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University of Surrey
School of Biological Sciences

Two-Stage Chemostat Studies of
Hybridoma Growth, Nutrient Utilisation
and Monoclonal Antibody Production | 1994

PhD Thesis by David Venables
Microbial Physiology Group
May 1994

*“Read not to contradict and confute, nor to believe and
take for granted . . . but to weigh and consider”*

Francis Bacon

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To Mum and Dad
and
Alicia

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ABBREVIATIONS

β	Fraction of a culture which is viable
$^{\circ}\text{C}$	Temperature in degrees centigrade
D	Dilution rate
D_2	Dilution rate in second stage chemostat
dl	Decilitre(s)
DMEM	Dulbecco's Modification of Eagles Medium
DOT	Dissolved oxygen tension
dp	Change in product concentration
dt	Change in time
dx	Change in cell concentration
dx_t	Change in total cell concentration
ELISA	Enzyme-Linked Immunosorbent Assay
F	Flow rate
FACScan	Fluorescence activated cell scanner
FCS	Foetal calf serum
G0	Gap 0 cell cycle phase
G1	Gap 1 cell cycle phase
G2	Gap 2 cell cycle phase
g	Gravitational constant
g	Gram(s)
GIDH	Glutamate dehydrogenase
h	Hour(s)
HPLC	High pressure liquid chromatography
Ig	Immunoglobulin
INT	Iodonitro tetrazolium chloride
l	Litre(s)
ln	Logarithm
$\ln x_0$	Logarithm of cell concentration at time zero
$\ln x_t$	Logarithm of total cell concentration
LOD	L-lactate:Oxidoreductase
μ	Specific growth rate
μ_2	Specific growth rate in second stage chemostat
μg	Microgram(s)
μl	Microlitre(s)
μ_{max}	Maximum specific growth rate
M	Mitosis cell cycle phase
MEM	Minimum Eagles Medium
mg	Milligram(s)
ml	Millilitre(s)
mM	Millimolar
NAD	Nicotinamide-adenosine dinucleotide
nm	Nanometers
P	Concentration of product

PITC	Phenylisothiocyanate
P_r	Concentration of product in the medium feed
PTC	Phenylcabamyl
q_{antibody}	Specific antibody production rate
q_p	Specific production rate
q_s	Specific rate of substrate utilisation
rpm	Revolutions per minute
S	Concentration of substrate in the culture
S	Stationary cell cycle phase
S_r	Concentration of substrate in the medium feed
t	Time
V	Volume of culture
v/v	Volume/volume
w/v	Weight/volume
x	Cell concentration
x_0	Cell concentration at time zero
x_1	Cell concentration in first stage chemostat
x_2	Cell concentration in second stage chemostat
x_t	Total cell concentration
x_{t0}	Total cell concentration at time zero
x_{t1}	Total cell concentration in stage one chemostat
x_{t2}	Total cell concentration in stage two chemostat
x_{tt}	Total cell concentration at time t
x_v	Viable cell concentration
x_{v0}	Viable cell concentration at time zero
x_{v2}	Viable cell concentration in stage two chemostat

SUMMARY

Growth and monoclonal antibody production kinetics of the hybridoma cell line NB1 were studied in batch, chemostat and two-stage chemostat culture.

Growth associated production kinetics were observed in batch culture when glutamine was the first nutrient to be depleted. The growth phase was extended and production increased by supplementing the culture with extra glutamine, essential and non-essential amino acids. Monoclonal antibody was produced by the viable cell population with no evidence for its release from lysing cells.

Increasing the concentration of individual amino acids in batch culture appeared to have a negative effect on cell growth and in some cases a positive effect on antibody production. The effect on production may have been indirect, due to the effect on growth rate. Under conditions of leucine and isoleucine limitation the kinetics of antibody production in batch culture were changed to growth dissociated with the majority of the antibody being produced during the decline phase of the culture. The kinetics of production were therefore dependent on the nutrient limiting growth.

In single-stage chemostat culture monoclonal antibody production was negatively growth rate associated, in contrast to the positively growth associated production generally observed in batch culture. Therefore the feasibility of optimising negatively growth associated antibody production in the second stage of a two-stage chemostat was studied.

Steady states in terms of cell concentration, nutrient utilisation and antibody production were observed in both stages of a two-stage chemostat. Monoclonal antibody production in both stages of a two-stage chemostat was negatively growth rate associated. Specific production rate increased with decreasing dilution rate. The specific production rate in the second stage was less than the first stage at an equivalent dilution rate even though the growth rate was less, except at dilution rates below 0.02 h^{-1} when the growth rate in stage two was negative.

Feeding the second stage of a two-stage chemostat with amino acids increased the growth rate but decreased the specific production rate. Attempts to decrease the growth rate of a fed second stage by increasing the volume and therefore decreasing the dilution rate relative to the first stage were unsuccessful. Growth rate remained high, however volumetric production was significantly increased.

The concentration of an amino acid in the medium appeared to influence its rate of utilisation in batch and chemostat culture. Increasing the concentration of an individual amino acid tended to have a negative effect on cell growth. Decreasing the concentration of an individual amino acid reduced the extent to which it was utilised. Increasing the concentration of all amino acids increased their rate of utilisation without increasing the cell concentration.

1.0 INTRODUCTION

1.1 Development of Animal Cell Culture

The *in vitro* cultivation of animal cells was a natural progression from the early successes of tissue explantation. Wilhelm Roux (1885) reported the first successful explantation when he was able to maintain the medullary plate of a chick embryo in warm saline for a few days. However, Harrison (1907) is generally regarded as the father of tissue culture because of his experiments with nerve fibres. Harrison demonstrated that it was possible to keep tissues alive *in vitro* and also that the technique was capable of making a fundamental contribution to biological knowledge. The first paper to describe true cell culture was published by Rous and Jones (1916) who cultured individual chick cells rather than explanted tissue. Cells were dispersed from cultured pieces of tissue by digesting with the proteolytic enzyme trypsin. Suspensions of individual cells were obtained. However for continued growth and division the cells required the solid support of a plasma clot. The characteristics of growth observed were typical of an anchorage dependent cell, that is, losing their spherical shape, putting out processes and proliferating until the scattered cells connected with each other to form a monolayer. Owens, Gey and Gey (1954) were the first to grow cells in suspension. This was achieved by culturing the cells in a rotating drum. As a result of this it was noticed that some of the cells underwent a 'transformation' after which they grew spontaneously in suspension in stationary flasks. Animal cells can therefore be classified into one of two groups depending on their requirement or not for a surface on which to grow in culture. Anchorage dependent cells require a surface to grow on and are typified by primary and diploid cells. Suspension cells do not require a surface to grow on and are typified by transformed cell lines. However, primary cells which grow in suspension *in vivo*, such as

lymphoid cells, continue to grow in suspension *in vitro*. This classification is not absolute as cells such as Chinese hamster ovary (CHO) and baby hamster kidney (BHK) are able to grow in suspension and attached to surfaces.

1.2 Types of Animal Cell in Culture

Animal cells are divided in their classification not only by their requirement or not for a surface on which to grow but also by their life span in culture. When a cell is first isolated from animal tissue or an organ, a primary culture is established. Such a culture retains the morphology, karyotype and function observed *in vivo* and grows for only a few generations. If this primary culture successfully adapts to the *in vitro* conditions it may continue to grow for generally up to fifty generations after which it loses its capacity for continued replication. Such a culture is known as a cell line (for definition of terms see Schaeffer, 1984).

Cell lines sometimes spontaneously 'transform' to become a continuous cell line, or are transformed by infecting with viruses. Continuous cell lines tend not to have the same morphology as the original cell *in vivo*, they are aneuploid, have a relatively short doubling time, grow to higher cell concentrations than cell lines, and do not appear to have a limited life span. Continuous cell lines can also originate directly from tissue where they tend to be associated with tumours. The characteristics of continuous cell lines, in particular their ability to grow to high cell concentrations, their indefinite life span, and their generally less demanding requirements in terms of nutrition, have made them the favoured vehicle for the production of various reagents for research, medical, veterinary and industrial use.

The characteristics of continuous cell lines have now been widely exploited in mammalian cell culture. The exploitation ranges from using continuous cell lines as research tools in virology, toxicology and genetics laboratories (Klausner, 1987; Ramabhadran, 1987; Sedivy, 1988; Tartaglia and Paoletti, 1988) to their industrial exploitation, as in the large scale production of foot-and-mouth disease vaccine (Radlet *et al.*, 1985). However, one of the most widespread examples of the industrial use of continuous cell lines is their use in the production of monoclonal antibodies by hybridoma cell lines, developed over the last fifteen years.

1.3 Monoclonal Antibodies

Köhler and Milstein (1975) pioneered the science of hybridoma technology from which monoclonal antibodies were developed. Antibodies are produced *in vivo* by B lymphocytes in a specific response to a foreign antigen. However in culture, B lymphocytes exhibit the properties of all primary cell lines, that is limited life span and slow growth rate. These drawbacks were overcome by fusing a B lymphocyte with a continuous cell of the immune system, a myeloma, resulting in the production of a hybrid or hybridoma cell. Hybridomas express the lymphocyte's property of specific antibody production, and the myeloma's properties of indefinite life and rapid growth rate. It is now theoretically possible to produce virtually unlimited amounts of highly pure antibody of defined and consistent antigen specificity originating from a single clone of cells.

The specificity of monoclonal antibodies combined with the ability to produce them in unlimited quantities has resulted in their industrial

exploitation in a number of areas. Monoclonal antibodies have had possibly their biggest impact in the field of *in vitro* diagnostics with a U.S. market expected to be in excess of \$500 million in the 1990's. Other areas under extensive investigation are the use of monoclonal antibodies for affinity purification, *in vivo* imaging, and immunotherapy with an estimated demand for kilogram amounts. For this reason efforts are being made at universities and by industry to develop efficient methods for their large scale production (Reuveny and Lazar, 1989).

1.4 Mammalian Cell Culture Systems

A wide range of cultivation systems for propagating monoclonal antibody secreting hybridomas have been developed in recent years (Randerson, 1985; Lydersen, 1987), however, in each system the restraints associated with large scale cell culture remain similar. Shevitz *et al.*, (1989) identified the following areas:

1. Defining the nutrient needs of the culture.
2. Accumulation of metabolic by-products and problems associated with their removal in continuous culture.
3. Oxygen mass transfer.
4. Sensitivity of cells to physical and physiological constraints to growth.
5. Process analysis and control.
6. Maintaining cultures at an optimum cell concentration and productivity by limiting excessive cell growth.

The present culture systems for suspension cell cultures such as hybridoma cells fall into one of two categories: 1. homogeneous systems in well agitated reactors; and 2. heterogeneous systems which include hollow fibre reactors, ceramic matrix reactors, entrapment and

microencapsulation (Lambe and Walker 1987). Methods of operating each of the culture systems varies between batch, perfusion and continuous mode.

1.4.1 Heterogeneous Cell Culture

In heterogeneous systems the cells are retained in an enclosed chamber at a high concentration into which growth medium is perfused. The advantages of such a system are that a high cell concentration is maintained in a small volume, thereby mimicking the *in vivo* environment to a certain extent. The cells are entrapped or immobilised and therefore not damaged by mechanical agitation. Feeding and waste product removal can be achieved without disrupting the cells. The high cell concentration and low volume results in a concentrated product. The heterogeneity of the system is its major disadvantage. Concentration gradients are generated inside the growth chamber resulting in non-homogeneity in terms of cell concentration, viability and productivity making it nearly impossible to take a representative sample from the reactor. Therefore the monitoring and control of key parameters such as pH, dissolved oxygen tension, glutamine and other metabolites in the microenvironment of the cell culture are difficult.

Hybridoma cell cultures have been grown in a number of different heterogeneous culture systems. Microencapsulation has been developed as a compromise between the desire for highly concentrated perfused cultures and homogeneity, resulting in a semihomogeneous system in which the cells are entrapped inside relatively large capsules. This allows easy separation of cells from culture supernatant for continuous harvesting of product as well as the advantages of a concentrated cell culture. Cellular microencapsulation was first developed by Lim and

Sun, (1980) and has been applied to monoclonal antibody production (Jarvis and Gardina, 1983). The technique consists of placing living cells inside an alginate sphere, followed by bonding of a polymer to the sphere surface to create a semi-permeable membrane. The membrane allows diffusion of nutrients and waste metabolites to support high cell densities and retains monoclonal antibodies (Rupp *et al.*, 1987). Alternatively, cells have been entrapped in agarose beads which, due to their porous nature, allow the diffusion of antibody into the medium (Nilsson, 1987). The difference between the two systems is the method of harvesting the product. Another method of entrapping hybridoma cells has been pioneered by the Verax Corporation using macroporous collagen microcarriers. Collagen microspheres containing cells are cultured in fluidised-bed reactors up to concentrations greater than 10^8 viable cells/ml of matrix. Cell specific productivity in such a system has been reported to be 5 times higher than batch culture and 1.8 times higher than chemostat culture (Runstadler *et al.*, 1992).

As previously stated, one of the aims of a nonhomogeneous perfusion system is to achieve high cell densities by mimicking the *in vivo* environment. This is typified by hollow-fibre reactors, first described by Knazek *et al.* (1972). Cells are grown in the space outside bundles of semipermeable fibres through which medium flows. Nutrients and metabolic waste products pass through the membrane while cells and product are retained in the extracapillary space. The cells can grow to very high concentrations and be maintained for extended periods of time, resulting in very high monoclonal antibody titre (Hirschel and Gruenberg, 1987). Other heterogeneous perfusion systems which have been applied to monoclonal antibody production include flat membrane reactors (Klement *et al.*, 1987), in which cells are located between two

membranes. An ultrafiltration membrane on one face of the chamber separates cells from medium so that nutrients diffuse into the cell growing space while a microfiltration membrane on the opposite face separates cells from product, allowing cell free product to be harvested. Ceramic matrix reactors (Marcipar *et al.*, 1983) and fixed bed reactors (Lazar *et al.*, 1987) have also been developed in which the cells are immobilised within a solid support through which medium is perfused.

1.4.2 Homogeneous Cell Culture

In homogeneous systems the cells are grown in agitated suspension with the major advantage being culture homogeneity. Samples representative of the whole reactor can be readily obtained, the macroenvironment of the culture can be monitored and controlled while the microenvironment is reduced to a minimum. Homogeneous culture systems, due to their ease of monitoring and control, have been more widely used in research laboratories to study the parameters affecting cells in culture than heterogeneous systems. In the industrial production of mammalian cell products, homogeneous systems have so far proved more popular than heterogeneous systems, largely due to the transfer of reliable tried and tested methods from microbial fermentation processes.

