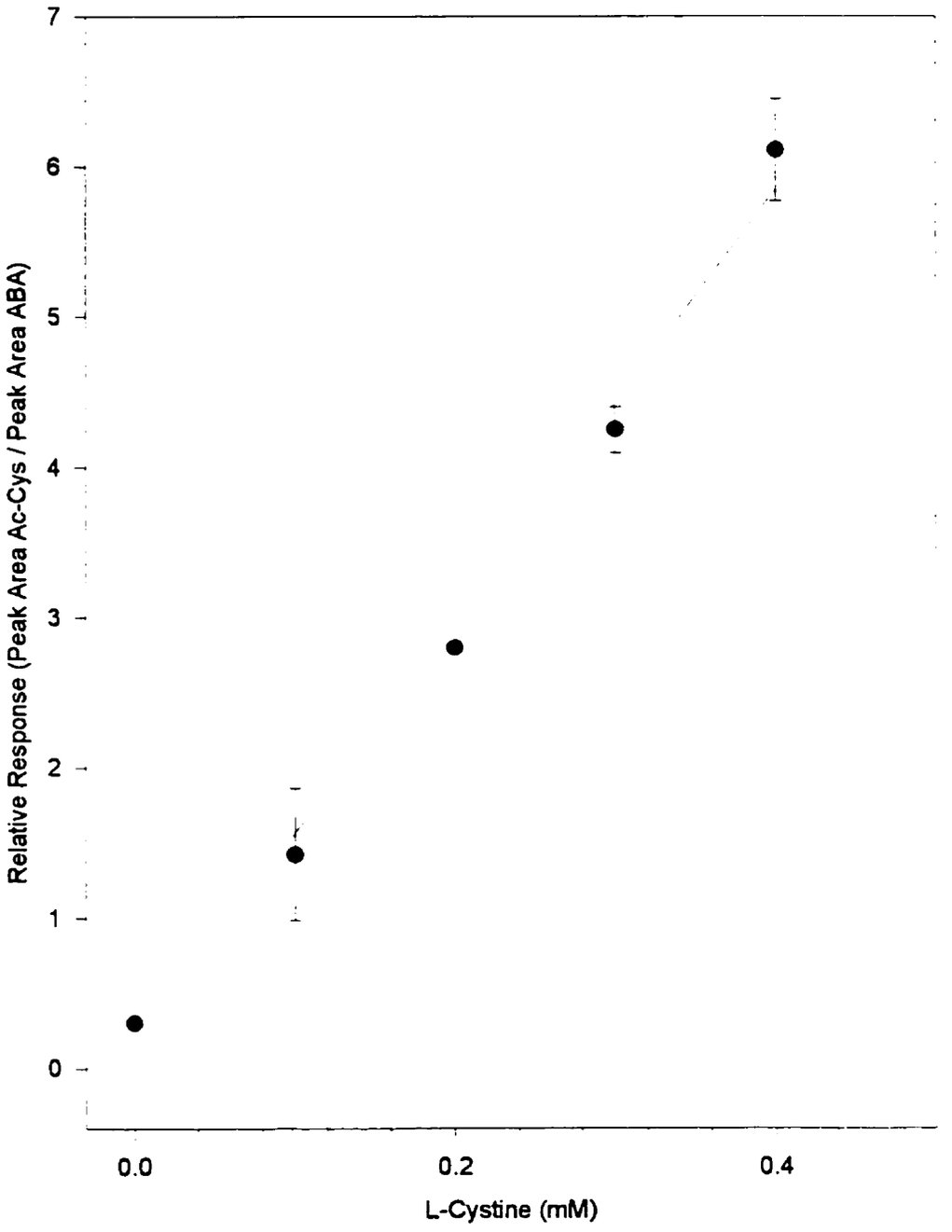


Figure 3.4.2 HPLC relative response standardization for iodoacetic acid labeling of L-cystine in SFM. L-cystine (0.1-0.4 mM) was added to SFM. Samples were then reduced (6 mM DTT) and then derivatized using iodoacetic acid (680 mM). The samples were then loaded onto reverse phase C-18 HPLC to assess range of linearity. Response was assessed relative to control amino butyric acid (ABA, 500*u*M). (n=2, S.E.M.)



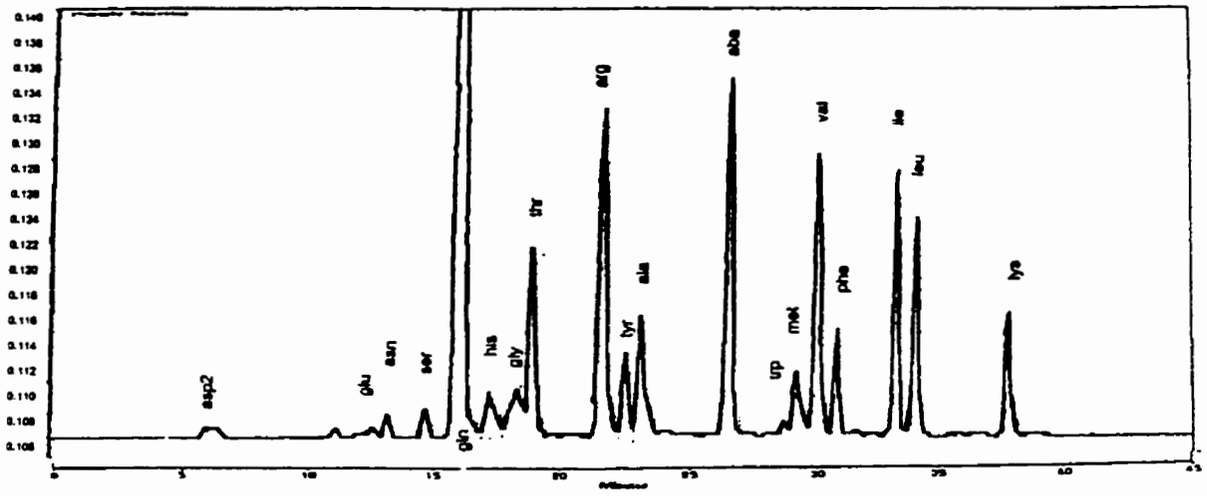
a retention time of 11.6 minutes (Figure 3.4.3 Chromatogram a). Other amino acids in SFM were not affected by the IAA derivatization process when compared to a control chromatogram of SFM which was not subjected to IAA derivatization (Figure 3.4.3 Chromatogram b). Observing no significant difference between chromatograms a and b (Figure 3.4.3), with the exception of the Ac-Cys peak, the technique was adopted to assess changes in cystine utilization in SFM with increased glutamate concentrations.

