THE USE OF GLUTAMATE AND DIPEPTIDES AS SUBSTITUTES FOR GLUTAMINE IN ANIMAL CELL CULTURES

BY

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A Thesis Submitted to the
Faculty of Graduate Studies in Partial Fulfillment
of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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FACULTY OF GRADUATE STUDIES

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Abbreviations

ATCC	American Type Culture Collection
BME	Eagle's Basal Medium
conc	concentration
DMEM	Dulbecco's Modified Eagle's Medium
D-PBS	Dulbecco's Phosphate Buffered Saline
HPLC	high performance liquid chromatography
hr	hours
mAb	monoclonal antibody
OPA	*o*-phthaldialdehyde
r.p.m.	revolutions per minute

Enzymes

ALT	alanine aminotransferase (also known as glutamic pyruvic transaminase or GPT)
GDH	glutamate dehydrogenase
GS	glutamine synthetase
LDH	lactate dehydrogenase
PAG	phosphate-activated glutaminase

Metabolites

ala	alanine
ala-gln	alanyl-glutamine
arg	arginine
asn	asparagine
asp	aspartate

ATP	adenosine triphosphate
frc	fructose
frc-6-P	fructose-6-phosphate
glc	glucose
GlcN-6-P	glucosamine-6-phosphate
gln	glutamine
glu	glutamate
gly	glycine
gly-gln	glycyl-glutamine
his	histidine
ile	isoleucine
lac	lactate
leu	leucine
lys	lysine
met	methionine
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
PCA	pyrrolidone-carboxylic acid
phe	phenylalanine
pyr	pyruvate
ser	serine
thr	threonine
trp	tryptophan
tyr	tyrosine
UDP-GlcNAc	UDP-*N*-acetylglucosamine

UDP-GalNAc	UDP-*N*-acetylgalactosamine
UDP-GNAc	sum of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine
val	valine

Nomenclature for equations

ΔC	Change in medium component concentration (nmoles/ml)
D	doubling time (hours)
d	diameter (μmeters)
μ	specific growth rate (hr^{-1})
N	cell density at a specified time point (10^6 cells/ml)
N_f	final cell density (10^6 cells/ml)
N_i	initial cell density (10^6 cells/ml)
ΔP	change in product (nmoles)
q	specific consumption or production (nmoles 10^{-6} cell hr^{-1})
q_{gln}	specific glutamine consumption
q_{glu}	specific glutamate consumption
ΔS	change in substrate (nmoles)
t	time (hours)
t_f	final time point (hours)
t_i	initial time point (hours)
x	cell number at a specified time point (10^6 cells)
Y	growth yield (cells/pmole)
Y_{lac}	growth yield on lactate
$Y_{NH_4^+}$	growth yield on ammonium

Abstract

The Use of Glutamate and Dipeptides as Substitutes for Glutamine in Animal Cell Cultures

by Andrew D. Christie

The chemical decomposition and cellular metabolism of glutamine in animal cell cultures results in ammonium accumulation in the growth medium. Less ammoniagenic substrates including glutamate and dipeptides (ala-gln and gly-gln) were investigated as substitutes for glutamine in cell culture. The CC9C10 murine hybridoma and BHK-21 cells were sensitive to ammonium. Consequently, these two cell lines were used as models to evaluate the effects of substitutes on growth and metabolism and the factors influencing the ability to utilize glutamate and dipeptides.

Glutamate was found to inhibit the growth of CC9C10 cells although ala-gln and gly-gln supported the growth of the hybridoma. High cell densities were obtained in the presence of 6 mM ala-gln and 20 mM gly-gln. The final cell density in gly-gln was 14 % higher than in glutamine medium although monoclonal antibody production was not improved. Substrate utilization and metabolism were affected by the dipeptides, particularly with gly-gln. The accumulation of ammonium and lactate was significantly lower. The higher concentration of gly-gln was required because a cellular peptidase had a lower affinity for gly-gln. An investigation of the mechanism of dipeptide utilization implicated extracellular hydrolysis following release of peptidase into the culture medium. The dipeptides were also effective as substitutes for BHK cells although high gly-gln concentrations (up to 40 mM) were required. In addition, glutamate, asparagine and a low level of glutamine or dipeptides supported high growth rates and cell yields of BHK cells. Significant changes in metabolism were characterized in glutamate-based medium including markedly reduced ammonium production although growth was not improved with respect to glutamine-based medium.

Cell yields were limited by surface area or nutrient depletion in batch cultures. In fed-batch microcarrier cultures, glutamate substitution resulted in the accumulation of inhibitory lactate levels. Growth in glutamate-based medium was not limited by glutamate uptake. Changes in intracellular enzymes such as glutamine synthetase were probably more important for the adaptation to utilize glutamate.

Substitutes for glutamine are effective for controlling ammonium levels in culture. However, achieving substantial increases in growth and productivity may depend on the regulation of other substrates in the medium, particularly glucose.

Chapter 1

Introduction

1.1 A brief history of animal cell culture

The dawn of animal cell culture may be traced back to the work of Roux (1885) who described the survival of chick embryo tissue in warm saline for a number of days. However, the actual beginning of cell culture is perhaps more accurately dated to the first decade of the twentieth century when it was realized that animal tissue could divide, grow and function outside of the animal host. Jolly (1903) documented cell division of salamander leukocytes in hanging drops. Later, Harrison (1907) reported the growth of nerve fibers from frog cells over a duration of several weeks using the hanging drop method. Embryonic frog nerve tissue was embedded in a clot of frog lymph on a coverslip which was then inverted and placed over a well in a microscope slide. The coagulated protein in the clot served as a matrix or substrate on which the cells could grow while the lymph fluid provided nourishment to the cells. The hanging drop procedure was developed further by Burrows (1910) who used a plasma clot in place of lymph.

Over the next several years, numerous contributions to the field were made by Alexis Carrel. His earliest studies had shown that chick embryo extract, used as a supplement to plasma, strongly stimulated growth of certain cells (Carrel, 1913). Carrel also introduced aseptic methods and successfully demonstrated that animal cells could be cultured *in vitro* for numerous generations provided precautions were employed to prevent contamination and overgrowth by microbes. His invention of the Carrel flask in 1923 further reduced the risk of contamination by other microorganisms.

One of the most significant advances establishing animal cell culture as a routine laboratory technique was the discovery of antibiotics in the 1940s. Carrel's insistence on strict and elaborate procedures to maintain asepsis had fostered the

belief that culturing animal cells was a very difficult process. However, the addition of antibiotics to culture medium helped reduce the incidence of bacterial contamination, facilitated the isolation of new cell lines and made cell culture a more generally accessible procedure in the laboratory.

The use of trypsin for passaging anchorage dependent cells marked another significant development in cell culture. Trypsin was first used in 1916 by Rous and Jones to free cells from the extracellular matrix of tissue yielding a suspension of single cells (Butler, 1991). Moscona and Moscona (1952) developed the technique further and the current methodology for trypsin-aided passaging of cells is based on their work. Its use has permitted the isolation of homogeneous cell populations from tissue and the technique is commonly used to disperse and subculture monolayers of anchorage dependent cell lines.

A turning point where animal cell culture evolved towards a science rather than an art was marked by the introduction of chemically defined media in the 1950s. The impetus to define the essential nutrients and design an appropriate medium that could sustain cells was due to the inconsistent growth arising from the poorly defined and variable mixtures of plasma and embryo extracts normally used as a medium. At the forefront of those studies was Harry Eagle who analyzed the nutritional requirements of mouse L and HeLa cells. The mouse L fibroblast line was derived by chemical transformation and could be grown indefinitely in culture (Sanford *et al.*, 1948) while the HeLa line was isolated from a human carcinoma (Gey *et al.*, 1952). Eagle identified the essential amino acids, vitamins, salts, carbon source and other components as well as the concentrations required to support optimal growth of the two cell lines (Eagle, 1955). The resulting medium came to be known as Eagle's basal medium (BME). Numerous modifications of this original formulation followed and were designed to satisfy the specific nutritional requirements of other cell lines. These media not only reduced the variability associated with the use of biological fluids previously used to

grow animal cells but were also easier to sterilize and consequently less susceptible to contamination by microorganisms. However, they still required supplementation with animal serum, usually at concentrations of 10 %, which provided undefined growth factors, hormones and other proteins necessary for growth. Work on producing completely defined media to eliminate the need for serum began about the same time BME was developed. These studies have resulted in the identification of a number of the most important growth-promoting components of serum. By the beginning of the 1980s, serum-free media for a number of cell lines had been described (Barnes and Sato, 1980).

The development of animal cell culture over the last century has contributed considerably to the understanding of the growth and metabolism of animal cells both *in vitro* and *in vivo*. Principles of cell culture have been applied to further basic medical research in the fields of virology, neoplasia, cell biology, immunology and genetics. The knowledge gained in these areas has synergystically formed the basis for the industrial-scale production of medical, veterinary and diagnostic products derived from animal cells. The potential use of animal cell culture technology for large-scale generation of biological products had become apparent with reports that poliomyelitis virus could be grown in human embryonic cell cultures (Enders *et al.*, 1949). By 1954, polio vaccine was the first licensed commercial product derived from animal cell cultures on a large-scale. Vaccines for measles, rabies, mumps and rubella are among others that followed in the 1960s.

In 1976, Köhler and Milstein revolutionized many areas of the biosciences as they introduced the technology to produce monoclonal antibodies (mAbs) (Köhler *et al.*, 1976). Monoclonal antibodies have been widely used for diagnostic applications since the early 1980s. The first mAb was licensed for therapeutic use in 1986. Hybridoma technology has since continued to evolve towards the production of more efficacious monoclonals for human therapeutic use. Advances in this area include methods for

creating human hybridomas and human antibodies expressed and secreted by high producing recombinant cell lines. Because of their high specificity, monoclonal antibodies have also been widely exploited for the purification of other biologically active molecules on both the laboratory and industrial levels. To accommodate the growing demand, monoclonals are already produced commercially in culture vessels thousands of litres in size. This trend is sure to continue as additional applications are discovered and new mAbs are approved for therapeutic consumption.

Other products derived from animal cells which are licensed or are in clinical trials include interferons, interleukins and other lymphokines, tissue plasminogen activator (tPA), erythropoietin (EPO), blood clotting factors and human growth hormone. Animal cell culture has, in fact, grown to a multibillion dollar industry and will continue to expand as research progressively reveals the molecular basis of disease and as new treatments are uncovered. However, shrinking health care budgets demand cost-effective production of diagnostic and therapeutic agents. Consequently, the general objective of this project has been to optimize animal cell culture medium to limit accumulation of inhibitors. The desired result is to improve cell yields and reduce associated production costs.

1.2 Culture conditions and cellular growth requirements

1.2.1 Culture vessels and cell attachment substrates

Animal cells are cultured in a variety of different vessels including multiwell plates, Petri dishes, roller-bottles, T-flasks and spinner flasks. The vessel used is determined by factors such as the culture volume and cell yield required and whether the cell line grows in suspension or is anchorage dependent.

Most animal cell lines require a substrate on which to grow and divide and are thus referred to as anchorage dependent. The substrate must allow efficient cell attachment following inoculation into the culture medium. The initial attachment

between the cell membrane and substrate surface is mediated by electrostatic and van der Waals forces (Absolom *et al.*, 1983). Calcium ions (Ca^{+2}) in the culture medium and basic proteins produced by the cell or present in the serum form a layer or cation bridge between the negatively charged cell membrane and substrate surface to facilitate the adhesion process. Both glass and plastic can provide an appropriate negative charge density for the formation of the bridging layer. Plastics, particularly sulfonated polystyrene, are widely used to manufacture disposable culture vessels.

Cell attachment is followed by growth and spreading of the cells across the substrate surface. Growth normally continues *in vitro*, barring a nutrient limitation or inhibition by accumulation of waste products, until the cells have covered the available surface area and have formed a confluent monolayer. Thus, if high cell densities are to be attained, the culture system must provide maximum surface area for a given culture volume. T-flasks allow a surface to volume (s/v) ratio up to 5 cm^2 per millilitre of culture volume. Roller bottles increase this ratio 2-3-fold by allowing cells to grow around the cylinder's walls. Nevertheless, surface area limitations are realized even in roller bottles. Further increases in cell yield would therefore require increasing the number of roller bottles which is costly in terms of materials and time. Other designs have been developed to further optimize the surface to volume ratio but most are limited by difficulties in scale-up to industrial production levels. The development of microcarriers, however, proved to be an innovation that combines maximum surface to volume with ease of scale-up.

Microcarriers are small beads 100-200 μm in diameter with a charged surface to promote cell attachment. DEAE-Sephadex had originally been used as a microcarrier (van Wezel, 1967) but the high negative charge on this ion exchange resin proved toxic to the cells. Modification of the beads to reduce the charge density eliminated this effect (Levine *et al.*, 1979). Microcarriers are now available from commercial suppliers and are made from a number of different materials including polystyrene,

polyacrylamide and Sephadex. The beads are typically added to the culture medium at 1-5 g/L without adversely affecting the cells and increasing the s/v at least 6-fold compared to T-flasks. Spinner flasks equipped with a magnetic stirring bar and paddle assembly are commonly used to provide gentle agitation to microcarrier cultures. This provides aeration and maintains the beads in a uniform suspension while minimizing cell damage from shear effects between colliding beads. Scale-up to increase cell yields is also possible and may be achieved by increasing bead concentrations and the volume of the culture vessel. Because of these advantages, microcarriers, primarily Cytodex 1 (Pharmacia), have been used in the following investigations for high density anchorage dependent cell cultures. Cytodex 1 is produced from Sephadex - a cross-linked dextran bead with charged DEAE groups attached to the surface.

Some cell lines do not require an attachment substrate and grow freely suspended in the culture medium. They are usually derived from hemopoietic cells or in some cases may have originated from a transformed anchorage dependent line. Suspension cells such as hybridomas are not subject to surface area limitations and scale-up is normally achieved by an increase in culture vessel volume.

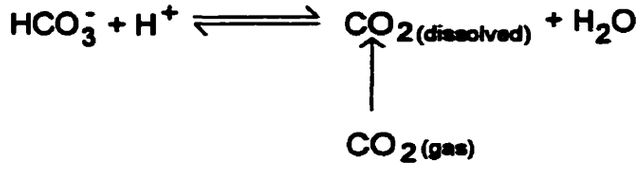
1.2.2 Temperature

Mammalian cell lines are generally cultured at the normal body temperature of 37 ^{o}C although may demonstrate varying degrees of growth from 33-39 ^{o}C (Freshey, 1983). The optimal temperature within this range varies according to the species and tissue from which the cell line was established. Temperatures higher than 39.5 ^{o}C, however, are rarely tolerated and induce a rapid decline in cell viability. Consequently, precise temperature control is essential for the culture of animal cells and is normally achieved with water-jacketed incubators.

1.2.3 pH

Most animal cells grow well at physiological pH from 7.2 to 7.4 but the pH optimum may encompass a broader range depending on the particular cell line (Eagle, 1973). Growth for the majority of cell lines, though, is normally limited beyond pH 6.8-7.8.

The pH of the culture medium is usually maintained with a bicarbonate-carbon dioxide buffering system. The addition of sodium bicarbonate to the medium and gassing of the incubation chamber with CO_2 establishes the following equilibrium:



The pH of the medium can be related to the Henderson-Hasselbalch equation:

$$pH = pK_a + \log[HCO_3^-]/[CO_2\ (dissolved)]$$

where the pK_a is 6.3. The CO_2 dissolved in the culture medium is a function of the percentage of CO_2 in the atmosphere. A combination of 24 mM $NaHCO_3^-$ and a 5 % CO_2 atmosphere at 37 ^oC buffers at about pH 7.4. Some medium formulations also call for 44 mM $NaHCO_3^-$ with 10 % CO_2 in the gas phase to allow for increased buffering capacity. Although the HCO_3^-/CO_2 pair is not the most effective buffering system in the physiological pH range, it is the most widely employed because it is non-toxic, relatively inexpensive and serves as a nutrient. Bicarbonate or carbon dioxide is utilized for biosynthetic reactions, particularly when the cell density is low and consequently metabolically derived CO_2 is limited (Ham and McKeehan, 1979).

1.2.4 Oxygen

Oxygen is an essential nutrient for all animal cells because of its role as the terminal electron acceptor in the electron transport chain and the means by which ATP is generated via oxidative phosphorylation. Apparent cellular oxygen requirements vary among different cell lines. Oxygen uptake rates (OUR) have been reported to range from 0.045-0.47 mmol O_2 L^{-1} hr^{-1} per 10^6 cells/ml (Fleischaker and Sinskey, 1981). A particular cell line, however, may grow in a range of oxygen concentrations. Mouse LS cells, for instance, grow at oxygen tensions of 40-100 mm Hg which corresponds to 5-13 % of air saturation (Kilburn and Web, 1968 and Kilburn *et al.*, 1969). Outside of the optimal range, though, low oxygen limits growth and high levels are toxic. Balin *et al.* (1976) examined growth of WI-38 cells at different oxygen levels. Normal growth was observed at 26, 44 and 134 mm Hg. At 7.8 mm Hg, lower growth rates and cell densities were noted as well as increases in glucose consumption and lactate production. An increase to 291 mm Hg again resulted in depressed cell growth and yields and dramatic elevations in lactate production while 560 mm Hg completely inhibited cell proliferation. Numerous studies have established that high oxygen concentrations result in free-radical damage that can induce cell death. Extremes in the oxygen concentration should thus be avoided. Most monolayer and suspension cultures grow well in T-flasks with an atmosphere of 90-95% air and the balance consisting of CO_2. A large surface area of the culture medium is exposed to the gas phase under these conditions allowing oxygen to diffuse readily to the cells. In cylindrical culture vessels with a lower aspect ratio (width/height of culture) such as spinner flasks, agitation or stirring is required to supply enough oxygen for cell growth. In vessels with a volume greater than 1 L, surface aeration is no longer adequate to maintain a sufficient supply of dissolved oxygen. For these larger cultures, aeration of the medium with sparging systems is normally required to provide enough oxygen to support cell growth.

1.2.5 Osmolarity

The culture medium should provide an isotonic environment for cell growth. Media are formulated to emulate the osmolarity of plasma *in vivo* which would normally be about 300 mOsmol/kg. Most cells are quite tolerant of deviations from their optimal osmolarity and generally will grow in a range from 260-320 mOsmol/kg (Waymouth, 1970 and Freshney, 1983). However, extreme changes in osmotic pressure have been shown to result in reduced growth rates and cell densities and increased death rates (Ozturk and Palsson, 1991). The salt concentration in the medium may therefore require adjustment to reduce osmolarity when additional supplements are added to the culture medium, particularly for those cell lines that are sensitive to osmotic stress. The productivity of a culture, however, may not be reduced by higher medium osmolarity. Specific monoclonal antibody production rates from hybridomas were reported to increase in the presence of high osmolarity (up to 435 mOsmol/kg) (Ozturk and Palsson, 1991 and Ozturk *et al.*, 1992). Final antibody concentrations, though, were similar to cultures with normal osmotic pressure because lower growth rates and cell densities were obtained with higher osmolarity. On the other hand, Oh *et al.* (1993) found the specific productivity and the final monoclonal antibody yields for another hybridoma line were increased when adapted to medium with elevated osmolarity (350 and 400 mOsmol/kg).

1.2.6 Culture medium composition

Since Eagle's early studies on the growth requirements of animal cells, numerous media have been developed, many of which are based on his original BME formulation. Modifications were introduced to support growth of different cell lines. Despite the differences in composition, the medium in all cases is intended to provide a relatively isotonic environment at physiological pH with a suitable complement of essential nutrients to promote optimal growth. Dulbecco's Modified Eagle's Medium or

DMEM (Dulbecco and Freeman, 1959) is among the most widely used because it is rich in nutrients and supports growth of many cell types. DMEM is the basal medium used throughout these investigations and its ingredients are provided in Appendix A along with BME for comparison.

1.2.6.1 Inorganic salts

Inorganic salts are quantitatively the most significant constituents of cell culture medium. Eagle's early studies on the nutritional requirements of HeLa and mouse fibroblast cells revealed that sodium, potassium, calcium, magnesium, chloride and phosphate are essential for the growth of animal cells *in vitro* (Eagle, 1955). They are necessary for the formation of electrochemical gradients, to maintain correct osmotic balance and most serve as enzyme cofactors in a variety of biosynthetic reactions. Bicarbonate is also added for both its buffering role and nutritional value as discussed previously. In addition, cells require trace amounts of iron which is necessary in a number of metalloproteins. Other minerals are also vital but despite their apparent omission, deleterious effects on the cells are often not observed. Essential trace elements are commonly present in sufficient levels as contaminants in other medium additives or are found in the serum supplement (McKeehan *et al.*, 1976).

1.2.6.2 Carbohydrates

Glucose has been the most widely used carbohydrate in culture media since the introduction of chemically defined formulations. Successful attempts have been made to replace glucose with other sugars such as fructose, galactose, mannose and maltose although these alternatives may not be effective for all cell lines (Eagle, 1955, Eagle *et al.*, 1958, Burns *et al.*, 1976 and Imamura *et al.*, 1982).

Because of its high utilization rate by animal cells, glucose is typically the most abundant nutrient in the medium, aside from the salts, at concentrations of 5-25 mM.

The rapid consumption suggested it was a major energy and carbon source for animal cells *in vitro* (Levintow and Eagle, 1961 and Morell and Froesch, 1973). Further studies have demonstrated, however, that a majority of glucose is metabolized via glycolysis instead of the more efficient and higher energy yielding TCA cycle (Reitzer *et al.*, 1979). In some cases, glucose may be required primarily as a carbon source for the production of ribose (via the pentose phosphate pathway) which is subsequently used for nucleotide biosynthesis (Reitzer *et al.*, 1980).

1.2.6.3 Amino acids

Thirteen amino acids are required for survival and growth of most cultured animal cells. These essential amino acids include arginine, cyst(e)ine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine and are standard constituents in culture media (Eagle, 1955). Nonessential amino acids such as serine, asparagine, proline and glycine may also be necessary or beneficial for growth of certain cell lines. The optimal amino acid concentrations depend on the cell line and the culture conditions, although certain generalizations hold in a majority of cases. Glutamine, with few exceptions, is consumed from the medium at the highest rate, usually 5-20 times that of other amino acids (Eagle, 1955, Griffiths and Pirt, 1967 and Butler and Thilly, 1982). Other essential amino acids are consumed at low or moderate rates while alanine and glycine are often by-products of cellular metabolism which increase in the medium during the course of the culture period (Thomas, 1986).

The high rates of glutamine consumption are attributed to its role as both an anabolic precursor and an important energy source in cultured animal cells. Glutamine is used for the biosynthesis of amino acids, proteins, nucleotides and perhaps lipids (Tate and Meister, 1973, Engstrom and Zetterberg, 1984 and Reed *et al.*, 1981). Energy is derived from glutamine via oxidation in the TCA cycle (Pardridge *et al.* 1978,

Reitzer *et al.*, 1979 and Zielke *et al.*, 1984). The contribution of glutamine metabolism to the total cellular energy or ATP levels, however, varies among cell lines and culture conditions and will be discussed in further detail in a later section.

Most other essential amino acids are probably required mainly for incorporation into cellular protein and consequently are not consumed at high rates (Eagle, 1959). This is reflected by their relatively low concentration in the culture medium. Certain amino acids such as isoleucine, leucine and valine may additionally be utilized to some extent for energy production but would supply only a small fraction of that derived from glutamine (Pardridge *et al.*, 1981).

1.2.6.4 Vitamins

Standard culture media contain choline and most of the water-soluble B group vitamins. Choline is required for lipid biosynthesis while folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin and thiamine are precursors of coenzymes necessary for catalyzing numerous reactions. Eagle (1955) described these nutrients as essential as their absence from the medium resulted in cellular degeneration within days and eventually death of the culture. Inositol, a substrate for the production of lipids, was subsequently identified as an essential medium component for all human cell lines investigated (Eagle *et al.*, 1957). Biotin, vitamin B_{12} and ascorbate are also included in some medium formulations to meet the demands of certain cell lines.

1.2.6.5 pH indicators

Phenol red is used as a pH indicator in culture media because it is non-toxic at low concentrations and is sensitive to changes in pH outside of the optimal range of growing animal cells. At pH 7.4, the medium is red while a purple coloration is produced at pH 7.8. An elevation in pH is symptomatic of a partially sealed culture vessel and incomplete gassing of the medium with CO_2. Alternatively, a decrease in pH can be

visually monitored by a change in colour to orange at pH 7.0 and yellow at pH 6.5. A gradual decline in medium pH is normal for growing animal cells as lactic acid is generated as a metabolic by-product. A rapid change in the medium colour, however, is evidence of microbial contamination of the culture.

1.2.7 Serum supplements

The growth requirements of animal cells are more complex than the combination of salts, amino acids, carbohydrates and vitamins provided in the basal medium. A number of additional factors are necessary for the proliferation of animal cells *in vitro* including growth factors and hormones, carrier proteins, attachment factors and trace elements. These may be provided by supplementing the medium with 5-10 % serum (v/v), normally derived from equine or bovine blood. Calf and fetal calf serum are most commonly used and efficacious even for fastidious cell lines because of the high content of growth factors.

1.3 Glucose and glutamine metabolism in cell culture

In vivo, energy is derived from the oxidation of numerous organic compounds including glucose, fatty acids, amino acids and ketone bodies. In cultured animal cells, glucose and glutamine are the primary energy and carbon sources. Glucose is normally present at high concentrations in the growth medium and most is catabolized via glycolysis yielding lactate as the predominant by-product. Glutamine is now accepted as the main substrate of aerobic energy metabolism *in vitro* and supplies a significant proportion of the cellular energy requirements. The pathway by which glutamine is oxidized involves the TCA cycle and produces CO_2, ammonium, carboxylic acids and amino acids as major end products. In addition to the catabolic role, glucose, glutamine and intermediate metabolites of glycolysis and glutaminolysis are utilized for the

biosynthesis of numerous cellular components. Figure 1-1 presents a simplified overview of the pathways of glucose and glutamine metabolism.

Glucose and glutamine utilization in culture has been the subject of numerous investigations because they are the major source of ATP and NADPH in the cell. Together ATP and NADPH drive the anabolic reactions required for cellular biosynthesis and growth. Furthermore, lactate and ammonia, the end products of glycolysis and glutaminolysis, are inhibitors of cell proliferation. Thus, glucose and glutamine utilization are closely linked to growth and productivity of a cell culture. Regulation of these substrates in the medium may be essential for optimal yields of a desired product from the culture. Consequently, glucose and glutamine metabolism will be examined below in some detail.

1.3.1 Glucose metabolism

The main pathways of glucose metabolism are glycolysis, the tricarboxylic acid (TCA) cycle and the pentose phosphate pathway (PPP). The sequential action of glycolysis and the TCA cycle would be the most efficient means of glucose catabolism, yielding 38 moles ATP/mole of glucose. However, a number of investigations have concluded that a relatively small fraction of glucose carbon enters the TCA cycle and is oxidized to CO_2 in cultured cells (Morell and Froesch, 1973, Schrek *et al.*, 1973, Tildon, 1973 and Donnelly and Scheffler, 1976). Instead, the majority of glucose is anaerobically metabolized by glycolysis to pyruvate which is subsequently reduced to lactate, producing only 2 moles ATP/mole glucose (Reitzer, 1979). High glycolytic rates are characteristic of tumor cells and rapidly proliferating non-transformed cell lines (Hume *et al.*, 1978, Stanisz *et al.*, 1983 and Newsholme *et al.*, 1985). The glycolytic pathway is shown in Figure 1-2.

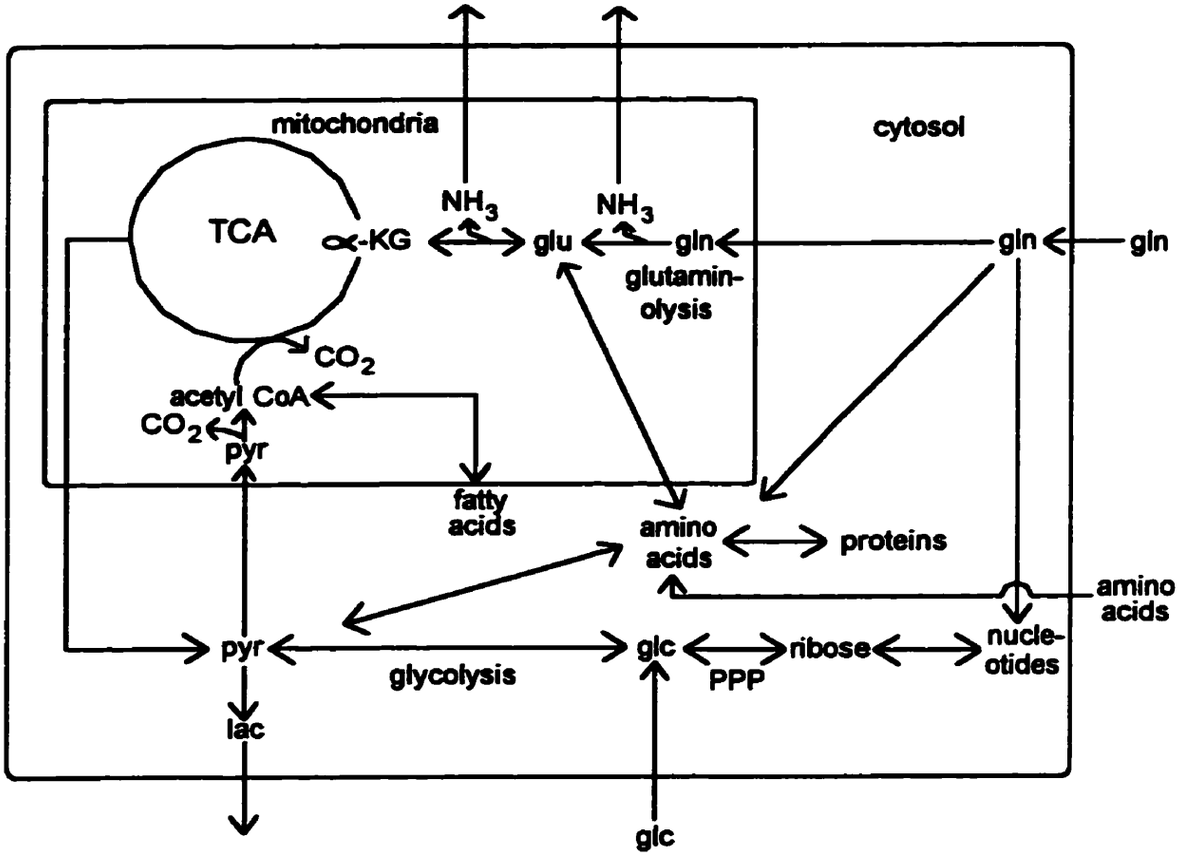


Figure 1-1. Pathways of glucose and glutamine metabolism in cultured animal cells. Glucose, glutamine and other amino acids are transported into the cell. Glucose is catabolized primarily by glycolysis. A fraction of the resulting pyruvate escapes reduction to lactate and is decarboxylated to acetyl-CoA and oxidized in the TCA cycle. A small proportion of the glucose also enters the pentose phosphate pathway (PPP) which generates ribose and reducing equivalents for biosynthetic reactions. The bulk of glutamine entering the cell is metabolized via a process known as glutaminolysis. The initial steps of glutaminolysis involve deamidation and deamination reactions with the concomitant release of ammonium or transfer of the amide and amine nitrogen to other molecules (not shown). The resulting α-ketoglutarate is oxidized by reactions common to the TCA cycle yielding CO_2 and pyruvate among other products. The remaining glucose and glutamine carbon enters a number of biosynthetic pathways to produce other cellular building blocks or macromolecules. Abbreviations: glc = glucose, gln = glutamine, glu = glutamate, α-KG = α-ketoglutarate, lac = lactate and pyr = pyruvate. Adapted from Batt and Kompala, 1989

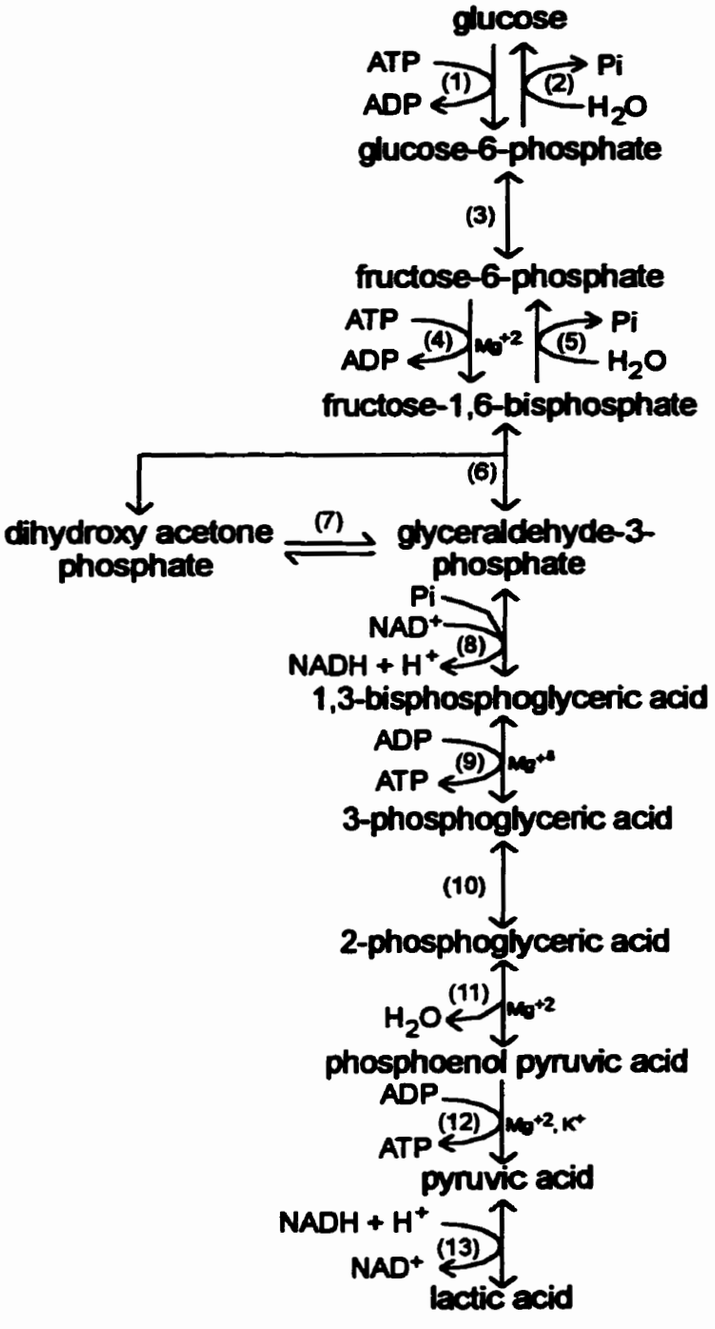


Figure 1-2. The glycolytic pathway. Enzymes catalyzing the reactions are: (1) hexokinase, (2) glucose-6-phosphatase, (3) phosphohexoisomerase, (4) phosphofructokinase, (5) fructose-bisphosphatase, (6) aldolase, (7) triosephosphate isomerase, (8) glyceraldehyde-3-phosphate dehydrogenase, (9) phosphoglyceryl kinase,(10) phosphoglyceryl mutase, (11) enolase, (12) pyruvic kinase and (13) lactate dehydrogenase. Reproduced from Conn *et al.*, 1987.

Although cultured cells may derive a significant portion of their energy requirements from glycolysis, there is convincing evidence that high rates of glucose consumption are not pertinent for cell growth. These conclusions are supported by several studies demonstrating the actual glucose requirements for a number of cell lines are minimal. For instance, Zielke *et al.* (1978) reported identical growth rates for human diploid fibroblasts grown in medium with glucose maintained at very low levels of 25 to 40 μM and a much higher concentration of 5.5 mM. Furthermore, higher glucose consumption and lactate production were noted with increasing hexose in the medium. These observations indicated that the rate of glycolysis but not the growth rate was dependent on the glucose concentration. Other investigators have further shown that glycolytic activity can be virtually eliminated in different cell lines, without adversely affecting growth, by replacing glucose with other carbohydrates. Reitzer *et al.* (1979) reported that for HeLa cells cultured in the presence of high glucose concentrations (≥ 1 mM), 80 % of the glucose was converted to lactate, 4-5 % entered the TCA cycle and 8 % was utilized in the pentose phosphate pathway. However, when glucose was replaced with 2 mM fructose, very little glycolytic activity was observed and at least 90 % of the consumed sugar was routed through the pentose cycle. Most of the fructose was found to be utilized for ribose biosynthesis and subsequent incorporation into nucleic acids (Reitzer *et al.*, 1980). These results suggested that, at least in some cases, glucose or another carbohydrate source is vital only for an anabolic role in the synthesis of nucleotides. This hypothesis is consistent with reports that a number of cell types are capable of growth in medium in which glucose has been replaced with different combinations of nucleosides such as inosine, thymidine, uridine and cytidine (Wice *et al.*, 1981 and Zielke *et al.*, 1984).

Together, the studies above illustrate that the rate of glycolysis appears to proceed faster than is possible for cultured cells to utilize intermediates of this pathway for oxidative energy metabolism or biosynthesis when glucose is present at high levels.

The result is the accumulation of high concentrations of lactate in the medium. Other end products of glucose metabolism include pyruvate, alanine and citrate although yields are considerably lower than lactate (Lanks, 1987 and Lanks and Li, 1988). The generation of large quantities of incompletely oxidized by-products in the medium has been referred to as overflow metabolism (Ljunggren and Häggström, 1992). Nevertheless, high glucose levels in the medium may be necessary to maintain optimal rates of DNA synthesis (Hume *et al.*, 1978). Thus, caution must be exercised when restricting glucose in the medium to prevent deleterious effects on cell growth.

1.3.2 Glutamine metabolism

Glutamine is an important anabolic precursor and serves as a nitrogen donor in numerous biosynthetic reactions. The amide of glutamine is the source of nitrogen in position 3 and 9 of the purine ring, the amine group of guanine, cytidine and glucosamine and the amide nitrogen of asparagine. The amine of glutamine provides the amine group of other amino acids via transamination of α-keto acids. Glutamine is the source of nitrogen in carbamyl phosphate and aspartate which are precursors in the synthesis of the pyrimidine ring. Carbamyl phosphate is additionally utilized for the biosynthesis of arginine and urea. A more complete review of the anabolic role of glutamine is available elsewhere (Tate and Meister, 1973).

The high rate of glutamine consumption by cultured cells is due not only to its requirement in numerous biosynthetic reactions but also because it is a major oxidative substrate and energy source. This conclusion is drawn from several lines of evidence indicating that glutamine accounts for a majority of oxygen uptake and CO_2 production in cultured cells. For instance, high rates of oxygen consumption were observed in rat hepatoma cells when glutamine was provided as a substrate (Kovacevic and Morris, 1972). Similarly, glutamine oxidation was reported to be responsible for 70-80% of oxygen consumption in lymphoma cells while less than 10 % was attributed to glucose

(Lavietes *et al.*, 1974). These investigators and others have further demonstrated that CO_2 is a major end product of glutamine metabolism in transformed mammalian cells (Kovacevic and Morris, 1972, Lavietes *et al.*, 1974, Stoner and Merchant, 1972 and Reitzer *et al.*, 1979). The high rate of aerobic metabolism of glutamine, though, is not restricted to transformed cells but instead appears to be characteristic of normal rapidly proliferating cells. Zielke *et al.* (1984) indicated glutamine is oxidized at least 50 times more rapidly than any other substrate examined including glucose, fatty acids and ketone bodies in diploid human fibroblasts. Significant levels of glutamine oxidation have likewise been observed in lymphocytes (Ardawi and Newsholme, 1982a), isolated enterocytes (Watford, 1994) and other cell types (McKeehan, 1986).

When it became clear that glutamine provided energy for the cell, efforts to elucidate the pathways of glutamine catabolism soon followed. Glutamine may be oxidized to CO_2 and other metabolites as outlined in Figure 1-3. The first step is the removal of the amido nitrogen of glutamine. This is catalyzed in most cases by phosphate-activated mitochondrial glutaminase (Windmueller, 1982, Kovacevic and McGivan, 1983 and McKeehan, 1986). The products of the reaction are glutamate and free ammonium. Alternatively, the amide group may be removed by amidotransferases which are generally located in the cytosol (Buchanan, 1973). These enzymes transfer the amide nitrogen from glutamine to other molecules rather than releasing ammonium. Amidotransferases include glutamine-phosphoribosyl pyrophosphate amidotransferase, carbamoyl phosphate synthase II and asparagine synthase and are involved in synthesizing some of the products from glutamine described above. The next step is the removal of the amine nitrogen from glutamate. The reaction is catalyzed by aminotransferases or glutamate dehydrogenase (GDH) which both produce α-ketoglutarate as an end product. Transaminase activity has been found in both the cytosol and mitochondria while GDH has only been observed in the mitochondria (Kovacevic, 1972). Transamination is mediated by aspartate and alanine aminotrans-

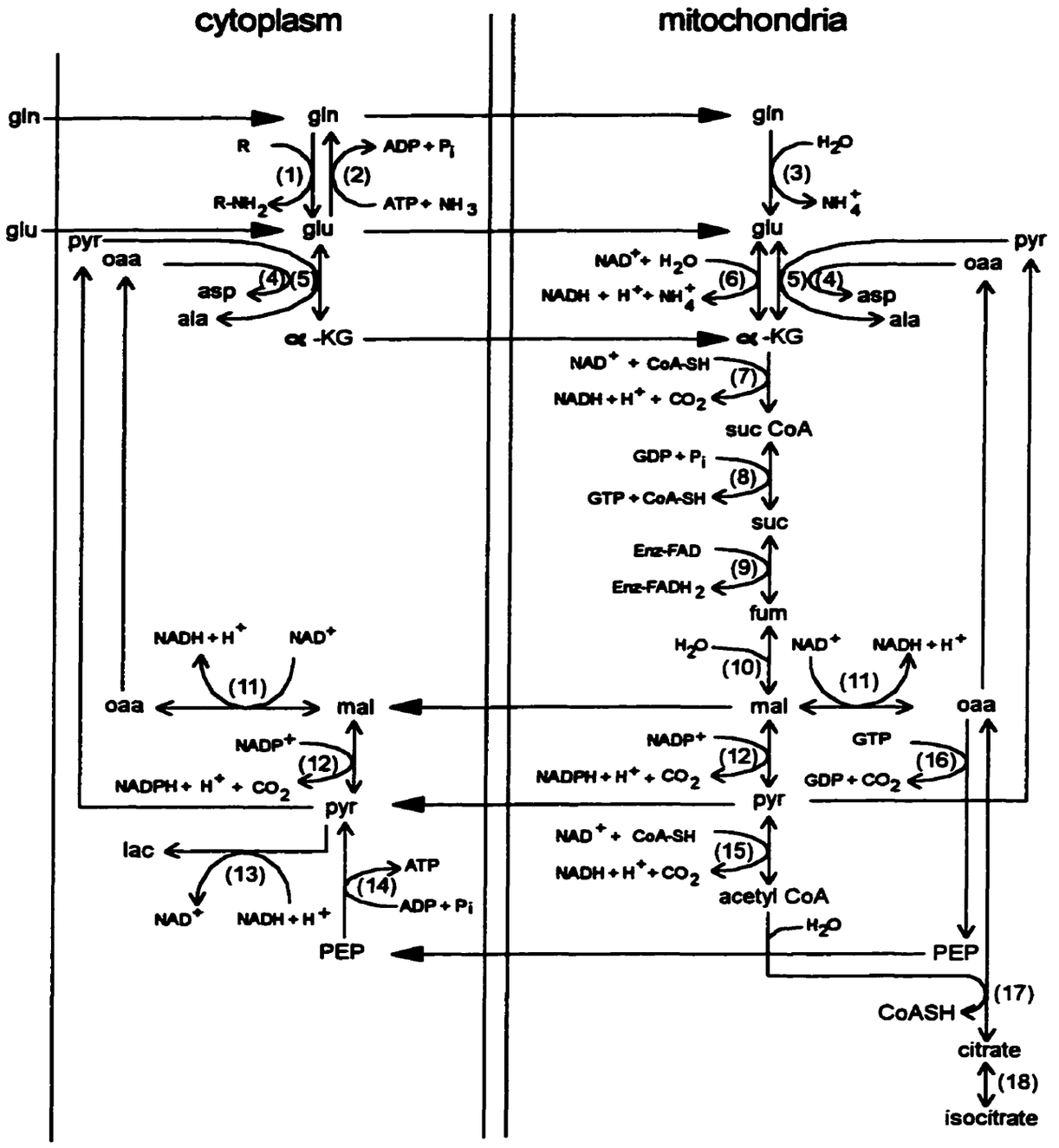


Figure 1-3. Pathways of glutamine metabolism in animal cells. Participating enzymes are: (1) glutamine amidotransferases, (2) glutamine synthetase, (3) glutaminase, (4) aspartate aminotransferase, (5) alanine aminotransferase, (6) glutamate dehydrogenase, (7) α-ketoglutarate dehydrogenase, (8) succinate thiokinase, (9) succinate dehydrogenase, (10) fumarase, (11) malate dehydrogenase, (12) malic enzyme, (13) lactate dehydrogenase, (14) pyruvate kinase, (15) pyruvate dehydrogenase complex, (16) phosphoenol pyruvic carboxykinase, (17) citrate synthase and (18) aconitase. Adapted from McKeehan (1986).

ferase as suggested by their high activity in most cells and tissues investigated (McKeehan, 1986). The amine nitrogen would therefore be sequestered in the form of aspartate or alanine instead of being released as ammonium into the culture medium as is the case with GDH. Following deamination, the resulting α-KG can be oxidized partially or completely to CO_2 in the mitochondria by enzymes of the TCA cycle. Partially oxidized end products result when malate, for instance, is decarboxylated to pyruvate by malic enzyme (Simpson and Estabrook, 1969, Hansford and Lehninger, 1973, Sauer and Dauchy, 1978 and Moreadith and Lehninger, 1984a). The NADPH produced in this reaction is believed to be a major source of reducing equivalents for biosynthesis (Simpson and Estabrook, 1969 and Reitzer *et al.*, 1980). The conversion of glutamine to pyruvate and CO_2 via the linear segment of the TCA cycle in conjunction with malic enzyme is referred to as glutaminolysis (McKeehan, 1982). The resulting pyruvate is then subject to transamination to alanine, decarboxylation to acetyl CoA for further oxidation in the TCA cycle or reduction to lactate in the cytosol. Malic enzyme, however, may play a minor role in some cell lines. Moreadith and Lehninger (1984b) reported insignificant conversion of malate to pyruvate by malic enzyme in ascites tumour mitochondria. Instead, malate derived from glutamine or glutamate was stoichiometrically converted to oxaloacetate by malate dehydrogenase and subsequently transaminated to aspartate.

As illustrated above, a number of possible routes are available for the metabolism of glutamine. The relative contribution of each is of interest because they generate different energy (ATP) yields and metabolic by-products, some of which may be inhibitory to cell growth. Discerning the predominant pathway(s) of glutamine metabolism may thus reveal important parameters to facilitate optimization of growth and productivity of cultured cells. The activities of glutaminase, amidotransferases, aminotransferases and GDH, for instance, together determine the yield of ammonium from glutamine. Steet *et al.* (1993) employed $^{1}H/^{15}N$ n.m.r. to examine the products of

nitrogen metabolism in mammalian cell cultures using ^{15}N-labelled glutamine. Their studies with HeLa and CHO cells demonstrated that all ammonium produced in culture originated from the amide nitrogen of glutamine rather than the amine group. The amine moiety was primarily found in glutamate and alanine with some labeling of aspartate, glycine and pyrrolidone-5-carboxylic acid also detected. These results indicate glutamine is deamidated largely by glutaminase. Furthermore, GDH appears to play an insignificant role in deamination which is instead largely achieved via transamination, particularly by alanine aminotransferase and, to a lesser extent, aspartate aminotransferase. Other investigators have presented results that are consistent with minimal deamination by GDH in the pathway of glutamine catabolism. Low GDH activity was reported in lymphocytes (Ardawi and Newsholme, 1982b) and tumour cells (Filc-DeRicco *et al.*, 1990). In contrast to the work of Street *et al.* (1993), Ardawi and Newsholme (1982a and 1983) observed higher production of aspartate than alanine from glutamine in lymphocytes. This indicated a more prominent role for deamination by aspartate aminotransferase in these cells. The pathway of glutamine metabolism, however, is probably dependent on both the cell line and growth conditions. Baverel and Lund (1979) suggested GDH was more important for transamination than aminotransferases in isolated rat enterocytes. Other workers have proposed the origin of glutamate influences the route of deamination. Glutamate derived from the deamidation of glutamine in the mitochondria is deaminated mainly by GDH while an external supply of glutamate, from the culture medium for instance, is metabolized by transamination (Kovacevic, 1971 and Schoolwerth and LaNoue, 1980).

The reactions following deamidation and deamination largely determine the energy yield from glutamine metabolism. Repetitive rounds of the glutamine carbon skeleton through the TCA cycle is the most efficient means of catabolism, producing 27 moles ATP/mole glutamine. Incomplete oxidation to pyruvate followed by transamination to alanine or reduction to lactate results in 9 moles ATP/mole glutamine.

Similarly, oxidation to oxaloacetate, followed by transamination to aspartate generates 9 moles ATP/mole glutamine (Schneider *et al.*, 1996). The contribution of glutamine to cellular energy metabolism can thus be evaluated by identifying the operative metabolic pathway(s) in cultured cells. A limited number of reports have described almost complete oxidation of glutamine (Sauer *et al.*, 1980 and McKeehan, 1982). Other investigators have indeed found CO_2 was a major product of glutamine metabolism (Lavietes *et al.*, 1974, Stoner and Merchant, 1972, Kovacevic and Morris, 1972 and Reitzer *et al.*, 1979). For instance, Reitzer *et al.* (1979) reported HeLa cells converted 35 % of glutamine carbon to CO_2, 13 % to lactate, 18-25 % to macromolecules and perhaps another 15 % to amino acid and pyrimidine pools. However, complete oxidation of a majority of glutamine in the TCA cycle would have resulted in a substantially higher percentage of carbon appearing as CO_2. This conclusion is in agreement with the work of Zielke *et al.* (1978) in which evolution of $^{14}CO_2$ from [1-^{14}C] and [5-^{14}C] glutamine from human diploid fibroblasts was monitored. Labelled CO_2 from oxidation of [1-^{14}C] glutamine was more than 4-fold higher than [5-^{14}C] glutamine indicating most glutamine was incompletely oxidized rather than repeatedly cycling through the TCA cycle. The generation of end products other than CO_2 observed in numerous investigations further implies a significant proportion of glutamine is incompletely oxidized and utilized in anabolic reactions. Zielke *et al.* (1980) found human fibroblasts metabolized up to 13 % and 18% of glutamine to lactate and glutamate, respectively. Lanks (1987) observed glutamate, aspartate and citrate were the predominant products of glutamine metabolism in L929 cells. Lanks and Li (1988) investigated glutamine and glucose metabolism in cultured cells, including both normal and transformed lines. End product formation greatly exceeded oxidation of glutamine to CO_2 in 12 cell lines examined and the authors suggested this metabolic pattern may be generally applicable to cultured cells. High levels of glutamine in the medium

appears to promote overflow metabolism in which excessive quantities of ammonium and partially oxidized end products are generated (Ljunggren and Häggstöm,1992).

The studies cited above along with other reports indicate that in most cases the bulk of glutamine utilized by cultured cells is incompletely oxidized through part of the TCA cycle. Nevertheless, limited oxidation is sufficient to provide a significant proportion of the cellular energy demands. The fraction of energy derived from glutamine, however, is probably dependent on the cell line, culture conditions and the exact pathway(s) of glutamine metabolism. For instance, glutamine oxidation was calculated to supply at least 30 % of the energy requirements of human diploid fibroblasts (Zielke *et al.*, 1978) and 40 % in Chinese hamster fibroblasts (Donnelly and Scheffler, 1976) while glucose provided the balance. Glutamine oxidation contributed 70 % of cellular energy in HeLa cells cultured with high glucose but the level rose to more than 98 % when glucose was eliminated from the medium and replaced with fructose or galactose (Reitzer *et al.*, 1979). Clearly, the type and availability of hexose in the medium is a key variable that influences the energy supplied by glutamine.

1.3.3 Interaction of glucose and glutamine metabolism

Cultured mammalian cells utilize both glucose and glutamine as principal carbon and energy sources when each is readily available in the medium. Because their roles coincide, it is not unexpected that interactions or coordinate control of carbohydrate and glutamine metabolism have been observed. The interrelationship between the two substrates, however, appears to vary among cell lines.

Reciprocal regulation of glucose and glutamine has been reported in a number of cases, where a glucose limitation induces increased glutamine uptake and metabolism or a limited glutamine supply results in increased glucose consumption. Reitzer *et al.* (1979) noted that glutamine consumption in HeLa cells increased when glucose was eliminated from the medium and replaced with fructose or galactose. In

isolated colonocytes, the presence of glutamine reduced glucose consumption but not vice-versa (Ardawi *et al.*, 1985). In CHO cells (Donnelly and Scheffler, 1976) and human fibroblasts (Zielke *et al.*, 1978), either of the substrates may affect the utilization or metabolism of the other. In each of these cell lines, metabolism of one of the substrates presumably increases to compensate for a deficiency of energy resulting from the depletion of the other. The mechanisms underlying the reciprocal control of glucose and glutamine utilization are not well understood. Glacken (1988), however, proposed that glucose may regulate glutamine metabolism by its effect on the intracellular P_i concentration. Elevated glycolytic activity induced by higher glucose concentrations is expected to increase ATP synthesis with a corresponding reduction in P_i levels within the cell. This, in turn, would result in a decline in phosphate-activated glutaminase activity. This model is consistent with a decrease in intracellular P_i upon addition of glucose to HeLa and myeloma cells perfused with glutamine (Sri-Pathmanathan *et al.*, 1990). Glucose had a comparable effect on intramitochondrial P_i in Ehrlich ascites tumour cells (Medina *et al.*, 1988).

In addition to reciprocal regulation, another form of control of glucose and glutamine metabolism has been observed in isolated rat lymphocytes. Ardawi and Newsholme (1983) described an increase in glucose and glutamine consumption by the cells when both substrates were present in the incubation medium. The mechanisms responsible for this pattern of cooperative coordinate regulation have not been resolved. It has been hypothesized, however, that glutamine stimulates glycolysis by enhancing 6-phosphofructokinase activity. Glutamine may elicit this response by increasing cellular AMP or aspartate levels, both activators of the enzyme in lymphoid tissue.

The close, but variable, interaction between glucose and glutamine utilization in many cell lines warrants careful monitoring of both substrates when the concentration of either in the culture medium is changed.

1.4 Growth limitations in culture

Growth limitations in animal cell cultures can arise due to limitations in surface area or depletion of essential nutrients. In addition, the accumulation of inhibitory levels of waste products can result from the metabolism of glucose and glutamine. The use of microcarriers prevents growth restrictions of anchorage dependent cell lines resulting from the limited availability of surface area. Higher cell densities are achieved by simply increasing the microcarrier concentration in the medium. Thus, nutrient depletion and inhibition by waste products are more problematic if high cell densities and productivities are to be achieved.

Early investigations by Eagle (1955) on the nutritional requirements of animal cells had established that omission of glucose or a single essential amino acid or vitamin resulted in cellular degeneration and eventually death. Nutrient depletion in culture is characterized by a rapid cessation of exponential growth and entry into the stationary or decline phase. Batch cultures, in particular, are susceptible to this type of growth limitation. Fed-batch strategies, though, are effective in controlling the problem by replenishing nutrients with complete medium or selected nutrients (Duval *et al.*, 1991).

Oxygen limitation is another form of nutrient depletion that must be considered in high density cultures. In T-flask cultures, sufficient oxygen may be supplied by diffusion due to the large surface area of medium exposed to the atmosphere. Providing an adequate supply of oxygen has been a challenge in larger culture volumes because of its low solubility in solution (Fleischaker and Sinskey, 1981) and the fragility of animal cells. Gentle agitation coupled with air or oxygen sparging ensures the oxygen transfer rate into the medium exceeds cellular uptake even in large volume, high density cultures. Improved bioreactor designs also incorporate bubble-free aeration systems to minimize mechanical damage to the cells and limit foaming of the medium.

Controlling the accumulation of waste metabolites is an ongoing problem in cell culture that has proven more difficult to resolve. Lactate and ammonium are the predominant waste products formed by mammalian cells and both have been implicated as inhibitors. Consequently, the effects of each on cell growth and possible mechanisms of their inhibitory action are reviewed below.

1.4.1 Lactate inhibition

Lactate is produced in greater quantities than any other metabolite because of the high rates of glycolysis in animal cells. Concentrations up to 35 mM may accumulate in batch cultures (Miller *et al.*, 1988), exceeding the optimal buffering range of the medium and resulting in considerable decreases in culture pH. Eagle (1973) suggested cell growth was limited by this metabolic acidification of the medium. Later studies, though, have shown that lactate can limit growth even in cultures with pH control. Lactate, therefore, has additional inhibitory activities, apparently due to increases in medium osmolarity or other more specific chemical effects (Ozturk *et al.*, 1992). However, these were found to have a limited impact except at very high lactate concentrations, beyond that which would normally accumulate in batch and in some cases even in fed-batch cultures. Miller *et al.* (1988) also observed no inhibition of a murine hybridoma at a lactate concentration of 40 mM. Glacken *et al.* (1988) noted up to 70 mM lactate was required to produce inhibition in another hybridoma while 40 mM had little effect.

Lactate accumulation in culture can be controlled by regulating the type and availability of carbohydrate in the medium. Replacing glucose with other sugars (Reitzer *et al.*, 1979) or reducing glucose in the medium (Zielke *et al.*, 1978) results in lower lactate yields. However, the tolerance of cells to lactate suggests that limiting its production alone would produce minimal improvements in cell growth. Furthermore, attempts to regulate glucose can result in increased glutamine utilization due to

reciprocal control of the two substrates. This, in turn, introduces the possibility of glutamine depletion and increased ammonium production (Kurokawa *et al.*, 1994). The relative sensitivities of animal cells to lactate and ammonium must therefore be considered carefully when formulating a strategy to improve growth by manipulating substrate levels in the culture.

1.4.2 Ammonium inhibition

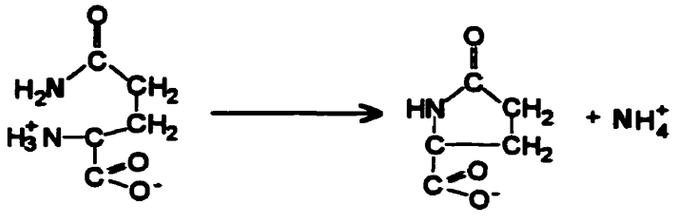
In vivo, ammonium must be removed from the blood stream because it is toxic to cells. Consequently, mammals possess elaborate mechanisms to prevent its accumulation. The urea cycle is the primary means and the enzymes of this pathway are located in the liver. In this organ, excess ammonium is converted to urea which is subsequently excreted in the urine. In cell culture, with a homogeneous population of cells, the urea cycle would not be operational resulting in the accumulation of ammonium in the medium. Relatively low ammonium concentrations have been found to cause inhibition in culture. Thus, numerous studies have been devoted to elucidating the source of ammonium, its effect on cell growth and productivity and the mechanisms of its inhibitory action.

1.4.2.1 Sources of ammonium in culture

Ammonium is produced primarily from glutamine, via cellular metabolism or chemical decomposition. As indicated previously, ammonium is produced during the deamidation and deamination of glutamine by the sequential action of the enzymes phosphate-activated glutaminase and glutamate dehydrogenase, respectively. This could potentially yield up to 2 moles of ammonium per mole of glutamine metabolized. However, the ratio is normally much lower because of the competing amido- and aminotransferase reactions (McKeehan, 1986 and McQueen and Bailey, 1990). The final yield of ammonium due to cellular metabolism is also related to the availability of

glutamine in the medium. Glacken *et al.* (1986) observed higher specific glutamine utilization rates by MDCK cells with an increasing glutamine concentration in the medium. This, in turn, could result in increased ammonium production.

The ammonium derived from glutamine decomposition is due to the instability of this amino acid in aqueous solutions. Tritsch and Moore (1962) described the break-down of glutamine to pyrrolidone-carboxylic acid (PCA) and ammonium:



glutamine PCA

An irreversible reaction between the amine nitrogen and the carbonyl carbon of the amide group results in the formation of the pyrrolidone ring and the release of the amide nitrogen as free ammonium. The reaction follows first order kinetics resulting in an exponential decrease of glutamine with time and can be described by an equation (Schneider *et al.*, 1996):

$$[gln]_t = [gln]_0 \cdot e^{-k \cdot t} \qquad (1)$$

where t is time, $[gln]_0$ and $[gln]_t$ are the glutamine concentrations at time 0 and t, respectively, and k is the first order rate constant. The rate increases with temperature and at 37 ^{o}C the half-life may be as low as 6.5 days (Tritsch and Moore, 1962). In addition, pH and the chemical composition of the medium appear to have a considerable influence on the reaction kinetics (Seaver *et al.*, 1984 and Ozturk and Palsson, 1990). Thus, the loss of glutamine due to degradation depends on the culture conditions but is generally quite significant for a batch culture which has a typical duration of 3-5 days.

In some cases, ammonium may also originate from glutaminase and arginase activity present in the serum (Wein and Goetz, 1973). Lin and Agrawal (1988) observed the half-life of glutamine decreased with higher serum content in the medium, although other investigations indicated it had little effect (Seaver *et al.*, 1984 and Ozturk and Palsson, 1990). Thus, the loss of glutamine as a result of serum enzymatic activity probably varies among batches of serum and methods of preparation and pretreatment. Furthermore, its contribution to accumulation of ammonium is likely minor compared to the effects of cellular metabolism and chemical degradation of glutamine.

Final ammonium concentrations of 5.5 mM have been reported in batch cultures (Reuveny *et al.*, 1986) and levels have even been observed to reach 2.3 mM in perfusion systems (Butler *et al.*, 1983). However, the ammonium yield in each particular case is determined by the concentration of glutamine in the medium, the cell line and its metabolic activity and culture conditions.

1.4.2.2 Effects of ammonium on growth and productivity of cell cultures

The most widely characterized effect of ammonium on cultured cells is the inhibition of growth with both reduced cell numbers and specific growth rates observed. Lower cell numbers can, in turn, diminish yields of products from the culture such as monoclonal antibodies (mAb), recombinant proteins and vaccines, thus increasing the costs associated with their production. Ammonium concentrations as low as 2 mM limit growth of animal cells (Holley *et al.*, 1978, Butler and Spier, 1984 and Hassell *et al.*, 1991). However, the sensitivity to ammonium varies among cell lines (Hassell *et al.*, 1991) and culture conditions (Doyle and Butler, 1990). Consequently, the extent of inhibition and the impact on product yields due to ammonium accumulation may also vary.

The effect of ammonium on the growth and monoclonal antibody production has been investigated in hybridoma cells. Reuveny *et al.* (1986) observed the addition of

>2mM ammonium chloride to exponentially growing murine hybridoma cells resulted in a significant reduction in cell numbers and mAb concentration. They therefore reasoned that ammonium concentrations which reached up to 5.5 mM in the later stages of a batch culture could limit cell numbers and mAb yield. Ozturk *et al.* (1992) found a decrease in maximum viable cell density and a 50 % reduction in the specific growth rate for another murine hybridoma cell line with the addition of 3.75 mM NH_4Cl to the growth medium. The final mAb yield decreased with increasing ammonium concentration in the medium, although the specific antibody production rate was unaffected. This indicates ammonium reduced antibody levels by decreasing viable cell numbers rather than limiting the antibody produced per cell. Yields of other cellular proteins have similarly been found to be reduced by ammonium. Ito and McLimans (1981) described a reduction in interferon production by ammonia. Hansen and Emborg (1994) reported ammonium up to 8 mM had no effect on growth of CHO cells in continuous culture but decreased production of recombinant t-PA from the cells. Ammonium concentrations may thus affect cellular productivity without growth inhibition. Ammonium also has the potential to restrict vaccine production from cultured cells. Propagation and yields of different viruses have been reduced in the presence of ammonium (Eaton and Scala, 1961, Jensen and Liu, 1961 and Canning and Fields, 1983). This has obvious implications for virus production in cell culture.

In addition to the effects on growth and productivity of cells, ammonium can induce qualitative changes in cellular products. Ammonium altered the glycosylation of IgM secreted by mouse plasma cells (Thorens and Vassalli, 1986) and recombinant proteins expressed by CHO cells (Borys *et al.*, 1994 and Andersen and Goochee, 1995). The extent of glycosylation is an important consideration because the oligosaccharide chains of glycoproteins can affect the structure and biological activity of proteins used for diagnostic and therapeutic purposes. Ammonium may cause these effects by changing the pH of intracellular organelles where post-translational

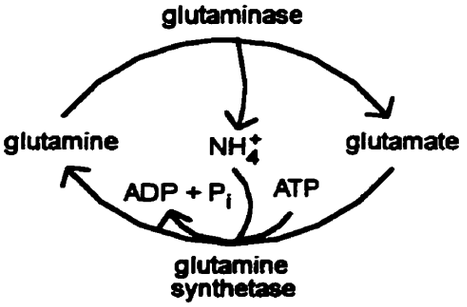
processing occurs or introducing an imbalance in cellular aminohexose pools as described below.

1.4.2.3 Mechanisms of ammonium inhibition

An explanation of the effects of ammonium on cell growth and productivity requires a thorough understanding of the mechanisms of ammonium inhibition. A number of hypotheses have recently been introduced and the most plausible are discussed.

1.4.2.3.1 Energy consumption by futile cycles

A futile cycle results from the simultaneous operation of metabolic reactions which produce and consume the same substrates with only a net hydrolysis of ATP. Glacken (1988) suggested glutaminase and glutamine synthetase may be involved in such an unproductive, energy dissipating process in cultured cells:



Although this futile cycle remains speculative, the conditions that drive these competing reactions are observed in culture. During the end of the exponential phase of a batch culture, the glutamine concentration is often low and ammonium levels are elevated due to cellular metabolism as well as degradation of glutamine. Glutaminase has been shown to be activated by high ammonium levels (McGivan and Bradford, 1983 and Verhoeven *et al.*, 1983) and glutamine synthetase is stimulated by low

glutamine concentrations (Feng *et al.*, 1990, Street *et al.*, 1993 and McDermott and Butler, 1993).

The concomitant action of glutaminase and glutamine synthetase could increase the cellular maintenance energy requirements which is the ATP utilized to sustain the cells in the absence of growth. Higher glutamine or glucose consumption may be necessary to provide the additional energy resulting in increased ammonium and lactate yields, thereby fueling the futile cycle. Eventually, growth and biosynthesis of cellular products may be limited by the reduced availability of ATP.

1.4.2.3.2 Alteration of intracellular pH and ionic gradients

A model has been proposed which suggests ammonium inhibits cells by disturbing intracellular pH and K^+ gradients (Martinelle and Häggström, 1993). This could affect the activities of intracellular enzymes and deplete cellular energy levels. The model is based on the equilibrium between NH_4^+ and NH_3 and the different diffusion and transport characteristics of each of the species across cell membranes. Ammonium acts as a weak acid in solution and forms the following equilibrium:

$$\underset{\text{ammonium}}{NH_4^+} \rightleftharpoons \underset{\text{ammonia}}{NH_3} + H^+$$

Ammonia, the conjugate base, exists as a gas dissolved in the aqueous environment. The concentration of ammonium and ammonia depends on the pH of the solution and the equilibrium is established according to the Henderson-Hasselbalch equation:

$$pH = pK_a + \log [NH_3]/[NH_4^+] \qquad (2)$$

Due to the high pK_a of ammonium (pK_a is 9.3 at 37 ^oC), NH_4^+ is the predominant form under physiological conditions; at pH 7.2, for instance, less than 1 % is in the form of NH_3. In addition, ammonium and ammonia contrast sharply in their rates of diffusion through cell membranes. Ammonium diffuses very slowly as do other ions as a result of

their charge. The diffusion rate of ammonia, however, is 4-5 orders of magnitude higher because it is a small, neutral, lipophilic molecule (Knepper *et al.*, 1989). However, NH_4^+ is believed to readily enter the cell via transport proteins in the plasma membrane such as the Na^+K^+-ATPase and the $Na^+K^+2Cl^-$-cotransporter (Martinelle and Häggström, 1993). Presumably, NH_4^+ is transported in place of K^+ because their hydrated ionic radii are comparable (Kirkeri *et al.*, 1989 and Knepper *et al.*, 1989). Figure 1-4 illustrates how these properties together could result in growth inhibition by ammonium that has been produced by cellular metabolism and the decomposition of glutamine.

The metabolism of glutamine and glutamate via glutaminase and glutamate dehydrogenase in the mitochondria results in the production of ammonium (Figure 1-4a). The NH_4^+ rapidly equilibrates with NH_3 and H^+ according to the mitochondrial pH. The NH_3 subsequently diffuses across the mitochondrial membranes into the cytoplasm and then into other organelles or the extracellular environment (the culture medium). Once outside of the cell, NH_3 equilibrates with NH_4^+ which can be transported back into the cytoplasm by the Na^+K^+-ATPase or $Na^+K^+2Cl^-$-cotransporter. Thus, H^+ accumulates in the mitochondria because NH_3 diffuses out leaving H^+ in the matrix. The cytoplasmic proton concentration also increases because of the outward flux of NH_3 and inward transport of NH_4^+. While both the mitochondrial matrix and the cytoplasm are acidified, other organelles such as lysosomes suffer elevated pH as NH_3 (the conjugate base) diffuses into them. Alternatively, ammonium originating from an extracellular source such as the decomposition of glutamine or the direct addition of ammonium salts to culture medium (Figure 1-4b) is predicted to increase the mitochondrial pH. The cytoplasmic pH is also decreased in this case as the NH_4^+ is transported into the cell. However, NH_4^+ rapidly equilibrates with NH_3 which subsequently diffuses into other organelles including the mitochondria.

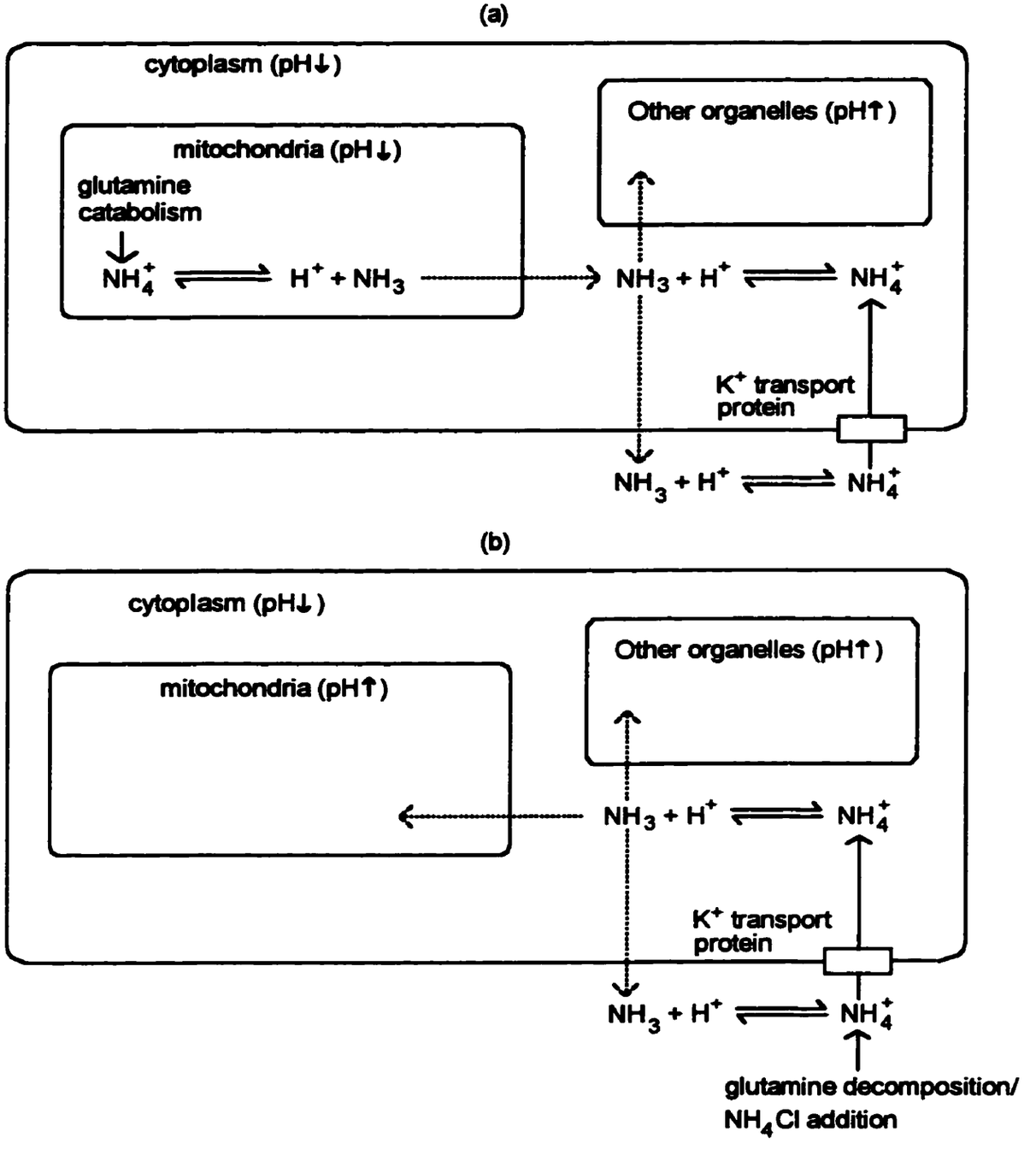


Figure 1-4. A model for ammonium inhibition by disruption of intracellular pH and K^+ gradients. (a) Ammonium produced by metabolism of glutamine and glutamate in the mitochondrial matrix. (b) Ammonium produced by decomposition of glutamine or addition of NH_4Cl to culture medium. Adapted from Schneider *et al.*, 1996.