The simplest and therefore most common method of homogeneous animal cell culture is stirred batch culture. Batch culture of hybridoma cells has been described by numerous workers and a typical pattern of cell growth has emerged. When the cells are inoculated into a medium containing all the nutrients essential for growth, in an environment controlled within limits permissive for growth in terms of temperature, oxygen concentration and pH, the cell population will grow and divide at a maximum rate until a nutrient essential for growth becomes

depleted and/or a growth inhibitory waste metabolite accumulates to a significant level. The cell population then enters a brief stationary phase followed by a decline phase. In terms of cell and antibody yields, batch culture tends to be the least efficient production process available (Reuveny and Lazar, 1989; Shevitz *et al.*, 1989; Runstadler *et al.*, 1992) therefore several modifications have been devised.

Fed-batch culture, in which the culture is fed intermittently or continuously with whole medium or specific limiting nutrients, results in higher cell concentrations, prolonged viability and a resultant increase in antibody titre. Growth and production of hybridoma cells have been compared in batch and fed-batch systems (Reuveny *et al.*, 1986; Reuveny and Lazar, 1989), in each instance higher antibody titres were obtained in fed-batch compared to batch culture.

Perfusion culture may be seen as a natural progression of fed-batch culture. However, in this case batch culture is fed with fresh medium while maintaining a constant volume. The cells are physically retained in the reactor therefore the cell concentration constantly increases until it becomes limited at a high cell concentration. The advantage of such a system is the increased volumetric productivity achieved as a result of the increased cell concentration. Methods of retaining cells within the reactor are numerous, including rotating filters, hollow fibre bundles, nylon cloth and various membrane configurations (Reuveny and Lazar, 1989). Total cell retention in perfusion systems results in a steadily decreasing viability of the culture, therefore methods for sustaining viability have been developed which include the selective recycle of viable cells (Hulscher *et al.*, 1992) and the inclusion of a cell bleed rate (Hiller *et al.*, 1993).

1.4.3 Continuous Chemostat Culture

During batch and perfusion culture the cell population experiences continually changing environmental conditions in terms of nutrient depletion and waste metabolite and product accumulation. This results in changes in the cell physiology, growth rate, which is constant for only a short period of time during the exponential growth phase, and production kinetics. Such a constantly changing system is difficult to analyse and control. Chemostat culture differs in that it allows the cells to grow at a constant growth rate under environmental conditions which do not change with time. The culture is generally started as a batch system and then a continuous medium feed is initiated during the exponential phase of growth. Culture medium, containing cells and cell products, is removed at the same rate as the feed. The result is that the cells achieve steady state conditions in which a state of exponential growth is maintained. The cell population density is maintained in steady state by the depletion of an essential nutrient or by accumulation of a waste product at a toxic level. The specific growth rate is controlled by varying the rate of medium feed. Provided the dilution rate does not exceed the maximum growth rate of the cells then a physiological steady state is established in which any measured parameter should be constant with respect to time. Operating single or multiple stage chemostats under steady state conditions provides the opportunity to examine the cellular physiology of cultures growing at various rates and under various environmental conditions. The ability to control the growth rate allows the reproduction of physiological and environmental conditions observed only transiently during batch culture.

Theoretically, to maximise the productivity for strictly growth-

associated production the chemostat should be operated at high dilution rates. For a product which follows partially growth-associated or growth-dissociated production the dilution rate should be low to optimise production. Because growth is a prerequisite for steady state chemostat operation, such a culture system is inappropriate for the production of metabolites which can only be synthesised by non-growing cells. The use of multiple-stage chemostats adds a versatility to this mode of operation which allows more in depth study of growth-dissociated phenomena. Because the first stage of a multiple chemostat culture provides a continuous inoculum for subsequent stages, growth is only required in the first stage. Subsequent stages can therefore be used for the study of non growth-associated product formation. Also, because conditions optimal for growth and monoclonal antibody production are often different, variations in operating conditions between stages which could enhance productivity are possible.

1.5 Requirements for the Growth of Hybridoma Cells in Culture

In order for animal cells to grow in culture, a number of minimum conditions must be met in terms of the physical and chemical environment. However, factors which influence the growth and viability of cells in culture also affect the production of monoclonal antibody by hybridoma cells. The growth requirements of animal cells in culture will, therefore, be discussed with particular reference to hybridoma cell lines and the influence of culture conditions on antibody production.

In order for cells to be grown *in vitro* the environment must mimic conditions *in vivo* . This entails provision of a controlled environment in terms of temperature, dissolved oxygen tension and pH. In addition, suspension cultures must be stirred to maintain homogeneity. The

nutritional requirements of the cells are met by the components of the cell culture medium. The basic constituents of media include amino acids, glucose, vitamins, inorganic ions and a buffering system to maintain a stable pH. Although such media may successfully maintain cells in a viable condition, they are not suitable for prolonged or optimal cell growth. Growth factors are also generally required to stimulate cell proliferation. The requirement for these factors has traditionally been met by supplementing the basal medium with animal serum although a number of serum substitutes have now been identified allowing cells to be grown in a more defined, serum-free medium.

1.6 Effect of Culture Conditions on Hybridoma Growth and Antibody Production

a. Serum

The necessity of providing serum in cell culture media to promote cell proliferation was established (Willmer, 1958). and the role of serum constituents in cell proliferation is now well documented (Griffiths, 1986; Maurer, 1986). Serum provides the cells with a variety of essential nutrients. These include lipids, carried by albumin, various growth factors, such as platelet derived growth factor and epidermal growth factor, the requirements for which are cell line dependent. Serum also contains various hormones that stimulate cell proliferation, cell attachment factors, transport proteins, various trace elements and cell growth inhibitors. The major disadvantages of supplementing media with animal serum are its complex and undefined nature and potential batch to batch variability, high cost and interference in down-stream processing as well as risks of contamination with virus, mycoplasma and bacteriophage. For these reasons a number of serum-free and protein-free defined media have been developed. (Barnes and Sato, 1980;

Franek *et al.*, 1992; Inouye *et al.*, 1992). The success of these media is very much dependent on the individual requirements of cell lines.

Hybridoma cells have been grown in serum-containing and serum-free media and the effect of serum on antibody production appears to be cell line dependent. Reducing the concentration of serum in the medium has been reported to have a positive effect on antibody production (Tharakan *et al.*, 1986; Schliermann *et al.*, 1987), a negative effect (Low and Harbour, 1985) or no effect at all (Renard *et al.*, 1988; Lee *et al.*, 1989). The fact that antibody production has been observed when hybridoma cells were grown in serum-free media (Tharakan *et al.*, 1986; Cole *et al.*, 1985; Glassy *et al.*, 1987) demonstrates that serum is not essential for antibody production.

b. Amino Acids

The amino acids required for the growth of animal cells in culture were originally determined by Eagle, (1955 and 1959) who identified the essential and non-essential requirements of mouse L cells. Thirteen essential amino acids were required, while sufficient non-essential amino acids could be made by the cell for them not to be necessary in the culture medium. The thirteen essential amino acids identified by Eagle are arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine. Non-essential amino acids which are also commonly added to media are alanine, asparagine, aspartic acid, proline, glycine and serine. The classification of amino acids into essential and non-essential differs between cell lines. For example serine, normally considered non-essential, is required by lymphoblastoid cell lines (Birch and Hopkins, 1977).

The role of amino acids in the culture media is the same as that in the blood stream of the whole animal, i.e. to act as precursors for protein, purine and pyrimidine synthesis as well as being involved in many other biochemical pathways (see Stryer, 1981). There is nothing to suggest that their role in animal cell culture differs from their role *in vivo* except for the extent to which glutamine is involved in supplying the energy necessary for growth as opposed to glucose (Reitzer *et al.*, 1979).

The rates of utilisation and production of amino acids varies between different cell lines (reviewed by Thomas, 1986). However, the rate of glutamine utilisation is consistently 5 - 10 times higher than all other amino acids (Griffiths and Pirt, 1967; Blaker *et al.*, 1971; Lambert and Pirt, 1975). The reason for this high rate of utilisation is likely to be the use of glutamine as an energy source. Approximately 40% of the energy requirement of Chinese hamster fibroblasts (Donnelly and Scheffler, 1976) and 30% of the energy requirement of human diploid fibroblasts (Zielke *et al.*, 1978) were found to be met by glutamine in the presence of excess glucose.

The supplementation of hybridoma cultures with extra essential amino acids has been reported to increase antibody titre in batch culture (Rupp, 1985; Luan *et al.*, 1987; Duval *et al.*, 1989 and 1991). However, the specific role of individual amino acids in antibody production is not yet known, neither is it known whether the positive effect of supplementing batch cultures with extra amino acids has a positive effect on antibody production directly or indirectly through its effect on cell viability and growth rate. For example, Rupp and Geyer, (1985) suggested that the

beneficial effect of increased amino acid concentration in the medium on antibody production was due to the increased osmolality.

c. Glucose

Glucose is present in the medium as an energy source and is also implicated in the formation of intermediates for nucleotide, amino acid and lipid biosynthesis (Zielke *et al.*, 1978; Pouysseguy *et al.*, 1980; Romano and Connell, 1982). As with amino acids, the effect of glucose concentration on antibody production would appear to be indirect through its effect on growth. Therefore Birch *et al.*, (1985) and Miller *et al.*, (1988a) observed increased antibody production at low glucose concentrations in glucose limited chemostats, however, this also coincided with reduced growth rates. Glucose concentration has also been shown to have no effect on antibody production (Tharakan and Chau, 1986). Duval *et al.*, (1992) observed that feeding fructose instead of glucose during the stationary phase of a culture prolonged the life span of the culture which had a positive effect on antibody production, and demonstrated that glucose was not essential to antibody production.

d. Vitamins

The requirement for vitamins by animal cells has been reviewed by Higuichi, (1973). Members of the B group of vitamins are the most commonly included in cell culture media. Mostly they are cofactors for enzymes. Folic acid, nicotinamide, pantothenic acid, pyridoxine, riboflavin and thiamine are required by most cultured cells and included in most media. Although vitamins are present in the media little is known concerning their turnover or possible exhaustion during a culture. However, Duval *et al.*, (1992) observed vitamin limitation during fed batch cultures of hybridoma cells. Supplementation of the

culture with daily feeds of vitamin solution resulted in increased cell survival without any significant increase in antibody production.

e. Inorganic Ions

Inorganic ions are included in animal cell culture media where they have essential physiological functions, such as maintaining osmotic pressure and membrane potentials and buffering, as well as promoting cell attachment or acting as cofactors for enzyme reactions. Concentrations of inorganic ions have traditionally been based upon those in general purpose balanced salt solutions. Inorganic ions essential for the growth of HeLa cells were shown to be sodium, potassium, calcium, magnesium, phosphate, bicarbonate and chloride (Eagle, 1955). Increased concentrations of inorganic ions have been added to hybridoma cultures to increase the osmolarity of the medium. Under conditions of osmotic stress the cells have been observed to increase their specific antibody production rate and decrease their specific growth rate (Oyass *et al.*, 1989; Reddy *et al.*, 1992; Ho *et al.*, 1993).

1.7 Kinetics of Monoclonal Antibody Production

The kinetics of product formation in microbial cultures has been discussed by Pirt (1975). The relationships between growth and product formation observed in microbial cultures may also be observed in animal cell cultures, therefore the discussion of Pirt is equally applicable here. In general, the relationship between growth and production falls into one of two classes, growth-linked and non-growth-linked. When a product is growth-linked, or growth-associated, the amount of product formed is directly proportional to the biomass formed, i.e. the specific production rate is proportional to the growth rate. This is demonstrated in batch culture by an increase in product titre during the exponential

phase of growth, with no further increase once the population enters the decline phase. In chemostat culture growth-associated production is demonstrated by increasing specific production with increasing growth rate. Growth-linked processes are generally those which are essential to the function of the organism. Non-growth-linked, or growth-dissociated, product formation can be of two types, either specific production rate is independent of growth rate or it varies with specific growth rate in a complex way. In batch culture, such non-growth-linked product formation is demonstrated by changing specific production rate or no production during the exponential phase during which growth rate is constant, and increased production during the stationary and decline phases of the growth cycle. Non-growth -associated product formation in chemostat culture is demonstrated by product output that remains constant over a range of dilution rates, or that varies in a more complex way. In chemostat culture specific production rate which is inversely proportional to growth rate has also been observed where production is negatively growth-associated rather than growth-dissociated. This is demonstrated by an increase in specific production rate with decreasing growth rate.

Several types of production kinetics of antibody in batch culture have been described by various investigators and these have been classified into three groups by Merten *et al.*, (1987) as follows:

- Type 1: the highest specific production rate of antibody occurs before the beginning of the exponential growth phase, i.e. during the lag phase.
- Type 2: the highest specific production rate of antibody occurs at the beginning of the exponential growth phase and during the stationary and decline phases.

Type 3: a constant specific production rate of antibody is seen only in the exponential growth phase, with no production during stationary and decline phases.

Type 1 has been described by Merten *et al.* (1985) for one cell line and is explained by feedback inhibition. However, this type of production kinetic is not very common.

Type 2 is the most commonly observed form of antibody production kinetic. During the exponential phase a relatively low concentration of antibody is produced, this level is greatly increased during the stationary and decline phases. Such observations have led to the proposal that antibody production is growth-dissociated, i.e. growth of the cell is not necessary for antibody production.

Type 3 is often referred to as growth-associated production as antibody is produced only during the growth phase of the cell population. This implies that antibody can only be produced while the cell is actively dividing, and when growth ceases so does production.

A large number of studies have been carried out in order to try to optimise antibody production by a growth-associated or dissociated producer cell line without really understanding what causes a cell to demonstrate growth-associated or dissociated production characteristics. Therefore production decisions are made based on batch culture results in which the cells are growing in a constantly changing environment of depleting nutrients and increasing inhibitory metabolites. For example, perfusion systems (which hold the cell at the stationary state) are used for hybridoma cell lines showing Type 2 production kinetics, and batch

or fed-batch systems (in which the cells are in the exponential growth phase for much of the time) for hybridoma cell lines showing Types 1 and 3 production kinetics (Merten *et al.*, 1987; Reuveny and Lazar, 1989). A long term optimisation strategy which seeks to understand what causes a cell to follow a particular production profile in batch culture, and demonstrates whether it is more than just an artifact of the culture system but an inherent characteristic of the cell line would be of obvious benefit.

Various studies on the kinetics of monoclonal antibody synthesis in batch culture have reached different conclusions to explain growth-associated and dissociated production.

1.7.1 Growth-Associated Monoclonal Antibody Production

It has been proposed that production of monoclonal antibody is not growth-dissociated but is produced during the growth of the cell, stored in cytoplasmic vesicles and released during the death phase when the dead cells break up. Al-Rubeai, Rookes and Emery (1989) used immunogold anti-mouse IgG labelling to show that cells store the synthesised IgG in vesicles, and suggest that only a small amount is secreted during the life of the cell and that most is released after cell death. However, the antibody levels measured by ELISA in freeze thawed samples of cells taken throughout the growth cycle of a hybridoma cell line showing increased specific rate of monoclonal antibody secretion during the decline phase of growth proved to be small and insufficient to account for the observed kinetics (Birch *et al.*, 1987).