3.4.2 Metabolic Profiles for CC9C10 Hybridoma Growth in Increasing Glutamate Concentrations

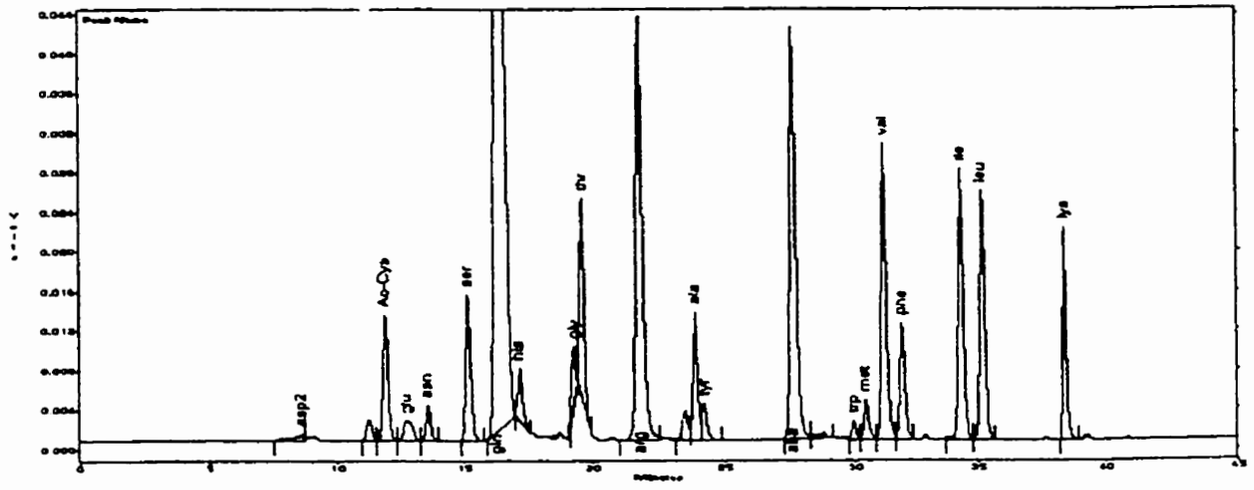
The growth of CC9C10 hybridoma cells in SFM containing elevated glutamate levels or in media where glutamine was replaced with glutamate SFM has been shown to be greatly inhibited over that of controls. To generate further insight into what is affecting cell growth, it was decided to briefly examine the metabolic status of the cells. The cells were examined under varied growth conditions (fixed glutamine 6 mM, increasing glutamate 0-12 mM; Figure 3.1.2) over a 96 hour period. Media samples were collected every 24 hours during growth and the spent media examine for specific rates of consumption or production of amino acids. Specific rates were calculated by comparing the change in amino acid concentrations (24-96 hours) to the integral of the change in cell density with respect to time (24-96 hours). The samples were subjected to IAA derivatization so that information as to the metabolic status of cystine

Figure 3.4.3 Chromatogram of SFM and SFM containing derivatized L-cystine. L-Cystine (0.6 mM) was added to SFM, reduced with DTT (6 mM) and derivatized with iodoacetic acid (680 mM). Sample was then load onto reverse phase C-18 HPLC. The derivatized L-cystine (Ac-Cys chromatogram b) elutes as a unique peak at 11.6 minutes. Chromatogram a) is SFM with no IAA derivatization.

a) Chromatogram of SFM



b) Chromatogram of SFM with cystine derivatized to Ac-cysteine by IAA



could be determined. Table 3.4.1 gives a summary of specific rates over the 96 hour period under increasing glutamate concentrations.

Of the aliphatic amino acids (valine, leucine and isoleucine), at 1 mM glutamate concentrations there was a slight decrease in consumption rates relative to controls (pooled p=0.088, 0.267 and 0.228 respectively, Figure 3.4.4). Alanine on the other hand, was observed to increase in production with increasing glutamate concentration, achieving a maximum rate of approximately 60 *n*moles/hr./10^6 cells (pooled p=0.001; Figure 3.4.4).

The acidic amino acid glutamate was found to have increased rates of consumption with increasing glutamate concentration, relative to the control (pooled p=0.176; Figure 3.4.5). Aspartate was consumed in the control, but with increasing glutamate concentrations aspartate switched to production, with a maximum production rate of approximately 1.5 *n*moles/hr./10^6 cells (Figure 3.4.5). Glutamine was consumed in all conditions examined at a rate not significantly different from the control (pooled p=0.795). Asparagine was produced by cells in all conditions examined and was found to have a maximum value of approximately 4.5 *n*moles/hr./10^6 cells (pooled p=0.035; Figure 3.4.5)

In the hydroxyl- or sulphur-containing side chain group of amino acids serine was found to change from consumption (control) to production, which increased with increasing glutamate concentration to a maximum of

Table 3.4.1 Amino acid production (+) or consumption (-) rates for CC9C10 hybridoma cells under inhibitory and non-inhibitory conditions. Media samples were analyzed over 96 hour growth period for cells grown in increasing concentrations of glutamate (0-12 mM). Samples were derivatized using iodoacetic acid and run on reverse phase C-18 HPLC from which specific consumption or production rates (*n*moles/hr/10^6 cells) of amino acids were calculated. (n=3, Standard error)

Amino Acid	Control SFM	1 mM Glu	3 mM Glu	6 mM Glu	9 mM Glu	12 mM Glu
u (Specific Growth Rate, $hr.^{-1}$)	0.024	0.024 ($p = 1.0$)	0.021 ($p = 0.015$)	0.021 ($p = 0.009$)	0.021 ($p = 0.008$)	0.017 ($p = 0.007$)
Alanine	24.1±2.1	31.9±1.8	38.0±2.3	52.4±7.7	61.3±8.7	62.0±4.3
Arginine	-4.9±1.7	-1.2±1.3	-3.2±0.7	-4.0±1.3	-4.4±0.8	-3.9±0.9
Asparagine	1.9±0.5	3.1±0.3	4.8±0.3	3.6±0.9	6.3±1.8	6.4±3.4
Aspartate	-1.2±0.3	1.5±0.5	2.1±0.6	2.5±0.1	0.1±0.8	0.9±0.7
Acetyl-Cysteine	-4.6±0.5	-1.3±0.2	-2.2±0.2	-3.1±0.4	-3.2±0.7	-2.3±1
Glutamate	0.5±1.4	-9.9±6.9	-15.2±8.5	-22.2±13.4	-56.1±44.9	-55.8±47.9
Glutamine	-102.6±18.7	-81.0±11.7	-92.8±9.3	-106.3±17.7	-100.5±22.5	-106.2±16.8
Histidine	-0.3±0.3	-0.1±0.3	-1.1±0.9	-1.5±1.1	0.1±0.3	0.5±0.3
Isoleucine	-2.6±0.6	-0.4±0.8	-1.6±0.5	-1.9±0.8	-2.3±1.0	-1.2±1.6
Leucine	-3.7±0.7	-1.5±0.8	-2.8±0.4	-3.3±0.7	-3.3±1.0	-2.3±1.7
Lysine	-6.0±1.4	-3.2±0.7	-3.7±1.2	-4.0±0.9	-4.4±1.7	-2.6±2.8
Methionine	-3.1±0.8	-2.1±0.7	-1.5±0.4	-2.2±1.2	-3.5±0.8	-2.3±0.9
Phenylalanine	-1.9±0.4	-0.5±0.2	-0.8±0.3	-0.9±0.5	-0.9±0.6	-0.7±0.7
Serine	-2.8±1.2	2.2±0.9	2.8±0.8	3.8±1.2	7.6±0.8	6.2±1.1
Tyrosine	-4.2±1.6	-2.9±1.3	-3.9±1.4	-5.1±2.1	-5.8±2.4	-6.2±2.9
Threonine	-3.3±1.0	-2.0±1.0	-2.8±1.4	-2.3±1.0	-1.6±0.5	-1.5±1.1
Valine	-3.5±0.7	-0.9±0.8	-1.9±0.5	-2.4±0.7	-2.5±0.9	-0.7±1.8
Net Production	27±4	39±4	48±4	62±9	75±12	76±9
Net Consumption	-145±30	-107±27	-134±26	-159±41	-189±78	-186±80

Figure 3.4.4 Rates of consumption or production by CC9C10 hybridomas for aliphatic amino acids. Cells were grown in SFM with increasing glutamate concentrations (0-12 mM). Changes in rates were assessed over a growth period of 72 hours. (n=3, Standard error)