The model presented above is consistent with a correlation between the addition of NH_4Cl to the extracellular environment and an observed decrease in intracellular pH sufficient to cause growth inhibition (McQueen and Bailey, 1991). Moreover, ammonia and other amines have been shown to increase the intralysosomal pH and inhibit receptor mediated endocytosis of growth factors (Poole and Ohkuma, 1981, King *et al.*, 1980, King *et al.*, 1981 and Cain and Murphy, 1986). The changes in pH of the cytoplasm and intracellular organelles could disrupt numerous enzyme activities and cellular processes. Energy metabolism in the mitochondria, lysosomal digestion of macromolecules and glycosylation of proteins, for instance, may all be affected by accumulation of ammonium and ammonia. Furthermore, in addition to the pH affect, transport of NH_4^+ in place of K^+ likely necessitates additional hydrolysis of ATP to maintain the K^+ gradient. This may place a greater demand on cellular energy reserves, potentially limiting growth and cellular biosynthetic capacity. A final important feature of the model is inhibition is attributed to the combined effects of both ammonium and ammonia. The term 'ammonium inhibition' used in the present studies therefore refers collectively to the effects of NH_4^+ and NH_3.

1.4.2.3.3 Disturbance of intracellular UDP-aminohexose pools

Recently, an alternative inhibitory mechanism has been proposed based on the ammonium induced increases in intracellular UDP-GNAc levels. UDP-GNAc refers to the combined concentration of UDP-GlcNAc and UDP-GalNAc which have been found to be elevated in different cell lines at the end of the exponential phase of growth (Ryll and Wagner, 1992). The high UDP-GNAc concentration has been correlated with elevated extracellular ammonium concentrations (Ryll *et al.*, 1994) that would occur during the later stages of batch or fed-batch cultures. According to this scenario, ammonium may not be the actual inhibitor but rather an effector while the UDP-sugars are the actual inhibitory metabolites. UDP-GlcNAc and UDP-GalNAc are the activated

forms of GlcNAc and GalNAc, respectively, used for the synthesis of oligosaccharide side chains of glycoproteins. Ryll *et al.* (1994) proposed the increased UDP-GNAc pools result in altered glycosylation of cellular proteins, culminating in growth inhibition. In addition, transient reductions in pyrimidine nucleotides available for nucleic acid synthesis may occur because of the diversion of UTP into UDP-sugars. Ammonium has been implicated in eliciting these responses by serving as a substrate in UDP-GNAc synthesis as shown in Figure 1-5.

Ammonium is presumed to enter the pathway for UDP-GNAc biosynthesis via glucosamine-6-P deaminase, catalyzing the production of GlcN-6-P from Frc-6-P and ammonium. Normally, glucosamine-6-P deaminase catalyzes the deamination reaction (Comb and Roseman, 1958) while GlcN-6-P is produced by glutamine-fructose-6-P transaminase using glutamine as the amino group donor (Gryder and Pogell, 1959 and Ghosh *et al.*, 1960). However, when coupled with glucosamine-6-P acetylase and a high ammonium concentration, glucosamine-6-P deaminase may favor the synthesis of GlcN-6-P. High levels of ammonium could thus promote excessive UDP-GlcNAc and UDP-GalNAc production (as well as reduced pyrimidine levels), particularly if GlcN-6-P synthesis is ordinarily a rate limiting reaction in the pathway. The variation in the concentration of UDP-sugars could affect the glycosylation of proteins (Wice *et al.*, 1985). Alterations in UDP-GNAc pools have further been attributed with growth inhibition (Krug *et al.*, 1984). In addition, inhibition of viral replication has been linked to increases in glucosamine which is also a precursor of UDP-GNAc (Figure 1-5) (Scholtissek *et al.*, 1975). As already discussed, ammonium elicits similar responses and is consistent with its proposed role as the effector of detrimental changes in cellular UDP-aminohexose levels.

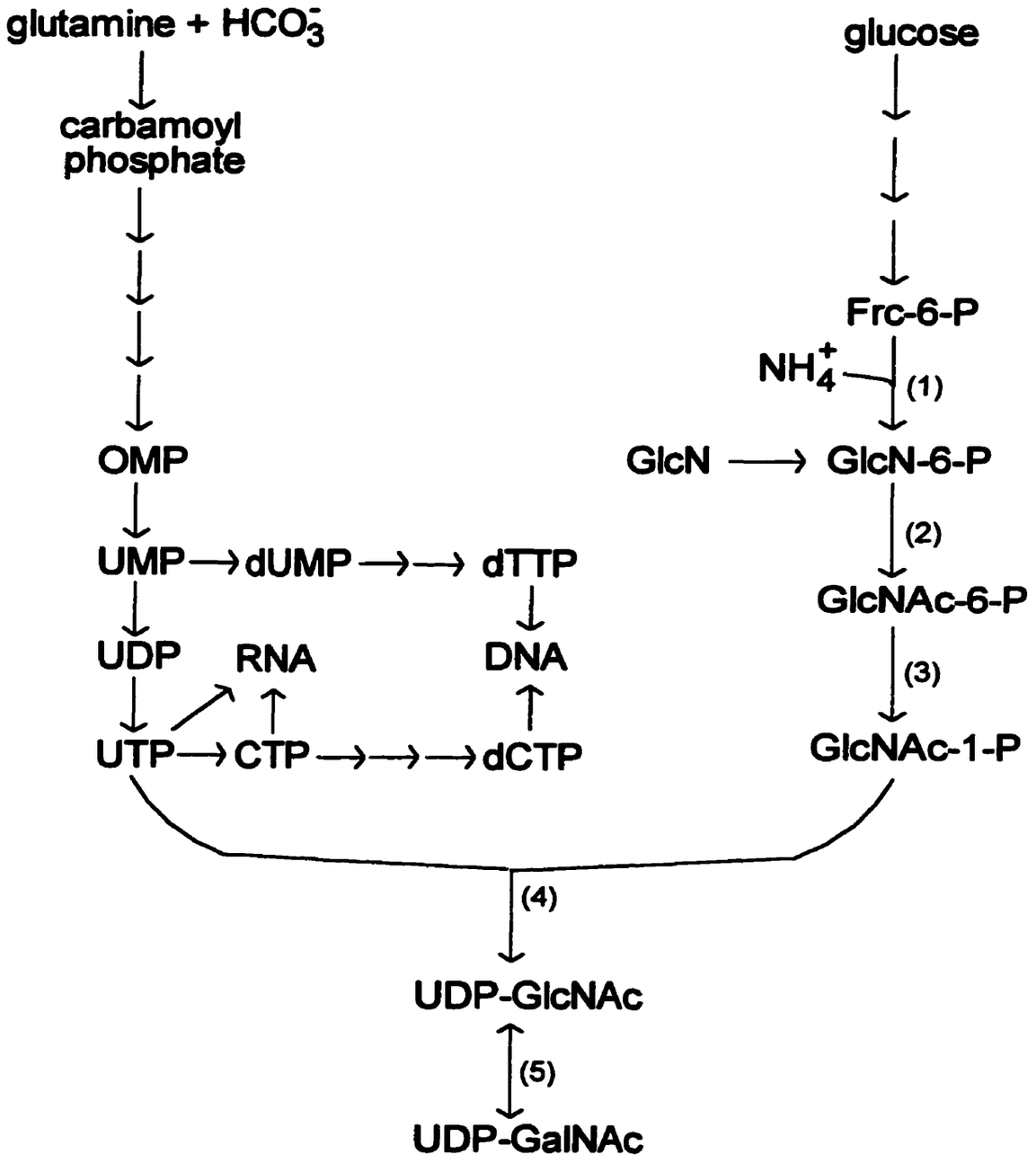


Figure 1-5. Biosynthesis of UDP-GNAc in the presence of high ammonium concentrations. Ammonium is presumed to serve as a substrate for (1) glucosamine-6-phosphate deaminase which produces GlcN-6-P. Formation of GlcNAc-6-P and GlcNAc-1-P is catalyzed by (2) glucosamine-6-P acetylase and (3) acetylglucosamine phosphomutase, respectively. Incorporation of UTP is catalyzed by (4) UDP-glucosamine pyrophosphorylase. UDP-GlcNAc and UDP-GalNAc are interconvertible via (5) UDP-*N*-acetyl-D-glucosamine 4-epimerase. Adapted from Ryll and Wagner, 1994.

The mechanisms presented above provide only a brief summary of the continuing efforts to define the basis of ammonium inhibition. It is also noteworthy that the models are not mutually exclusive. Additional studies, however, will be necessary to identify the predominant mode of inhibition or if each contributes to the observed effects differentially in each cell line.

1.5 Strategies to control ammonium accumulation in culture

The control of ammonium levels in animal cell cultures is important to ameliorate growth inhibition. In addition, glycosylation and therefore the quality and efficacy of cellular products may be influenced considerably by ammonium in the medium. Various methods have been introduced to minimize accumulation of this inhibitor and involve either removal of ammonium or limiting the availability of glutamine in the medium.

1.5.1 Ammonium removal

Ion-exchange resins and gas-permeable hydrophobic membranes have both been used to remove ammonium from cultures. Studies with hybridoma cultures have demonstrated modest increases in cell yields with these systems although monoclonal antibody production was not improved (Capiaumont *et al.*, 1995). In addition, the use of ion-exchange and hydrophobic membranes may be cumbersome, susceptible to clogging, costly and difficult to scale-up. Thus, implementation on a large-scale would likely be impractical.

A continuous or perfusion culture strategy may be employed to control ammonium and other inhibitors as well as prevent nutrient depletion. In these systems, fresh culture medium is continuously added to the culture while waste medium is removed at the same rate. Cells are removed along with the medium effluent in continuous cultures, while in perfusion cells are retained in the growth vessel. These

systems permit prolonged culture periods with high viable cell densities and productivities. Unfortunately, these processes involve the turnover of large volumes of medium and may be expensive approaches to increasing the productivity of cultures. In addition, Butler *et al.* (1983) reported relatively high ammonium levels (2.3 mM) in perfused MDCK cultures and ammonium therefore remained a potential growth inhibitor.

1.5.2 Limitation of glutamine in the medium

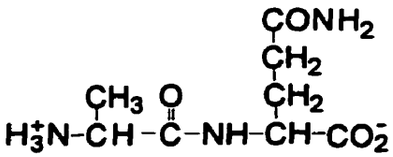
The most efficient and cost-effective means of limiting the formation of inhibitors in culture involves preventing their production in the first place. This is achieved by regulating the concentrations of substrates or precursors in the medium from which the waste metabolites are derived. In the case of ammonium, this of course means that glutamine, the primary source of this inhibitor in culture, must be controlled.

One method of regulating levels of glutamine is a glutamine-limited fed-batch strategy. This involves adding low levels of glutamine to the medium continuously or at regular intervals. This prevents ammonium accumulation due to both decomposition and high rates of cellular metabolism expected in the presence of high glutamine levels in the medium. Ljunggren and Hägströmm (1990) used this type of regular glutamine feeding regime to successfully reduce ammonium accumulations by nearly 50 % in a myeloma culture.

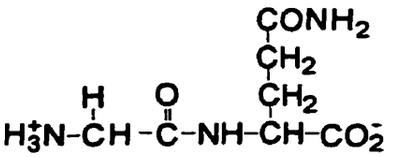
Another method of reducing the glutamine content in the medium involves complete or partial replacement with less ammoniagenic substrates or analogues. This approach offers a number of potential advantages compared to glutamine-limited fed-batch cultures. Substitution of glutamine may be less labor intensive than daily feeding regimes, reduces the risk of depletion of an essential nutrient and increases the thermostability of the medium. Both glutamate and glutamine-containing dipeptides have been used as substitutes for glutamine.

Early studies indicated high concentrations of glutamate could substitute for glutamine in HeLa cell cultures but not with mouse L cells (Eagle *et al.*, 1956). A combination of glutamate and a low level of glutamine were later described to produce increased cell yields in mouse LS cells (Griffiths and Pirt, 1967) and in the human diploid lines WI-38 and MRC-5 (Griffiths, 1973) compared to medium with the normal complement of glutamine. Hassell and Butler (1990) examined the ability of three anchorage dependent cell lines to adapt to a glutamate-based medium. McCoy cells were found to adapt readily while BHK and Vero required longer periods of adaptation. Higher cell yields were obtained for the McCoy cells along with reduced ammonium production in the presence of glutamate.

Cumulatively, the studies with glutamate demonstrate this amino acid may be advantageous as a substitute for growth of some cell lines because of its stability and inherently less ammoniagenic nature. However, some cells display a limited capacity to adapt to glutamate. In these cases, the dipeptides alanyl-glutamine (ala-gln) and glycyl-glutamine (gly-gln) may be considered as alternative replacements. The structures of the dipeptides are shown below:



ala-gln



gly-gln

The peptide bond at the amine nitrogen of glutamine prevents the formation of pyrrolidone carboxylic acid and ammonium. Consequently, the dipeptides are thermally stable at 37 ^{o}C and at higher temperatures, thus permitting heat sterilization. The dipeptides have been used as replacements for glutamine in human, hamster and mouse cell lines. Both ala-gln and gly-gln appear to be equally effective as substitutes for human cells and promote growth rates comparable to medium with a normal

glutamine complement (Roth *et al.*, 1988). However, ala-gln was found to be a better replacement for glutamine in murine cultures (Minamoto *et al.*, 1991). Gly-gln produced low cell yields at the concentrations examined for a murine hybridoma (0.7 and 3.5 mM). On the other hand, normal growth, reduced ammonium production and increased productivity of tPA were reported for recombinant CHO cells in medium with a mole for mole substitution of glutamine with gly-gln (4 mM) (Holmlund *et al.*, 1992). Thus, the appropriate substitute for glutamine appears to vary depending on the cell line. Nevertheless, glutamate or dipeptides are anticipated to support the growth of a range of cells in medium with a substantially reduced glutamine content. The factors controlling the ability of cell lines to utilize either of these substitutes, however, have not been fully identified in the literature. Additional studies are also required to characterize the effects of substitutes on growth, metabolism and productivity of animal cells.

1.6 Project objectives

A continuing challenge in animal cell culture on both the laboratory and industrial levels is to optimize culture parameters to maximize cell growth. This is necessary to improve the yields of products derived from the cells including monoclonal antibodies, native or recombinant proteins and vaccines. The net result is to reduce the associated cost of generating a cellular product so large-scale production is financially feasible. Attaining and maintaining high cell densities, however, has been problematic. Growth is generally considered to be limited by nutrient depletion or the accumulation of waste products arising from cellular metabolism or decomposition of medium components. Nutrient depletion may be remedied by adding heavily consumed nutrients to the medium at the beginning or throughout the course of the culture. Reducing waste products, however, is more difficult to achieve. Hydrogen ions, lactate and ammonium have all been identified as inhibitory waste by-products. In processes with pH control, ammonium has been cited as the most potent of the known inhibitors. Furthermore, ammonium may affect products derived from cultured cells by altering protein glycosylation. Thus, limiting ammonium accumulation was considered imperative to prevent growth limitations, reduce production costs and ensure consistent product quality. The approach involved reducing or replacing glutamine with less ammoniagenic substrates in the culture medium. The two cell lines used as models throughout the investigations were a murine hybridoma (CC9C10) and baby hamster kidney cells (BHK-21). The project objectives are summarized below:

i) Investigate the use of glutamate and dipeptides (ala-gln and gly-gln) as substitutes for glutamine in animal cell cultures.

ii) Characterize the effects of the alternative substrates on growth and metabolism of cell lines demonstrating sensitivity to ammonium.

iii) Explore the mechanisms of dipeptide and glutamate utilization and identify the factors mediating growth in the presence of the substitutes.

Ideally these studies will contribute to a long term goal of ensuring products from animal cell cultures used for diagnostic and therapeutic purposes are readily available and affordable.

Chapter 2

General Materials and Methods

2.1 Cell lines

All cell lines were purchased from the American Type Culture Collection (ATCC). CC9C10 (ATCC No. HB123) is a murine hybridoma derived from the Sp2/0 myeloma and secretes a monoclonal antibody of class IgG_1 against insulin (Schroer *et al.*, 1983). CC9C10 grows freely suspended in culture medium. BHK-21 (C-13) (ATCC No. CCL-10) is a clone derived from the kidneys of baby Syrian hamsters (MacPherson and Stoker, 1962 and MacPherson, 1963). Vero cells (ATCC No. CCL-81) were isolated from the kidney of an African green monkey (Yasumara and Kawakita, 1963). BHK and Vero cells have a fibroblast-like morphology and are anchorage dependent. Both exhibit abnormal karyotypes and grow continuously in cell culture.

2.2 Chemicals

All chemicals and reagents were obtained from the Sigma Chemical Company unless otherwise indicated. All additions to the culture medium were cell culture grade or of the highest purity available.

2.3 Culture medium

The basal medium in all cases was Dulbecco's Modified Eagle's Medium (Gibco, 23800-022) with 25 mM glucose and 44 mM sodium bicarbonate. DMEM was provided in powdered form and was prepared with water purified by reverse osmosis and either distillation or Milli-Q filtration. A 0.2 μm filter (Gelman) was used to sterilize medium which was subsequently stored at 4 ^{o}C. Glutamine, glutamate, dipeptides and other supplements were added to the stock DMEM at concentrations specified in each experiment and the medium was again sterilized by 0.2 μm membrane filtration. Just

prior to cell inoculation, 10 % bovine serum (v/v) was added to the medium. The serum supplements used were FetalClone (HyClone, A-6165-L), Calf Serum (supplemented, iron enriched) (Gibco, 16201-022) or Donor Calf Serum with iron (Gibco, 10371-029) as specified for each experiment.

2.4 Culture conditions

Cells were routinely cultured in a CO_2 incubator (NuAire, IR Autoflow) at 37 ^oC with a 10 % CO_2/90 % air atmosphere. Experiments were performed in disposable plastic 24-well plates as well as 25 cm^2, 75 cm^2 and 150 cm^2 T-flasks (Corning). Culture medium volumes in these vessels were 1 ml (per well), 10 ml, 30 ml and 70 ml, respectively, unless otherwise indicated. Larger cultures required the use of glass 100 ml spinner flasks (Bellco) or a 1.5 L bioreactor (New Brunswick, CelliGen) with volumes of 100 ml and 1.25 L, respectively. For the anchorage dependent BHK cells, microcarriers were used as the substrate for cell attachment when grown in the spinner flasks or bioreactor. Further details on the use microcarriers are provided below.

2.5 Maintenance of cell stocks and subculture procedure

Working cell stocks were maintained in 25 cm^2 T-flasks at a volume of 10 ml. Cells were subcultured every 3-4 days, prior to onset of stationary phase. The split ratio or dilution factor was chosen to correspond with this desired interval between passages. CC9C10 simply required a 1/10-1/20 dilution of the cell suspension in fresh medium. BHK and VERO cells are anchorage dependent and required detachment from the T-flask by trypsinization prior to subculture. The trypsinization procedure involved removing the culture medium from the flask, washing cells with Dulbecco's Phosphate Buffered Saline (D-PBS) (Gibco, 21600-10) to remove proteins and incubation with 0.5 ml trypsin/EDTA (0.25 % trypsin, 1mM EDTA•4Na in Hanks' balanced salt solution) (Gibco, 25200-72) for 5-10 minutes at room temperature. The

bottom of the flask was tapped vigorously to dislodge the cells. The trypsin reaction was stopped by addition of 10 ml D-PBS with 10 % bovine serum (v/v). The suspension was transferred to a 15 ml plastic centrifuge tube (Corning) and centrifuged at 330 g for 5 minutes. The supernatant was then removed and the cell pellet resuspended in 10 ml of D-PBS or DMEM. The cell suspension was diluted 1/30 for BHK cells and 1/20 for Vero cells in 10 ml of fresh medium. Once the medium was inoculated, the T-flask was placed in the CO_2 incubator with the cap loosened to allow equilibration with the atmosphere.

Greater quantities of cells required for experimental work were provided by subculture into larger volumes of media in a number of 75 or 150 cm^2 T-flasks. BHK and VERO cells were trypsinized by the procedure outlined above although the volume of trypsin used increased in proportion to the size of the flask. With the exception of the centrifugation, all steps of the subculture procedure and other experimental work involving direct manipulation of the cells were performed under aseptic conditions in a laminar flow hood (NuAire). Trypsin, PBS and any other reagents used in culture processes were supplied sterile by the manufacturer or sterilized by 0.2 μm membrane filtration.

2.6 Microcarrier cultures

Cytodex 1 microcarriers (Pharmacia, 17-0448-01) were used for growth of BHK cells in 100 ml spinner flasks and the bioreactor. The beads were prepared by a procedure based on that provided by the manufacturer. First, 10 g of the microcarriers were hydrated in 500 ml of D-PBS (pH 7.2) for at least 3 hours. An additional wash was provided by decanting the supernatant and making up to 500 ml with fresh PBS. The beads were allowed to settle and the supernatant again removed. The volume was made up to 250 ml with PBS and sterilization was achieved by autoclaving at 120 ^oC, 110 kPa for 20 minutes. After cooling, the volume was adjusted to 250 ml resulting in a

stock microcarrier solution of 40 g/L. Microcarriers were added to culture medium from the stock to yield a final concentration of 5 g/L. The bottle used to prepare the microcarriers and the culture vessels were siliconized with Sigmacote (Sigma) according to manufacturer's instructions to prevent beads adhering to glass surfaces.

2.7 Cell storage

Master stocks of each cell line were stored frozen in liquid nitrogen (-196 ^{o}C). Cells were prepared for storage by suspension in a freezing medium at $4X10^6$ - $1X10^7$ cells/ml. The freezing medium was purchased from Gibco (11101-011) or was an in-house formulation consisting of sterile DMEM/20 % calf serum/10 % DMSO (v/v). The cells were then aliquoted in 1 ml volumes into cryovials (Nunc) which were placed in a plastic storage box (Nalgene). This, in turn, was packed in a covered Styrofoam box to ensure more gradual cooling during incubation at -70 ^{o}C overnight. The boxes were transferred into a liquid nitrogen container (Thermolyne, Locator 8) the next day.

Cells stored in liquid nitrogen were withdrawn to replace stock cells that had been passaged 30-40 times or were demonstrating anomalous growth. The vials were rapidly thawed in a 37 ^{o}C water bath and the contents immediately dispensed into 10 ml of fresh growth medium. When the cells were nearly confluent (usually 1-2 days later), they were subcultured according to the procedure described previously.

2.8 Sampling of culture medium and cell enumeration

Samples were collected from the growth medium in regular intervals, usually 12 or 24 hours, for evaluation of cell concentrations and medium components. The sample was generally 1-5 ml, depending on the volume of the culture vessel.

Cell growth was monitored by counting cells, either with a Neubauer haemocytometer or Coulter counter (Model Z_F). Viable cell concentrations were determined using the trypan blue exclusion method (Patterson, 1979) and a

haemocytometer. Briefly, equal volumes of the cell sample and a solution of 0.2 % trypan blue in D-PBS were mixed (normally 200 µl of each). A few microlitres of the sample were then added to both chambers of the haemocytometer. The unstained cells were counted in each chamber to determine the viable cell concentration. The Coulter counter was used for rapid determination of total cell concentrations for a large number of samples. Cell preparation for counting with this method involved mixing 200 µl of the cell suspension with 20 ml of Isoton II (Coulter Electronics, 7546719).

For CC9C10, part of the growth medium sample was used directly for cell counts. If analysis of the medium was necessary, the remaining fraction was centrifuged at 330 g for 5 minutes to pellet the cells. The supernatant was then removed and stored at -20 ^{o}C for subsequent quantification of medium components.

Anchorage dependent cells required trypsinization before counts were performed. Thus, BHK and Vero cultures grown in T-flasks were trypsinized and resuspended in PBS or DMEM/10 % bovine serum (v/v), comparable to the procedure previously described for subculture. Modifications of the trypsinization protocol were required for cells grown in 24-well plates or microcarrier cultures. For 24-well plates, the medium was first removed and each well was washed with 1 ml PBS followed by the addition of 150 µl of trypsin/EDTA. The plate was placed on a shaker bed at 150 r.p.m. at room temperature for 5-10 minutes. The reaction was stopped and cells resuspended with the addition of 850 µl of PBS/10 % bovine serum (v/v) to each well. All wells were then counted in duplicate with the Coulter counter. The procedure for counting microcarrier cultures began with the removal of 1 ml sample from the culture vessel which was added to a 1.5 ml microfuge tube (Fisher). The beads were allowed to settle for at least 2 minutes and the supernatant was then withdrawn and stored at -20^{o}C or discarded. The beads were washed with 1.2 ml of PBS and after they had settled, the supernatant was removed and discarded. Trypsin/EDTA was added to a total volume of 1 ml and the sample incubated at 37 ^{o}C for 5-10 minutes. The reaction

was stopped by mixing equal volumes of the trypsinized sample and PBS or DMEM/ 20 % bovine serum (v/v). Viable cell concentrations were determined with trypan blue staining and the haemocytometer. Supernatants removed from T-flasks, 24-well plates or microcarrier cultures were stored at -20°C for further analysis if necessary.

2.9 Parameters for evaluation of growth performance

Cultures were routinely compared by specific growth rates (μ) during the exponential phase. This value was determined from a plot of the natural log of the cell densities (lnN) against the time of culture. The slope of the best straight line during the period of exponential growth was the specific growth rate and expressed in units of hr^{-1}.

Doubling times were used in some cases as a measure of the rate of cell growth. This value was determined according to the following equation:

$$D = \frac{(t_f - t_i)\log 2}{\log(N_f / N_i)} \qquad (3)$$

where D is the doubling time (hours), t_f and t_i are the final and initial time points, respectively, and N_f and N_i are the final and initial cell densities, respectively.

2.10 Determination of cellular protein

Protein from cellular extracts was determined with the bicinchoninic acid protein assay kit from Sigma (BCA-1). The method, described by Smith *et al.* (1985), is based on the ability of proteins to reduce Cu^{+2} to Cu^{+1} under alkaline conditions. The resulting cuprous ion reacts with bicinchoninic acid, forming a stable purple colored complex which absorbs maximally at 562 nm. The protein concentration is proportional to the production of the chromogenic product. The reagents and procedure adapted for a 96-well plate assay are provided below.

I Reagents

i) Bicinchoninic Acid Solution (Reagent A): The solution containing bicinchoninic acid, sodium carbonate, sodium bicarbonate and sodium tartrate in 0.1 M NaOH (pH 11.25) was prepared by Sigma (B-9643).

ii) Copper (II) Sulfate Pentahydrate Solution (Reagent B): 4 % cupric sulfate• $5H_2O$ (w/v) solution was provided by Sigma (C-2284).

iii) Protein Stock Solution: 1.0 mg/ml of bovine serum albumin (BSA) prepared in 0.15 M NaCl and preserved with 0.05 % NaN_3 was stored at -20 ^oC until required.

II Assay procedure

i) A series of BSA protein standards ranging in concentration from 0.2 - 1.0 mg/ml were prepared from the Protein Stock solution.

ii) Samples were diluted up to 1/40 to fall within the concentration range of the standards.

iii) 10 µl of standards and samples were added in duplicate to the wells of a 96-well plate (Nunc).

iv) The protein determination reagent was prepared just before use by mixing 1 part of Reagent B with 50 parts of reagent A. The assay was initiated by adding 200 µl of this reagent to the wells of the microtitre plate. The plate was incubated for 30 minutes at 37 ^oC and allowed to cool to room temperature (at least 1 hour).

v) The absorbance of each well was read at 550 nm with a plate reader (SLT-Labinstruments Australia, EAR 400 AT). Protein concentrations in samples were determined from a standard curve produced from a best straight line fit of the protein standards. A sample standard curve is shown in Appendix B.

2.11 Analysis of media components

2.11.1 Glucose and lactate

Glucose and lactate concentrations in culture medium were determined by a YSI Industrial Analyzer or colorimetric assays in a microtitre plate, as specified for a particular experiment. Each of the methods relies on the specific interaction between an enzyme and cognate substrate for accurate quantification.

2.11.1.1 YSI Industrial Analyzer

The YSI Model 27 Industrial Analyzer (Yellow Springs Instrument Company) measures concentrations of different compounds using an enzyme electrode sensor. This consists of a sensor probe covered with a thin, enzyme impregnated membrane mounted in a measurement chamber. The probe is fitted with either a glucose oxidase (YSI, 2365) or lactate oxidase (YSI, 2329) membrane for determination of glucose and lactate, respectively. Oxidation of these compounds at the membrane yields hydrogen peroxide as a by-product. The H_2O_2 is oxidized at the probe's platinum anode according to the following reaction:

$$H_2O_2 \xrightleftharpoons{\text{anode}} 2H^+ + O_2 + 2e^-$$

The resulting current generated between the platinum electrode and a silver/silver chloride reference electrode is proportional to the concentration of glucose or lactate in the sample. The signal current is converted to a voltage, scaled relative to standards containing known concentrations of the substrates and the resulting concentration value displayed on a digital meter of the analyzer.

The instrument was calibrated with 0 and 27.7 mM glucose standards and 0, 5 and 15 mM lactate standards. The volume injected into the measurement chamber was 25 µl for both standards and samples.

2.11.1.2 Glucose oxidase assay for glucose

The glucose oxidase assay was provided as a kit from Sigma (510-DA) for determination of glucose in biological fluids and is based on the procedure of Raabo and Terkildsen (1960). The method combines simultaneous enzyme reactions catalyzed by glucose oxidase and peroxidase with the use of *o*-dianisidine, a chromogenic oxygen acceptor:

$$\text{D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-dianisidine} \xrightarrow{\text{peroxidase}} \text{oxidized o-dianisidine (brown)}$$

The absorbance of the oxidized *o*-dianisidine measured at 425-475 nm is proportional to the glucose present in the sample. The reagents used and the modified Sigma procedure adapted for use in 96-well plates is outline below.

I Reagents

i) PGO Enzyme Solution: A capsule containing 500 units of glucose oxidase, 100 Purpurogalin units of peroxidase and buffer salts (Sigma, 510-6) was dissolved in 100 ml of water. The solution was stored at 4 ^{o}C.

ii) Color Reagent Solution: One vial containing 50 mg of *o*-dianisidine dihydrochloride (Sigma, 510-50) was dissolved in 20 ml of water and stored at 4 ^{o}C.

iii) Combined Enzyme-Color Reagent Solution: 1.6 ml of Color Reagent Solution was mixed with 100 ml of PGO Enzyme Solution and stored at 4 ^{o}C until required.

iv) Stock Glucose Solution: A 100 mg/dL (5.56 mM) glucose solution preserved with 0.1 % benzoic acid (Sigma, 635-100) was provided with the kit.

II Assay procedure

i) A series of 2-fold dilutions of the Stock Glucose Solution was prepared in water, producing a range of glucose standard concentrations from 0.017 - 1.112 mM.

ii) Samples were diluted 1/20 or 1/40 in water.

iii) 20 µl of each standard or sample were added in duplicate and triplicate, respectively, to the wells of a 96-well plate.

iv) 200 µl of Combined Enzyme-Color Reagent Solution was added to each well and the plate incubated at 37 ^{o}C for 30 minutes.

v) The absorbance of each well at 450 nm was measured in a THERMO$_{max}$ plate reader (Molecular Devices). Sample glucose concentrations were determined from a standard curve produced from the best straight line fit of the glucose standards (see Appendix B for a typical standard curve).

2.11.1.3 Lactate dehydrogenase assay for lactate

Lactate dehydrogenase was used to determine lactate concentrations based on the method described by Gutmann and Wahlefeld (1974). The reaction catalyzed by the enzyme is as follows:

$$\text{L-lactate} + NAD^{+} \overset{\text{LDH}}{\rightleftharpoons} \text{pyruvate} + NADH + H^{+}$$

The production of NADH measured spectrophotometrically at 340 nm is proportional to the initial lactate present in the sample. The reagents and procedure required for a 96-well plate assay are provided below.

I Reagents

i) Glycine/Hydrazine Buffer: 0.83 M glycine and 5.1 % hydrazine hydrate (v/v) preserved with 0.05 % NaN_3. The solution was stored at 4 ^{o}C.

ii) NAD Solution: 17.2 mM NAD stored at -20 ^{o}C. Aliquots of the solution were thawed as needed.

iii) LDH Solution: Concentrated lactate dehydrogenase (Sigma, L-2881) was diluted to 119 units/ml in Glycine/Hydrazine Buffer just before use.

iv) Stock Lactate Solution: 100 mM lactate stored at -20 ^{o}C until needed.

II Assay procedure

i) The Stock Lactate Solution was used to prepare standards ranging in concentration from 0.062 - 2 mM in 2-fold dilutions.

ii) Samples were diluted up to 1/160 in water, within the concentration range of the standards.

iii) 40 µl of standards or samples were added in duplicate to the wells of a 96-well plate, followed by 40 µl NAD Solution, 130 µl Glycine/Hydrazine Buffer and 40 µl of LDH Solution. The plate was incubated at 37 ^{o}C for 30 minutes.

iv) The absorbance of each well at 340 nm was read with the THERMO$_{max}$ plate reader. A best straight line fit of the standards produced a standard curve that was used to determine sample lactate concentrations. An example of a standard curve is shown in Appendix B.

2.11.2 Ammonium

The total concentration of ammonium and ammonia in samples was measured with an ammonia electrode (Orion, Model 95-10). Thus, reference to the "ammonium concentration" throughout these studies actually includes the combined concentration of ammonium and ammonia. The electrode is comprised of sensing and reference elements immersed in a filling solution separated from the sample by a hydrophobic membrane. A sample is made basic by addition of a strong alkali, converting ammonium ion to ammonia gas. The ammonia from the sample diffuses through the membrane and into the filling solution until the partial pressure on either side of the membrane is equal. The partial pressure of ammonia is proportional to its concentration in the alkaline sample. A fraction of the ammonia reacts with the water in the filling solution as shown below:

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-$$

The reaction results in a change in potential between the sensing and reference elements that is related to the ammonia concentration in the sample. The potential difference is read by a digital meter (Fisher Scientific, Model 25 pH meter). The sample concentration is determined from a calibration curve of the electrode potentials for a series of standard ammonium solutions.

I Reagents

i) Alkali Solution: 10 M NaOH.

ii) Stock Ammonium Solution: 0.1 M ammonium chloride stored at -20 ^{o}C until required.

II Procedure

i) Calibration standards from 10^{-1} to 10^{-5} were prepared by 10-fold serial dilutions of the Stock Ammonium Solution in water.

ii) Samples were diluted 1/4 in water for a total volume of 1 ml.

iii) 10 µl of Alkali Solution was added to 1 ml of standards or samples just before each measurement. The tip of the electrode was immersed in the stirred solution and the millivolt output allowed to stabilize before recording the final value. The readings for the standard solutions were fit to the best straight line and the sample concentrations determined from the standard curve. A typical standard curve is provided in Appendix B.

2.11.3 Monoclonal antibodies

Monoclonal antibodies in hybridoma supernatants were determined with a ProAnaMabs column (Biolytica) and an HPLC system. The column consists of a silica matrix coupled with the bacterial Fc receptor. A pre-column filter was attached to the inlet to remove particulate material and prevent fouling of the main column. Monoclonal antibodies were selectively bound and eluted from the column and quantified by U.V. absorbance. The HPLC system included an LKB controller (Model 2152), two pumps (Model 2150), high pressure mixing chamber, solvent conditioner (Model 2156),

rheodyne valve, rapid spectral detector (Model 2140) and a computer with EZChrome software (Shimadzu).

I Reagents

i) Binding and Elution Buffers: Buffers were supplied as 20X concentrates (HyClone, JH9-8423-D) and diluted just before use. The pHs of the diluted Binding and Elution Buffers were adjusted to 5.0 and 1.6, respectively, if necessary.

ii) Antibody Standards: The ProAnaMAbs IgG standard was supplied in lyophilized form (HyClone) and dissolved in water to a concentration of 3.0 mg/ml. This was diluted in Binding Buffer to provide IgG standards of 12.5, 25, 50, 100 and 200 μg/ml.

II Procedure

i) A 1 ml volume of samples (diluted 1 in 4 in Binding Buffer) or standards were injected into the column via the rheodyne valve. Buffers were delivered to the high-pressure mixing chamber, then to the rheodyne valve and column by separate pumps and was regulated by the controller. Degassing of the buffers with helium was governed by the solvent conditioner. The analytical cycle consisted of binding, elution and re-equilibration phases as indicated below:

Time (min)	Mobile Phase	Flow Rate (ml/min)
0-2	Binding Buffer	2
2-4	Elution Buffer	3
4-7	Binding Buffer	2

ii) The effluent from the column was routed through the spectral detector and the absorbance at 280 nm monitored. The detector output was recorded with the computer and analyzed with the EZChrome software. Thus, the monoclonal antibody peak produced during the elution phase was identified from the retention time and quantified by the peak area according to the IgG standard curve (see Appendix B).

2.11.4 Amino acids

Glutamine concentrations in a large number of samples were determined rapidly by a colorimetric glutaminase enzyme assay in microtitre plates. Derivatization of samples with OPA and HPLC analysis was required for quantification of other amino acids or confirmation of glutamine concentrations obtained with the plate assay.

2.11.4.1 Glutaminase assay for glutamine

The enzymatic method for determination of glutamine was adapted from the procedure described by Lund (1985). The method couples the glutaminase reaction with glutamate dehydrogenase:

$$\text{L-glutamine} + H_2O \xrightarrow[H^+]{\text{glutaminase}} \text{L-glutamate} + NH_4^+$$

$$\text{L-glutamate} + NAD^+ + H_2O \underset{\text{ADP}}{\overset{\text{GDH}}{\rightleftharpoons}} \alpha\text{-ketoglutarate} + NH_4^+ + NADH$$

The glutamine present in the sample is proportional to the production of NADH and thus the increase in absorbance at 340 nm. The glutaminase reaction has a pH optimum of 5.0 and is carried out under acidic conditions. The equilibrium of the glutamate dehydrogenase reaction favors the production of glutamate and NAD^+. However, the forward reaction may be forced in a basic buffer with a high NAD concentration and a trapping agent for α-ketoglutarate. ADP is also included to activate the enzyme. The reagents and protocol for a 96-well microtitre plate assay are outlined below.

I Reagents

i) Acetate Buffer: A 0.5 M acetate buffer, pH 5.0, was prepared by mixing 0.5 M sodium acetate and 0.5 M glacial acetic acid in appropriate proportions to obtain the desired pH. The solution was stored at 4 °C.

ii) Glutaminase Solution: Lyophilized glutaminase (Sigma, G 5382) was dissolved in 10-fold diluted Acetate Buffer to a concentration of 10 units/ml. Aliquots were stored at -70 ^{o}C until required.

iii) Hydroxylamine Solution: 20 mM hydroxylamine, stored at 4 ^{o}C.

iv) Tris/Hydrazine Buffer: 0.15 M Trizma base, 3 mM EDTA and 7.5 % hydrazine hydrate (v/v) was prepared and the pH adjusted to 9.0. The solution was stable at 4 ^{o}C for up to 1 week.

v) NAD Solution: 30 mM NAD, stored at -20 ^{o}C. Aliquots were thawed as required.

vi) ADP Solution: 100 mM ADP (sodium salt) prepared in water and neutralized with 2 M NaOH. Aliquots were stored at -20 ^{o}C until needed.

vii) GDH Solution: A 2500 unit/ml glutamate dehydrogenase preparation was obtained from Sigma (G 2626) and stored at 4 ^{o}C until required.

viii) Glutamine Stock Solution: 100 mM glutamine, stored at -20 ^{o}C until required.

ix) Reagent A: 2160 µl Acetate Buffer, 1080 µl Hydroxylamine Solution and 1155 µl of water were mixed and warmed in a water bath at 37 ^{o}C. Immediately before use, 108 µl of Glutaminase Solution was added.

x) Reagent B: 2160 µl Acetate Buffer, 1080 µl Hydroxylamine Solution and 1265 µl water were mixed and warmed in a 37 ^{o}C water bath.

xi) Reagent C: 9.6 ml of Tris/Hydrazine Buffer and 3.1 ml of water were mixed and warmed in a 37 ^{o}C water bath. Immediately before use, 1440 µl NAD, 144 µl ADP and 90 µl GDH Solutions were added.

<u>II Assay procedure</u>

i) Glutamine standards with concentrations of 0.031 - 1 mM in 2-fold dilutions were prepared from the Glutamine Stock Solution.

ii) Samples were diluted up to 1/10 in water to fall within the concentration range of the standards.

iii) Two separate sections were demarcated on the microtitre plate. Standards and samples were added in duplicate at a volume of 70 µl/well to each of the two halves of the plate in an identical pattern.

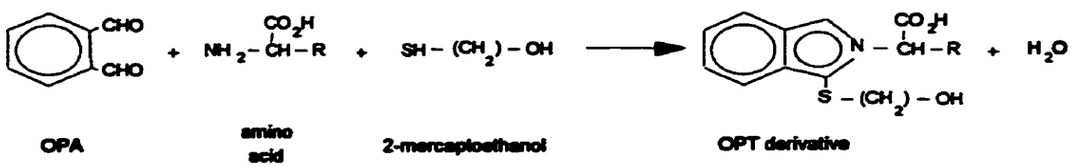
iv) The glutaminase reaction was initiated with the addition of 50 µl Reagent A to each well on the first half of the plate. In the second half of the plate, 50 µl of Reagent B was added to each well. The plate was incubated at 37 ^{o}C for 75 minutes.

v) To each of the wells, 120 µl Reagent C was added and the plate was incubated at 37 ^{o}C for 30 minutes.

vi) The absorbance of each well at 340 nm was measured with the THERMO$_{max}$ plate reader. The increase in absorbance in the first half of the plate was due to the presence of both glutamine and glutamate in the sample. The second half of the plate did not contain glutaminase and the absorbance increase was due to the presence of glutamate. Thus, to obtain the increase in absorbance due to glutamine alone, the absorbance for each well in the second half of the plate was subtracted from the corresponding well in the first half. A calibration curve was constructed from the standards and the glutamine concentrations in the samples were determined. An example of a calibration curve is available in Appendix B.

2.11.4.2 OPA derivatization and HPLC analysis

Amino acid concentrations in culture medium were determined by *o*-phthaldialdehyde (OPA) derivatization followed by separation on an HPLC reverse phase column. OPA reacts with primary amines including amino acids and a thiol such as 2-mercaptoethanol yielding an *o*-phthaldialdehyde-thiol (OPT) derivative at alkaline pH. The chemical reaction is as follows:



The heterocyclic isoindole moiety of the derivative is highly fluorescent. This permits very sensitive quantification of nanomole levels of amino acids with a fluorescence detector. The hydrophobic ring structure of the derivative also results in retention by the reverse phase column. The net hydrophobicity, however, is a function of the R group of the amino acid. This, of course, determines the retention time of each amino acid derivative on the column and is the key determinant in their separation. Separation is facilitated by applying a gradient of increasing organic content or hydrophobicity to the column during elution of the derivitized sample.

The HPLC system consisted of the same basic hardware used for monoclonal antibody analysis except a Shimadzu SIL-9A auto-injector replaced the rheodyne valve and an LKB fluorescence detector (Model 2144) was used to monitor the effluent. An Alltech ODS C-18 0.24 X 20 cm reverse phase column with Waters guard column was used for separation of the amino acid derivatives. The method of amino acid derivatization of medium samples and separation is based on a method described by Jones and Gilligan (1983). The procedure is described below.

I Reagents

i) OPA Reagent: 54 mg of *o*-phthaldialdehyde was dissolved in 0.5 ml HPLC grade methanol. Next, 10 ml of 0.1 M borate, 100 µl 2-mercaptoethanol and 200 µl Brij were added and the solution filtered through a 0.2 µm syringe filter (Nalgene). The reagent was prepared fresh, only as needed.

ii) Elution Buffers: Mobile Phase A was 50 mM sodium acetate/0.5 % tetrahydrofuran, pH 5.7, prepared only as needed. Millipore water and HPLC grade tetrahydrofuran (Fisher) were used and the solution passed through a 0.2 µm filter to minimize

contaminants and particulate matter that could degrade separation. Mobile Phase B was 100 % HPLC grade methanol (Fisher).

II Procedure

i) An aqueous amino acid standard containing 20 amino acids and the internal standard L-α-amino-n-butyric acid (aba) was prepared, each at a concentration of 500 μM. Alanyl-glutamine (ala-gln) and glycyl-glutamine (gly-gln) were included in some cases but at a concentration of 1.0 mM. A concentrate of 17 amino acids, each at 2.5 mM, was purchased (Sigma, AA-S-18) and simply required dilution in water. Asparagine, glutamine, tryptophan, the internal standard and dipeptides were added from separate 25 mM stock solutions.

ii) The internal standard, amino butyric acid, was added to each sample at a final concentration of 500 μM.

iii) Standards and samples were treated with an equal volume of ice-cold 10 % trichloroacetic acid (TCA) (w/v) followed by centrifugation at 14 000 r.p.m. for 6 minutes at 4 ^{o}C. This process precipitated the protein from the samples. The supernatant was removed and neutralized with an equal volume of 0.2 M borate buffer (pH 10.4). The neutralized sample was again microfuged at 14 000 r.p.m. for 6 minutes at 4 ^{o}C.

iv) 250 μl of neutralized standards and samples were dispensed into 500 μl microfuge tubes (Ependorf) and placed in HPLC sample vials.

v) An equal volume (250 μl) of OPA Reagent was mixed with the prepared standard or sample. Following mixing and an incubation of 1.7 minutes at room temperature, 50 μl of derivitized sample was injected into the column. This process was automated with the auto-injector.

vi) Derivatized amino acids were eluted from the column using a gradient of increasing hydrophobicity formed by mixing the aqueous acetate buffer (Mobile Phase A) and the methanol (Mobile Phase B). Degassing of the mobile phases with helium was regulated by the solvent conditioner. A separate pump was used to deliver each of the mobile

phases to the high-pressure mixing chamber and subsequently to the column at a flow rate of 1 ml/min. The gradient was determined by the relative proportions of the two mobile phases delivered to the mixing chamber which was regulated by the HPLC controller. The elution gradient is shown below:

Time (minutes)	Mobile Phase B (%)
0-5	0-20
5-35	20-65
35-40	65-100
40-45	100
45-50	100-0
50-55	0

vii) The effluent from the column was monitored by the fluorescence detector with excitation and emission filters of 340 nm and 455 nm, respectively. The output from the detector was recorded and analyzed by the computer. The EZChrome software identified peaks via retention times, determined their areas and calculated response factors from the amino acid standards. The amino acid concentrations were then determined in samples based on the appropriate response factor and peak area relative to the internal standard. The method outlined above permits detection and quantification of all amino acids in culture medium with the exception of proline and cysteine. The former is a secondary amine which does not react with the OPA reagent while the latter yields a poor fluorescent response. A chromatogram of a standard amino acid run and the equations used to calculate amino acid concentrations are available in Appendix C.

2.11.5 Specific rates of consumption or production

Cell specific consumption and production rates (q) of media components were calculated using the equation:

$$q = [\Delta C/(t_f - t_i)]/[(\ln N_f - \ln N_i)/(N_f - N_i)] \qquad (4)$$

where ΔC is the change in component concentration, t_f and t_i are the final and initial time points, respectively, and N_f and N_i are the final and initial cell densities, respectively.

An integral method was used as an alternative for the determination of specific consumption or production rates. At each time point of the culture, the integral of the cell number was evaluated using the trapezoidal rule with SigmaPlot (Jandel Scientific). This is referred to as the cell index and is represented by the following equation:

$$\text{cell index} = \int_0^t x\,dt \qquad (5)$$

where x is the cell number and t is the time. The change from the initial quantity of substrate (ΔS) or product (ΔP) up to each time point was then plotted against the corresponding value for the cell index. The points from a linear portion of the exponential phase of the culture were fit to the best straight line. The slope of the line was equivalent to the specific consumption or production rate during this period of growth. The error associated with the resulting value was represented by the standard error of the line (determined by SigmaPlot). An example of this method is provided in Appendix D. The specific rate values using both the equation and integration approach were expressed as nmoles of substrate utilized or product produced per 10^6 cells per hour (nmol 10^{-6} cells hr^{-1}).

2.12 Nucleotide analysis

The analysis of nucleotides was based on the method described by Ryll and Wagner (1991). The HPLC procedure involves separation of nucleotides on a C-18 reverse phase column and detection and quantification by absorbance at 254 nm. The HPLC system was as described for amino acid analysis except that the spectral detector was used to monitor the column effluent.

I Reagents

i) Elution Buffers: Mobile Phase A consisted of 96.5 mM potassium phosphate buffer/8.0 mM tetrabutylammonium hydrogen sulfate, pH 5.3. The solution was prepared with Millipore water and particulates removed with a 0.2 μm filter. Mobile Phase B was comprised of 70 % Mobile Phase A and 30 % HPLC grade methanol.

ii) Nucleotide Standard Solution: The nucleotide standard included NAD, UDP-Glc, UDP-GalNAc, UDP-GlcNAc, AMP, GDP, CTP, UTP, ADP, GTP and ATP, each at a concentration of 2×10^{-5} M. The solution was prepared by adding 40 μl of a 1×10^{-3} M stock of each nucleotide to 1170 μl water and 390 μl Mobile phase A for a total volume of 2 ml.

II Procedure

i) The Nucleotide Standard Solution required no further pretreatment prior to analysis. Samples were prepared by diluting 1:1 with Mobile Phase A and particulate material was removed by centrifugation in a microfuge (14 000 r.p.m. for 5 minutes).

ii) Standards and samples (100 μl) were dispensed into 500 μl microfuge tubes (Ependorf) and placed in HPLC sample vials. A 50 μl volume of each was delivered to the column by the auto-injector.

iii) An elution gradient was formed by mixing the two mobile phase solutions, gradually increasing the proportion of Mobile Phase B. The flow rate was 1.5 ml/min and the gradient is provided below:

Time (minutes)	Mobile phase B (%)
0-5	0-20
5-15	20-24
15-20	24-40
20-28.5	40-100
28.5-40.5	100
40.5-44.5	100-0
44.5-52.5	0

iv) The column effluent was routed through a spectral detector and the absorbance measured at 254 nm. Detector output was recorded and analyzed by the computer.

EZChrome was used to identify peaks by retention times and determine areas. The software also determined response factors from the standard run which were used to evaluate nucleotide concentrations in the samples. A sample chromatogram and the calculations required to determine nucleotide concentrations are provided in Appendix E.

2.13 Assays for intracellular enzyme activities

2.13.1 Glutamine synthetase/glutamine transferase (GS)

In addition to the synthesis of glutamine from glutamate and ammonium, glutamine synthetase catalyzes a non-physiological γ-glutamyl transfer reaction (Rowe *et al.*, 1970). Glutamine and hydroxylamine form γ-glutamylhydroxymate and ammonia in the presence of manganese ions and arsenate in the following reaction:

$$\text{L-glutamine} + NH_2OH \xrightarrow[Mn^{+2},\ As_i]{GS} \text{L-}\gamma\text{-glutamylhydroxymate} + NH_3$$

The product of the reaction, γ-glutamylhydroxymate, forms a colored complex with ferric ions which can be detected colorimetrically (535 nm). The assay outlined below is based on a procedure described by Meister (1985).

I Reagents

i) Reaction Buffer: 0.123 M imidazole-HCl, 62 mM L-glutamine, 77 mM hydroxylamine, 0.25 mM $MnCl_2$, 12 mM sodium arsenate and 0.49 mM ADP. The pH of the final solution was adjusted to pH 7.2.

ii) Ferric Chloride Solution: 0.37 M $FeCl_3$, 0.67 M HCl and 0.20 M trichloroacetic acid.

iii) γ-Glutamylhydroxymate Solution: 10 mM γ-glutamylhydroxymate in 50 mM ascorbic acid.

II Assay procedure

i) The γ-Glutamylhydroxymate Solution was used to prepare a series of standards from 0 - 1.0 μmoles in two-fold dilutions in a volume of 100 μl.

ii) The assay was initiated with the addition of 400 μl Reaction Buffer to 100 μl of standards and samples followed by incubation at 37 °C for 30 minutes.

iii) The reaction was terminated with 0.75 ml Ferric Chloride Solution. After centrifugation in a microfuge (14 000 r.p.m. for 5 minutes), the absorbance was measured at 535 nm using water as a blank. Product formation in samples was determined from a linear standard curve (see Appendix B) constructed from the absorbance of known quantities of γ-glutamylhydroxymate. Activity was expressed as μmoles γ-glutamylhydroxymate formed per minute per mg of cell protein.

2.13.2 Phosphate-activated glutaminase (PAG)

The phosphate-activated glutaminase assay was based on a procedure described by Kvamme *et al.* (1985) and adapted for use in 96-well plates. The method couples the glutaminase and glutamate dehydrogenase reactions:

$$\text{glutamine} + H_2O \xrightarrow[Pi]{PAG} \text{glutamate} + NH_4^+$$

$$\alpha\text{-ketoglutarate} + NH_4^+ + \text{NADH} \xrightleftharpoons{GDH} \text{glutamate} + NAD^+ + H_2O$$

The glutaminase activity can thus be monitored colorimetrically (340 nm) by the rate of NADH oxidation. The reagents and assay procedure are provided below.

I Reagents

i) Reaction Buffer: 0.125 M potassium phosphate buffer (pH 8.0), 12.5 mM glutamine, 6.25 mM α-ketoglutarate, 0.25 mM EDTA, 0.39 mM NADH and 1.25 units/ml glutamate dehydrogenase. The GDH was diluted from a concentrated stock (2451 units/ml) (Sigma, G 2626). The pH of the final solution was adjusted to 8.0.

ii) 0.10 M Ammonium Chloride Solution

II Assay Procedure

i) Standards were prepared from the Ammonium Chloride Solution in a series of 2-fold dilutions from 0 - 0.25 μmoles in a volume of 50 μl. Samples (50 μl) were also added to the wells of the microtitre plate.

ii) The reaction was initiated with the addition of 200 μl of Reaction Buffer to the standards and samples. The plate was incubated at 25 ^{o}C in the THERMO$_{max}$ plate reader for 15 minutes. The absorbance at 340 nm was measured automatically every 30 seconds with the instrument in the kinetic mode.

iii) The maximum change in absorbance (V_{max}) during the incubation was determined by the plate reader SOFTmax software. A linear standard curve (see Appendix B) of the maximum absorbance change versus μmoles of ammonium was plotted. The standard curve was used to determine the ammonium produced in the samples. The reaction was also independently monitored by glutamate production quantified by HPLC analysis. Activity was expressed as μmoles of glutamine deaminated to glutamate and ammonium per minute per mg of cell protein.

2.13.3 Alanine aminotransferase/glutamic pyruvic transaminase (ALT/GPT)

Alanine aminotransferase (ALT) was determined in cell extracts using a kit obtained from Sigma (505-P). The method was introduced by Tonhazy *et al.* (1950) and simplified by Reitman and Frankel (1957). Aminotransferase activity is detected colorimetrically (505 nm) from the reaction of pyruvate, a product of the enzyme reaction, with 2,4-dinitrophenylhydrazine:

$$\text{alanine} + \alpha\text{-ketoglutarate} \xrightarrow{\text{ALT}} \text{pyruvate} + \text{glutamate}$$

$$\text{pyruvate} + \text{2,4-dinitrophenylhyrazine} \longrightarrow \text{pyruvic-phenylhyrazone derivative}$$

The absorbance of the phenylhydrazone product at 505 nm is related to the ALT activity in the sample. Although α-ketoglutarate also forms a colored hydrazone, the absorption maximum is considerably different from that of pyruvate (Kachmar, 1970) and does not interfere with the assay. The reagents and procedure are essentially as described by Sigma but the reaction volume was reduced by 4-fold.

I Reagents

i) Alanine-α-KG Substrate: 0.2 M DL-alanine and 1.8 mM α-ketoglutarate in phosphate buffer, pH 7.5 (Sigma, 505-51).

ii) Color Reagent: ~20 mg/dl 2,4-dinitrophenylhydrazine in acid solution (Sigma, 505-2).

iii) Calibration Solution: 1.5 mM sodium pyruvate in phosphate buffer, pH 7.5 (Sigma, 505-10).

II Assay procedure

i) Standards were prepared by combining the solutions indicated in Table 2-1.

Table 2-1. Preparation of standards for the ALT/GPT assay.

Test tube number	Calibration Solution (μl)	Ala-α-KG Substrate (μl)	Water (μl)	ALT Activity (SF Units/ml)
1	0	250	50	0
2	25	225	50	23
3	50	200	50	50
4	75	175	50	83
5	100	150	50	125

ii) A 250 μl volume of Color Reagent was added to each tube, shaken gently and incubated at room temperature for 20 minutes.

iii) 2.5 ml of 0.4 M NaOH was added to all tubes and inverted to mix.

iv) After 5 minutes, the absorbance of each tube at 505 nm was determined in a cuvet (1 cm light path) using water as a blank.

v) The absorbance of Tube 1 was subtracted from each of the tubes and the net absorbance in each tube was plotted against the ALT activity specified in Table 2-1.

The standard curve (see Appendix B) was produced by fitting the points to the best hyperbolic equation using a curve fitting algorithm in SigmaPlot.

vi) Aminotransferase activity was next determined in the samples. A 50 µl volume of each sample was added to a test tube followed by 250 µl Ala-α-KG Substrate pre-warmed at 37 ^{o}C. Tubes were shaken gently and incubated for 30 minutes at 37 ^{o}C.

vii) 250 µl of Color Reagent was added to each of the samples followed by an inversion to mix and incubation at room temperature for 20 minutes.

viii) The reaction was stopped with the addition of 2.5 ml 0.4 M NaOH and tubes were inverted to mix.

ix) After 5 minutes, the absorbance at 505 nm was determined using water as the blank. The absorbance of Tube 1 was subtracted from each of the samples and the standard curve used to determine the ALT activity. Activities in the samples were converted from Sigma-Frankel (SF) Units/ml to µmoles glutamate formed/ml. One SF Unit produces $4.82X10^{-4}$ µmoles glutamate/minute at pH 7.5 and 25 ^{o}C (Sigma Procedure No. 505). Thus, activity was expressed as µmoles of glutamate formed per minute per mg of cell protein.

2.13.4 Glutamate dehydrogenase (GDH)

The assay for glutamate dehydrogenase was adapted for a 96-well plate from the procedure described by Schmidt (1974). The reaction catalyzed by the enzyme is shown below and activity measured by the oxidation of NADH to NAD^{+}.

$$\alpha\text{-ketoglutarate} + NH_4^{+} + NADH + H^{+} \xrightleftharpoons{GDH} \text{glutamate} + NAD^{+} + H_2O$$

<u>I Reagents</u>

i) Reaction Buffer: 58.3 mM triethanolamine buffer (pH 8.0), 3.00 mM EDTA, 0.136 M ammonium acetate, 1.37 mM ADP, 0.28 mM NADH and 2.75 units/ml lactate

dehydrogenase. The LDH was diluted from a concentrated stock (4848 units/ml) (Sigma, L 2881). The pH of the final solution was adjusted to 8.0.

ii) 43.7 mM α-KG Solution

iii) GDH Solution (2451 units/ml) (Sigma, G 2626)

II Assay procedure

i) A series of standards were prepared from the GDH Solution in 2-fold dilutions from 0 - 0.25 units/ml. One unit reduces 1 μmole of α-KG to glutamate per minute at pH 7.3 and 25 ^{o}C. A 50 μl volume of the standards and samples were added to the wells of the microtitre plate.

ii) Next, 160 μl of Reaction Buffer was added to each well and the plate incubated at 25 ^{o}C for 10 minutes to remove any pyruvate in the sample.

iii) The reaction was initiated with the addition of 40 μl α-KG Solution. During a 15 minute incubation at 25 ^{o}C in the THERMO$_{max}$ plate reader, the absorbance at 340 nm was determined every 30 seconds with the instrument in kinetic mode.

iv) The maximum absorbance change (V_{max}) during the incubation was determined by the SOFTmax software. A standard curve (Appendix B) was derived from a plot of the maximum change in absorbance against the known activity of the GDH standards. The points were fit to a quadratic equation by SOFTmax. Activity of the samples were determined from the standard curve and expressed as μmoles α-ketoglutarate reduced to glutamate per minute per mg of cell protein.

2.14 Statistical analysis

Unless otherwise indicated, cultures and sample analyses were performed at least in duplicate. Values were expressed as the average of the replicates plus or minus the standard error (SE) of the mean. The standard error was calculated as the standard deviation divided by the square root of the number of samples. Statistically significant differences were indicated by standard errors which exceeded the difference

between the means of compared samples. Student's *t*-tests ($P<0.05$) were also performed where indicated using SigmaPlot. Equations for standard deviation, standard error and *t*-tests are available in Appendix F.

Chapter 3

Ammonium and Lactate Inhibition of BHK, Vero and CC9C10 Cells

3.1 Introduction

The purpose of the following studies was to explore the use of less ammoniagenic substrates than glutamine in animal cell cultures. The selection of appropriate cell lines to illustrate the effects and any possible benefits of reduced ammonium production, however, required preliminary experiments to establish their sensitivity to ammonium.

Cell lines have been characterized as very sensitive, moderately sensitive and tolerant of ammonium. The particular degree of sensitivity depends on both the cell line and culture conditions. Thus, it was necessary to establish the response of cells to ammonium under the culture conditions employed throughout the present investigations. Commercially valuable cell lines exhibiting some degree of ammonium inhibition would clearly be ideal for studies of low-ammoniagenic media. The three lines initially chosen were BHK, Vero and CC9C10 cells. BHK cells are routinely used for production of recombinant proteins and veterinary vaccines. Vero cells are widely used for preparation of human vaccines. The CC9C10 line is a typical murine hybridoma and potentially useful for elucidating the effects of reduced ammonium on monoclonal antibody production.

The procedure commonly used to study the effects of ammonium inhibition on cells is to include varying initial concentrations of an ammonium salt, such as ammonium chloride, in the culture medium. For reasons discussed later, this strategy may not provide a representative measure of the inhibition due to ammonium produced by cellular metabolism. Nevertheless, it yields an indication of relative sensitivities of different cell lines under equivalent culture conditions. A comparable method was used to examine lactate inhibition although other workers have demonstrated a limited effect

on cell growth compared to ammonium. Lactate appears to elicit its inhibitory effects by reducing the pH or substantially increasing the osmolarity of the growth medium. The chemical effects of lactate inhibition were distinguished from those induced by pH or osmolarity changes by neutralizing the medium after addition of lactate and comparing growth to cultures with equivalent concentrations of NaCl.

3.2 Materials and methods

3.2.1 Ammonium inhibition

BHK and Vero cells were grown in DMEM/10 % calf serum with 3 mM glutamine, 1.2 mM serine and a range of ammonium chloride concentrations. CC9C10 was grown in DMEM/10 % FetalClone with 4 mM glutamine and increasing levels of NH_4Cl. The desired series of NH_4Cl concentrations for each cell line were prepared from two stock solutions of growth media, one with no added ammonium salt and the other with the maximum concentration tested. These stocks were mixed together in appropriate proportions to produce medium with a specific NH_4Cl concentration. The highest concentration of NH_4Cl examined was 13.5 mM, 40 mM and 20 mM for BHK, Vero and CC9C10 cells, respectively. Ammonium chloride of the highest purity available was used in these studies to minimize inhibitory effects from impurities. BHK and Vero cells were cultured in triplicate for each concentration of NH_4Cl in 24-well plates at a volume of 1 ml per well. The initial seed density was 0.64 X 10^5 total cells/ml for BHK and 0.80 X 10^5 total cells/ml for Vero cells. The hybridoma was seeded at an initial density of 2.75 X 10^5 total cells/ml in duplicate 25 cm^2 T-flasks with a medium volume of 10 ml for each NH_4Cl concentration. All cultures were incubated under the standard conditions defined in section 2.4.

3.2.2 Lactate inhibition

The effects of lactate on the growth of BHK cells were determined with a method analogous to that used to examine ammonium inhibition. The growth medium was DMEM/10 % donor calf serum with 1.2 mM serine, 3 mM asparagine, 4 mM glutamine and various additions of sodium lactate. Two stock solutions of growth media with 0 and 125 mM sodium lactate were mixed to provide the array of lactate concentrations. The pH of the stock medium containing 125 mM lactate was adjusted to pH 7.4 to eliminate the effects of growth inhibition due to reduced pH caused by the lactate. An identical set of growth media was prepared with additions of NaCl in place of lactate. The cells were seeded at an initial concentration of 1.0×10^5 total cells/ml in a 1 ml volume in 24-well plates with duplicate samples for each lactate and NaCl concentration. Incubation conditions were described previously (section 2.4).

3.3 Results

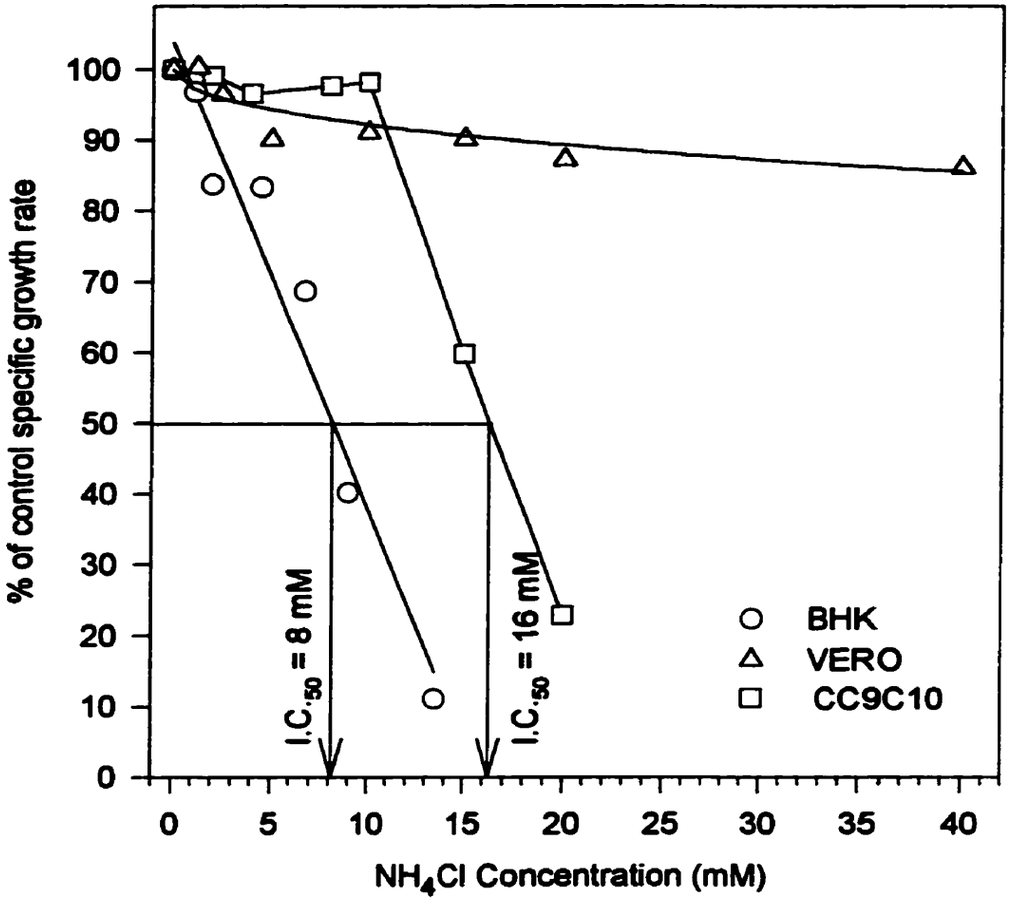
3.3.1 Ammonium inhibition

The effects of ammonium chloride on growth rates and cell densities were examined for each of the three cell lines. In Figure 3-1a, the exponential phase specific growth rate at each NH_4Cl concentration is expressed as a percentage of the control which contained no initial addition of ammonium salt. Each of the cell lines exhibits a different response to the added ammonium. The growth rate of BHK cells decreased linearly with increasing NH_4Cl concentrations. The Vero growth rate declined marginally over the wide range of NH_4Cl concentrations examined. The hybridoma was tolerant to ammonium up to 10 mM but the growth rate rapidly decreased beyond this concentration. The sensitivity of each of the cell lines can be defined more quantitatively with the assignment of an $I.C._{50}$ value. The $I.C._{50}$ designates the inhibitory concentration of NH_4Cl which reduces the specific growth rate by 50 %. The $I.C._{50}$ values were 8 mM and 16 mM for BHK and CC9C10 cells, respectively. A value

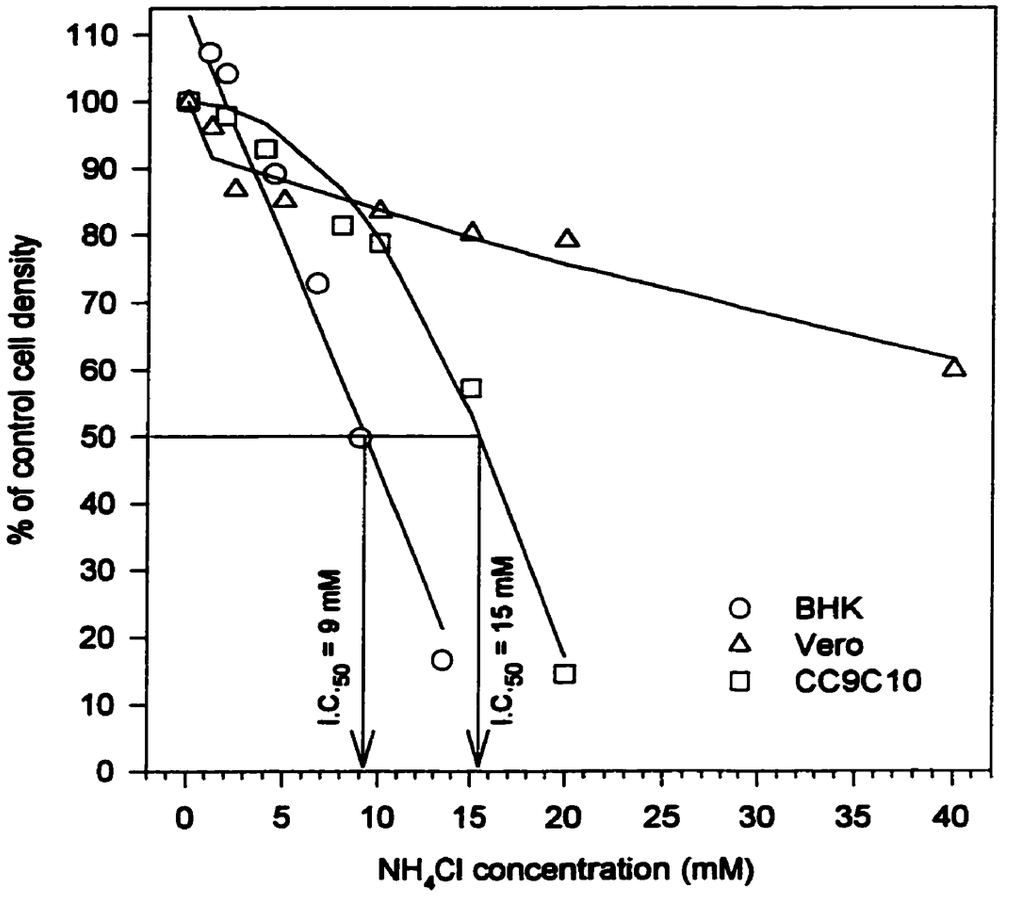
could not be determined for Vero cells because ammonium chloride as high as 40 mM decreased the specific growth rate by less than 15 %. Figure 3-1b illustrates the cell densities after ~3 days for BHK cells and ~4.5 days for Vero and CC9C10 cells at each NH_4Cl concentration as a percentage of the control with no added ammonium. The effect of ammonium on cell density was similar to that observed for the growth rate. An $I.C._{50}$ of 9 mM for BHK and 15 mM for CC9C10 cells was comparable to that obtained from the specific growth rate. Once again, an $I.C._{50}$ could not be derived for Vero cells as densities were reduced by only 40 % in the presence of 40 mM NH_4Cl. Vero cell concentrations, however, were affected to a greater extent by ammonium than was noted for the specific growth rate as were CC9C10 densities below 10 mM NH_4Cl.