Walker *et al.*, (1987) investigated the timescale of antibody secretion

and their results indicate continuous secretion of antibody with negligible storage. Five mouse hybridoma cell lines of diverse background were used in pulse-chase experiments where radiolabelled methionine was traced to extracellular antibody. The rate of radioactive antibody secretion reached a peak within two hours of the pulse; this was followed by a rapid decline and a negligible rate of labelled antibody appearance over the following days during which the cells died.

Velez *et al.* (1986) observed that a significant amount of monoclonal antibody was produced during the decline phase when the cells had already stopped growing, however they found a direct correlation between the concentration of living cells in the culture during the latter stages of propagation and the amount of monoclonal antibody produced. It was concluded that maintenance of cell viability was more important than growth rate in production of antibody.

1.7.2 Growth-Dissociated Monoclonal Antibody Production

The growth-dissociated mode of antibody production is supported by the fact that conditions optimal for hybridoma growth are not necessarily optimal for monoclonal antibody production. Reuveny *et al.*, (1986) found that the highest cell concentration of a certain hybridoma was obtained at a dissolved oxygen tension of 60% air saturation, while the highest titre of monoclonal antibody was obtained at 25%. Miller *et al.*, (1988) found that specific monoclonal antibody production rate was higher at extreme pH values (pH 6.8 and 7.7) than in the pH range for optimal cell growth (pH 7.1 - 7.4). Merten *et al.*, (1985) found that cultivating cells below 37°C resulted in higher titres of monoclonal antibody because the cells remained viable for longer while Reuveny *et*

al., (1986) observed an increase in cell viability but a decrease in monoclonal antibody production rate. It has been generally observed that the growth rate of hybridomas in serum-free media is lower than in serum-supplemented media while the specific rate of monoclonal antibody secretion is higher (Glassy *et al.*, 1988). Therefore, hybridomas are more efficient in terms of monoclonal antibody production but less efficient in terms of growth under serum-free conditions.

The precise relationship between cell growth and antibody production has so far not been elucidated, however several possible theories have been proposed.

1.7.3 Stress and Monoclonal Antibody Production

Animal cells are frequently exposed to environmental stress under their research and production conditions (reviewed by Goochee and Passini, 1988). A simple definition of stress is a change in the cells environment which results in a reduction in its growth rate. Stress may be induced by the change in environment that accompanies inoculation into fresh medium. Cells may experience a variety of stressful environmental conditions as they circulate in a large scale culture vessel, including exposure to local variations in dissolved oxygen concentration and hydrodynamic shear stress. In batch culture, nutrient depletion or the accumulation of toxic metabolic byproducts are sources of stress. The overproduction of a protein, for example monoclonal antibody, could also be stressful. The exposure of cells to many types of environmental stress results in the increased synthesis of intracellular proteins that apparently counteract the detrimental effects of the stress. In response to a sudden increase in temperature, a set of 'heat shock' proteins is

induced in bacteria, yeast, insect, plant, and mammalian cells. In bacteria, many additional stress agents have been identified that cause the increased synthesis of specific subsets of intracellular proteins, including amino acid/energy limitation (the stringent response), and DNA damage (the SOS response). In addition to the heat shock proteins, a set of 'glucose regulated' proteins have been identified in eucaryotic cells subjected to glucose starvation. The induction of stress in hybridoma cultures has been shown to have a positive effect on the specific rate of monoclonal antibody production. This has been demonstrated for hyperosmotic stress (Oyass *et al.*, 1989; Ozturk *et al.*, 1992), nutritional stress (Miller *et al.*, 1988; Linardos *et al.*, 1991) and environmental stress (Miller *et al.*, 1988). Linardos *et al.*, (1991) claim that the pattern of monoclonal antibody production in their experiments bears some important similarities with the production of stress proteins in mammalian cells, and that one of the glucose regulated stress proteins is identical to an immunoglobulin heavy chain binding phosphoprotein (BiP) which is found associated with Ig heavy and light chains in the endoplasmic reticulum of antibody secreting hybridoma cells (Knittler and Haas, 1992). However, an increase in the stress on a cell population results in a decrease in the growth rate. The effect of stress on monoclonal antibody production may therefore be indirect and the increase in production be due to the decreasing growth rate.

1.7.4 The Cell Cycle and Monoclonal Antibody Production

There are four successive phases of the cell cycle, M, G1, S and G2. The M phase consists of nuclear division (mitosis) and cytoplasmic division (cytokinesis) resulting in two daughter cells which begin the interphase of the next cell cycle. Interphase begins with the G1 phase, in which the cells, whose biosynthetic activities have been greatly slowed during

mitosis, resume a high rate of biosynthesis. The S phase begins when DNA synthesis starts, and ends when the DNA content of the nucleus has doubled and the chromosomes have replicated. The cell then enters the G2 phase, which ends when mitosis starts. The M phase begins with mitosis and ends with cytokinesis.

DNA synthesis in animal cells is confined to the S phase of the cell cycle while RNA synthesis is continuous throughout, except during mitosis when synthesis appears to stop. Protein synthesis also occurs continuously with only a reduction in the rate during mitosis and not a complete cut off.

The few reports that are available on the relationship between the cell cycle and monoclonal antibody synthesis suggest that the relationship is cell line dependent. Alshuler *et al.* (1986) reported the detection of intracellular antibody throughout the cell cycle in mouse hybridomas while Al-Rubeai *et al.* (1989) claimed production was G1 phase-specific. Suzuki and Ollis (1989) proposed a cell cycle model based on experimental evidence which showed that antibody production was restricted to the late G1 and S phases. The rate of monoclonal antibody production would therefore be related to the proportion of cells in those particular phases of the cell cycle which would in turn be affected by the growth rate. The model showed good correlation with the experimental data. However, Hayter *et al.* (1992) have shown for another hybridoma cell line that monoclonal antibody production is not restricted to the G1 and S phases. Linardos *et al.* (1991) suggest that high levels of environmental stress can lead to a higher percentage of cells in the G1 phase and therefore higher monoclonal antibody production rates based on reports by Suzuki *et al.* (1988, cited Linardos, 1991) that cells

arrested in G1 phase produce more antibody than cells in other phases of the cycle.

1.7.5 Control or Competition in Monoclonal Antibody Production

The observed profiles of growth and monoclonal antibody production in the batch culture of a large number of hybridomas demonstrating growth-dissociated production suggest that while growth of the cell is uninhibited by nutrient limitation or toxin accumulation, monoclonal antibody production occurs at a low level. Once growth becomes inhibited the rate of monoclonal antibody production increases until it too becomes inhibited by nutrient depletion, toxin accumulation or cell lysis. Such an observation may be the result of competition or control. These two concepts are discussed below.

a) Control

The production of monoclonal antibody may be under the careful control of the cell. During the exponential phase of the culture's growth the cell may be actively repressing the whole or a part of the monoclonal antibody production pathway and this repression ceases once the cell stops growing, or in a particular phase of the cell cycle, or when the cell experiences stressful conditions. However, hybridomas result from the fusion of two cells one of which supplies the capacity for infinite growth while the other supplies the genes necessary for monoclonal antibody production. As the resulting cell line has not undergone evolutionary pressure any form of global control exerted over monoclonal antibody production would occur by chance. It may be more feasible to look at growth and production in terms of competition rather than control.

b) Competition

The pathways of growth and antibody production may be competing for a common pool of energy, nutrients, precursors or enzymes, resulting in the most efficient pathway being favoured. If the two pathways of growth and production have a greater or lesser requirement for a limiting nutrient or a greater or lesser tolerance to a toxic catabolite then one pathway may continue after the other has been inhibited. Therefore, under optimum conditions for growth, monoclonal antibody production is out competed because growth is essential to the survival of the cell while antibody production is not. However as conditions change during batch culture they become suboptimal for growth and the monoclonal antibody production pathway becomes the more competitive. Thus monoclonal antibody production increases as growth decreases. This would account for the positive effect of stress on monoclonal antibody production, and could explain observed growth phase specific monoclonal antibody production where certain phases require less resources for growth processes making more available for monoclonal antibody production.

The true relationship between growth and monoclonal antibody production can best be studied in chemostat culture in which the growth rate of the cell can be controlled while its environment is kept constant. Two-stage chemostat culture is theoretically the ideal medium for studying the growth-dissociated kinetics of antibody production. The aim of this project was therefore to identify the kinetics of growth and production of the NB1 hybridoma cell line in batch culture and to assess the feasibility of using two-stage chemostat culture to study in greater depth the factors affecting the kinetics of antibody production. If stable steady states could be demonstrated in two-stage chemostat culture and

antibody production was shown to be growth-dissociated, the possibility of optimising production independently of growth in the second stage would be explored.

2.0 MATERIALS and METHODS

2.1 Cell Line

The cell line used in this study was the mouse hybridoma, NB1, derived from a fusion between mouse spleen cells primed by human red blood cell group B antigen and a mouse myeloma NS1 cell line (Sacks and Lennox, 1981). The antibody produced was an IgM which has agglutinating activity against the group B antigen of human red blood cells.

2.2 Cell Propagation

2.2.1 Storage and Recovery of Frozen Cell Stocks

Cells cultured in serum-supplemented or serum-free media were centrifuged (400g for 5 minutes) and resuspended in cell storage medium at a concentration of between 1 and 2×10^7 viable cells/ml. Cell storage medium consisted of DMEM supplemented with 20% (v/v) foetal calf serum and 10% (v/v) dimethyl sulphoxide (Sigma, D-5879). The cells were then dispensed into 2ml labelled freezing ampoules and placed at -70°C overnight before being transferred to liquid nitrogen, -196°C .

Recovery of cells from liquid nitrogen storage was achieved by rapidly thawing an ampoule and slowly diluting the cells to 10ml with growth medium. After centrifugation (400g for 5 minutes) the cells were resuspended in 10ml of fresh growth medium, transferred to a tissue culture flask, gassed with 5% carbon dioxide in air and incubated at 36.5°C .

2.2.2 Growth Medium

Cell stocks were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented either with 5% foetal calf serum (Imperial

Laboratories) or a serum-free supplement mix. The DMEM was supplied in powdered form by Gibco (Cat. No. 074-02100) and was made up following their recommendations after which it was sterilised by filtration through a 0.2 μm filter. DMEM was used in all batch and chemostat experiments performed unless otherwise stated. The formulation of DMEM is given in Appendix 1. Where specified, culture medium was supplemented with MEM amino acid solution (Appendix 2.), MEM Non-essential amino acid solution (Appendix 3.) and MEM Vitamin solution (Appendix 4.), all supplied by Gibco.

2.2.3 Cultivation of a Cell Stock

A 100ml stock of growing cells was maintained in a spinner culture consisting of a 250ml Duran containing a magnetic flea placed on a stirrer base and incubated in a 5% carbon dioxide air atmosphere at 36.5°C with a loosened cap to maintain the pH between 7.0 and 7.2. Diffusion of carbon dioxide into the bottles did not always adequately maintain the pH, in which case cultures were directly gassed with a 5% carbon dioxide in air mixture and the bottles sealed. The cells were diluted at intervals to maintain a concentration between 1.0 and 6.0 $\times 10^5$ viable cells/ml. Cells were removed from the stock culture to initiate expansion cultures.

2.2.4 Determination of Cell Concentration

Cell concentration was determined using a Fuchs-Rosenthal haemocytometer. The culture samples were first diluted in 0.04% w/v solution of erythrosin B stain (Sigma, E-7379) in saline, the dilution varying between 1:4 and 1:10 depending on the concentration of cells present. Dead cells stained red while live cells remained colourless. All counts were performed in duplicate. The standard deviation of all cell

counts was within $\pm 10\%$.

2.2.5 LH Fermentation 2000 Series Fermenter

Batch and chemostat experiments were performed using an LH 2000 series fermenter fitted with a two litre working volume glass vessel. Temperature was controlled at a set-point of 36.5°C by passing water through a finger from a thermostatically controlled supply. The culture was stirred at a rate of 60 rpm by a top driven agitator fitted with a single Rushton turbine impeller. Dissolved oxygen tension (DOT) was measured using an autoclavable polarographic oxygen electrode (Ingold). The DOT was controlled at 40% of air saturation by the sparging of air into the culture when required. pH was monitored by a pH electrode (Ingold) and controlled between setpoints of 7.0 - 7.2 by the addition of carbon dioxide or alkali (1M NaOH). Carbon dioxide was used to reduce the pH of the culture after it was discovered that 1M HCl resulted in death of the cells possibly due to leaching out of chromium ions from the stainless steel ports.

A single-stage chemostat was set up by using a peristaltic pump to feed medium from a refrigerated reservoir into the culture vessel. The culture volume was kept constant by pumping excess culture out through a weir in the culture vessel.

A two-stage chemostat consisted of two LH 2000 fermenters in series where the overflow from the first stage was fed into the second stage (Figure 2.1).

Figure 2.1: The configuration of a Two-Stage Chemostat

Medium feed rates were measured by a pipette fitted to the feed line via a 'T' piece connector.

2.3 Biochemical Analyses

During the course of a fermentation samples were removed from the culture and, after centrifugation to remove cells and cellular debris, were dispensed into 1.5ml vials and stored at -20°C. These samples were used for biochemical analyses and determination of antibody concentration.

2.3.1 Glucose Assay

Glucose analysis was carried out using a GM7 analyser (Analox Instruments Ltd.). The GM7 analyser functions by measuring the oxygen change when oxidoreductase enzymes (oxidases) react with their substrate under controlled semi-anaerobic conditions. Thus, for the glucose/glucose oxidase (GO) system:

Under appropriate conditions the resultant oxygen change is monitored by an oxygen sensor. The total change and the maximum rate of change are both proportional to the substrate concentration initially present. The analyser was calibrated with a 8 mM (144.1 mg/dl) standard and checked with a 300 and 500 mg/dl standard. A 10µl sample was used and each sample was assayed in duplicate with a standard deviation of no more than $\pm 10\%$. The method followed was as supplied by Analox Instruments Ltd (Analox Instruments Ltd Technical Note Glu/6.90).

2.3.2 L-Lactate Assay

Lactate analysis was carried out using a GM7 analyser, employing the same principles as for the glucose analysis. In this case L-lactate:Oxidoreductase (LOD) catalyses the oxidation of L-lactate to pyruvate:

LOD is highly specific for L-lactate and under appropriate conditions the maximum rate of oxygen consumption is directly related to lactate concentration. A 7µl sample was used and calibration was carried out with a 8.0 mM (72.1 mg/dl) lactate standard. Calibration is checked by a second standard of 40 mg/dl. Each sample was assayed in duplicate with a standard deviation of no more than $\pm 10\%$. The method followed was as supplied by Analox Instruments Ltd (Analox Instruments Ltd Technical Note LII/2.91).