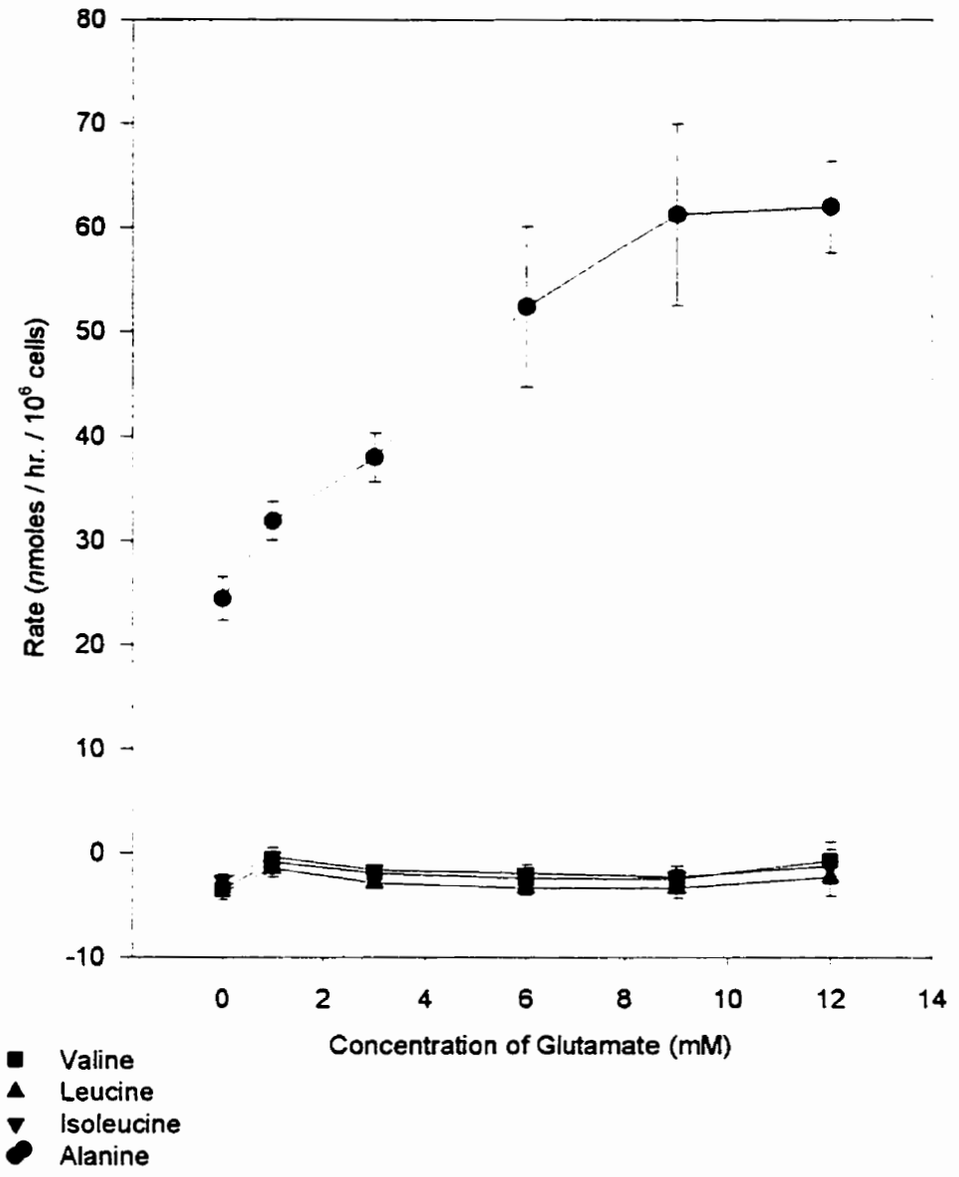
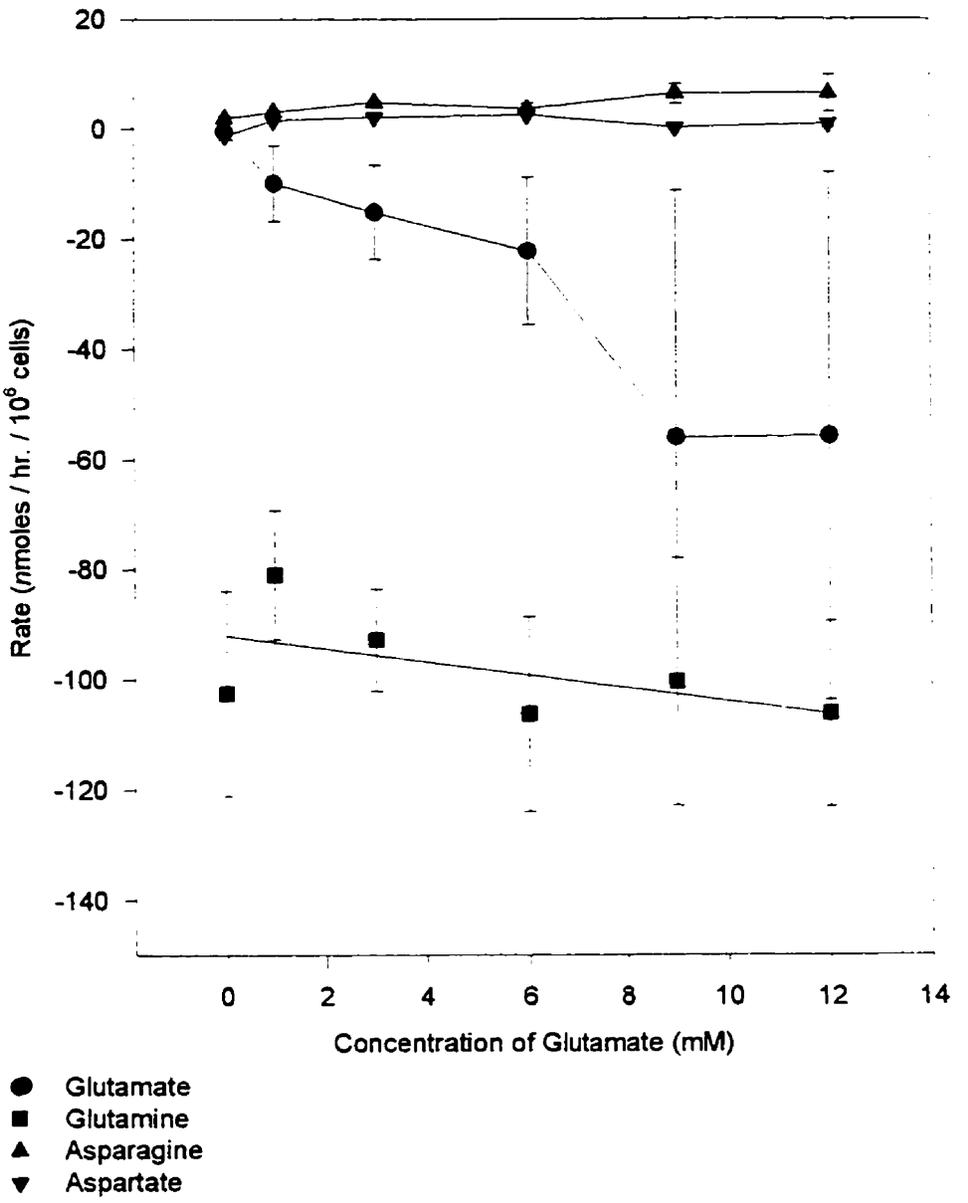


Figure 3.4.5 Rates of consumption or production by CC9C10 hybridomas for acidic amino acids and their amides. Cells were grown in SFM with increasing glutamate concentrations (0-12 mM). Changes in rates were assessed over a growth period of 72 hours. (n=3, Standard error)



approximately 6 *n*moles/hr./10^6 cells (pooled p=0.00002), Figure 3.4.6). Acetyl-cysteine was found to exhibit reduced consumption rates relative to control with increasing glutamate concentrations (pooled p=0.060, Figure 3.4.6). Threonine and methionine were consumed by cells at a rate not significantly different from that of controls, at all levels of glutamate examined (pooled p=0.339 and 0.351 respectively, Figure 3.4.6).