Figure 3-1a-b. The effect of NH_4Cl on (a) specific growth rates and (b) cell densities of BHK, Vero and CC9C10 cells. BHK and Vero cells were cultured in triplicate (n=3) in 24-well plates. Total cell concentrations were evaluated by counting each well twice by Coulter counter. CC9C10 cells were grown in duplicate T-flasks (n=2) for each NH_4Cl concentration. Total cell concentrations were determined by counting samples from each flask twice by haemocytometer. The specific growth rate (μ) was calculated from exponential growth corresponding to 20-44 hours for BHK, 22-93 hours for Vero and 23-45 hours for CC9C10 cells. The effect on cell densities were evaluated after 68 hours for BHK, 117 hours for Vero and 112 hours for CC9C10 cells. The percent of control growth rate and cell densities were calculated relative to the cultures with no added NH_4Cl. In all cases, points representing an average of replicate counts were plotted and the standard error of each was <10 % with respect to the mean.

(a)


% of control specific growth rate
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
NH4Cl Concentration (mM)
I.C.50 = 8 mM
I.C.50 = 16 mM
BHK
VERO
CC9C10

(b)

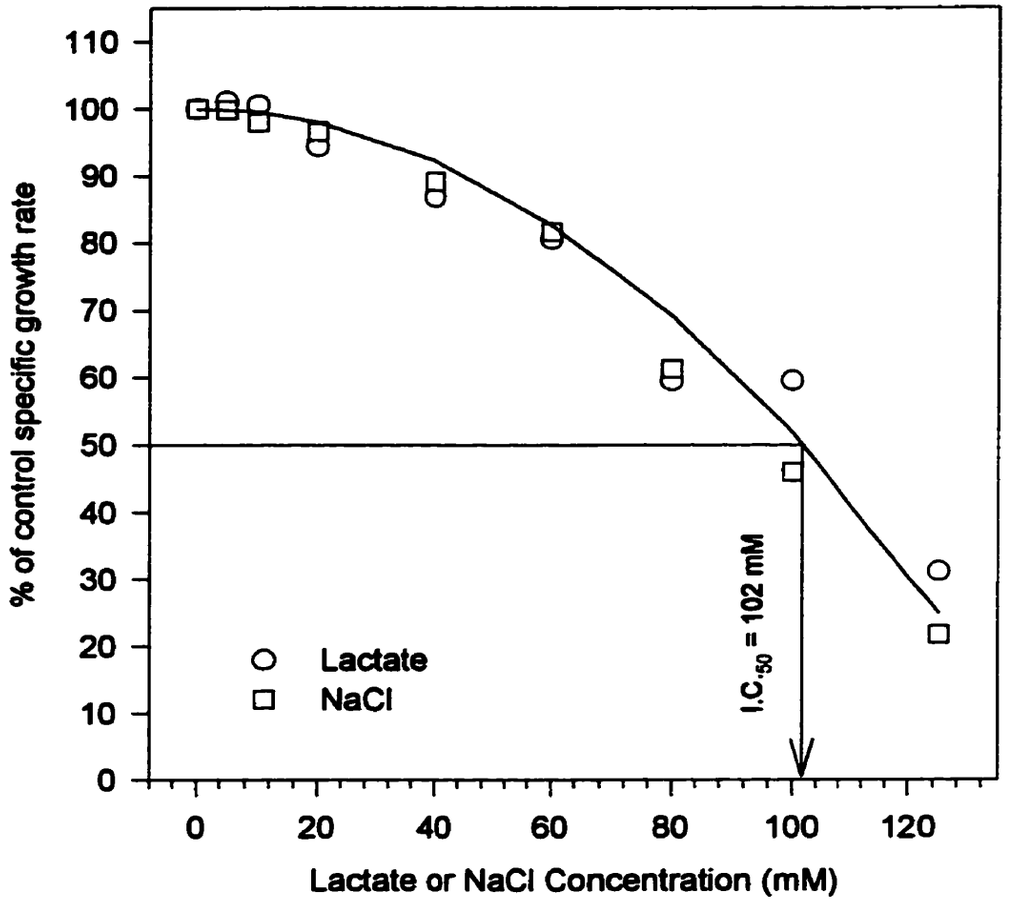

% of control cell density
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
NH4Cl concentration (mM)
I.C.50 = 9 mM
I.C.50 = 15 mM
BHK
Vero
CC9C10

3.3.2 Lactate inhibition

The effects of sodium lactate and NaCl on the exponential phase specific growth rate of BHK cells were very similar as depicted in Figure 3-2a. The $I.C._{50}$ was 102 mM for both lactate and NaCl which is an order of magnitude higher than observed for NH_4Cl. Inhibition of cell densities measured after ~3 days of growth is shown in Figure 3-2b. The impact on cell densities was more marked than observed for the specific growth rate. In addition, lactate had a more significant effect on cell densities than NaCl. Lactate yielded an $I.C._{50}$ of 54 mM while the value for NaCl was slightly higher at 70 mM. The $I.C._{50}$ values for NH_4Cl, lactate and NaCl are summarized in Table 3-1.

Figure 3-2a-b. Effect of lactate and NaCl on (a) specific growth rate and (b) cell density of BHK cells. Duplicate cultures (n=2) for each lactate and NaCl concentration were grown in 24-well plates. Total cell concentrations were determined by counting each well twice with a Coulter counter. The specific growth rate was calculated from exponential growth corresponding to 23-46 hours for lactate and 22-45 hours for NaCl. The effect on cell density was determined from the cell concentration after 71 hours for lactate and 69 hours for cells grown with NaCl. The percent of control growth rate and cell density were expressed as a percentage relative to the medium with no additions of inhibitor. Points are an average of replicate counts and standard error for each was <10 % relative to the mean.

(a)


% of control specific growth rate
110
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Lactate or NaCl Concentration (mM)
Lactate
NaCl
I.C.50 = 102 mM

(b)

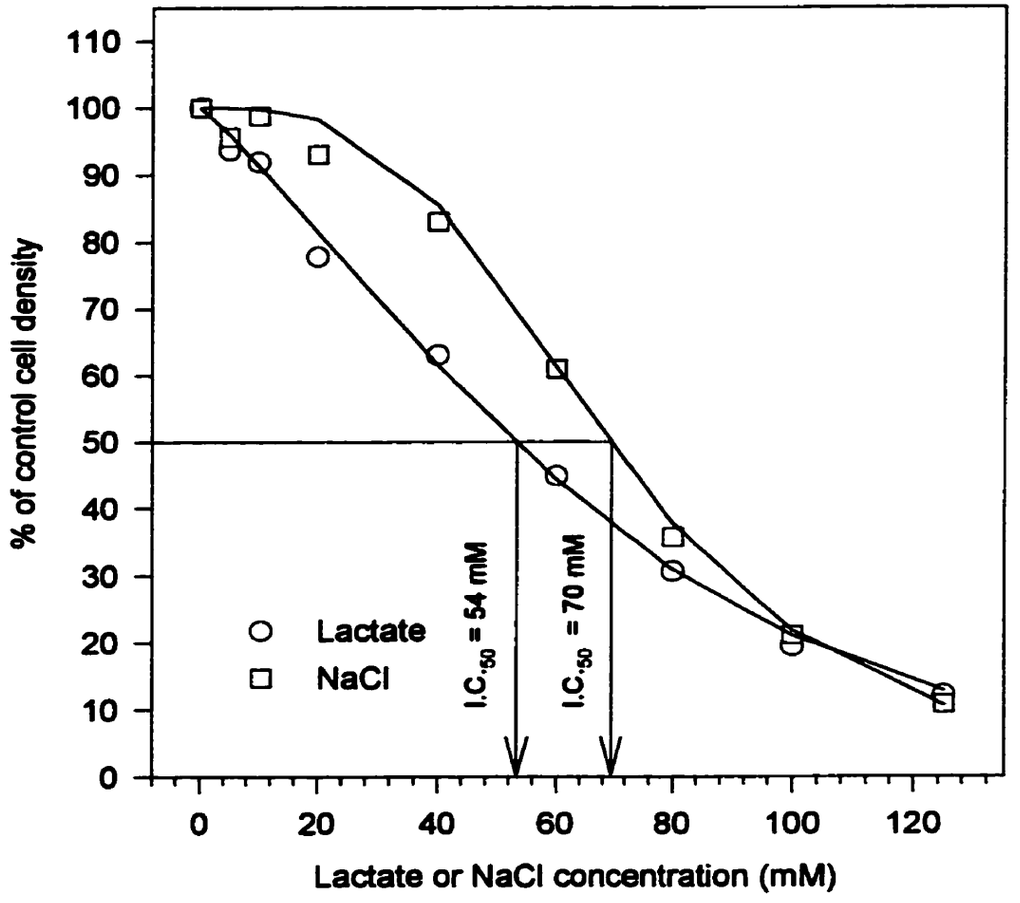

% of control cell density
110
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Lactate or NaCl concentration (mM)
Lactate
NaCl
I.C.50 = 54 mM
I.C.50 = 70 mM

Table 3-1. Summary of $I.C._{50}$ values for inhibition of BHK, Vero and CC9C10 cells by NH_4Cl, lactate and NaCl. The $I.C._{50}$ determined from the effect on specific growth rate (μ) and the total cell concentrations (N) are listed for each cell line.

	Growth parameter used to determine $I.C._{50}$					
	μ	N	μ	N	μ	N
Inhibitor	BHK		Vero		CC9C10	
NH_4Cl	8 mM	9 mM	>40 mM	>40 mM	16 mM	15 mM
lactate	102 mM	54 mM	N.D.	N.D.	N.D.	N.D.
NaCl	102 mM	70 mM	N.D.	N.D.	N.D.	N.D.

N.D. is not determined

3.4 Discussion

Ammonium has been considered for some time as a primary inhibitor of animal cell cultures (Ryan and Cardin, 1966, Butler *et al.*, 1983, Glacken *et al.*, 1983 and Butler and Spier, 1984). The effects on cell growth and cell densities were assessed by the addition of ammonium chloride directly to the culture medium as shown in Figure 3-1. The different sensitivities of BHK, Vero and CC9C10 cells to ammonium (Table 3-1) were expected as other investigators have noted varying degrees of inhibition among cell lines. Hassell *et al.* (1991) studied ammonium inhibition in 8 cell lines and categorized each according to the effect of a 2 mM NH_4Cl addition to the culture medium on cell yields. The first group was characterized by a tolerance to ammonium with little or no growth inhibition (<14 %) compared to controls. The second group was moderately sensitive, demonstrating reductions in cell densities by 50-60 %. The third group was very sensitive to the NH_4Cl with decreases of >75 %. Among the cell lines they studied, Vero cells were placed in the first category, as no reduction in cell yields were apparent at 2 mM NH_4Cl and the $I.C._{50}$ was >2.5 mM. Alternatively, the cell density of BHK cells was reduced 80 % by 2 mM NH_4Cl and the $I.C._{50}$ was 1.3 mM. Thus, the BHK line was an example of the third group which is most sensitive to ammonium inhibition. On the other hand, an $I.C._{50}$ of 2.5 mM was reported for BHK cells in another study (Butler and Spier, 1984) and an even higher value was derived in the present investigation. The $I.C._{50}$ determined from the effect on cell density was 9 mM and >40 mM for BHK and Vero cells, respectively (Table 3-1). Thus, the cells appeared to be much less sensitive to ammonium than noted in earlier investigations. Despite the elevated $I.C._{50}$ values shown here, BHK and Vero cells were clearly at opposite extremes regarding their tolerances to ammonium. In other words, the same relative sensitivities observed by other workers were still apparent, the former being very sensitive while the latter is tolerant. CC9C10 demonstrated an intermediate level of

sensitivity between these two cell lines. Hybridomas are typically moderately sensitive or tolerant to ammonium with $I.C._{50}$ values of 2-10 mM (Ozturk *et al.*, 1992).

The variable sensitivities to ammonium, not only among different cell lines but also by the same cell line in different studies, suggest that culture conditions and the history of the cell line may have a profound influence on the response to ammonium. For instance, Wentz and Schügerl (1992) reported a reduction in the specific growth rate of suspension BHK cells grown in Roux flasks from 0.051 hr^{-1} to 0.011 hr^{-1} upon addition of 17.7 mg/L NH_4Cl. This is equivalent to an $I.C._{50}$ of less than 0.33 mM and a degree of sensitivity much greater than observed by other investigators. In contrast to the Roux cultures, however, the addition of 100 mg/L (1.87 mM) NH_4Cl had no effect on the BHK cells grown in a stirred bioreactor. The authors surmised the pH control in the bioreactor and displacement of NH_3 from the vessel by indirect aeration resulted, at least in part, in the reduced sensitivity to ammonium. Doyle and Butler (1990) had, in fact, previously indicated the degree of ammonium inhibition was related to the pH of the culture medium. A negative correlation was found between the $I.C._{50}$ and the pH of the medium. This was postulated to be the result of an increased proportion of NH_3 in the medium, which readily diffuses through cell membranes and into cells, as the pH is raised. It was further speculated that the variable response of cell lines to ammonium may be to some degree related to the differences in culture conditions such as medium pH. Other workers have found that the composition of the culture medium affects sensitivity to ammonium. Iio *et al.* (1985) described a 64 % reduction in the cell numbers of a murine myeloma grown in serum-free medium in contrast to a 30 % decrease in the presence of serum. The higher $I.C._{50}$ values obtained for BHK and Vero cells in the current study may be indicative of more favorable or optimal culture conditions. This could result in the reduced sensitivity to ammonium compared to earlier investigations.

The methodology of adding inhibitors directly to the growth medium introduces an additional factor that affects the sensitivity of cells to ammonium. Martinelle and Häggström (1993) have suggested ammonium produced from cellular metabolism may be more inhibitory than that originating externally from decomposition of glutamine or addition of ammonium salts to the culture medium. The NH_4^+ generated intracellularly by mitochondrial enzymes such as glutaminase and glutamate dehydrogenase results in an acidification of the mitochondrial matrix. This could weaken the proton gradient across the mitochondria and thus reduce ATP production. Externally supplied NH_4^+, conversely, induces an increase in the pH of the mitochondria, perhaps enhancing the proton gradient and improving the energy status of the cell. In contrast, a recent report by Newland *et al.* (1994) demonstrated cells may adapt more readily to the gradual increases in ammonium which would be generated by cellular metabolism compared to the sudden increases resulting from the addition of ammonium salts to the medium. They found the SP01 hybridoma was not inhibited by a low rate of continuous addition of NH_4Cl up to a concentration of 12.5 mM in a stirred bioreactor. In contrast to this fed-batch system, the cells had an $I.C._{50}$ of 3 mM for ammonium in batch culture. In continuous culture, step-wise increases in ammonium resulted in a transient inhibition followed by stabilization of cells at a lower density. Thus, the dynamic conditions in the culture also influence the sensitivity of cells to the inhibitor which may not be reflected by its immediate addition to the medium. A number of competing variables are therefore likely to determine the overall response of each cell line to ammonium and are likely to vary, at least to some degree, in each experiment. The result is manifested as contrasting $I.C._{50}$ values reported by different laboratories. Nevertheless, the inhibition studies described above established the importance of reducing ammonium accumulation in the medium and provided the rationale for examining the response of cultured cells to a low-ammoniagenic medium.

The addition of lactate to animal cell cultures followed by adjustment of the medium pH to neutrality is a common practice to distinguish the chemical effects of lactate from the inhibition due to acidification of the medium. Lactate inhibition studies were similarly performed with BHK cells as shown in Figures 3-2 and Table 3-1. Figure 3-2a reveals the effect of sodium lactate on the specific growth rate of BHK cells. The $I.C._{50}$ was >100 mM, 10-fold greater than observed for ammonium. The effects of NaCl were virtually indistinguishable from those of the lactate. In addition, equivalent concentrations of sodium lactate and NaCl induced identical increases in medium osmolarity suggesting inhibition by the former may be entirely due to elevation of osmolarity. However, Figure 3-2b indicates lactate is somewhat more inhibitory than NaCl based on their effects on cell density with $I.C._{50}$'s of 54 and 70 mM, respectively. These contrasting values can be explained by differences in the extent of the lag phase, growth rate and duration of the exponential phase induced by each of the substances. Most of the inhibitory action of lactate can be attributed to osmotic effects but a minor component appears to be due to other uncharacterized biochemical effects. These conclusions are similar to those reported by Ozturk *et al.* (1992) where the effect of lactate on murine hybridoma cell line 167.4G5.3 was examined. Lactic acid was added to the culture medium at various concentrations up to 69 mM and neutralized to compensate for the decrease in pH. An initial lactate concentration of 23 mM had minor effects on the specific growth rate. Inhibition was noted at higher levels but appeared to be the result of increased osmolarity at lactate concentrations up to 40 mM. Inhibition of growth rate due to direct chemical effects of lactate became apparent only at levels of 40-50 mM. The chemical effects of lactate and the mechanisms of inhibition, however, were not defined.

Although cell yields were affected to a greater extent than growth rates, neutralized lactate remained a relatively mild inhibitor compared to ammonium. These findings are consistent with previous studies. Hassell *et al.* (1991) described lactate

inhibition in three cell lines with different sensitivities to ammonium. Lactate concentrations up to 20 mM had negligible effects on the cell yields of BHK, McCoy and Vero cells which represented cell lines with a high, moderate and a low degree of ammonium sensitivity, respectively. Wentz and Schügerl (1991) also reported no inhibition of BHK cells at lactate concentrations up to 2.5 g/L (28 mM) and only a much higher level of 9.5 g/L (105 mM) caused significant reductions in viable cell concentrations in Roux flasks. They also observed lactate had little effect on growth rate and cell densities at 4.5 g/L (50 mM) in a stirred reactor with pH control. High concentrations of lactate have similarly been found to have little or no inhibitory effect on murine hybridomas (Reuveny *et al.*, 1986, Glacken *et al.*, 1988 and Miller *et al.*, 1988). Ozturk *et al.* (1992) reported the $I.C._{50}$ of 55 mM for lactate with the mouse hybridoma 167.4G5.3 was more than 10-fold higher than the value observed for NH_4Cl.

The present studies, supported by previous investigations, suggest that ammonium in animal cell cultures would limit growth in advance of the accumulation of inhibitory levels of lactate. With pH control or cultures with supplementary buffers, the effects of lactate are minimized. Consequently, the reduction of ammonium by replacing glutamine with less ammoniagenic substrates appeared a more plausible strategy for improving growth and productivity of cultured cells and was the focus of subsequent studies. CC9C10 and BHK cells were selected as model cell lines in this type of medium because of their sensitivity to ammonium. Vero cells were not considered further as reduced ammonium yields would have a limited impact on an ammonia-tolerant cell line.

Chapter 4

Growth and Productivity of a Murine Hybridoma in Dipeptide Media

4.1 Introduction

Following the selection of appropriate cell lines susceptible to ammonium inhibition, efforts were directed toward development of low-ammoniagenic media. Other investigators have considered replacing glutamine with more stable and less ammoniagenic analogues. In some cases, glutamate may be an acceptable substitute for glutamine (Griffiths and Pirt, 1967 and Hassell and Butler, 1990). However, not all cell lines adapt to glutamate-based media (Eagle *et al.*, 1956). This may be the result of a deficiency in the inducibility of glutamine synthetase (Griffiths, 1973) or an inability to transport the amino acid into the cell at a sufficient rate to satisfy cellular demand (McDermott and Butler, 1993). Glutamine-containing dipeptides such as ala-gln and gly-gln have been considered as alternative replacements (Roth *et al.*, 1988, Minamoto *et al.*, 1991 and Holmlund *et al.*, 1992).

The suitability of both glutamate and glutamine-containing dipeptides as substitutes for glutamine in murine hybridoma cultures was investigated using CC9C10 as a model cell line. An acceptable substitute should reduce ammonium accumulation and sustain high growth rates and cell yields without limiting the productivity of the culture over numerous generations. These criteria established dipeptides as the most effective replacement for glutamine. The changes in growth and metabolism associated with the reduced availability of glutamine and lower levels of the ammonium inhibitor were analyzed in dipeptide media. In addition, the potential of the dipeptides to improve the stability of the medium was investigated.

4.2 Materials and methods

4.2.1 Culture conditions

The ability of CC9C10 to grow in the presence of glutamate was examined in DMEM/10 % FetalClone with 4 mM gln and 10 mM L-glu or D-glu. The effect of a range of L-glu concentrations up to 10 mM was also examined in DMEM/10 % FetalClone with 4 mM glutamine. Dipeptide media consisting of DMEM/10 % FetalClone with either 6 mM ala-gln, 6 mM gly-gln or 20 mM gly-gln were evaluated relative to the control containing 6 mM gln. These cultures were designated DMEM/ala-gln, DMEM/gly-gln (6 mM or 20 mM) and DMEM/gln, respectively. The glutamine concentration of FetalClone (which is derived from bovine serum) was determined to be less than 50 μM (see Appendix G) and did not contribute significantly to glutamine levels in the complete media. Cells were allowed to adapt to the dipeptides by subculturing in the presence of each substrate for at least 6 passages (~24 generations) prior to the experimental work. For all experiments, cells were cultured at the standard temperature and atmosphere described previously (section 2.4).

4.2.2 Cell enumeration

Cell counts were determined by the trypan blue exclusion method and the Neubauer haemocytometer or with the Coulter counter (section 2.8) as specified for each experiment.

4.2.3 Analysis of media components

Amino acid concentrations were evaluated by HPLC with the C-18 reverse phase column following derivatization with *o*-phthaldialdehyde (section 2.11.4.2). Glutamine was measured independently with the glutaminase assay (section 2.11.4.1). Ammonium was determined with the gas-sensing electrode (section 2.11.2). Glucose and lactate were analyzed with the YSI industrial analyzer fitted with appropriate

membranes (section 2.11.1.1). Monoclonal antibody concentrations were determined with the ProAnaMabs column (section 2.11.3). Specific consumption and production rates of metabolites were calculated by Equation 4 (section 2.11.5).

4.2.4 Determination of intracellular amino acids and dipeptides

Cells were seeded in duplicate T-150 flasks in DMEM/gln, DMEM/ala-gln and DMEM/gly-gln (20 mM) (50 ml/ flask) at a density of ~1 X 10^6 viable cells/ml. Following incubation for 24 hours, cells (~1 X 10^8) were pelleted at 250 g (saving supernatant for extracellular metabolite analysis), washed with D-PBS and pelleted once again. The pellets were resuspended in 1 ml of 5 mM Tris-HCl, pH 7.5 with 0.15 % SDS and sonicated 3 X for 30 seconds on ice. The resulting lysates were centrifuged at 31 000 g for 90 minutes at 4 ^oC. Supernatants were lyophilized, resuspended in 2 ml of water and stored at -20 ^oC until ready for analysis. Amino acid and dipeptide concentrations in the supernatant were determined by HPLC analysis (section 2.11.4.2). Intracellular metabolite calculations were based on estimating the total volume of the cells lysed using the method of Schmid and Blanch (1992). Cells were assumed to be spheres with an average diameter (d) of 16.5 µm determined by measurement with a micrometer. Cell volume was determined from the equation:

$$V = \pi d^3/6 \qquad (6)$$

4.2.5 Stability of dipeptides

The suitability of dipeptides for heat-sterilization was evaluated. Aqueous solutions with 6 mM glutamine or dipeptides were autoclaved (120 ^oC at 110 kPa for 20 minutes) while control samples remained on ice. The remaining dipeptide concentrations were evaluated from the glutamine released following incubation with 0.1 mg/ml cell lysate protein in a 250 µl volume of D-PBS/50 mM HEPES, pH 7.4, for 8 hours. The cell lysates contained peptidase activity which hydrolyzed the dipeptide. The CC9C10 cell lysate was prepared by sonication of ~3 X 10^8 cells (3 X 30 seconds on

ice) in D-PBS with 50 mM HEPES, pH 7.4. Cell debris was removed by centrifugation (31 000 g for 90 minutes at 4 ^{o}C) and filtration of the supernatant through a 0.2 μm filter. The protein content of the lysate was evaluated by the bicinchoninic acid assay outlined previously (section 2.10). After incubation with the lysate, samples were assayed for glutamine using the specific enzymatic method described previously (section 2.11.4.1).

The chemical stability of glutamine and dipeptides was compared in DMEM/gln, DMEM/ala-gln and DMEM/gly-gln (6 and 20 mM) with and without 10 % FetalClone. Each medium was maintained at pH 7.4 with 20 mM HEPES. Each medium was added to duplicate 75 cm^2 T-flasks (25 ml/flask). Following a 4 day incubation at 4 ^{o}C and 37 ^{o}C, ammonium concentrations in each sample were measured (section 2.11.2).

The susceptibility of dipeptides to hydrolysis by serum enzymatic activity was also studied. Peptidase activity in the serum supplement was investigated in DMEM with dipeptides (100 mM ala-gln or gly-gln) and containing either no serum or 10 % untreated or heat-treated FetalClone. DMEM with untreated serum and no added substrate was included for comparison. The heat-treated serum was incubated at 65 ^{o}C for 30 minutes before addition to the medium. The pH of the media were controlled at 7.4 with 50 mM HEPES. Samples were added in duplicate to the wells of a 96 well plate (100 μl/well) and incubated 24 hours at 37 ^{o}C. The increases in glutamine content in the samples after the incubation were determined by the glutaminase assay (section 2.11.4.1).

4.3 Results

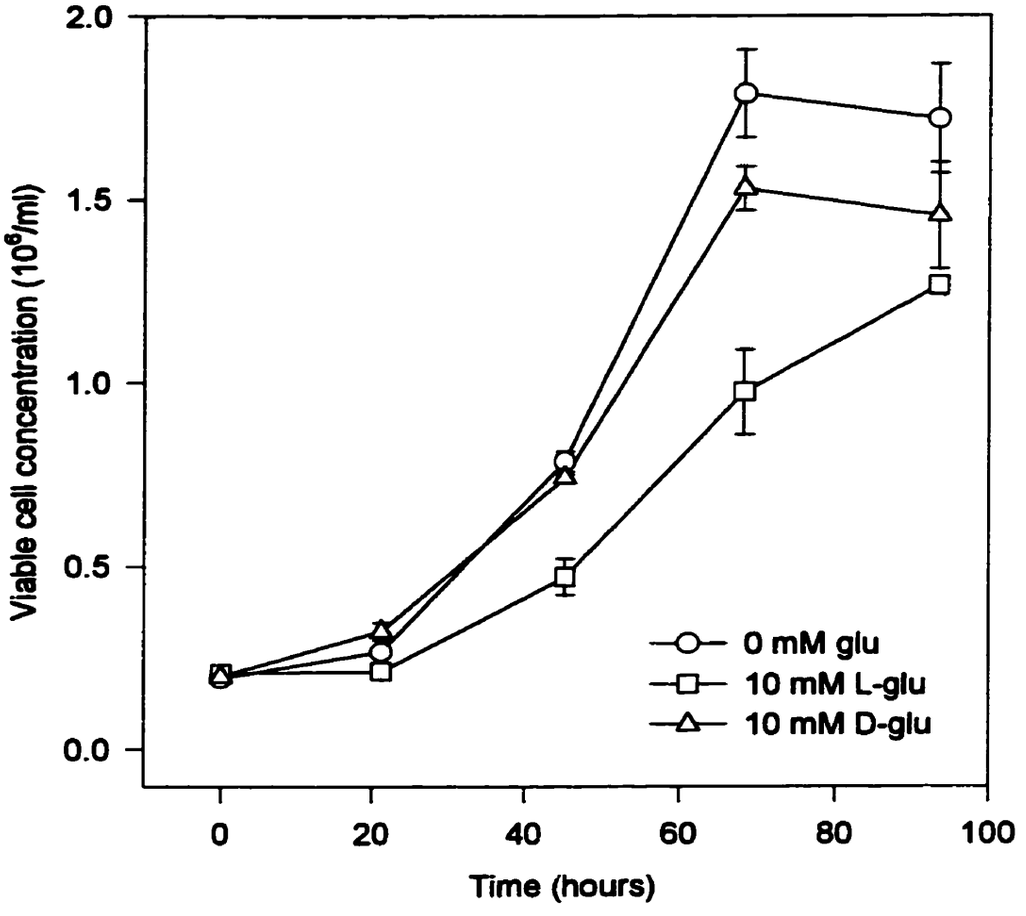
4.3.1 Growth in glutamate medium

Initial attempts to adapt CC9C10 to a medium substituted with glutamate were performed by subculturing cells into a medium containing both glutamine and a high concentration of glutamate. The glutamine level would subsequently be reduced with each subculture until it was eliminated, thereby allowing cells to adapt over numerous passages. Figure 4-1a shows the growth of the hybridoma in medium containing 4 mM gln and either 10 mM L-glu or D-glu. The inclusion of L-glu in the medium exacerbated the lag phase and reduced the growth rate and final cell density achieved compared to the control without glutamate. On the other hand, the nonbiological enantiomer D-glu elicited a less pronounced inhibition of cell growth. This implies inhibition by L-glu is primarily due to specific biochemical effects rather than a nonspecific mechanism such as a change in medium osmolarity also induced by D-glu.

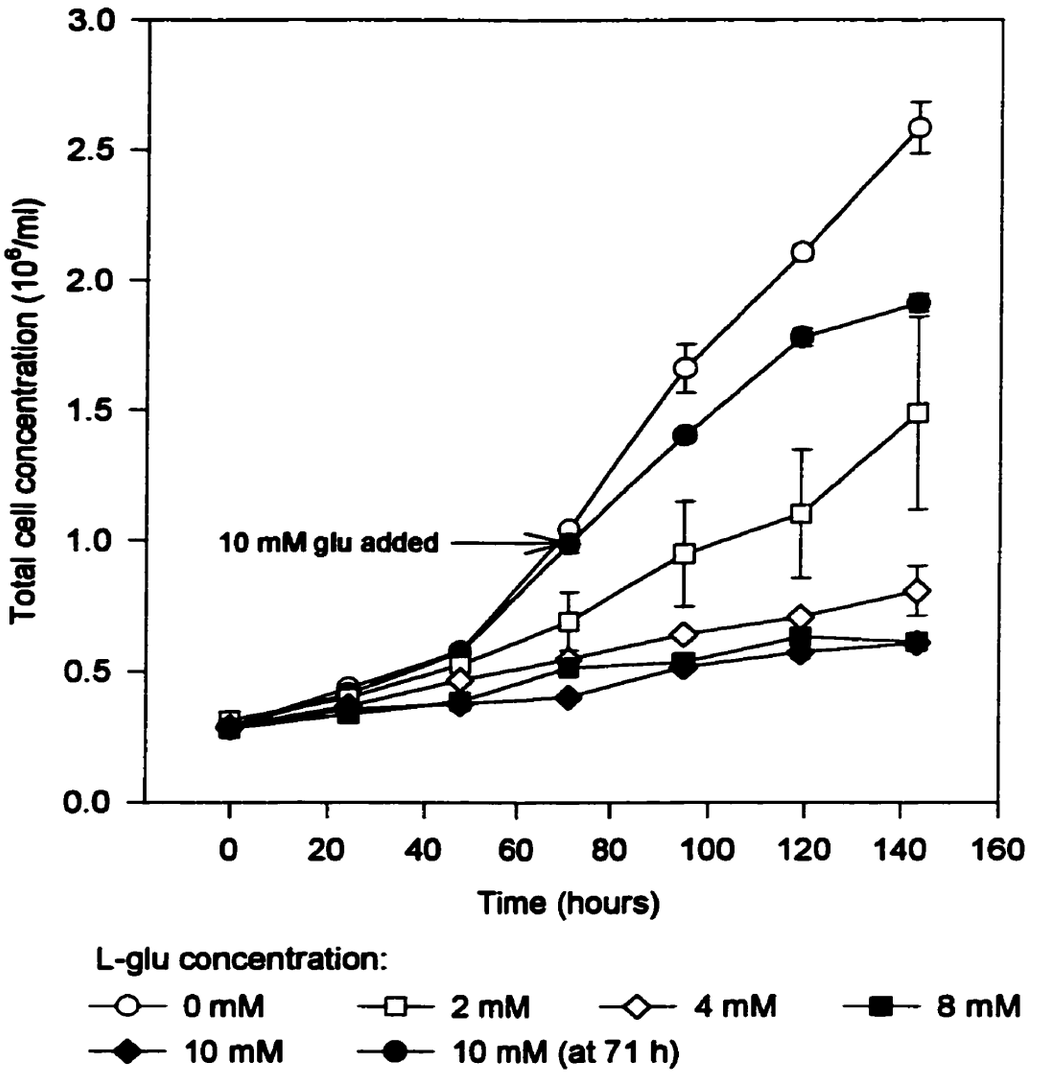
Additional studies demonstrated that reducing the level of glutamate did not improve growth (Figure 4-1b). Concentrations as low as 2 mM L-glu produced a significant growth reduction while 10 mM in this case was almost completely inhibitory. Adding 10 mM glutamate after ~3 days resulted in a normal lag phase and initial growth rate compared to the control (0 mM glutamate) although the inhibitory effects were evident after the addition. Inhibition appeared more extensive than the previous experiment at 10 mM L-glu indicating some variability in response to glutamate with culture conditions or the state of the cell inoculum. However, the inhibitory effect observed even at low concentrations suggested glutamate would not be a viable replacement for glutamine. This was confirmed when attempts to adapt cells to lower glutamate with gradual reductions in glutamine concentration were ineffective. Further efforts to adapt cells to glutamate medium were therefore discontinued.

Figure 4-1a-b. The effect of glutamate on the growth of CC9C10 cells. (a) The culture medium was DMEM/10% FetalClone with 4 mM glutamine and either 0 mM glu (○), 10 mM L-glu (□) or 10 mM D-glu (△). Viable cell concentrations were determined by haemocytometer counts. (b) Cells were also cultured in the same medium with 4 mM gln and a range of L-glu concentrations: 0 mM (○), 2 mM (□), 4 mM (◊), 8 mM (■), 10 mM (◆) and 0 mM initially with 10 mM added after ~3 days (71 hours) (●). Total cell concentrations were evaluated with the Coulter counter. The hybridoma was grown in duplicate (n=2) 25 cm^2 T-flasks for each of the cultures in both experiments. Duplicate counts were averaged and plotted ± standard error (SE).

(a)


2.0
1.5
1.0
0.5
0.0
Viable cell concentration (10^6/ml)
0
20
40
60
80
100
Time (hours)
0 mM glu
10 mM L-glu
10 mM D-glu

(b)


3.0
2.5
2.0
1.5
1.0
0.5
0.0
Total cell concentration (10^6/ml)
10 mM glu added
0
20
40
60
80
100
120
140
160
Time (hours)
L-glu concentration:
0 mM
2 mM
4 mM
8 mM
10 mM
10 mM (at 71 h)

4.3.2 Dipeptide medium

4.3.2.1 Cell growth and productivity

The CC9C10 hybridoma grew in media supplemented with glutamine (6 mM), ala-gln (6 mM) or gly-gln (20 mM) to maximum densities >2 X 10^6 viable cells/ml within 5 days (Figure 4-2). Cell yields of the dipeptide cultures were slightly higher (14 % for DMEM/gly-gln) compared to DMEM/gln. In cultures containing 6 mM ala-gln or 20 mM gly-gln, no deterioration of cell growth was apparent even after more than 40 passages. However, growth and cell yield in gly-gln (6 mM) was poor with an insignificant increase in cell concentration before 5 days. Consequently, medium with a low gly-gln level was not considered acceptable for the growth of the hybridoma.

A decrease in viable cell concentration occurred during a lag phase (initial 24 hours) in the ala-gln and gly-gln cultures. This resulted in the maximum cell yield occurring later in the culture compared to the glutamine-based control. The lag period was characterized by a substantial decrease in viability (%) of the cells grown in dipeptide-based media as measured by trypan blue exclusion (Figure 4-3). This suggests that there was a loss in membrane integrity and possibly cell lysis during this period. The lag period was also associated with dipeptide hydrolysis and a concomitant increase in glutamine concentration in the medium (discussed below).

The possibility that a low initial concentration of glutamine could reduce the lag phase in the presence of a dipeptide was investigated by the addition of 0.5 mM gln to a DMEM/gly-gln (20 mM) culture. As shown in Figure 4-4, this low-level supplement of glutamine eliminated the lag phase and allowed the culture to reach a maximum cell density 24 hours earlier.

Monoclonal antibody concentrations were measured daily throughout the culture period. In all cultures, the mAb concentration was proportional to the integral of the viable cell concentration and time (viable cell index). The mAb production was not significantly different between DMEM/gln, DMEM/ala-gln and DMEM/gly-gln (20 mM)

cultures (Figure 4-5) reaching a maximum concentration of ~200 μg/ml and a specific production rate of 1.0-1.3 μg per 10^6 cells per hour.

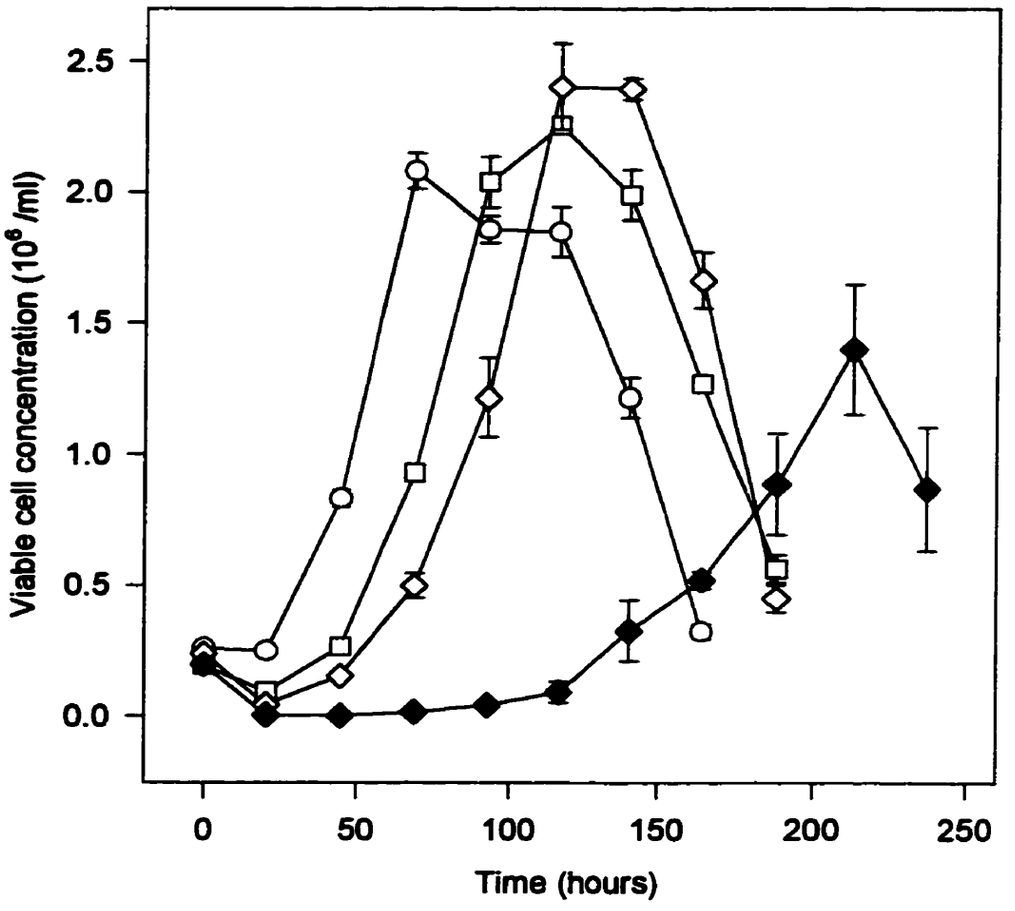


Figure 4-2. CC9C10 cell growth in media supplemented with glutamine or dipeptides. The hybridomas were grown in media with 6 mM gln (○), 6 mM ala-gln (□), 6 mM gly-gln (◆) or 20 mM gly-gln (◊) in duplicate 25 cm^2 T-flasks (n=2). Viable cell concentrations were determined by daily counting of each flask twice with a haemocytometer. Each point represents an average of replicate counts from each medium ± SE.

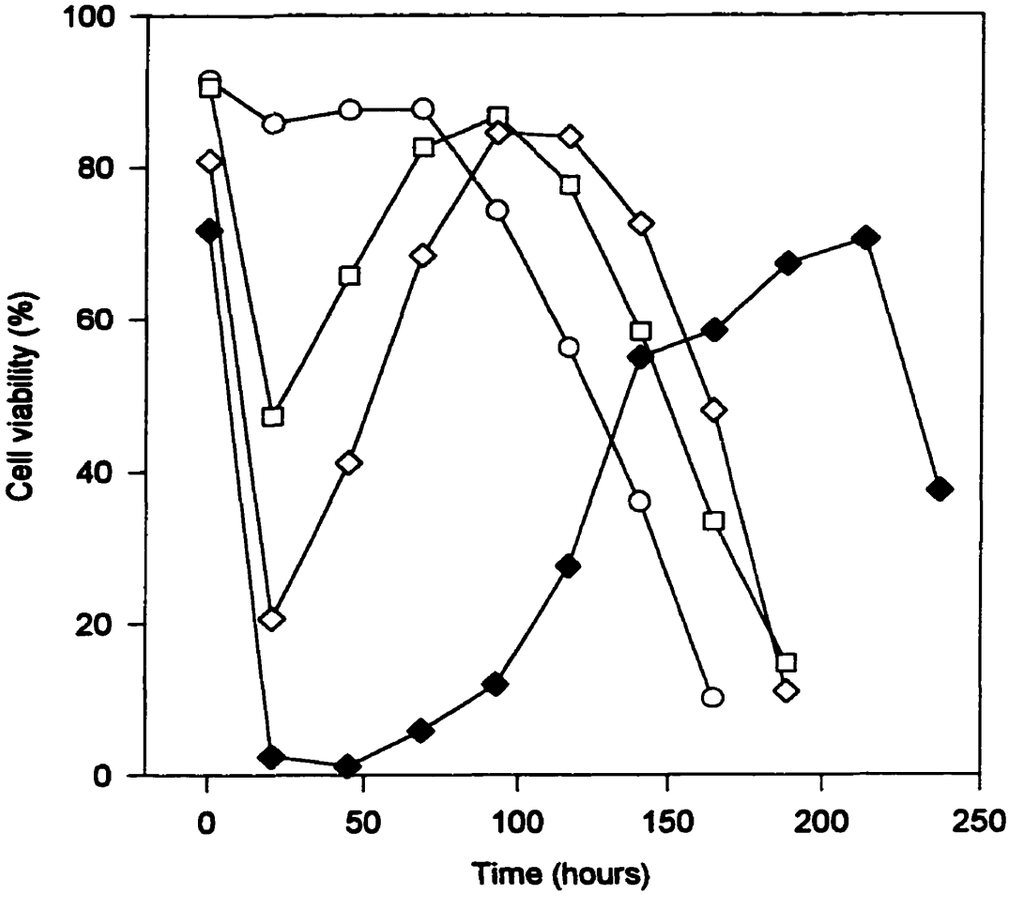


Figure 4-3. The viability of CC9C10 cells in glutamine and dipeptide cultures. Cells were grown in media supplemented with 6 mM gln (○), 6 mM ala-gln (□), 6 mM gly-gln (◆) or 20 mM gly-gln (◇) in duplicate 25 cm^2 T-flasks (n=2). The viability (%) was determined from the number of viable cells (which excluded trypan blue) as a percentage of the total cells during the counting procedure. The duplicate flasks from each medium were counted twice and each point represents the average. The standard error associated with most points was <10 % of the mean.

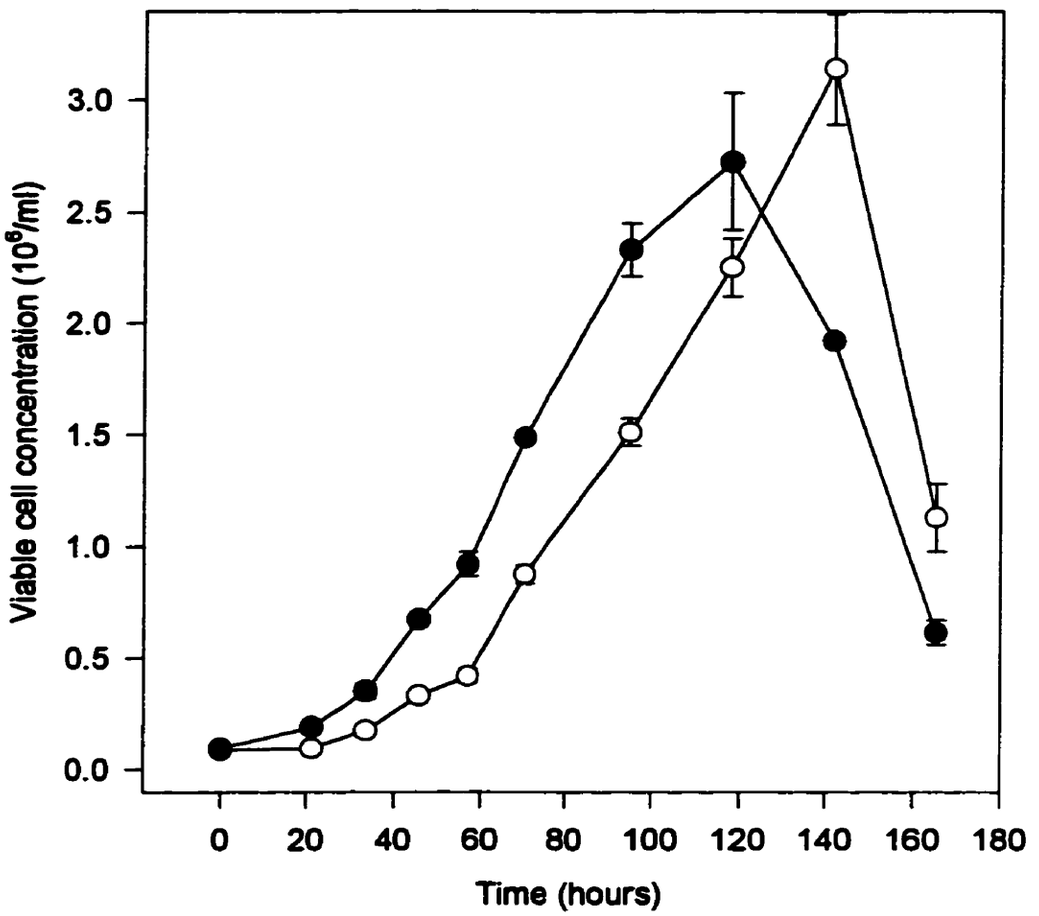


Figure 4-4. CC9C10 cell growth in DMEM/gly-gln with and without a low level of glutamine. The cells were grown in media supplemented with 20 mM gly-gln with (●) and without (○) the addition of 0.5 mM glutamine in duplicate 25 cm^2 T-flasks. Viable cell concentrations were determined daily by haemocytometer counts. Duplicate points were averaged and plotted ± SE.

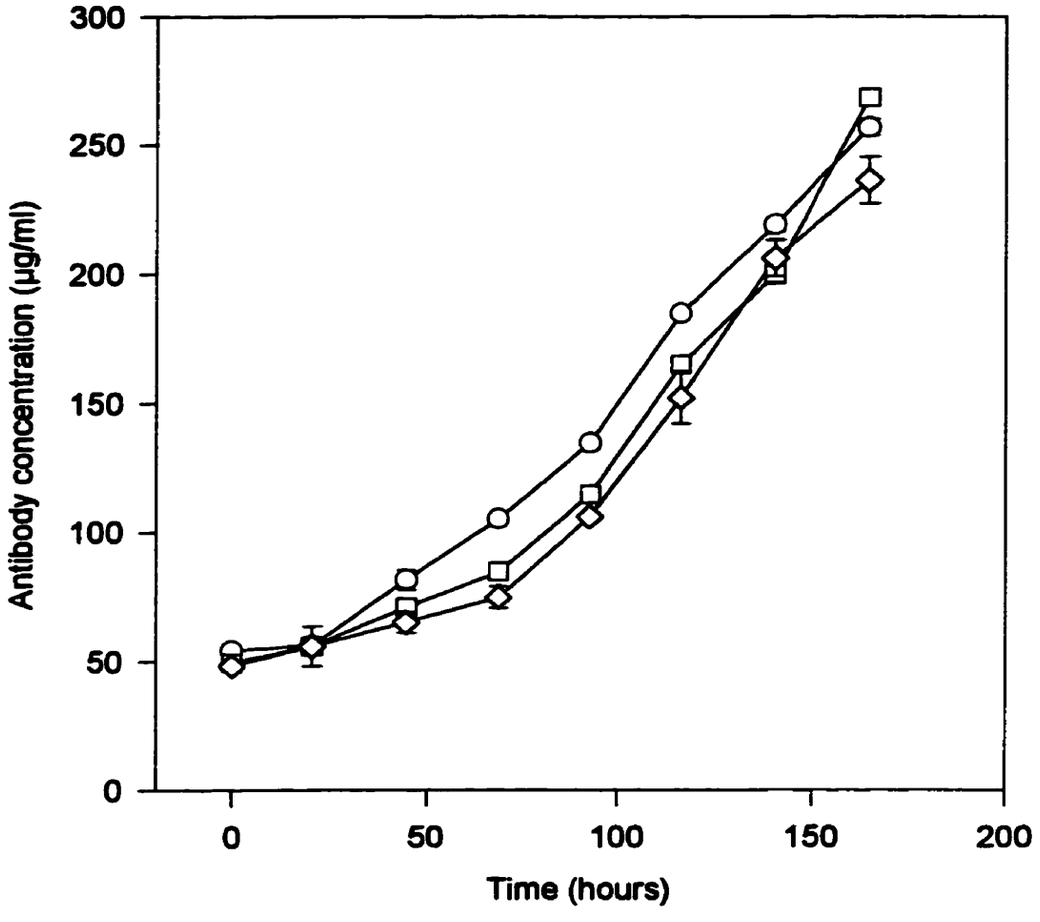


Figure 4-5. Monoclonal antibody production by CC9C10 cells in glutamine and dipeptide media. The media were supplemented with 6 mM glutamine (○), 6 mM ala-gln (□) or 20 mM gly-gln (◊) in duplicate 25 cm^2 T-flasks (n=2). Antibody concentrations were determined from daily samples from each of the different media. The duplicate values were averaged and points plotted ± SE.

4.3.2.2 Substrate utilization and by-product formation

4.3.2.2.1 Glutamine and ammonium

Concentrations of glutamine and ammonium were determined from daily samples of the three cultures shown previously including DMEM/gln, DMEM/ala-gln and DMEM/gly-gln (20 mM) (Figure 4-6). In DMEM/gln, the glutamine content of the medium was completely depleted in 116 hours with a concomitant increase in ammonium concentration to 4.4 mM. This can be expressed as a metabolic coefficient (ammonium/glutamine) of 0.73 which is typical for glutamine-based cultures (Hassell and Butler, 1990). In DMEM/ala-gln, the glutamine concentration increased to 4.2 mM. This may be associated with a period of rapid dipeptide hydrolysis occurring in the lag phase. The initial increase in glutamine concentration in DMEM/gly-gln was considerably lower (1.4 mM) despite the higher original content of dipeptide (20 mM). This indicates that the enzymatic activity associated with peptide hydrolysis has a higher specificity for ala-gln than gly-gln.

A specific glutamine consumption rate (91.6 nmol per 10^6 cells per hour) was determined for DMEM/gln (Table 4-1). Values could not be determined for dipeptide cultures because of the simultaneous process of dipeptide hydrolysis and glutamine consumption by the cells. The glutamine content of the dipeptide cultures increased rapidly in the first 24 hours of the culture. However, it is apparent that during the exponential growth phase, the cellular consumption of glutamine from dipeptide cultures was equal to or greater than the rate of its release by dipeptide hydrolysis. The increase in glutamine concentration during the decline phase of the DMEM/gly-gln culture may be explained by a period in which the rate of dipeptide hydrolysis was higher than the cellular glutamine consumption.

Table 4-1 indicates that the specific ammonium production during the exponential phase of growth was slightly lower in DMEM/ala-gln (43 nmol per 10^6 cells per hour) compared to DMEM/gln (57 nmol per 10^6 cells per hour), although the final

ammonium concentration (4.5-5 mM) was not reduced. In DMEM/gly-gln, the specific ammonium production (23 nmol per 10^6 cells per hour) was substantially lower (60 %) compared to DMEM/gln as was the final ammonium concentration (3.5 mM).

4.3.2.2.2 Glucose and lactate

Daily measurements were made of glucose and lactate concentrations in the three cultures. In all cases, the glucose was almost completely consumed with a concomitant increase in lactate concentration. For DMEM/gln, the glucose was nearly depleted in 5 days with an associated metabolic coefficient (lactate/glucose) of 1.7 (Figure 4-7). The lactate/glucose coefficient for the dipeptide cultures was similar with a value of 1.6. During exponential cell growth, the specific glucose consumption rate of the DMEM/gly-gln culture at 176 nmol per 10^6 cells per hour was nearly 50 % lower than in the DMEM/gln culture (Table 4-1). The equivalent rate in DMEM/ala-gln (295 nmol per 10^6 cells per hour) was 13 % lower than for DMEM/gln. The reduced glucose consumption in DMEM/gly-gln resulted in almost a 50 % decrease in specific lactate production (373 nmol per 10^6 cells per hour).

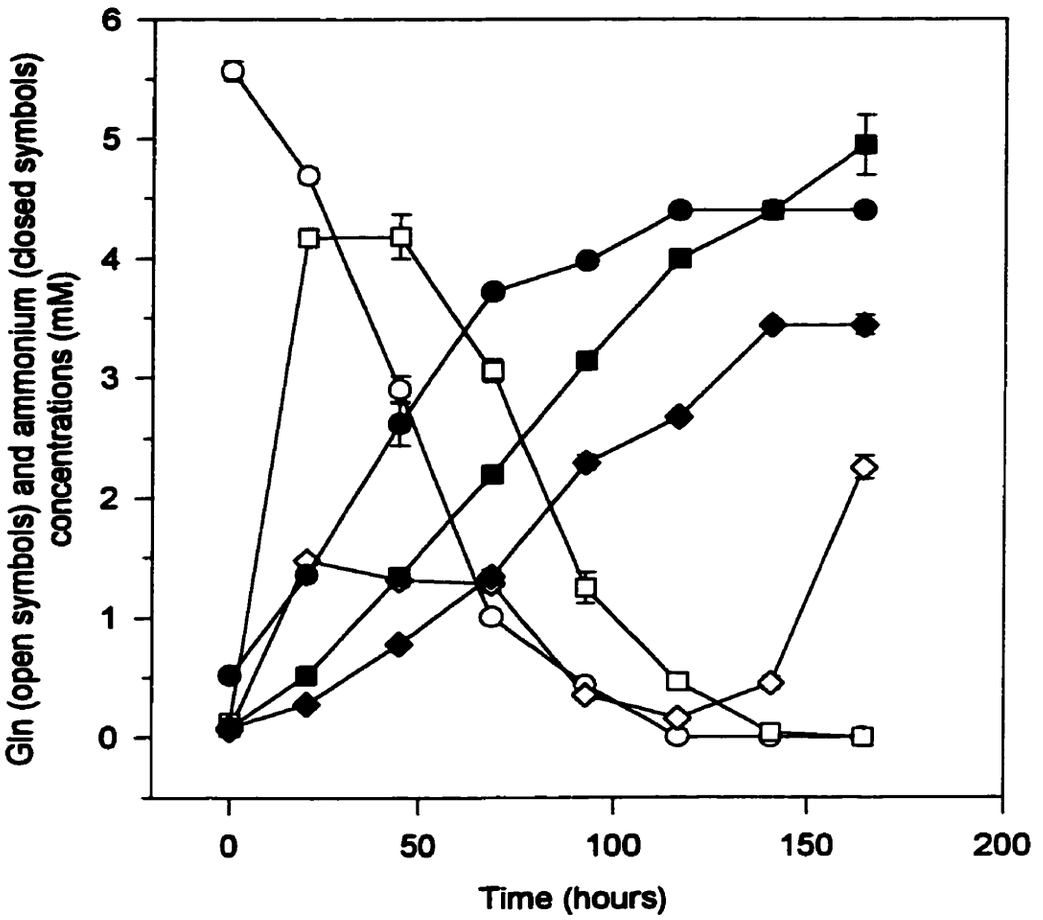


Figure 4-6. Glutamine and ammonium concentrations in CC9C10 cultures. The hybridomas were grown in media containing 6 mM glutamine (○,●), 6 mM ala-gln (□,■) or 20 mM gly-gln (◊,◆) in duplicate 25 cm^2 T-flasks (n=2). Glutamine (open symbols) and ammonium (closed symbols) were determined from daily samples taken from the cultures. Duplicate samples were averaged and plotted ± SE.

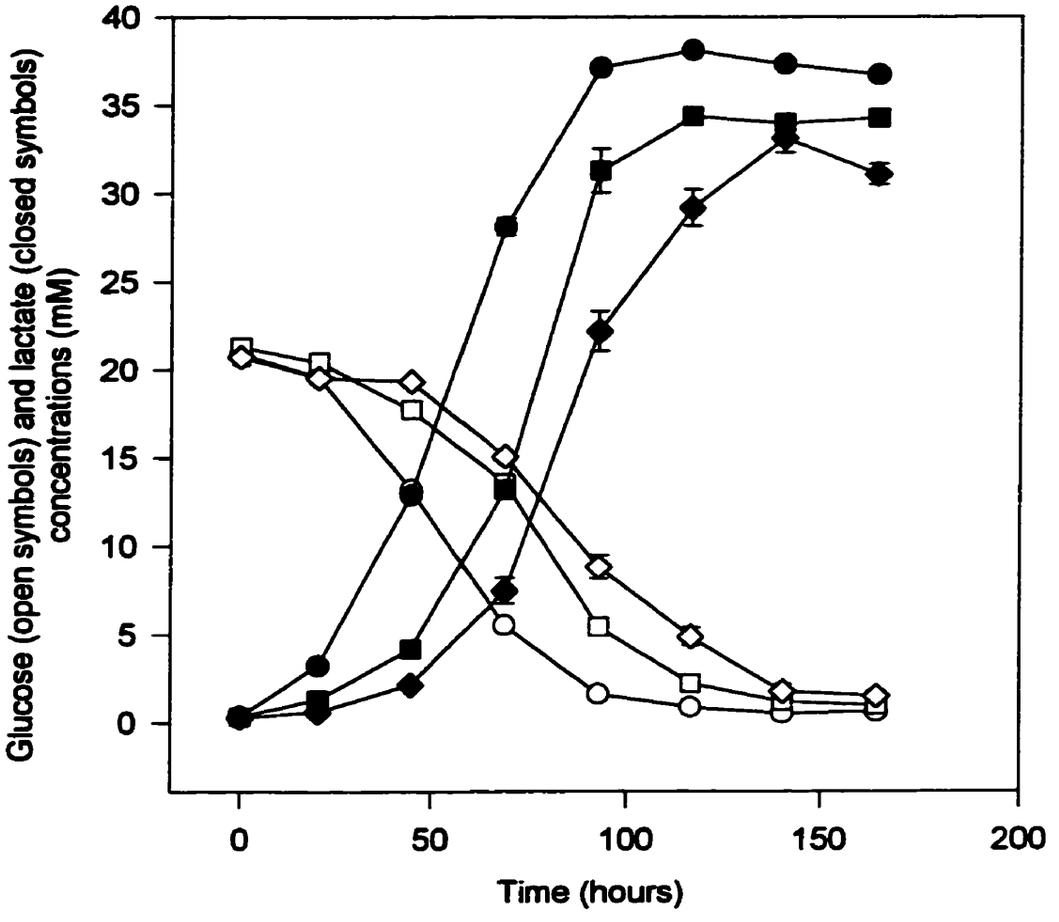


Figure 4-7. Glucose and lactate concentrations in CC9C10 cultures. The cells were grown in media containing 6 mM glutamine (○,●), 6 mM ala-gln (□,■) or 20 mM gly-gln (◇,◆) in duplicate 25 cm^2 T-flasks (n=2). Glucose (open symbols) and lactate (closed symbols) were determined from daily samples taken from the cultures. Duplicate samples were averaged and plotted ± SE.

Table 4-1. Specific rates of metabolite consumption (-) and production (+) during the exponential phase of CC9C10 cells grown in glutamine and dipeptide media. The culture media included DMEM + 10 % FetalClone with 6 mM gln, 6 mM ala-gln or 20 mM gly-gln. The exponential phase in DMEM/gln, DMEM/ala-gln and DMEM/gly-gln corresponded to 20-68 hours, 45-93 hours and 68-117 hours, respectively. Values are the means ± SE of duplicate samples (n=2).

	Specific production (+) and consumption (-) rates per 10^6 cells (nmol 10^{-6} cells hr^{-1})		
	DMEM/gln	DMEM/ala-gln	DMEM/gly-gln
ala	+34.6 ± 1.0	N.D.[a]	+14.8 ± 2.0
arg	-6.29 ± 0.26	-6.40 ± 0.45	-3.80 ± 0.33
asn	+1.17 ± 0.01	+1.68 ± 0.06	N.D.[b]
asp	+0.17 ± 0.03	+0.12 ± 0.02	N.D.[b]
glu	+1.90 ± 0.22	+1.75 ± 0.02	+2.27 ± 0.24
gln	-91.6 ± 2.0	N.D.[a]	N.D.[a]
gly	+0.54 ± 0.87	-0.77 ± 0.54	N.D.[a]
his	-2.33 ± 0.14	-1.62 ± 0.30	N.D.[c]
ile	-7.37 ± 0.45	-6.01 ± 0.33	-5.98 ± 0.50
leu	-9.18 ± 0.40	-7.91 ± 0.37	-6.58 ± 0.45
lys	-7.87 ± 0.57	-6.31 ± 0.60	-4.66 ± 0.65
met	-2.43 ± 0.13	-2.21 ± 0.09	-1.87 ± 0.51
phe	-0.98 ± 0.83	-1.30 ± 0.77	-0.92 ± 0.34
ser	-3.70 ± 0.60	-0.46 ± 0.49	-3.48 ± 0.28
thr	-7.96 ± 0.46	-1.98 ± 0.66	-3.25 ± 0.68
trp	-0.80 ± 0.07	-0.76 ± 0.08	-0.59 ± 0.08
tyr	-2.82 ± 0.37	-1.64 ± 0.20	-1.74 ± 0.33
val	-7.24 ± 0.42	-5.66 ± 0.48	-5.28 ± 0.42
glc	-340 ± 6	-295 ± 2	-176 ± 11
lac	+601 ± 12	+650 ± 27	+373 ± 27
NH_4^+	+57 ± 1	+43 ± 1	+23 ± 2

N.D. is not determined

[a] Cellular production or consumption rates could not be determined due to additional production of amino acid from hydrolysis of dipeptide.

[b] Consumption and/or production rates were small and variable

[c] Could not be quantified by HPLC analysis due to co-elution with gly-gln peak

4.3.2.2.3 Amino acid consumption or production

The specific rates of consumption or production of amino acids in three cultures (DMEM/gln, DMEM/ala-gln and DMEM/gly-gln) were determined from daily measurements of the amino acid content of the culture medium (Table 4-1). Apart from glutamine, 6 amino acids showed high rates of cellular consumption (>5 nmol per 10^6 cells per hour) in the three cultures - arg, ile, leu, lys, thr and val. For these 6 amino acids, the specific rates of consumption were significantly lower in DMEM/gly-gln compared to DMEM/gln. These amino acids (except arg) also showed lower specific consumption rates in DMEM/ala-gln compared to DMEM/gln.

There was a high net production of alanine in DMEM/gln (34.6 nmol per 10^6 cells per hour), although the rate was significantly lower (57 %) in DMEM/gly-gln. The equivalent cellular production rate of alanine could not be determined in DMEM/ala-gln due to the additional release from dipeptide hydrolysis. Four other amino acids (asn, asp, glu and gly) increased in concentration during the culture but no significant differences in calculated specific production rates were observed between cultures.

4.3.2.3 Intracellular dipeptide and amino acid concentrations

Intracellular and extracellular concentrations of 18 amino acids and dipeptides were measured after a 24 hour incubation of cells (10^6/ml) in DMEM/gln, DMEM/ala-gln and DMEM/gly-gln (Table 4-2). This would indicate if dipeptides or their hydrolytic products accumulated intracellularly.

The concentrations of glutamine and the dipeptides decreased substantially in the culture supernatant over the incubation period. However, the dipeptides were not detected in the intracellular samples of either DMEM/ala-gln or DMEM/gly-gln. This is compatible with the idea that the dipeptides are hydrolyzed in the culture medium prior to uptake of the products into the cells (discussed in the following chapter). The hydrolytic products ala and gly were substantially higher in the intracellular samples of

DMEM/ala-gln and DMEM/gly-gln at 9.6 and 27.6 mM, respectively, corresponding to elevated extracellular concentrations of these amino acids.

In most cases (except where indicated in Table 4-2), the amino acid concentrations were significantly higher in the intracellular environment compared to the culture supernatant. Such accumulation against a concentration gradient is typical for active transport of the amino acids into the cell. For 12 amino acids (asn, asp, glu, gln, his, ile, leu, lys, met, phe, ser and val), the intracellular concentrations in DMEM/ala-gln and DMEM/gly-gln were significantly lower than in DMEM/gln. This is compatible with the data presented in Table 4-1, although the differences between cultures may be greater for this short incubation experiment (24 hours) because it covers the lag period of the dipeptide cultures.

Table 4-2. Extracellular and intracellular metabolite concentrations of CC9C10 cells grown in glutamine and dipeptide media. Concentrations were measured after 24 hours in cultures with DMEM/10% FetalClone and 6 mM gln, 6 mM ala-gln or 20 mM gly-gln. Values are the means ± SE (n=2).

	Extracellular concentration (μM)			Intracellular concentration (μM)		
	Medium : DMEM/					
	gln	ala-gln	gly-gln	gln	ala-gln	gly-gln
ala	854 ± 37	3780 ± 323	307 ± 19	5940±621	9630±1280	3930±472
arg	161 ± 8[b]	171 ± 14	192 ± 14	127 ± 39	81 ± 31[c]	99 ± 27[c,d]
asn	52 ± 2	36 ± 2	0[a]	441 ± 58	149 ± 30	147 ± 32[d]
asp	0[a]	0[a]	0[a]	1300±166	697 ± 134	302 ± 111
glu	100 ± 2	94 ± 6	124 ± 9	5380±613	2490 ± 389	752 ± 125
gln	2100 ± 90	1710 ± 140	108 ± 8[b]	412 ± 62	113 ± 20	132 ± 25[d]
gly	328 ± 14	322 ± 21	1600 ± 65	8350±808	5420 ± 871	27600 ± 2840
his	121 ± 6	114 ± 8[b]	109 ± 7	309 ± 57	144 ± 24	170 ± 35[d]
ile	417 ± 22	404 ± 33	348 ± 27	577 ± 79	322 ± 45	489 ± 65
leu	371 ± 19	359 ± 26[b]	318 ± 20	1080±148	371 ± 65	588 ± 91
lys	461 ± 19	435 ± 28	472 ± 29[b]	823±110	302 ± 49	508 ± 87
met	103 ± 4	95 ± 7	104 ± 6	552 ± 73	180 ± 34	355 ± 61
phe	249 ± 10	238 ± 18[b]	243 ± 16	587 ± 79	258 ± 40	440 ± 62
ser	184 ± 9	171 ± 13	108 ± 9	1810±235	728 ± 156	1350 ± 192
thr	496 ± 22	475 ± 31	518 ± 33	2200±227	1300 ± 168	5080 ± 610
trp	50 ± 3	54 ± 7[b]	53 ± 4	132 ± 26	66 ± 18[c]	110 ± 21[c,d]
tyr	250 ± 12	242 ± 18	243 ± 16	762± 97	360± 65	748±113[c,d]
val	461 ± 24	437 ± 34[b]	410 ± 29	966 ±130	427 ± 62	724 ± 99
ala-gln		1260 ± 75			0[a]	
gly-gln			13500±360			0[a]

[a] Concentration was below the threshold of detection (<10 μM for amino acids and < 50 μM for dipeptides)

[b] The extracellular concentration is not significantly different from the intracellular according to a *t*-test comparison (P<0.05).

[c] The intracellular concentration is not significantly different from the intracellular concentration in DMEM/gln (P<0.05).

[d] The intracellular concentration is not significantly different from the intracellular concentration in DMEM/ala-gln (P<0.05).

4.3.2.4 Storage and stability of dipeptide media

The thermal stability of glutamine and dipeptides was examined by autoclaving 6 mM solutions of each for 20 minutes. Final glutamine concentrations were quantified by the glutaminase assay. Dipeptide concentrations were determined by incubating dipeptide solutions with cell lysates for 8 hours prior to performing the assay. Figure 4-8 shows that glutamine was almost completely degraded after autoclaving with only 10 % of the initial concentration remaining. However, the dipeptide concentrations were not significantly reduced by autoclaving. For both ala-gln and gly-gln, the final concentration was >95 % of the original, a change within the error limits of the assay system.

The decomposition of dipeptides and production of ammonium was compared with that of glutamine by incubation of culture media at 4 ^{o}C and 37 ^{o}C for 4 days (Figure 4-9). The ammonium concentration of glutamine-containing DMEM at 37 ^{o}C accumulated to 1.66 mM which represents 28 % decomposition of glutamine, assuming only deamidation has occurred. Glutamine degradation in the presence of serum or at 4 ^{o}C was reduced. Both dipeptides were much less ammoniagenic than glutamine. The production of ammonium from ala-gln, however, was significantly greater than from gly-gln in the presence of serum. The enhanced ammonium production due to serum, particularly at 37 ^{o}C, was presumably due to hydrolysis of the dipeptides by a serum derived peptidase. The presence of peptidase activity in serum is further illustrated by Figure 4-10. Fresh, cell-free culture medium supplemented with 10 % untreated or heat-treated FetalClone was incubated with 100 mM of each of the dipeptides while substrate was omitted from the control sample. After 24 hours, the increase in glutamine concentration was measured in each of the samples. In the absence of serum, the hydrolysis of dipeptide and release of glutamine was minor. The addition of serum resulted in the production of 0.47 mM and 0.37 mM glutamine in medium containing ala-gln and gly-gln, respectively. However, hydrolytic activity was reduced

markedly by heating the serum prior to incubation with the dipeptides (78 % for ala-gln and 82 % for gly-gln). This further suggests the presence of a heat-labile enzyme in the serum catalyzing dipeptide hydrolysis. The ideal storage conditions for the dipeptide-based media appear to be at 4 ^{o}C without serum supplementation to minimize hydrolysis of the dipeptide and the resulting breakdown of glutamine to ammonium.