2.3.3 Glutamine Assay

Glutamine analysis was carried out using the Boehringer Mannheim glutamic acid assay kit (Cat. No. 130 092). In the presence of the enzyme glutamate dehydrogenase (GIDH), L-glutamic acid (L-Glutamate) is deaminated oxidatively by nicotinamide-adenosine

dinucleotide (NAD) to α -ketoglutarate. In the reaction catalysed by diaphorase and the NADH formed converts iodonitro tetrazolium chloride (INT) to formazan which is measured in the visible range at 492 nm. This assays for L-glutamate. To assay for L-glutamine, asparaginase (Grade V Asparaginase. Sigma Cat. No. A4887) was added to the above reaction mixture, converting L-Glutamine into L-Glutamate resulting in the formation of more formazan and a subsequent increase in absorbance at 492 nm. The method followed was as supplied by the manufacturers (Boehringer Mannheim method sheet 691.150.248 932). Each sample was assayed in duplicate with a standard deviation of no more than $\pm 10\%$.

2.3.4 Ammonia Assay

Ammonia concentration was determined using a Sigma Diagnostics ammonia determination assay (Cat. No. 170-10). The method is based on the reductive amination of 2-oxoglutarate, using glutamate dehydrogenase (GLDH) and reduced nicotinamide adenine dinucleotide (NADH) as follows:

The decrease in absorbance at 340 nm, due to the oxidation of NADH, is proportional to the ammonia concentration. The method followed was as supplied by the manufacturers (Sigma Diagnostics Procedure No. 171-UV). Each sample was assayed in duplicate with a standard deviation of no more than $\pm 10\%$.

2.3.5 Amino Acid Analysis

The amino acid concentrations in culture supernatant were analysed using a Waters PICO.TAG Amino Acid Analyser system (Buzsàky, Zachrisson and Boork, 1993). This is an automated gradient liquid

chromatography system designed for analysis of amino acids.

The method involves two steps:

1. pre-column derivitisation of the sample
2. analysis by reverse phase high pressure liquid chromatography (HPLC).

In the first stage the samples were derivitised with phenylisothiocyanate (PITC) to produce phenylcabamyl (PTC) amino acids. In the second stage the amino acids were separated using a C18 column and their elution detected at 440nm. The procedure followed is given in Appendix 5.

2.4 Double-Antibody Sandwich ELISA for Estimation of Mouse IgM

The concentration of IgM monoclonal antibody in cell culture supernatant was determined by double-antibody sandwich ELISA. A standard protocol was followed (supplied by Celltech) which was optimised for the particular reagents used in our laboratory. The ELISA was optimised for antibody coating concentration, conjugate concentration, standard concentration and substrate incubation time (Catty and Raykundalia, 1988).

1. Coating Antibody: less than adequate coating of the solid phase will reduce the sensitivity of the ELISA and allow non-specific binding of the conjugate to the plastic. Over-coating may lead to non-specific trapping of the conjugate. The optimum concentration of coating antibody was therefore determined.
2. Antibody-Enzyme Conjugate: a conjugate should show a) high antibody titre and avidity so that the conjugate can be used at a high dilution to give minimal non-specific binding effects; b) specificity at a working dilution; c) low background readings; d) adequate enzyme activity. The conjugate antibody concentration was therefore optimised

in terms of working dilution, low background reading and specificity.

3. Standard Antigen Dilutions: the concentrations of standard antigen which gave a linear standard curve was determined.

4. Substrate Incubation Time: the optimal incubation time results in the greatest difference between positive and negative readings, and between positive readings of different concentrations. Keeping to the same incubation time also allows standardisation between tests. The ELISA protocol is given in Appendix 6.

2.5 Cell Cycle Analysis

Analysis of the percentage of a cell population in various stages of the cell cycle was performed on a Becton Dickson FACScan System. This is an automated cell analyser consisting of a bench-top flow cytometer and computer system. The system identifies and characterises cells as they pass through a focused laser beam one at a time. DNA cell cycle analysis was carried out using single colour fluorescence, performed on a single cell suspension stained with propidium iodide.

A cell suspension of approximately 1×10^6 cells/ml was fixed in 70/30 ethanol and PBS followed by centrifugation and resuspension in PBS containing DNase free RNase (Boehringer Mannheim, Cat. No. 1119) and propidium iodide (Sigma). All RNA present is digested while the DNA is stained by the propidium iodide. The amount of dye that binds is proportional to the amount of DNA present in the cell which is related to the stage of the cell cycle the cell has reached. The cell suspension was then analysed by the FACScan and the fluorescence measurements were accumulated to form a distribution of DNA content. The final results were analysed by the SFIT software program which calculates the percentage of the cell population present in the G1/G0, S,

and G2/M phases of the cell cycle.

3.0 KINETICS OF GROWTH, ANTIBODY PRODUCTION AND NUTRIENT UTILISATION IN BATCH CULTURE

3.1 SUMMARY

The kinetics of growth, antibody production, and nutrient utilisation demonstrated by the NB1 hybridoma cell line in batch culture were studied. A typical batch culture was compared with cultures fed with extra glutamine and supplemented with extra essential and non-essential amino acids. The effects of the various nutrient additions on growth and antibody production kinetics were studied. Different methods of analysing the data for specific growth rate and specific production rate were compared. Amino acid analysis was performed on samples from a typical batch culture and glutamine-fed batch culture. Specific rates of utilisation or production of the various amino acids were calculated. The distribution of cells in the cell cycle during the course of a typical batch culture was also studied. Glutamine was an essential nutrient for cell growth and monoclonal antibody production. Feeding a culture with extra glutamine and other essential and non-essential amino acids resulted in an increase in both cell growth and antibody produced. The majority of the antibody was produced during the growth phase of the culture and was related to the time integral of viable cell concentration (viable cell hours) in a growth-associated manner. Accumulation of the cell population in the G1/G0 phase of the cell cycle towards the end of the batch culture did not result in an increased rate of monoclonal antibody production.

3.2 INTRODUCTION

In batch culture of hybridoma cells, when the cells are inoculated into a medium containing all the nutrients essential for growth, in an environment optimised in terms of temperature, oxygen and pH, the cell population will grow and divide at a maximum rate until a nutrient essential for growth becomes depleted and/or a growth inhibitory waste

metabolite accumulates to a significant level. The cell population then enters a brief or prolonged stationary phase followed by a decline phase.

The various types of production kinetics of antibody in batch culture have been classified into three groups by Merten *et al.* (1987) as follows:

- 1) the highest specific production rate of antibody occurs at the beginning of the exponential growth phase.
- 2) the highest specific production rate of antibody occurs at the beginning of the exponential growth phase and during the stationary and decline phases.
- 3) a constant specific production rate of antibody is seen only in the exponential growth phase, with no production during stationary and decline phases.

Batch cultures of the NB1 hybridoma were carried out in serum-supplemented and serum-free media and the growth and monoclonal antibody production profiles determined. The effects of increased concentrations of various essential and non-essential amino acids on cell growth and production were also investigated. Specific rates of growth, monoclonal antibody production, nutrient utilisation and waste metabolite production were calculated.

3.3 AIM

The aim of this set of experiments was to ascertain the growth and production kinetics of the NB1 hybridoma cell line under batch culture conditions, and to compare different methods of analysing the data.

3.4 RESULTS AND DISCUSSION

A series of experiments was set up in order to study the effects of increases in glutamine concentration, and other essential and non-essential amino acids on growth and production in batch culture as follows:

1. Control; cells inoculated into DMEM plus serum.
2. Glutamine-Fed; 4mM of glutamine added towards the end of the exponential phase of growth.
3. Supplemented with 2X MEM amino acids and glutamine-fed.
4. Supplemented with 2X MEM amino acids and 1X MEM non-essential amino acids and glutamine-fed.
5. Supplemented with 2X MEM amino acids and 1X MEM non-essential amino acids without being glutamine-fed.

3.4.1 Typical Batch Culture

A typical unfed batch culture is shown in Figure 3.1. Increasing viable cell concentration was accompanied by decreasing glucose and glutamine concentrations and increasing lactate, ammonia and monoclonal antibody concentration. The point at which the culture entered the decline phase coincided with the point at which glutamine became depleted. After this point there was no further increase in viable or total cell concentration, glucose utilisation, lactate or ammonia production. Monoclonal antibody production occurred predominantly during the growth phase with 30% being produced during the decline phase.

Figure 3.1a Batch Culture of NB1 Cells. Profiles of cell concentration and antibody accumulation.

Figure 3.1b Batch Culture of NB1 Cells. Profiles of glutamine and glucose utilisation and ammonia and lactate production.

This result indicates that glutamine was essential for cell growth and the requirement for glutamine was not being met by another source.

The majority of the monoclonal antibody production occurred during the growth phase of the culture, typical of growth-associated production. However, production did not cease immediately the culture entered the decline phase but continued for a short while afterwards. This may have been due to production continuing through a brief stationary phase, or a delay between synthesis and secretion of the antibody, or simply that although the majority of antibody is produced during the growth phase of the culture, growth and production are not limited to the same extent by depletion of some nutrient (such as glutamine) or accumulation of a toxin. Dalili, Sayles, and Ollis (1990) observed that the time of exhaustion of glutamine coincided with the cessation of both cell growth and monoclonal antibody production up to a certain initial concentration of glutamine. When glutamine was present in the medium at a low concentration antibody production was growth-associated. When the initial concentration of glutamine was increased, glutamine was still present in the medium when the culture entered the decline phase and production continued in a growth-dissociated manner. They suggested that the presence of glutamine, rather than continued cell growth, was necessary for monoclonal antibody production.

3.4.2 Batch Culture With Glutamine Feed

To assess whether cessation of growth and production was due to glutamine depletion a batch culture of NB1 hybridomas was fed with extra glutamine. The results are shown in Figure 3.2. The extra glutamine was added as a single 'shot' during the batch culture (at the

end of 'exponential phase') to reduce the amount lost through spontaneous decomposition in the medium into pyrrolidone-carboxylic acid and ammonia (Tritsch and Moore, 1962). Rather than entering a rapid decline phase immediately after the growth phase as in the unfed culture, the viable cell population stabilised in a prolonged stationary phase. During the stationary phase the extra glutamine was utilised, as was glucose to a lesser extent. Monoclonal antibody production continued through the stationary phase in what appeared to be a growth-dissociated manner as the viable cell population was stationary. However, the increase in total cell concentration indicates that although the viable cell population was stationary, individual cells were still dividing.

Figure 3.2a Glutamine-Fed Batch Culture of NB1 Cells. Profiles of cell concentration and antibody accumulation.

Figure 3.2b Glutamine-Fed Batch Culture of NB1 Cells. Profiles of glutamine and glucose utilisation and ammonia and lactate production.

Monoclonal antibody production was therefore still associated with a growing viable cell population. This suggests that glutamine was essential for maintaining a viable cell population and that the continued production of monoclonal antibody depended on the presence of a viable cell population and/or on the presence of glutamine, and continued growth.

3.4.3 Amino Acid Supplemented Batch Culture

A batch culture of NB1 hybridoma cells was also carried out in DMEM.FCS supplemented with extra minimum essential medium (MEM) essential amino acids (Appendix 2) and non-essential amino acids (Appendix 3) and the effect on the kinetics of growth and production were observed.

When a batch culture was supplemented with extra MEM essential amino acids and fed with extra glutamine the cells entered a prolonged stationary phase, similar but on a larger scale to the glutamine-fed culture, during which a large amount of monoclonal antibody was produced (Figure 3.3).

Figure 3.3 Glutamine-Fed Batch Culture of NB1 Cells Supplemented with 2X MEM Amino Acids. Profiles of cell concentration and antibody accumulation.

Once again the total cell concentration was increasing even though the viable cell population was stationary or decreasing. Monoclonal antibody production therefore appeared to be associated with the viable cell population once again.

When extra MEM essential and non-essential amino acids were present in a culture which was fed with glutamine, an increased maximum viable cell concentration was observed compared with the control and

non-supplemented glutamine-fed cultures, as was an increased maximum monoclonal antibody concentration (Figure 3.4).

Figure 3.4 Glutamine-Fed Batch Culture of NB1 Cells Supplemented with 2X MEM and 1X Non-Essential Amino Acids. Profiles of cell concentration and antibody accumulation.

A brief stationary phase was observed followed by a decline phase. However, although glutamine was depleted by the end of the culture, the start of the decline phase did not coincide with glutamine depletion and may have been associated with depletion of another nutrient or accumulation of an inhibitory waste metabolite. Although there was some monoclonal antibody production during the decline phase, the majority was produced during the stationary phase.

When extra essential and non-essential amino acids were present in a culture which was not fed with extra glutamine (Figure 3.5), the

maximum viable cell concentration was slightly less than the control while total monoclonal antibody production was slightly greater. Once again growth and production ceased when glutamine became exhausted.

Figure 3.5 Batch Culture of NB1 Cells Supplemented with 2X MEM and 1X Non-Essential Amino Acids. Profiles of cell concentration and antibody accumulation.

Similar results to these have been obtained by Luan *et al.* (1987) who extended the stationary phase of cell growth for several days, and thereby increased the final monoclonal antibody concentration, by adding essential amino acids, glutamine and vitamins. However, in the control culture growth was limited by an essential amino acid other than glutamine. Similar results have also been reported by Duval *et al.* (1989 and 1991)

3.4.4 Growth Kinetics

Before discussing nutrient utilisation and monoclonal antibody production kinetics in batch culture it is important to have a clear understanding of what is meant by growth and how rates of growth are calculated. Growth, according to the Concise Oxford Dictionary, is "increase in size or value", this is described in biological terms by Brock *et al.* (1984) as "an orderly increase in all the cellular constituents and structures of an organism". In most microorganisms and animal cell cultures this increase continues until the cell divides into two new cells.

An important distinction must be made between the growth of individual cells and the growth of populations of cells. The growth of a cell is an increase in its size and weight and is usually a prelude to cell division. Population growth, on the other hand, is an increase in the number of cells as a consequence of cell growth and division. Because of the difficulty in studying the growth of individual cells quantitatively almost all growth and production studies of hybridomas are population studies. For this reason Type 2 production kinetics described by Merten *et al.* (1987) may appear to be growth-dissociated because monoclonal antibody production occurs principally during the lag and stationary phases of batch culture when the viable cell population is not growing but individual cells may be. This would be demonstrated by an increasing total cell population provided that the rate of increase in total cell concentration was greater than the rate of lysis of dead cells. Type 3 production kinetics are generally referred to as growth-associated because most of the monoclonal antibody is produced during the exponential phase of the culture and production ceases when population growth ceases due to nutrient depletion or metabolite inhibition.