In aromatic amino acids phenylalanine was found to have a reduced consumption rate relative to control. The reduced rate of consumption was similar at all levels of glutamate examined, being approximately 1 *n*moles/hr./10^6 cells (pooled p=0.016, Figure 3.4.7). Tyrosine demonstrated a change from consumption in control to production at all levels of glutamate examined but was not significantly different from control (pooled p=0.793).

The basic amino acids lysine and arginine exhibited decreased consumption rates which were significantly different from the control (pooled p=0.139 and 0.041 respectively, Figure 3.4.8). Histidine was found to be consumed by cells exposed to increasing glutamate concentrations, but was not significantly different from control (pooled p=0.879, Figure 3.4.8)

In summary, with increasing glutamate concentrations in the media, the amino acids which appear to undergo changes in their specific rates of production or consumption are alanine, asparagine, glutamate, cystine (acetyl-cysteine), serine, aspartate, lysine, arginine, valine, isoleucine, and phenylalanine.

Figure 3.4.6 Rates of consumption or production by CC9C10 hybridomas for hydroxyl- or sulphur-containing amino acids. Cells were grown in SFM with increasing glutamate concentrations (0-12 mM). Changes in rates were assessed over a growth period of 72 hours. (n=3, Standard error)

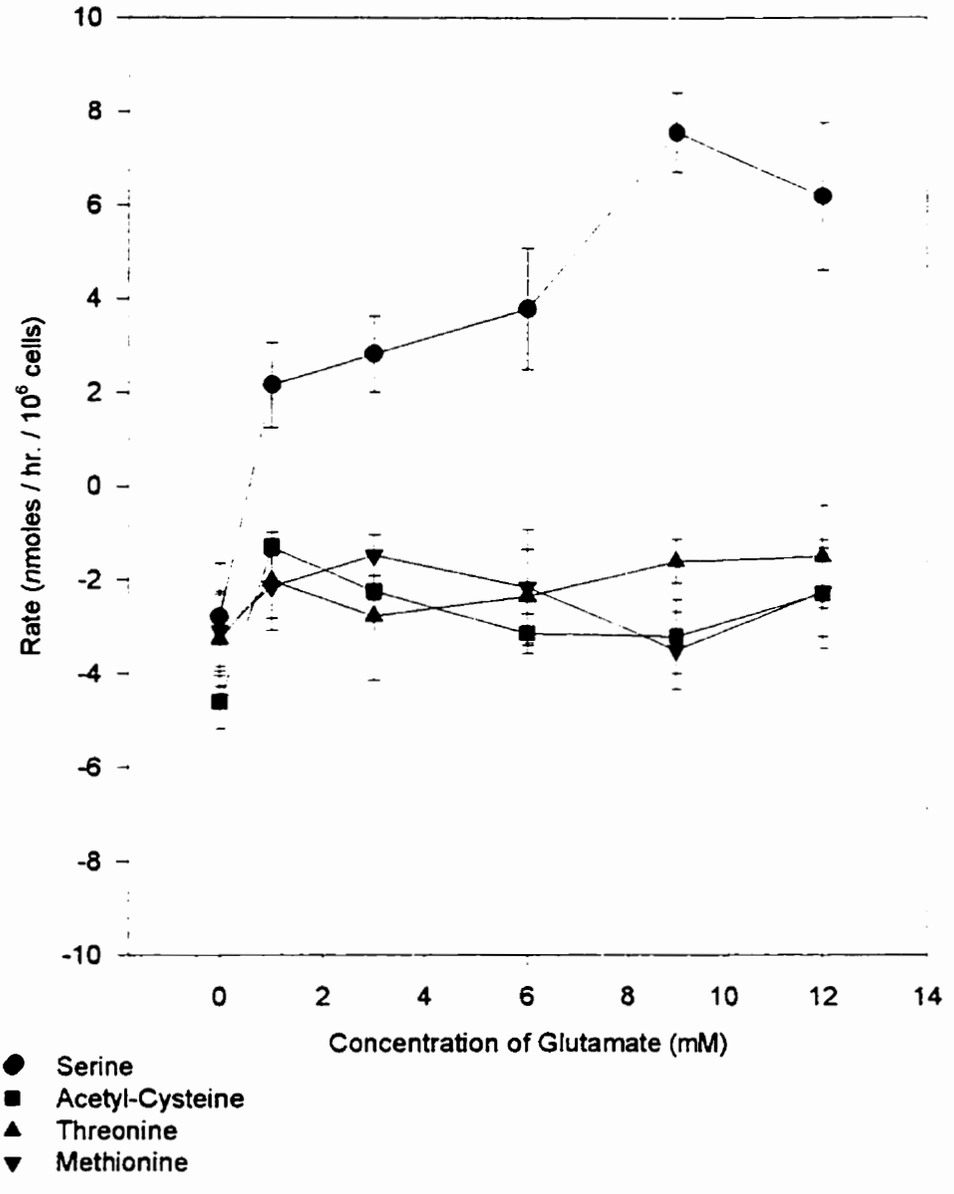


Figure 3.4.7 Rates of consumption or production by CC9C10 hybridomas for aromatic amino acids. Cells were grown in SFM with increasing glutamate concentrations (0-12 mM). Changes in rates were assessed over a growth period of 72 hours. (n=3, Standard error)