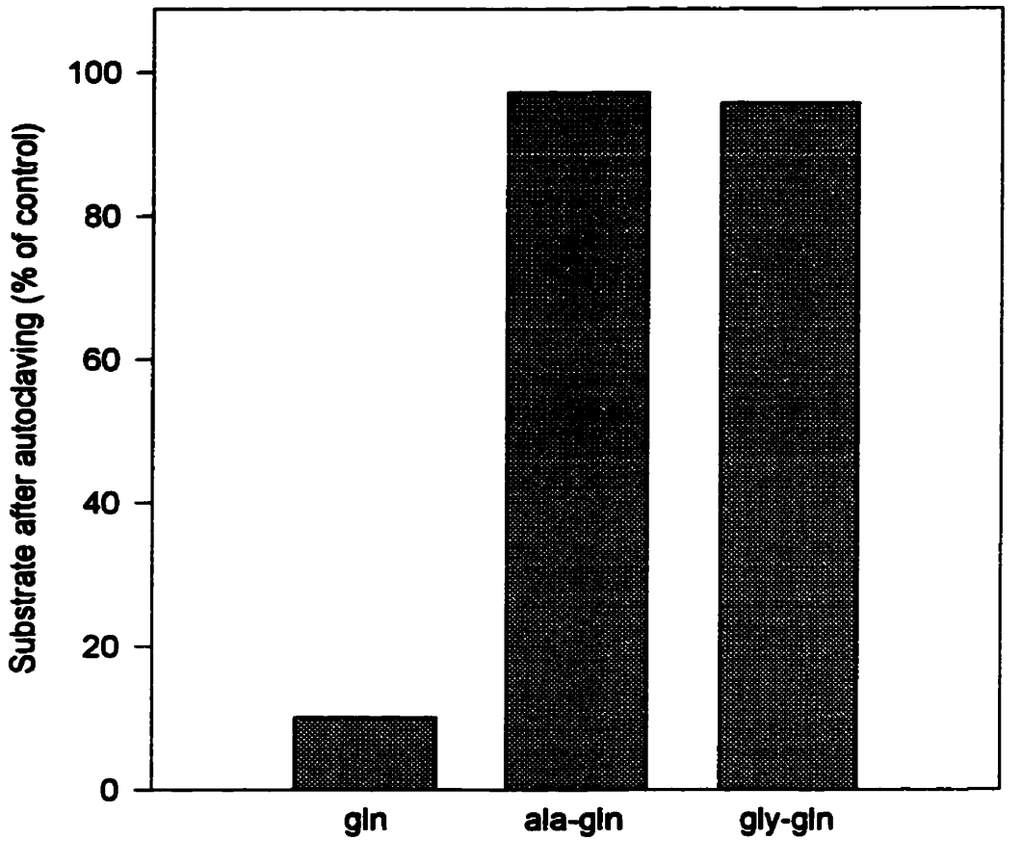


Figure 4-8. Effect of autoclaving on glutamine and dipeptides. Solutions of 6 mM glutamine, ala-gln and gly-gln were treated at 120 ^{o}C and 110 kPa for 20 minutes. Dipeptides were hydrolyzed by cell lysates and glutamine concentrations determined by enzyme assays. The error associated with the procedure was <10 %.

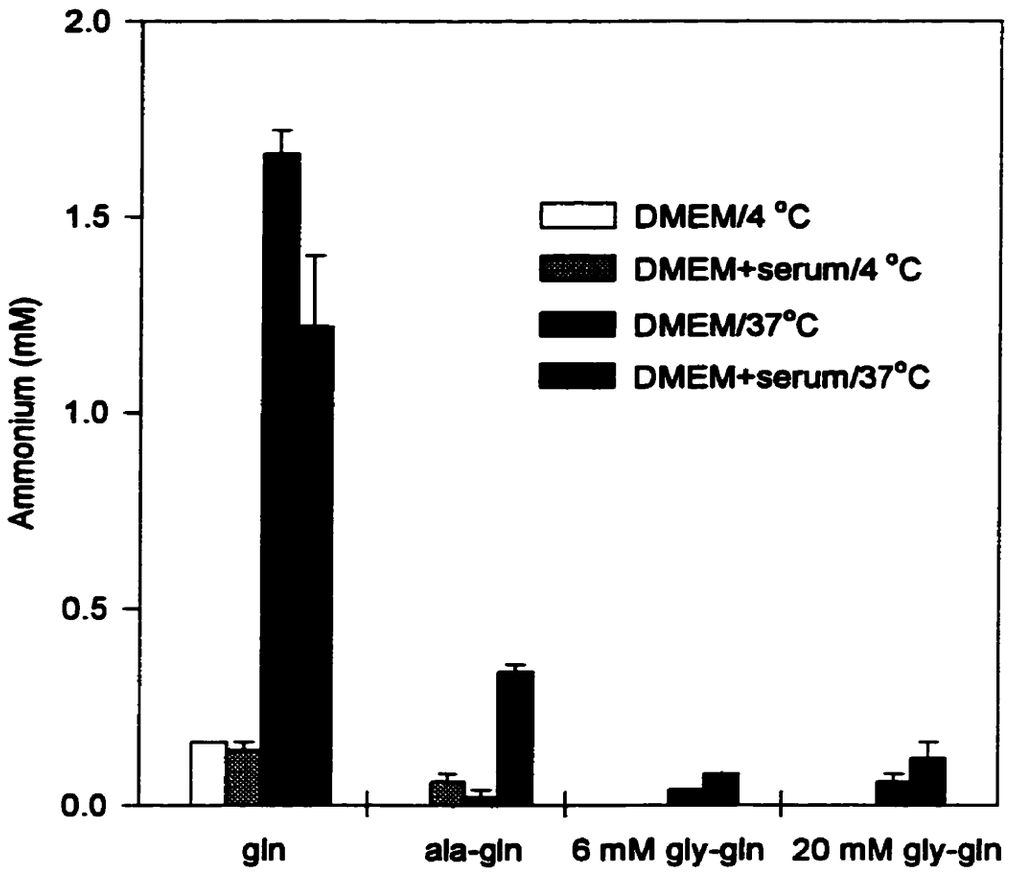


Figure 4-9. Chemical decomposition of glutamine, ala-gln and gly-gln. Culture media (25 ml DMEM with 20 mM HEPES, pH 7.4) with or without 10 % FetalClone and containing glutamine (6 mM), ala-gln (6 mM) or gly-gln (6 and 20 mM) were incubated at 4 °C or 37 °C in duplicate 75 cm^2 T-flasks (n=2). After 4 days, the ammonium concentration was measured and averages of duplicate flasks plotted ± SE.

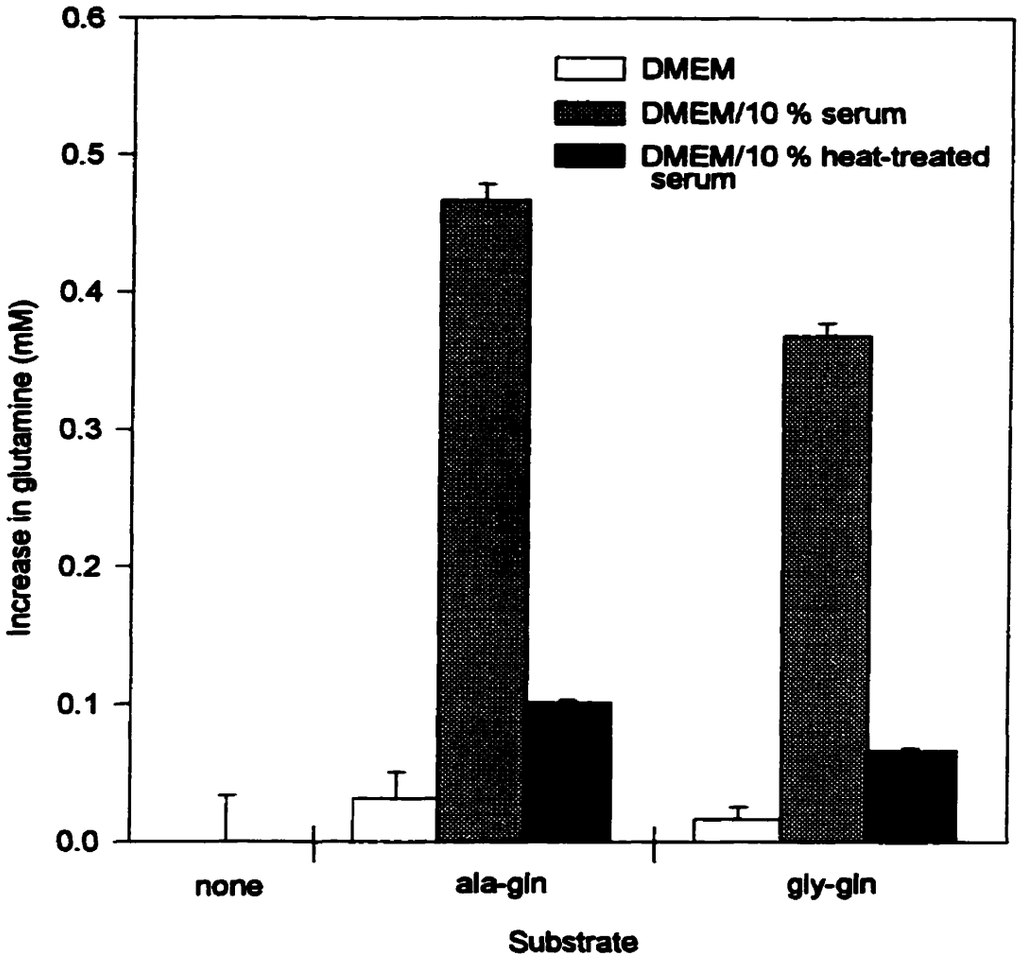


Figure 4-10. Peptidase activity in serum (FetalClone). DMEM with 100 mM ala-gln or 100 mM gly-gln was prepared containing untreated and heat-treated 10 % FetalClone. The dipeptide substrate was omitted from the control which contained only DMEM/10 % FetalClone. Each sample was added in duplicate (n=2) to the wells of a 96-well plate and incubated for 24 hours at 37 ^{o}C. The glutamine content of samples before and after the 24 hour incubation was measured by the glutaminase assay. The increase in glutamine concentration from duplicate wells was averaged and plotted ± SE.

4.4 Discussion

Although some cell lines can utilize glutamate in place of glutamine, adaptation to glutamate-based medium has proven variable among cultured cells. Some cell lines require higher initial glutamate concentrations and extended periods of adaptation to assume normal growth in the absence of glutamine (Hassell and Butler, 1990) while others are unable to adapt at all (McDermott and Butler, 1993). However, the limited growth of the CC9C10 hybridoma in the presence of both glutamine and glutamate (Figure 4-1) was unexpected. Inhibition by glutamate has not been widely characterized as a problem in the adaptation of cells to glutamate medium. Other studies, though, have described inhibition or toxicity of glutamate in culture and may also account for the observations in the present investigation.

Glutamate inhibition has been attributed to its interference with the uptake of cystine, another essential amino acid. Bannai and Kitamura (1980) described a unique transporter in human diploid fibroblasts specific for glutamate and cystine. Cystine uptake was competitively inhibited by glutamate and vice versa in this Na^+-independent carrier, designated System CG. The addition of high glutamate concentrations to the culture medium may thus result in a deficiency of cellular cystine. Depletion of cystine has been shown to result in cell death (Bannai *et al.*, 1977). Similar findings have been reported in other cell lines. Murphy *et al.* (1989) described a cys/glu antiporter in a neuronal hybridoma. Glutamate and cystine again demonstrated mutual competitive inhibition. Cytotoxic effects were observed at millimolar glutamate concentrations with 200 μM cystine present in the basal culture medium (DMEM). Glutamate cytotoxicity was also found to be more pronounced with decreasing cystine concentrations. Furthermore, the morphological changes associated with cellular degeneration prior to cell lysis in low cystine medium were indistinguishable from those induced by glutamate. Cell death apparently results from oxidative or free radical damage due to reduced production of glutathione (May and Gray, 1985 and Murphy *et al.*, 1989).

Cysteine, a precursor of glutathione, would be limited by the decreased uptake of cystine in the presence of glutamate.

Attempts to adapt the CC9C10 hybridoma to glutamate-based medium proved unsuccessful and may be the result, at least in part, of a cystine deficiency. Reducing glutamate in the medium, however, did not fully ameliorate the inhibitory effect. Furthermore, increasing the cystine concentration would be of little benefit because of its limited solubility in solution. The solubility of cystine in water is equivalent to only 0.47 mM at 25 ^{o}C and 0.99 mM at 50 ^{o}C (Windholz *et al.*, 1983). Thus, the use of dipeptides was investigated as an alternative strategy for reducing the glutamine content and preventing ammonium accumulation in the culture medium.

The ability of dipeptides to promote cell growth and normal mAb productivity was shown clearly for the CC9C10 cultures. Cell yields in the dipeptide media were slightly but significantly higher than the glutamine-based culture. This was most apparent in medium with the 20 mM gly-gln supplement where the cell yield was 14 % higher (Figure 4-2). Monoclonal antibody production was comparable in all three cultures (Figure 4-5). Human leukemia (Roth *et al.*, 1988), human lymphoma (Minamoto *et al.*, 1991) and Chinese hamster ovary (CHO) cells (Holmlund *et al.*, 1992) have all been grown successfully in ala-gln or gly-gln at a concentration of 2-4 mM. However, Minamoto *et al.* (1991) found poor cell yields when a murine hybridoma was grown in gly-gln at a concentration up to 3.5 mM. This was probably due to the low concentration of dipeptide and confirms the data in the present study that a higher concentration of gly-gln (20 mM) is required to obtain growth of murine cells. An insufficient gly-gln concentration was characterized by a considerable decrease in viability during the lag phase and reduced cell yields (Figures 4-2 and 4-3).

The effect of the dipeptides on cellular metabolism was analyzed from the pattern of substrate utilization and by-product formation. The glutamine concentration of the ala-gln and gly-gln cultures increased in the first day (Figure 4-6), presumably

due to dipeptide hydrolysis. This corresponded to a lag period of cell growth which could be prevented if a minimal concentration of glutamine was added initially (Figure 4-4). During the growth phase, the rate of glutamine release by dipeptide hydrolysis was offset by rapid cellular consumption. This resulted in a low glutamine concentration in gly-gln cultures until the stationary phase when the glutamine level began to increase. The low concentration of glutamine could be explained by a low dipeptide hydrolysis rate in the gly-gln cultures which, in turn, resulted in a reduced specific rate of ammonium production (60 % reduction compared to the control containing glutamine) (Table 4-1). In addition, the total ammonium produced after 164 hours in the 20 mM gly-gln culture was 22 % lower than the glutamine control (Figure 4-6). This can be compared with a glutamine-limited fed-batch culture in which a low concentration of glutamine (<0.5 mM) was maintained (Ljunggren and Häggström, 1990). In this report, the total glutamine utilized by Sp2/0 murine myeloma cells was reduced and the final ammonium concentration was 50 % lower than an equivalent batch culture with an initial glutamine concentration of 4 mM.

The dipeptides had a significant effect on the glucose consumption of CC9C10 cells. Although the total glucose utilized was similar in all three cultures (Figure 4-7), the specific consumption rate during the exponential phase was 13 % and 50 % lower in ala-gln and gly-gln medium, respectively (Table 4-1). This phenomenon may be explained by the need for an increased glycolytic flux in glutamine-supplemented cultures to sequestrate the higher intracellular ammonium. Further evidence for this hypothesis is the significantly lower (57 %) specific production of alanine in the gly-gln grown CC9C10 cultures (Table 4-1). The alanine may be generated by transamination of glycolytically derived pyruvate as required for growth at higher glutamine concentrations or in the presence of added ammonium (Hassell and Butler, 1990 and Butler *et al.*, 1991).

Significant decreases also occurred in the specific rates of consumption of amino acids in dipeptide media, particularly for the six amino acids arg, ile, leu, lys, thr and val which showed a pattern of rapid utilization (Table 4-1). This decrease was especially noticeable in the gly-gln culture and to a lesser extent in ala-gln medium compared to the glutamine-based control. In addition, the intracellular concentration of the 12 amino acids asn, asp, glu, gln, his, ile, leu, lys, met, phe, ser and val were reduced in dipeptide cultures (Table 4-2). No specific explanations are offered for this but it does indicate how a change in the availability of a major substrate can alter the overall utilization pattern of the cells. The intracellular analysis of metabolites also revealed no traces of dipeptides which is consistent with an extracellular hydrolysis prior to uptake and will be discussed further in the following chapter.

The stability of dipeptides has been recognized as a major advantage for their use in cell culture media. Thus, heat sterilization or long-term storage of dipeptide-based serum-free media at 37 ^{o}C has no deleterious effects on growth promotion (Minamoto *et al.*, 1991 and Roth *et al.*, 1988). The stability of ala-gln and gly-gln was confirmed in the present study (Figure 4-8), although some ammonium was produced in dipeptide media stored in the presence of serum, particularly at 37 ^{o}C (Figure 4-9). This may be attributed to the presence of peptidase activity in serum (Figure 4-10).

The dipeptides have proven an acceptable replacement for glutamine in murine hybridoma cultures. Slightly higher cell concentrations were achieved in gly-gln which may, in part, be due to the reduced ammonium accumulation in the cultures. Cell growth and productivity may be further enhanced for hybridoma lines demonstrating a greater degree of sensitivity to ammonium.

Chapter 5

Characterization of Peptidase Activity and the Mechanism of Dipeptide Utilization

5.1 Introduction

The dipeptides ala-gln and gly-gln are effective substitutes for glutamine in CC9C10 cultures. However, relatively little is known about how the dipeptides are utilized by hybridomas. Minamoto *et al.* (1991) described an aminopeptidase activity in a murine hybridoma with a higher specificity for ala-gln than gly-gln. This accounted for the poor growth observed in relatively low concentrations of gly-gln. However, a more thorough characterization of the peptidase and the mechanism of dipeptide utilization was necessary. This would provide a more complete explanation for the differences in ala-gln and gly-gln concentrations required to support growth of the hybridomas.

The investigation of the peptidase began with studies to determine the cellular location of the activity in CC9C10 cells. The specificity and kinetic properties of peptidase were subsequently examined in the cell fraction containing the highest specific activity. The mechanism of dipeptide utilization was explored by attempts to localize dipeptide hydrolysis to either the intra- or extracellular environment. This involved an assay for the release of peptidase into the culture medium. The nature of peptidase expression in CC9C10 was also investigated to discern if activity was constitutive or adaptation was required and induced by the dipeptides. Finally, some of the factors which influence peptidase activity were examined to identify conditions which could potentially limit growth in dipeptide medium.

5.2 Materials and methods

5.2.1 Cell fractionation

CC9C10 lysates were prepared based on a method described by Howell *et al.* (1992). Cells were grown to mid-exponential phase in DMEM/ala-gln, washed with D-PBS and suspended in 5 mM Tris-HCl (pH 7.4). Cells (~3 X 10^8) were lysed with 12 passes of a cooled Potter-Elvehjem homogenizer. The resulting homogenate was fractioned by three centrifugation steps at 4 ^oC: 3 000 g for 10 minutes, 31 000 g for 90 minutes and 100 000 g for 90 minutes. The pellets (P1-P3) were washed with D-PBS and resuspended in 50 mM HEPES, pH 7.4. The supernatant (S1-S3) and pellet (P1-P3) fractions were stored at -70 ^oC prior to analysis in protein and enzyme assays. The S2 (microsomal/cytosolic) fraction was passed through a 0.2 μm filter before use. This fraction was used in experiments to characterize the peptidase enzyme.

5.2.2 Peptidase assays

5.2.2.1 Peptidase activity in cell fractions

For the standard assay of peptidase activity in cell fractions, 0.05 mg/ml protein was incubated with 2 mM dipeptide in 50 mM HEPES buffer, pH 7.4, in a total volume of 250 μl. The incubations were for 1 hour at 37 ^oC in 96-well microtitre plates. The reaction was stopped by the addition of an equal volume of cold 10 % TCA. The precipitated proteins were pelleted in a microfuge at 14 000 r.p.m. for 10 minutes at 4 ^oC. Supernatants were neutralized with an equal volume of 0.32 M Tris, pH 10.5. The rate of reaction was determined from measurements of the formation of free glutamine quantified by the glutaminase assay or OPA derivitization and HPLC analysis (section 2.11.4).

5.2.2.2 Substrate specificity of peptidase activity

The specificity of the peptidase for various dipeptide substrates was determined using the standard assay system with a minor modification. Ala-gln, gly-gln, gly-glu, glu-trp and gly-D-phe were each incubated at a concentration of 6 mM with 0.05 mg/ml lysate protein (S2 fraction). Hydrolysis of dipeptides after the incubation was evaluated by HPLC analysis.

5.2.2.3 Determination of peptidase V_{max} and K_m

V_{max} and K_m values with respect to ala-gln or gly-gln were determined by varying the dipeptide concentration (0-100 mM), keeping the S2 fraction protein concentration constant at 0.025 mg/ml. The reaction was performed in 100 µl PBS/50 mM HEPES, pH 7.4, for 1 hour at 37 ^{o}C. Reactions were stopped and neutralized as described above and assayed for glutamine with the glutaminase assay. V_{max} and the Michaelis constant were determined by fitting the rate data to a hyperbolic equation using SigmaPlot.

5.2.2.4 Peptidase activity in conditioned media

Peptidase activity in conditioned media was measured from cells cultured in DMEM/gln, DMEM/ala-gln and DMEM/gly-gln (20 mM). Cells were seeded at ~1 X 10^{6}/ml and incubated 24 hours. Samples of the cultures before and after the growth period were centrifuged at 200 g, and the supernatants filtered through a 0.2 µm filter to remove remaining cells or membranous material. Samples (150 µl) from each of the different types of media were diluted 1:1 (v/v) with PBS/100 mM HEPES (pH 7.4) with no further additions or with 200 mM ala-gln or gly-gln and incubated 24 hours at 37 ^{o}C. Reactions were stopped, neutralized and assayed for glutamine with the glutaminase assay.

5.2.2.5 Peptidase activity in adapted and non-adapted cells

S2 fractions were prepared from cells adapted to dipeptides (grown in DMEM/ala-gln or DMEM/gly/gln for ≥6 passages) and non-adapted cells (grown in DMEM/gln). Peptidase assays were performed according to the standard method for cell fractions described previously except the lysate protein concentration from each of the different types of media was only 0.015 mg/ml. Glutamine was measured by HPLC analysis after incubation with each of the ala-gln and gly-gln substrates.

5.2.2.6 Influence of media components and metal ions on peptidase activity

The effects of DMEM and its various components on the activity of the peptidase was determined. S2 lysate fractions (0.05 mg/ml) were incubated with 2 mM gly-gln in D-PBS, DMEM or D-PBS containing 2X MEM amino acids, 2X MEM non-essential amino acids, 4X MEM vitamins or a combination of these amino acids and vitamin solutions. The 50X MEM amino acids (Gibco, 320-1130) and 100X vitamins (Gibco, 320-1120) stocks were diluted 1/25 in the PBS resulting in concentrations of most amino acids and vitamins comparable to those in DMEM. The 100X MEM non-essential amino acids (Gibco, 320-1140) were diluted 1/50 in the PBS. Glucose, sodium bicarbonate, $CaCl_2$ and phenol red were added to the PBS in concentrations equivalent to DMEM and the pH of final solutions adjusted to 7.4. After a 24 hour incubation, glutamine release was determined by HPLC analysis.

The effects of EDTA and various metal ions on peptidase activity were also examined. The S2 lysates (0.05 mg/ml) were incubated in D-PBS/2mM gly-gln (pH 7.4) with 4 μM EDTA or 0.2 mM $FeNO_3$, $CaCl_2$, $MgSO_4$, $CuSO_4$ or $ZnSO_4$. The lysates were also pre-incubated for 2 hours at room temperature with 0.2 mM EDTA before addition to the PBS/2 mM gly-gln with the metal ions. This resulted in a final EDTA concentration of 0.02 mM in the reaction mixture while the metal ions were in 10-fold excess at 0.2 mM. Reactions were allowed to proceed for 24 hours at 37 ^{o}C. Glutamine

concentrations were then determined by HPLC analysis. Activity was expressed as a percentage of glutamine produced in each sample relative to the control containing only PBS/substrate and lysate.

5.3 Results

5.3.1 Cellular location of the peptidase

The location of the peptidase activity required for hydrolysis of ala-gln and gly-gln was investigated by differential centrifugation of the cell lysate. Three pellet fractions were characterized as follows: P1 (nuclei and plasma membrane sheets), P2 (smaller organelles and plasma membrane fragments) and P3 (all other non-cytosolic material). These fractions and corresponding supernatant fractions (S1-S3) were tested for peptidase activity by their ability to hydrolyze ala-gln and gly-gln to free amino acids. The results (Table 5-1) showed that there was minimal enzymatic activity in the pellet fractions compared to supernatant fractions. Ultracentrifugation at 100 000 g eliminates all membranous material to leave only cytosolic components in supernatant sample S3, which showed the highest overall specific activity (0.605 μmol per mg protein per minute). This indicates that the peptidase activity is not membrane bound but is present in the cytosol.

In further experiments to characterize the enzyme, the S2 fraction was used. This was the microsomal/cytosolic fraction with relatively high specific activity and which could be prepared easily.

5.3.2 Specificity and kinetics of the peptidase activity

The specificity of the peptidase activity was investigated by incubating the S2 cell lysate fraction with various dipeptides. Of five dipeptides tested, those containing glutamine showed the highest activity (Table 5-2). The rate of ala-gln hydrolysis was

nearly 2 X compared to gly-gln at the substrate concentration (6 mM) used in this assay.

The peptidase activity was further characterized with respect to ala-gln and gly-gln by measurements of rates of hydrolysis at varying substrate concentrations (0-100 mM). The enzyme showed typical Michaelis-Menten kinetic behavior with respect to either substrate (Figure 5-1). The apparent V_{max} and K_m values were determined by curve fitting of the data to a hyperbolic equation (Table 5-3). The K_m is at least an order of magnitude higher for gly-gln (14.0 mM), indicating that the peptidase has a substantially higher affinity for ala-gln (K_m = 1.21 mM), although the V_{max} is higher for gly-gln.

The relatively higher affinity of the peptidase for ala-gln compared to gly-gln could indicate why a higher concentration of gly-gln is required to maintain normal cell growth. From the Michaelis-Menten plot (Figure 5-1), it can be shown that the rate of hydrolysis of ala-gln at 6 mM is equivalent to the rate of hydrolysis of gly-gln at 21 mM under the described assay conditions. These dipeptide concentrations were found to support normal growth rates of CC9C10 cells.

Table 5-1. Hydrolytic activity in fractions of CC9C10 isolated by differential centrifugation. Protein from each fraction (0.05 mg/ml) was incubated with 2 mM ala-gln or gly-gln. Glutamine produced was measured by the glutaminase assay or HPLC analysis after a 1 hour incubation at 37 °C. Specific hydrolytic activity is expressed as the μmoles dipeptide hydrolyzed per mg of cellular protein per minute. Values are the means ± SE (n=2).

Fraction	Specific hydrolytic activity (μmol mg^{-1} min^{-1})	
	ala-gln	gly-gln
P1	0.007 ± 0.002	0[a]
P2	0.090 ± 0.007	0.028 ± 0.001
P3	0[a]	0[a]
S1	0.468 ± 0.018	0.133 ± 0.002
S2	0.552 ± 0.023	0.155 ± 0.007
S3	0.605 ± 0.030	0.192 ± 0.020

[a] Hydrolytic activity was below the threshold of detection (<0.003 μmol mg^{-1} min^{-1})

Table 5-2. Specificity of peptidase activity in CC9C10 cell lysate. Each dipeptide (6 mM) was incubated with 0.05 mg/ml of lysate protein from the S2 fraction. Following a 1 hour incubation at 37 °C, hydrolysis to individual amino acids was determined by HPLC analysis and used to express the specific activity. Values are the means ± SE (n=2).

Dipeptide	Specific rate of hydrolysis (μmol mg^{-1} min^{-1})
ala-gln	0.742 ± 0.043
gly-gln	0.382 ± 0.002
gly-glu	0.068 ± 0.002
glu-trp	0.243 ± 0.001
gly-D-phe	0[a]

[a] Rate of hydrolysis was below the threshold of detection (<0.003 μmol mg^{-1} min^{-1})

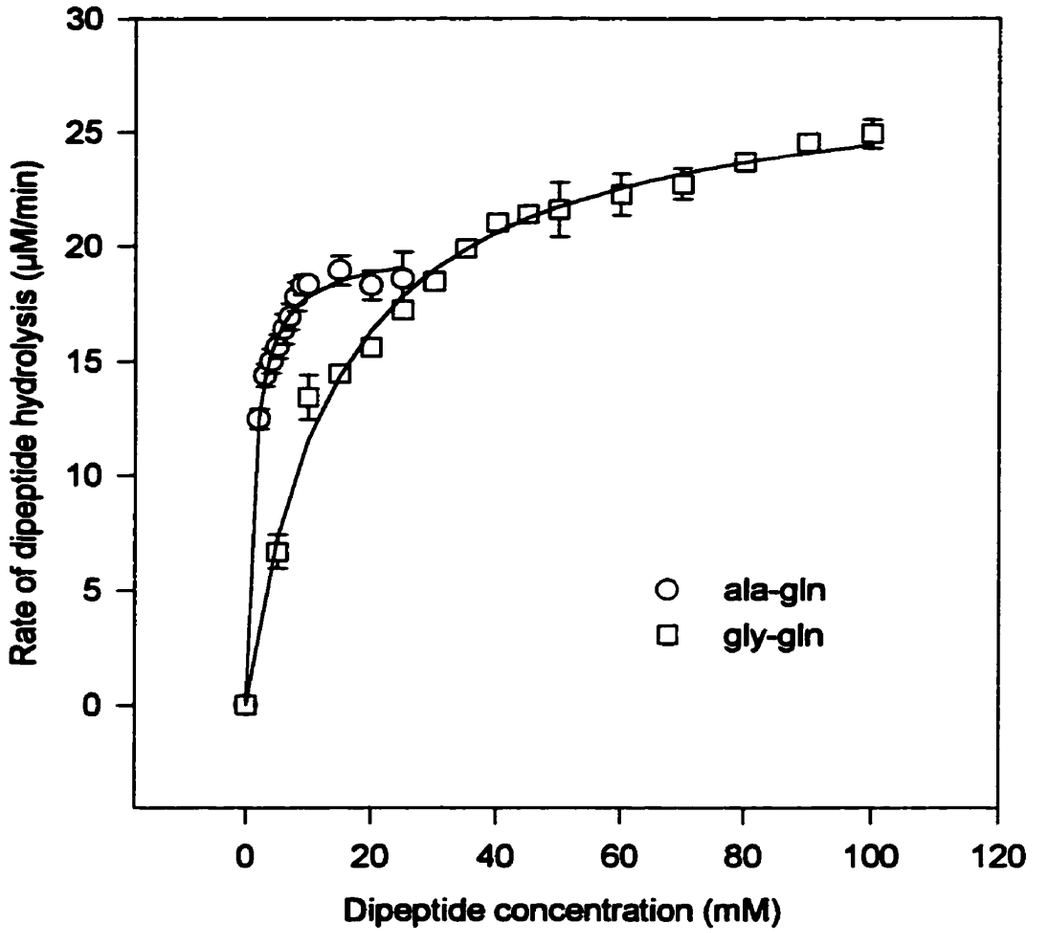


Figure 5-1. Michaelis-Menten plot of peptidase activity in CC9C10 cell lysate. The rate of ala-gln (O) or gly-gln (□) hydrolysis was determined at various substrate concentrations (0-100 mM) incubated with 0.025 mg/ml protein from the S2 fraction. After a 1 hour incubation at 37 ^{o}C, glutamine release was determined by the glutaminase assay. Values are means ± SE (n=3).

Table 5-3. V_{max} and K_m values for CC9C10 lysate peptidase hydrolysis of ala-gln and gly-gln. A curve fitting program (SigmaPlot) was used to fit the rate data (Figure 5-1) to a hyperbolic equation and determine the constants ± SE.

Substrate	V_{max} (µmol min^{-1})[a]	K_m (mM)
ala-gln	0.799 ± 0.027	1.21 ± 0.25
gly-gln	1.114 ± 0.049	14.0 ± 2.3

[a] Rate of hydrolysis is expressed per mg of protein

5.3.3 Peptidase activity in conditioned media

Peptidase activity was assayed in culture medium conditioned by cell growth in order to substantiate the possibility of extracellular hydrolysis of the dipeptides. In this experiment, cell-free media was isolated following a 24 hour incubation of cells (10^6/ml) in DMEM/gln, DMEM/ala-gln and DMEM/gly-gln. These samples of conditioned media were then tested for peptidase activity by incubation with 100 mM added ala-gln or gly-gln for 24 hours after which the change in glutamine concentration was determined. Controls without addition of substrate showed little increase in glutamine concentration since the dipeptide available for hydrolysis from the medium alone was relatively low.

The results of this experiment (Table 5-4) showed significant peptidase activity in all conditioned media samples even from DMEM/gln cultures, with a maximum increase of 2.77 mM glutamine. This indicates that the enzyme is released into the supernatant during culture. However, the activity in the conditioned media was estimated to be a small fraction of the maximum activity available in the original culture:

1. The maximum specific activity available was based on a V_{max} value for ala-gln of 0.799 μmol min^{-1} mg^{-1} (Table 5-3) and a protein content determined to be 0.116 mg/10^6 cells (in the S2 fraction).

$$\text{maximum activity} = 0.799\ \mu\text{mol min}^{-1}\ \text{mg}^{-1} \times 0.116\ \text{mg}\ (10^6\ \text{cells})^{-1}$$

$$= 9.3 \times 10^{-2}\ \mu\text{mol min}^{-1}\ (10^6\ \text{cells})^{-1}$$

Note that the maximum activity determined from an S2 fraction was considered representative of that present in an unfractioned sample. This is because a V_{max} value would be smaller but the cellular protein content higher in the unfractioned preparation. Thus, applying the calculation described above, an unfractioned sample should yield a similar maximum activity of ~9.3×10^{-2} μmol min^{-1} $(10^6$ cells$)^{-1}$.

2. The highest level of dipeptide hydrolysis in conditioned media occurred in DMEM/gln using ala-gln as a substrate. Table 5-4 indicates that 2.77 mM ala-gln was hydrolyzed in the 150 µl sample volume used in the assay during the 24 hour incubation. This was used to determine the specific activity in the conditioned medium.

µmoles of ala-gln hydrolyzed $= 2.77 \text{ mmol } L^{-1} \text{ day}^{-1} \times 150 \times 10^{-6} \text{ L} \times (1 \times 10^{3} \text{ µmol/mmol})$

$= 0.416 \text{ µmol day}^{-1}$

number of cells in sample volume $= 1 \times 10^{6} \text{ cells/ml} \times 0.150 \text{ ml}$

$= 1.5 \times 10^{5} \text{ cells}$

specific activity in conditioned medium $= (0.416 \text{ µmol day}^{-1} / 1.5 \times 10^{5} \text{ cells})$

$= 2.77 \times 10^{-6} \text{ µmol day}^{-1} \text{ cell}^{-1}$

$= 1.9 \times 10^{-3} \text{ µmol min}^{-1} (10^{6} \text{ cells})^{-1}$

3. The peptidase activity released by the cells into the culture medium was expressed as a percentage of the maximum available.

$$\text{\% activity in conditioned medium} = \frac{(1.9 \times 10^{-3} \text{ µmol min}^{-1} (10^{6} \text{ cells})^{-1})}{(9.3 \times 10^{-2} \text{ µmol min}^{-1} (10^{6} \text{ cells})^{-1})} \times 100$$

$= 2.0 \%$

The glutamine produced in the other conditioned medium samples was less than that from cells grown in DMEM/gln. Thus, the activity in conditioned media was ≤ 2 % of the maximum content in CC9C10 cells.

Table 5-4 also shows that peptidase hydrolysis in conditioned media was significantly higher (up to 3.1 X) for ala-gln as a substrate compared to gly-gln. This would seem to contradict data from the kinetic assay and the apparent higher V_{max} with respect to gly-gln. This might be explained by the presence of components in conditioned media which interfere with the peptidase activity, particularly when gly-gln is the substrate. This explanation is further substantiated by the lower glutamine produced in DMEM/gly-gln (20 mM) cultures compared to DMEM/ala-gln (6 mM) (Figure 4-6), even though the rates of hydrolysis of the two substrates at these concentrations

are equivalent in D-PBS (Figure 5-1). It is also noteworthy that the measured peptidase activity in serum would account for only a portion of the dipeptide hydrolysis observed in the conditioned media. In DMEM with 10 % FetalClone, 0.47 mM and 0.37 mM glutamine was produced when incubated for 24 hours at 37 ^{o}C with 100 mM ala-gln and gly-gln, respectively (Figure 4-10).

Table 5-4. Change in glutamine concentration following incubation of conditioned growth media (DMEM/gln, DMEM/ala-gln or DMEM/gly-gln) without dipeptide or with 100 mM ala-gln or gly-gln. After the 24 hour incubation at 37 °C, glutamine was determined by the glutaminase assay and values expressed as means of duplicate samples (n=2) ± SE. An increase in glutamine concentration is indicated as a positive (+) value while a decrease is shown as a negative (-) value.

	Change in glutamine concentration (mM)		
Substrate	DMEM/gln	DMEM/ala-gln	DMEM/gly-gln
none	-0.20 ± 0.07	+0.26 ± 0.05	+0.18 ± 0.08
ala-gln	+2.77 ± 0.41	+2.37 ± 0.17	+1.88 ± 0.07
gly-gln	+0.88 ± 0.28	+1.06 ± 0.07	+0.71 ± 0.13

5.3.4 Expression of peptidase activity

To determine if the peptidase was constitutively expressed or inducible, activity was measured in cells grown in dipeptides for numerous passages and those cultured in glutamine-containing medium. Both ala-gln and gly-gln were used as substrates for the enzyme in lysate protein (S2 fraction) derived from cells grown in each of the different types of media. Figure 5-2 shows that the specific activities of the peptidase isolated from cells grown in DMEM/ala-gln and DMEM/gly-gln were comparable. However, peptidase activity from cells grown in DMEM/gln was significantly higher than that from cells derived from the dipeptide cultures (by at least 21 % and 58 % when ala-gln and gly-gln were the substrates, respectively). The rate of hydrolysis of the gly-gln substrate was found to be lower than that for ala-gln, for each of the different lysates, as would be expected in the presence of the low dipeptide concentration (2 mM). These results indicate that CC9C10 cells grown in the absence of dipeptides express the peptidase constitutively. Consequently, murine hybridomas are likely to require minimal adaptation prior to the substitution of glutamine with an appropriate concentration of either of the dipeptides.

5.3.5 Factors affecting peptidase activity

The reduced hydrolysis of gly-gln compared to ala-gln in the conditioned medium experiment suggested that DMEM may contain components that interfere with peptidase activity. This possibility was investigated further by examining the hydrolysis of gly-gln in PBS containing additions of amino acids and vitamins that approximated the concentration of most components in DMEM. Figure 5-3 shows the activity (measured by production of glutamine) in DMEM was lower (by >70 %) compared to the PBS control. The presence of MEM amino acids in PBS resulted in a 28 % decrease in hydrolysis of the dipeptide while non-essential amino acids and vitamins alone had little effect. However, the combination of MEM essential and non-essential amino acids and

vitamins yielded a 47 % drop relative to the control. This was less than observed in DMEM but nevertheless demonstrates the presence of components in complete medium that interfere with the activity of the peptidase.

To determine if the peptidase had any obvious cofactor requirements, the effect of EDTA and various metal ions on the enzyme activity was studied. Figure 5-4 shows low concentrations of EDTA (4 μM) abolished the hydrolysis of 2 mM gly-gln by the cell lysate. In addition, copper and zinc ions acted as inhibitors of the peptidase, completely eliminating activity at concentrations of 0.2 mM. This suggested that the peptidase was a metalloprotein and that copper and zinc may displace the normal metal cofactor. Thus, the cell lysate was pre-incubated with EDTA (0.2 mM) before addition to the reaction buffer (PBS/2 mM gly-gln) containing different metal ions. Only ferric ion was found to partially restore peptidase activity to a significant degree. These results indicate the availability of iron in the culture medium and minimal copper and zinc concentrations may be important criteria for growth in dipeptide media.

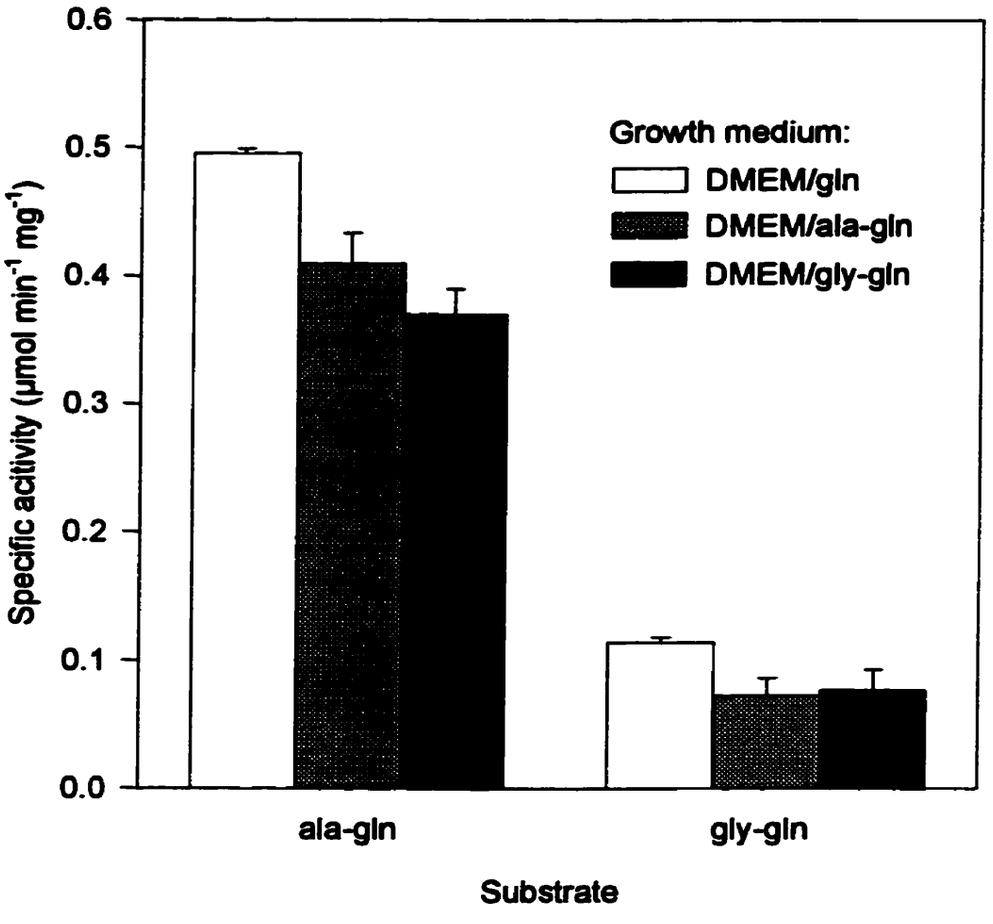


Figure 5-2. Peptidase activity in CC9C10 cells cultured in glutamine medium and adapted to dipeptide media. Lysates from cells (0.015 mg/ml protein from the S2 fraction) grown in DMEM/gln, DMEM/ala-gln and DMEM/gly-gln were incubated with 2 mM ala-gln or gly-gln as substrate for 1 hour at 37 °C. Glutamine was determined by HPLC analysis and specific activity calculated. Values plotted are means ± SE (n=2).

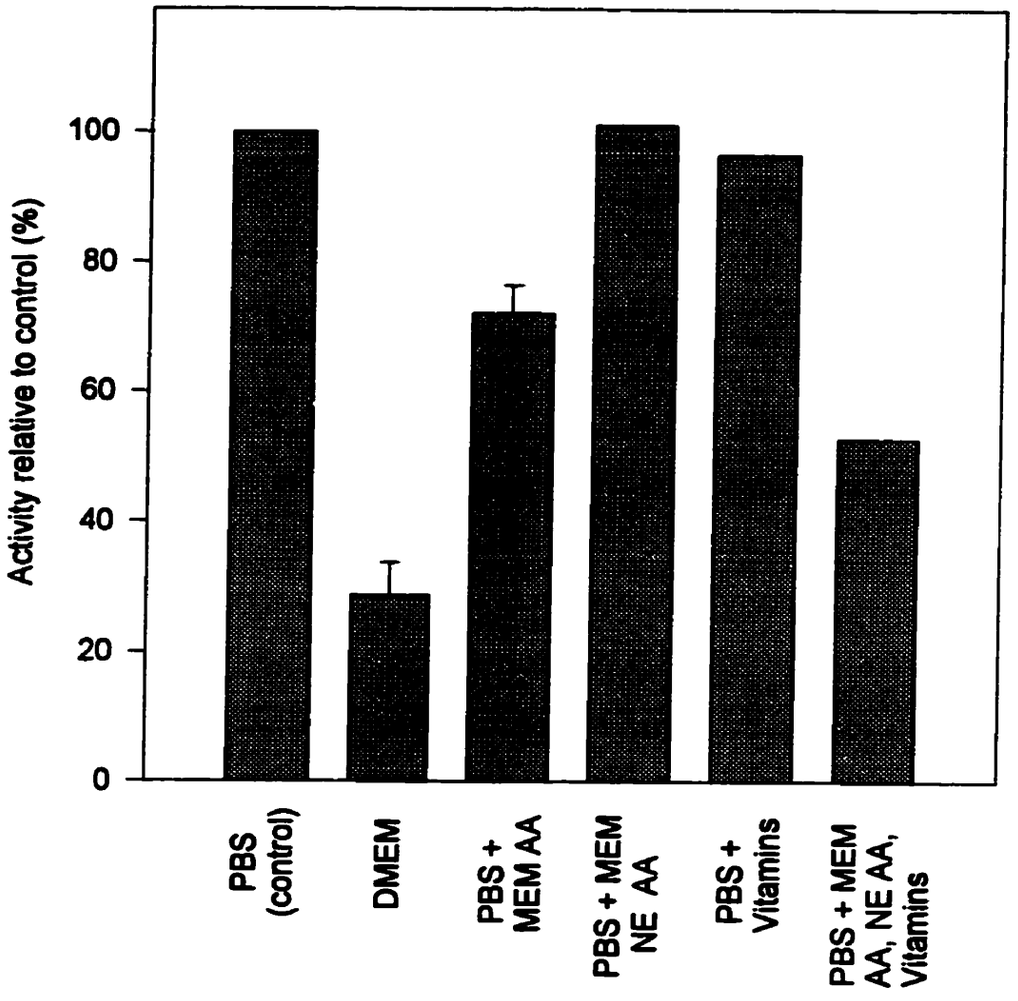


Figure 5-3. The effect of DMEM and its components on peptidase activity. S2 lysate fractions from CC9C10 cells were incubated with 2 mM gly-gln substrate in D-PBS, DMEM or PBS containing 2X MEM amino acids (MEM AA), 2X MEM non-essential amino acids (MEM NE AA), 4X MEM vitamins or a combination of the amino acid and vitamin solutions. Glutamine production was measured by HPLC analysis after the 24 hour incubation and expressed relative to that of the PBS control. Values plotted are means ± SE (n=2).

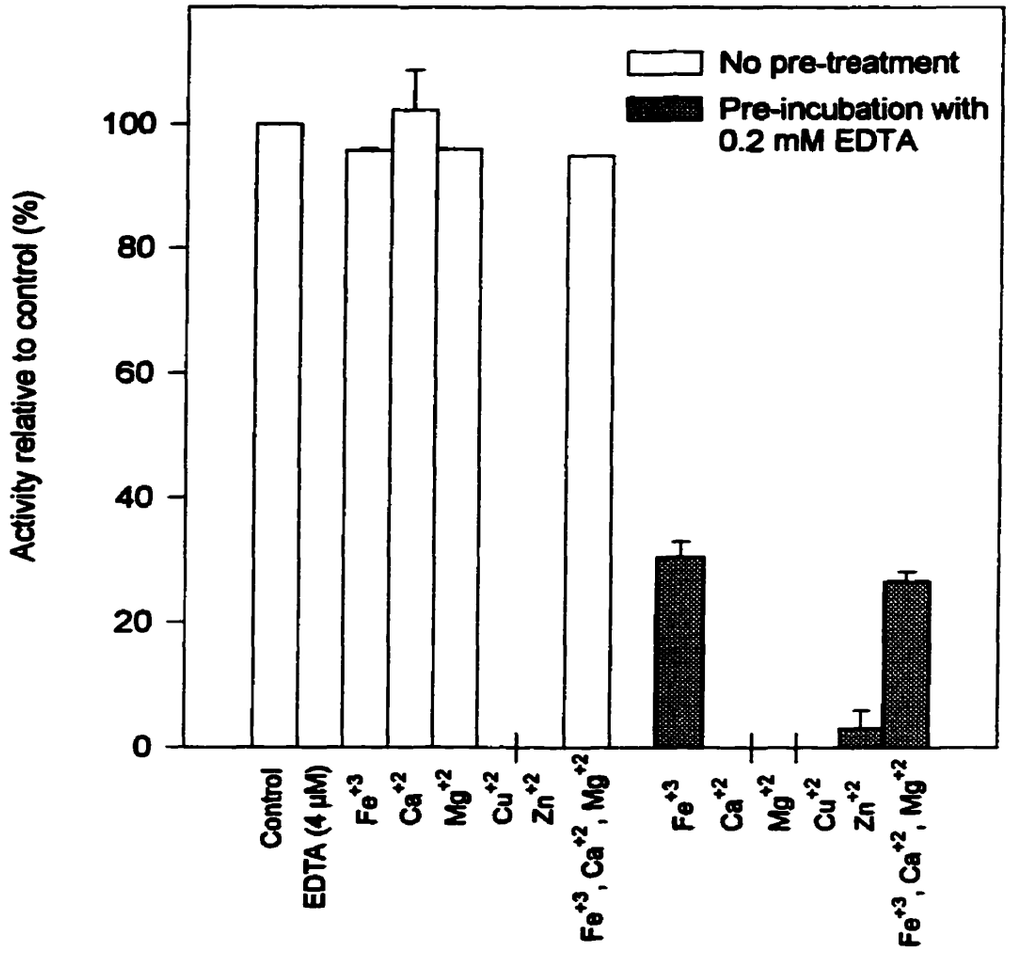


Figure 5-4. The effect of EDTA and metal ions on peptidase activity. CC9C10 lysates (S2 fraction) were incubated with 2 mM gly-gln in PBS containing additions of 4 µM EDTA or 0.2 mM $FeNO_3$, $CaCl_2$, $MgSO_4$, $CuSO_4$, $ZnSO_4$ or a combination of the iron, calcium and magnesium salts (0.2 mM of each). Lysates were also pre-incubated with 0.2 mM EDTA prior to the addition to the reaction buffer containing substrate and the metal ions (resulting in a final EDTA concentration of 0.02 mM). Activity was determined by the glutamine produced (measured by HPLC) after the 24 hour incubation period and expressed relative to the control containing only PBS/substrate and lysate. Values represent the average ± SE (n=2).

5.4 Discussion

The mechanism by which murine hybridomas utilize dipeptides has not been adequately characterized in the literature and was thus investigated in the present study. Evidence for hydrolysis of the dipeptides was indicated by the rapid increase of glutamine in the medium following cell inoculation in dipeptide media (Figure 4-6). Also, significantly higher levels of alanine and glycine were found both extra- and intracellularly following incubation in DMEM/ala-gln and DMEM/gly-gln, respectively (Table 4-2). This suggested the presence of peptidase activity although three possible mechanisms of dipeptide utilization were possible: (a) dipeptide is transported intact into the cell and metabolized intracellularly by a peptidase; (b) dipeptide is hydrolyzed extracellularly by a plasma membrane-bound peptidase; or (c) dipeptide is hydrolyzed extracellularly by a peptidase released into the medium.

The first mechanism would require dipeptide transport through the cell membrane. Dipeptide transport has been reported in cells of the intestine (Ganapathy *et al.*, 1984 and Takuwa *et al.*, 1985) and kidney (Daniel *et al.*, 1991). However, cell types from sources including skeletal muscle, liver and red blood cells have been found to lack a peptide transport system (Lochs *et al.*, 1990). Evidence against dipeptide transport as a major mechanism of utilization in the CC9C10 cultures is implied by: (a) the substantial rate of hydrolysis of the dipeptides in the culture medium and (b) the inability to detect intact dipeptides in cell lysates following a 24 hour incubation in dipeptide-based media (Table 4-2). Thus, the possibility of dipeptide transport in CC9C10 cells could only be entertained if hydrolysis were to occur extremely rapidly following uptake.

The second mechanism suggested above is that the dipeptide is hydrolyzed extracellularly by a plasma membrane bound enzyme. Cell surface peptidases have been observed and characterized in human colon cell lines (Howell *et al.*, 1992). However, the differential centrifugation of CC9C10 lysates showed low activity of

peptidase in cellular pellet fractions P1 and P2 which would be expected to contain membrane (Table 5-1). In fact, the highest specific peptidase activity was found in the final supernatant fraction S3, which is representative of the cytosol. The peptidase enzyme found in CC9C10 may be related to the aminopeptidase activity which has been described recently in the human T-cell lymphoma line, Jurkat (Murray *et al.*, 1994). A high proportion (89 %) of the aminopeptidase of Jurkat cells was found in the cytosol and had distinct properties from a previously reported cell surface aminopeptidase in lymphoid tissue (Bowes and Kenny, 1987).

The significant peptidase activity found in cell-free culture media which had been conditioned by incubation with cells for 24 hours (Table 5-4) provides experimental data which weighs heavily in support of the third mechanism of dipeptide utilization; that the dipeptides are hydrolyzed by an enzyme released extracellularly. This may occur by secretion from viable cells or by lysis of a proportion of the cell population. The possibility of cell lysis cannot be ruled out because of the significant lag phase that occurs following inoculation and the substantial loss in viability of the dipeptide grown cells during this phase (Figure 4-3).

The kinetic properties of the peptidase enzyme were analyzed from a cell lysate fraction (S2). Clearly, the peptidase has a high affinity for dipeptides containing glutamine (Table 5-2). Furthermore, the peptidase has the highest affinity for ala-gln as is evident by the ten-fold lower K_m value compared to gly-gln (Table 5-3). In fact, the kinetic data indicates that the rate of hydrolysis of gly-gln at 21 mM was equivalent to the rate of ala-gln at 6 mM. These were also the approximate dipeptide concentrations that allowed successful growth of the CC9C10 cells in culture. The indicated dipeptide concentrations should also replace glutamine without an extended period of adaptation as the peptidase activity was constitutively expressed in the hybridoma (Figure 5-2). In fact, higher enzyme activity was observed in lysates from cells grown in DMEM/gln.

The presence of factors in culture medium which affect peptidase activity was suggested by the differential rates of increase in glutamine concentration in DMEM/ ala-gln (6 mM) and DMEM/gly-gln (20 mM) (Figure 4-6). Further evidence for this comes from the higher rate of ala-gln hydrolysis in conditioned media at a high substrate concentration (100 mM) (Table 5-4). This is apparently contradictory to the effect observed in the assay system with PBS/HEPES buffer in which gly-gln has a higher V_{max} (Figure 5-1). Peptidase activity in DMEM was indeed found to be lower than in PBS buffer and MEM amino acids were identified as one of the group of medium components that may inhibit the enzyme (Figure 5-3). The activity of the enzyme may additionally be affected by metal ions in the culture medium. The peptidase appears to require iron as a cofactor and is inhibited by copper and zinc (Figure 5-4).

Studies with the CC9C10 hybridomas has demonstrated that high cell yields can be obtained with murine hybridoma cultures containing either ala-gln or gly-gln providing the concentrations are optimal for extracellular hydrolysis by a peptidase derived from the cytosol. The optimal dipeptide concentrations, though, may differ with cell type. Furthermore, the type and composition of the medium may influence the dipeptide requirements. The use of gly-gln is particularly effective in reducing ammonium accumulation in cultures. However, the lower specificity of the enzyme for this dipeptide, especially in culture medium, warrants careful determination of the optimal concentration when used as a replacement for glutamine with other cell lines.

Chapter 6

Development and Optimization of Low-Ammoniagenic Medium for Growth of BHK Cells

6.1 Introduction

The CC9C10 cell line responded favorably to the replacement of glutamine with the less ammoniagenic dipeptides. However, the improvement in growth with dipeptide media may have been limited by the nature of the cell line which exhibited only a moderate sensitivity to ammonium. BHK cells were considered more ideally suited to examine the effects of low-ammoniagenic medium on cell growth and metabolism. One would predict that BHK cells, which have been established as more sensitive to ammonium, would respond to an even greater extent with enhanced growth when lower levels of the inhibitor are produced. Investigating this possibility first required the development of the low-ammoniagenic medium that supports the growth of BHK cells. The method employed focused on the reduction of glutamine and replacement with dipeptides, glutamate and other supplements. Glutamate has previously been used as a replacement for glutamine in BHK cultures (Hassell and Butler, 1990). The present study extends the earlier investigations and more closely analyzes the most effective combination of substitutes for glutamine. Low-ammoniagenic medium was optimized to support maximal growth rates and cell yields in batch cultures. This provided a foundation for experiments in the subsequent chapter where the growth, metabolism and adaptation of cells in low-ammoniagenic medium were investigated in stationary and microcarrier cultures.

6.2 Materials and methods

6.2.1 Culture media and growth conditions

The medium used to examine the growth of BHK cells was DMEM/10 % calf serum with 1.2 mM serine and other supplements added as specified in each

experiment. The glutamine requirements were first assessed by adding glutamine to this medium in a range of concentrations and analyzing the effect on growth. The initial stage in the development of the low-ammoniagenic medium involved substitution of glutamine directly with dipeptides or glutamate. Prior to the growth experiments using dipeptides as a substitute, cells were allowed to adapt to ala-gln or gly-gln for at least 6 passages. Cells were more gradually adapted to glutamate by first subculturing cells into medium with a high glutamate and a low glutamine concentration and reducing the levels of each after sequential passages. Thus, cells were subcultured 2 passages each in medium with 1 mM, 0.5 mM and then 0 mM glutamine with 20 mM glutamate in all cases. Glutamate was then reduced to 10 mM, 6 mM and as low as 3 mM, with 2 passages at each concentration. The subculture ratio was normally 1:19 with each passage. The effects of ammonium, aspartate and asparagine on growth in glutamate medium were investigated without prior adaptation. The most effective combination was established to reduce the glutamine or dipeptide requirements in the glutamate-based medium. For all experiments, cells were grown in 24-well plates under the standard culture conditions defined in section 2.4.

6.2.2 Cell enumeration and evaluation of growth performance

Total cell concentrations were determined with the haemocytometer or Coulter counter (section 2.8). Cultures with different supplements were grown in at least duplicate samples in 24-well plates and replicate counts averaged. Growth performance was typically evaluated by the specific growth rate (μ) (section 2.9). The cell yield after ~3 days was also considered. At this point, cultures were normally within the exponential phase and cell viabilities remained high. Cell yields were expressed as cells/ml based on a medium volume of 1 ml/well.

6.2.3 Analysis of media components

Glutamine and glutamate concentrations were determined by HPLC analysis of OPA derivatized samples (section 2.11.4.2). Ammonium was measured with the gas-sensing electrode (section 2.11.2).

6.3 Results

6.3.1 Glutamine requirements of BHK cells

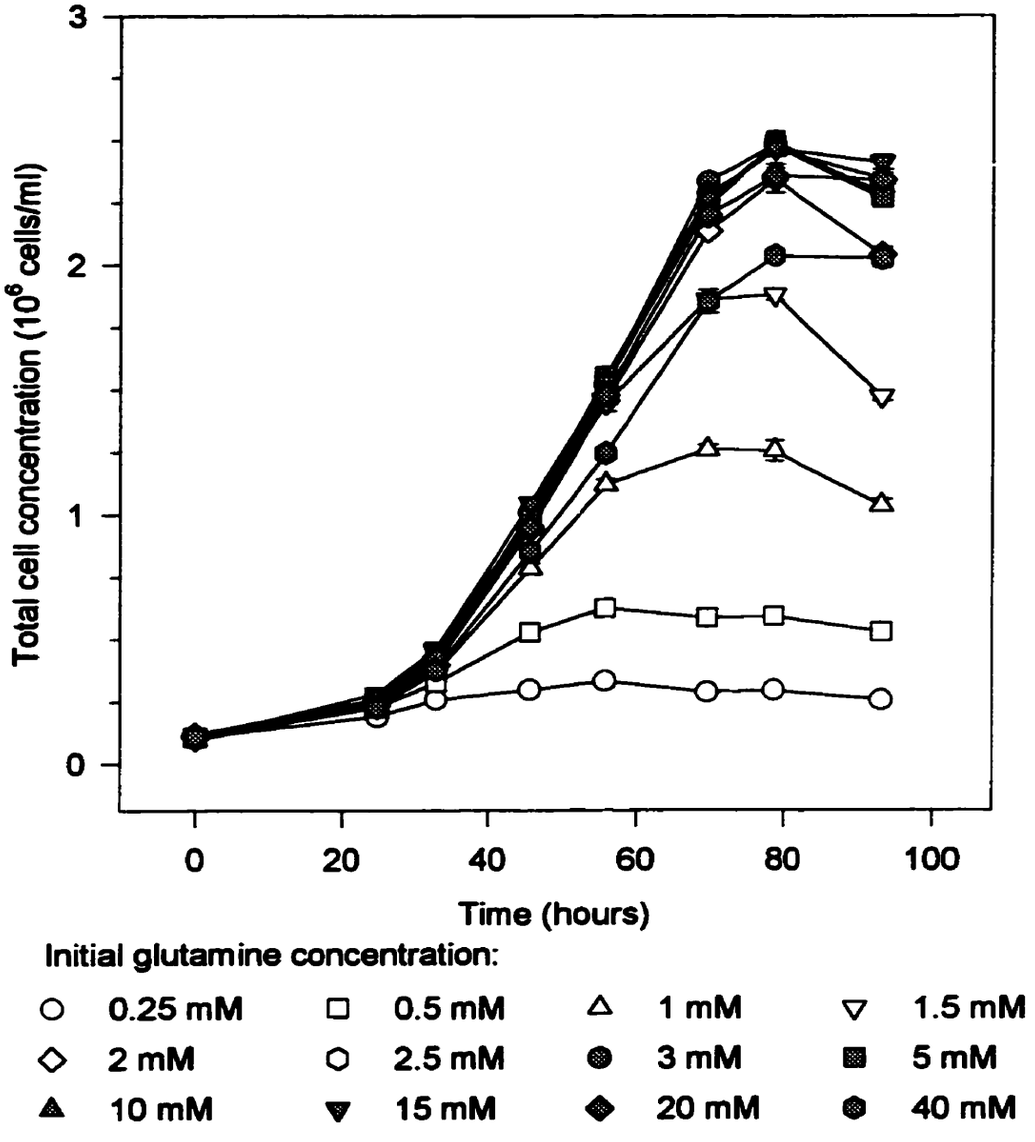
Glutamine is a vital component in standard culture media but is unstable in solution and decomposes to produce pyrrolidone carboxylic acid and ammonium. Consequently, glutamine should not be included in the medium at concentrations beyond that required to support the growth of the cells in order to prevent unnecessary accumulation of ammonium. Thus, the glutamine requirements of BHK cells in batch culture were characterized prior to growing cells in media with a modified complement of glutamine.

BHK cells were grown in a range of glutamine concentrations from 0.25 to 40 mM. Figure 6-1a illustrates that the cells were dependent on glutamine as minimal growth was observed in the presence of low concentrations (≤0.5 mM). High growth rates were achieved at glutamine levels as low as 1.5 mM although cells prematurely entered the stationary phase. A glutamine concentration of 2-3 mM supported both high growth rates and cell yields. Very high glutamine additions (40 mM) clearly resulted in reduced growth. The glutamine concentrations required to promote optimal growth are more clearly depicted in figure 6-1b. The specific growth rate and cell yield after ~3 days increased rapidly with glutamine additions up to 1.5-2.0 mM while further improvements were not realized beyond 3 mM glutamine. In fact, glutamine concentrations above 5 mM resulted in a gradual decline in the growth rates and cell concentrations.

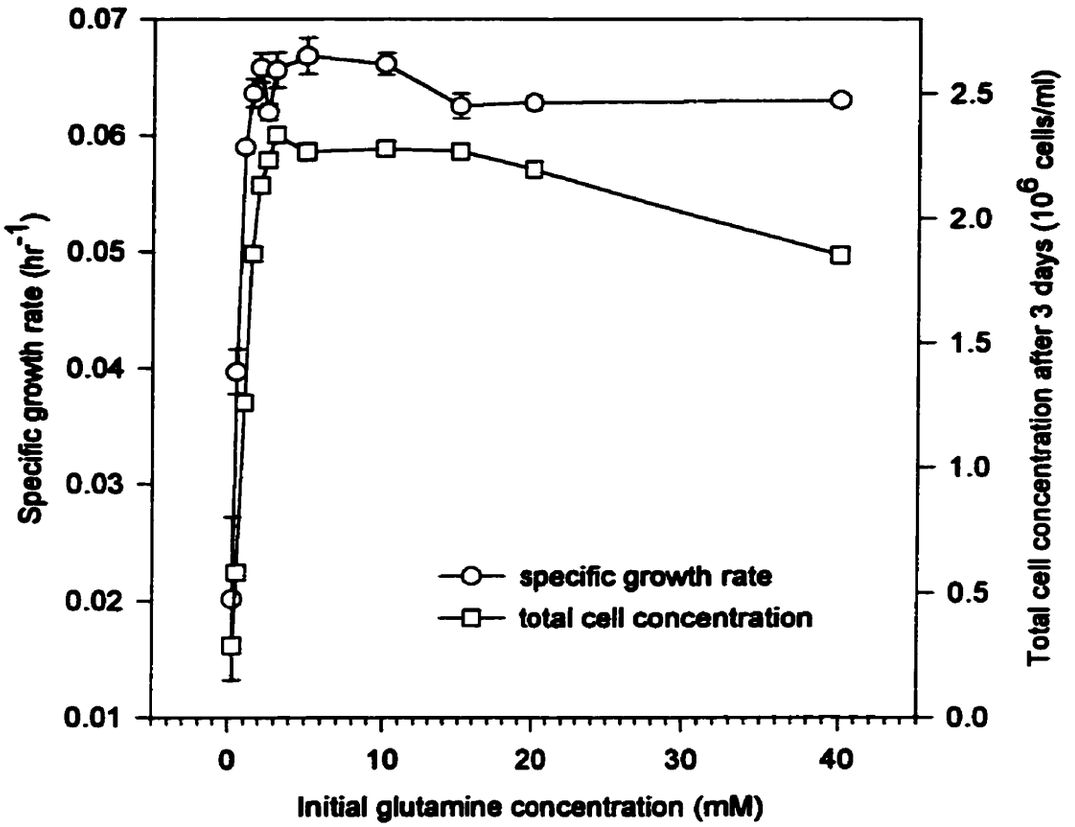
The minimum effective glutamine level was also established by determination of the glutamine and ammonium concentrations after ~3 days of culture. Figure 6-1c indicates glutamine was completely consumed after this period in medium containing initial concentrations <2 mM. The depletion of an essential nutrient explains the early onset of stationary phase in cultures with low initial glutamine levels (≤1.5 mM). The reduced growth at very high glutamine concentrations, meanwhile, can be attributed to the accumulation of inhibitory ammonium levels. Figure 6-1d depicts the ammonium after ~3 days of incubation for cultures and cell-free medium with equivalent initial glutamine concentrations. In the 40 mM glutamine culture, 8.1 mM ammonium was produced. This was clearly sufficient to cause the 4 % reduction in specific growth rate and the 21 % decrease in cell concentration after 3 days compared to the culture with 3 mM glutamine in which only 2.1 mM ammonium accumulated. It was also obvious from the cell-free medium that an increasing initial glutamine concentration resulted in greater ammonium levels from chemical decomposition of the amino acid. Thus, the proportion of ammonium produced due to the degradation of glutamine increased compared to that arising from cellular metabolism. By comparing BHK cultures and cell-free medium with equivalent initial glutamine concentrations, the proportion of ammonium arising from the chemical decomposition of glutamine was determined. This value increased from 41% to 72 % with initial glutamine concentrations of 3 and 40 mM, respectively. Thus, a glutamine concentration of 3 mM allowed optimal growth and minimized ammonium production from decomposition in batch cultures of BHK cells.

Figure 6-1a-d. Growth, glutamine utilization and ammonium accumulation in BHK cultures with various initial glutamine supplements. (a) Growth of cells in medium with 0.25-40 mM glutamine as indicated in the legend. Cells were cultured in duplicate wells (n=2) of 24-well plates at each concentration. Samples were counted by Coulter counter and each point represents the average of replicate counts at each glutamine concentration ± SE. (b) The effect of glutamine concentrations on specific growth rate and cell yield. The specific growth rate (○) was determined during the exponential phase of growth at each glutamine concentration from 25-46 hours and plotted ± SE. Total cell concentrations (□) are shown after 70 hours (~3 days) of growth for each glutamine concentration and points represent the average of counts ± SE. (c) Glutamine concentrations in the cultures after ~3 days of growth with different initial additions of glutamine. Glutamine was measured after 70 hours in the duplicate samples and the average plotted ± SE. (d) Ammonium concentrations measured after 70 hours for duplicate samples of cultures (○) and in cell-free medium (●). The average ammonium concentrations were plotted ± SE.