To determine the effect of growth rate on nutrient utilisation and

monoclonal antibody production it is first necessary to assess how growth rate is defined. Most of the following equations are taken from Pirt (1975).

In batch culture of hybridoma cells, when the cells are inoculated into a medium containing all the nutrients essential for growth, in an environment that is permissive for growth, the cell population will grow and divide at a maximum rate until a nutrient essential for growth becomes depleted and/or a growth inhibitory waste metabolite accumulates to a significant level. The cell population then enters a brief or prolonged stationary phase followed by a decline phase.

The basic measure of growth rate is the specific growth rate (μ) which states that the increase in biomass (dx) is proportional to the amount present (x) and to the time interval (dt), provided that the time interval is infinitely small:

$$\mu = dx/dt \cdot 1/x \quad (\text{equation 3.1})$$

In reality it is very difficult to perform an experiment in which samples are taken during an infinitely small time period, making this equation difficult to apply. Alternatively, an approximation to μ can be obtained by replacing d with delta, however this assumes linear growth between two time points and introduces inaccuracies into the calculation. When μ is constant, integration and rearrangement of equation 3.1 gives:

$$\mu = (\ln x_t - \ln x_0)/t \quad (\text{equation 3.2})$$

where x_t is biomass concentration at time (t), and x_0 is biomass

concentration at time (0).

This allows the calculation of specific growth rate between two time points, assuming exponential growth. A plot of $\ln x$ against time should therefore give a straight line with slope μ . The validity of these assumptions was tested against the unfed (Figure 3.6) and glutamine-fed (Figure 3.7) batch cultures as shown below.

Figure 3.6a Batch Culture of NB1 Cells. $\ln x$ (viable cells) against Time with a slope of μ .

Figure 3.6a demonstrates that during the growth phase of the batch culture a constant exponential growth rate of 0.039h^{-1} was observed, as indicated by the high correlation between $\ln x$ and time.

In order to test whether the value for μ accurately described the growth rate of the population, the derived viable cell concentration (calculated

from the known concentration of viable cells at the beginning of the culture and the calculated growth rate from Equation 3.2) and the actual concentration of viable cells were compared (Figure 3.6b).

Figure 3.6b Batch Culture of NB1 Cells. Observed and calculated values for viable cell concentration.

Up to 35 hours the derived growth is an accurate reflection of the actual growth, while between 35 and 48 hours the growth is underestimated and overestimated between 48 and 60 hours. Use of the growth equation is obviously not applicable to the decline phase of the culture because cell death is not necessarily exponential.

In the glutamine-fed culture (Figure 3.7), equation 3.2 was once again shown to accurately derive the growth of the viable cell population during the exponential phase, but was not applicable to the stationary and decline phases as neither demonstrate exponential growth or death.

Figure 3.7a Glutamine-Fed Batch Culture of NB1 Cells. $\ln X$ (viable cells) against Time with a slope of μ .

Figure 3.7b Glutamine-Fed Batch Culture of NB1 Cells. Observed and calculated values for viable cell concentration.

The validity of this method of calculating specific growth rate may be questioned when a culture is not growing at a constant exponential rate, but is also not a true reflection of the specific growth rate of a culture which is less than 100% viable. This is demonstrated most noticeably by the glutamine-fed batch culture (Figure 3.7) when during the 'stationary' phase the viable cell population is not increasing and therefore is calculated as a negative growth rate, but the total cell population is still increasing significantly. For a more accurate estimation of specific growth rate equations 3.1 and 3.2 are modified to allow for the generation of viable and non-viable cells from the viable biomass (Pirt, 1975) as follows:

$$\mu = dx_t/dt \cdot 1/x_v \quad (\text{Equation 3.3})$$

$$\mu = (\ln (x_{tt} - x_{t0} + x_{v0}) - \ln x_{v0})/t \quad (\text{Equation 3.4})$$

Therefore the change in total biomass ($x_{tt} - x_{t0}$) between time 0 and time t results from the viable cell biomass present at time 0 (x_{v0}). A plot of $\ln (x_{tt} - x_{t0} + x_{v0})$ against time should therefore have a slope of μ .

Equation 3.4 was tested against the data from the unfed (Figure 3.8) and glutamine-fed (Figure 3.9) batch cultures.

Equation 3.4 was derived from Equation 3.3 assuming that there was no change in the number of non-viable cells, between t_0 and t_t , or in the death rate constant.

Figure 3.8a Batch Culture of NB1 Cells. $\ln(X_{tt}-X_{t0}+X_{v0})$ against Time with a slope of μ , accounting for the generation of viable and non-viable cells.

Figure 3.8b Batch Culture of NB1 Cells. Observed and calculated total cell concentration.

The validity of equation 3.4 is demonstrated by the high correlation exhibited in Figure 3.8a, and the closeness of fit between observed and calculated total cell concentrations shown in Figure 3.8b. However, even this calculated growth rate slightly underestimates the cell concentration at 48 hours. The deviation between the calculated and observed cell concentrations may be accounted for by error in cell counts which are typically $\pm 10\%$.

Figure 3.9a Glutamine-Fed Batch Culture of NB1 Cells. $\ln(X_{tt} - X_{t0} + X_{v0})$ against Time with a slope of μ , accounting for the generation of viable and non-viable cells.

Figure 3.9b Glutamine-Fed Batch Culture of NB1 Cells. Observed and calculated total cell concentration.

Figure 3.9 demonstrates the validity of the application of equation 3.4 to a culture with an extended stationary phase, as in the glutamine-fed batch culture.

A disadvantage of the above method of calculating growth rate is that by taking logarithms small deviations are eliminated and a straight line is forced where a straight line does not necessarily exist. This method is therefore insensitive to small fluctuations in growth rate during the exponential phase as well as the point at which the cell population enters the stationary phase. Equation 3.3 is by its nature responsive to fluctuations in growth rate but is not particularly practical. Its application may be made practical however by fitting a curve to the data points and applying the equation to the curve. This has been achieved by employing a partial cubic spline computer program (Oner *et al.*, 1986,

Bushell *et al.*, 1993, McDermot *et al.*, 1993). The program fits an equation through three points, i.e. 1, 2, and 3. It then fits an equation through points 2, 3, and 4 and so on through all the points. As the curve fitting proceeds the slopes are calculated in order to yield the rates of change by applying equation 3.1. This method allows the estimation of both volumetric and specific rates of growth. Figure 3.10 shows the curve of specific growth rate obtained by this method for unfed and glutamine-fed batch cultures applying equation 3.3 to the partial cubic spline curve (95% fit).

Figure 3.10a. Batch Culture of NB1 Hybridoma Cells.
Specific growth rate calculated by partial cubic spline method

Figure 3.10b. Glutamine-Fed Batch Culture of NB1 Hybridoma Cells.
Specific growth rate calculated by partial cubic spline method

As would be expected, maximum growth rate calculated in this way is higher than maximum growth rate calculated using Equation 3.4, as it is much more sensitive to fluctuations in the growth rate as shown in the Table 3.1.

Table 3.1

Comparison of μ max Calculated Using Partial Cubic Spline and Equation 3.4		
Batch Culture	Partial Cubic Spline	Equation 3.4
Unfed	0.050	0.040
Gln Fed	0.050	0.040
Essential Amino Acids and Gln Fed	0.055	0.035
Essential and Non Essential Amino Acids	0.060	0.036
Essential and Non-Essential Amino Acids and Gln Fed	0.060	0.039

3.4.5 Kinetics of Monoclonal Antibody Production

From the graphs shown above (Figures 3.1a and 3.1b) it is apparent that the majority of the monoclonal antibody is produced during the exponential phase of cell growth, and once glutamine becomes depleted growth ceases, shortly followed by production. In a culture fed with extra glutamine at the end of the exponential phase, growth and production continued during the stationary phase. The association between growth and antibody production would therefore not appear to

be strictly growth-associated, nor necessarily growth-dissociated. The characteristics of production may in fact be dependent on the supply of glutamine in the medium. Whether production can occur in the absence of growth can only be tested in a non-growing culture in the presence of all the nutrients necessary for production.

A theory has been proposed that monoclonal antibody is not actively secreted by viable cells but stored in vesicles and released by dead cells in some cell lines (Al-Rubeai, Rookes and Emery, 1989). If this were the case for this cell line, monoclonal antibody production would not be expected to cease shortly after the cells entered the decline phase in the unfed culture. It is therefore likely that in the glutamine-fed culture monoclonal antibody is being actively secreted from the viable cell population rather than being released from dead cells breaking up.

Renard *et al.* (1988) related monoclonal antibody production kinetics to the time integral of viable cell concentration in batch culture to provide evidence that monoclonal antibody is secreted by viable cells rather than released by dead cells. If their hypothesis is correct then the titre of antibody in the supernatant at any time should be proportional to the time integral of viable cell concentration. Thus, the production curve and the integral curve should show analogous profiles and regression of one over the other should be linear. This hypothesis has been tested against these data and the results are shown in Figure 3.11. The results show that this holds true for the glutamine-fed and unfed cultures.

Figure 3.11 The relationship between the time integral of viable cell concentration and antibody concentration in batch culture of NB1 cells.

Evidence for the growth-associated nature of monoclonal antibody production is obtained from a partial cubic spline plot of volumetric antibody production and volumetric viable cell production showing that peaks in the two curves coincide (Figure 3.12). The peaks are displaced in the glutamine-fed batch culture because growth of viable cells in the stationary phase is not accounted for.

Figure 3.12a. Batch Culture of NB1 Hybridoma Cells
Volumetric Viable Cell and Antibody Production Rates

Figure 3.12b. Glutamine-Fed Batch Culture
Volumetric Viable Cell and Antibody Production Rates

3.4.6 Specific Production Rate of Monoclonal Antibody

The specific rate of product formation during an infinitely small time interval (dt) in batch culture is given by:

$$q_p = dp/dt \cdot 1/x_v \quad (\text{Equation 3.5})$$

If q_p is constant it may be calculated from the following equation (Pirt 1975):

$$q_p = p - p_0 / x_0(e^{\mu t_1} - 1) / \mu \quad (\text{Equation 3.6})$$

This may be simply calculated from the slope of the curve when the time integral of viable cells is plotted against antibody production, as in Figure 3.11.

q_p was therefore calculated by applying equation 3.5 to a partial cubic spline plot of the viable cell and product data, and by calculating the slope of the curve from Figure 3.11. The results for the glutamine-fed and unfed batch cultures are shown in Figure 3.13 and Table 3.2 and 3.3.

Figure 3.13 is a plot of the specific growth rate and specific antibody production rate for the glutamine-fed and unfed batch culture. Table 3.2 gives the data for specific antibody production rate calculated from the slope of the curve of viable cell hours against antibody concentration shown in Figure 3.11 for the glutamine-fed and unfed batch culture. Table 3.3 lists the maximum specific rates of monoclonal antibody production in all of the variously supplemented and fed batch cultures calculated using the partial cubic spline and the time integral of viable

cells against antibody concentration methods.

Table 3.2

Specific Antibody Production Rate in Glutamine-Fed and Unfed Batch Culture, Calculated From The Slope Of The Viable Cell Hours Against Antibody Curve		
Culture Time (hours)	Specific Antibody Production Rate (ug/10 ⁶ viable cells/h)	
	Unfed	Glutamine-Fed
0 - 22.5	0.56	0.55
22.5 - 34.5	0.74	0.96
34.5 - 47.5	0.76	0.63
47.5 - 58.5	0.28	0.31
58.5 - 71.5	0.59	0.71
71.5 - 83.0	-0.08	0.49
83.0 - 98.5	0.53	0.88
98.5 - 123.0	1.50	0.57
123.0 - 146.0	-6.61	0.45

The results for specific antibody production rate calculated by the two different methods using Equation 3.5 and Equation 3.6, are shown in Table 3.2 and Figure 3.13. Both methods show similar general trends in the rate of antibody production during the course of the batch culture. One of the benefits in using the partial cubic spline method is that it plots the trend in the data, rather than forcing a curve through each point. It is also possible to state the percentage fit of the line to the data points, thereby compensating for the normal error experienced in assays

such as an ELISA. Undue significance is therefore not placed on a particular data point in isolation, but the trend between several points is considered simultaneously. Equation 3.6 is only valid when production rate is constant between two time points. It does not allow for any standard deviation around a data point and does not consider any underlying trend. Therefore, calculated rates of production can fluctuate wildly, as for the unfed batch culture after 58.5 hours.

Figure 3.13a. Batch Culture of NB1 Hybridoma Cells
Specific Rates of Growth and Antibody Production

Figure 3.13b. Glutamine-Fed Batch Culture of NB1 Hybridoma Cells
Specific Rates of Growth and Antibody Production

In unfed batch culture the peaks in specific growth rate and specific antibody production rate coincide, providing further evidence for the growth-associated nature of antibody production. The glutamine-fed batch culture shows two peaks in specific production, the first coincided with the peak in specific growth rate while the second occurred during the decreased growth rate of the prolonged stationary phase. This double peak in specific production rate was observed in all glutamine-fed batch cultures and suggests that monoclonal antibody production is not always strictly growth-associated.

Table 3.3

Maximum Specific Rate of Monoclonal Antibody Production in Batch Culture. Comparison Between Partial Cubic Spline And The Slope Of The Time Integral Of Viable Cells Against Antibody Curve				
Batch Culture Conditions	Exponential Phase		Stationary Phase	
	Partial Cubic Spline	Cell Hours Against Antibody	Partial Cubic Spline	Cell Hours Against Antibody
Unfed	0.81	0.76	No Stationary Phase	
Gln Fed	0.84	0.96	0.72	0.88
Gln Fed and Essential Amino Acid	1.00	0.99	0.90	1.20
Essential and Non Essential Amino Acids	1.41	1.22	1.41	1.22
Gln Fed, Essential and Non-Essential Amino Acid	1.14	0.94	1.15	1.14

Table 3.3 compares the maximum specific rates of antibody production in batch culture calculated using Equation 3.5 and Equation 3.6. Similar

conclusions may be drawn as for the comparison between results presented in Table 3.2 and Figure 3.13. The partial cubic spline method of calculating specific production rate, by plotting the trend with a percentage fit to the data points, gives values for specific rates of production which may differ from specific production rates calculated by applying Equation 3.6. However, the extent of the difference between calculated values, in the majority of cases is less than ten percent of the mean..

From the results presented for specific growth rate (Table 3.1) and specific production rate, calculated using partial cubic spline, (Table 3.3) it would appear that in batch culture, an increasing specific growth rate resulted in an increasing specific production rate during the exponential phase of growth. This is typical of growth-associated product formation. However when the growth rate fell during the stationary phase a corresponding fall in specific production rate was not observed suggesting that the increased specific production rate was possibly due to the increased concentration of amino acids rather than the increased growth rate.