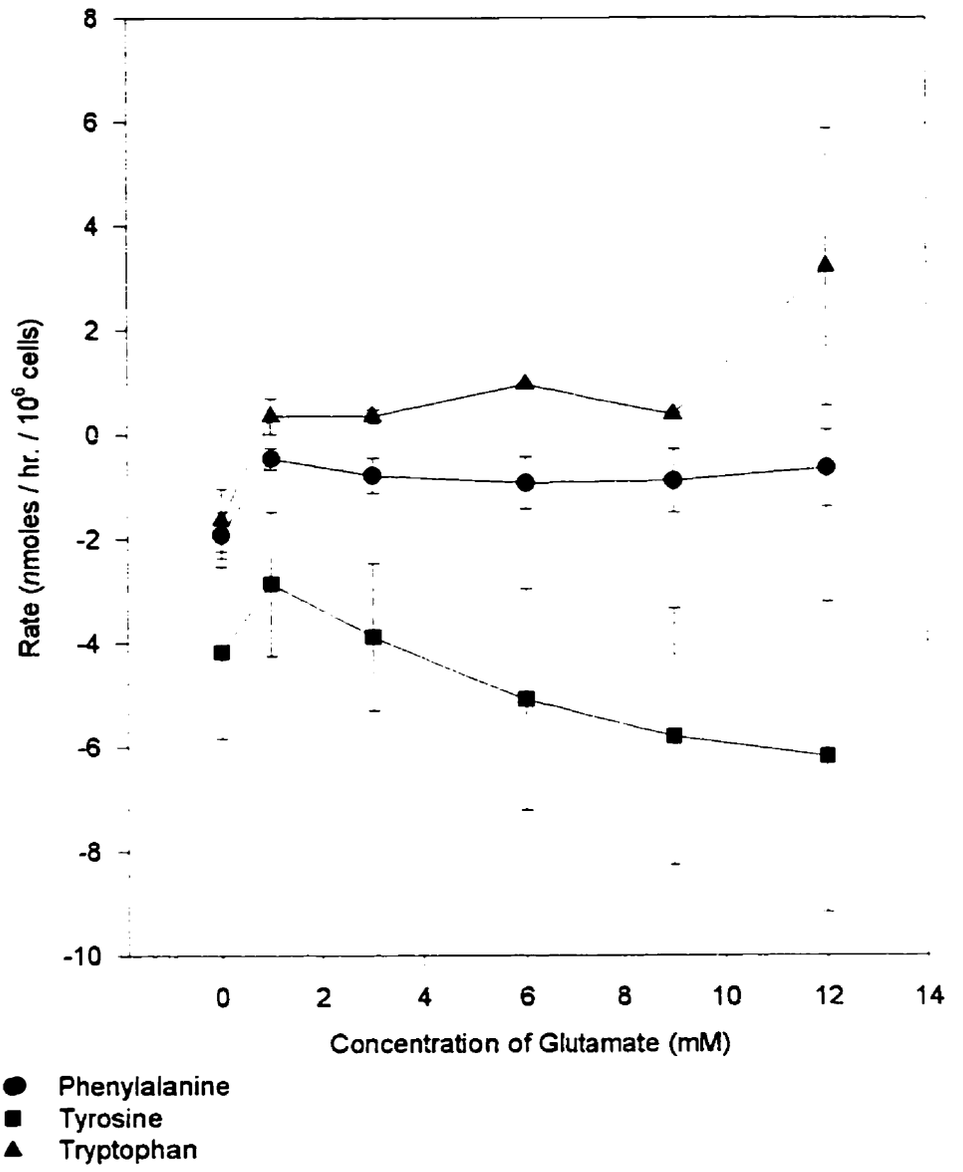
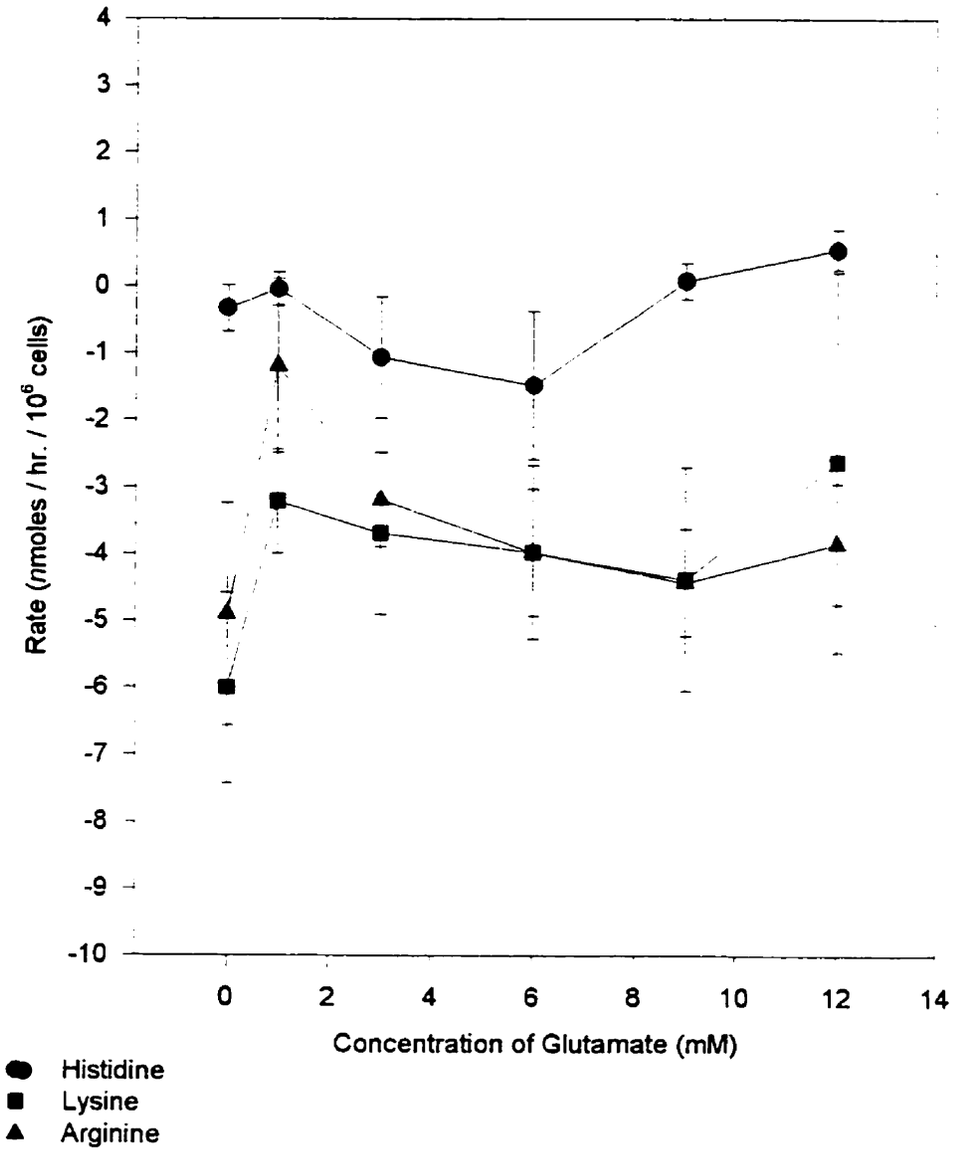


Figure 3.4.8 Rates of consumption or production by CC9C10 hybridomas for basic amino acids. Cells were grown in SFM with increasing glutamate concentrations (0-12 mM). Changes in rates were assessed over a growth period of 72 hours. (n=3, Standard error)



Chapter 4. Discussion

Chapter 4. Discussion

4.1 Effect of Glutamate on CC9C10 Hybridoma Cells

4.1.1 Overview of Completed Research

The research was undertaken to examine whether or not glutamate could be used as a suitable catabolic substitute for glutamine. Glutamate has been used successfully to support growth of various cells lines (Christie unpublished; Hassell & Butler, 1990; McDermott & Butler, 1993). The advantage of having cells utilize glutamate over glutamine would be a proposed reduction in ammonia levels in the culture medium. Ammonia build up in cell culture can be both growth limiting and/or end product limiting.

Growth of CC9C10 hybridoma cells in SFM supplemented with 6 mM glutamine and increasing amounts of glutamate (0-20 mM) resulted in decreased cell densities with increasing glutamate. Similar inhibition of cell growth by elevated glutamate levels has been observed by Murphy *et al.* (1989) and McDermott & Butler (1993). The apparent inhibition of glutamate was even more pronounced when glutamate was used to replace glutamine on a mole for mole basis. At this point it was hypothesized that glutamate inhibited cystine uptake which could result in decreased glutathione levels. Glutathione protects cells from oxidative stress, and if absent could lead to cell death which would explain the observed decreased cell densities. Attempts to alleviate glutamate inhibition by cystine supplementation, or by addition of reducers 2-mercaptoethanol or glutathione proved unsuccessful.

Aminoadipate, which is similar in structure to glutamate and is a known competitor of glutamate uptake, was utilized to reproduce the pattern of glutamate inhibition of cell growth. This is important as it indicated that the reduction in observed cell densities was due to elevated glutamate levels and that the mechanism most likely involved glutamate uptake.

In order to assess whether or not cells could first utilize glutamate it was necessary to perform a kinetic examination for the uptake of glutamate. The uptake revealed that cells did indeed take up glutamate from the external medium, which is important if cells are to utilize glutamate as a catabolic source of energy. The uptake of glutamate was sodium independent, stereo-specific, and subject to competitive inhibition by cystine and homocysteic acid. This implied that the mechanism of glutamate inhibition at elevated levels could be due to competition of glutamate with cystine for uptake. A cursory examination of cystine uptake verified that both cystine and glutamate are taken up by the same system in a sodium independent manner.

Having established a connection between glutamate uptake and cystine uptake, and knowing that elevated glutamate levels resulted in reduced cell densities, an examination of cellular metabolism was performed. Specific rates of consumption or production revealed altered metabolic profiles with increasing glutamate concentrations, most notably a reduction in the rate of consumption of cystine by cells.