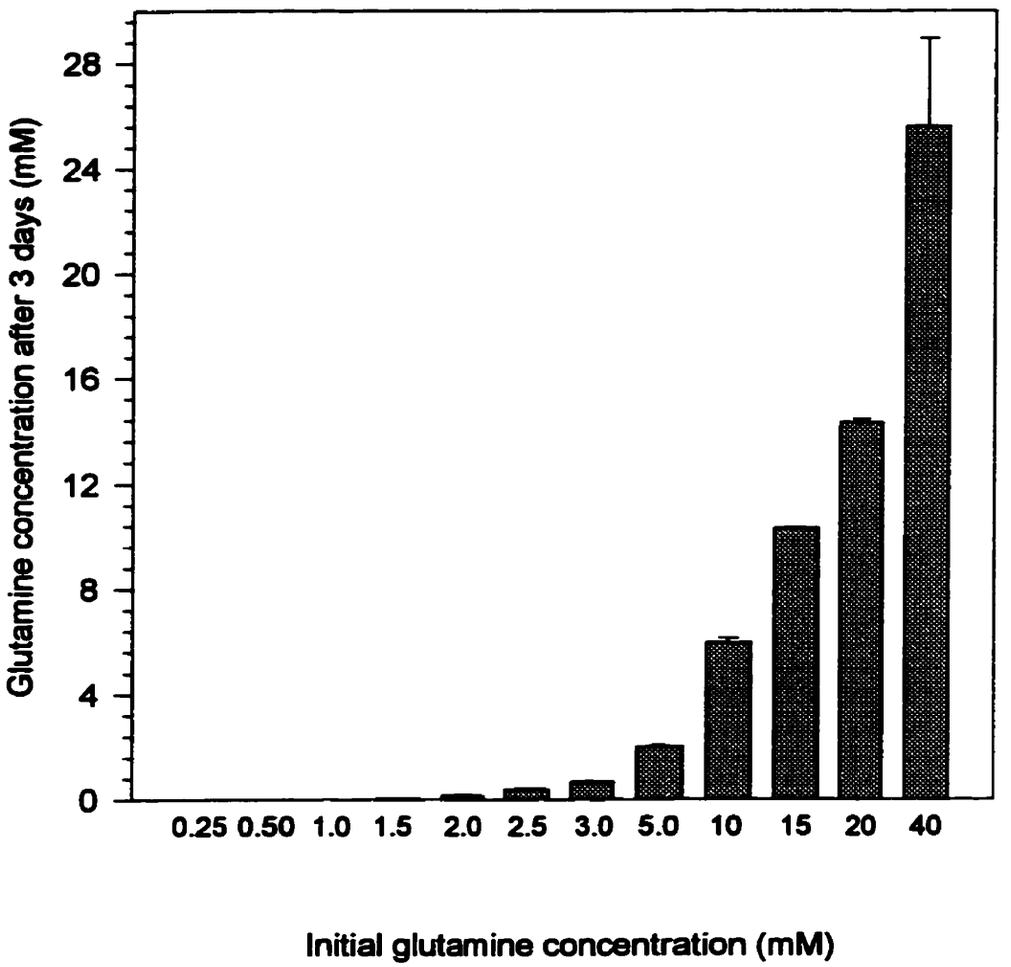
(a)


Total cell concentration (10^6 cells/ml)
3
2
1
0
0
20
40
60
80
100
Time (hours)
Initial glutamine concentration:
0.25 mM
0.5 mM
1 mM
1.5 mM
2 mM
2.5 mM
3 mM
5 mM
10 mM
15 mM
20 mM
40 mM

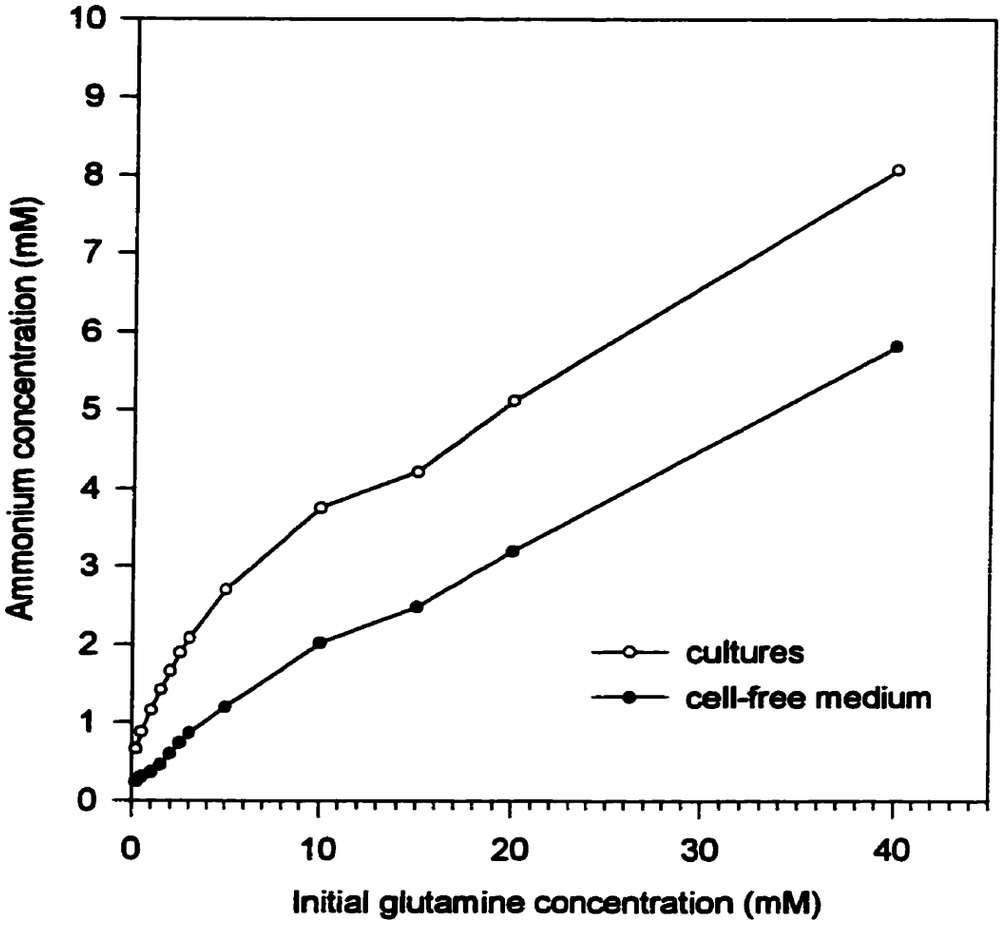
(b)


0.07
0.06
0.05
0.04
0.03
0.02
0.01
Specific growth rate (hr-1)
2.5
2.0
1.5
1.0
0.5
0.0
Total cell concentration after 3 days (10^6 cells/ml)
specific growth rate
total cell concentration
0
10
20
30
40
Initial glutamine concentration (mM)

(c)


Glutamine concentration after 3 days (mM)
0
4
8
12
16
20
24
28
0.25 0.50 1.0 1.5 2.0 2.5 3.0 5.0 10 15 20 40
Initial glutamine concentration (mM)

(d)


Ammonium concentration (mM)
Initial glutamine concentration (mM)
cultures
cell-free medium
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40

6.3.2 BHK growth in dipeptide media

Dipeptides were investigated as possible replacements for glutamine in BHK cultures. Studies with the CC9C10 hybridoma revealed that growth was influenced by the dipeptide concentration in the medium. Consequently, BHK cells were grown with a range of ala-gln and gly-gln concentrations to determine if a minimal level of each was required to promote optimal growth performance.

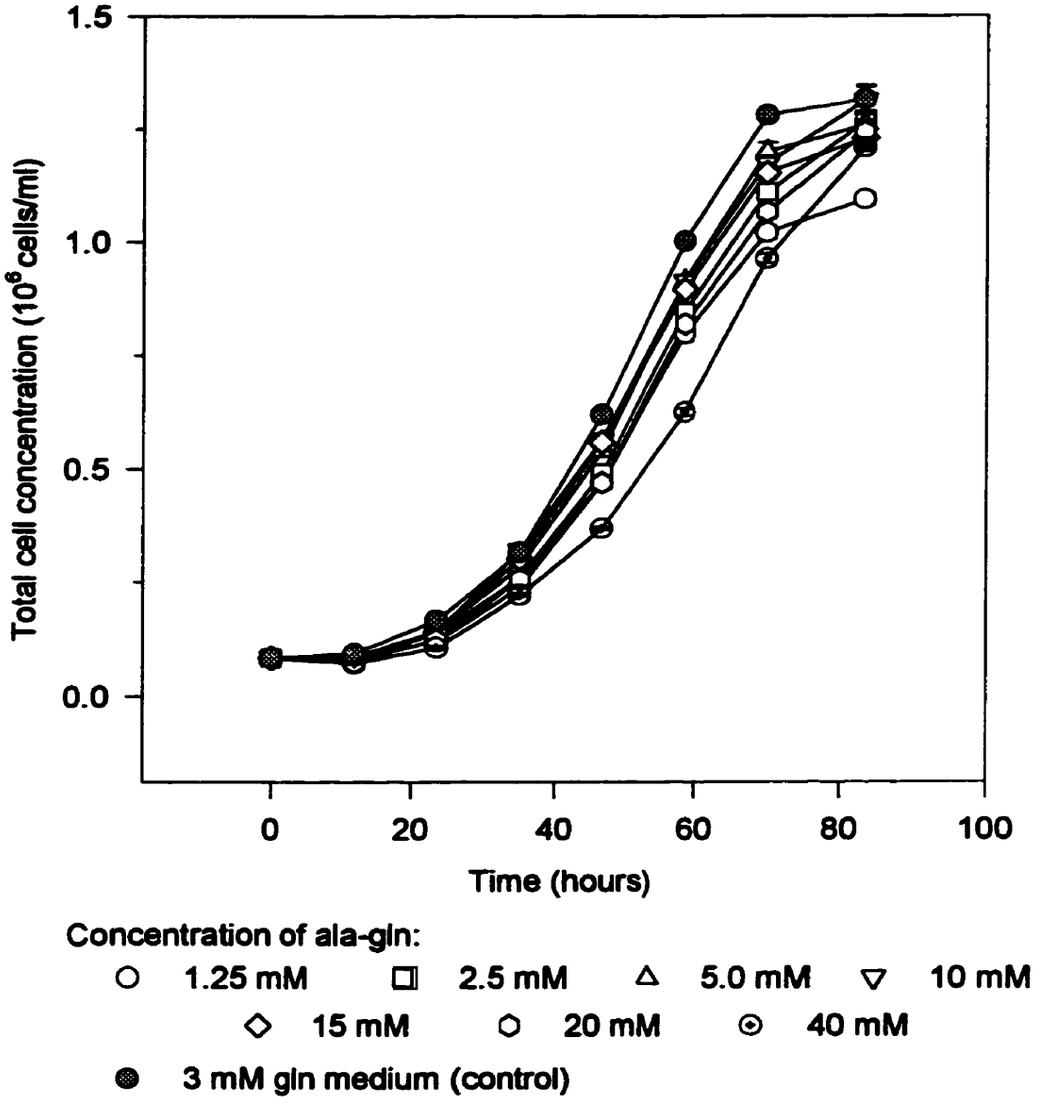
Glutamine was replaced with ala-gln concentrations ranging from 1.25 to 40 mM and growth compared to a control containing 3 mM glutamine. Figure 6-2a demonstrates after ~3 days, the cell yield was significantly higher in the DMEM/gln culture compared to those supplemented with ala-gln. The cell yield increased with ala-gln concentrations up to 5 mM but further improvements were not observed beyond this concentration. In fact, very high ala-gln levels (40 mM) resulted in an apparently slower growth rate, perhaps due to increased medium osmolarity.

The media from cultures with varying ala-gln concentrations were analyzed to determine if the use of this dipeptide resulted in lower glutamine in the medium and, consequently, lower ammonium production. As shown in figure 6-2b, glutamine in the cultures after ~3 days increased with higher ala-gln levels in the medium. With 1.25 mM ala-gln, glutamine remained <0.37 mM throughout the culture period and was eventually almost depleted. This would account for the reduced cell densities at this ala-gln concentration. Glutamine remained low in 2.5 mM ala-gln as well, ranging from 0.50-0.65 mM after the first 24 hours of culture. However, this concentration was apparently not so low as to limit growth to the same extent as the 1.25 mM culture. With 5 mM dipeptide, glutamine further increased to a range of 0.60-1.70 mM after the first day and appears to supply sufficient glutamine throughout the culture period to ensure both high growth rates and cell concentrations. Ala-gln from 10-20 mM resulted in a further jump in glutamine available in the medium and was similar in each of these cultures, ranging from 0.70-3.20 mM for most of the culture duration. As expected,

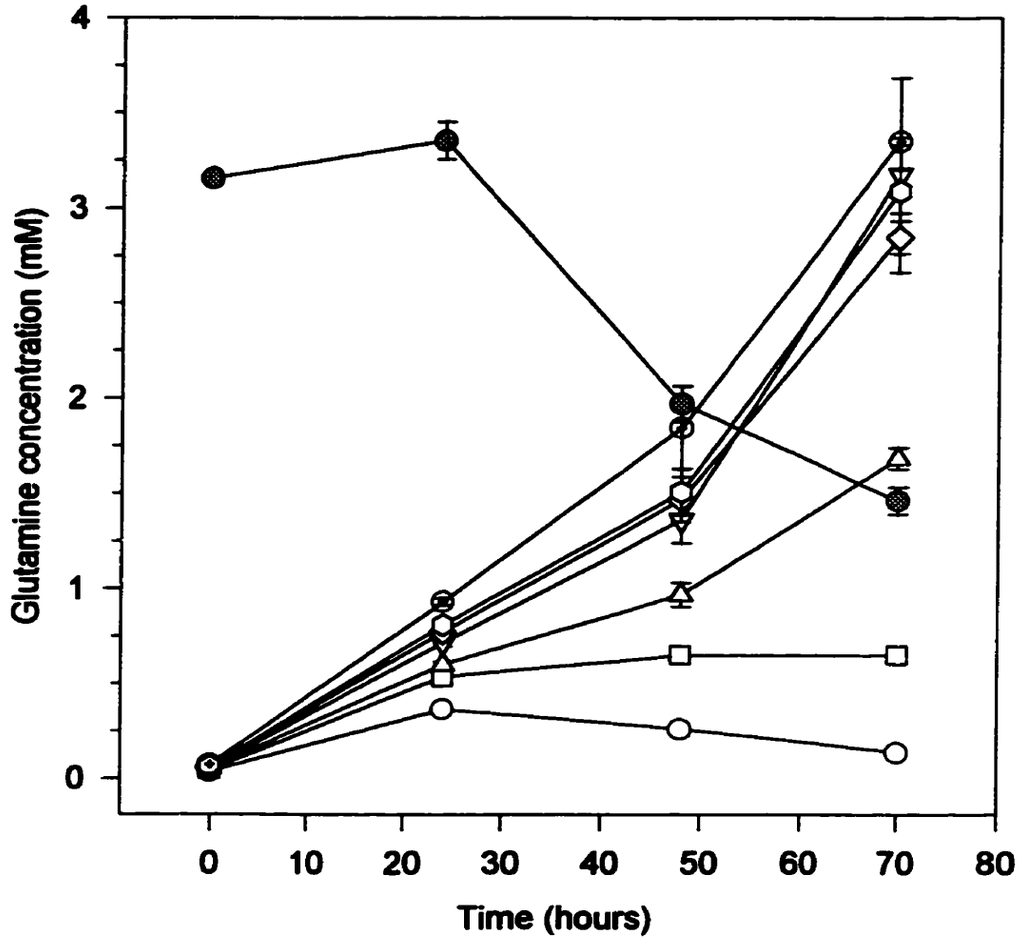
40 mM ala-gln resulted in the highest release of glutamine into the medium, from about 0.90 mM after the first day of culture up to 3.4 mM by the third day. The increased glutamine availability with the higher ala-gln concentrations was also associated with increased ammonium yields. Figure 6-2c illustrates the ammonium concentrations after ~3 days of culture which increased progressively with a higher initial ala-gln concentration in the medium. Note that an increase in ala-gln from 1.25 mM to 5 mM resulted in a significant improvement in cell concentrations yet the difference in ammonium production between them was marginal. Ala-gln greater than 5 mM, on the other hand, resulted in higher ammonium levels without the benefit of significant improvements in growth. Ammonium was 1.44 mM after 3 days with 5 mM ala-gln compared to 2.13 mM in the culture with the 3 mM glutamine supplement, a difference of 32 %. Further increases in the dipeptide up to 40 mM resulted in ammonium yields comparable to the glutamine-based medium. Thus, 5 mM ala-gln promoted high growth rates and cell densities with reduced ammonium accumulation although cell densities were slightly lower than the 3 mM glutamine culture.

Figure 6-2a-c. Growth and concentrations of glutamine and ammonium in BHK cultures with a range of initial ala-gln supplements. (a) Growth of cells in medium with 1.25-40 mM ala-gln or 3 mM glutamine as indicated by the symbols in the legend. Each culture was grown in triplicate (n=3) in 24-well plates and samples were counted with a Coulter counter. Replicate counts were averaged and plotted ± SE. (b) Glutamine concentrations in the cultures for the first 3 days of growth. Glutamine was determined in samples at each time point from each of the replicate wells. Replicates were averaged and plotted ± SE. (c) Ammonium concentrations in each of the cultures after ~3 days (70 hours) of growth. Ammonium was determined for each of the triplicate wells and the average plotted ± SE.

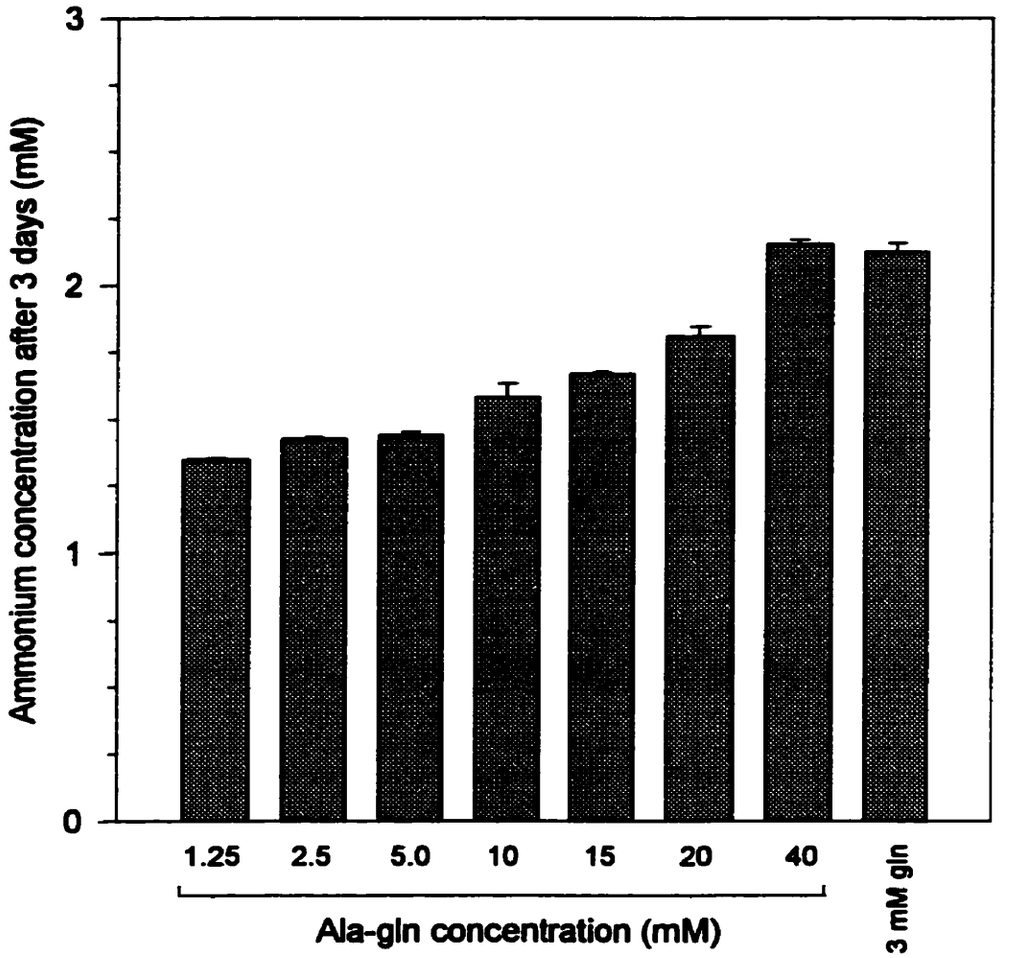
(a)


1.5
1.0
0.5
0.0
Total cell concentration (10^6 cells/ml)
0
20
40
60
80
100
Time (hours)
Concentration of ala-gln:
1.25 mM
2.5 mM
5.0 mM
10 mM
15 mM
20 mM
40 mM
3 mM gln medium (control)

(b)


Glutamine concentration (mM)
4
3
2
1
0
0
10
20
30
40
50
60
70
80
Time (hours)
Concentration of ala-gln:
1.25 mM
2.5 mM
5.0 mM
10 mM
15 mM
20 mM
40 mM
3 mM gln

(c)


Ammonium concentration after 3 days (mM)
3
2
1
0
1.25
2.5
5.0
10
15
20
40
3 mM gln
Ala-gln concentration (mM)

Studies with the CC9C10 cell line demonstrated that acceptable growth rates of the hybridoma could only be sustained in gly-gln medium when the dipeptide was present at high concentrations (20 mM). BHK cells were similarly found to rely on elevated levels of gly-gln as shown in Figure 6-3. Cells were grown in a range of gly-gln concentrations from 2.5 to 40 mM. Low levels of gly-gln (2.5-10 mM) resulted in prolonged lag phases, reduced growth rates and lower cell yields. This suboptimal growth may be explained by a phenomenon already described for the hybridoma where gly-gln, at low concentrations, is not hydrolyzed at a sufficient rate to meet the cellular glutamine requirements. A minimum of 15 mM gly-gln appeared necessary to supply glutamine at a level where BHK growth was not considerably impaired. Further improvements were possible by increasing gly-gln up to 40 mM. At this concentration, the lag phase was still slightly more pronounced and the cell yield after ~3 days was lower than the control with glutamine. The cell density eventually reached a level similar to that of the DMEM/gln medium but this occurred after 5 days. The addition of gly-gln to growth medium at concentrations as high as 40 mM, however, is undesirable because its eventual hydrolysis could release high levels of glutamine into the medium, culminating in increased ammonium production. In addition, the dipeptide is expensive and would substantially increase the medium osmolarity. Further efforts were therefore directed toward examining the efficacy of replacing glutamine with glutamate in BHK cultures.

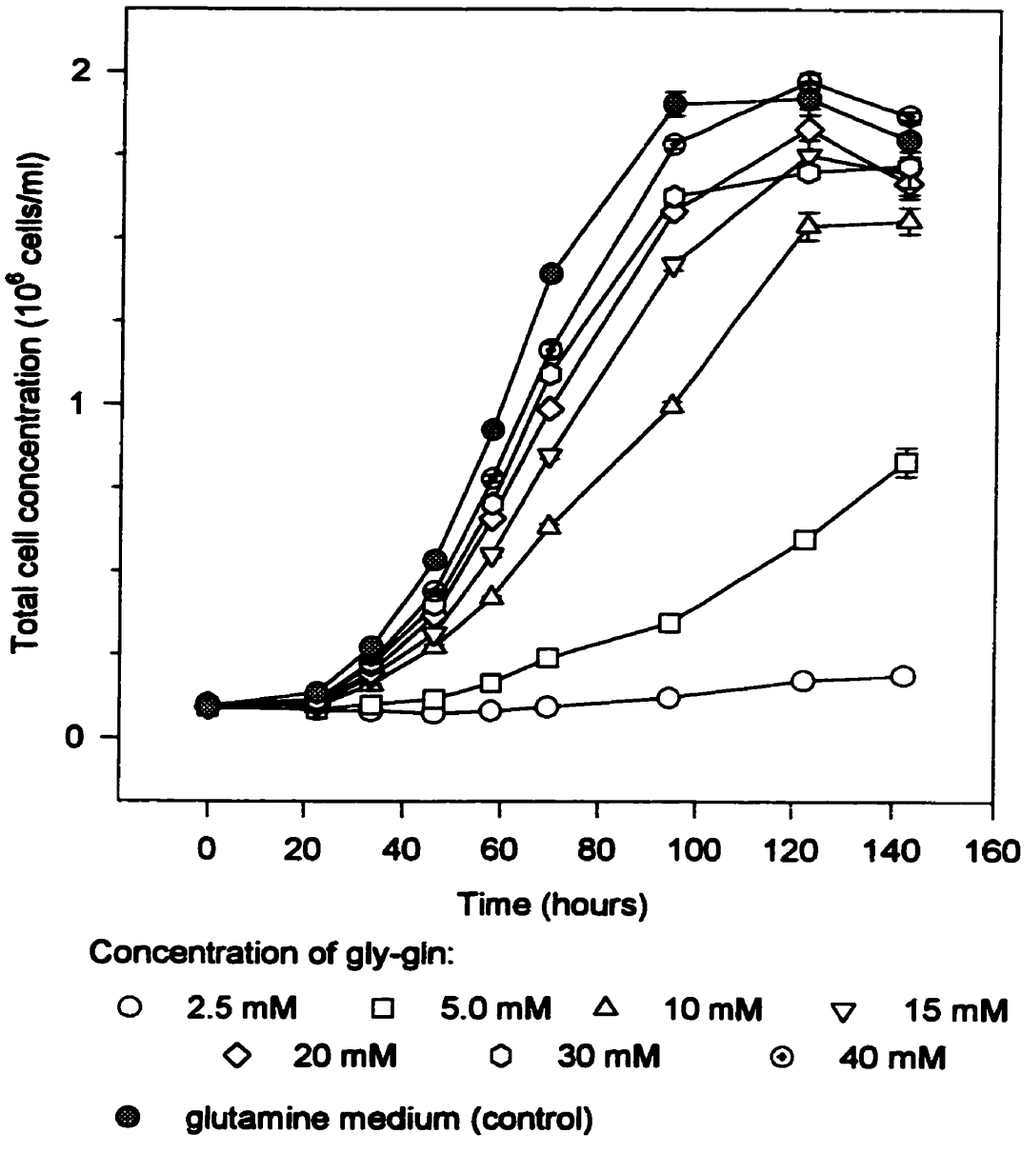


Figure 6-3. Growth of BHK cells in medium with various gly-gln concentrations. The culture medium was supplemented with the indicated additions of gly-gln or 3 mM glutamine. Cells were cultured in triplicate wells (n=3) of 24-well plates at each concentration. Wells were counted by Coulter counter and replicates averaged and plotted ± SE.

6.3.3 Optimization of growth in glutamate medium

BHK cells were adapted and grown in medium in which glutamine had been replaced with a low (6 mM) and high (20 mM) concentration of glutamate. Amino acid analysis confirmed that minimal glutamine was available in the serum (Appendix G) and the glutamate medium was thus virtually glutamine-free. BHK growth overall was not significantly different at the two glutamate concentrations and was characterized by prolonged lag phases, reduced growth rates and lower cell yields compared to glutamine medium (Figure 6-4). The poor growth could not be attributed to an insufficient period of adaptation since cells were cultured in medium containing glutamate at the different concentrations for at least 6 passages prior to the start of the experiment. Inhibitory effects of glutamate were also ruled out as growth was normal in medium containing both glutamate and glutamine during the adaptation of cells to glutamate medium. Thus, glutamate could not be directly substituted for glutamine on a mole-for-mole basis without a significant impact on growth. Further refinements in culture methods and medium were essential to improve growth in the presence of a limited availability of glutamine.

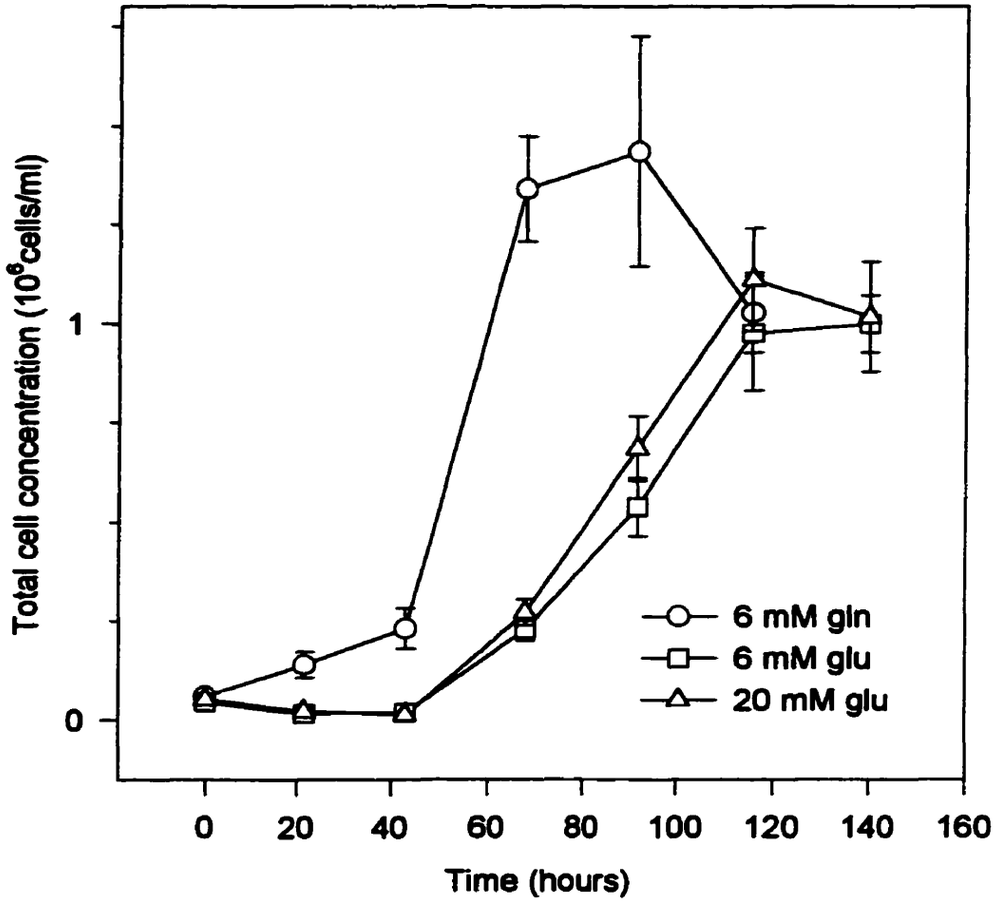


Figure 6-4. Growth of BHK cells in glutamine or glutamate medium. Cells were cultured in 24-well plates in medium with 6 mM glutamine (○), 6 mM glutamate (□) or 20 mM glutamate (Δ). The initial inoculation density was ~0.5 X 10^5 cells/ml. Eight replicate wells were used for each medium (n=8) and total cell concentrations evaluated by haemocytometer counts. Replicate counts were averaged and plotted ± SE.

6.3.3.1 Effect of seed density on growth in glutamate medium

The possibility that a low initial seed density contributed to the suboptimal growth in glutamate medium was investigated by seeding the cells at increasing concentrations from 0.32 X 10^5 to 2.56 X 10^5 cells/ml. Although the cell density in the culture inoculated at 0.32 X 10^5 cells/ml eventually surpassed those seeded at higher concentrations, it suffered the most severe lag phase and slowest growth rate (Figure 6-5). An inoculation density of 1.28 X 10^5 cells/ml reduced the lag and increased the growth rate significantly compared to the lower inoculum concentrations. An increase to 2.56 X 10^5 cells/ml further improved the lag phase at the expense of a lower growth rate. Thus, glutamate medium was normally inoculated with $\geq$1 X 10^5 cells/ml in subsequent optimization experiments. This cell density was expected to mitigate the lag phase to some degree and increase the growth rate in glutamate medium although limitations compared to glutamine-based medium were still evident.

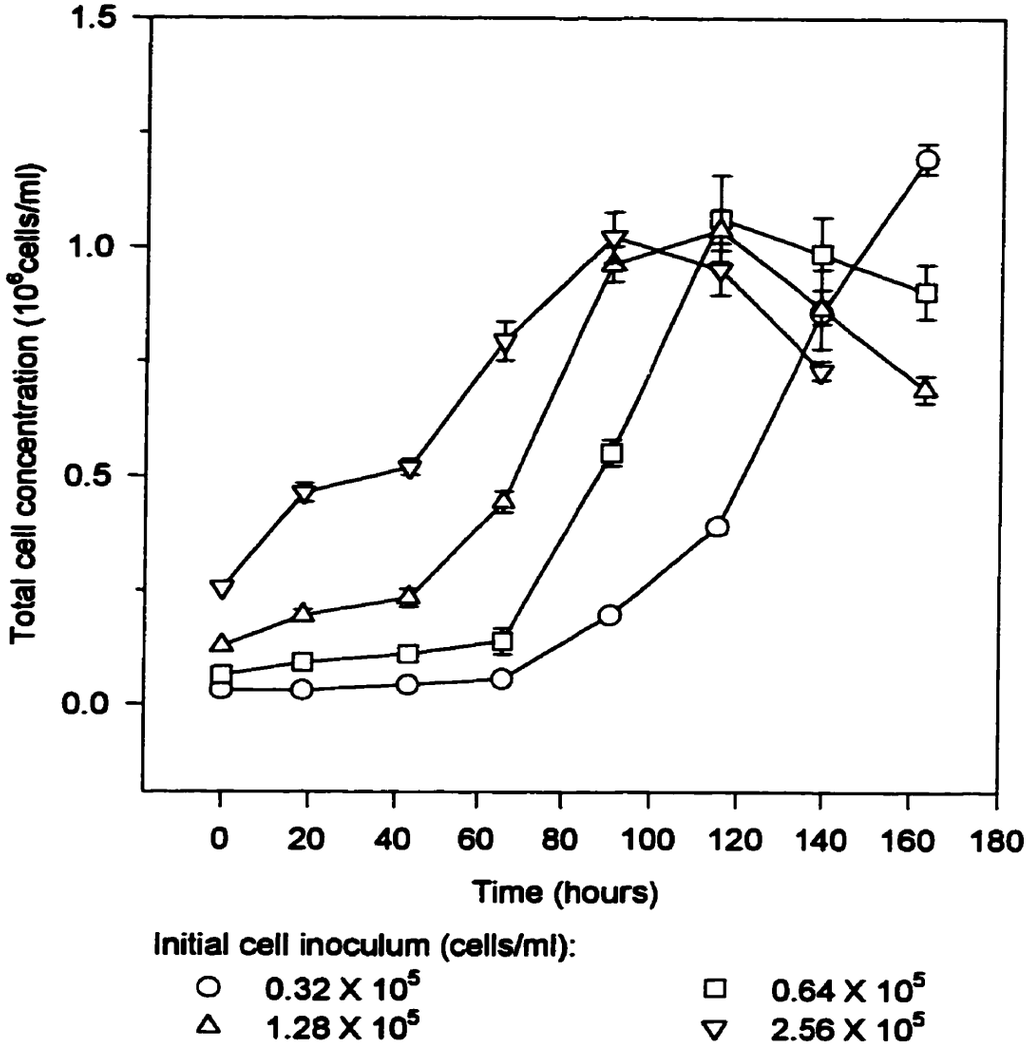


Figure 6-5. Growth of BHK cultures inoculated at different cell densities in glutamate medium. Cells were cultured in 24-well plates in medium with a 6 mM glutamate supplement. Duplicate wells (n=2) were seeded for each cell concentration of 0.32 X 10^5 (○), 0.64 X 10^5 (□), 1.28 X 10^5 (△) and 2.56 X 10^5 (▽) cells/ml. Total cell concentrations were evaluated with a haemocytometer and replicate counts averaged and plotted ± SE.

6.3.3.2 Effect of ammonium, aspartate and asparagine supplements in glutamate medium

The lower growth rates and cell yields observed in glutamate medium suggested a nutritional limitation was induced by the elimination of glutamine. An attempt was made to compensate for the deficiency by the addition of nutrients or metabolites derived from glutamine to the medium.

Although glutamine was excluded from glutamate medium, its synthesis was still essential because of its role in various cellular processes described previously. However, the absence of glutamine in the medium introduced the possibility of a limited availability of ammonium as a substrate for glutamine production via glutamine synthetase, at least during the early stages of the culture. BHK cells were therefore grown in glutamate medium (3 mM) with the addition of NH_4Cl concentrations from 0.5-2.0 mM. Ammonium up to 2 mM had a negligible effect on growth in glutamate medium or the control cultures containing 3 mM glutamine compared to those with no initial addition of NH_4Cl (Figure 6-6). The cells were clearly not limited by a deficiency of ammonium when glutamate was substituted for glutamine.

An attempt was also made to improve growth in glutamate medium by including aspartate. Aspartate is not a standard constituent of DMEM and would normally be derived from the metabolism of glutamine. In glutamine-free medium, however, a low rate of aspartate production could limit growth. Thus, aspartate was added to 3 mM glutamate medium in a range of concentrations up to 20 mM. Figure 6-7 demonstrates aspartate did not reduce the extended lag phase, enhance the growth rate or increase the cell yield after ~3 days. In fact, increasing aspartate concentrations resulted in slower growth. The final cell density was slightly higher in medium with 1.25-5 mM aspartate compared to that with only the glutamate (0 mM aspartate) but only after ~6 days of growth. A limitation was still apparent compared to the culture containing

glutamine. Obviously, aspartate was not a limiting nutrient and its addition to glutamate medium was unnecessary.

Asparagine was considered as an additional supplement in glutamate medium. The biosynthesis of asparagine is expected to involve an amidotransferase reaction with aspartate and glutamine as substrates, the latter serving as amide donor. The absence of glutamine in glutamate-based medium could thus reduce the rate of cellular asparagine production culminating in a growth limitation. Asparagine was added to 3 mM glutamate medium at concentrations from 0.06-3.0 mM and the effect on growth examined. As shown in Figure 6-8a, an asparagine concentration as low as 0.12 mM resulted in considerable improvements in both the growth rate and cell yield. Further increments in asparagine concentration up to 3 mM resulted in slight but significant improvements in growth. However, growth rates and cell concentrations were still lower than the culture containing glutamine. Nevertheless, μ increased 25 % from 0.028 hr^{-1} with no asparagine addition (0 mM) to 0.035 hr^{-1} with 3 mM asparagine. The cell yield increased 85 % from 0.33 X 10^6 cells/ml to 0.61 X 10^6 cells/ml after ~3 days culture with 0 and 3 mM asparagine, respectively. These values compare with a specific growth rate of 0.038 hr^{-1} and a cell yield of 1.38 X 10^6 cells/ml after ~3 days in glutamine medium. A combination of asparagine and aspartate in glutamate medium did not result in further improvements in growth (Figure 6-8b). Growth in glutamine-free medium was obviously limited, in part, by asparagine but could be circumvented by its direct addition to glutamate medium. These results also suggested the biosynthesis of other nutrients, and hence growth, could similarly be limited by the availability of glutamine. Thus, the complete elimination of glutamine without compromising growth performance would likely require identification of these nutrients and their addition to glutamate medium. Instead, a less complicated and more efficient approach was employed and involved minimizing rather than replacing glutamine in the medium.

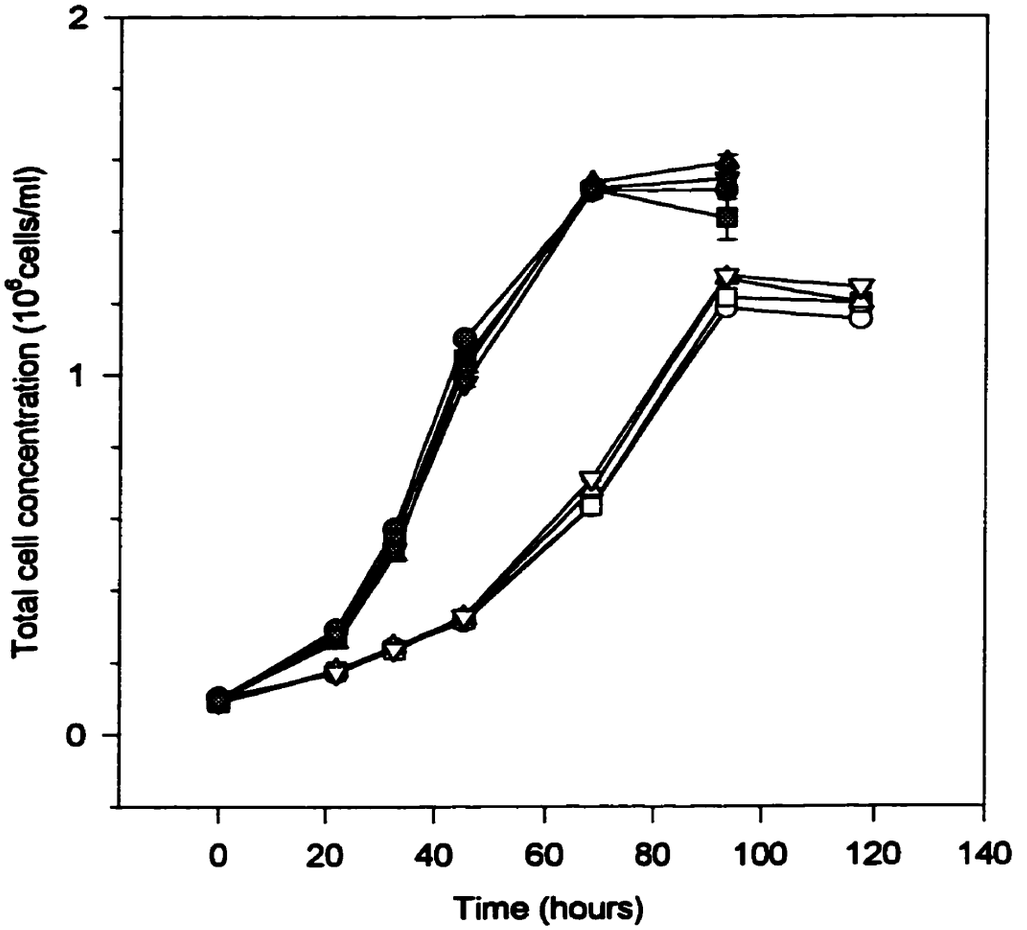


Figure 6-6. Effect of ammonium addition on BHK cell growth in glutamate medium. Cells were grown in medium with supplements of either 3 mM glutamate (open symbols) or 3 mM glutamine (closed symbols) and the NH_4Cl concentrations specified above. Duplicate cultures (n=2) for each ammonium concentration were added to 24-well plates. Total cell concentrations were evaluated by Coulter counter and replicates counts averaged and plotted ± SE.

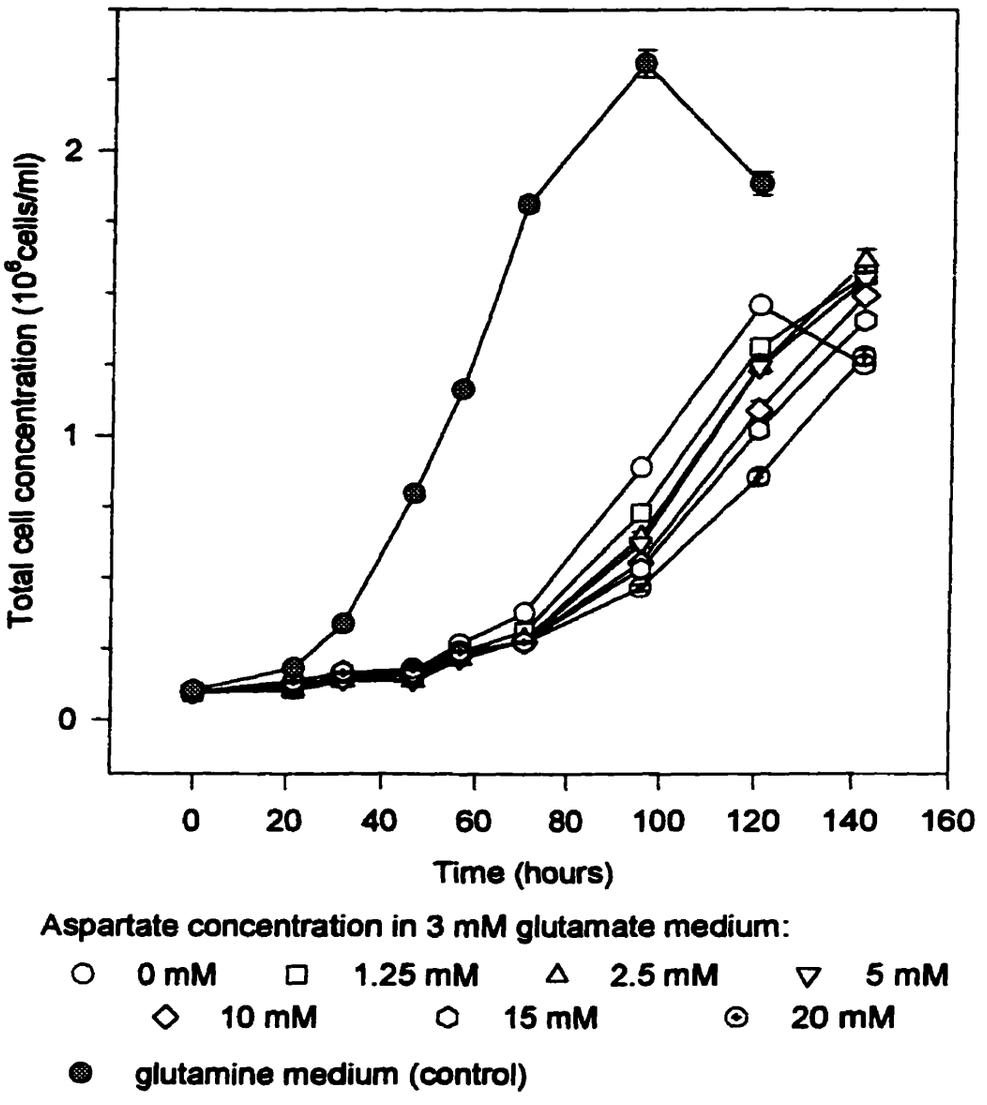
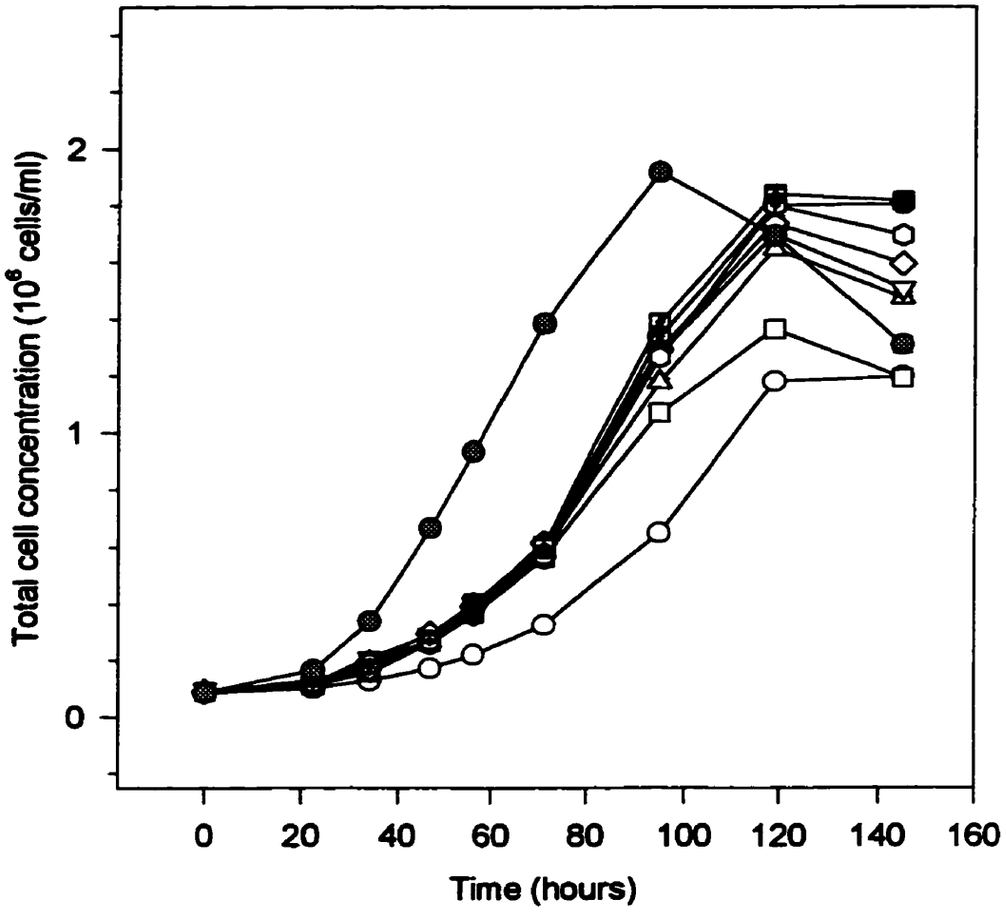


Figure 6-7. Growth of BHK cells in glutamate medium supplemented with various aspartate concentrations. The medium contained 3 mM glutamate and the indicated concentrations of aspartate while the control had a 3 mM glutamine supplement. Cultures were grown in triplicate (n=3) in 24-well plates. Total cell concentrations were determined by Coulter counter and replicates averaged and plotted ± SE.

Figure 6-8a-b. (a) Growth of BHK cells in glutamate medium with various concentrations of asparagine. The medium contained 3 mM glutamate and the indicated concentrations of asparagine. Cultures with asparagine up to 1 mM were grown in triplicate (n=3) while the remaining cultures were in duplicate (n=2) wells of 24-well plates. (b) Growth in glutamate medium with asparagine and varying aspartate concentrations. The medium contained 3 mM glutamate, 3 mM asparagine and the indicated concentrations of aspartate. Triplicate cultures (n=3) were grown in 24-well plates. In both experiments, the control contained a 3 mM glutamine supplement. Total cell concentrations were determined by Coulter counter and replicates averaged and plotted ± SE.

(a)



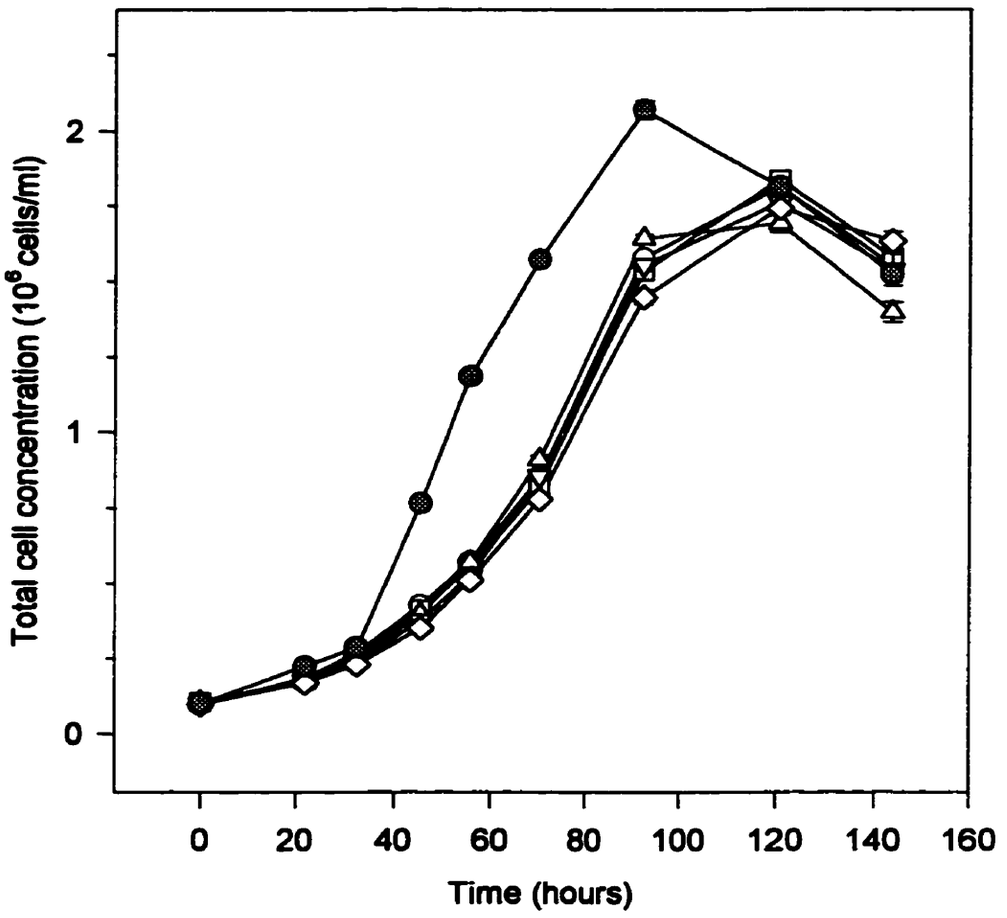
Asparagine concentration in 3 mM glutamate medium:

○ 0 mM □ 0.06 △ 0.12 mM ▽ 0.25 mM

◇ 0.50 mM ⬡ 1.0 mM ⊙ 2.0 mM ⊡ 3.0 mM

● glutamine medium (control)

(b)


Total cell concentration (10^6 cells/ml)
2
1
0
0
20
40
60
80
100
120
140
160
Time (hours)
Aspartate concentration in 3 mM glutamate and 3 mM asparagine medium:
0 mM
0.25 mM
0.5 mM
1.5 mM
3 mM
glutamine medium (control)

6.3.3.3 Glutamine and dipeptide supplements in glutamate-based medium

Efforts to reduce the concentration of glutamine in culture media and thereby limit ammonium production were focused on complementing glutamate medium with asparagine and low levels of glutamine. A range of glutamine concentrations (0.12-3.0 mM) were added to medium containing 3 mM glutamate. Figure 6-9a demonstrates glutamine at a concentration as low as 0.12 mM substantially improves growth in glutamate. An immediate reduction in the duration of the lag phase and an increase in the growth rate were observed relative to the culture without a glutamine supplement (0 mM). The trend of improved growth continued with increasing glutamine concentrations up to 1 mM where growth was comparable to the 3 mM glutamine control culture (without glutamate). For glutamate cultures with 0 and 1 mM glutamine, μ was increased 50 % from 0.026 hr^{-1} to 0.039 hr^{-1}, respectively. The cell yield after ~3 days growth was 227 % higher in the culture with 1 mM glutamine (1.60 X 10^6 cells/ml) compared to that without glutamine (0.49 X 10^6 cells/ml). Glutamine supplements above 1 mM in glutamate medium did not result in further improvements of growth rates and cell concentrations. An additional reduction in glutamine requirements was anticipated by including asparagine in the medium. Cells were cultured in glutamate medium with a range of low glutamine concentrations (0.25-1 mM) with and without 3 mM asparagine (Figure 6-9b). In the presence of asparagine, 0.25 mM glutamine resulted in a growth rate and cell yield after ~3 days that was comparable to the control culture (medium with 3 mM glutamine). The low level of glutamine was clearly suboptimal without the asparagine supplement. Asparagine, on the other hand, had little effect at higher glutamine concentrations (>0.25 mM). However, routinely including asparagine with a slightly higher glutamine concentration (0.5 mM) would compensate for variations in minimum glutamine requirements in glutamate medium caused by differences in culture conditions. Some variability, for instance, was apparent by comparing Figures 6-9a and 6-9b in which the

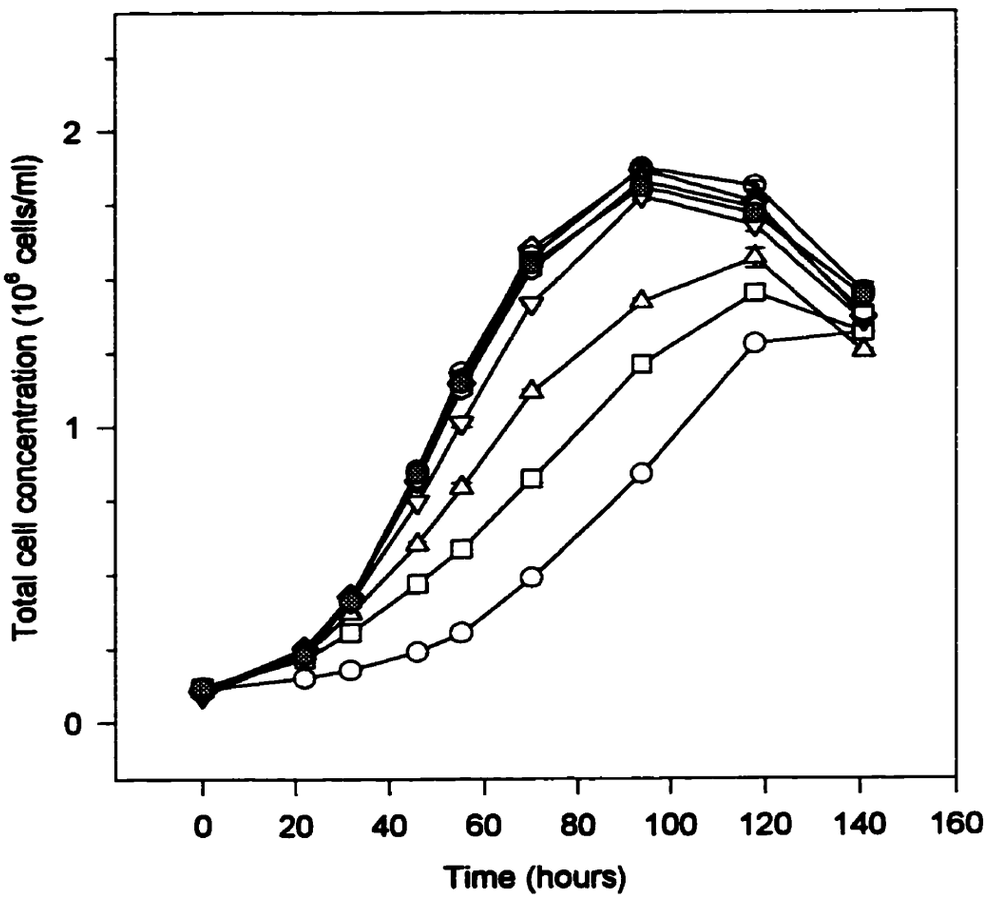
minimal levels of glutamine required for normal growth were 1 mM and 0.5 mM, respectively, in glutamate medium without asparagine. Thus, the combination of 3 mM glutamate and asparagine would permit at least a 6-fold reduction in the amount of glutamine required to sustain normal growth of BHK cells in batch cultures (from 3 mM in glutamine-based medium to 0.5 mM in glutamate-based medium).

The possibility that relatively low concentrations of ala-gln or gly-gln could be used in place of glutamine in glutamate-based medium was also investigated. BHK cells were grown in medium supplemented with glutamate, asparagine and a series of different dipeptide concentrations (Figure 6-10a-c). Figure 6-10a shows the cells cultured in the glutamate-based medium with an increasing ala-gln supplement. Growth in 0.25-0.50 mM ala-gln was characterized by a more pronounced lag phase resulting in reduced cell yields after ~3 days compared to the control culture with 3 mM glutamine. However, growth in 1 mM ala-gln was comparable to the control. Thus, in the presence of glutamate and asparagine, the ala-gln requirement was reduced 5-fold, from 5 mM to 1 mM. Including these amino acids in the medium also permitted a dramatic decrease in the gly-gln requirement. Figure 6-10b illustrates growth of cells with glutamate, asparagine and gly-gln ranging from 2.5 to 40 mM. Remarkably, it was found that at 2.5 mM, the lowest concentration tested, gly-gln produced growth that was similar to the control with 3 mM glutamine. Concentrations higher than 2.5 mM did not improve growth characteristics but, in fact, appeared to eventually inhibit growth at 30 and 40 mM. Another experiment was performed to address the question of how low gly-gln could be reduced before growth was significantly impaired. Cells were grown in the presence of 3 mM glutamate and gly-gln concentrations from 0.5 to 2.5 mM. Some of these cultures contained asparagine while it was omitted from others to ascertain its effect on growth under low gly-gln conditions (Figure 6-10c). In medium supplemented with both glutamate and asparagine, gly-gln concentrations <2.5 mM produced reduced cell yields after ~3 days compared to the glutamine control. When asparagine was

omitted, further reductions in growth were observed for the range of gly-gln concentrations examined (0.5 to 2.5 mM). This again illustrates the benefit of asparagine under conditions where glutamine is apt to be present at low levels. The net effect was that in medium supplemented with both glutamate and asparagine, up to a 16-fold reduction in the gly-gln concentration was possible, from 40 to 2.5 mM, without compromising growth.

Figure 6-9a-b. Growth of BHK cells in glutamate medium with varying glutamine concentrations with and without asparagine. (a) Cells were grown in medium with 3 mM glutamate and the indicated concentrations of glutamine while the control culture contained 3 mM glutamine without glutamate. Each of the cultures were grown in triplicate (n=3) in 24-well plates. (b) Growth in medium supplemented with 3 mM glutamate and the indicated glutamine and asparagine concentrations. The control, once again, contained 3 mM glutamine. Cultures with both glutamate and glutamine were grown in triplicate (n=3) and the remainder in duplicate (n=2) in 24-well plates. In both experiments, cells were counted by Coulter counter, replicates averaged and plotted ± SE.

(a)



Glutamine concentration in 3 mM glutamate medium:

○ 0 mM ▭ 0.12 mM △ 0.25 mM ▽ 0.50 mM

◇ 1.0 mM ⬡ 1.5 mM ⊙ 3.0 mM

● glutamine medium (control)

(b)

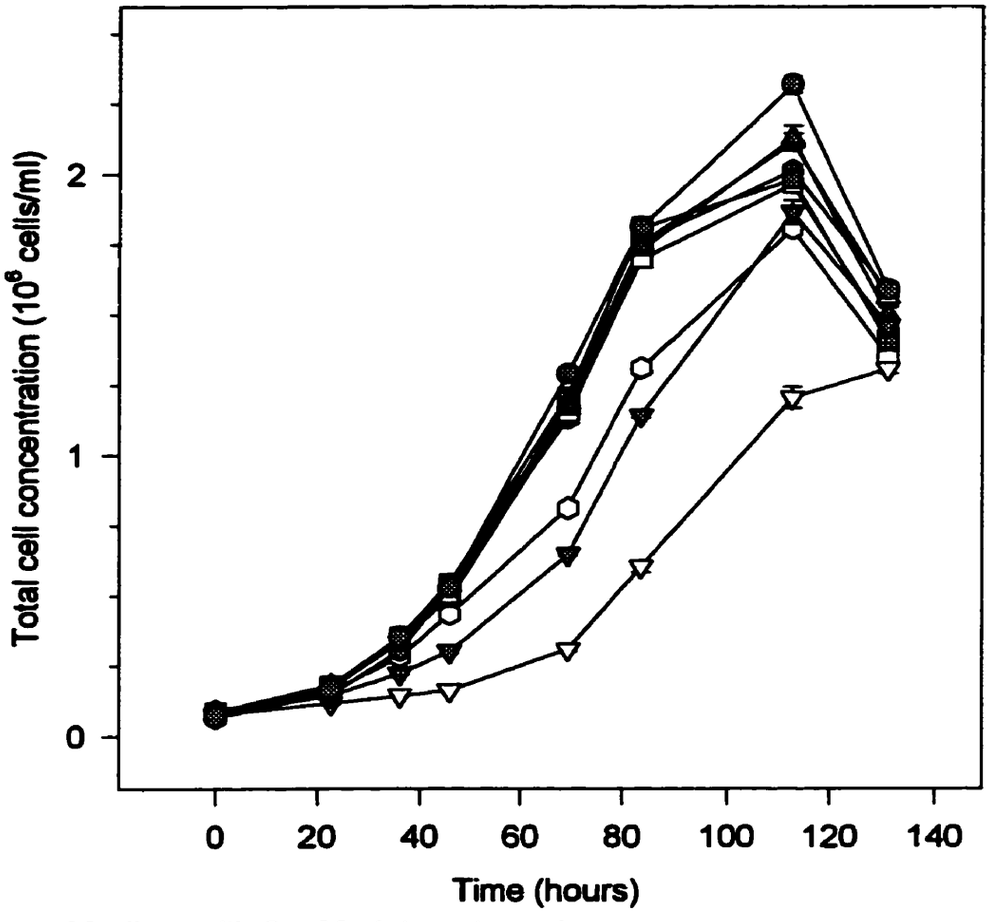
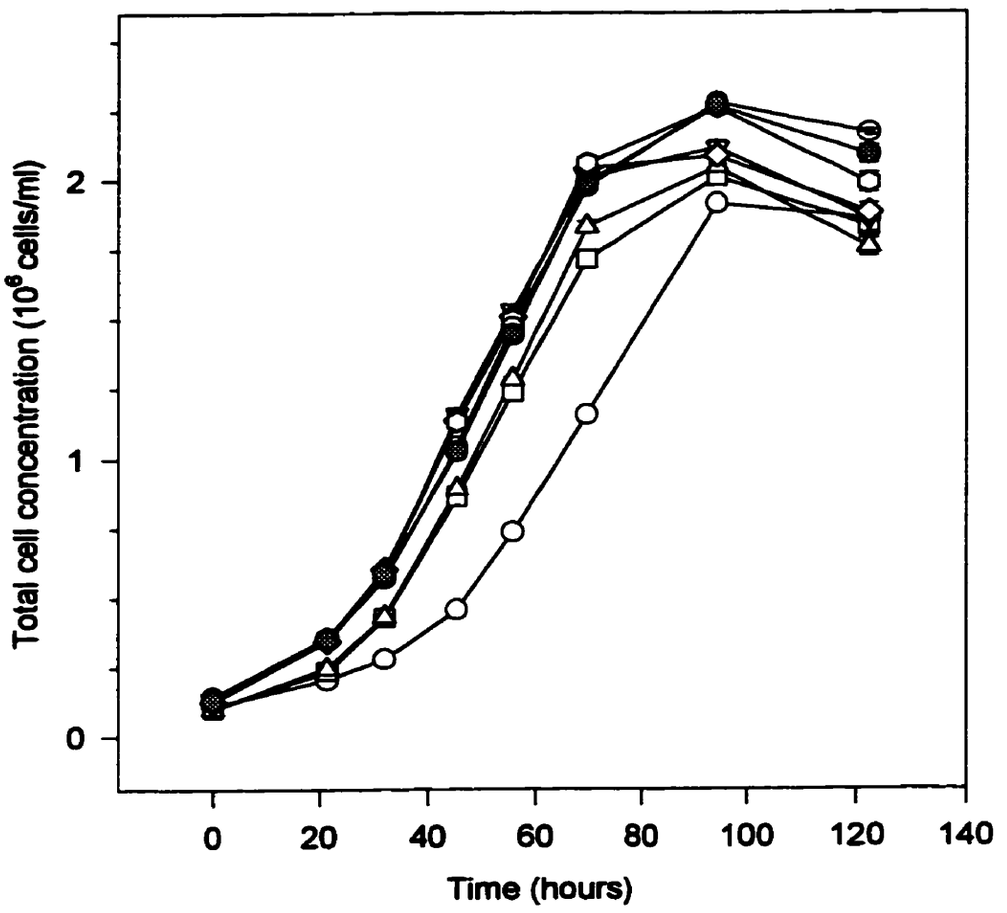

Total cell concentration (10^6 cells/ml)
0
1
2
0
20
40
60
80
100
120
140
Time (hours)
Medium with 3 mM glutamate and:
0 mM gln and
0.25 mM gln and
0.50 mM gln and
1.0 mM gln and
0 mM asn
0 mM asn
0 mM asn
0 mM asn
3 mM asn
3 mM asn
3 mM asn
3 mM asn
glutamine medium (control)

Figure 6-10a-c. Growth of BHK cells in glutamate-based medium with various dipeptide concentrations. Cells were cultured in medium with 3 mM glutamate, 3 mM asparagine and the specified concentrations of (a) ala-gln or (b) gly-gln. (c) Growth in medium with 3 mM glutamate, either 0 or 3 mM asparagine and the indicated gly-gln concentrations. The control in all cases contained a 3 mM glutamine supplement. Each of the cultures was grown in triplicate for (a) and (b) and duplicate for (c) in 24-well plates. Cell concentrations were determined by Coulter counter and replicates averaged and plotted ± SE.

(a)



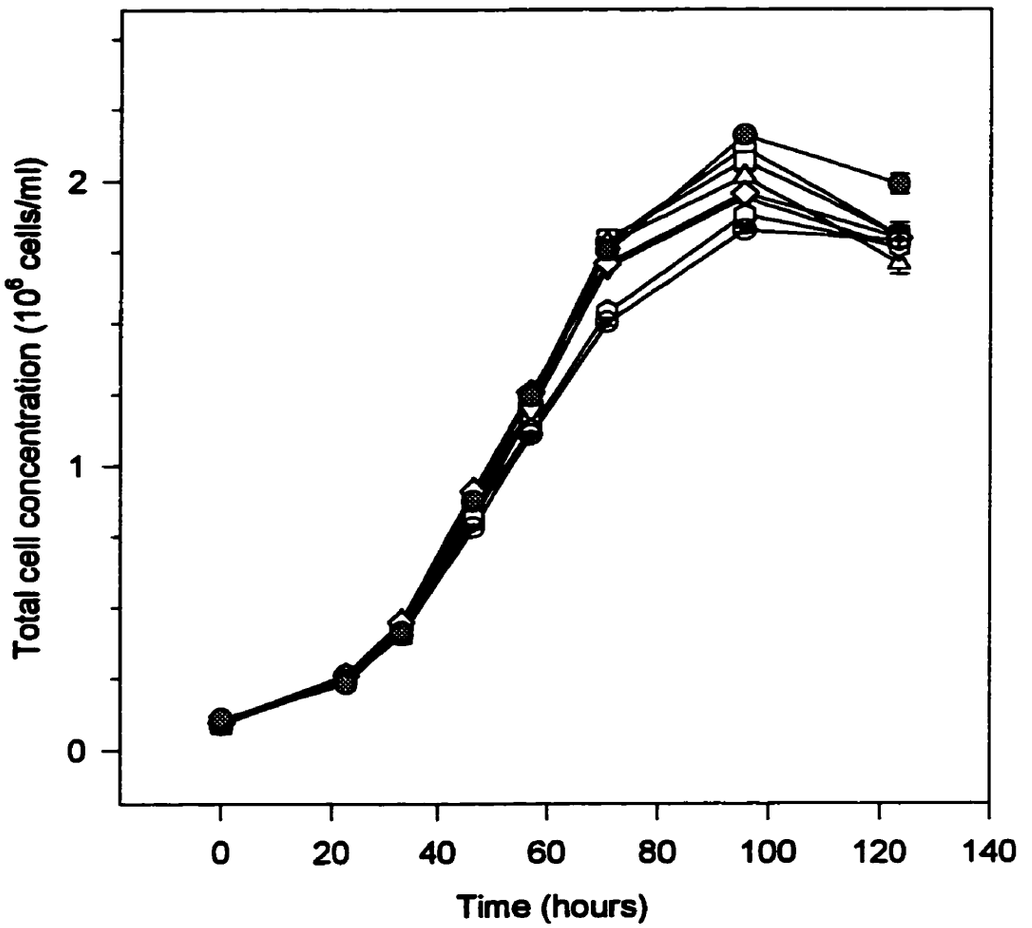
Concentration of ala-gln in medium with 3 mM glutamate and 3 mM asparagine:

○ 0 mM □ 0.25 mM △ 0.50 mM ▽ 1.0 mM

◇ 1.5 mM ⬡ 3.0 mM ⊙ 5.0 mM

● glutamine medium (control)

(b)



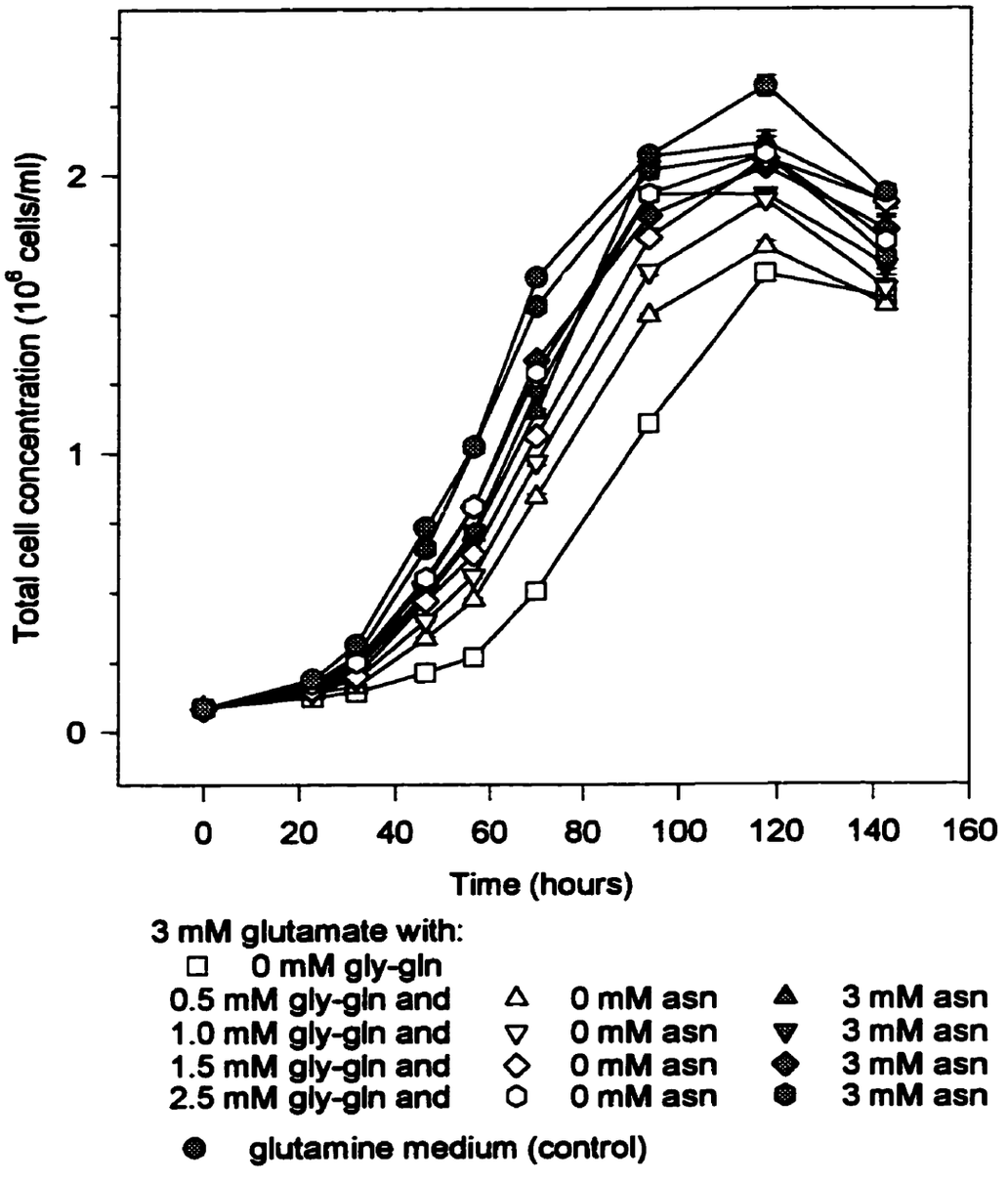
Concentration of gly-gln in medium with 3 mM glutamate and 3 mM asparagine:

○ 2.5 mM □ 5.0 mM △ 10 mM ▽ 15 mM

◇ 20 mM ⬡ 30 mM ⊙ 40 mM

● glutamine medium (control)

(c)


Total cell concentration (10^6 cells/ml)
0
1
2
0
20
40
60
80
100
120
140
160
Time (hours)
3 mM glutamate with:
0 mM gly-gln
0.5 mM gly-gln and 0 mM asn
3 mM asn
1.0 mM gly-gln and 0 mM asn
3 mM asn
1.5 mM gly-gln and 0 mM asn
3 mM asn
2.5 mM gly-gln and 0 mM asn
3 mM asn
glutamine medium (control)

6.3.3.4 Glutamate requirements in glutamate-based medium

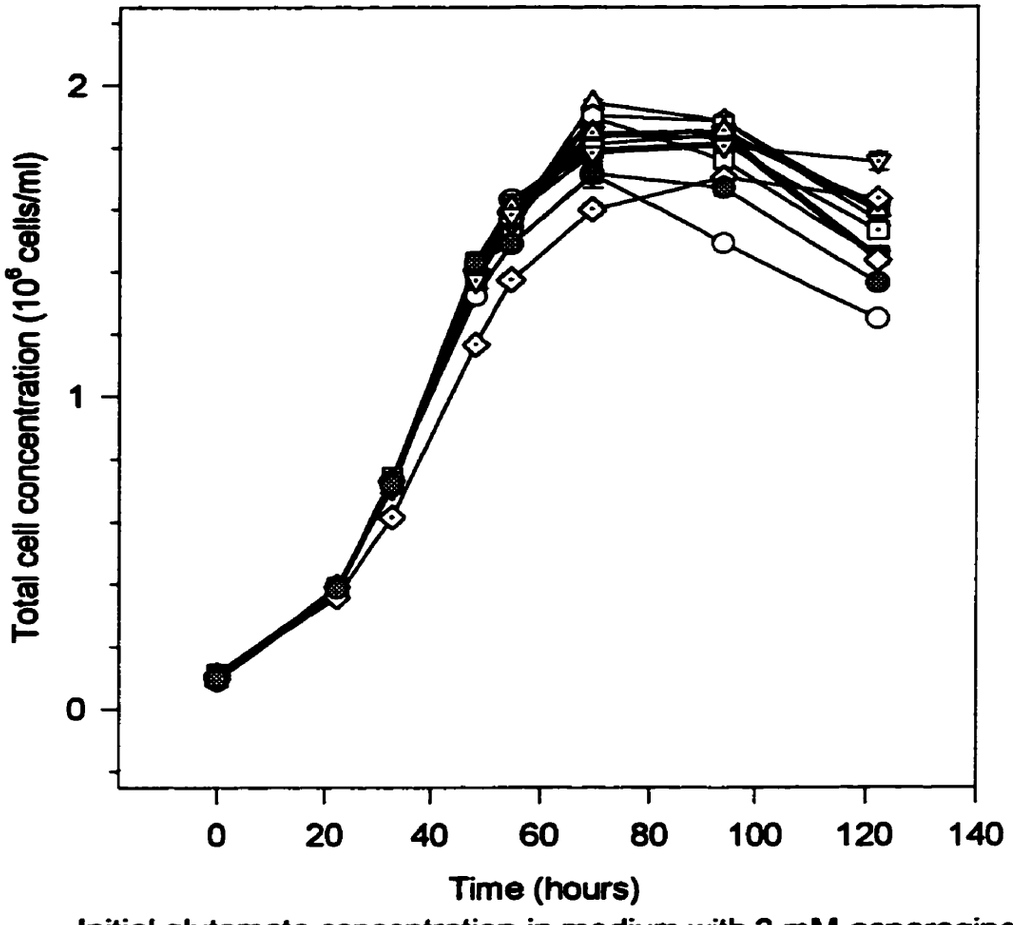
Growth of BHK cells was examined in medium supplemented with 3 mM asparagine, 0.5 mM glutamine and a range of glutamate concentrations from 1.0-20 mM (Figure 6-11a). This would establish the minimum level of glutamate required for normal growth with a reduced availability of glutamine in batch culture. Glutamate concentrations from 2.0-15 mM produced cell yields after ~3 days that were slightly but significantly higher than those observed in the control with 3 mM glutamine. The culture with the lowest glutamate concentration (1.0 mM) had the same cell yield as the glutamine control but entered a more pronounced decline phase after 3 days. A 3 mM glutamate supplement appeared optimal and produced the highest cell yield after ~3 days at 1.94 X 10^6 cells/ml. This compares with 1.72 X 10^6 cells/ml in the glutamine control at the same time point. Increases in the glutamate concentration beyond 3 mM did not improve growth. In fact, a decline in growth rate and cell concentration was observed at 20 mM glutamate.