3.3.7 Amino Acid Utilisation

Amino acid analysis of the unfed batch culture and the glutamine-fed batch culture revealed that amino acids can be classified into three groups; a) those which are produced; b) those which are alternately consumed and produced; c) those which are consistently consumed by the cells. The classification of individual amino acids is shown in the Table 3.4.

Table 3.4

Classification of Amino Acids in Terms of Consumption and Production in Batch Culture		
Consumed Only	Produced Only	Consumed and Produced
ARG CYS GLN HIS ILE LEU LYS MET PHE THR TRP VAL	ALA* PRO*	ASN* ASP* GLU* GLY SER TYR

*Amino Acids not present in DMEM

Similar classifications have been arrived at by other workers (Griffiths and Pirt, 1967; Duval *et al.*, 1989; Duval *et al.*, 1991), however the distribution of amino acids between the three groups appears to differ for each cell line.

In the unfed batch culture glutamine was the only amino acid which was totally exhausted from the medium, while in the glutamine-fed culture methionine and cystine were also exhausted and histidine, arginine and tryptophan were depleted to low levels (data shown in Appendix 7).

3.3.8 Specific Rates of Amino Acid Utilisation

Volumetric and specific rates of utilisation of amino acids were plotted using the partial cubic spline program (Figure 3.14). The peak in

volumetric utilisation rate coincided with the peak in growth rate. The specific utilisation rate was high at the beginning of the culture and gradually decreased as the batch culture proceeded. In the glutamine-fed batch culture, the volumetric utilisation rate peaked twice. The first peak coincided with the peak in growth rate, while the second coincided with the stationary phase of the culture. The specific utilisation rate began high and gradually decreased as the batch culture proceeded, levelling off to an almost constant rate during the stationary phase. This general trend was observed for all amino acids which were consumed by the cell and is shown for valine in Figure 3.14. A high specific uptake rate of amino acids during the initial phase of growth has been observed before (Griffiths and Pirt, 1967). This correlates well with the peak in specific rates of glucose uptake observed in this study, and protein, RNA and DNA synthesis observed by other workers (King *et al.* 1959; Ruekert and Mueller 1959; Salzman 1959; and Swaffield and Foley 1960).

The maximum specific utilisation rates of amino acids in unfed and glutamine-fed batch culture are shown in Table 3.5.

Figure 3.14a. Unfed Batch Culture of NB1 Hybridoma Cells
Volumetric and Specific Rates of Valine Utilisation

Figure 3.14b. Glutamine-Fed Batch Culture of NB1 Hybridoma Cells
Volumetric and Specific Rates of Valine Utilisation

Table 3.5

Maximum Specific Utilisation Rates Of Amino Acids In Non Glutamine-Fed And Glutamine-Fed Batch Culture			
Amino Acid	Non GLN Fed ($\mu\text{moles}/10^6$ viable cells/h) Growth Phase	GLN Fed ($\mu\text{moles}/10^6$ viable cells/h)	
		Growth Phase	Stationary Phase
SER	0.0025	0.0016	0.0000
GLN	0.1000	0.1100	0.0310
GLY	0.0045	0.0070	0.0000
HIS	0.0020	0.0010	0.0009
ARG	0.0045	0.0026	0.0022
THR	0.0076	0.0076	0.0034
ALA	-0.0250	-0.0280	-0.0100
PRO	-0.0050	-0.0060	-0.0040
TYR	0.0021	0.0066	0.0013
VAL	0.0100	0.0078	0.0026
MET	0.0030	0.0024	0.0015
CYS	0.0024	0.0010	0.0004
ILE	0.0110	0.0060	0.0030
LEU	0.0130	0.0087	0.0029
PHE	0.0037	0.0024	0.0022
TRP	0.0030	0.0009	0.0006
LYS	0.0090	0.0072	0.0024

The rate of utilisation of glutamine was approximately 10-50 times the rate for other amino acids. This is typical for cultures of mammalian cells (for example: Mohberg and Johnson, 1963; Griffiths and Pirt, 1967; Blaker et al., 1971; Lambert and Pirt, 1975). The reason for this high rate of utilisation is related to the use of glutamine as an energy source, as demonstrated by Donnelly and Scheffler (1976) and Zielke *et al.* (1978). The branched chain amino acids, leucine, isoleucine and valine, also disappeared from the medium at relatively high rates, but at much slower rates than glutamine. These amino acids, along with others, as well as being incorporated into protein, can also function as energy sources besides glutamine.

3.3.9 Glucose Utilisation.

Glucose utilisation followed the same general trend as amino acid utilisation (data not shown). Volumetric utilisation rate peaked during the growth phase of the culture while specific utilisation was initially high but decreased as the culture progressed. The trend in glucose utilisation was mirrored in the trend for lactate production (data not shown).

There was no obvious change in the rate of glucose utilisation when glutamine became exhausted to suggest that it could be used as an alternative energy source as has been demonstrated for some cell lines (Zielke *et al.*, 1978). However, glucose may have been able to replace glutamine as an energy source but not as a precursor in protein synthesis, therefore growth ceased not due to the lack of an energy source but due to a lack of an essential protein precursor. Further experiments are necessary in order to determine this.

3.3.10 Metabolite Production: Alanine, Proline, Ammonia and Lactate

Alanine, proline and ammonia were consistently produced during the course of a batch culture. These waste metabolites are all end products of amino acid transamination (Stryer, 1981). Alanine has also been shown to derive from glucose metabolism in L929 cells (Lanks, 1987).

A proportion of amino acids utilised by the cell which are not used for the synthesis of proteins and other biomolecules are used as metabolic fuel. The α -amino group is removed and the carbon skeleton is converted into acetyl CoA, acetoacetyl CoA, pyruvate, or one of the intermediates of the citric acid cycle. The α -amino group of many amino acids is transferred to α -ketoglutarate to form glutamate, which

is then oxidatively deaminated to yield ammonia. The transient appearance of glutamate during the exponential phase of batch culture when amino acid utilisation is at its highest rate was therefore not surprising.

Glutamate is involved in the synthesis pathways of all the other amino acids which were not originally present in DMEM but were produced during a batch culture. Alanine is synthesised from pyruvate and glutamate, while aspartate is synthesised from oxaloacetate and glutamate which can then lead on to the synthesis of asparagine. Glutamate is also the precursor of proline. It is therefore likely that the appearance of these amino acids simply reflects the transamination of other amino acids. Utilisation rates and requirements for such amino acids are difficult to determine since it is not known to what extent they are produced and utilised by the cell without ever appearing in the medium.

Tyrosine can be produced simply by the hydroxylation of phenylalanine or by a more complex pathway involving chorismate, an intermediate in the biosynthesis of phenylalanine, tyrosine and tryptophan. Serine is synthesised from 3-phosphoglycerate, an intermediate in glycolysis, and acts as a precursor in the synthesis of glycine. These three amino acids were both consumed and produced in batch culture.

Ammonia concentrations in the range of 2.0 - 5.0 mM were observed at the end of batch cultures supplemented with extra glutamine and other amino acids. This is typical for batch culture of hybridoma cells (Ozturk *et al.*, 1992). However, the effect of such concentrations of ammonia does vary between cell lines in general and between hybridoma cell lines

in particular. Several studies have shown that ammonia inhibits cell growth, however the sensitivity of the growth rate to ammonia concentration varied among the cell lines used. The ammonia concentration required to inhibit cell growth rate by 50% of the maximum is in the range of 2 to 10 mM (Reuveny *et al.*, 1987; Thorpe, 1989; Miller *et al.*, 1988; Truskey *et al.*, 1990; McQueen and Bailey, 1990; Ozturk *et al.*, 1992). Reports on the effect on specific antibody production rates suggest that there is no change with increasing ammonia concentration (McQueen and Bailey, 1990; Ozturk *et al.*, 1992).

Studies with this cell line have shown that ammonia concentrations up to 5.8 mM reduced the maximum viable cell concentration by 17% while the growth rate was reduced by 25% (Boraston, 1990). The effect on the specific production rate of antibody was not reported.

Lactate is mainly produced from glucose metabolism but can also be produced in small amounts from glutamine (Zielke *et al.*, 1990). In this set of batch experiments final lactate concentrations between 19 and 26 mM were observed, which, from the data of Boraston (1990), translates into a possible 20% decrease in maximum viable cell concentration and a 25% decrease in growth rate. However, as these concentrations were only observed towards the end of the decline phase, any negative effect during the growth phase was likely to be small. Studies on other hybridoma cell lines have revealed no inhibition at concentrations up to 40 mM lactate (Thorpe, 1988; Miller *et al.*, 1988), stimulation of growth at concentrations up to 22 mM, followed by inhibition above 28 mM (Reuveny *et al.*, 1986). Ozturk *et al.*, (1992) observed an increasing specific rate of antibody production with increasing lactate

concentration, however this was ascribed to an increasing osmolarity of the medium.

3.3.11 Cell Cycle Analysis

The cell cycle through a typical unfed batch culture was analysed, the results are shown in Table 3.6.

Table 3.6

Cell Cycle Distribution During The Course Of A Typical Batch Culture				
Culture Age (hours)	Culture Viability (%)	% Of Cells In Stages Of The Cell Cycle		
		G1/G0	S	G2/M
0	95	33.95	41.68	24.66
45	92	36.08	47.93	12.98
66	93	48.17	41.87	10.40
78	85	48.88	37.52	14.11
89	51	68.33	24.85	7.40
101	21	81.74	14.07	2.73

During the exponential growth phase the percentage of cells in G1/G0 phase of the cell cycle increased. The increase in G1/G0 was offset by a decreasing percentage of cells in the S phase and G2/M phases. Cell cycle analysis of hybridoma cell lines by other workers is in agreement with this general observation of cells accumulating in the G1/G0 phases of the cell cycle as the culture ages (Schliermann *et al.*; Linardos *et al.* 1991). In many cell lines of lymphoid origin the production of antibody appears to be restricted to the G1 and S phases of the cell growth cycle (Byars and Kidson, 1970; Buell and Fahey, 1969; Takahashi *et al.*, 1969; Garatun-Tjeldsto *et al.*, 1976; Turner *et al.*, 1985). However, with this cell line, cells accumulated in the G1/G0 phases of the cell cycle during

the decline phase of the culture but with no increase in the specific rate of antibody production. Studies by Hayter *et al.* (1992) demonstrated that antibody production was not restricted to a particular cell cycle phase for their cell line. There is obviously therefore no simple relationship between specific antibody production rate and cell cycle with all hybridoma cell lines. It is possible in this case that increased specific antibody production does occur during the G1 phase of the cell cycle, but that under the nutrient depleted conditions experienced during the decline phase, the nutrients required for antibody production are limiting. As glutamine appears to be essential for both growth and antibody production, and the batch culture was glutamine depleted, then this is a distinct possibility.

3.5 CONCLUSIONS

- Glutamine is an essential nutrient for both growth and monoclonal antibody production. When glutamine runs out growth ceases, shortly followed by production.
- Death of a cell population in DMEM.FCS is due to nutrient depletion, not inhibitor accumulation, demonstrated by continued growth when extra nutrients were added. However the drop in viability during extended stationary phases may be due to stress or inhibitor accumulation.
- Growth and production can be extended and increased by supplementing and feeding the culture with glutamine, essential and non-essential amino acids.

- Monoclonal antibody appears to be produced by the viable cell population with no evidence of large scale release by lysing cells. Peaks in specific growth rate and specific production rate coincide suggesting growth-associated production, however, production does continue into the decline phase of batch culture but at a reduced rate.
- Accumulation of the cell population in the G1/G0 phase of the cell cycle does not result in increased monoclonal antibody production under glutamine depleted conditions.

4.0 SINGLE-STAGE CHEMOSTAT CULTURE

The results reported in this chapter are the results of the first stage of a two-stage chemostat experiment reported in Chapter 5.

4.1 SUMMARY

Single-stage chemostat culture of the NB1 hybridoma cell line was performed. The effects of various dilution rates on specific growth rate, cell concentration, specific production rate of monoclonal antibody, and specific rate of nutrient utilisation and waste metabolite production were studied. Steady states were maintained at various dilution rates over a number of volume changes. Monoclonal antibody production was negatively growth rate associated and specific rates of amino acid utilisation decreased with decreasing dilution rate along with a decreasing viable cell concentration and viability.

4.2 INTRODUCTION

Chemostat culture has been extensively used in the study and exploitation of microbial processes, however its use in animal cell culture has, until recently, been limited.

The theory of chemostat culture was first established in 1950 by Monod and at the same time, independently, by Novick and Szilard. A chemostat culture is established by feeding a culture, initiated as a batch culture, with medium in which a single substrate, or inhibitory catabolite is at a concentration which will limit growth. The flow of medium into the vessel is equal to the flow of medium and culture out, thereby maintaining a constant volume with a dilution rate, D , of medium feed rate, F , divided by culture volume, V :

$$D = F/V$$

Equation 4.1

A self regulating steady state is established during which the specific growth rate of the culture is equal to the dilution rate provided that the

culture is 100% viable:

$$\mu = D \quad \text{Equation 4.2}$$

This occurs because growth of the culture is reliant on the supply of the growth-limiting substrate, which is determined by the dilution rate. When a culture is 100% viable the growth rate is equal to the dilution rate, however, if a culture is not 100% viable then the specific growth rate (μ) exceeds the dilution rate (because the viable cell population generates the non-viable as well as the viable cells) according to the equation:

$$\mu = D/\beta \quad \text{Equation 4.3}$$

where D is the dilution rate and β is the fraction of the culture which is viable (Pirt 1975).

In chemostat culture under steady state conditions specific rates of substrate utilisation (q_s) are estimated according to the equation:

$$q_s = D.(S_r - S)/x \quad \text{Equation 4.4}$$

where D is the dilution rate, S is the concentration of substrate in the culture, S_r is the concentration in the medium feed (for example, amino acid concentration) and x is the viable cell concentration.