4.1.2 Kinetic Characterization of Glutamate Uptake

Uptake of glutamate by mammalian cells is known to occur via three distinct and separate systems. System X^-_{AG} is a sodium dependent system shared with aspartate. System x^-_G is a sodium independent system that takes up glutamate and analogs, but excludes aspartate. System x^-_C is a sodium independent system that takes up glutamate and competes/exchanges cystine with glutamate. As there was no literature on the uptake of glutamate by CC9C10 hybridoma cells it was necessary to characterize this uptake kinetically, so that the mechanism of glutamate inhibition might further be understood.

The uptake of glutamate by CC9C10 hybridoma cells was found to occur in a sodium independent manner. A study of amino acid competition for this system revealed: 1) a V_{max} of 5 ± 1 *n*moles glutamate/10^6 cells/min (which is equivalent to 12.5 ± 2.5 *n*moles glutamate/ mg. cell protein / min. if 10^6 cells equals 0.4 mg. cell protein, see Petch, 1994) and a k_m of 20 ± 5 mM; 2) the system was stereo-specific for glutamate; 3) cystine competed with glutamate for this system; and 4) homocysteic acid competed with glutamate for uptake by this system. These results lend support to the system of uptake responsible for glutamate being system x^-_C. System x^-_C was first described in the literature by Bannai and Kitamura in fetal human diploid fibroblasts (1980). The k_m for glutamate uptake was 0.2 mM with an approximate V_{max} 0.11 *n*moles glutamate/mg protein/minute. In this case cells were cultured in Eagle's basal medium supplemented with 10% fetal calf serum. It is known that values for k_m

and V_{max} vary with general culture conditions, cell type, and uptake buffer. Table 4.1 is a compiled list of literature values for glutamate uptake by system x^-_c in various cell lines and culture conditions. In comparison to literature values the experimentally derived V_{max} (12.5 *n*moles/mg. cell protein/min.) is not significantly different from that of Bannai & Kitamura (1982). However, it is important to observe that there is a great difference between V_{max} values for different cells lines grown under very different conditions. The experimental k_m was found to be two orders of magnitude higher than those in published literature (range 0.00126-0.38 mM). The difference in magnitude most likely stems from cell and culture conditions. No reference in the literature was made as to what phase of growth (log, mid-log, stationary) cells were examined in. All experiments performed in this were done in mid-log phase of growth when cells are actively growing. Examinations of different phase of growth would yield different experimental values. Another possible factor is the inclusion of serum in the basal growth media of all published literature. The addition of serum markedly changes the growth patterns of cells as it not only acts to protect cells from mechanical damage, but also provides a vast array of nutrients, peptides, and hormones which may not be included in a serum free formulation. Any or all of these factors could alter kinetically determined values.

Table 4.1 Comparison of anionic uptake system x^-_c in various cell lines and culture conditions for the uptake of glutamate.

Cell Line	Culture Conditions	[1]k_m	[2]V_{max}	Reference
CC9C10 hybridoma	Serum Free Media	20	12.5	This work
IMR-90 human diploid fibroblasts	Eagle's basal media + 10% FCS	0.20	0.11	Bannai & Kitamura (1980)
HAIN-6 human diploid fibroblast derived from embryonic lung	EBM + 10% FCS with cystine added without cystine	 0.32 0.38	 4.8 10	Bannai & Kitamura (1982)
CHO-K1	45% DMEM, 45% Ham's F12, 5% HS, 5% CS	0.063	0.33 - 0.62	Ash *et al.* (1993) & Ash & Igo (1993)
Bovine pulmonary artery endothelial cells	RPMI-1640 + 10% FCS	N.D.	[3]39.51	Deneke (1992)
1- & 2- cell mouse conceptuses	Brinster's medium	1: 0.070 2: 0.054	[4]1: 18.4 2: 4.1	van Winkle *et al.* (1992)
N18-RE-105 neuronal hybridoma	DMEM + 5% FCS	0.053	0.205	Murphy *et al.* (1989)
human diploid fibroblasts derived from skin biopsies	Medium 199 + 10% FCS starved unstarved	 0.28 0.32	 [5]0.12 [5]0.067	Dall'Asta *et al.* (1983)
Synaptic membrane vesicle	N.A.	0.00126	0.00055	Koyama *et al.* (1995)

N.D. Not Determined
N.A. Not Applicable
[1] k_m measured in mM
[2] V_{max} measured in *n*moles/mg. cell protein/min
[3] V_{max} *p*moles/10^6 cells/min.
[4] V_{max} *f*moles /conceptus/min.
[5] V_{max} *u*mol/min/ml cell water
[•] value converted from *n*moles/10^6 cells/min assuming 0.4 mg of cellular protein is equivalent to $1X10^6$ cells (Petch, 1994)

4.1.3 Kinetic Characterization of Cystine Uptake

Uptake of cystine in the majority of mammalian cell lines is achieved primarily by system x^-_c. In nervous tissue there exists a second uptake system which is sodium dependent, but is unique to nervous tissues. Upon entering the cells cystine is thoroughly reduced to cysteine by glutathione and cycled into glutathione production, anabolic biosynthesis, or released back into the external medium as cysteine. In order to assess if the mechanism of observed glutamate inhibition was due to glutamate affecting cystine uptake, it was necessary to kinetically characterize cystine uptake.

The uptake of cystine by CC9C10 hybridoma cells was found to occur in a sodium independent manner. A cursory examination revealed: 1) a V_{max} of 0.9 *n*moles cystine/mg. cell protein/min. (assuming 10^6 cells is equivalent to 0.4 mg. cell protein; Petch, 1994) and a k_m of 0.87 mM; 2) glutamate competed with cystine for uptake. These results, in conjunction with those of glutamate, establish that system x^-_c is solely responsible for the uptake of glutamate and cystine in CC9C10 hybridoma cells.

Uptake of cystine by system x^-_c was first described in the literature by Bannai and Kitamura (1980) in fetal human diploid fibroblasts. The k_m for cystine uptake was 0.043 mM with an approximate V_{max} of 0.66 *n*moles/mg protein/min. Table 4.2 is a compiled list of literature values for the uptake of cystine by system x^-_c in various cell lines and culture conditions. Again, note that values vary between cell lines and culture conditions.

Table 4.2 Comparison of anionic uptake system x^-_c in various cell lines and culture conditions for the uptake of cystine.

Cell Line	Culture Conditions	[1]k_m	[2]V_{max}	Reference
CC9C10 hybridoma	Serum Free Media	0.87	*0.9	This work
IMR-90 human diploid fibroblasts derived from fetal lung	Eagle's basal media + 10% FCS	0.043	0.66	Bannai & Kitamura (1980)
HAIN-6 diploid fibroblasts derived from embryonic lung	EMEM + 15% FCS 1) with cystine added 2) without cystine	 0.060 0.058	 1.3 2.6	Bannai & Kitamura (1982)
IMR-90	Eagle's basal media + 10% FCS	N.D.	0.25-1.66	Bannai (1986)
N-18-RE-105 neuronal hybridoma	DMEM + 5% FCS	0.020	0.052	Murphy et al. (1989)
Bovine pulmonary artery endothelial cells	RPMI-1640 + 10% FCS	N.D.	[3]114.8	Deneke (1992)
1-Cell mouse conceptuses	Brinster's medium	0.067	[4]12.7	van Winkle et al. (1992)
Mouse peritoneal macrophages	RPMI-1640 + 10% FBS	N.D.	0.12	Sato et al. (1995)
PaTu 8902 pancreatic duct cells	DMEM + 10% FCS	0.086	1.82	Sweiry *et al.* (1995)

N.D. Not Determined
[1] k_m measured in mM
[2] V_{max} measured in *n*moles/mg. cell protein/min
[3] V_{max} measured in *p*moles/10^6 cells/10 min.
[4] V_{max} measured in *f*moles/conceptus/min
* value converted to *n*moles/mg. cell protein/min assuming 0.4 mg of cellular protein is equivalent to $1X10^6$ cells (Petch, 1994)

In comparison to literature values, the experimentally derived V_{max} (0.90±0.07 *n*moles/mg. cell protein/min.) fits within the range of published values (0.052 - 2.6 *n*moles/mg. cell protein/min.). The experimental k_m found to be an order of magnitude higher than the range of published literature values (0.020 - 0.087 mM). Again these difference could be attributed to different culture conditions, cells lines, growth phase and uptake protocols.