Amino acid analysis revealed that glutamate was consumed by the BHK cultures at a high rate. Figure 6-11b shows glutamate was depleted after ~3 days culture with an initial concentration <4 mM. The complete consumption of glutamate would explain the abrupt onset of the decline phase in the culture with the lowest glutamate concentration. Other factors, however, appeared to limit growth with higher glutamate concentrations (>3 mM). Ammonium was also determined at each of the glutamate concentrations after ~3 days for cultures and cell-free medium (Figure 6-11c). Ammonium in the cultures with a range of glutamine concentrations originally depicted in Figure 6-1d were included for contrast. In the 1 mM glutamate culture, the highest ammonium level was observed at 1.08 mM. Ammonium actually decreased to as low as 0.52 mM with increasing glutamate in the medium. In glutamine-based medium, conversely, a progressive increase in ammonium occurred with higher initial glutamine supplements. For comparison, the 3 mM glutamine culture reached 2.08 mM

ammonium after 3 days while that containing the equivalent glutamate concentration was 63 % lower at 0.76 mM. The cell density was 17 % higher in the glutamine medium after ~3 days but this alone would not account for the much higher ammonium level. Clearly, cellular ammonium production was significantly reduced in the glutamate medium. The greater stability and lower ammoniagenic nature of glutamate was also demonstrated by the low and constant ammonium (~0.2 mM) in the cell-free glutamate medium. This contrasted sharply with glutamine medium in which decomposition accounted for a significant fraction of the ammonium produced in the cultures.

Figure 6-11a-c. Growth, glutamate utilization and ammonium accumulation in BHK cultures supplemented with asparagine, a low level of glutamine and a range of initial glutamate concentrations. (a) Growth of cells in medium with 3 mM asparagine, 0.5 mM glutamine and glutamate concentrations from 1-20 mM as indicated in the legend. The control contained 3 mM glutamine without glutamate or asparagine. Each culture was grown in duplicate wells of 24-well plates. Cell concentrations were evaluated by Coulter counter and replicate counts averaged and plotted ± SE. (b) Glutamate concentrations in the cultures after ~3 days (69 hours) of growth. Glutamate from each of the duplicate samples was determined and the average plotted ± SE. (c) Ammonium concentrations in the glutamate cultures (□) and cell-free glutamate medium (■) after 69 hours. Ammonium in cultures grown with various glutamine concentrations (○) and cell-free glutamine medium (●) after 69.5 hours were included for comparison. The concentration of ammonium was determined in duplicate samples, averaged and plotted ± SE.

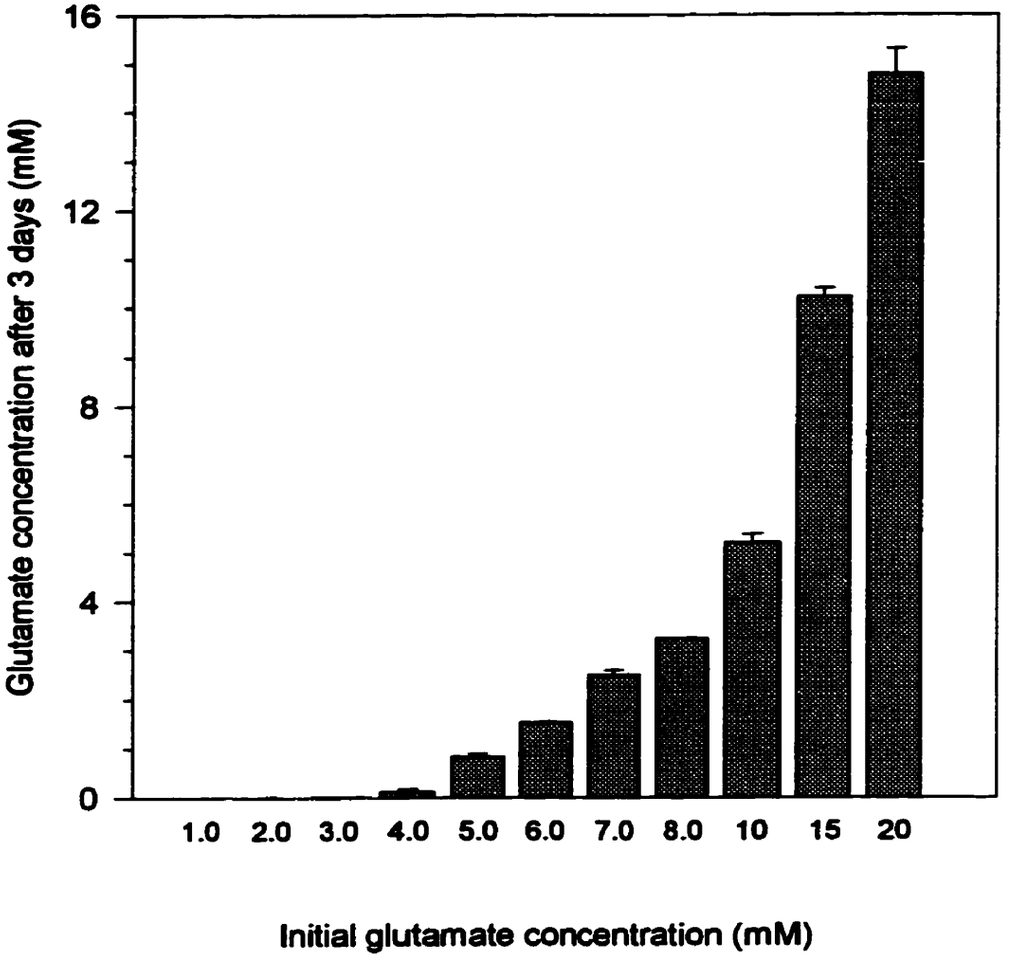
(a)



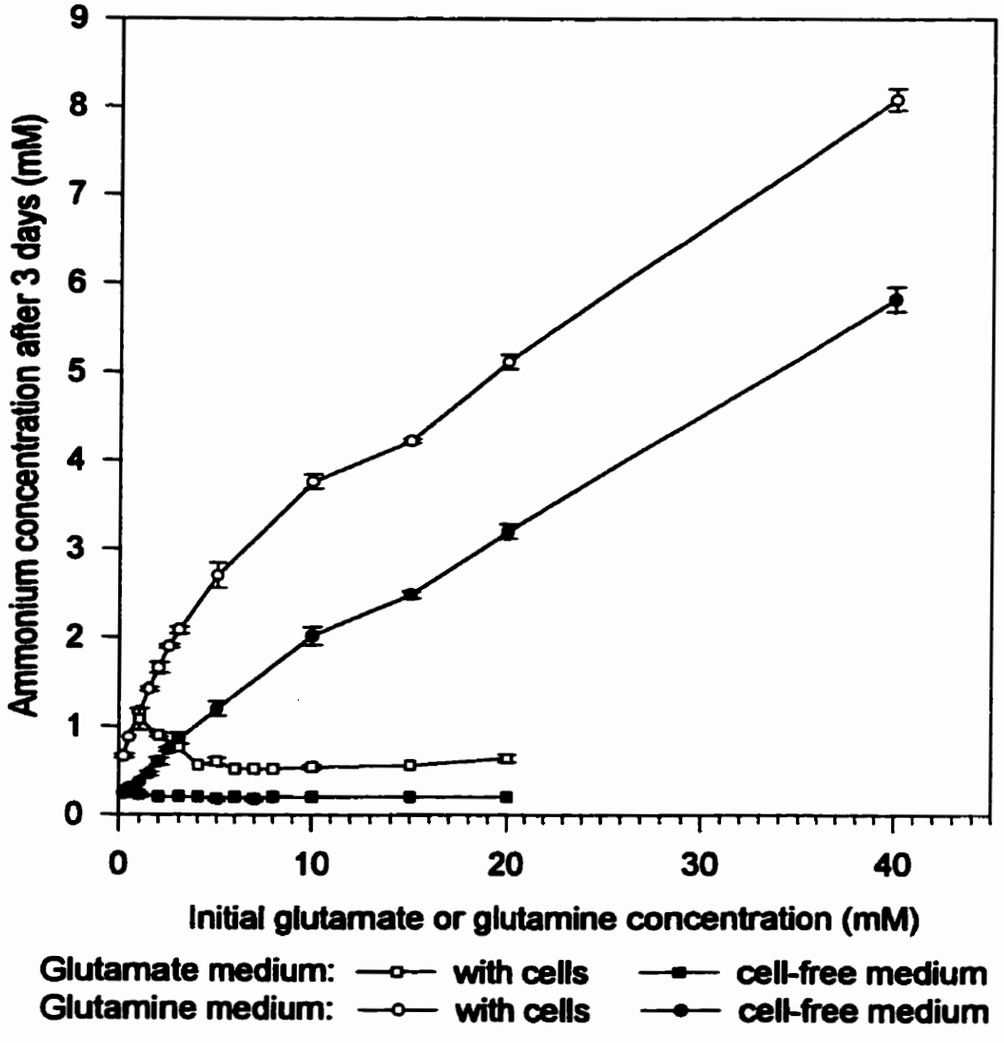
Initial glutamate concentration in medium with 3 mM asparagine and 0.5 mM glutamine:

○ 1 mM	□ 2 mM	△ 3 mM	▽ 4 mM glu
◇ 5 mM	⬡ 6 mM	⊙ 7 mM	⊡ 8 mM
◬ 10 mM	▽ 15 mM	◈ 20 mM	
● glutamine medium (control)			

(b)


16
12
8
4
0
Glutamate concentration after 3 days (mM)
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
10
15
20
Initial glutamate concentration (mM)

(c)


Ammonium concentration after 3 days (mM)
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
Initial glutamate or glutamine concentration (mM)
Glutamate medium: with cells
cell-free medium
Glutamine medium: with cells
cell-free medium

6.4 Discussion

Prior to the development of the low-ammoniagenic medium, the minimum glutamine concentration which could support optimal growth of BHK cells was investigated. This would provide an indication of the extent of the dependence of the cell line on glutamine and the potential for replacement with other substrates. Furthermore, unnecessarily high levels of glutamine in the medium would exaggerate the contribution of ammonium inhibition to growth limitation in batch cultures. Other studies have established the importance of controlling the availability of glutamine in culture medium to reduce the accumulation of ammonium. Glacken *et al.* (1986) demonstrated the specific glutamine utilization rate increased with a higher initial glutamine supplement in the medium. Ljunggren and Häggström (1994) reported very low glutamine levels (<0.28 mM) could be maintained in murine hybridoma cultures, without affecting the growth rate, by continuously feeding the amino acid at a low rate. Limiting glutamine in the medium is therefore a feasible strategy to alleviate ammonium production from both decomposition and rapid metabolism of glutamine.

The present study revealed a glutamine concentration of 3 mM was sufficient to promote maximum growth rates and high cell yields in batch cultures (Figure 6-1a-b). Glutamine as low as 1.5 mM was able to sustain maximal exponential growth but a glutamine limitation (Figure 6-1c) eventually resulted in a premature entry into the stationary phase and a lower cell yield. Alternatively, a growth limitation due to ammonium inhibition only became apparent at very high glutamine concentrations. Ammonium increased as the initial glutamine concentration in the medium was raised, reaching in excess of 8 mM after 3 days in the 40 mM glutamine culture (Figure 6-1d). This level of ammonium approximated the $I.C._{50}$ of BHK cells determined previously (Figure 3-1a-b). In the 40 mM glutamine culture, though, the specific growth rate and cell concentration after 3 days were only 4% and 21 % lower, respectively, than for the culture with 3 mM glutamine. Thus, a greater degree of inhibition might have been

expected. However, the determination of the $I.C._{50}$ value does not consider the additional ammonium produced during the course of cell growth. Furthermore, the more gradual accumulation of ammonium from decomposition of glutamine and cell metabolism was probably less inhibitory than the immediate addition of NH_4Cl directly to the medium. Obviously, ammonium inhibition could be minimized with an initial glutamine concentration of 3 mM in the medium although growth was eventually limited by other factors. A culture with a 3 mM glutamine supplement served as a reference for normal growth rates, cell yields and ammonium production in batch cultures during the development of a low-ammoniagenic medium.

Previous work with the CC9C10 hybridoma introduced the possibility that dipeptides may also substitute for glutamine with BHK cells. An optimal ala-gln concentration of 5 mM in BHK cultures (Figure 6-2a) was similar to the 6 mM which promoted normal growth rates and high cell yields with the hybridoma (Figure 4-2). Significantly lower ala-gln resulted in a limited availability of glutamine in the medium (Figure 6-2b) which was earlier shown to result in reduced growth rates and cell concentrations (Figure 6-1). Higher gly-gln supplements (≥15 mM) were required to prevent a substantial decline in growth performance of BHK cells (Figure 6-3) which is consistent with previous observations in CC9C10 cultures. Improvements in growth continued up to the highest gly-gln concentration examined (40 mM). Again, a peptidase with a reduced affinity for gly-gln appears operative and the mechanisms of dipeptide utilization are probably analogous in each of the cell lines. An obvious difference, however, was the more gradual increase of glutamine in ala-gln medium with BHK cultures (Figure 6-2b) compared to the rapid dipeptide hydrolysis noted with CC9C10 cells (Figure 4-6). This could be explained by a reduced sensitivity of BHK cells to the low initial glutamine levels in dipeptide medium resulting in a less severe drop in cell viability and release of the peptidase.

The studies above demonstrated 5 mM ala-gln or up to 40 mM gly-gln can substitute for glutamine in BHK cultures. The indicated ala-gln concentration also resulted in a significant reduction (32 %) in ammonium after 3 days of culture relative to the control with a 3 mM glutamine supplement (Figure 6-2c). Even more substantial reductions in ammonium levels, though, were expected in medium substituted with glutamate. Following hydrolysis of dipeptides, the released glutamine is still subject to metabolic deamidation and decomposition. Furthermore, the development of a glutamate-based medium could eliminate the need for the costly additions of high levels of gly-gln.

Early efforts to replace glutamine with glutamate demonstrated growth of HeLa cells in the substituted medium but only when a high glutamate level (20 mM) was used (Eagle *et al.*, 1956). Even at the high glutamate concentration, a marked lag phase was observed for HeLa cells while another line, mouse L fibroblasts, were unable to grow. Hassell and Butler (1990) later described the growth of different cell lines in glutamate medium. McCoy, BHK and Vero cells were each able to grow in medium with an equimolar (4 mM) substitution of glutamate for glutamine. However, the period of adaptation required for the cells to assume normal growth rates in glutamine-free medium varied among the cell lines. BHK cells depended on an adaptation period of 15-20 days and a higher initial glutamate level which was gradually reduced over several passages.

The present study indicates suboptimal growth of BHK cells when glutamine was substituted with glutamate at either high (20 mM) or low (6 mM) concentrations without additional supplements. Regardless of the initial level of glutamate, more pronounced lag phases, slower growth rates and lower cell yields were obtained compared to the medium containing glutamine (Figure 6-4). Growth also did not appear to improve in glutamate despite adaptation and prolonged culture in the medium considerably longer than 20 days. This poor performance of BHK cells in glutamate

may not have been witnessed in the earlier report which indicated doubling times of 22-25 hours in glutamine medium (Hassell and Butler, 1990). A doubling time of 12-14 hours is possible with these cells and a longer period indicates suboptimal culture conditions. This may have obscured the actual differences between growth in glutamine and glutamate medium. Thus, the growth response in glutamate medium is not only dependent on the cell line but may also vary within the same line depending on the specific culture conditions.

The present study demonstrates glutamine cannot be completely eliminated from the medium without a considerable impact on the growth performance. Nevertheless, cells were capable of growing in glutamate and means of improving the growth characteristics required further attention. The effect of the cell inoculum on the growth in glutamate medium was first examined because a low initial density could contribute to a reduction in growth performance. This possibility has not been considered by other investigators. The duration of the lag period was reduced and the exponential growth rate improved with increasing inoculation densities up to 1.28×10^5 cells/ml (Figure 6-5). A seed concentration of ~1×10^5 cells/ml in glutamate medium was selected for future experiments to reduce the lag phase while ensuring an adequate growth rate. However, further improvements required closer scrutiny of additional nutritional requirements of BHK cells cultured in glutamate medium.

Efforts to ameliorate a possible nutritional deficiency in glutamate medium focused on supplementing the medium with metabolites normally produced from glutamine. Eagle *et al.* (1956) found that adding 0.5-1 mM NH_4Cl to medium substituted with glutamate usually improved the growth of HeLa cultures. Presumably, the ammonium served as a substrate along with glutamate and enhanced the cellular synthesis of glutamine via the glutamine synthetase reaction. The addition of up to 2 mM NH_4Cl had little effect on either the lag phase or growth rates of BHK cells cultured in glutamate medium (Figure 6-6). Although lower ammonium production was

expected, it was evidently not a growth limiting factor in this case. Sufficient ammonium was probably generated by the metabolism of glutamate to meet the demands of existing levels of glutamine synthetase.

Aspartate and asparagine are also derived from glutamine (Tate and Meister, 1973 and McKeehan, 1986) and are not standard components of DMEM. Cells must therefore rely on endogenous production of these amino acids and a low rate of biosynthesis in glutamate medium could limit growth. This process could also place further demands on a potentially limited cellular capacity to produce glutamine. Previous reports have indicated aspartate or asparagine supplements are important in cultures with a limited availability of glutamine or when cells are unable to utilize glutamine as a substrate. Glacken (1988) suggested ammonium could be substantially reduced in bioreactors by controlling glutamine at low levels and supplying aspartate and asparagine. This was compatible with the report by Donnelly and Scheffler (1976) indicating CHO cells could grow in the presence of a respiration inhibitor (and were therefore unable to oxidatively metabolize glutamine), provided asparagine was included in the medium. Asparagine has also been used as a substitute for glutamine in CHO cell cultures (Kurano *et al.*, 1990). Consequently, aspartate and asparagine were investigated as additional supplements in glutamate medium for BHK cultures.

Aspartate, throughout a range of concentrations examined (1.25-20 mM), was of no benefit in improving the lag phase or growth rate of BHK cells in glutamate medium (Figure 6-7). On the contrary, the lag phase appeared to be exacerbated with an increasing concentration of aspartate. The addition of asparagine, alternatively, resulted in marked improvements in growth (Figure 6-8a). At all concentrations considered (0.06-3.0 mM), the lag phase was ameliorated and the specific growth rate and cell yield increased in response to the asparagine. A concentration of 3 mM appeared optimal, resulting in 25 % and 85 % increases in growth rate and cell yield after 3 days, respectively, compared to glutamate medium without the asparagine

supplement. The relatively high concentration of asparagine would prevent its depletion and promote high consumption rates. Addition of aspartate to medium supplemented with both glutamate and asparagine resulted in no further improvements in growth (Figure 6-8b). These experiments suggested the rate of cellular aspartate production was sufficient to meet the demands of BHK cells grown in glutamate medium. Presumably, aspartate was readily produced from the abundant supply of glutamate. However, the biosynthesis of asparagine was apparently a growth limiting reaction, probably resulting from the restricted availability of glutamine required for the amidation of aspartate. This was offset by including asparagine directly in the medium. Growth performance was nevertheless considerably reduced in the presence of both glutamate and asparagine compared to cultures containing the usual glutamine supplement. This implies a deficiency of other products derived from glutamine also contributes to the growth limitation in glutamate medium. Defining each of the limiting nutrients and including them as supplements would increase the complexity and cost and perhaps also reduce the stability of the medium. The more practical approach taken here was to minimize and replace a majority of the cellular glutamine requirements in glutamate medium.

In glutamate-based medium, the minimum glutamine level necessary for normal growth of BHK cells was determined with and without asparagine. In the presence of 3 mM glutamate, glutamine could be reduced to 1 mM without a deleterious effect on growth (Figure 6-9a). The specific growth rate and cell yield were 50 % and 227 % higher, respectively, than the culture lacking glutamine. Even the lowest concentration examined (0.12 mM) substantially improved growth although limitations were still apparent until glutamine reached 1 mM. The addition of 3 mM asparagine to the glutamate medium further reduced the minimum glutamine level to as low as 0.25 mM (Figure 6-9b). However, 0.5 mM would more likely accommodate potential variations in

glutamine requirements (or loss of glutamine during prolonged incubation or storage) and was still 6 X lower than the 3 mM necessary in glutamine-based medium.

The benefits of supplementing glutamate medium with low levels of glutamine may be related to a report by Griffiths and Pirt (1967). These authors described the growth of mouse LS cells adapted to medium substituted with 8 mM glutamate. Adding a nominal quantity of glutamine (0.7-0.8 mM) to the glutamate medium significantly reduced the lag phase and increased the growth rate compared to cultures lacking the glutamine. In their experiments, an additional supplement with asparagine did not result in further improvements in growth in the glutamate/glutamine medium. However, asparagine may have a limited effect with increasing glutamine levels as observed for BHK cells (Figure 6-9b).

In the presence of glutamate and asparagine, only a minimal glutamine supplement was essential to sustain normal growth of BHK cells. It would also stand to reason that relatively low concentrations of dipeptides could be used in place of glutamine. Indeed, a combination of 3 mM glutamate, 3 mM asparagine and 1 mM ala-gln or 2.5 mM gly-gln resulted in growth rates and cell yields comparable to cultures containing 3 mM glutamine (Figure 6-10a-c). Thus, a 5- and 16-fold reduction in ala-gln and gly-gln, respectively, was possible and growth actually improved compared to media in which only the dipeptides were used as substitutes (Figure 6-2a and Figure 6-3). The dipeptides were also advantageous in ensuring the stability of the medium when used in conjunction with glutamate. A low glutamine supplement, on the other hand, could decompose to limiting levels with extended periods of storage. The use of gly-gln also became feasible at the lower concentration which would reduce costs considerably.

The final process in the development of the glutamate-based medium was to establish the optimal glutamate concentrations for BHK cells in batch culture and its efficacy in controlling ammonium formation. This was essential to prevent a growth

limitation resulting from its depletion and validate continued studies with this type of medium. In the presence of 3 mM asparagine and 0.5 mM glutamine, 3 mM glutamate was sufficient to maintain a growth rate and cell yield that was comparable to or exceeded the control culture with 3 mM glutamine (Figure 6-11a). A low glutamate concentration (1 mM) resulted in a premature entry into the decline phase. Although the glutamate was depleted after 3 days at an initial concentration of 3 mM, this did not appear to be the growth limiting factor. This was indicated by the experiments with cultures whose higher initial glutamate supplements were not completely consumed, but entered the stationary phase at the same point (Figure 6-11b). Ammonium was next measured in the cultures with different glutamate additions and found to be markedly reduced compared to those with an equivalent glutamine supplement (Figure 6-11c). With glutamate and glutamine each initially at 3 mM, for example, ammonium was 63 % lower in the former at 0.76 mM after 3 days of culture. In addition, increasing the initial glutamate supplement did not result in an elevation of ammonium as observed with glutamine. Furthermore, regardless of the concentration of the initial glutamate supplement, ammonium was low and almost a constant ~0.2 mM in the cell-free medium. Clearly, glutamate was more stable and losses due to decomposition were insignificant compared to glutamine-based medium. Thus, higher levels of glutamate may be included in the culture medium without the drawback of increased ammonium yields.

Together, the studies presented above demonstrate glutamate and asparagine effectively replace the majority of glutamine requirements of BHK cells. Only a minimal additional supplement with glutamine or dipeptides was essential to support normal growth rates and cell yields in the glutamate-based medium. However, growth was not enhanced compared to cultures with the standard complement of glutamine despite the much lower ammonium production when glutamate was utilized by the cells. An explanation for this was provided in the following chapter.

Chapter 7

Growth, Metabolism and Adaptation of BHK Cells in Glutamate-Based Medium

7.1 Introduction

Glutamate and, to a lesser extent, asparagine significantly reduce the glutamine requirements of BHK cells. However, the effect of the substitution on cellular metabolism warranted further consideration. Considerable reductions in ammonium production in glutamate medium have been shown by other investigators (Hassell and Butler, 1990) and this was confirmed in the current study. The impact on glucose, lactate, amino acid metabolism and intracellular nucleotides was less obvious and has not been covered extensively in the literature. Growth and metabolic analyses of BHK cells in glutamate medium were conducted with batch stationary cultures (multiwell plates or T-flasks). The potential for improved cell yields with glutamate-based medium was then evaluated in batch and fed-batch microcarrier systems. The microcarriers would eliminate the potential of growth limitations arising from a restricted availability of surface area.

Further investigations focused on the factors which influence growth, metabolism and adaptation in glutamate medium. Early studies suggested the activity of glutamine synthetase, and hence the conversion of glutamate to glutamine, limited growth in glutamate-substituted medium (Griffiths and Pirt, 1967). McDermott and Butler (1993) working with other cell lines concluded the rate of glutamate uptake and not glutamine synthetase activity dictated adaptability to glutamine-free medium. Each of these possibilities was investigated as a growth limiting parameter for BHK cells cultured in glutamate medium. Other enzymes involved in the initial stages of glutamine and glutamate metabolism including phosphate-activated glutaminase, alanine aminotransferase and glutamate dehydrogenase were also considered to explain some of the metabolic changes associated with growth in glutamate medium.

7.2 Materials and methods

7.2.1 Media and culture conditions

7.2.1.1 Batch stationary cultures (multiwell plates and T-flasks)

The glutamate-based media selected for metabolic analyses were based on formulations developed in the previous chapter which supported normal growth characteristics of BHK cells in batch culture. Unless otherwise indicated, the composition of the media and the controls with shorthand designations are listed below.

Glutamate-based media
DMEM/10 % calf serum/1.2 mM serine with:
i) 3 mM glutamate + 3 mM asparagine + 0.5 mM glutamine (DMEM/glu+gln)
ii) 3 mM glutamate + 3 mM asparagine + 1 mM ala-gln (DMEM/glu+ala-gln)
iii) 3 mM glutamate + 3 mM asparagine + 3 mM gly-gln (DMEM/glu+gly-gln)

Controls
DMEM/10 % calf serum/1.2 mM serine with:
iv) 3 mM glutamate (DMEM/glu)
v) 3 mM glutamine (DMEM/gln)

Media containing glutamate, including the optimized glutamate-based formulations and the DMEM/glu control, were collectively referred to as "glutamate-substituted" or simply "glutamate media". Cells were adapted to each of the different media for at least 6 passages before beginning the experiments unless otherwise indicated. Experiments performed in 24-well plates or T-flasks were designated as "stationary" cultures to distinguish them from growth in the stirred microcarrier cultures. Cells were inoculated at ~1 X 10^5 cells/ml in a volume of 1 ml/well for the multiwell plates. The T-flasks were used only for analysis of intracellular nucleotides and enzyme activities according to procedures described later (sections 7.2.4 and 7.2.5, respectively). Standard culture conditions, including incubation temperature and atmosphere, were stated in section 2.4.

7.2.1.2 Batch microcarrier cultures (spinner flasks)

Growth media used for the experiments in 100 ml spinner flasks were DMEM/glu+gln, DMEM/glu and DMEM/gln as described above with a few modifications. All media contained a 3 mM asparagine supplement. In addition, the glutamate concentration was increased to 4 mM in the glutamate-substituted media (DMEM/glu+gln and DMEM/glu) and 4 mM glutamine was included in DMEM/gln. Cytodex 1 was added to each flask at a concentration of 5 g/L and the final culture volume was 100 ml. Flasks were pre-incubated for ~2 hours to allow pH equilibration and then inoculated at ~1.5 X 10^5 viable cells/ml for each type of medium. The vessels were then placed on a stirring platform in the CO_2 incubator with a constant 40 r.p.m. stir rate. Standard incubation conditions were employed for the duration of the culture (section 2.4). The sample volume removed from the spinners for cell counts and medium analysis was 2 ml/day.

7.2.1.3 Fed-batch microcarrier cultures (bioreactor)

Fed-batch experiments were performed in a New Brunswick CelliGen 1.5 L bioreactor. The growth media used were DMEM/glu+gln and DMEM/gln essentially as described for stationary batch cultures, with 3 mM initial glutamate and glutamine concentrations, respectively. DMEM/gln also contained the same 3 mM asparagine supplement that was added to DMEM/glu+gln. Cytodex 1 was included in the medium at 5 g/L and the final culture volume for each medium was 1.25 L. Cells were inoculated at ~1.5 X 10^5 viable cells/ml in each medium. The daily sample volume removed from the bioreactor for cell counts and medium analysis was ~5 ml. The temperature, pH and dissolved oxygen (D.O.) concentrations were regulated automatically by the bioreactor controller system. The temperature was maintained at 37 ^oC by a heating mat surrounding the bioreactor vessel. The pH of the medium was controlled at 7.2 by the addition of 0.2 M Na_2CO_3 from a peristaltic pump and CO_2

from a mixed gas sparging system. Oxygen was regulated at 50 % air saturation by infusion of a balance of oxygen and nitrogen. Pressurized CO_2, O_2 and N_2 were added through an aeration cage in the stirring assembly. The stirring rate was 40 r.p.m. for the first 24 hours and 60 r.p.m. for the remainder of the culture period. The cultures were fed daily from concentrated nutrient solutions. Nutrients consumed at the highest rates were provided in Feed Solution 1: glucose, glutamate, glutamine and serine for DMEM/glu+gln and glucose, glutamine and serine for DMEM/gln. Feed Solution 2 consisted of 50X MEM amino acids (Gibco, 320-1130). The composition of each of these solutions and the increase in nutrient concentration resulting from the feed are listed in Table 7-1. The volumes added were 11.5 ml Feed Solution 1 and 12.7 ml Feed Solution 2 at 22 and 118 hours. This was increased to 20.7 ml and 25.3 ml for Feed Solutions 1 and 2, respectively, at 46, 70 and 96 hours. Pumps were used to add the feed solutions and the correct volume was dispensed according to the measured flow rate. The sample volume removed daily from the bioreactor was ~5 ml.

Table 7-1. Feed solutions and concentrations for fed-batch microcarrier cultures.

DMEM/	Nutrient	Feed stock conc. (mM)	Conc. increase after feed (mM) 22 and 118 h	46-96 h
glu+gln	Feed Solution 1			
	glc	500	4.6	8.3
	glu	200	1.8	3.3
	gln	33.3	0.3	0.6
	ser	100	0.9	1.7
gln	Feed Solution 1			
	glc	500	4.6	8.3
	gln	200	1.8	3.3
	ser	100	0.9	1.7
glu+gln and gln	Feed Solution 2			
	arg•HCl	30	0.30	0.60
	cys	5	0.05	0.10
	his•HCl•H_2O	10	0.10	0.20
	ile	20	0.20	0.40
	leu	20	0.20	0.40
	lys•HCl	20	0.20	0.40
	met	5	0.05	0.10
	phe	10	0.10	0.20
	thr	20	0.20	0.40
	trp	2.5	0.025	0.05
	tyr	10	0.10	0.20
	val	20	0.20	0.40

7.2.2 Cell enumeration

Total cell densities were evaluated using a Coulter counter. Viable cell concentrations were determined with trypan blue staining and a haemocytometer as described previously (section 2.8).

7.2.3 Analysis of media components

Amino acid concentrations were determined by OPA derivatization of samples with separation and quantification by reverse phase HPLC (section 2.11.4.2). Ammonium was analyzed with a gas-sensing electrode (section 2.11.2). Glucose and lactate were quantified with the glucose oxidase (section 2.11.1.2) and lactate dehydrogenase (section 2.11.1.3) assays , respectively.

7.2.4 Intracellular nucleotide analysis

Intracellular levels of UDP-GNAc (UDP-GlcNAc + UDP-GalNAc) and ATP were determined for BHK cells cultured in glutamate- and glutamine-based media (DMEM/glu+gln and DMEM/gln, respectively). The procedure was adapted from a method described by Ryll and Wagner (1991). Cells were seeded at an initial concentration of ~0.5 X 10^5 cells/ml in triplicate 25 cm^2 T-flasks for each medium. After 4 days of growth, cells were trypsinized according to standard procedures (section 2.5) and resuspended in PBS. Each of the replicate cultures were treated independently and contained 4 to 5 X 10^6 viable cells. Cells were centrifuged at 330 g for 8 minutes. The pellet was resuspended in 120 µl of cold 6 % TCA (v/v) and sonicated for 15 seconds on ice. After 20 minutes of cooling in an ice bath, cell debris was removed by a 5 minute centrifugation in a microfuge at 14 000 r.p.m. and 4 ^oC. A 100 µl volume of supernatant was collected and neutralized with 27 µl 0.5 M Tris (pH 9.0) and 8-10 µl 2 M NaOH. Nucleotides were determined by separation and quantification with reverse phase chromatography as outlined in section 2.12.

7.2.5 Preparation of cellular lysates for enzyme assays

The activities of glutamine synthetase, phosphate-activated glutaminase, alanine aminotransferase and glutamate dehydrogenase were determined in BHK cells grown in glutamate-based medium (DMEM/glu+gln) and controls (DMEM/glu and DMEM/gln). The media were essentially as described in section 7.2.1.1 except the glutamate supplement was increased to 4 mM in DMEM/glu+gln and DMEM/glu and 4 mM glutamine was included in DMEM/gln. DMEM/glu also contained 3 mM asparagine for this experiment. Cultures were grown for 3 days to mid-exponential phase in 7 X 150 cm^2 T-flasks for each of the media. Cells were trypsinized and cultures of the same medium composition were pooled. For each medium, 5 to 8 X 10^7 viable cells were harvested. The cells were washed with PBS, centrifuged (330 g for 5 minutes) and resuspended in 1.5 ml extraction buffer (0.25 M sucrose, 5 mM HEPES and 1 mM EGTA, pH 7.4). Each of the samples was sonicated 4 X 15 seconds on ice and then centrifuged at 31 000 g for 5 minutes at 4^oC. The supernatant was the cytosolic fraction. The pellet corresponded to the membrane fraction and was washed with PBS, centrifuged again at 31 000 g and resuspended in the extraction buffer. Both the cytosolic and membrane fractions were stored in aliquots at -70 ^oC. Protein was determined in each of the samples by the bicinchoninic acid procedure (section 2.10) and the enzyme assays performed as described in section 2.13.

7.3 Results

7.3.1 Growth and metabolism of BHK cells in batch stationary cultures with glutamate-based media

7.3.1.1 Growth performance

Batch cultures are useful for initial comparisons of growth performance and metabolism under different culture conditions. Growth in each of the glutamate-based media and the glutamine control could be directly compared in multiwell plates as shown in Figure 7-1. Cells adapted to each of the glutamate-based media were also subcultured into the same medium but without glutamate (0 mM). This would indicate whether the cells were utilizing glutamate as a replacement for glutamine or instead metabolized lower levels of glutamine more efficiently. Growth in medium with 1 mM ala-gln was similar with and without the glutamate supplement. This indicates that cells previously cultured in glutamate may use glutamine more effectively or metabolize alanine in place of glutamine to some extent. However, the importance of glutamate was apparent in medium with 0.5 mM glutamine and 3 mM gly-gln from which the glutamate had been omitted. In the former, cells prematurely entered the stationary phase resulting in a low cell yield and was undoubtedly due to glutamine depletion. The latter was characterized by a markedly reduced growth rate and could be explained by a glutamine limitation throughout the exponential phase.

For a more quantitative evaluation of growth in glutamate-based media (DMEM/glu+gln, DMEM/glu+ala-gln and DMEM/glu+gly-gln) and controls (DMEM/glu and DMEM/gln), cell yields (after 3 days), doubling times and specific growth rates were determined and provided in Table 7-2. The cell yields showed only minor differences among the glutamate-based media which reached ~2 X 10^6 cells/ml. Cell concentrations were <10 % higher in the control culture with 3 mM glutamine. Cells were confluent after 3 days and the wells were completely covered in all media except DMEM/glu which attained peak density 24 hours later. This indicated surface area was

a limiting factor in batch stationary cultures. The doubling times were not significantly different in DMEM/glu+gln, glu+ala-gln and glu+gly-gln at ~20 hours and only slightly higher than the 18.5 hours in DMEM/gln. The specific growth rates were also comparable among these media at 0.035-0.038 hr^{-1}. Growth in DMEM/glu demonstrated an obvious limitation, exemplified by the lower cell yield, longer doubling time and reduced growth rate as previously observed (Figure 6-4). The glutamate-based media with minimal glutamine or dipeptide additions all produced acceptable growth and were selected for a detailed metabolic analysis. The ability of BHK cells to grow with a considerably reduced availability of glutamine immediately suggested alterations in metabolism had occurred. To more precisely define and characterize those changes, an array of metabolites and nutrients were analyzed in the cultures grown in multiwell plates.

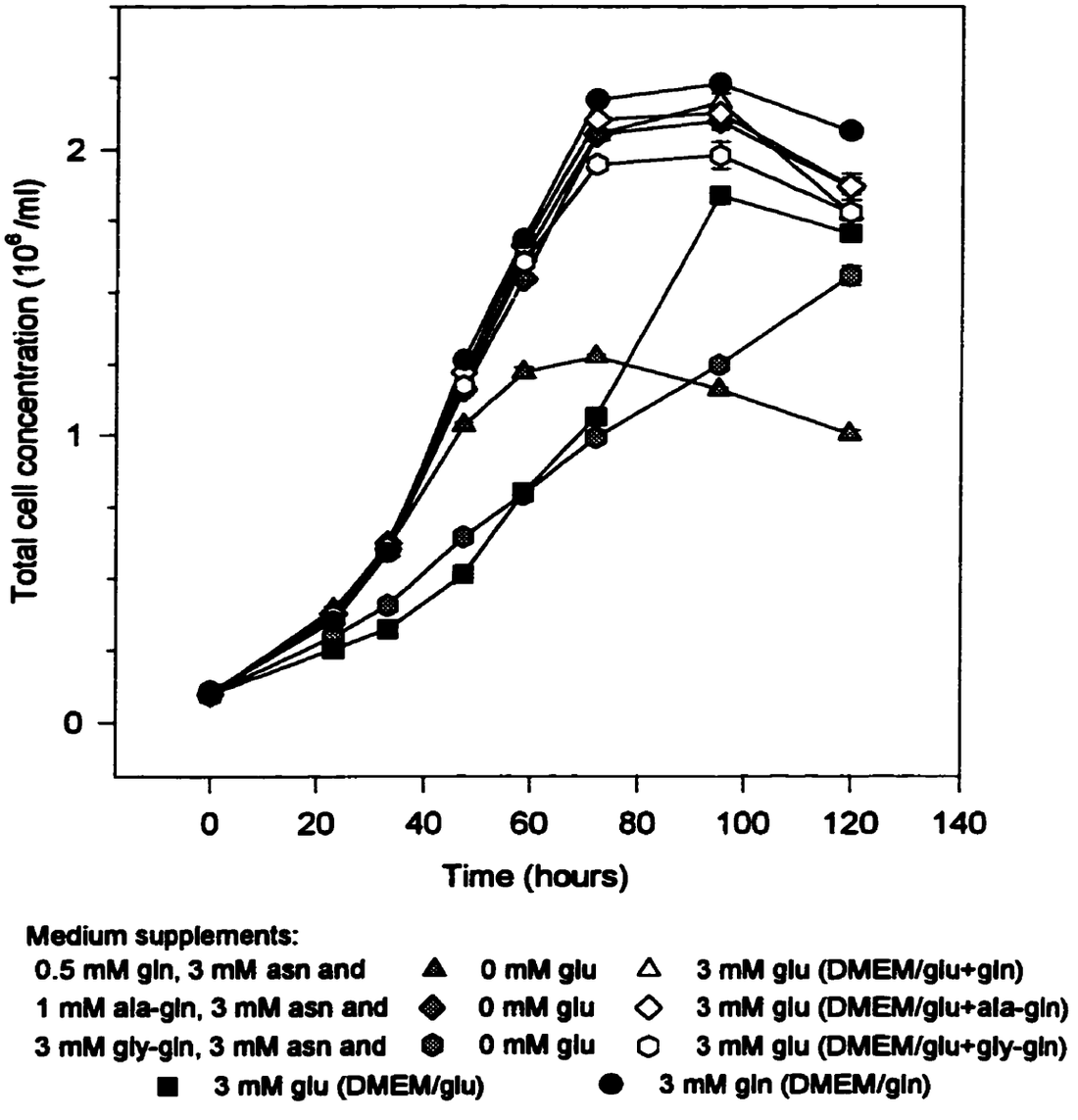


Figure 7-1. Growth of BHK cells in batch stationary cultures with glutamate-based media and controls. Supplements included glutamate, asparagine and glutamine or dipeptides as specified in the legend. Controls contained 3 mM glutamate or 3 mM glutamine without further additions. For each medium, cultures were grown in triplicate (n=3) in 24-well plates and counted with a Coulter counter. Replicate counts were averaged and plotted ± SE.

Table 7-2. Growth parameters for BHK cells cultured in glutamate-based media and controls. The cell yield in all cases indicates the cell concentration after 3 days (72 hours) of growth. Doubling times and specific growth rates were determined from 47-95 hours for DMEM/glu and 23-72 hours for the remaining cultures. Values are derived from the average of three replicate cultures (n=3) ± SE.

DMEM/	Cell Yield (10^6 cells/ml)	Doubling Time (hours)	Specific Growth Rate ($hour^{-1}$)
glu+gln	2.05 ± 0.02	19.5 ± 0.5	0.036 ± 0.004
glu+ ala-gln	2.10 ± 0.02	19.7 ± 0.4	0.036 ± 0.004
glu+gly-gln	1.95 ± 0.00	20.4 ± 0.5	0.035 ± 0.004
glu	1.06 ± 0.02	26.0 ± 1.1	0.026 ± 0.002
gln	2.17 ± 0.01	18.5 ± 0.3	0.038 ± 0.005

7.3.1.2 Ammonium production

Ammonium concentrations were measured in the glutamate-based media and control cultures. This would demonstrate if the low levels of glutamine or dipeptide supplements resulted in increased ammonium production. Figure 7-2 shows that in all the glutamate-based media, ammonium levels were similar to DMEM/glu and considerably reduced with respect to the DMEM/gln culture. DMEM/glu+gln produced slightly more ammonium towards the later stages of the culture period than the other glutamate cultures. Nevertheless, after 3 days of growth, ammonium was reduced by more than 60 % in all of the glutamate-based media with concentrations ranging from 0.49-0.73 mM compared to 1.97 mM in DMEM/gln.

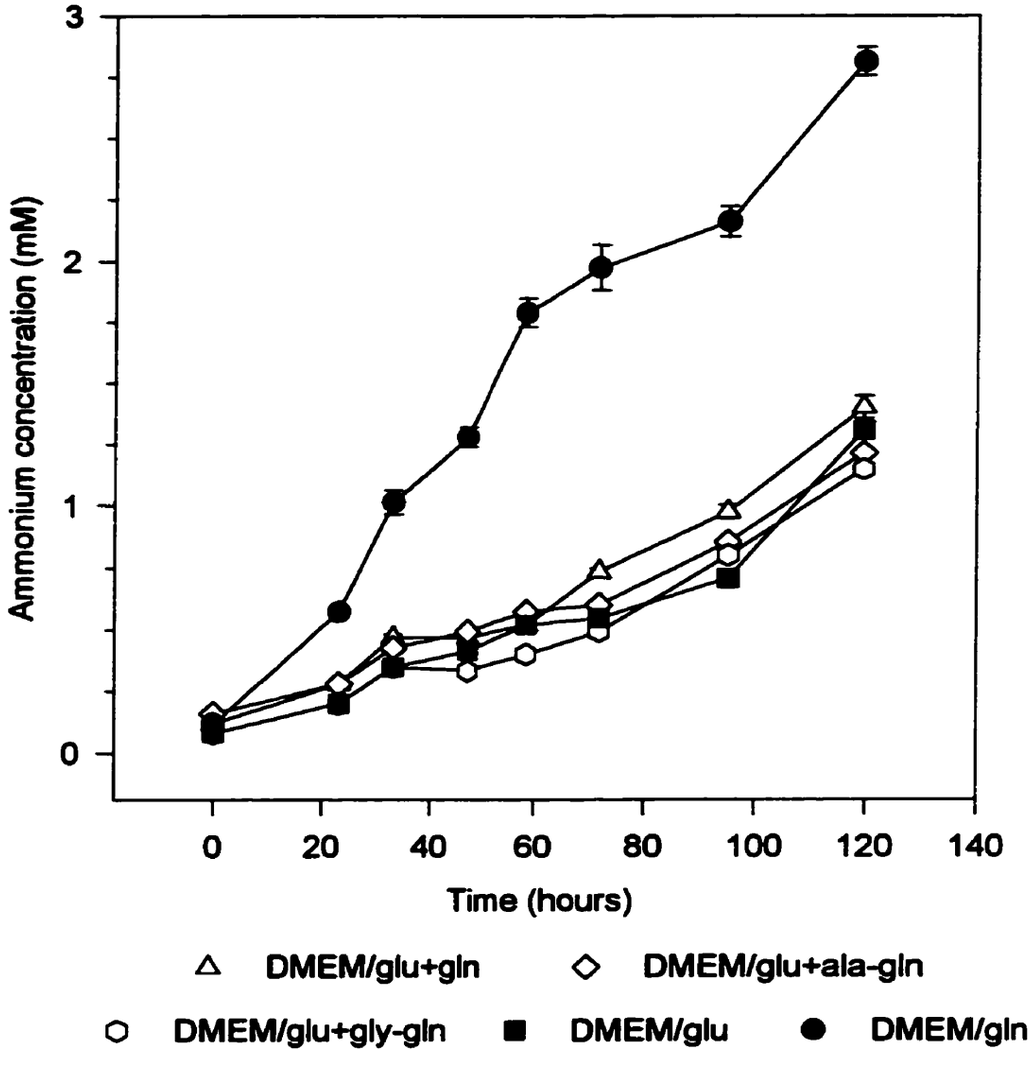


Figure 7-2. Ammonium concentrations in batch stationary cultures of BHK cells grown in glutamate-based media and controls. Ammonium was determined in three replicate cultures (n=3) from each of the media indicated in the legend. The average of the replicates was plotted ± SE.

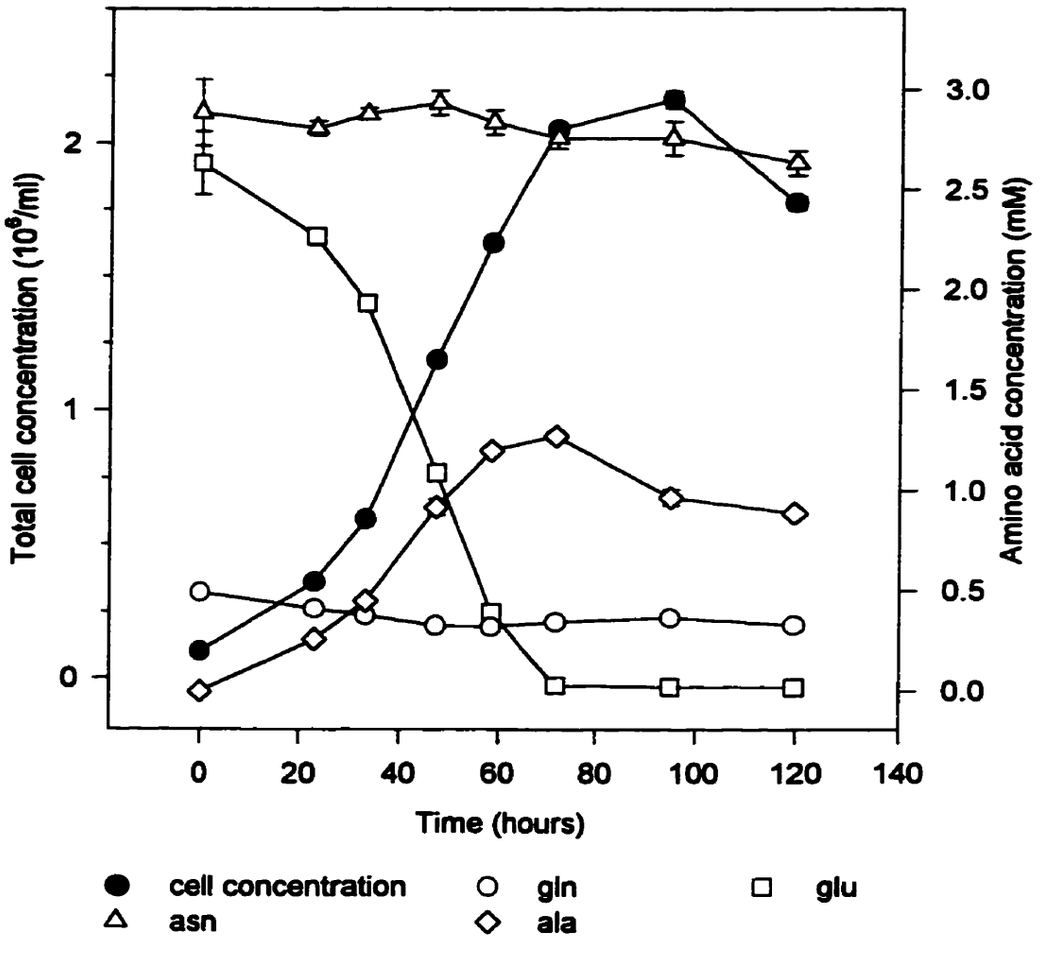
7.3.1.3 Amino acid and dipeptide metabolism

The low-ammoniagenic nature of glutamate-based media was obviously due to the decreased availability of glutamine. This was demonstrated with amino acid analysis of the cultures. The concentrations of glutamine, glutamate, other selected amino acids and dipeptides are presented in Figure 7-3a-e. The reduced requirement for glutamine could be directly observed in DMEM/glu+gln (Figure 7-3a). The glutamine concentration, originally at 0.5 mM, decreased by only about 0.15 mM after 3 days of culture. Glutamine consumption was minimal for the remainder of the culture period and was not depleted. In DMEM/glu+ala-gln, a decrease in the concentration of dipeptide corresponded to an increase in glutamine in the medium (Figure 7-3b). Glutamine was therefore supplied by the hydrolysis of the dipeptide but only a fraction of the released glutamine was consumed. The highest rate of ala-gln hydrolysis occurred during the lag phase. This was also observed with the growth of the CC9C10 hybridoma in medium containing this dipeptide and was probably due to the release of peptidase from non-viable cells during the lag period. By the end of the culture period, there was complete hydrolysis of the 1 mM ala-gln and the final glutamine concentration reached about 0.9 mM. Thus, a maximum of only 0.1 mM glutamine was utilized which was consistent with the low consumption in DMEM/glu+gln. In DMEM/glu+gly-gln, only a fraction of the dipeptide was hydrolyzed (Figure 7-3c). Glutamine increased in the medium at a relatively slow and constant rate throughout the culture period which was not unexpected considering a low peptidase affinity for gly-gln described previously with the hybridoma line. The amount of glutamine consumed was very low as in the ala-gln medium but could not be measured precisely due to the relatively low level of gly-gln hydrolysis and the error associated with the quantification of the dipeptide. In contrast to the glutamate-based media, glutamine was consumed at a higher rate than any other amino acid in DMEM/gln, decreasing by >2 mM in the first 3 days (Figure 7-3e) and was 13.6 X that utilized in DMEM/glu+gln.

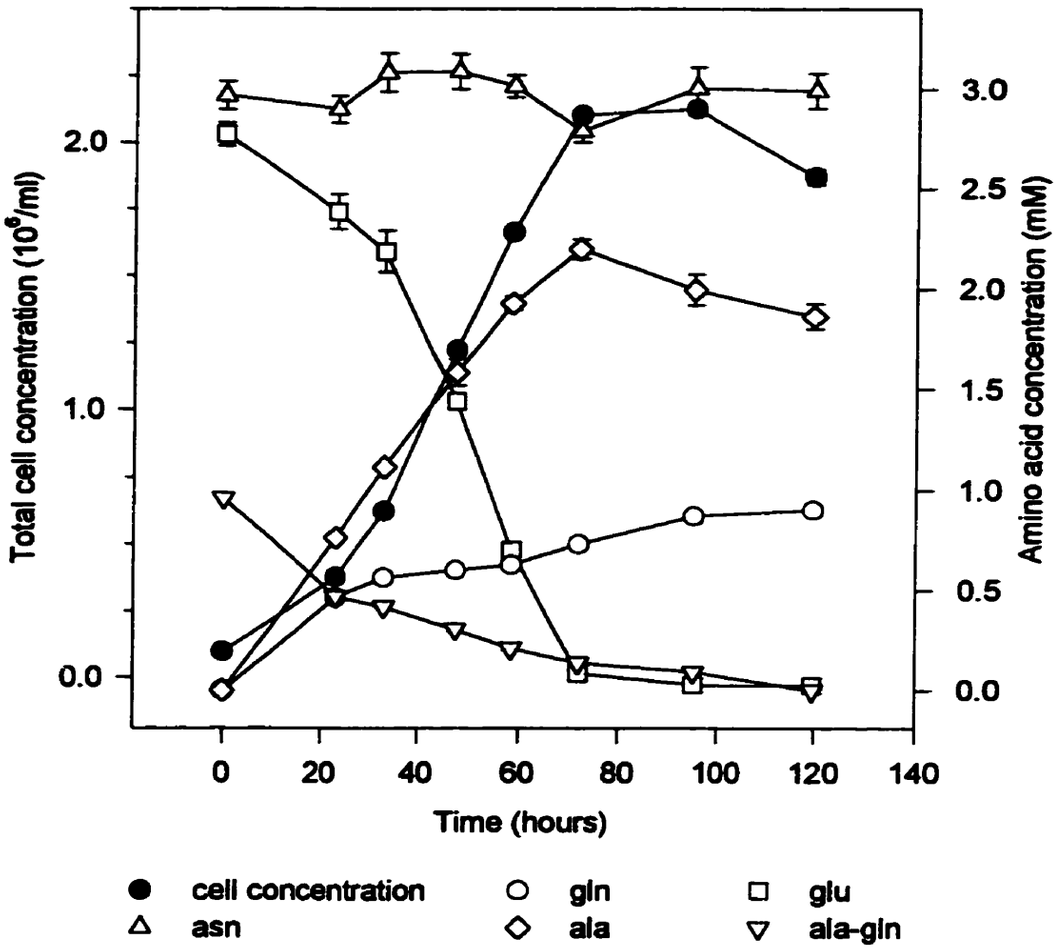
Glutamine was reduced to 0.5 mM in DMEM/gln by the end of the culture period but was not depleted. In the glutamate-based cultures, glutamate was clearly utilized at a high rate in place of glutamine and was essentially depleted after 3 days (Figure 7-3a-c). It was also completely consumed in the DMEM/glu control but not for another 24 hours due to the slower growth rate (Figure 7-3d). The extent of asparagine utilization in the glutamate-based media was also considered and found to be minimal. Asparagine clearly plays a less important role in replacing glutamine than glutamate. Another important observation was alanine levels were much higher in the cultures containing glutamate. Alanine reached 1.27 mM and 1.34 mM after 3 days in DMEM/glu+gln and DMEM/glu+gly-gln, respectively. The alanine concentration was even higher in DMEM/glu+ala-gln at 2.20 mM after 3 days but was similar to the other two cultures if the alanine arising from dipeptide hydrolysis was subtracted. Even in DMEM/glu, alanine reached 0.94 mM after 3 days despite the lower cell yield in this medium. This compares with 0.54 mM on day 3 with DMEM/gln. The implications of differences in alanine production and their relationship to glutamine and glutamate metabolism are considered below in further detail.

Figure 7-3a-e. Selected amino acid and dipeptide concentrations in batch stationary cultures of BHK cells grown in glutamate-based media and controls. The metabolites examined in (a) DMEM/glu+gln, (b) DMEM/glu+ala-gln, (c) DMEM/glu+gly-gln, (d) DMEM/glu and (e) DMEM/gln included glutamine, glutamate, asparagine, alanine, ala-gln and gly-gln as specified in the legend. Concentrations were determined in each of the triplicate cultures (n=3) for each medium. Replicate values were averaged and plotted ± SE.

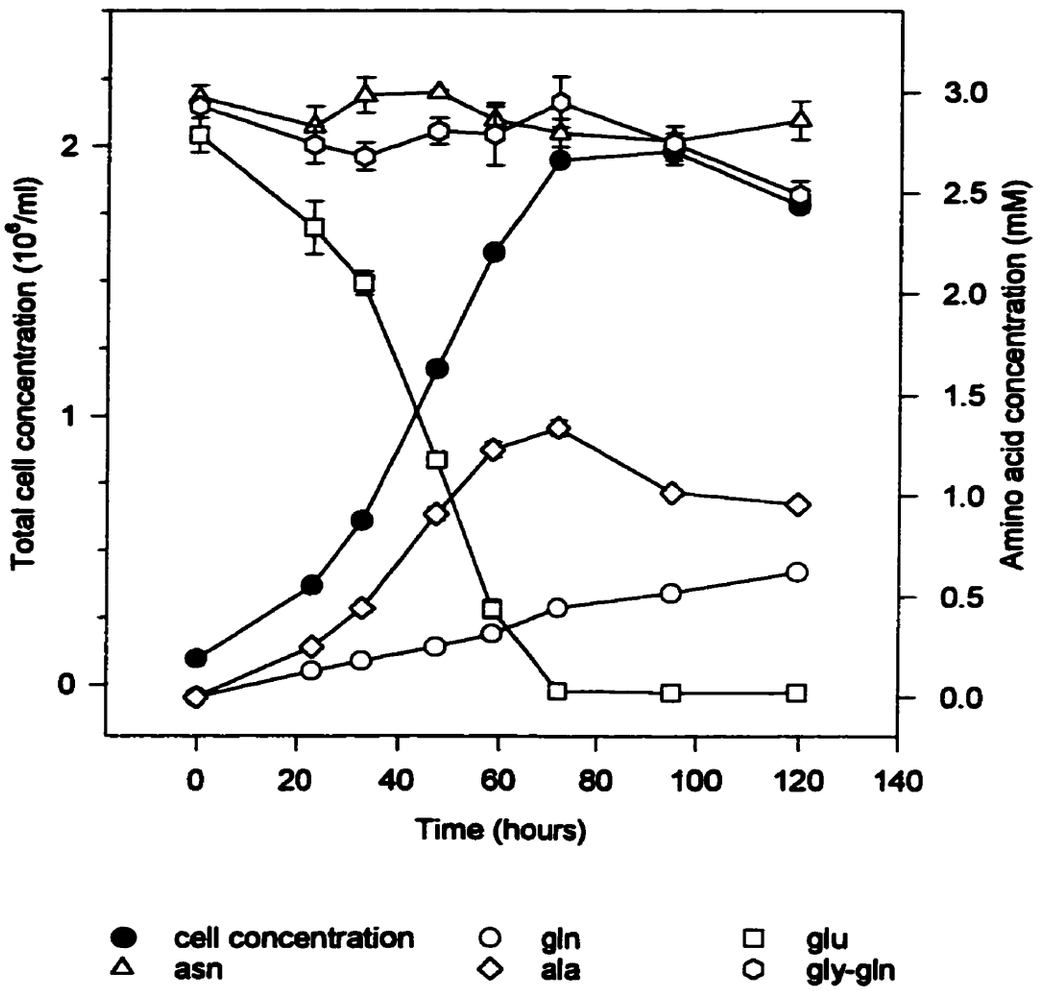
(a)


Total cell concentration (10^6/ml)
Amino acid concentration (mM)
Time (hours)
0
20
40
60
80
100
120
140
0
1
2
0.0
0.5
1.0
1.5
2.0
2.5
3.0
cell concentration
gln
glu
asn
ala

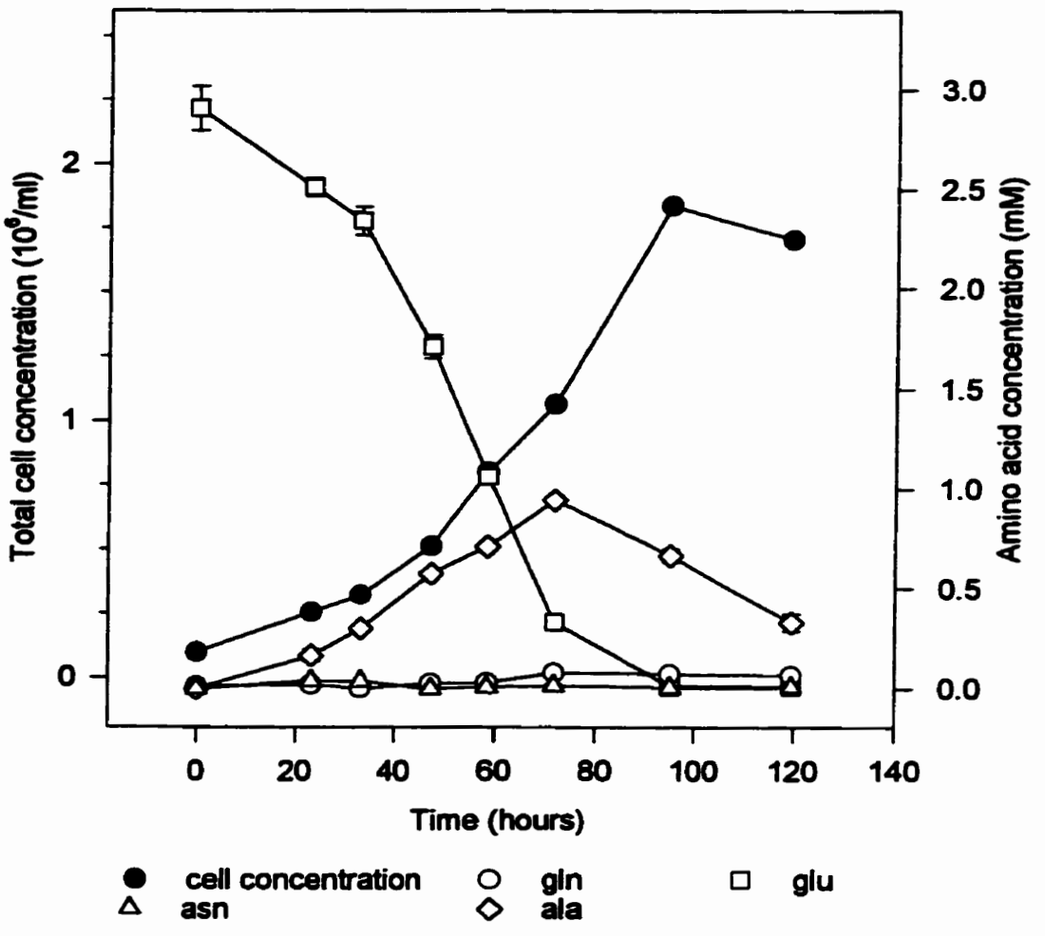
(b)


Total cell concentration (10^6/ml)
Amino acid concentration (mM)
Time (hours)
0.0
1.0
2.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
20
40
60
80
100
120
140
cell concentration
gln
glu
asn
ala
ala-gln

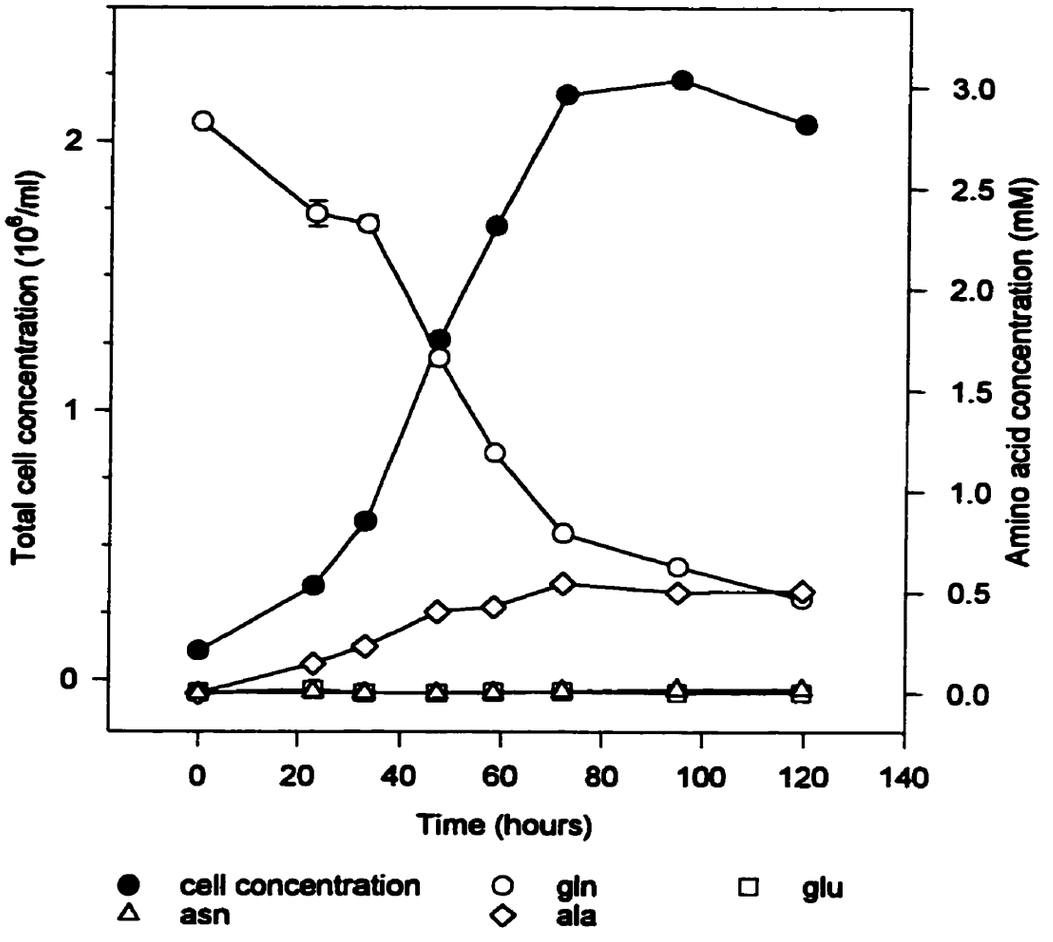
(c)


Total cell concentration (10^6/ml)
Amino acid concentration (mM)
Time (hours)
0
20
40
60
80
100
120
140
0
1
2
0.0
0.5
1.0
1.5
2.0
2.5
3.0
cell concentration
asn
gln
ala
glu
gly-gln

(d)


Total cell concentration (10^6/ml)
Amino acid concentration (mM)
Time (hours)
0
20
40
60
80
100
120
140
0
1
2
0.0
0.5
1.0
1.5
2.0
2.5
3.0
cell concentration
gln
glu
asn
ala

(e)


Total cell concentration (10^6/ml)
Amino acid concentration (mM)
0
1
2
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
20
40
60
80
100
120
140
Time (hours)
cell concentration
gln
glu
asn
ala

A more complete profile of metabolism is provided in Table 7-3 which lists the specific consumption and production rates of all analyzed substrates and by-products during the exponential phase in each medium. To simplify analysis, the metabolites can be considered in categories of low (<1 nmol 10^{-6} cell hr^{-1}), moderate (1-5 nmol 10^{-6} cell hr^{-1}) and high (> 5 nmol 10^{-6} cell hr^{-1}) consumption or production.

A few amino acids were consumed at very high rates; in decreasing order of magnitude, these were glutamate (in the three glutamate-based media and DMEM/glu), glutamine (in DMEM/gln) and serine (in all media). Only glutamate was consumed at a rate which resulted in depletion by the end of the exponential phase (3-4 days). Specific glutamate utilization (q_{glu}) was not significantly different among the glutamate-based media and only slightly higher in DMEM/glu. A key observation was that glutamate was consumed at an even higher specific rate (up to 68.5 nmol 10^{-6} cell hr^{-1}) than glutamine (37.8 nmol 10^{-6} cell hr^{-1}) which indicates the glutamate uptake rate was unlikely a growth limiting factor in DMEM/glu. It further suggests that glutamate is utilized less efficiently than glutamine since the former was consumed at a greater rate but produced similar cell yields to the DMEM/gln culture. In other words, the growth yield (Y) (cells produced/pmole of substrate consumed) was lower for glutamate than glutamine. Another important point illustrated by the table was the low specific glutamine utilization (q_{gln}) value in DMEM/glu+gln. There was a net low consumption of glutamine in DMEM/glu+ala-gln and DMEM/glu+gly-gln as well although the table indicates glutamine was produced due to the hydrolysis of the dipeptide. The minimal glutamine utilization is reflected by a specific ammonium production more than 3-fold lower in the glutamate-based media (3.07-5.19 nmol 10^{-6} cell hr^{-1}) compared to DMEM/gln (17.8 nmol 10^{-6} cell hr^{-1}). The specific rates for ammonium were not significantly different among the glutamate-based media. The high serine consumption was very similar in all types of media at about 13 nmol 10^{-6} cell hr^{-1}. Thus, the 1.2 mM serine supplement was required in glutamate and glutamine media to prevent depletion

of this amino acid. Most of the remaining amino acids were utilized at low to moderate rates in glutamate-based media and, with some exceptions, were similar to the DMEM/glu control. In DMEM/gln, however, certain amino acids were consumed at higher rates including ile, leu and val. These nutrients may thus be utilized more efficiently in the glutamate-substituted media.