Specific rates of product synthesis (q_p) are calculated according to the equation:

$$q_p = D.(p - p_r)/x \quad \text{Equation 4.5}$$

where p is the product concentration in the culture and p_r is the concentration in the medium feed (zero in the case of antibody). The productivity of a chemostat can be determined by calculating volumetric production rate according to the following equation:

$$\text{volumetric production} = D.(p - p_r) \quad \text{Equation 4.6}$$

The first published account of an attempt to cultivate animal cells in chemostat culture did not appear until 1958 (Cooper *et al.*, 1958). An embryo rabbit kidney (ERK) cell line was used but steady states were not observed and the system was not self balancing so that if the cells overgrew they then entered a stationary phase and were washed out. However the chemostat achieved its desired purpose of providing a continuous supply of cells for agar-suspension plaque assays and suspension growth of poliovirus in a more efficient and less troublesome way than batch culture. The same authors reported their attempts at continuous flow culture of animal cells in more detail in another paper (Cooper *et al.*, 1959) in which they combined the principles of chemostat culture with turbidostat culture as a safety device to overcome the problems of irreversible fluctuations in the steady states. A successful attempt at maintaining steady states in chemostat culture of animal cells was reported by Cohen and Eagle (1961) with HeLa cells. However, steady states were only observed at high dilution rates. Low dilution rates resulted in a cyclic variation in cell concentration and the growth-limiting factor was not identified. Pirt and Callow (1964) made the first successful attempt to identify the growth-limiting nutrient in their chemostat cultures of L cells and ERK cells. They were also able

to study the effect of pH on steady state cell concentration and on the specific utilisation rate of glucose. Moser and Vecchio (1967) were able to obtain stable steady states at low dilution rates using mouse ascites mast cells in chemostat cultures. However, long adjustment periods were necessary before oscillations were totally eliminated. The growth-limiting nutrient was not identified. Griffiths and Pirt (1967) used chemostat culture in their study of the uptake of amino acids by mouse LS cells. They were able to demonstrate steady and reproducible metabolic states, and show for the first time a relationship between cell growth rate and nutrient requirements. Other workers also reported the successful cultivation of animal cells in chemostat culture around this time (Holmstrom, 1968; Kilburn and Van Wezel, 1970; Tovey *et al.*, 1973; Tovey *et al.*, 1975). The first detailed analysis of the chemostat culture of animal cells was reported by Tovey and Brouty-Boye (1976). Reproducible and stable steady-state cultures of mouse L 1210 cells over a wide range of growth rates were obtained with glucose as the growth-limiting substrate. The problems encountered previously, such as failure to obtain steady states, cyclic variations in cell number at low dilution rates, and wide variations between different cultures at the same dilution rate, were overcome.

De St. Groth (1983) was the first to report the growth of hybridoma cells in continuous culture in what he termed a 'Cytostat'. The cytostat differed from a chemostat in that the growth rate was not specifically controlled by a growth-limiting nutrient, but medium was supplied at a rate which matched the maximum growth rate of the cells, thus maintaining the cells at a constant concentration (i.e. a turbidostat). He successfully grew ten hybridomas in this form of culture. Boraston *et al.* (1984) successfully grew a hybridoma cell line in a true chemostat

under oxygen-limited steady state conditions. From the data obtained, values for the specific respiration rate and growth yield from oxygen over a range of growth rates could be determined. In a later paper, Birch *et al.* (1985) presented chemostat data for a hybridoma cell line which demonstrated that antibody synthesis was not growth-associated because as the growth rate was slowed the specific rate of antibody secretion increased. Several studies of hybridoma growth and monoclonal antibody production in chemostat culture have since been carried out (Miller *et al.*, 1988 and 1989; MacMichael, 1989; Harbour *et al.*, 1989; Ray *et al.*, 1989; Frame and Hu, 1990, and 1991a and b; Hayter *et al.*, 1992). In each case stable steady states were obtained, and monoclonal antibody production kinetics appeared to be cell line dependent.

In this study, single-stage chemostat studies of the NB1 cell line were carried out in DMEM supplemented with 5% FCS over the dilution range of 0.05 to 0.01 h⁻¹.

4.3 Aim

The aim of this set of experiments was to determine the monoclonal antibody production kinetics exhibited by the hybridoma cell line NB1 in chemostat culture.

4.4 RESULTS AND DISCUSSION

Single-stage chemostat culture of NB1 hybridoma cells was carried out at dilution rates of 0.05, 0.04, 0.03, 0.02 and 0.01 h⁻¹. Steady state conditions in terms of cell, antibody, glucose, glutamine, lactate and ammonia concentrations, was generally observed after four volume changes. Figure 4.1 shows the steady state obtained after four volume

changes and maintained for four volumes changes at a dilution rate of 0.03 h^{-1} for viable, non-viable and total cell and monoclonal antibody concentration.

Figure 4.1 Single-Stage Chemostat Culture. The steady states observed at a dilution rate of 0.03 h^{-1} for total, viable and non-viable cell concentrations and monoclonal antibody titre.

4.4.1 Cell Concentration

In single-stage studies steady state total cell concentration remained fairly constant across the dilution rate range, while viable cell concentration decreased (Figure 4.2).

Figure 4.2 Single-stage chemostat culture. Viable and total cell concentrations observed between dilution rates of 0.05 - 0.01h⁻¹.

The viability of the culture therefore fell with decreasing dilution rate.

The effect of dilution rate on hybridoma cell concentration would appear to be cell line dependent. Viable cell concentration has been reported to remain constant across a dilution rate range (Ray *et al.*, 1989; Hayter *et al.*, 1992; Martens *et al.*, 1993), increase with increasing dilution rate (Frame and Hu, 1991; Linardos *et al.*, 1991) or show a maximum concentration at an intermediate dilution rate, possibly because the higher dilution rates were approaching washout (Miller *et al.*, 1988). Total cell concentration has been reported to increase with increasing dilution rate (Frame and Hu, 1991), decrease with increasing dilution rate (Miller *et al.*, 1988; Martens *et al.*, 1993) or reach a maximum concentration at an intermediate dilution rate (Ray *et al.*,

1989). Viability of hybridoma cells in chemostat culture appears to always decrease with decreasing dilution rate, except in the case of Hayter *et al.*, (1992) where it remained constant.

Figure 4.3 shows the theoretical growth rate based on the dilution rate (Equation 4.2) and the specific growth rate after taking into account culture viability (Equation 4.3). Based on similar results obtained by other workers (Tovey and Brouty-Boye, 1976; Boraston *et al.*, 1984; Miller *et al.*, 1988) it has been suggested that there is a minimum required growth rate to maintain cell viability. Pirt (1975) discussed the possibility of a minimum growth rate for bacteria in chemostat culture. The possibility of part of the population differentiating into dormant cells allowing a minimum growth rate to be maintained by the growing fraction of the population was proposed. The results of Boraston *et al.* (1984), using the same cell line as in this study, suggested the possibility of a minimum growth rate of around 0.02h^{-1} for NB1 hybridoma cells. However, although a growth rate below 0.02h^{-1} was not observed here, it was not possible to conclude on the likelihood of a minimum growth rate without reducing the dilution rate further. There is no suggestion that animal cells are able to enter a dormant stage in chemostat culture unless the G0 phase of the cell cycle were classed as the equivalent, and its existence in a large proportion of the cell population at low dilution rates demonstrated. FACScan cell cycle analysis carried out in this study (Section 5.4.6) demonstrated the accumulation of cells in the G1/G0 phase of the cell cycle at low dilution rates but was unable to distinguish between the G1 and G0 fraction of the cell population.

Figure 4.3 Single-Stage Chemostat Culture. The effect of dilution rate on specific growth rate. Theoretical growth rate, based on $\mu = D$, and specific growth rate based on culture viability (Equation 4.3)

The maximum growth rate observed in chemostat culture was 0.053h^{-1} at a dilution rate of 0.05h^{-1} . The fact that a steady state was observed at this dilution rate indicates that the maximum growth rate of the cell line was not reached. At a dilution rate greater than μ_{max} washout of the cell population from the chemostat occurs because removal of cells by the dilution rate exceeds the replacement of cells by the growth rate. In batch culture the maximum growth rate observed when the cells were grown in serum-supplemented DMEM was only 0.05h^{-1} .

4.4.2 Glutamine Utilisation and Ammonia Production

The total amount of glutamine utilised and ammonia produced was relatively constant over the dilution rate range (Figure 4.4). The change in the concentration of residual glutamine between different dilution

rates was in fact only 0.00 - 0.18 mM out of an original concentration of 4.00 mM. Glutamine was the most likely candidate for growth-limiting substrate and the linearity results because almost all of the glutamine was utilised at all dilution rates.

Figure 4.4 The concentration of glutamine utilised and ammonia produced in single-stage chemostat culture between dilution rates of 0.01 and 0.05h⁻¹.

The specific rate of glutamine utilisation and ammonia production (Equation 4.4 and 4.5) decreased with decreasing dilution rate (Figure 4.5). This is in keeping with the results of other workers (Griffiths and Pirt, 1967; Miller *et al.*, 1988; Hayter *et al.*, 1992).

Figure 4.5 Single-Stage Chemostat Culture. The effect of dilution rate on specific rates of glutamine utilisation and ammonia production between dilution rates of 0.01 and 0.05h⁻¹.

The ratio of glutamine consumed to ammonia produced, shown in Table 4.1 below, remained fairly constant across the dilution rate range and showed that for every mole of glutamine utilised half a mole of ammonia was produced out of a theoretical maximum of two. The remaining ammonia was either incorporated into the cellular biomass or into waste metabolites such as alanine and proline.

Table 4.1

Ratio of Ammonia Produced to Glutamine Utilised in Single-Stage Chemostat Culture	
Dilution Rate (1/h)	Molar Ratio
0.05	0.55
0.04	0.56
0.03	0.59
0.02	0.55
0.01	0.54

During the growth phase of batch culture a value of 1.5 mM ammonia per mole of glutamine was generally observed. Glutamine utilisation in the chemostat therefore resulted in less ammonia being released than in batch culture. The fact that the ammonia concentration was almost the same for all dilution rates means that any inhibitory effect caused by ammonia on growth or production was the same at all dilution rates.

4.4.3 Glucose Utilisation and Lactate Production

Glucose showed a general trend of decreasing specific utilisation rate with decreasing growth rate and lactate production mirrored this trend (Figure 4.6). This is in agreement with the observations of other workers (Robinson and Memmert, 1991; Hayter *et al.*, 1992; Miller *et al.*, 1988; Ray *et al.*, 1989).

Figure 4.6 Single-Stage Chemostat Culture. Specific rates of glucose utilisation and lactate production between dilution rates of 0.01 and 0.05h^{-1} .

The specific rates of glucose utilisation and lactate production in Figure 4.6 shows the relationship between lactate production and glucose consumption as well as showing that specific glucose utilisation is non-linear. A non-linear rate of glucose consumption has been observed by other workers (Miller *et al.*, 1988). The specific rate of glucose utilisation appears to have become saturated above a dilution rate of 0.03h^{-1} . This is possibly because the demand for glucose was limited by some other factor, or because the efficiency of glucose utilisation increased with decreasing growth rate, demonstrated by a general trend of decreasing glucose:lactate ratio, particularly at the lower dilution rates, which has a theoretical maximum of 2.0 (Table 4.2). The ratio of glucose consumed to lactate produced at the higher dilution rates is comparable to the ratio of 1.53 seen during the growth phase of batch culture. The ratio seen at lower dilution rates although lower than the

growth phase ratio is not as low as the batch culture stationary phase ratio of 0.69.

Table 4.2

Ratio of Lactate Produced to Glucose Utilised in Single-Stage Chemostat Culture			
Dilution Rate (1/h)	Glucose Utilised (mM)	Lactate Produced (mM)	Molar Ratio
0.05	18.34	30.00	1.64
0.04	18.86	29.69	1.57
0.03	22.67	38.30	1.69
0.02	16.17	16.27	1.01
0.01	14.24	14.99	1.05

The concentration of lactate in the medium decreased dramatically at the two lowest dilution rates. Judging from the data showing the effect of lactate concentration on growth (Boraston, 1990) this should have a positive effect on growth rate.

For some cell lines it has been demonstrated that glucose and glutamine can be used interchangeably as an energy source (Zielke *et al.*, 1978). If this were the case for this cell line it would be expected that as glutamine became more limiting at the lower dilution rates then the specific rate of glucose utilisation would increase. In fact the specific utilisation rate of glucose decreased with glutamine concentration. (data shown in Appendix 8). However, the ratio of lactate produced to glucose utilised decreased therefore less glucose was more fully metabolised making it difficult to determine what the actual glucose demand of the cells was.

4.4.4 Monoclonal Antibody Production

Monoclonal antibody concentration increased with decreasing dilution rate (Figure 4.7), as did the specific rate of monoclonal antibody production. This has been described as typical of growth-dissociated production kinetics by several workers (Miller *et al.*, 1988; Hayter *et al.*, 1992), however as this implies that specific growth rate and specific production rate should vary independently of each other it is more accurately described as inversely (or negatively) growth-associated and is in contrast to the growth-associated production kinetics observed in batch culture.

Figure 4.7 Single-Stage Chemostat Culture. Volumetric antibody titre and specific rates of antibody production between the dilution rates of 0.01 and 0.05h⁻¹.

In general the characteristics of specific monoclonal antibody production observed in batch culture have been reflected in chemostat

culture resulting in reports of positively growth-associated antibody production (Low *et al.*, 1987; MacMichael, 1989), inversely growth-associated production (Boraston *et al.*, 1984; Reuveny *et al.*, 1986; Miller *et al.*, 1988; and Frame and Hu, 1991), as well as growth-dissociated production (Ray *et al.*, 1989; Hiller *et al.*, 1991). Besides this case, only Goergen *et al.* (1992) have reported seeing a positive association between growth and monoclonal antibody production in batch culture, and a negative association in glucose limited chemostat culture. However their chemostat data were based not on steady states but on the transient phase leading up to steady state. The effect of varying the dilution rate on steady state monoclonal antibody production was not reported.

4.4.5 Amino Acid Utilisation

As with batch culture, in single-stage chemostat culture amino acids could be classified in terms of those which were utilised only, those which were utilised and produced and those which were produced only. Alanine and proline were produced only, glycine, tryptophan, asparagine, aspartic acid and glutamic acid were produced and consumed while all other amino acids were consumed only. An example from each of these three categories is shown in Figure 4.8 in which negative values indicate amino acid production.

Figure 4.8 Single-Stage Chemostat Culture. Specific rates of histidine, tryptophan and proline production between dilution rates of 0.01 and 0.05h⁻¹.

The amino acids which were utilised only all followed the general trend of decreasing specific utilisation rate with decreasing dilution rate and growth rate, as has been reported by other workers (Miller *et al.*, 1989; Hiller *et al.*, 1991). Glycine and tryptophan, the two amino acids present in DMEM which were produced and consumed, both followed a trend of increasing specific utilisation with decreasing dilution rate. While tryptophan was produced at the highest dilution rates, glycine was produced at the lowest dilution rate. Alanine and proline were produced only and both followed a trend of decreasing production rate with decreasing dilution rate.