4.1.4 Kinetic Characterization of Glutamine Uptake

Uptake of glutamate by mammalian cells is known to occur via 4 possible uptake systems, namely A, ASC, L, and N^m. System A (Pro, Gly, Ala, Ser, Met, and Gln), ASC (Ala, Ser, Cys, Thr, and Gln) and N^m (Gln, Asn and His) are sodium dependent, whereas system L (Leu, Phe, Met, Cys, and Gln) is sodium independent. A preliminary examination of glutamine uptake by CC9C10 hybridomas revealed 1) a V_{max} of 3 *n*moles/mg. cell protein/min. (assuming 10^6 cells is equivalent to 0.4 mg. cell protein; Petch, 1994) and a k_m of 6.58 mM; 2) the system was largely sodium dependent; and 3) of the amino acids tested serine, cysteine, arginine, and methionine were the most extensive inhibitors of uptake.

In comparison to literature the k_m is within the range of published values (0.174-12.7 mM; Table 4.3). The V_{max} is within the range of published values (1.156 - 50 *n*moles/mg cell protein/min.). Again though, there is vast range of values for different cell lines under different culture conditions. Further work is required to elucidate exactly which system is responsible for glutamine uptake and further analysis of the sodium independent fraction of uptake also requires further investigation.

Table 4.3 Comparison of glutamine kinetic parameters for various cells lines and culture conditions.

Cell Line	Culture Conditions	[1]k_m	[2]V_{max}	Reference
CC9C10 hybridoma	SFM (DMEM/F-12)	6.58±0.03	*3.0±0.3	This work
Bovine Lymphocytes	RPMI +10% autologous serum + 2 mM Gln	1 (Hill Coeff. 1) 4.7 (2.3) 12.7 (9.5)	4.5 6.0 9.0	Piva *et al.* (1992)
IMR-90 human diploid fibroblasts	BME + 10% FBS	0.25	50.00	Bannia & Ishii (1988)
Myoblast Myotube	25% M199, 65% DMEM +10% FCS	0.197±0.038 0.174±0.047	1.165±0.06 1.435±0.47	Tadros *et al.*, (1993)
Rat Hindlimb	N.A.	9.25±1.15	1.156±0.193	Hundal *et al.*, (1987)

N.A. Not Applicable
[1] mM
[2] *n*moles/mg cell protein/min.
* values converted to *n*moles/mg cell protein/min. assuming 0.4 mg of cellular protein is equivalent to $1X10^6$ cells (Petch, 1994)

4.1.5 Amino Acid Metabolic Profiles of CC9C10 Hybridomas in Elevated Glutamate

An examination of amino acid utilization rates by CC9C10 hybridoma cells in elevated glutamate levels revealed that relative to utilization rates in control, those of alanine, asparagine, glutamate, cystine, serine, aspartate, and phenylalanine all underwent specific changes in rates of production or consumption (Refer to Section 3.4.2). As the glutamate concentration was increased in the media, the overall rate of consumption was not significantly different from control (-144.7 *n*moles/hr./10^6 cells). The overall rate of production however, was found to increase and reach a maximum of approximately 71.2 *n*moles/hr./10^6 cells.

Alanine and aspartate were found to undergo an increased rate of production reaching maximum rates of approximately 60 and 1 *n*moles/hr./10^6 cells respectively. The control for alanine was found to have a rate of production of 24 *n*moles/hr./10^6 cells, and for aspartate consumption of -1.2 *n*moles/hr./10^6 cells. The production of aspartate could also be larger, as there is a noted increase in the production of asparagine (maximum production rate of 6.4 *n*moles/hr./10^6 cells). Aspartate produced in the transamination step, described below, could then be used in a deamination of glutamine. The increase in alanine and aspartate production could be attributed to an increase in pyruvate, either from glycolysis or glutaminolysis. Pyruvate and glutamate are converted to alanine or aspartate and α-ketoglutarate via alanine-glutamate or aspartate-glutamate transamination (McKeehan, 1986; Fitzpatrick *et al.*, 1993; Petch & Butler, 1994). Most literature suggests that the majority of alanine is produced via glutaminolysis, as very little glucose is believed to enter the TCA cycle (Reitzer *et al.*, 1979; Zielke *et al.*, 1984; McKeehan, 1986). Also, the rate of glutamate conversion to α-ketoglutarate is near equilibrium (McKeehan, 1986), and it is believed that the major

conversion of glutamate to α-ketoglutarate is via the transaminations described above. Therefore an increase in alanine and aspartate production could be construed as evidence for increased α-ketoglutarate.

If it is glycolysis (glucose consumption) that is being increased, as mentioned above, then alanine could be produced via pyruvate, aspartate could be produced through phosphoenolpyruvate with/without subsequent amination to asparagine, and serine could be produced via 3 phosphoglycerate (Mathews & van Holde, 1990). This is perhaps the most likely scenario, as it explains the increased production of all three amino acids, however a much more detailed study involving analysis of enzyme activity and metabolic flux would need to be undertaken to elucidate this further.

As glutamate levels were elevated, there was an observed increase in the consumption of glutamate ($p = 0.17$) with a perceived maximum rate of consumption of approximately 20 *n*moles/hr./10^6 cells. With the increased glutamate consumption, there was an observed decreased cystine consumption ($p = 0.06$) with increasing glutamate levels. This is to be expected as system x^-_c is thought to function by exchanging 1 mole of cystine for 1 mole of glutamate in either direction (into or out of the cell; Bannai & Ishii, 1988). Therefore at elevated levels of glutamate in the media, glutamate would out compete cystine for uptake by this system which would result in the concurrent loss of cystine from within the cell resulting in the observed decreased consumption rates.

Similar decreases in consumption rates of valine, leucine, isoleucine, lysine and arginine, with the exception of phenylalanine, were also observed in that of Christie & Butler (1994, Table 4.4) where glutamine in the media was replaced with dipeptides. It

Table 4.4 Comparison of amino acid production (+) or consumption (-) rates (*n*moles/10^6 cells/hr) for various hybridoma cell lines.