The rates for the dipeptides are also presented in Table 7-3. In this case, the numbers refer to a specific rate of change in the dipeptide concentrations and do not infer that the dipeptide has been consumed by the cell (only hydrolysis may have occurred). A moderate rate was observed for ala-gln at -7.03 nmol 10^{-6} cell hr^{-1}. A low rate in the decrease of gly-gln certainly occurred although the table indicates an increase due to difficulties in quantifying this dipeptide.

Only two amino acids accumulated in the medium as major metabolic by-products during the growth of BHK cells. Alanine and glycine were produced in all media but were higher in the glutamate-substituted formulations. Alanine was not significantly different among the cultures containing glutamate and was generated at a very high rate of 26.0-30.6 nmol 10^{-6} cell hr^{-1}. Note that the rate in DMEM/glu+ala-gln was not the highest as might have been expected. This can be explained by the most rapid rate of ala-gln hydrolysis (which generates alanine) occurring during the lag and not the exponential phase for which the specific rate was determined. Alanine production contrasted sharply in DMEM/gln at only 6.42 nmol 10^{-6} cell hr^{-1}. This was at least 4-fold lower than observed in the other cultures. Specific glycine production was not significantly different in DMEM/glu+gln, DMEM/glu+ala-gln and DMEM/glu at 5.28-6.06 nmol 10^{-6} cell hr^{-1}. The rate was more than double in DMEM/glu+gly-gln since glycine was produced not only via normal cell metabolism but also from the hydrolysis of gly-gln throughout the exponential phase. Glycine had only a moderate production rate (2.79 nmol 10^{-6} cell hr^{-1}) in DMEM/gln. Increased glutamate utilization in glutamate-based media appears to result in overflow metabolism and higher

production of incompletely oxidized end-products such as alanine and perhaps, to a lesser extent, glycine.

The most pertinent changes in ammonium and amino acid metabolism in the stationary cultures were related with product yield ratios determined after 3 days of growth (Table 7-4). The NH_4^+/gln value of 0.91 indicates almost 1 mole of ammonium was produced per mole of glutamine metabolized in DMEM/gln. Glutamate was considerably less ammoniagenic with only 0.24 mole NH_4^+/mole glu in DMEM/glu+gln. This ratio was even lower in the other glutamate-based media and DMEM/glu. The higher ammonium in DMEM/gln can be largely attributed to deamidation of glutamine via glutaminase activity. However, an increased proportion of the glutamine amino nitrogen may have been released as ammonium (via glutamate dehydrogenase) rather than being sequestered in the form of alanine via alanine aminotransferase. This is suggested by the lowest ala/gln value of 0.27 in DMEM/gln. This compares with 0.49 in DMEM/glu+gln and DMEM/glu+gly-gln which was nearly two-fold higher. The reduced alanine production and increased ammonium formation in DMEM/gln translates into an ala/NH_4^+ ratio up to an order of magnitude higher in glutamate-based media.

Cumulatively, the metabolic analysis of amino acids indicates glutamate and, to a lesser extent, asparagine can replace up to 90 % of cellular glutamine requirements. This allows glutamine to be maintained at a lower level in the growth medium and substantially reduces ammonium formation. Glutamate appears to be a less ammoniagenic substrate not only because of the fewer nitrogen moeities but also because it may be deaminated by a less ammoniagenic route. This possibility was later investigated with an examination of enzyme activities involved in glutamine and glutamate metabolism (section 7.3.4.2).

Table 7-3. Specific metabolite consumption (-) and production (+) rates (q) by BHK cells grown in batch stationary cultures in glutamate-based media and controls. Specific rates were determined from a plot of the change in substrate concentration versus the cell index. The values are derived from the best straight line ± SE during the exponential phase (33-58 hours for glutamate-based media and DMEM/gln and 47-72 hours for DMEM/glu unless otherwise indicated).

	Specific production or consumption (nmol/10^{-6} cell hr^{-1}) in				
	Glutamate-based media: DMEM/			Controls: DMEM/	
	glu+gln	glu+ala-gln	glu+gly-gln	glu	gln
ala	+26.0 ± 5.4	+27.6 ± 4.0	+27.6 ± 4.6	+30.6 ± 8.0[b]	+6.42 ± 3.30
ala-gln		-7.03 ± 0.71			
arg	-2.36 ± 0.00	-2.81 ± 0.38	-2.59 ± 0.12	-3.32 ± 1.05	-3.92 ± 0.26
asn	-1.62 ± 2.97	-2.37 ± 1.38	-4.48 ± 2.81	+0.68 ± 0.16	+0.07 ± 0.03
asp	-1.93 ± 0.02	-1.65 ± 0.10	-1.92 ± 0.09	+0.10 ± 0.34	0[a]
glu	-53.8 ± 6.2	-50.6 ± 3.1	-56.9 ± 6.0	-68.5 ± 7.8	0[a]
gln	-1.98 ± 0.93	+2.18 ± 0.21	+4.83 ± 0.38	0[a]	-37.8 ± 6.1
gly	+5.55 ± 0.96	+6.06 ± 1.32	+14.41 ± 0.48	+5.28 ± 0.39	+2.79 ± 1.66
gly-gln			+3.75 ± 3.10		
his	-0.59 ± 0.06	-1.65 ± 0.43	-0.83 ± 0.42	-1.55 ± 0.73	-2.04 ± 0.30
ile	-2.41 ± 0.89	-2.09 ± 0.63	-3.17 ± 1.47	-2.81 ± 0.77	-6.86 ± 0.77
leu	-4.71 ± 0.12	-4.18 ± 0.23	-5.35 ± 0.59	-3.92 ± 0.07	-6.77 ± 0.55
lys	-6.15 ± 1.80	-4.71 ± 1.14	-5.75 ± 0.20	-3.15 ± 0.79	-2.98 ± 0.21
met	-1.12 ± 0.03	-1.19 ± 0.09	-1.36 ± 0.17	-1.29 ± 0.46	-1.63 ± 0.32
phe	-1.82 ± 0.19	-1.58 ± 0.07	-1.75 ± 0.33	-1.92 ± 0.64	-2.28 ± 0.18
ser	-13.8 ± 1.4	-13.0 ± 1.4	-13.4 ± 1.2	-13.1 ± 1.1	-12.5 ± 1.5
thr	-1.97 ± 1.18	-1.99 ± 0.53	-1.90 ± 0.23	-2.71 ± 0.06	-3.78 ± 0.11
trp	-0.42 ± 0.11	-0.35 ± 0.14	-0.79 ± 0.16	-0.32 ± 0.19	-0.56 ± 0.17
tyr	-1.08 ± 0.10[c]	-0.94 ± 0.05[c]	-1.24 ± 0.21[c]	-2.82 ± 1.04	-1.64 ± 0.63[c]
val	-2.67 ± 1.06	-2.70 ± 0.06	-3.22 ± 1.22	-3.59 ± 1.59	-7.02 ± 0.87
NH_4^+	+5.19 ± 1.40	+3.17 ± 0.77	+3.07 ± 0.72	+4.98 ± 0.86	+17.8 ± 3.7
glc	-171 ± 27[d]	-194 ± 38[d]	-188 ± 31[d]	-184 ± 39[e]	-201 ± 37[d]
lac	+555 ± 75[d]	+492 ± 61[d]	+604 ± 111[d]	+444 ± 77[e]	+621 ± 23[d]

[a]production/consumption was small and variable
[b] 33-58 hours, [c] 47-72 hours, [d] 33-72 hours and [e] 47-95 hours were used to determine specific rates; these alternate periods were used because they represented the linear segment in a plot of the change in metabolite versus the cell index.

Table 7-4. Product yield ratios for BHK cells grown in batch stationary cultures with glutamate-based and control media. The quotient was determined from the net production or consumption of metabolites after 72 hours of culture. For glutamate-based media, NH_4^+/glu and ala/glu values were based on glutamate utilization alone and the minimal level of glutamine consumption was not considered in the calculations. In DMEM/gln, NH_4^+/gln and ala/gln ratios are provided. Values are expressed as an average of the three replicate cultures (n=3) for each type of medium ± SE.

DMEM/	Product Yields (mol/mol)			
	NH_4^+/glu or gln	ala/glu or gln	ala/NH_4^+	lac/glc
glu + gln	0.24 ± 0.02	0.49 ± 0.04	2.07 ± 0.09	3.47 ± 0.46
glu + ala-gln	0.16 ± 0.00	0.82 ± 0.04	5.00 ± 0.11	3.12 ± 0.19
glu + gly-gln	0.15 ± 0.01	0.49 ± 0.03	3.25 ± 0.19	3.90 ± 0.27
glu	0.18 ±0.01	0.37 ± 0.03	2.02 ± 0.11	4.91 ± 1.75
gln	0.91 ± 0.06	0.27 ± 0.01	0.29 ± 0.02	3.61 ± 0.38

7.3.1.4 Glucose consumption and lactate production

Glucose was consumed at a higher rate than any other nutrient but was not depleted and lactate was the predominant metabolic by-product in each of the medium formations (Figure 7-4 and Table 7-3). After 3 days of culture, glucose levels were not significantly different among the glutamate-based media and DMEM/gln and had decreased to concentrations of 10.3-11.0 mM. This is consistent with the specific glucose consumption rates which were not significantly different in these media at 171-201 nmol 10^{-6} cell hr^{-1}. Glucose was reduced to only 18.5 mM after the same period in DMEM/glu due to the lower cell concentrations. However, the specific consumption rate at 184 nmol 10^{-6} cell hr^{-1} was comparable to the other media. Lactate levels on day 3 were similar in each of the glutamate-based media (37.2-40.6 mM). Lactate was highest in DMEM/gln (44.5 mM) and lowest in DMEM/glu (23.9 mM). Specific lactate production was not significantly different among glutamate-based media and DMEM/glu (444-604 nmol 10^{-6} cell hr^{-1}) and similar in DMEM/gln (621 nmol 10^{-6} cell hr^{-1}). This reflects the similar rates of glucose consumption in the different media. These results contrast with the CC9C10 cultures grown in DMEM/gly-gln in which specific glucose consumption and lactate production were reduced.

In all media, the lac/glc product yield exceeded the theoretical maximum of double the glucose consumed in which each molecule of glucose is converted to two molecules of lactate via glycolysis (Table 7-4). The ratio was >3 and minor or insignificant differences in the values were observed among the media. Lactate is also a possible end-product of amino acid metabolism (particularly of glutamine and glutamate) which could contribute to the increased lactate production.

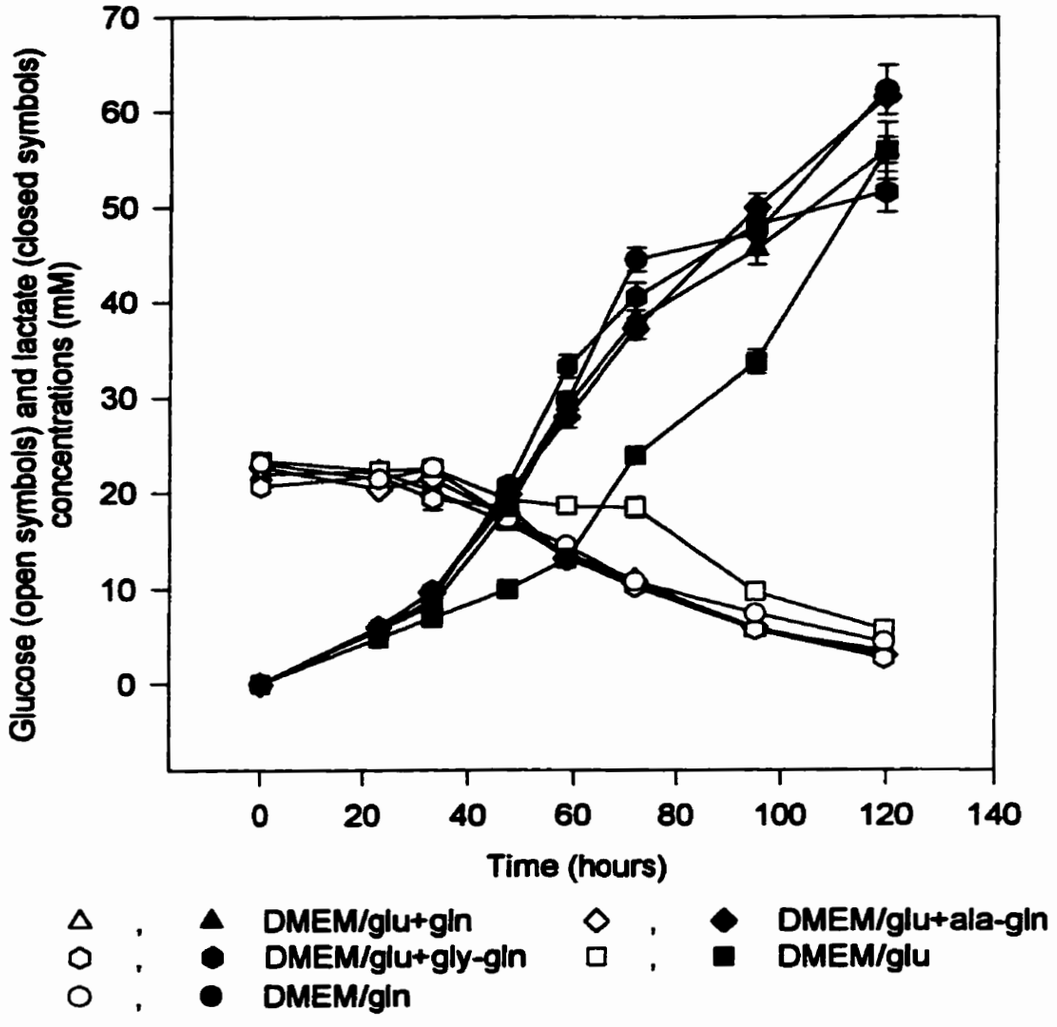


Figure 7-4. Glucose and lactate concentrations in batch stationary cultures of BHK cells grown in glutamate-based media and controls. Glucose (open symbols) and lactate (closed symbols) were determined in triplicate samples (n=3) for the cultures indicated in the legend. The average of the replicates were plotted ± SE.

7.3.1.5 Intracellular UDP-GNAc and ATP pools

Ammonium accumulation has been correlated with the elevation of intracellular levels of UDP-GlcNAC and UDP-GalNAc (collectively referred to as UDP-GNAc) and implicated as the underlying mechanism of ammonium inhibition in cultured cells (Ryll *et al.*, 1994). Consequently, the concentration of the UDP-aminohexoses were compared in BHK cells grown in glutamate- and glutamine-based media. In addition, ATP was examined to characterize the effects of a reduced availability of glutamine on cellular energy levels. The nucleotides were determined after ~4 days (95 hours) growth in DMEM/glu+gln and DMEM/gln. After this period, ammonium was nearly 3-fold lower in the former medium (Table 7-5). UDP-GNAc was 0.273 nmol/10^6 cells in DMEM/glu+gln which was significantly lower than the 0.389 nmol/10^6 cells in DMEM/gln ($P<0.05$). The cellular ATP, however, was identical in DMEM/glu+gln and DMEM/gln at 1.04 nmol/10^6 cells. Thus, the use of glutamate as an alternate substrate effectively prevented high cellular UDP-GNAc levels without compromising energy provisions. Growth, however, was limited by the availability of surface area, negating possible improvements in growth with the glutamate-substituted media. This problem was addressed with the use of microcarriers in batch and fed-batch cultures.

Table 7-5. Intracellular UDP-GNAc and ATP concentrations of BHK cells cultured in DMEM/glu+gln and DMEM/gln. Cells were initially seeded at ~0.5 X 10^5 cells/ml in triplicate (n=3) 25 cm^2 T-flasks for each type of medium and grown for 95 hours. Ammonium levels were measured and the nucleotide extraction performed separately on each of the replicate cultures. UDP-GNAc represents the sum of UDP-GlcNAc and UDP-GalNAc concentrations. The average of the replicate samples are shown ± SE.

	Ammonium Concentration (mM)	UDP-GNAc (nmol/10^6 cells)	ATP
DMEM/glu+gln	0.56 ± 0.00	0.273 ± 0.037[a]	1.04 ± 0.13
DMEM/gln	1.56 ± 0.00	0.389 ± 0.047	1.04 ± 0.09

[a]The cellular nucleotide concentration was significantly different from that of DMEM/gln ($P<0.05$)

7.3.2 Growth and metabolism in batch microcarrier cultures (spinner flasks) with glutamate-based medium

7.3.2.1 Growth performance

BHK cells were grown in 100 ml spinner cultures in both glutamate-based medium (DMEM/glu+gln) and control media (DMEM/glu and DMEM/gln). DMEM/glu+gln was used rather than the glutamate-based formulations containing dipeptides to allow more accurate monitoring of glutamine levels. A high microcarrier concentration (5 g/L Cytodex 1) was included to prevent surface area limitations. Glutamate and glutamine supplements were increased to 4 mM in glutamate media and DMEM/gln, respectively, in an attempt to prevent depletion of these essential nutrients. Growth in each of the cultures is shown in Figure 7-5. The specific growth rate in DMEM/glu+gln was 0.049 ± 0.007 hr^{-1} and not significantly different from DMEM/gln (0.058 ± 0.007 hr^{-1}) during the exponential phase (19-68 hours). As expected, a much lower value of 0.029 ± 0.001 hr^{-1} (determined from 44-92 hours) was observed in DMEM/glu. Viable cell densities peaked at 2.09 X 10^6, 1.81 X 10^6 and 3.70 X 10^6/ml in DMEM/glu+gln, DMEM/glu and DMEM/gln, respectively. The cell density in glutamine-based medium was considerably higher than the other cultures but rapidly declined following the exponential phase. The DMEM/glu+gln culture entered a stationary phase after 3 days, about 24 hours earlier than the glutamine-based control. As a result, the maximum cell density was much lower in the former. However, an extended stationary phase was observed in DMEM/glu+gln with little reduction in the viable cell concentration for the remainder of the culture. An explanation for the early onset of the stationary phase in the glutamate-based medium was apparent following a metabolic analysis of the growth medium.

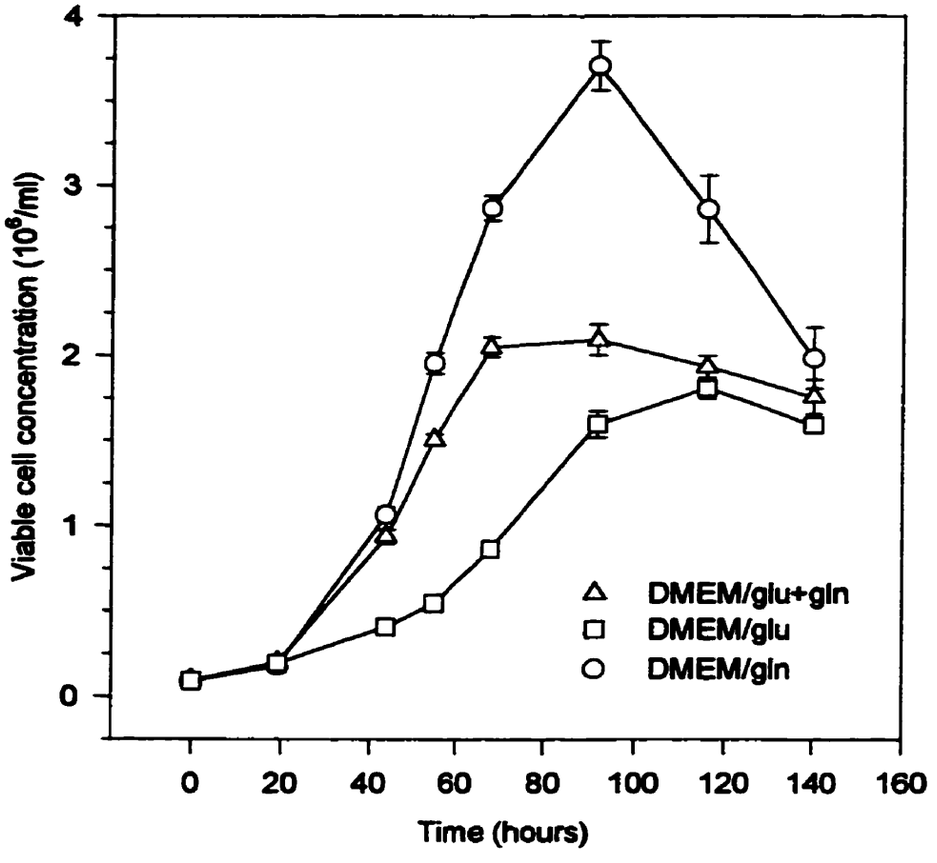


Figure 7-5. BHK cell growth in batch microcarrier cultures with glutamate-based medium and controls. Cultures were grown in triplicate 100 ml spinner flasks (n=3) for each of the media examined: DMEM/glu+gln (Δ), DMEM/glu (□) and DMEM/gln (○) containing 5 g/L Cytodex 1. Viable cell densities in each spinner were determined by counting with a haemocytometer. Replicate counts for each medium were averaged and plotted ± SE.

7.3.2.2 Ammonium and amino acid metabolism

Ammonium production for the microcarrier cultures is illustrated in Figure 7-6. After 3 days of growth, accumulation of ammonium was similar in the two glutamate-substituted cultures (0.82-1.00 mM) and reduced markedly (>70 %) compared to DMEM/gln (2.90 mM). Ammonium concentrations were increased in the spinner cultures compared to the equivalent medium in multiwell plates. This would be expected if higher viable cell densities were attained due to the availability of more surface area with microcarriers. In addition, the higher (4 mM) glutamine supplement in DMEM/gln with the spinner cultures (compared to 3 mM in the batch stationary cultures) would result in increased ammonium levels. However, the higher glutamate concentration would not be a similar contributing factor in the spinner cultures with DMEM/glu+gln and DMEM/glu. A previous experiment established an increasing glutamate supplement did not elicit a corresponding increase in ammonium accumulation (Figure 6-11c).

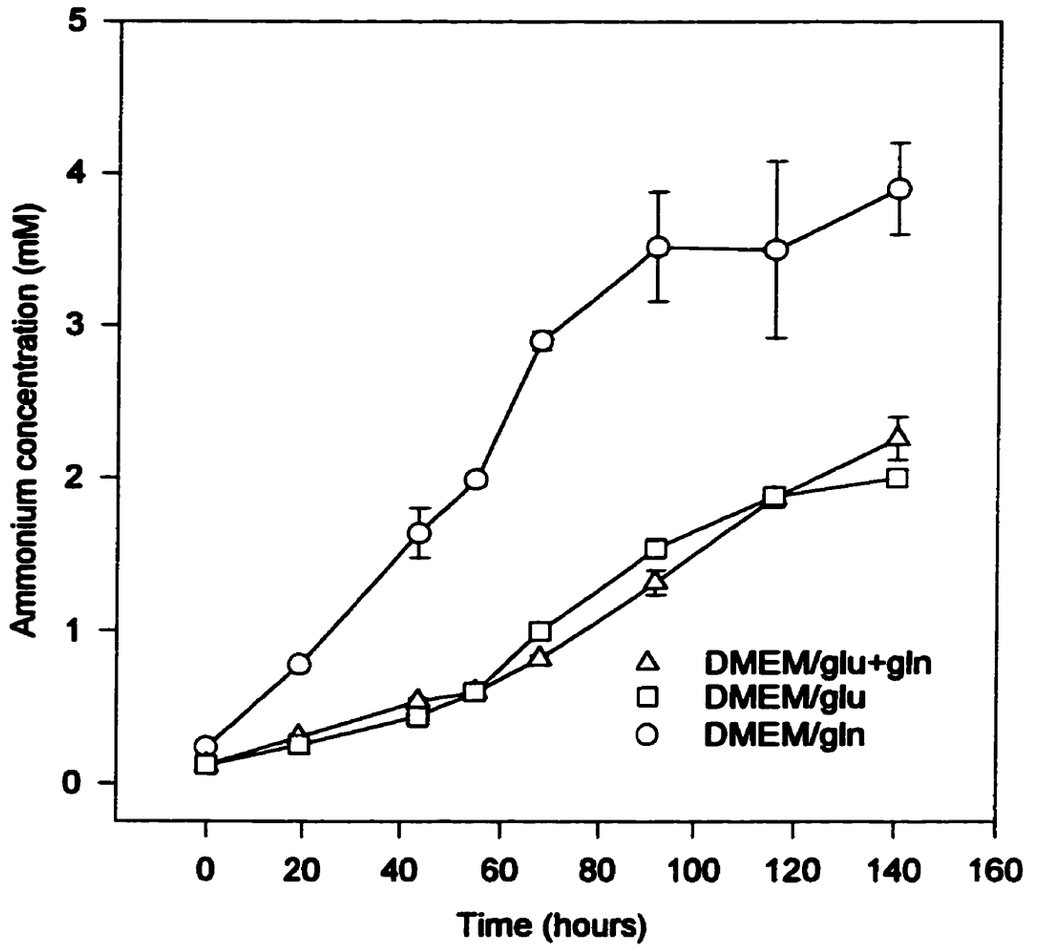


Figure 7-6. Ammonium concentrations in batch microcarrier cultures of BHK cells grown in glutamate-based medium and controls. Ammonium was measured in samples from each of the triplicate cultures (n=3) of DMEM/glu+gln (Δ), DMEM/glu (□) and DMEM/gln (○). Replicates were averaged and plotted ± SE.

The concentrations of selected amino acids in the microcarrier cultures are presented for DMEM/glu+gln, DMEM/glu and DMEM/gln (Figures 7-7a,b and c, respectively). In addition, specific consumption/production rates during the exponential phase for these amino acids and other metabolites are provided in Table 7-6. Glutamine was depleted in DMEM/glu+gln after less than 3 days of growth which contrasted with the minimal utilization in multiwell plates. Some glutamine was also initially present in DMEM/glu, perhaps due to low quantities present in the serum supplement and high levels of cellular glutamine synthetase activity. Glutamine was rapidly consumed for the first 3 days of growth in DMEM/gln but was not depleted. The concentration decreased at a lower rate after this point. The specific glutamine consumption during the exponential phase was about 2-fold higher in DMEM/gln (-37.8 nmol 10^{-6} cell hr^{-1}) compared to glutamate-based medium (-15.8 nmol 10^{-6} cell hr^{-1}). However, q_{gln} was increased in DMEM/glu+gln spinner cultures which could contribute to the higher rate of ammonium production (9.58 nmol 10^{-6} cell hr^{-1}) compared to the equivalent medium in multiwell plates (5.19 nmol 10^{-6} cell hr^{-1}). The reason for increased specific ammonium production in DMEM/glu with spinner cultures (24.5 nmol 10^{-6} cell hr^{-1}) compared to multiwell plates (4.98 nmol 10^{-6} cell hr^{-1}) was unclear.

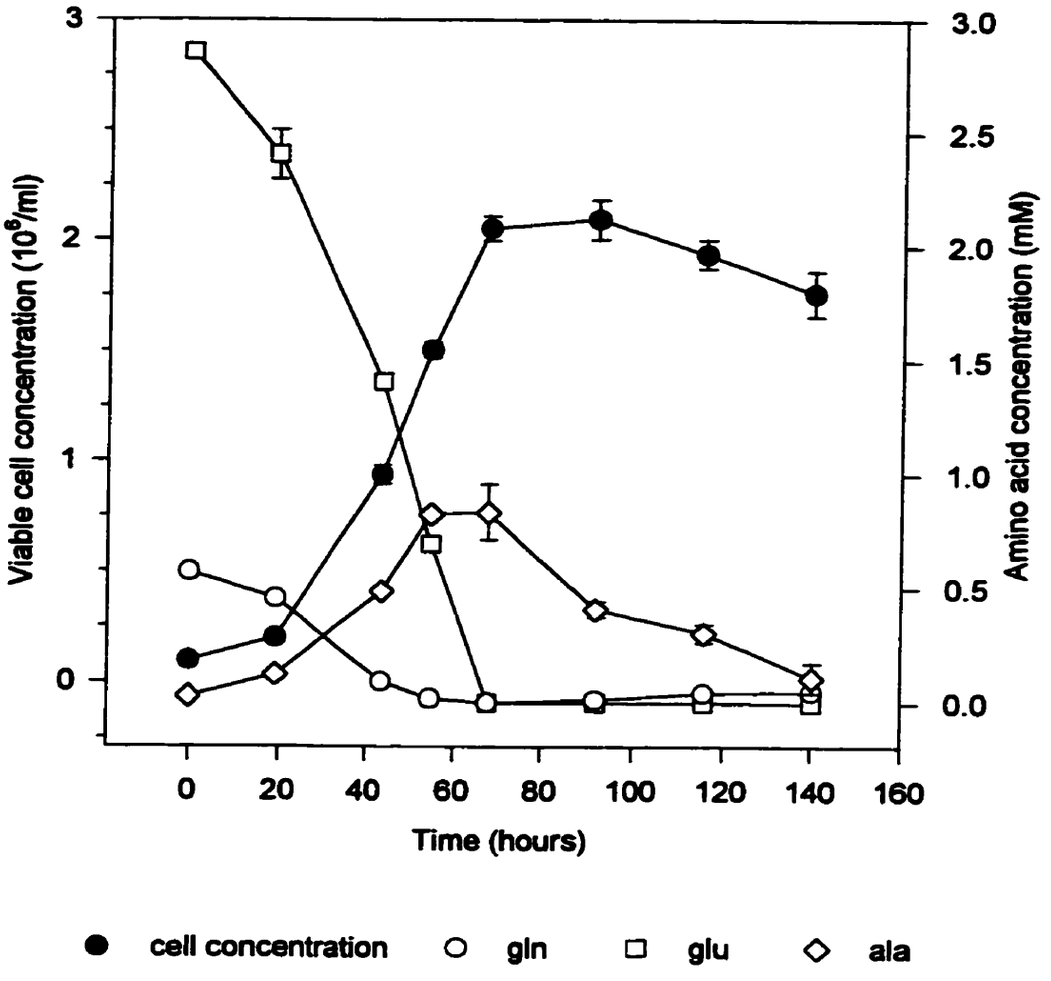
Although the 4 mM glutamate supplement was higher than provided for the cultures grown in multiwell plates, it was depleted after 3 days in DMEM/glu+gln and 24 hours later in DMEM/glu. This corresponded with the beginning of the stationary phase and explained the lower cell yields obtained in glutamate-based medium compared to DMEM/gln in the spinner cultures. The initial glutamate concentrations (0 hours) in the glutamate-substituted media were just under 3 mM according to amino acid analysis, considerably less than the 4 mM supplement added to the medium. A fraction of the glutamate may have escaped detection by complexing with serum proteins. In addition, there may have been high rates of cellular utilization after inoculation of the flasks and

before the first sample was collected. On the other hand, specific consumption during the exponential phase in DMEM/glu+gln (-46.6 nmol 10^{-6} cell hr^{-1}) was not significantly different from that observed in multiwell plates. Glutamate consumption actually appeared to be lower in DMEM/glu (-32.6 nmol 10^{-6} cell hr^{-1}) with the spinner cultures.

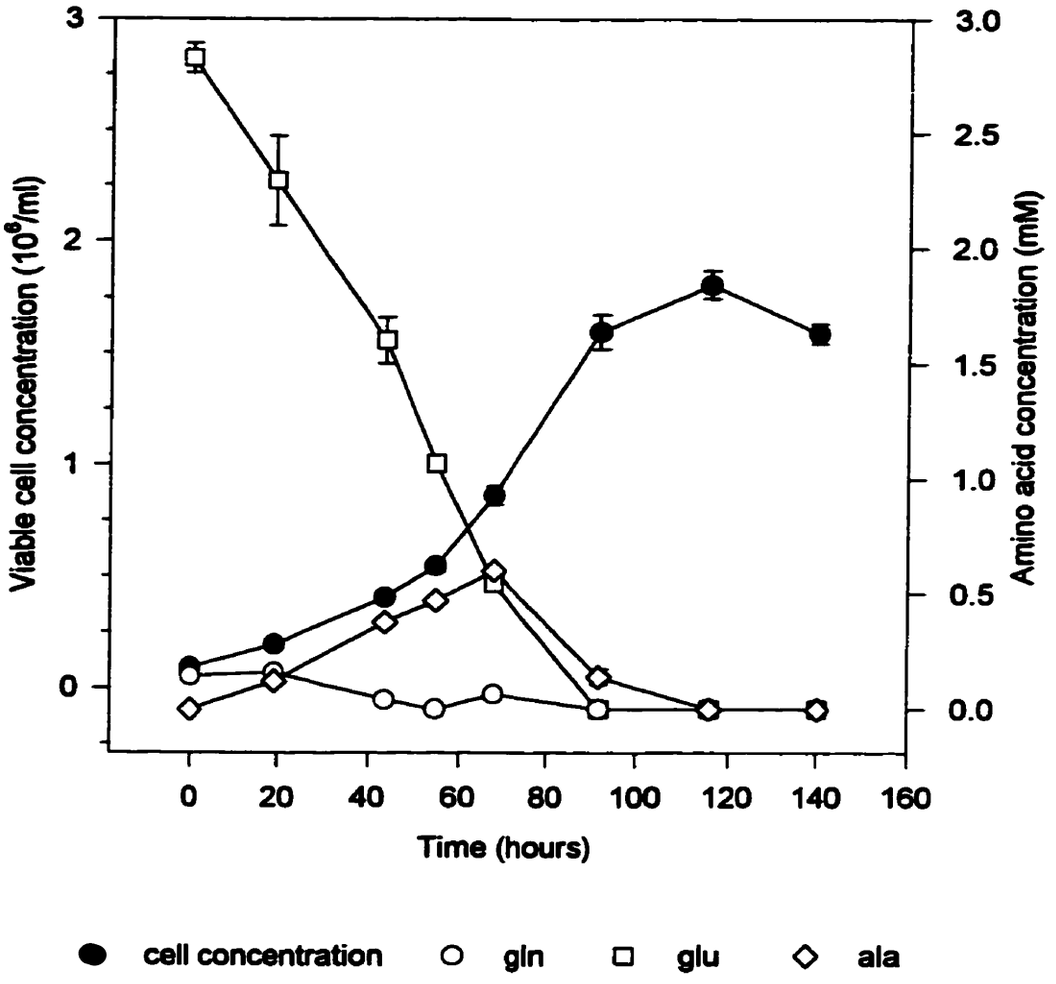
The pattern of alanine production in spinner flasks during the exponential phase was generally consistent with previous observations in multiwell plates. Again, this amino acid accumulated to the highest levels and had greater specific production rates in the glutamate-substituted cultures. However, the alanine accumulations were somewhat lower in the spinner vessels (35-50 %). Alanine also decreased substantially in each of the media during the latter stages of the exponential phase and the stationary phase. This may be the result of the reduced glutamine and glutamate concentrations available for transamination of pyruvate to form alanine. Thus, alanine previously excreted into the medium would be consumed to meet the cellular requirement for this amino acid.

Figure 7-7a-c. Selected amino acid concentrations in batch microcarrier cultures of BHK cells grown in glutamate-based medium and controls. Amino acids were determined in (a) DMEM/glu+gln, (b) DMEM/glu and (c) DMEM/gln. The concentrations of glutamine, glutamate and alanine are shown according to the symbols indicated in the legend. Points represent the average concentration of triplicate cultures (n=3) ± SE.

(a)


Viable cell concentration (10^6/ml)
Amino acid concentration (mM)
Time (hours)
0
20
40
60
80
100
120
140
160
0.0
0.5
1.0
1.5
2.0
2.5
3.0
cell concentration
gln
glu
ala

(b)


Viable cell concentration (10^6/ml)
Amino acid concentration (mM)
Time (hours)
0
20
40
60
80
100
120
140
160
0
1
2
3
0.0
0.5
1.0
1.5
2.0
2.5
3.0
cell concentration
gln
glu
ala

(c)

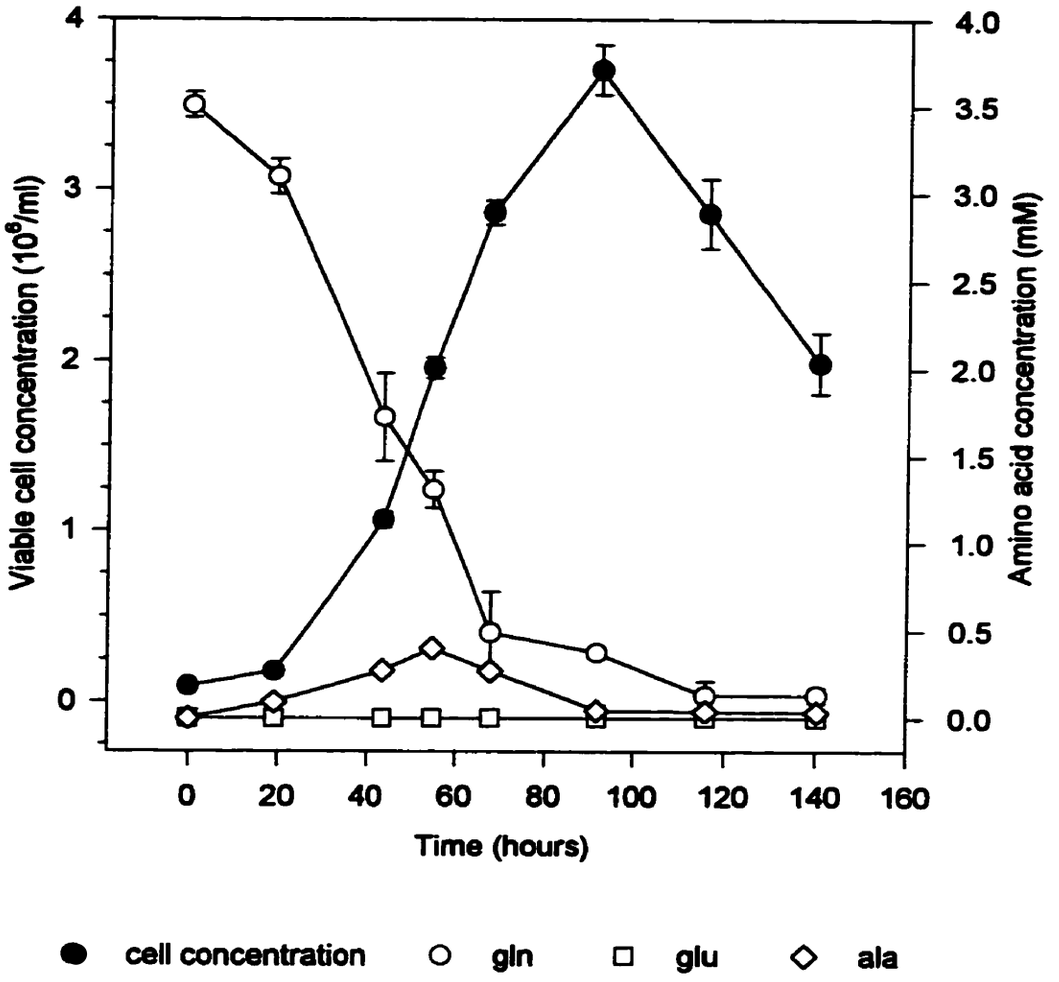

Viable cell concentration (10^6/ml)
Amino acid concentration (mM)
Time (hours)
0
20
40
60
80
100
120
140
160
0
1
2
3
4
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
cell concentration
gln
glu
ala

Table 7-6. Specific metabolite consumption (-) and production (+) rates by BHK cells grown in batch microcarrier cultures with glutamate-based medium and controls. Specific rates were determined from a plot of change in metabolite concentration versus the viable cell index. Values represent the best straight line fit during the exponential phase (19-68 hours unless otherwise indicated) ± SE of the line.

	Specific consumption or production (nmol 10^{-6} cell hr^{-1})		
	DMEM/glu+gln	DMEM/glu	DMEM/gln
ala	+25.4 ± 0.4[b]	+21.3 ± 3.7	+9.58 ± 1.27[b]
glu	-46.6 ± 7.0	-32.6 ± 9.1[d]	0
gln	-15.8 ± 6.0[b]	N.D.[a]	-37.8 ± 9.4
NH_4^+	+9.58 ± 1.41	+24.5 ± 3.4[d]	+31.6 ± 4.2
glc	-264 ± 16	-196 ± 33[d]	-198 ± 17
lac	+642 ± 192[c]	+474 ± 21[e]	+570 ± 100[c]

[a] Not determined: consumption or production was small and variable
[b] 19-55 hours, [c] 44-68 hours, [d] 44-92 hours, and [e] 55-92 hours were used to determine specific rates; these periods were used because they represented the linear segment in a plot of the change in metabolite versus the viable cell index.

Product yields were determined for the microcarrier cultures (Table 7-7) after 3 days of growth. For DMEM/glu+gln, the NH_4^+/glu or gln value in spinners was calculated using the sum of glutamate and glutamine consumed since glutamine was also utilized at a significant rate. The ratio of 0.20 in this medium was similar to that obtained in multiwell plates and more than 4-fold lower than the NH_4^+/gln value in DMEM/gln (0.88). In DMEM/glu, NH_4^+/glu was 0.37 and almost 2-fold higher than observed in the multiwell plates. An explanation for this was not apparent but was consistent with increased specific ammonium production in the spinners with DMEM/glu medium. The ala/glu or gln value in spinner cultures was again much higher in the glutamate-based medium (0.24) and reflects the higher alanine production compared to DMEM/gln (ala/gln=0.09). However, the value was about 2-fold lower in DMEM/glu+gln with spinners compared to the equivalent medium in multiwell plates. This was indicative of higher overall glutamate consumption and lower alanine production in the former culture system. The ala/NH_4^+ value was an order of magnitude higher in DMEM/glu+gln relative to the DMEM/gln control. This product yield in spinners, though, was much lower than in multiwell plates in all media because of the higher alanine and lower ammonium yields in the latter culture system.

Table 7-7. Product yield ratios for BHK cells grown in batch microcarrier cultures with glutamate-based medium and controls. The quotients were determined from the net consumption or production of metabolites following ~3 days (68 hours) of growth. For the DMEM/glu+gln medium, the sum of glutamate and glutamine consumed were used in the calculations of NH_4^+/glu or glu and ala/glu or gln. The ratios determined for DMEM/gln were NH_4^+/gln and ala/gln. Values are an average of the three independent cultures (n=3) for each medium ± SE.

	Product Yields (mol/mol)			
DMEM/	NH_4^+/glu or gln	ala/glu or gln	ala/NH_4^+	lac/glc
glu + gln	0.20 ± 0.01	0.24 ± 0.04	1.15 ± 0.21	1.93 ± 0.04
glu	0.37 ± 0.01	0.25 ± 0.01	0.68 ± 0.00	0.61 ± 0.18
gln	0.88 ± 0.07	0.09 ± 0.01	0.10 ± 0.01	1.85 ± 0.06

7.3.2.3 Glucose and lactate metabolism

Glucose concentrations decreased rapidly during exponential growth in DMEM/glu+gln and DMEM/gln (Figure 7-8). After 3 days, glucose had decreased to 7 mM and 6 mM in DMEM/glu+gln and DMEM/gln, respectively. This nutrient was almost depleted in these two media after 5 days and 24 hours later in DMEM/glu. The specific consumption rate was slightly but significantly higher during the exponential phase in DMEM/glu+gln (-264 nmol 10^{-6} cell hr^{-1}) compared to the other media (196-198 nmol 10^{-6} cell hr^{-1}) (Table 7-6). Lactate concentrations reached similar levels in glutamate- and glutamine-based media after 3 days (30.8 and 34.1 mM, respectively). Specific lactate production was not significantly different in DMEM/glu+gln (642 nmol 10^{-6} cell hr^{-1}) from the other media. Overall, however, lower maximum cell densities were attained in DMEM/glu+gln compared to DMEM/gln although lactate was similar in the two media. Thus, the growth yield for lactate was obviously lower in DMEM/glu+gln. Nevertheless, the lactate produced was reduced in the spinners compared to multiwell plates. This resulted in a lac/glc product yield <2 in all media for the spinners (Table 7-7) compared to a ratio in excess of 3 in the multiwell plates. Glucose metabolism thus appeared to be more efficient in the spinner flasks although the reason for this was unclear.

In glutamine-based medium, the use of microcarriers allowed a much higher maximum cell density to be achieved (3.70 X 10^6 viable cells/ml) compared to multiwell plates (2.23 X 10^6 total cells/ml). Further increases in cell density in DMEM/gln with microcarrier cultures was probably limited by a combination of factors. This would include high ammonium accumulations as well as low glucose and glutamine concentrations by the end of the exponential phase. The lower cell densities in glutamate-based medium, meanwhile, could be attributed to glutamate and glutamine depletion. Thus, a fed-batch feeding strategy was attempted to improve cell densities in the low-ammoniagenic glutamate-based medium.

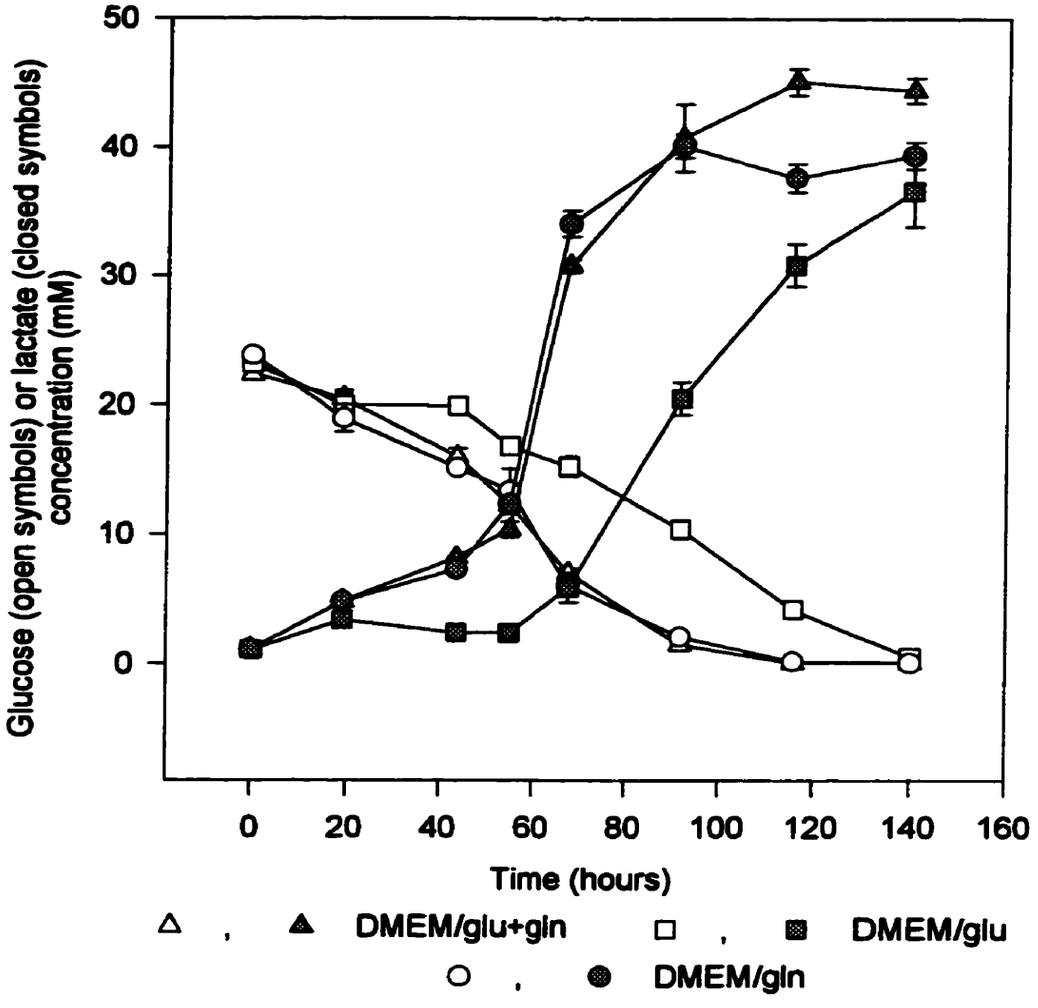


Figure 7-8. Glucose and lactate concentrations in batch microcarrier cultures of BHK cells grown in glutamate-based medium and controls. Glucose (open symbols) and lactate (closed symbols) were determined in DMEM/glu+gln, DMEM/glu and DMEM/gln as specified by the symbols in the legend. Points represent an average of the triplicate samples (n=3) from each medium ± SE.

7.3.3 Growth and metabolism in fed-batch microcarrier cultures (bioreactor) with glutamate-based medium

BHK cultures were grown in both glutamate- and glutamine-based medium with a feeding regime to prevent depletion of essential nutrients. This would demonstrate if improved yields could be generated in glutamate-based medium due to lower ammonium production after other limiting factors (surface area and nutrient depletion) had been ameliorated. The feeding strategy was designed to replenish the medium with at least 100 % of the initial levels of the most heavily consumed amino acids during exponential growth at 46, 70 and 96 hours. This included glutamate and glutamine in DMEM/glu+gln and DMEM/gln, respectively, and serine in both media. The rapid consumption of these nutrients was established in Table 7-3. Glutamine was also supplied at the same rate in DMEM/glu+gln in an attempt to preserve the original proportion of glu:gln. The concentrated MEM amino acids solution was used to replenish most remaining amino acids at 50 % of the initial content in the medium during exponential growth. Glucose was added at 33 % of the initial concentration in the medium during the exponential period. A higher rate of glucose feeding was avoided to prevent substantial increases in medium osmolarity. At 22 and 118 hours, each of the nutrients was fed at half of the rate specified during the exponential period. At 22 hours, cell concentrations were still relatively low and therefore the quantity of nutrients required was lower. At 118 hours, cells entered the decline phase and the feed was reduced to minimize further possible increases in osmolarity. The pH of the cultures were maintained at 7.2 and oxygen at 50 % of air saturation by the bioreactor controller system to prevent growth limitations from a low pH or oxygen tension.

Growth and ammonium production in the DMEM/glu+gln and DMEM/gln cultures is illustrated in Figure 7-9. Growth performance was similar in the two media and μ values determined during exponential growth (22-71 hours) were not significantly different at 0.055 ± 0.013 and 0.056 ± 0.003 in DMEM/glu+gln and DMEM/gln,

respectively. Maximum viable cell densities attained were ~3 X 10^6/ml for both cultures. This was a significant increase (40 %) for the glutamate-based medium compared to the batch microcarrier cultures. The maximum cell density in DMEM/gln, on the other hand, was 19 % lower than observed in the batch microcarrier cultures. Table 7-8 provides a summary of the growth rates and maximum cell densities with each of the different modes of culture (batch stationary, batch microcarrier and fed-batch microcarrier). Although growth was similar in the fed-batch cultures with each medium, ammonium production in DMEM/glu+gln was substantially lower with 1.58 mM compared to 5.48 mM in DMEM/gln at the end of the exponential phase (4 days).

Figure 7-10a-b shows the concentrations of selected amino acids in each medium during the course of the culture. Although glutamate was fed at a high rate, it was almost depleted from DMEM/glu+gln after 4 days (Figure 7-10a). The glutamine supplement, however, remained at a relatively constant level during exponential growth and increased in the stationary phase. In DMEM/gln, the glutamine concentration increased throughout most of the culture period up to a maximum of almost 6 mM (Figure 7-10b) due to its lower consumption rate than glutamate in DMEM/glu+gln. Alanine levels also contrasted markedly in the different media. Concentrations were over 2-fold higher in DMEM/glu+gln at 5.74 mM after 4 days compared to 2.40 mM in DMEM/gln. The elevated alanine production coincided with the high rates of glutamate utilization as noted previously in batch cultures.

Glucose consumption and lactate production for the fed-batch cultures are provided in Figure 7-11. Glucose was nearly exhausted in DMEM/glu+gln after 4 days but remained in excess in DMEM/gln (>10 mM). Higher glucose utilization resulted in much higher lactate concentrations in glutamate-based medium. In addition, lactate may have also been an end product of superfluous glutamate consumption. Lactate reached 105 mM after 4 days in DMEM/glu+gln compared to 55.5 mM in DMEM/gln. The excessive lactate production was sufficient to cause growth inhibition in glutamate-

based medium. Cell yields were previously shown to be reduced by 50 % in the presence of a 54 mM lactate addition (Figure 3-2b). The maximum viable cell density in the fed-batch system with DMEM/gln was reduced compared to the batch microcarrier culture, likely due to the higher ammonium levels and perhaps increased osmolarity resulting from the feeding.

Table 7-9 summarizes the growth yields (Y) of ammonium and lactate in multiwell plates and in batch and fed-batch microcarrier cultures after 3 days of growth. The cells produced per pmole of ammonium generated were consistently higher (more than 2.5-fold) in DMEM/glu+gln compared to DMEM/gln in all culture systems. Lower $Y_{NH_4^+}$ values with microcarriers compared to stationary cultures reflects the higher ammonium production in the former. In DMEM/glu+gln, lactate growth yields were significantly reduced compared to DMEM/gln in the microcarrier cultures. This was particularly evident in fed-batch mode in which Y_{lac} was 2-fold lower in DMEM/glu+gln due to the excessive lactate production. This further illustrates that attempts to improve growth in glutamate-based medium would be limited by the accumulation of lactate with conventional fed-batch approaches.

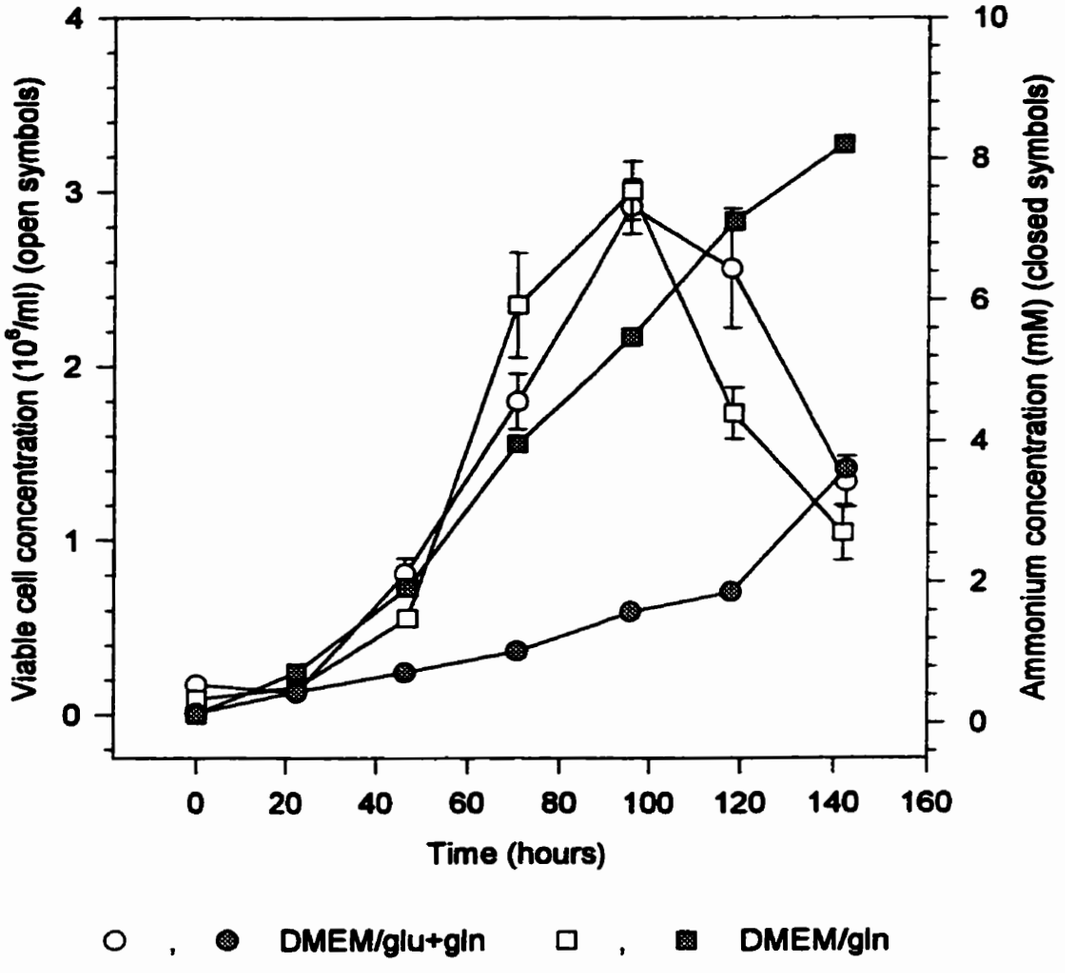


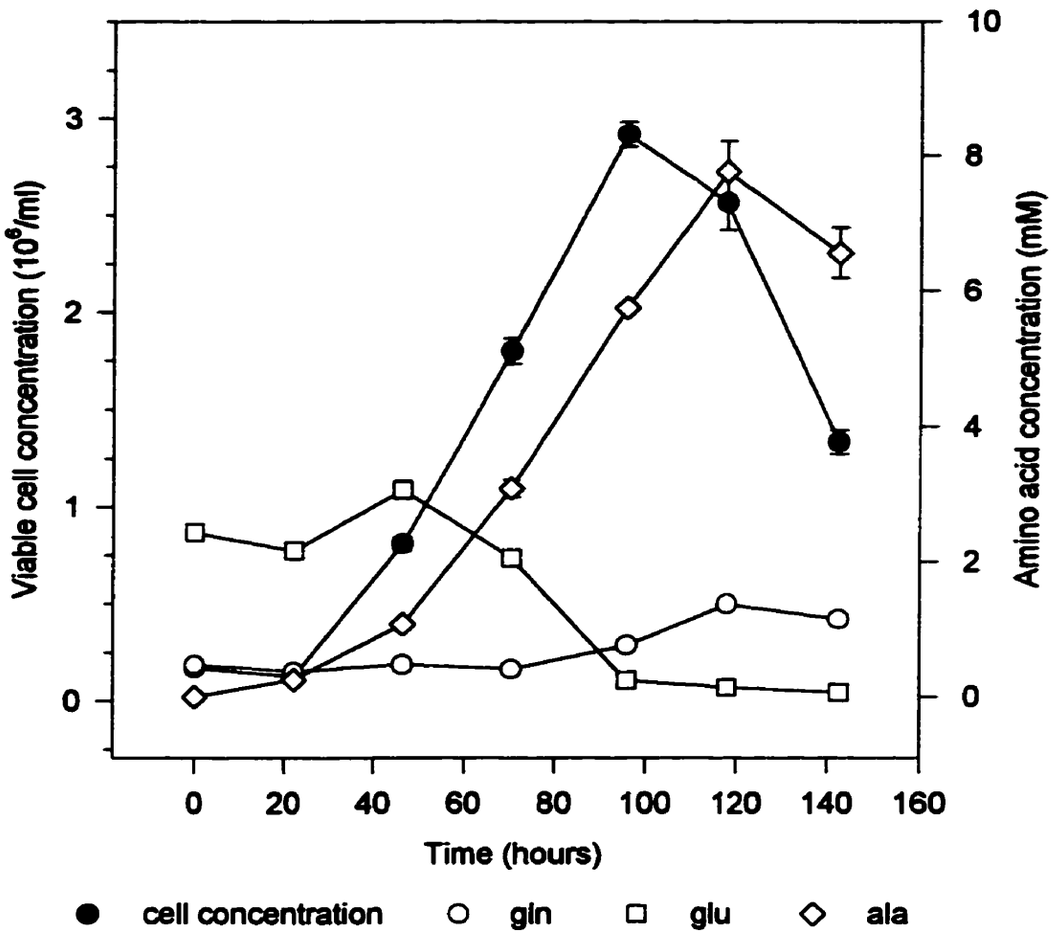
Figure 7-9. Growth and ammonium production in fed-batch microcarrier cultures of BHK cells with glutamate- and glutamine-based medium. Cultures were grown in a 1.5 L bioreactor with an initial volume of 1.25 L medium and 5 g/L Cytodex 1. Growth media were DMEM/glu+gln and DMEM/gln as indicated by the symbols in the legend. Cultures were fed on a daily basis with glucose and essential amino acids. Viable cell concentrations (open symbols) were determined by counting samples from each time point 6 times by haemocytometer. Replicate counts were averaged and plotted ± SE. Ammonium (closed symbols) was determined in each of the daily samples with a single measurement.

Table 7-8. Specific growth rates (μ) and maximum cell densities for BHK cells grown in multiwell plates, batch microcarrier and fed-batch microcarrier cultures with glutamate- and glutamine-based media. Specific growth rates were evaluated during a period of exponential growth (23-72 hours, 19-68 hours and 22-71 hours in multiwell plates, batch microcarrier and fed-batch microcarrier cultures, respectively). The maximum cell densities were achieved after 4 days for each of the culture systems. Note that total cells/ml are listed for multiwell plates while viable cells/ml were determined for the microcarrier cultures. In each case values are expressed ± SE.

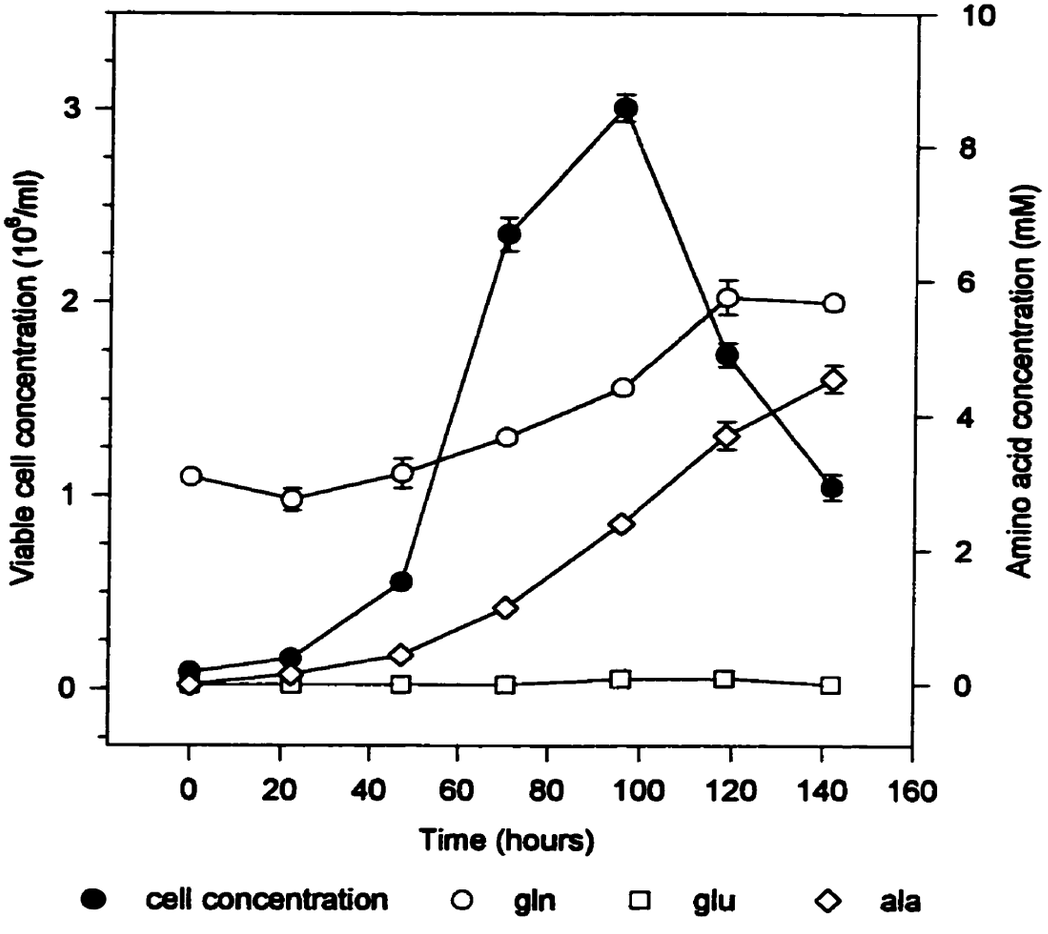
	multiwell plates	microcarrier (batch)	microcarrier (fed-batch)
μ (hr^{-1})			
DMEM/glu+gln	0.036 ± 0.004	0.049 ± 0.007	0.055 ± 0.013
DMEM/gln	0.038 ± 0.005	0.058 ± 0.007	0.056 ± 0.003
Max. Density (10^6 cells/ml)			
DMEM/glu+gln	2.16 ± 0.03	2.09 ± 0.09	2.92 ± 0.06
DMEM/gln	2.23 ± 0.01	3.70 ± 0.15	3.01 ± 0.07

Figure 7-10a-b. Selected amino acid concentrations of BHK fed-batch microcarrier cultures grown in glutamate- and glutamine-based media. Amino acids were determined in (a) DMEM/glu+gln and (b) DMEM/gln. The concentrations of glutamine, glutamate and alanine are shown and designated by the symbols indicated in the legend. Daily samples were analyzed in duplicate and the average concentration plotted ± SE.

(a)


Viable cell concentration (10^6/ml)
Amino acid concentration (mM)
Time (hours)
0
20
40
60
80
100
120
140
160
0
1
2
3
0
2
4
6
8
10
cell concentration
gln
glu
ala

(b)


Viable cell concentration (10^6/ml)
Amino acid concentration (mM)
Time (hours)
0
20
40
60
80
100
120
140
160
0
1
2
3
0
2
4
6
8
10
cell concentration
gln
glu
ala

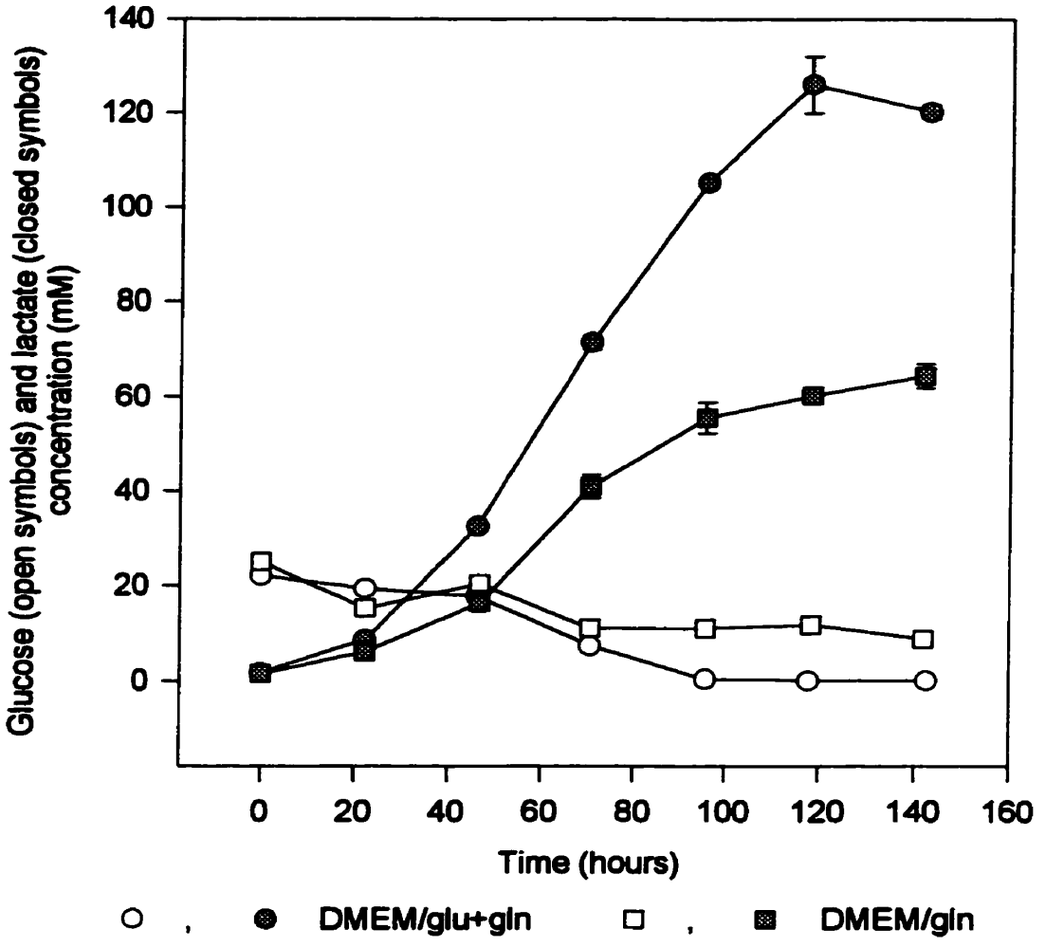


Figure 7-11. Glucose and lactate concentrations in fed-batch microcarrier cultures of BHK cells grown in glutamate- and glutamine-based media. Glucose (open symbols) and lactate (closed symbols) were determined in DMEM/glu+gln and DMEM/gln as indicated by the symbols in the legend. Daily samples from each medium were assayed in triplicate and quadruplicate for glucose and lactate, respectively. Points represent an average of the replicate determinations ± SE.

Table 7-9. Growth yield values for ammonium ($Y_{NH_4^+}$) and lactate (Y_{lac}) of BHK cells grown in multiwell plates and batch and fed-batch microcarrier cultures. The values were determined from the quotient of the net increase in viable cells per picomole of metabolite produced after 3 days of culture ± SE.

	Growth Yield (cells/pmol)			
	Ammonium		Lactate	
	DMEM/glu+gln	DMEM/gln	DMEM/glu+gln	DMEM/gln
multiwell plates	3.18±0.10	1.11±0.06	0.051±0.002	0.046±0.001
microcarrier (batch)	2.79±0.18	1.04±0.05	0.066±0.003	0.084±0.005
microcarrier (fed-batch)	1.85±0.09	0.59±0.02	0.023±0.002	0.057±0.006

7.3.4 Adaptation and enzyme activities of cells grown in glutamate medium

7.3.4.1 The adaptive growth response and glutamate uptake

The changes in BHK growth associated with the utilization of glutamate as an alternate substrate were investigated in DMEM/glu+gln, DMEM/glu and DMEM/gln. Cells cultured in each of these media for an extended duration (>6 passages) were subcultured into each of the other media and the impact on the specific growth rate and cell yield characterized (Table 7-10a-b). DMEM/glu+gln was advantageous in promoting high growth rates and cell yields with a minimal requirement for adaptation. The specific growth rate in DMEM/glu+gln was not significantly different at 0.038-0.041 hr^{-1} irrespective of the medium in which the inoculum cells had originally been cultured. The cell yield when subcultured from DMEM/gln into DMEM/glu+gln was 1.37 X 10^6 cells/ml after 3 days, only 12-15 % lower than cultures which had been adapted to glutamate-substituted media for numerous passages. On the other hand, marked differences were apparent when cells were subcultured into DMEM/glu. Cells inoculated from DMEM/gln had a considerably lower growth rate (0.021 hr^{-1}) and cell yield (0.14 X 10^6 cells/ml). This compares with cells previously cultured in DMEM/glu+gln and DMEM/glu with μ values of 0.028 hr^{-1} and 0.030 hr^{-1}, respectively. Cell yields for these two cultures were 0.32 X 10^6 cells/ml and 0.70 X 10^6 cells/ml, respectively. Growth performance was essentially identical when cells were subcultured into DMEM/gln regardless of the medium in which they had been previously grown. The results above suggest that inducible changes occurred in cells cultured in medium substituted with glutamate which supported growth in the absence of glutamine. Alternatively, the capacity to utilize glutamine was maintained despite prolonged culture in its absence.

Table 7-10a-b. (a) Specific growth rates and (b) cell yields of BHK cells during the first passage in DMEM/glu+gln, DMEM/glu and DMEM/gln. The inoculum cells had originally been grown in each of these media for at least 6 passages and then subcultured into the other medium formulations. Media were as described for batch stationary cultures except DMEM/glu contained a supplement of 3 mM asparagine. Cells were inoculated in all cases at ~1 X 10^5 cells/ml in 24-well plates. The specific growth rate was determined during the exponential phase (24-71 hours for DMEM/glu+gln and DMEM/gln and 48-97 hours for DMEM/glu). The cell yield refers to the total cell number/ml after ~3 days (71 hours) growth in each medium. Each of the cultures were grown in triplicate (n=3) and cell concentrations were evaluated with a Coulter counter. Values represent an average of the replicate samples ± SE.

(a)

Original Growth Medium	Specific Growth Rate (μ) (hr^{-1}) when subcultured in: DMEM/glu+gln	DMEM/glu	DMEM/gln
DMEM/glu+gln	0.041 ± 0.006	0.028 ± 0.002	0.041 ± 0.006
DMEM/glu	0.040 ± 0.007	0.030 ± 0.005	0.041 ± 0.006
DMEM/gln	0.038 ± 0.005	0.021± 0.002	0.039 ± 0.007

(b)

Original Growth Medium	Cell Yield (10^6/ml) in: DMEM/glu+gln	DMEM/glu	DMEM/gln
DMEM/glu+gln	1.62 ± 0.06	0.32 ± 0.01	1.55 ± 0.02
DMEM/glu	1.55 ± 0.03	0.70 ± 0.01	1.55 ± 0.01
DMEM/gln	1.37 ± 0.02	0.14 ± 0.00	1.55 ± 0.00

The possibility that glutamate uptake influenced adaptation to glutamate medium was investigated in BHK cells during the initial passages in DMEM/glu after subculture from DMEM/gln. Figure 7-12 illustrates the growth rate and cell yield gradually increased from passage 2-5 in DMEM/glu. By the sixth passage, no further improvements in growth were observed. Cells cultured >30 passages had a high initial growth rate but prematurely entered the stationary phase indicating growth without glutamine may be deleterious after a prolonged period. The specific glutamate consumption during the exponential phase in each of the cultures was determined and expressed as a percentage relative to the rate for passage 6 cells (Table 7-11). Enhanced glutamate uptake was not observed with an increasing number of passages in DMEM/glu. Rates were not significantly different among the cultures up to passage 6 and were slightly lower after >30 passages. Thus, adaptation to glutamate medium does not appear to involve an increase of glutamate transport across the plasma membrane. The adaptive process and the reduced growth in the absence of glutamine could therefore be related to the activity of enzyme(s) participating in the metabolism of glutamine and glutamate.

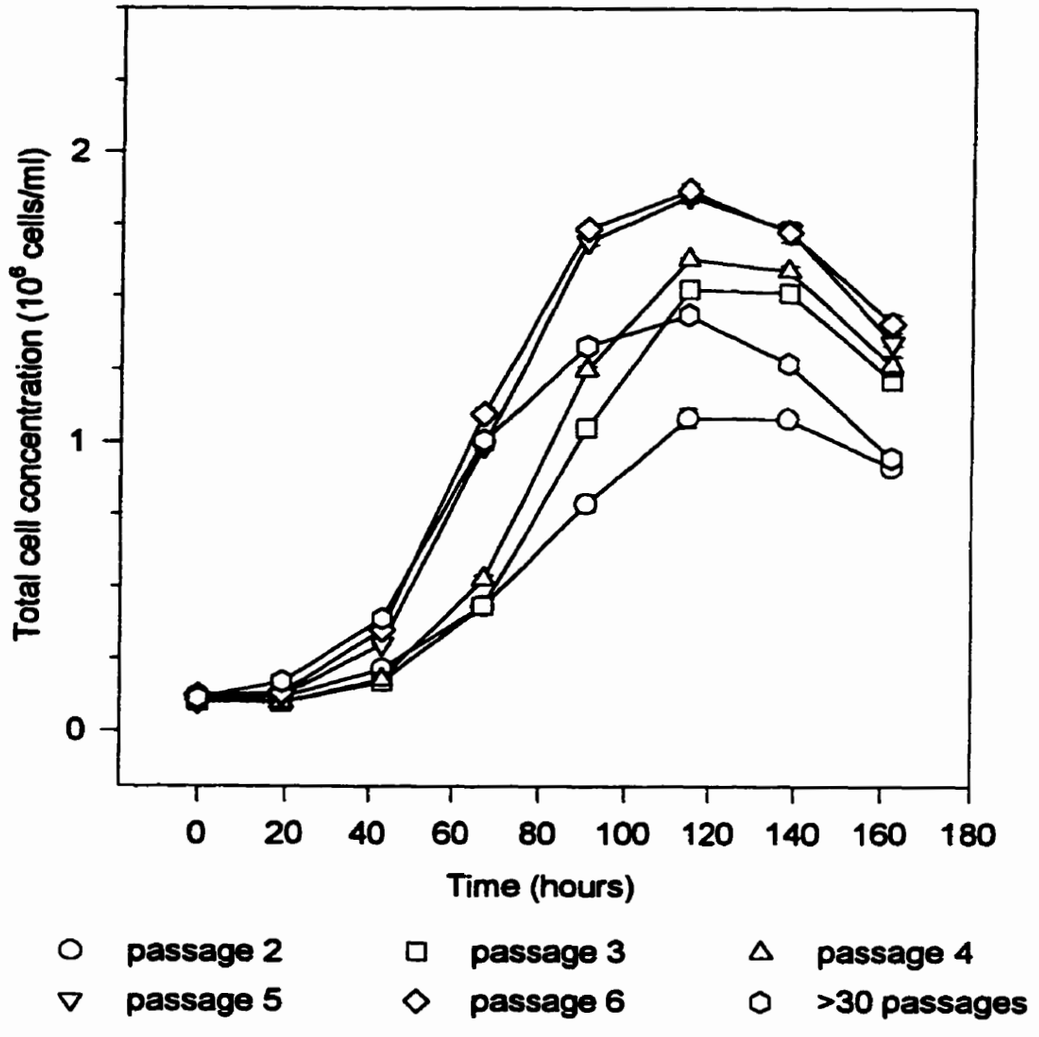


Figure 7-12. Growth of BHK cells at different numbers of passages in DMEM/glu following subculture from DMEM/gln medium. The DMEM/glu contained a 3 mM asparagine supplement for the experiment. Cell growth was examined at passages 2-6 and >30 passages as indicated in the legend. Each of these cultures were inoculated in triplicate (n=3) in 24-well plates and cell concentrations were determined with a Coulter counter. The average of replicate counts were plotted ± SE.

Table 7-11. Relative specific glutamate uptake by BHK cells during the initial passages in DMEM/glu. Glutamate concentrations were determined by HPLC analysis (section 2.11.4.2) for triplicate cultures (n=3) of cells grown in medium for 2-6 and >30 passages. The specific consumption rates during the exponential phase (19-67 hours) were determined using Equation 4 (section 2.11.5). These rates were normalized using passage 6 as a reference (100 %) resulting in the relative uptake. The averages of the replicate samples are shown ± SE.

Passage Number	Relative Glutamate Uptake (%)
2	105 ± 10
3	105 ± 5
4	106 ± 11
5	95 ± 5
6	100 ± 6
>30	87 ± 3

7.3.4.2 Enzyme activities of glutamine and glutamate metabolism

Four enzymatic activities were analyzed in extracts of cells grown in DMEM/glu+gln, DMEM/glu and DMEM/gln. These included glutamine synthetase (GS), phosphate-activated glutaminase (PAG), alanine aminotransferase (ALT) and glutamate dehydrogenase (GDH). Glutamine synthetase was assayed because it was expected to be a key enzyme mediating adaptation and growth with a limited availability of glutamine in the culture medium. The remaining enzymes were determined because they catalyze the initial reactions of glutamine/glutamate catabolism. Differences in ammonium and alanine metabolism between glutamate and glutamine media could be explained by changes in activities of PAG, ALT and GDH. The reactions catalyzed by the four enzymes are shown in Figure 1-3.

Glutamate in DMEM/glu+gln and DMEM/glu and glutamine in DMEM/gln were supplemented at a slightly higher concentration (4 mM) than in earlier experiments to prevent their depletion which could influence cellular enzyme activity. DMEM/glu also contained 3 mM asparagine for this experiment so the only difference between this medium and DMEM/glu+gln was the presence of a low glutamine level in the latter. Membrane and cytosolic fractions were prepared from BHK cells cultured in each of these media. This would indicate if the enzymes were primarily cytoplasmic in origin or associated with the membrane. The assays for GS, PAG, ALT and GDH were performed for fractions isolated from cells grown in each medium and the results are presented in Figure 7-13a-d.

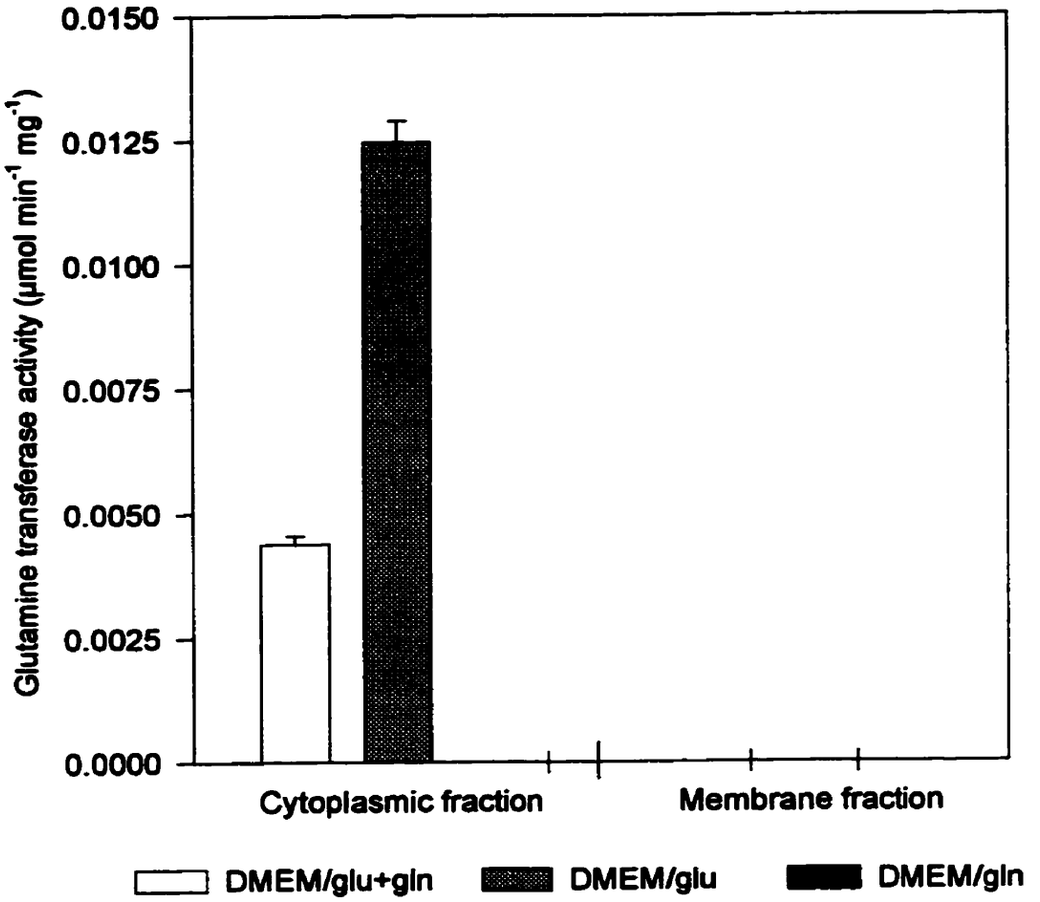
Glutamine synthetase was measured indirectly via glutamine transferase activity which was localized exclusively in the cytoplasmic fraction (Figure 7-13a). Activity was detected in extracts from both DMEM/glu+gln and DMEM/glu but was 2.8 X higher in the latter at 0.0125 $\mu mol\ min^{-1}\ mg^{-1}$. Predictably, GS was required at the highest level in DMEM/glu in which cells must synthesize all glutamine necessary for growth. In DMEM/glu+gln, at least some of the glutamine required was provided in the culture

medium. Cells may also adapt and grow in DMEM/glu+gln more readily than DMEM/glu because lower increases in glutamine synthetase are required in the former medium. There was no measurable activity in the cytoplasmic fraction isolated from cells grown in DMEM/gln. Clearly, there was little need for glutamine synthetase when glutamine was available in the medium at high concentrations. These results suggest the induction of this enzyme was an important adaptation in medium substituted with glutamate.

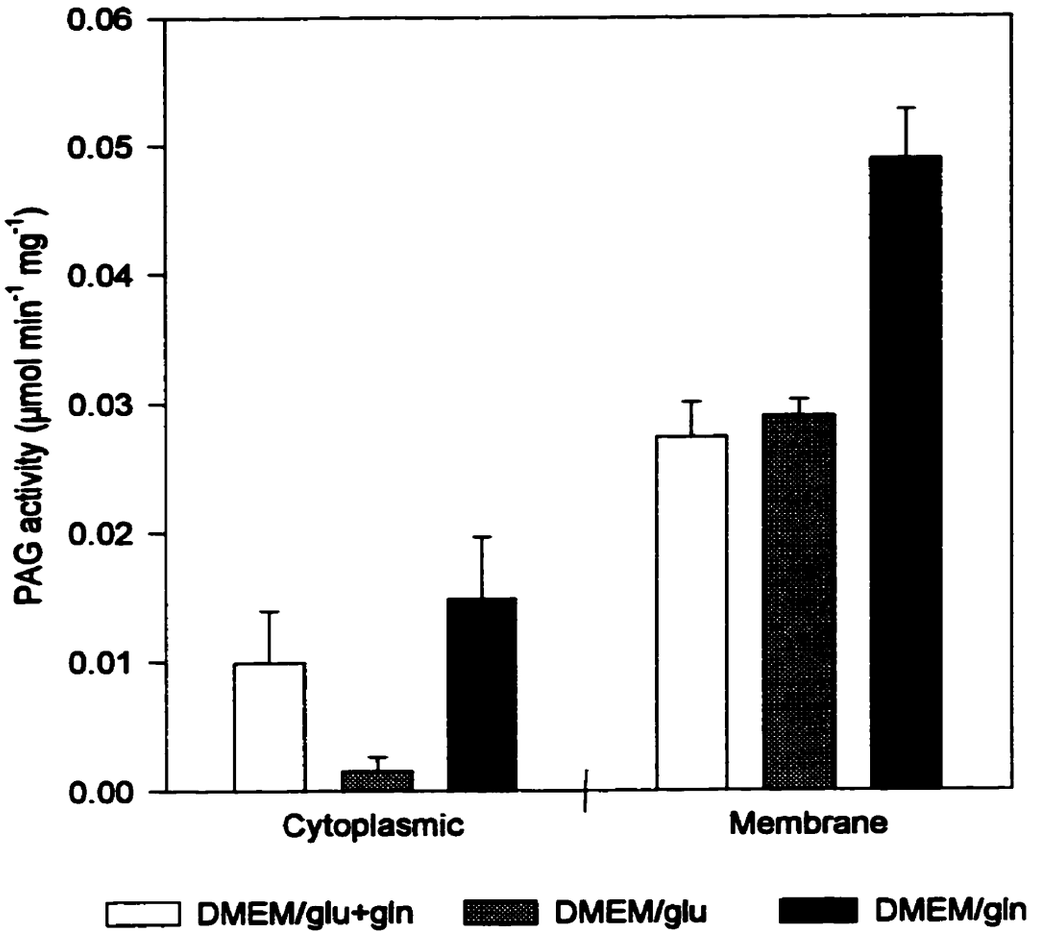
PAG, ALT and GDH are involved in the deamidation and deamination of glutamine and glutamate. These enzymes were all primarily membrane associated and the activities in only this fraction were considered. The PAG assay (Figure 7-13b) indicated enzyme activity was not significantly different in DMEM/glu+gln and DMEM/glu at 0.027-0.029 μmol min^{-1} mg^{-1}. This compared with levels 1.7-1.8 X higher in DMEM/gln at 0.049 μmol min^{-1} mg^{-1}. Nevertheless, PAG was relatively high in the extracts from DMEM/glu+gln and DMEM/glu considering glutamine, the substrate of the enzyme, was limited or absent in the medium. ALT was most active in membrane fractions isolated from DMEM/glu+gln (0.034 μmol min^{-1} mg^{-1}), and less so in DMEM/glu (0.024 μmol min^{-1} mg^{-1}) and DMEM/gln (0.013 μmol min^{-1} mg^{-1}) (Figure 7-13c). The reason for the difference between the extracts from DMEM/glu+gln and DMEM/glu was unclear. Nevertheless, ALT activity from cells grown in both of the glutamate-substituted media were significantly higher than in DMEM/gln. This would explain the lower specific alanine production and ala/gln ratio in glutamine-based medium. The assay for glutamate dehydrogenase revealed a somewhat higher activity in cells from DMEM/glu+gln of 0.037 μmol min^{-1} mg^{-1} compared to DMEM/glu and DMEM/gln which were not significantly different at 0.023-0.026 μmol min^{-1} mg^{-1} (Figure 7-13d). Although GDH was elevated in cells grown in DMEM/glu+gln, a major increase in ammonium production was not observed and was probably countered by the elevated ALT activity.

Figure 7-13a-d. (a) Glutamine synthetase/transferase, (b) phosphate-activated glutaminase, (c) alanine aminotransferase and (d) glutamate dehydrogenase activities in cytoplasmic and membrane fractions isolated from BHK cells grown in DMEM/glu+gln, DMEM/glu and DMEM/gln. Note that glutamine transferase activity was measured but was below the limits of detection for the DMEM/gln cytoplasmic fraction and for the membrane fractions from all of the media. Values shown are the average of at least duplicate determinations (n=2) ± SE.

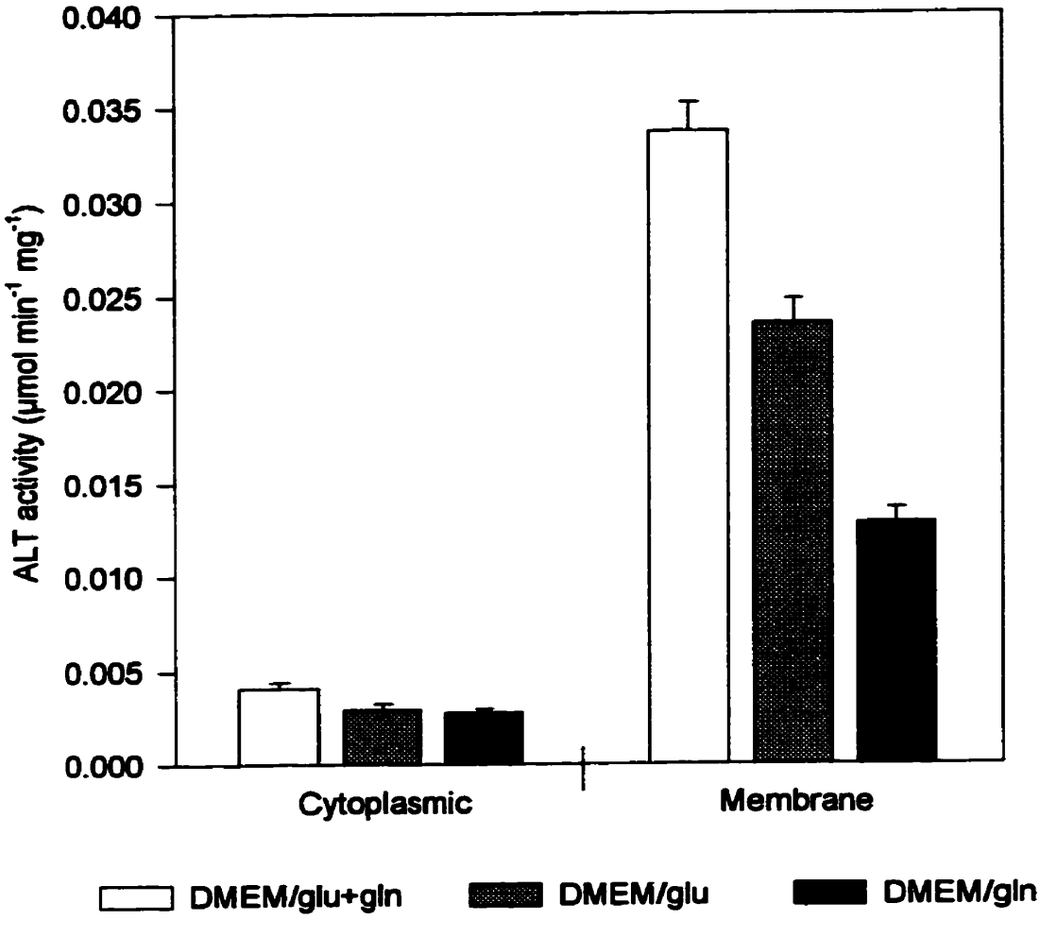
(a)


0.0150
0.0125
0.0100
0.0075
0.0050
0.0025
0.0000
Glutamine transferase activity (µmol min-1 mg-1)
Cytoplasmic fraction
Membrane fraction
DMEM/glu+gln
DMEM/glu
DMEM/gln

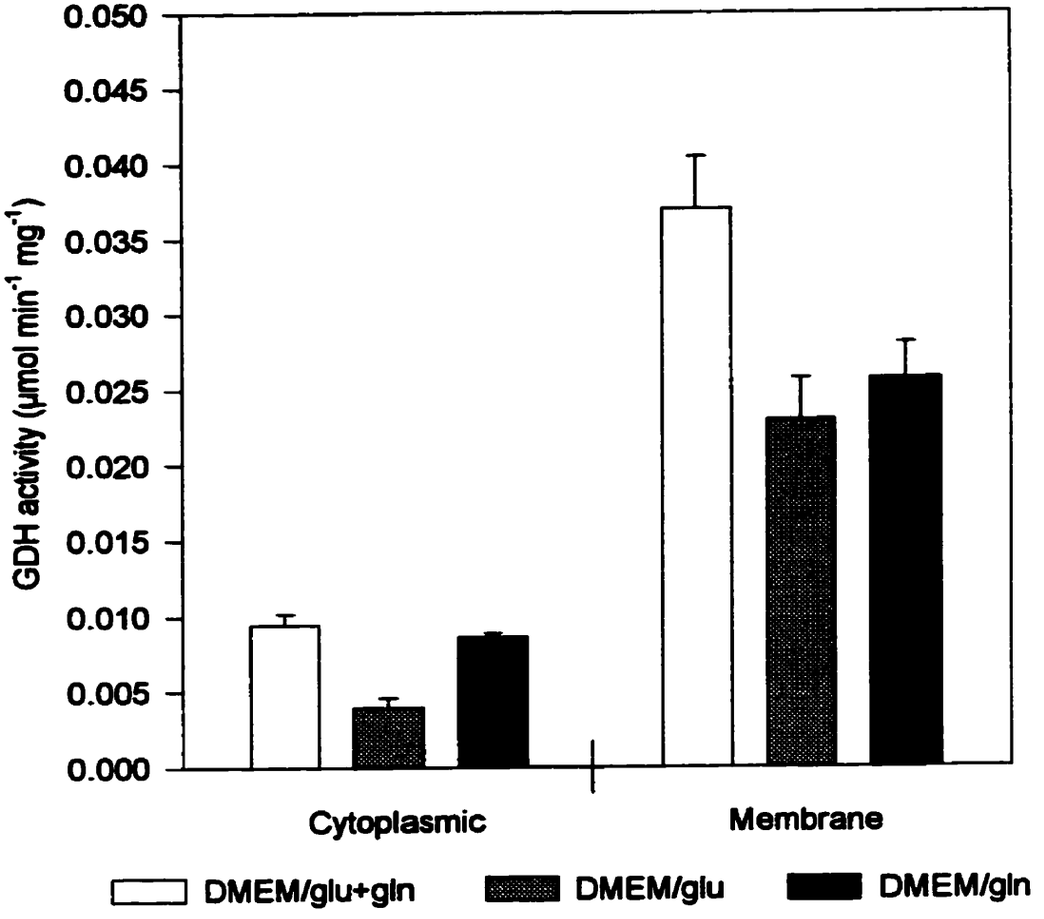
(b)


0.06
0.05
0.04
0.03
0.02
0.01
0.00
PAG activity (µmol min-1 mg-1)
Cytoplasmic
Membrane
DMEM/glu+gln
DMEM/glu
DMEM/gln

(c)


0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
ALT activity (µmol min-1 mg-1)
Cytoplasmic
Membrane
DMEM/glu+gln
DMEM/glu
DMEM/gln

(d)


0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
GDH activity (µmol min-1 mg-1)
Cytoplasmic
Membrane
DMEM/glu+gln
DMEM/glu
DMEM/gln

7.4 Discussion

Previous investigations have considered the effects of glutamate substitution on the growth of cultured cells. Most recently, Hassell and Butler (1990) reported significant reductions in ammonium yields for BHK cultures in glutamate-substituted medium. The cell yield in batch cultures (T-flasks), though, was not enhanced regardless of a 58 % decrease in the accumulation of ammonium. These results are consistent with the current study in which similar reductions in ammonium were achieved after the same period of growth (3 days) (Figure 7-2). Growth measured by cell yields, doubling times or specific growth rates were not improved in any of the glutamate-based media (DMEM/glu+gln, DMEM/glu+ala-gln and DMEM/glu+gly-gln) compared to medium with a normal complement of glutamine in multiwell plates (Figure 7-1 and Table 7-2). The relatively low accumulations of ammonium in batch stationary cultures proved not to be inhibitory in either glutamate- or glutamine-based media.

Although improved growth was not observed in batch cultures, the metabolic changes associated with growth in glutamate medium could be more thoroughly characterized than reported previously in the literature. Griffiths and Pirt (1967) had documented a high glutamate consumption for mouse LS cells cultured in medium with 8 mM glutamate and 0.7 mM glutamine. However, the glutamine was depleted during the first day of growth while about half of the glutamate was utilized over 5 days. This compared with glutamine-based medium in which an 8 mM glutamine supplement was almost completely consumed to produce a similar cell yield. Glutamate also proved a very effective replacement for glutamine with BHK cultures in the current investigation. This was evident from the high glutamate consumption and reduced glutamine utilization in glutamate-based medium. In fact, glutamate uptake was even higher than glutamine in DMEM/gln (Figure 7-3a-e and Table 7-3). Despite the high rate of glutamate utilization by BHK cells in glutamate-based medium, specific ammonium production was more than 3-fold lower than in DMEM/gln (Table 7-3). Glutamate was

obviously a less ammoniagenic substrate than glutamine. Furthermore, the low concentration of glutamine or dipeptides in glutamate-based media had a minimal impact on ammonium production when compared to the control culture supplemented with only glutamate (DMEM/glu). This was consistent with the low glutamine utilization in glutamate-based media. Glutamine may be required in only very small quantities as an essential anabolic precursor while glutamate replaces its role as a major oxidative substrate. This is analogous to reports suggesting that glucose is required at only low levels as a ribose precursor for nucleotide biosynthesis (Zielke *et al.*, 1984) and can even be replaced by the addition of nucleosides directly to the culture medium (Zielke *et al.*, 1976). Compared to glutamate, asparagine played a lesser metabolic role in minimizing glutamine requirements. The inclusion of asparagine probably reduced the need for its cellular biosynthesis from glutamine but it was not an important oxidative substrate. This was suggested by the nominal consumption of asparagine even though it was supplemented in the medium at the same concentration as glutamate.

Most other amino acids were consumed at similar rates in glutamate medium compared to the glutamine-based control. Obvious exceptions included decreased specific consumption for leucine, isoleucine and valine in glutamate-substituted media. These amino acids were also among those utilized at lower rates in CC9C10 cultures with the 20 mM gly-gln supplement. The reason for the reduced uptake of these amino acids with a limited availability of glutamine in the medium is presently unclear.

The substitution of glutamine with glutamate had a minimal effect on the glucose metabolism of BHK cells in the batch stationary cultures (Figure 7-4 and Table 7-3). Glucose was consumed at high rates which is typical of batch cultures. The rapid metabolism of both glucose and glutamine results in excessive production of waste metabolites such as lactate, alanine and ammonium (Ljunggren and Häggström, 1992 and 1994). The lac/glc product yield ratio was >3 in all media examined for BHK cultures grown in multiwell plates (Table 7-4). This suggests lactate was a significant

product of glutamine and glutamate metabolism in BHK cells. Unusually high alanine production in glutamate-substituted medium was also observed and can be attributed, in part, to the higher rates of glutamate uptake in this medium compared to glutamine in DMEM/gln. Unfortunately, glutamate was not an effective means of controlling overflow metabolism with BHK cells as has been achieved in glutamine-limited fed-batch cultures (Ljunggren and Häggström, 1994) or in batch cultures of CC9C10 grown in gly-gln medium. As indicated previously (section 1.3), overflow metabolism refers to the generation of large quantities of waste metabolites in the presence of high substrate levels. Although ammonium was reduced in glutamate-based medium, continued production of other overflow metabolites such as lactate could eventually result in a growth limitation. In batch stationary cultures, however, confluency was reached after 3-4 days and corresponded to the end of the exponential phase. Thus, a surface area limitation caused the cessation of growth in the multiwell plates in both glutamate- and glutamine-based media before other limitations became evident.

Although improved growth was not observed in the batch cultures of BHK cells, glutamate-based medium could be beneficial in producing a product of higher quality with more consistent glycosylation. The lower ammonium production in this medium introduced the possibility of maintaining reduced intracellular UDP-GNAc pools. Ryll *et al.* (1994) described ammonium (and glutamine) as substrates in the biosynthesis of UDP-GlcNAc and UDP-GalNAc. Ammonium accumulation could therefore promote increased UDP-aminohexose levels resulting in modified glycosylation of native and recombinant proteins. The possible consequences of this include growth inhibition and altered quality of protein products from the culture. Among the cell lines examined, the authors found the most pronounced increases in UDP-GNAc in BHK cells, consistent with their higher degree of ammonium sensitivity. The current study demonstrated lower UDP-GNAc levels in BHK cells grown in glutamate-based medium compared to DMEM/gln (Table 7-5). However, the extent of the reduction (30 %) was limited by the

relatively low ammonium concentrations, even in the glutamine-based medium. Greater differences could be expected in fed-batch cultures in which cell densities and ammonium accumulations are substantially higher.

The substitution of glutamine, a major oxidative substrate, with glutamate also could introduce an alteration in the cellular energy balance. The high alanine production in glutamate-substituted medium implicated that a large fraction of metabolized glutamate was incompletely oxidized. Less efficient energy metabolism could cause an energy limitation in this medium. This could be exacerbated by the additional ATP required by the glutamine synthetase reaction to produce glutamine from glutamate. Continued deamination of glutamate via alanine aminotransferase assumes stoichiometric conversion of α-ketoglutarate, the product of the reaction, to pyruvate. This is necessary to prevent depletion of the pyruvate as a substrate for subsequent transamination reactions. However, partial oxidation to pyruvate and transamination to alanine yields only 9 moles ATP/mole glutamine (or glutamate) (Glacken, 1988). Alternatively, deamination by glutamate dehydrogenase and complete oxidation produces up to 27 moles ATP/mole glutamine (or glutamate) (Ljunggren and Häggström, 1994). Thus, cellular ATP levels were measured in BHK cells cultured in glutamate- and glutamine-based media (DMEM/glu+gln and DMEM/gln, respectively). The analysis revealed identical ATP levels in both media (Table 7-5). Increased glutamate utilization appears to compensate, at least in part, for the potentially reduced energy available from incomplete oxidation of glutamate or from ATP consumption during glutamine synthesis. The lower ammonium levels may also place less demands on cellular energy reserves for maintenance energy (Martinelle and Häggstöm, 1993) in glutamate-based medium.

Microcarrier cultures were used in both batch and fed-batch systems to examine the potential of glutamate-based medium to enhance BHK cell densities. At 5g/L, the Cytodex 1 microcarriers are expected to offer up to 22 cm^2/ml of medium according to

specifications provided by the manufacturer. This area is an order of magnitude greater than that available in multiwell plates or T-flasks. Thus, surface area could be ruled out as a growth limitation under these conditions.

Maximum viable cell densities were 44 % lower in batch microcarrier cultures with glutamate-based medium compared to the glutamine control (Figure 7-5 and Table 7-8). Ammonium was 72 % lower in DMEM/glu+gln after 3 days of culture (Figure 7-6) but other limitations resulted from the replacement of glutamine with glutamate. Glutamate was consumed at a high rate resulting in glutamate depletion (Figure 7-7) and entry into the stationary phase while cell densities were comparatively low. The rapid glutamate and glucose utilization also led to the accumulation of overflow metabolites already described in multiwell plates. Reduced growth yields for lactate (Table 7-9) and higher alanine production (Table 7-6) were observed in DMEM/glu+gln compared to DMEM/gln. Glutamine medium, on the other hand, was subject to higher ammonium production which probably caused a growth limitation in conjunction with low glucose and glutamine levels.

A fed-batch feeding strategy was instrumental in improving cell yields in glutamate-based medium. Cells remained in exponential phase up to 24 hours longer than the batch microcarrier cultures resulting in a 40 % increase in viable cell concentrations from 2.09×10^6 to 2.92×10^6/ml. Ammonium was 71 % lower at the end of the exponential phase (4 days) in glutamate-based medium compared to DMEM/gln. At this point, however, glutamate and glucose were depleted or reduced to very low concentrations in DMEM/glu+gln despite the daily additions of these nutrients. Unfortunately, attempts to further improve growth with a higher feed rate to prevent these limitations would be impractical. This is because inhibitory lactate accumulations (>100 mM) resulted from the additions of glucose and glutamate to the fed-batch culture (Figure 7-11). Increase additions of these substrates would likely exacerbate the problem. Previous studies have shown interactive regulation of glucose and glutamine

metabolism and could explain this effect. Ardawi and Newsholme (1983) reported lactate production by lymphocytes was 2-fold higher in the presence of glucose and glutamine compared to cells supplied with only glucose. Glutamate may also stimulate glycolysis in BHK cultures and perhaps to a greater extent than glutamine because of the higher consumption rate of the former. This effect was not apparent in multiwell plates but could be related to the lower glucose and glutamate concentrations and viable cell concentrations under those conditions. The advantages of glutamate-based medium would be more obvious for cell lines in which the use of the alternate substrate is not associated with coordinate increases in glycolysis. Hassell and Butler (1990) described enhanced cell densities with McCoy cells in batch and fed-batch microcarrier cultures with glutamate-substituted medium compared to glutamine medium. However, the McCoy cells consumed less glucose and consequently generated lower lactate levels in the glutamate medium. In this case, the combination of reduced ammonium and lactate promoted higher viable cell densities.

Defining the factors controlling adaptation to glutamate is vital to facilitate an evaluation of the potential of a cell line to grow in glutamate-based medium. In some cases, the rate of glutamate uptake is a key determinant mediating the ability of cells to utilize glutamate as an alternate substrate. At least three systems for glutamate transport have been identified in human fibroblasts (Dall'Asta *et al.*, 1983) and high rates of uptake are possible in cultured cells. In addition, increases in glutamate transport have been reported in response to cystine (Bannai and Kitamura, 1982) or glutamine (Low *et al.*, 1994) starvation. In some cells, however, the capacity for glutamate uptake may be limited. McDermott and Butler (1993) investigated glutamate uptake and glutamine synthetase activity in two cell lines with different adaptabilities to glutamate-substituted medium (GMEM+glu). The McCoy cell line adapted quickly to glutamine-free medium while MDCK were unable to grow. Glutamine synthetase increased in both cell lines following depletion of glutamine or incubation in glutamate-

substituted medium. However, McCoy cells which had been adapted to GMEM+glu also responded with a 2-fold increase in the rate of glutamate uptake. On the other hand, glutamate transport was an order of magnitude lower in the MDCK cells. This was apparently insufficient to satisfy the cellular demand for growth in glutamine-free medium. Adaptation to glutamate was thus attributed to the rate of glutamate transport rather than glutamine synthetase activity in this particular cell line. The current studies have shown BHK cells grown in glutamate medium lacking a low supplementation with glutamine (or dipeptides) demonstrate a considerable decline in growth performance. Low specific growth rates and cell yields were particularly evident during the initial passages in DMEM/glu after subculture from DMEM/gln (Table 7-10 and Figure 7-12). However, growth was observed to improve over the course of 5 passages in DMEM/glu (Figure 7-12). This indicated adaptive changes had occurred but significant increases in glutamate uptake were not observed during the initial 5 passages in the DMEM/glu (Table 7-11). Thus, glutamate utilization was constitutively high in BHK cells and would have little influence on growth or adaptation in the substituted medium.

Increases in glutamine synthetase were also investigated as an adaptation required for growth in glutamate-substituted medium. Earlier studies with cultured skeletal muscle cells have shown this enzyme is highly regulated and responds with 3-4-fold increases in activity following glutamine depletion (Feng *et al.*, 1990). A similar response was reported when these cells were incubated in medium with glutamate or dexamethasone (Smith *et al.*, 1984). Kitoh *et al.* (1990) examined the ability of B and T lymphoblastoid cell lines to grow in glutamine-deficient medium. The B cells demonstrated limited growth whereas T cells proliferated in the presence of low glutamine concentrations. The difference was attributed to a significantly lower glutamine synthetase activity in the B cells. The authors described an inverse relationship between glutamine synthetase activity and dependency on glutamine. The current study was consistent with previous investigations as glutamine transferase

activity was related to the availability of glutamine and perhaps the high glutamate concentrations in BHK cultures. Activity was highest in DMEM/glu, reduced considerably in DMEM/glu+gln and undetectable in DMEM/gln (Figure 7-13a). The prolonged lag phases and reduced growth rates observed in DMEM/glu could be a consequence of insufficient glutamine biosynthesis despite the relatively high activity of the enzyme in BHK cells cultured in this medium. This was supported by the observed combination of lower glutamine transferase activity and significantly improved growth in DMEM/glu+gln compared to DMEM/glu despite the minimal levels of glutamine in the former. The process of adaptation to glutamate-substituted medium could also be attributed, at least in part, to the period required for induction of glutamine synthetase.

Other enzymes involved in glutamine and glutamate metabolism include phosphate-activated glutaminase, alanine aminotransferase and glutamate dehydrogenase. Together, these glutaminolytic enzymes are expected to regulate the balance of ammonium production by cultured cells. They may also influence growth and adaptation in medium substituted with glutamate.

High glutaminase activity is associated with rapidly proliferating cells and high rates of glutamine oxidation (Knox *et al.*, 1969, Linder-Horowitz *et al.*, 1969, Sevdalian *et al.*, 1980 and McKeehan, 1986). Phosphate-activated glutaminase was high in BHK cells even when cultured with a limited availability of substrate (glutamine) in glutamate-substituted medium (Figure 7-13b). Activity, however, was reduced in glutamate medium and may be regulated to some extent by product (glutamate) concentration. Smith *et al.* (1984) reported glutamine depletion had no effect on the enzyme but glutamate induced a 40 % decrease in glutaminase activity. Nevertheless, PAG appeared to be constitutively expressed in BHK cells which would ensure an ability to always utilize glutamine when available in the medium. Indeed, no apparent adaptation was involved when cells grown in DMEM/glu+gln or DMEM/glu were subcultured into DMEM/gln (Table 7-10). High levels of glutaminase further implicated the presence of a

glutamine synthetase/glutaminase futile cycle in BHK cells which could cause or exacerbate a glutamine limitation in DMEM/glu.

The relative contributions of aminotransferases and glutamate dehydrogenase in the deamination of glutamate varies among reports in the literature (McKeehan, 1986). BHK cells had significant alanine aminotransferase and glutamate dehydrogenase activities in glutamate- and glutamine-based media (Figure 7-13c and d). The former enzyme, however, was apparently more active in the deamination process in glutamate-substituted media where cells had increased ALT levels. This was indicated by the higher ala/glu product yield in the glutamate media compared to ala/gln in DMEM/gln (Table 7-4). In other words, a greater proportion of an external supply of glutamate (available from the medium) was deaminated by alanine aminotransferase rather than glutamate dehydrogenase compared to glutamate derived from the deamidation of glutamine. The high rates of glucose and glutamate metabolism in glutamate-based media could promote increased cellular pyruvate levels (the substrate of ALT) due to overflow metabolism and thus stimulate deamination by alanine aminotransferase. The net effect is that glutamate is a less ammoniagenic substrate than glutamine because it contains fewer nitrogen moeities and is deaminated via a less ammoniagenic metabolic pathway.

These studies define the differences between glutamate and glutamine metabolism when these substrates are provided in the culture medium. Furthermore, it is clear that the low-ammoniagenic nature of glutamate in substituted medium is not sufficient to achieve an increase in growth and productivity of BHK cultures. The work has established for the first time that improved growth and productivity for some cell lines must incorporate strategies to additionally control glucose metabolism in glutamate-substituted medium.

Chapter 8

General Discussion and Conclusions

The use of substitutes for glutamine consistently results in decreased ammonium production in animal cell cultures. Glutamate or dipeptides can be used alone or in combination as a replacement for glutamine in the medium.

Glutamine-containing dipeptides can be used as the main substitute for glutamine for cell lines which are unable to use glutamate. The ability of a cell line to grow in dipeptide medium depends on sufficient hydrolysis of ala-gln or gly-gln to satisfy the cellular glutamine requirements. This, in turn, depends on the expression of the cellular peptidase and the availability of adequate concentrations of dipeptide supplements in the medium. Dipeptides were able to promote growth of both the CC9C10 murine hybridoma and BHK cells and are likely effective for a range of cell lines. Higher cell densities and product yields may be achieved in some cases.

The use of glutamate as a substitute for glutamine is advantageous because of its stability, low ammoniagenic nature and limited cost. In addition, lower alanine or glycine accumulations are expected with glutamate compared to dipeptides. Glutamate can be used to replace a majority of cellular glutamine requirements but additional supplements may be necessary to maintain high growth rates and cell yields. Asparagine and low levels of glutamine or dipeptides may be used in conjunction with glutamate to support the growth of a wider range of cell lines than previously thought possible. The combination of glutamate and dipeptides also stabilizes the medium. This could be beneficial in large-scale production, allowing sterilization of the medium by heat treatment. Some cell lines, however, may be unable to grow in glutamate-substituted media. The current investigation with BHK cells along with other studies have established the main criteria required for adaptation and growth of cells in glutamate medium:

i) The cell line [illegible] vithou
inhibiting [illegible]
ii) Gluta[illegible] synthe[illegible] nesize
sufficien[illegible]
iii) Impro[illegible] ses i
other inhi[illegible] amino
with glut[illegible]

These guide[illegible] eased
producti[illegible] with oth[illegible] e as a
substitu[illegible] The use [illegible] doma
because [illegible] the inhi[illegible] dily in
optimize [illegible] media bu[illegible] erflow
metaboli[illegible]. Howeve[illegible] e both
growth [illegible] produci[illegible] ad fed-
batch [illegible]. This[illegible] and
glutama[illegible] in the me[illegible] olism
from bo[illegible] these[illegible] state,
ammoni[illegible] and ala[illegible] ld be
maintai[illegible] non-in[illegible] e not
been p[illegible] sly per[illegible] d the
growth [illegible] murine[illegible] ures.
They re[illegible] signifi[illegible] ue to
the red[illegible] available[illegible] umber
of cells [illegible] duced and[illegible] 1994)
similarly [illegible] med[illegible] murine
hybrido[illegible] culture.[illegible] -fold
increas[illegible] monoclo[illegible] f the

i) The cell line must possess a significant capacity for glutamate uptake without inhibiting the consumption of other essential nutrients such as cystine.

ii) Glutamine synthetase must also be active or inducible in order to synthesize sufficient glutamine from glutamate.

iii) Improvements in cell density depend on the absence of coordinate increases in other inhibitory metabolites such as lactate resulting from replacement of glutamine with glutamate.

These guidelines could be applied to evaluate the potential for increased productivity with other cell lines in batch or fed-batch cultures utilizing glutamate as a substitute. The use of glutamate was not feasible for the CC9C10 murine hybridoma because of the inhibitory effects on growth. Alternatively, BHK cells grew readily in optimized media but were subject to high rates of glutamate uptake and overflow metabolism. However, the use of glutamate has considerable potential to improve both growth and productivity of BHK cells in combined glucose- and glutamate-limited fed-batch cultures. This strategy would involve maintaining low levels of glucose and glutamate in the medium with regular low-level feeding to prevent overflow metabolism from both of these nutrients. The anticipated result would be reduced lactate, ammonium and alanine production. Thus, both lactate and ammonium could be maintained at non-inhibitory levels in glutamate-based medium. These studies have not been previously performed although Ljunggren and Häggström (1994) described the growth of a murine hybridoma in glucose- and glutamine-limited fed-batch cultures. They reported significant decreases in lactate, ammonium and alanine formation due to the reduced availability of glucose and glutamine. Slight increases in the total number of cells produced and monoclonal antibody yield were achieved. Kurokawa *et al.* (1994) similarly controlled both glucose and glutamine at low levels in another murine hybridoma culture. They achieved a 1.5-2.0-fold increase in cell density and a 2.7-fold increase in monoclonal antibody yield compared to cultures in which only one of the

carbon sources was maintained at a limited concentration. Xie and Wang (1994) used a stoichiometric feeding model to control by-product formation in fed-batch culture using a murine hybridoma. This strategy involved feeding nutrients including glucose, glutamine and other essential and non-essential amino acids according to cellular composition. In other words, the feed medium was formulated to match cellular composition or requirements to accurately supply the nutrients at a constant but low level. The result was a marked reduction in the accumulation of lactate and ammonium compared to batch cultures. Consequently, maximum viable cell densities and monoclonal antibody yields were increased 2.8-fold and 17-fold, respectively, in the fed-batch system. Ammonium, however, accumulated to relatively high levels (5.3 mM) in the fed-batch cultures. The authors attributed this, in part, to decomposition of glutamine and asparagine in the feed medium. Thus, further reductions in ammonium and improvements in growth and productivity may be possible using glutamate-substituted medium in conjunction with a stoichiometric feeding regime. Dipeptides may also be employed with this strategy for cell lines unable to utilize glutamate.

The use of substitutes for glutamine in nutrient-limited fed-batch cultures combined with the development of serum-free medium holds considerable potential for industrial production. The markedly reduced accumulation of by-products would promote high cell densities and product yields as well as consistent product quality. The serum-free medium is essential to maintain reproducible growth performance, eliminate undefined components from the medium and facilitate downstream processing and purification. The net result may be a more economical production process in animal cell culture.

Appendix A

Composition of DMEM and BME

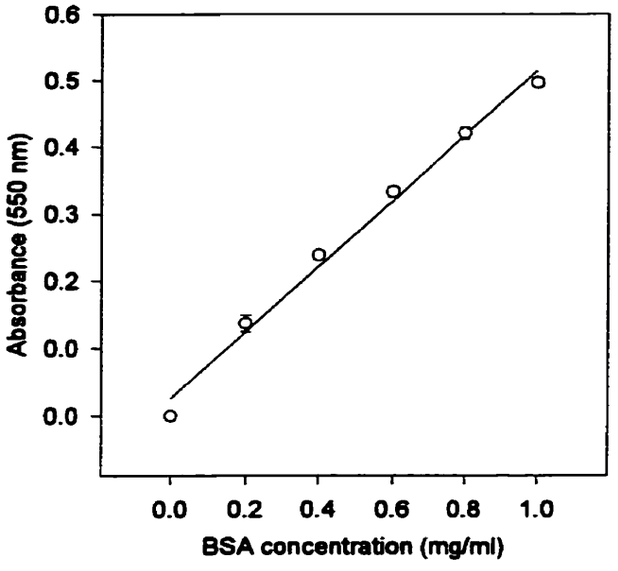
Component	Concentration BME	DMEM
Inorganic salts (mM)		
NaCl	100.0	109.5
$NaHCO_3$	20.0	44.0
KCl	5.0	5.4
$CaCl_2$	1.0	1.8
$MgCl_2$	0.5	—
$MgSO_4$	—	0.8
$NaH_2PO_4 \cdot H_2O$	1.0	—
$Na_2HPO_4 \cdot H_2O$	—	0.8
$Fe(NO_3)3 \cdot 9H_2O$	—	2.5×10^{-4}
Carbohydrate (mM)		
glucose	5.0	25.0
Amino Acids (mM)		
alanine	—	—
arginine	0.1	0.4
asparagine	—	—
aspartate	—	—
cystine	0.05	—
cystine•HCl	—	0.2
glutamate	—	—
glutamine	2.0	3.0-6.0
glycine	—	0.4
histidine	0.05	0.20
isoleucine	0.2	0.8
leucine	0.2	0.8
lysine	0.2	0.8
methionine	0.05	0.20
phenylalanine	0.1	0.4
proline	—	—
serine	—	—
threonine	0.2	0.8
tryptophan	0.02	0.08
tyrosine	0.1	0.4
valine	0.2	0.8

Composition of DMEM and BME (continued)

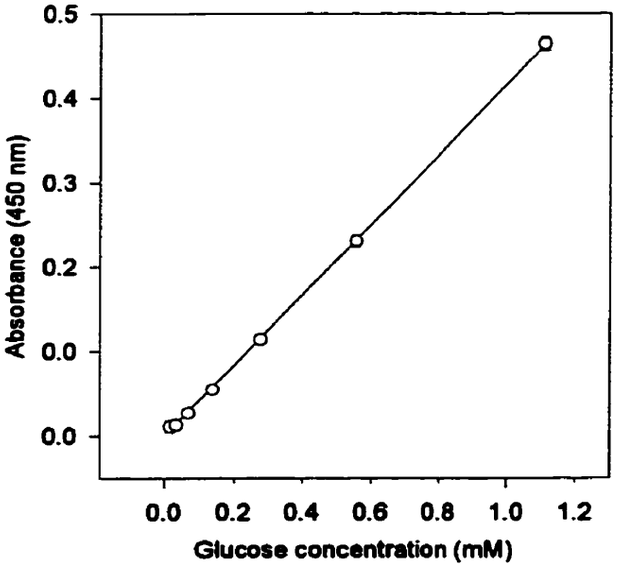
Component	Concentration	
	BME	DMEM
Vitamins(μM)		
biotin	4.1	—
choline	8.3	28.6
folic acid	2.3	9.1
inositol	—	38.9
nicotinamide	8.2	32.8
pantothenate	4.6	17.0
pyridoxal	6.0	19.6
riboflavin	0.27	1.10
thiamine	3.0	11.9
pH Indicators (μM)		
phenol red	14	12

Appendix B
Standard curves

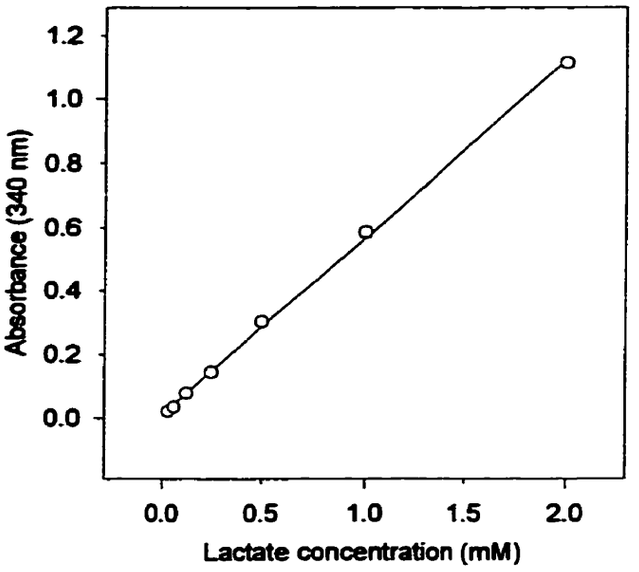
I Bicinchoninic acid protein assay



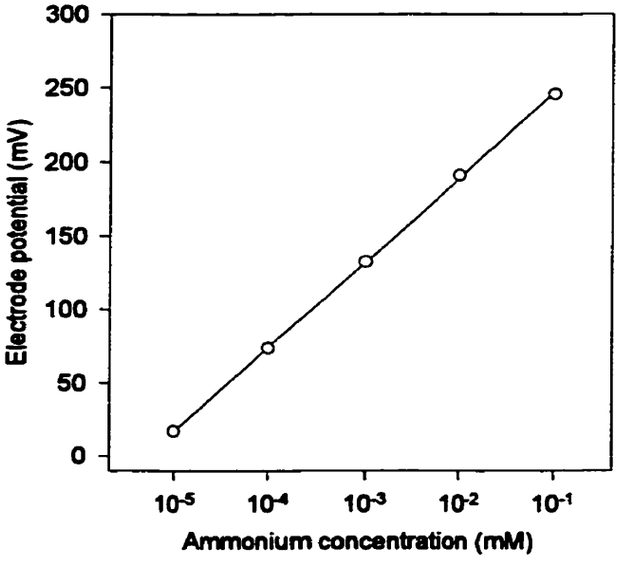
II Glucose oxidase assay for glucose



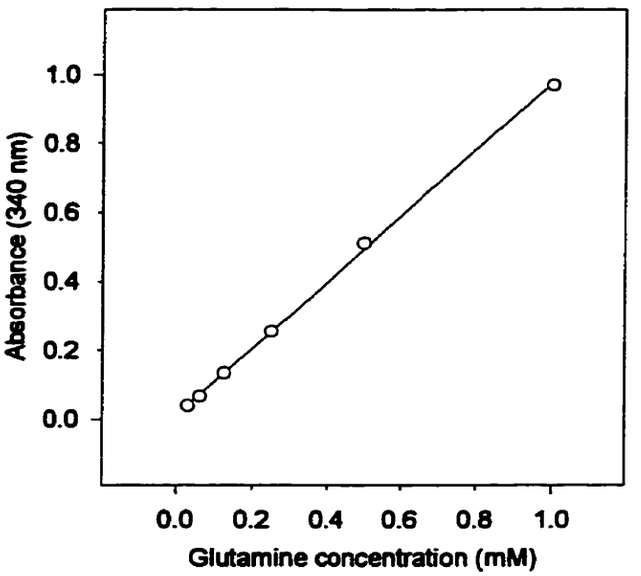
III Lactate dehydgrogenase assay for lactate



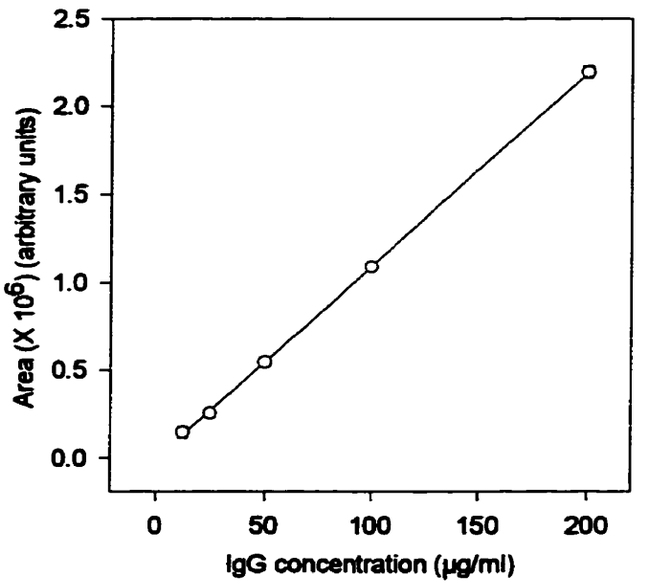
IV Ammonia electrode



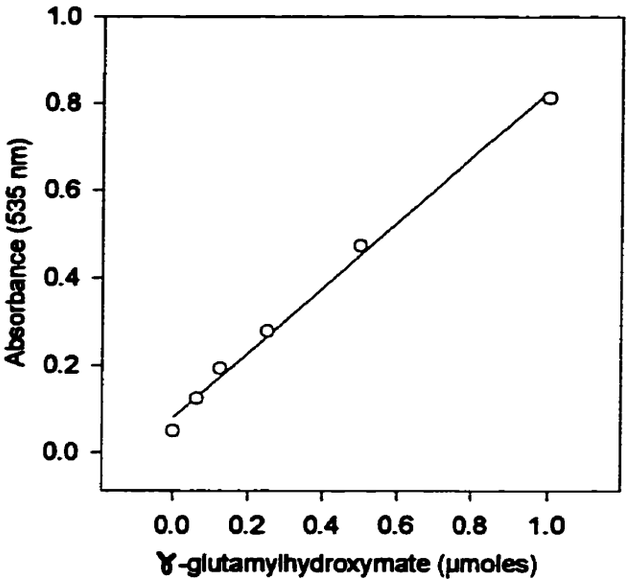
V Glutaminase assay for glutamine



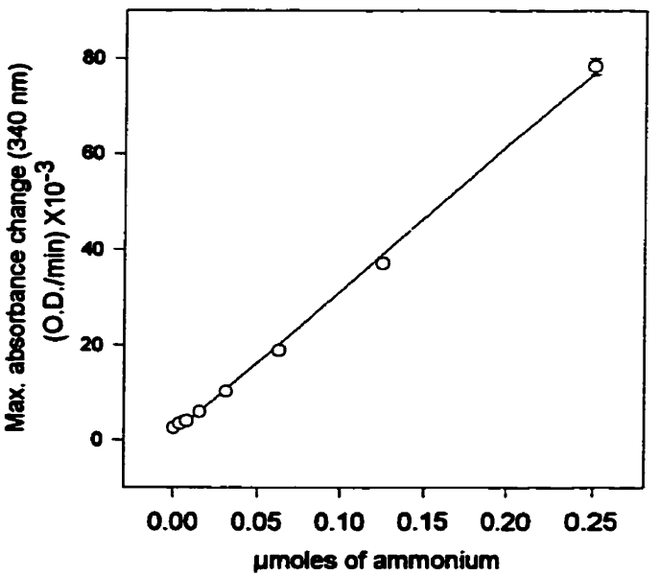
VI Monoclonal antibodies (ProAnaMabs column)



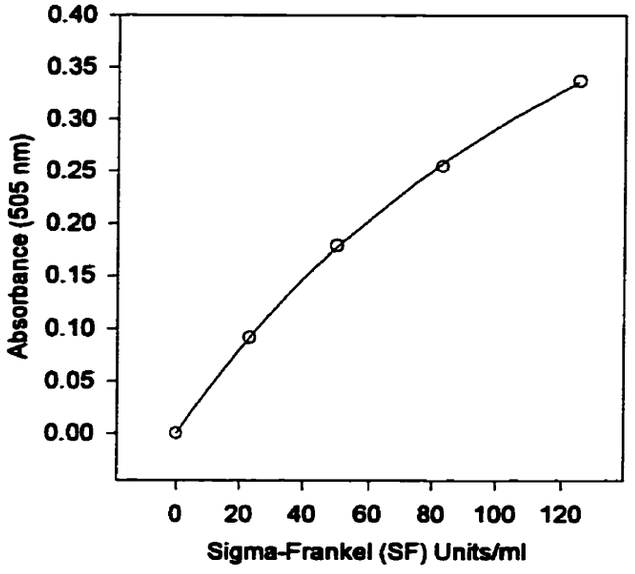
VII Glutamine synthetase/glutamine transferase assay



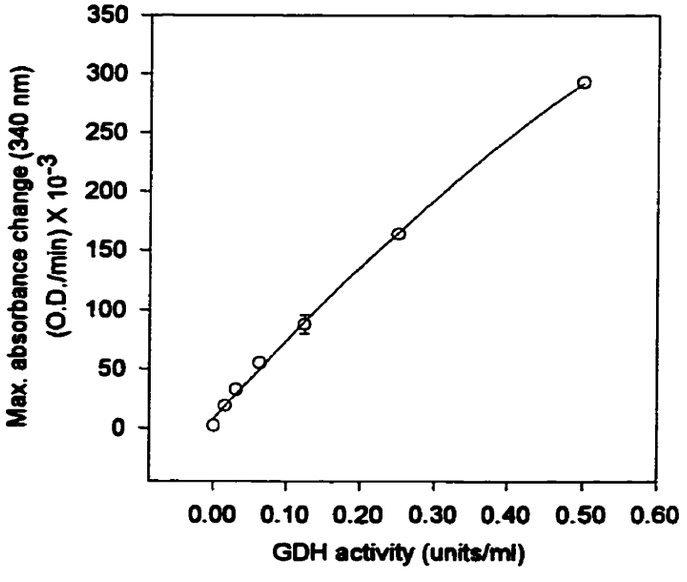
VIII Phosphate-activated glutaminase assay



IX Alanine aminotransferase/glutamic pyruvic transaminase assay

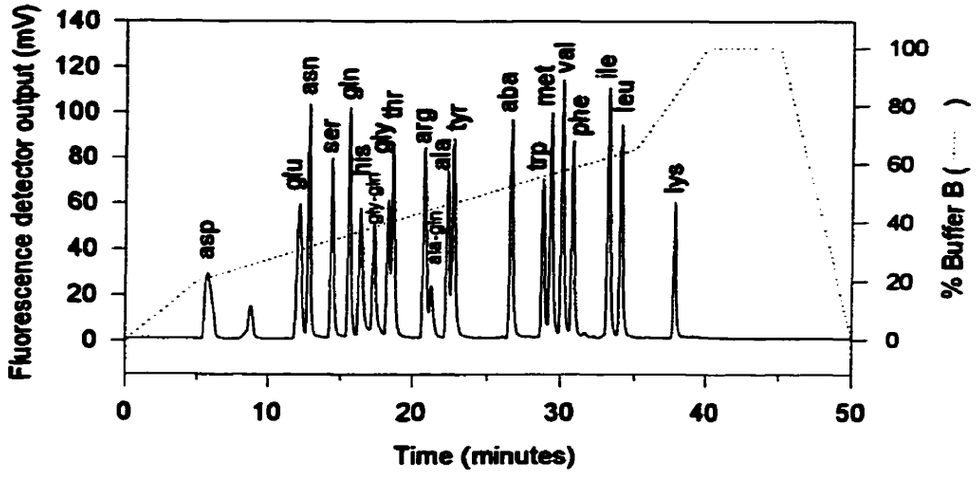


X Glutamate dehydrogenase assay



Appendix C
Determination of amino acid concentrations from HPLC analysis

I Sample chromatogram of a standard amino acid run.



II Peaks were identified and areas determined by computer integration of the standard run.

Amino acid	Ret. time (min)	Conc. (µM)	Area	RF
asp	5.79	500	1056051	1.30
glu	12.137	500	1300983	1.06
asn	12.817	500	1299448	1.06
ser	14.403	500	1130293	1.21
gln	15.61	500	1370862	1.00
his	16.407	500	1088186	1.26
gly-gln	17.317	1000	887918	3.09
gly	18.31	500	803364	1.71
thr	18.653	500	1285039	1.07
arg	20.813	500	1325642	1.04
ala-gln	21.287	1000	396205	6.93
ala	22.393	500	1073050	1.28
tyr	22.807	500	1311793	1.05
aba	26.643	500	1372757	1.00
trp	28.79	500	943447	1.46
met	29.327	500	1469358	0.93
val	30.093	500	1609805	0.85
phe	30.79	500	1213374	1.13
ile	33.22	500	1608449	0.85
leu	34.063	500	1352447	1.02
lys	37.727	500	764277	1.80

III Response factors were calculated from the fluorescent response of the internal standard (aba) relative to each amino acid in the standard run according to the following equation:

$$RF = (Area_{i.s.})/(Area_{a.a.}) \times (Conc._{a.a.})/(Conc._{i.s.})$$

where RF = response factor

$Area_{i.s}$ = area of the internal standard

$Area_{a.a}$ = area of amino acid

$Conc_{a.a.}$ = concentration of amino acid in the standard solution (500 μM)

$Conc._{i.s.}$ = concentration of internal standard (500 μM)

IV Samples containing a known addition of the internal standard were chromotographed. The concentration of each amino acid in samples was calculated from the appropriate response factor and the peak area relative to that of the internal standard by the equation below:

$$C_{a.a.} = (A_{a.a.}/A_{i.s.}) \times C_{i.s.} \times RF \times df$$

where $C_{a.a.}$ = concentration of the amino acid of interest

$A_{a.a.}$ = area of amino acid of interest

$A_{i.s.}$ = area of internal standard

$C_{i.s.}$ = known concentration of internal standard (500 μM)

RF = the response factor determined from the calibration run as described above

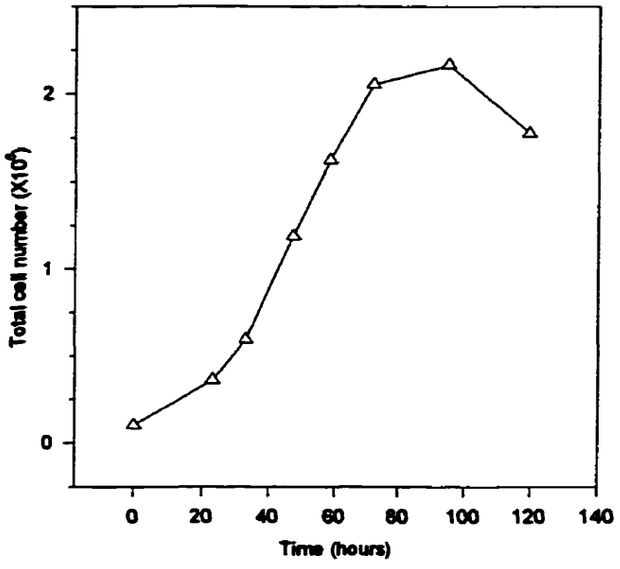
df = dilution factor of internal standard in sample (250 μl/245 μl)

Appendix D

Determination of specific consumption and production rates (integral method)

The specific glutamate consumption during exponential growth of BHK cells in DMEM/glu+gln in batch stationary cultures (see Table 7-3) is demonstrated as an example.

I Integration was performed on a plot of cell number versus time using the trapezoidal rule available as an algorithm in SigmaPlot.



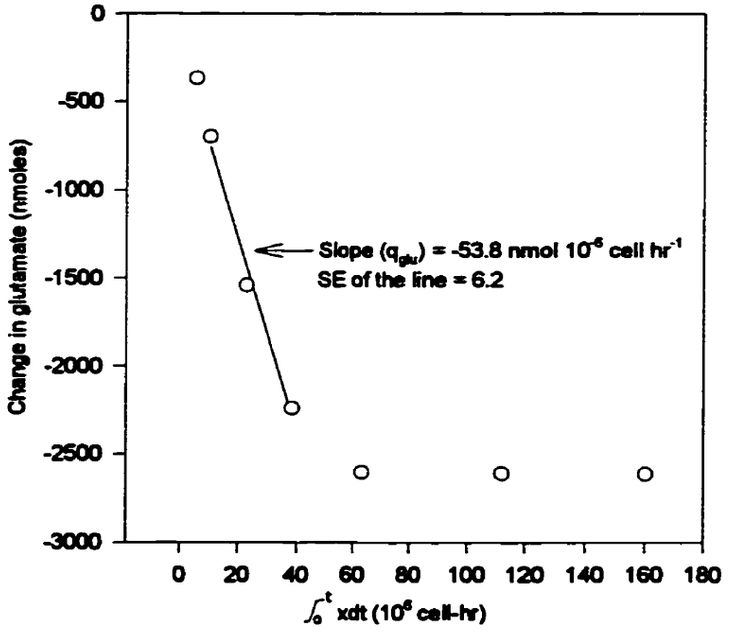
Integration

Time (hr)	Cell number (10^6 cells)	$\int_0^t x dt$ (10^6 cell hr)
0	0.10	
23	0.36	5.35
33	0.60	10.1
47	1.19	22.9
58	1.63	38.6
72	2.05	63.2
95	2.16	112
120	1.78	160

II The change in glutamate from the initial quantity in the medium at each time point of the culture was then determined.

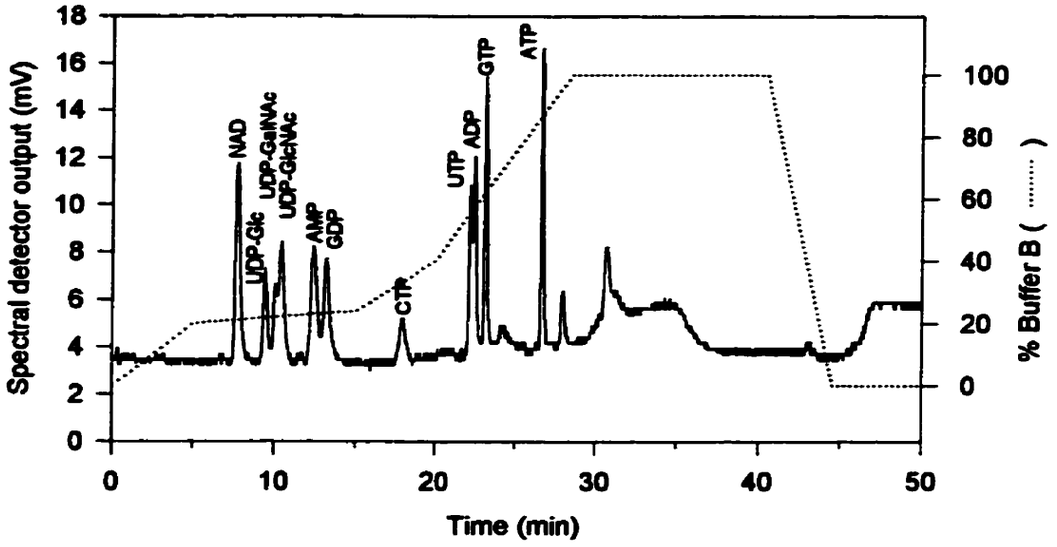
Time (hrs)	Glutamate in medium (nmoles)	ΔS_{glu} (nmoles)
0	2632	
23	2264	-368
33	1932	-700
47	1090	-1542
58	393	-2239
72	28	-2604
95	21	-2611
120	18	-2614

III The change in substrate (ΔS_{glu}) was plotted against the cell index ($\int_0^t$ xdt). The best straight line was fit to a linear region of the plot which corresponded to the exponential phase. The slope was determined (from 33-58 hours in this case) to produce the specific rate of glutamate consumption (q_{glu}).



Appendix E
Determination of nucleotide concentrations by HPLC analysis

I Sample chromatogram of a standard nucleotide run.



II Peaks were identified and computer integration used to determine the areas for known quantities of standards.

Nucleotide	Ret. time (min)	Conc. (X 10^{-5}) (M)	Area	RF (X 10^{-10})
NAD	7.79	2	166058	1.20
UDP-Glc	9.39	2	67197	2.98
UDP-GalNAc	10.11	2	47992	4.17
UDP-GlcNAc	10.43	2	117398	1.70
AMP	12.41	2	126369	1.58
GDP	13.16	2	107972	1.85
CTP	17.85	2	51342	3.90
UTP	22.23	2	94217	2.12
ADP	22.48	2	114705	1.74
GTP	23.18	2	130344	1.53
ATP	26.70	2	140621	1.42

III Response factors were calculated from an amount/area ratio for each nucleotide in the standard run.

$$RF = C_{std}/A_{std}$$

where RF = response factor

C_{std} = concentration of nucleotide in the standard solution = 2×10^{-5} M

A_{std} = area of nucleotide standard

IV The concentration of nucleotides in the sample were calculated based on the peak area of each nucleotide and the response factor determined above:

$$C = RF \times A \times df$$

where C = concentration of the nucleotide of interest

RF = response factor determined from the standard run

A = area of nucleotide of interest

df = dilution factor of sample with Mobile Phase A = 2

Appendix F
Statistical equations

Standard deviation :

$$s = [1/(n-1) \sum_{i=1}^{n} (x_i - \bar{x})^2]^{1/2}$$

where s = standard deviation,

n = the number of samples,

x_i = the value of the ith sample and

$\bar{x}$ = the mean of samples

Standard error:

$$SE = s/\sqrt{n}$$

Student's *t*-test:

$$t = \bar{D}/SE_D$$

where $\bar{D} = \bar{X} - \bar{Y}$,

$SE_D = [(\Sigma D_i^2 - (\Sigma D_i)^2/n)/(n(n-1))]^{1/2}$,

$\bar{X}$ and $\bar{Y}$ = the means of the X and Y data sets, respectively,

$D_i = X_i - Y_i$ and

X_i and Y_i = the value of ith sample from the X and Y data sets, respectively.

Appendix G
Amino acid concentrations in serum

The amino acid content of Calf Serum (Gibco), Donor Calf Serum (Gibco) and FetalClone (HyClone) was determined by HPLC analysis following OPA derivitization. Values are expressed as an average of triplicate samples (n=3) ± SE.

	Concentration (μM)		
	Calf Serum	Donor Calf Serum	FetalClone
ala	404	267 ± 10	110 ± 5
arg	242 ± 1	233 ± 7	10 ± 0
asn	<10	10	<10
asp	68	<10	<10
glu	374 ± 7	180 ± 4	75 ± 1
gln	48 ± 6	112 ± 4	40 ± 1
gly	484 ± 24	536 ± 33	51 ± 1
his	37 ± 1	24 ± 1	<10
ile	111 ± 4	111 ± 3	11 ± 1
leu	247 ± 2	149 ± 3	29 ± 1
lys	163 ± 6	120 ± 9	<10
met	<10	10 ± 2	<10
phe	85 ± 1	51 ± 2	11 ± 0
ser	131 ± 3	93 ± 5	12
thr	31 ± 0	<10	17 ± 8
trp	46 ± 1	40 ± 1	20 ± 0
tyr	309	44 ± 1	<10
val	256 ± 3	204 ± 6	30 ± 1

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