Amino Acid	[1]CC9C10 Hybridoma	[2]CC9C10 Hybridoma	[3]CC9C10 Hybridoma	[4]AB2-143.2 Hybridoma	[5]Murine Hybridoma
Acetyl-Cysteine (Cystine)	-4.6±0.5	-2.2±0.2	N.D.	N.D.	-2.7
Alanine	24.1±2.1	38.0±2.3	34.6±1	19.39	38.7
Arginine	-4.9±1.7	-3.2±0.7	-6.3±0.3	-2.31	-3.3
Asparagine	1.9±0.5	4.8±0.3	1.17±0.01	-0.26	2.2
Aspartate	-1.2±0.3	2.1±0.6	0.17± 0.03	0.13	7.7
Glutamate	0.5±1.4	-15.2±8.5	1.9±0.2	1.80	-8.3
Glutamine	-102.6±18.7	-92.8±9.3	-91.6±2	-47.90	-63.5
Histidine	-0.3±0.3	-1.1±0.9	-2.3±0.1	-1.28	-1.0
Isoleucine	-2.6±0.6	-1.6±0.5	-7.4±0.5	-3.21	-3.5
Leucine	-3.7±0.7	-2.8±0.4	-9.2±0.4	-3.98	-3.7
Lysine	-6.0±1.4	-3.7±1.2	-7.9±0.6	-2.83	-3.2
Methionine	-3.1±0.8	-1.5±0.4	2.4±0.1	-1.54	-1.6
Phenylalanine	-1.9±0.4	-0.8±0.3	-1.0±0.8	-1.28	-0.3
Serine	-2.8±1.2	2.8±0.8	-3.7±0.6	-1.28	3.7
Threonine	-3.3±1.0	-2.8±1.4	-8.0±0.5	-1.03	-0.5
Tyrosine	-4.2±1.6	-3.9±1.4	-2.8±0.4	-1.16	-1.1
Valine	-3.5±0.7	-1.9±0.5	-7.2±0.4	-3.47	-1.6
Net Production (+)	27±4	48±4	38±2	21.32	52.3
Net Consumption (-)	-145±30	-134±26	-150.57±7	-66.92	-94.3

N.D. Not Determined

[1] This work; batch culture conditions SFM (DMEM/F-12)

[2] This work; batch culture conditions SFM (DMEM/F-12) + 3 mM glutamate

[3] Christie & Butler (1994); batch culture conditions DMEM - Gln +10% FetalClone

[4] Schmid & Blanch (1992); batch culture conditions DME +10 FBS + 1% 100X MEM non-essential amino acids

[5] Bonarius *et al.* (1995); continuous culture conditions, Ham's F-12/Iscove's Serum Free low-protein, lipid free medium

remains unclear as to the mechanism responsible for the observed reductions in consumption. However, the reduction in consumption may be in direct relation to the observed decreased cell densities and overall health of the cells. Further metabolic studies would be required to elucidate the exact reasons for reduced consumption rates.

In comparison to other literature values (Refer to Table 4.4) for various hybridoma cell lines, it can be seen that under extreme inhibitory growth conditions (12 mM glutamate) the amino acid utilization rates are very similar to those of Bonarius *et al.* (1995) having production rates of alanine, aspartate, asparagine, and serine. In comparison to those production rates or the net production values, it may be that elevated production rates are an indication of overall cell health, and that observed high levels correspond to poor cell health.

4.2 BHK Fibroblast Kinetic Characterization of Glutamine and Glutamate Uptake

The kinetic characterization of glutamine and glutamate uptake was assessed in BHK fibroblasts as the cell line had been adapted to growth on a low glutamine, high glutamate media (Christie, unpublished). Therefore a brief study was undertaken to determine if any changes had occurred in the uptake systems when cells were adapted from glutamine based medium to a low glutamine/high glutamate medium.

The uptake of glutamine in BHK cells exhibited a more complex kinetic profile, than those observed for CC9C10. Simple Michaelis-Menten kinetics were not observed, but instead a sigmoidal curve was observed. The sigmoidal curve is suggestive of an allosteric effect involved in the uptake of glutamine and the Hill

coefficients were determined from the plots (1.34 parental cell line, 1.38 adapted cell line).

The uptake of glutamine was found to be larger in cells which were adapted to the low glutamine/high glutamate medium. The increased uptake over the parental cell line could result from a preference by cells for glutamine as a catabolic energy source and that under low glutamine conditions cells are essentially starved. When elevated levels are presented to the cells, an increased uptake occurs until cellular pools are replenished. Alternatively, observing the cell densities, the parental cell lines seemed to be high and it is possible that cells had entered into stationary phase. It is plausible that in stationary phase cells would have a reduced glutamine requirement as they are no longer in the active logarithmic phase of growth.

Similar curves to those observed experimentally, were observed for lymphatic cell lines (Piva *et al.*, 1992). In these cells, it was determined that the complex uptake pattern was triphasic, in that three systems were responsible for the uptake of glutamine. In order to verify if this is occurring, further studies involving a wider range of glutamine concentrations, sodium vs. sodium replaced, and starvation experiments would need to be undertaken.

The uptake of glutamate was also found to exhibit a complex uptake pattern. The observed uptakes were not simple Michaelis-Menten. The uptake was, like glutamine, suggestive of allosteric effects. The parental BHK cell line was observed to take up more glutamate, than the adapted cell line. However, the binding affinities were not significantly different, suggesting that one system was responsible for the uptake of glutamate. The observed allosteric effect, coupled with the Hill coefficients (1.45 ± 0.09 parental and 1.46 ± 0.10 adapted) suggest a transition from a weak binding

state to a stronger binding state which may involve one or more molecules. Further experimentation is required to elucidate the exact mechanisms of uptake, sodium dependence/independence of uptake, and number and type of uptake systems.

Chapter 5. Conclusion

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5.1 CC9C10 Hybridoma Conclusions

In conclusion the CC9C10 hybridoma cells fail to adapt to a low-glutamine or glutamate based medium. The failure to adapt is largely due in part to competition between glutamate and cystine for uptake into the cells by system x^-_c. Elevated glutamate levlels "outcompete" cystine for uptake into cells. A depletion of cystine in cells is likely to result in decreased glutathione content, which would lead to oxidative cell death.

Specifically it was shown that:

1) The cells take up L-glutamate in a sodium independent manner that was stereospecific for L-glutamate.
2) The uptake system was also found to be extensively and competitively inhibited by homocysteic acid and L-cystine, but notably not aspartate.
3) Uptake of glutamate under typical growth conditions, in SFM, was shown to be greatly inhibited compared to that of the uptake of glutamate in the sodium free buffer system.
4) Uptake of L-cystine was found to occur via a sodium independent uptake system which is competitively inhibited by glutamate.
5) Under typical media conditions (SFM) with elevated glutamate levels it was found that very little cystine is taken up by the cells.
6) L-Glutamate and L-cystine are taken up by a sodium independent system specific for these amino acids. This uptake system is x^-_c.

7) No evidence was found for the existence of uptake system X^-_{AG}, a system that has a large capacity for glutamate

Glutamine was found to be taken up by CC9C10 hybridomas in a sodium dependent manner, although there was evidence for a smaller sodium independent fraction. The uptake was found to correspond to Michaelis-Menten saturation. Uptake of glutamine was found to be inhibited by arginine, cysteine and serine, indicating that system ASC is taking up glutamine. Uptake system ASC has been shown to be a neutral amino acid uptake system which takes up alanine, serine and cysteine as well as glutamine. The inhibition of arginine on glutamine uptake is unclear and further characterization is necessary to assess if other systems are also taking up glutamine.

5.2 BHK Fibroblast Conclusions

BHK fibroblasts which were adapted to growth on glutamate had a greater amount of glutamate taken up over the 20 minute time period compared to BHK cells grown in glutamine. The kinetic analysis of glutamate uptake for both lines revealed a similar trend, the cells grown in glutamate had a greater proportion of glutamate uptake than those grwon in glutamine. This indicates that some form of adaptation had taken place. Both lines showed non-Michaelis-Menten saturation curves which indicated possible allosteric effects on glutamate uptake. The Hill plot interaction coefficient was found to approximately 1.5 for both cells lines and best fit to a ineteraction coefficient of 2. This mean that 2 molecules of glutamate are required for uptake into the cells. Further work is required to assess if multiple systems are operating and whether or not sodium is required for uptake.

The uptake of glutamine by the BHK cell line grown in 3 mM glutamine revealed that a larger proportion of glutamine was taken up over the 20 minute time range, when compared to cells grown in 3 mM glutamate. When a kinetic analysis was performed the reverse was found, i.e. the cells grown in 3 mM glutamate had a greater proportion of glutamine taken up. This seems unlikely and the work needs to be repeated to verify the observations. Also sodium dependence or independence needs to be assessed.

Chapter 6. Literature Cited

6.1 Literature Cited

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