Cystine was not detected in the spent medium. This may have been because cystine was the growth-limiting substrate and therefore exhausted from the medium. Addition of extra cystine to the culture

would therefore expect to result in an increase in cell concentration provided it was the sole growth-limiting substrate. Subsequent studies (reported in Chapter 6) demonstrated that addition of extra glutamine to a two-stage chemostat resulted in an increase in cell concentration. No further increase was observed however when both glutamine and cystine were added in excess, suggesting that cystine was not a growth-limiting substrate. The apparent depletion of cystine from the medium could have been due to its instability in the amino acid analysis method used (Sarah Boork, Personal Communication). Methionine was depleted below a dilution rate of 0.02 h^{-1} , tryptophan was depleted at a dilution rate of 0.01 h^{-1} .

Table 4.3 compares the specific utilisation rates of amino acids in chemostat culture with batch culture.

TABLE 4.3

Specific Rates Of Amino Acid Utilisation During The Growth Phase Of Batch Culture Compared To Single-Stage Chemostat Culture At $D = 0.03 \text{ h}^{-1}$		
Amino Acid	Batch Culture 0 - 58 hours $\mu\text{moles}/10^6 \text{ cells/h}$	Chemostat Culture $D = 0.03 \text{ h}^{-1}$ $\mu\text{moles}/10^6 \text{ cells/h}$
GLN	0.0767	0.0758
THR	0.0089	0.0095
LEU	0.0084	0.0092
LYS	0.0077	0.0080
VAL	0.0072	0.0074
ILE	0.0066	0.0066
TYR	0.0047	0.0045
ARG	0.0043	0.0041
PHE	0.0034	0.0033
SER	0.0033	0.0045
CYS	0.0029	0.0039
GLY	0.0028	0.0016
MET	0.0023	0.0025
HIS	0.0022	0.0021
TRP	0.0003	0.0002
PRO	-0.0042	-0.0055
ALA	-0.0195	-0.0168

At a dilution rate of 0.03 h^{-1} the specific rates of utilisation of amino acids were very similar to batch culture. Also, the order of amino acids in terms of rate of utilisation was very similar in batch and chemostat culture. This indicates that at an intermediate dilution rate, the metabolic rate of amino acid utilisation is no greater in chemostat culture than batch culture. As the specific utilisation rate tended to decrease with decreasing dilution rate, the amino acid metabolic rate was greater than batch culture at higher dilution rates, but was less than batch culture at low dilution rates. For other cell lines Griffiths and Pirt (1967) and Ozturk and Palsson (1990) have reported a lower rate of amino acid utilisation in chemostat culture compared to batch culture.

4.5 CONCLUSIONS

- Steady states in terms of cell concentration, antibody production, and nutrient utilisation were observed at all dilution rates.
- With decreasing dilution rate total cell concentration remained constant while the viable cell concentration and viability decreased.
- Specific rates of amino acid utilisation decreased with decreasing dilution rate.
- The specific growth rate did not fall below 0.02 h^{-1} , and therefore did not rule out the possibility of a minimum growth rate.
- Glutamine was the most likely candidate for growth-limiting substrate. The apparent depletion of cystine was possibly due to its instability in the HPLC assay used for amino acid analysis.
- Monoclonal antibody production was negatively growth rate associated.
- The results of the batch and chemostat experiments indicate that viable cell concentration and growth rate control are two key parameters in the optimisation of volumetric antibody productivity. As has been demonstrated, single-stage chemostat culture results either in high viability and high growth rate or low viability and low growth rate while maximum productivity would seem to require high viability and low growth rate. The feasibility of achieving this with two-stage chemostat culture was therefore explored.

5.0 TWO-STAGE CHEMOSTAT CULTURE

5.1 SUMMARY

Two-stage chemostat culture of the NB1 hybridoma cell line was performed. The effect of dilution rates between 0.01 h^{-1} and 0.05 h^{-1} on specific growth rate, cell concentration, specific production rate of monoclonal antibody, and specific rate of nutrient utilisation and waste metabolite production were studied. The distribution of cells in the various stages of the cell cycle at different dilution rates was also studied. Steady states were maintained at each dilution rate over a number of volume changes. Monoclonal antibody production was negatively growth rate associated. The specific monoclonal antibody production rate was greater in stage two compared to stage one only at the lowest dilution rates even though several amino acids were totally exhausted from the medium in stage two. A general trend of an increasing percentage of cells in the G1/G0 phase of the cell cycle with decreasing dilution rate was observed in stage one and stage two.

5.2 INTRODUCTION

If monoclonal antibody production is truly negatively growth-associated and the optima for growth and production are different it is logical to assume that this could be best studied and exploited in a two-stage chemostat system in which a growth stage feeds cells into a second production stage with the conditions in each being tailored to their specific function.

Within a few years of the establishment of chemostat culture it was realised that the productivity of many metabolites observed in batch culture was not always being matched in the chemostat. This led to the development of multi-stage chemostat systems, discussed initially in theory by Maxon (1955) and Deindorfer and Humphrey (1959) and

developed by Herbert (1961) and others. The reason for such low productivity may have been due to one or more of a number of possibilities. In a batch culture, each point on the growth curve is dependent on those preceding it, the culture therefore has a developmental history. However, in a single-stage chemostat the population is multiplying under constant environmental conditions in a steady state. The physiological state of the culture is therefore no longer dependent on its history but on the dilution rate. The biosynthesis of certain products is dependent on the passing of the cell population through certain physiological phases, for example, the induction of spore formation by *Bacillus cereus* was attempted in two-stage chemostat culture (Humphrey *et al.*, 1966). However the dependence of each physiological state on the previous states is so significant that spore formation was only observed in continuous culture when the organism was grown in a multistage chemostat consisting of six stages (Ricina, 1969).

Chemostat culture steady states tend to become easily destabilised at dilution rates close to the maximum growth rate of a culture, and at very low growth rates. If the maximum rate of product synthesis occurs during the growth phase of the population maximum productivity will be observed at maximum growth rate. To overcome the problem of studying product formation at growth rates close to the maximum, two-stage chemostat culture has been employed (Main *et al.*, 1969). Main *et al.* studied the effect of growth rate on enzyme activity in yeast and employed a multi-stage continuous system to overcome the problems of perturbations in steady state at values close to the maximum growth rate where small changes in the substrate concentration could result in complete washout. The dilution rate in the first stage was kept near to

the maximum growth rate. The cells were then passed on to the second stage which was also supplied with fresh medium. The conditions in the second stage were changed from low values of dilution rate to very high and the maximum attainable specific growth rate, nucleic acid and carbohydrate catabolising enzyme concentrations were determined. Wash out of the culture from the second stage did not occur because of the constant flow of inoculum from the first stage.

Two-stage chemostat culture is also applicable at the other end of the scale, when the specific rate of product formation is inversely proportional to the rate of growth and reaches its maximum, or only occurs, when the growth rate is equal to zero. Under most growth conditions *Bacillus* species produce extracellular protease only after the exponential growth phase. The production of protease was therefore studied in a two-stage chemostat (Fabian, 1969) and an industrially important strain of *Bacillus* was made to produce extracellular protease continuously in a two-stage fermenter for extended periods of time (Jensen, 1972). Single-stage chemostat culture of plant cells has generally resulted in poor secondary product synthesis (Tal and Goldberg, 1982). However, the total phenolic concentration obtained in a two-stage culture of *Nicotiana tabacum* was higher than that obtained in batch culture under identical nutrient conditions or in a single-stage system under identical conditions (Sahai and Shuler, 1984).

The versatility of multistage chemostat culture allows the study of cultures at maximum or zero growth rate, the development of culture history under steady state conditions, switches in the growth-limiting nutrient in different stages and changes in the physical conditions between stages such as temperature and pH. Although multistage

chemostat culture has been used in the study of bacteria, fungi, and plant cells there is not at present a report of its use in the study of the growth and physiology of animal cells. The advantages of using multistage culture in the study of hybridoma cell growth, metabolism and monoclonal antibody production are numerous and will be explored more fully in this study.

A two-stage chemostat culture, using DMEM supplemented with 5% foetal calf serum, with no extra feed to the second stage was set up as shown in the Materials and Methods (Figure 2.1). Steady states at dilution rates of 0.05, 0.04, 0.03, 0.02 and 0.01 h^{-1} were obtained.

5.3 AIMS

The aim of this set of experiments was to explore the possibility of culturing animal cells in a two-stage chemostat, and to observe the effects of such a culture system on cell growth and monoclonal antibody production.

5.4 RESULTS AND DISCUSSION

5.4.1 Cell Concentration

Total cell and viable cell concentration in the second stage compared to the first stage is shown in Figure 5.1a, and Figure 5.1b.

Figure 5.1a Two-Stage Chemostat Culture. Steady-state total cell concentration observed in stage one and stage two of a two-stage chemostat culture at dilution rates of 0.01 - 0.05h⁻¹.

Figure 5.1b Two-Stage Chemostat Culture. Steady-state viable cell concentration observed in stage one and stage two of a two-stage chemostat culture at dilution rates of 0.01 - 0.05h⁻¹.

At the highest dilution rates essential nutrients remaining from stage one were utilised in stage two resulting in higher total cell concentrations, while at lower dilution rates less nutrients were available from stage one resulting in decreased viable and total cell concentrations in stage two. Viable cell concentration was higher in stage two only at the highest dilution rate.

Growth rates in the second stage of a microbial two-stage chemostat culture with no extra feed to the second stage were calculated according to the following equation (Pirt, 1975):

$$\mu_2 = D_2(x_2 - x_1)/x_2$$

Equation 5.1

where D_2 is the dilution rate in stage two; x_2 is the biomass concentration in stage two and x_1 is the biomass concentration in stage one. However, this assumes that the culture is 100% viable. Determination of viable cell and total cell concentrations in animal cell culture allows growth rate to be calculated accounting for the viable cell population only.

Growth rate calculated in this way indicates that a positive growth rate is only observed in stage two at a dilution rate of 0.05 h^{-1} where viable cell concentration is greater in stage two than stage one. At the lower dilution rates, where the viable cell concentration in stage two is equal to or less than stage one, growth rate is zero ($D=0.04\text{h}^{-1}$) or negative ($D=0.03 - 0.01\text{h}^{-1}$).

However, although the viable cell population may show a negative growth rate there is no reason to suppose that individual cells are not growing and dividing. This would typically be shown by an increase in the total cell concentration between stage one and stage two, unless the dead cells were being broken up into small fragments. Dilution rates of 0.05 , 0.04 , and 0.03h^{-1} showed an increase in total cell concentration between stages one and two while only a dilution rate of 0.05 h^{-1} showed an increase in viable cell concentration. Dilution rates of 0.02 and 0.01 h^{-1} showed a decrease in total cell concentration.

Specific growth rate in stage two (μ_2) accounting for the generation of non-viable cells was calculated using Equation 5.2 which assumes that the change in total cell concentration between stage one and two results from the viable cell concentration in stage two as follows:

$$\mu_2 = D_2(x_{t2} - x_{t1})/x_{v2} \quad \text{Equation 5.2}$$

The calculation of growth rate in the second stage of the two-stage chemostat culture indicates that the cells have a minimum growth rate of around 0.02 h^{-1} as was suggested by single-stage culture both here and by other workers (Tovey and Brouty-Boye, 1976; Boraston *et al.*, 1984; Miller *et al.*, 1988). However, an unknown factor in the equation is the number of non-viable cells not accounted for due to their disintegration in the chemostat. The loss of non-viable cells from the count would make the growth rate appear to be lower than it actually is. Therefore, the negative growth rate calculated at dilution rates of 0.02 and 0.01 h^{-1} may be falsely depressed by disintegration of some of the non-viable population. The reality may be a growth rate which is positive or much closer to zero than has been calculated.

Figure 5.2 Two-Stage Chemostat Culture. The theoretical growth rate assuming 100% viability, and the calculated growth rates accounting for the generation of non-viable cells in stage one and stage two of a two-stage chemostat.

5.4.2 Monoclonal Antibody Production

The specific rate of antibody production, as well as the volumetric titre (steady state concentration), increased with decreasing dilution rate in stage one and stage two (Figure 5.3a and b).

Figure 5.3a The steady state monoclonal antibody titre in both stages of a two-stage chemostat culture at dilution rates of 0.01 - 0.05h⁻¹.

Figure 5.3b. Specific rate of monoclonal antibody production in both stages of a two-stage chemostat culture at dilution rates of 0.01-0.05h⁻¹.

At high dilution rates the specific production rate was greater in stage one, but at the lowest dilution rates was greater in stage two. By increasing the mean residence time of a culture due to the addition of a second stage, the time available for production by the cells was increased, therefore the antibody titre was much greater in a two-stage culture compared to a single-stage culture at the same dilution rate, with no extra cost in terms of medium, serum and preparation time. For example, at a dilution rate of 0.03h⁻¹ antibody titre in single-stage culture was 32.15 µg/ml while at the end of two-stage culture at the same dilution rate it was 54.07 µg/ml. The antibody titre of a single-stage chemostat at a dilution rate of 0.02h⁻¹ and a mean residence time of 50 hours was 33.04 µg/ml. A dilution rate of 0.04h⁻¹ in a two-stage chemostat gives a mean residence time equivalent to a single-stage

culture at 0.02h^{-1} , i.e. 50 hours, however the antibody titre of the two-stage system was $43.15\text{ }\mu\text{g/ml}$. A two-stage system can therefore be much more productive than a single-stage system. However, at lower dilution rates of 0.01 h^{-1} in single-stage and 0.02 h^{-1} in two-stage, mean residence times of 100 hours, the antibody titre was $88.19\text{ }\mu\text{g/ml}$ in single-stage and only $46.55\text{ }\mu\text{g/ml}$ in two-stage culture. The productivity of a fermenter can also be measured in terms of volumetric output. As a two-stage system has twice the volume of a single-stage system, to maintain or increase volumetric output antibody titre must be at least doubled in stage two compared to stage one. Volumetric output from stage two was less than stage one at all dilution rates.

The results for specific production rates at high dilution rates are consistent with observations in glutamine depleted batch culture, that is, antibody production stopped shortly after glutamine ran out, therefore we see reduced production in stage two where glutamine is depleted. However, the increase in production rate seen in stage two compared to stage one at a low dilution rate is not consistent with batch culture. This may be partly due to the fact that batch culture results in nutrient depletion, while chemostat culture results in nutrient limitation.

There are several other possible reasons why antibody production rate was greater in stage two at low dilution rates.

1. Antibody production was better able to compete with growth for common precursors or pathways because either a nutrient essential for growth but not for production was limiting; or toxins produced by the cells may have been inhibiting growth but not antibody production. This is supported by the fact that at dilution rates of

0.02 and 0.01h^{-1} when the growth rate was negative specific monoclonal antibody production was greater, indicating that when no cell growth occurs production is greatest.

2. The cells are accumulating in the G1/G0 phase of the cell cycle due to their slowed growth rate resulting in opportunity for increased production. This is supported by FACScan data which showed that the percentage of cells in the various stages of the cell cycle does change with growth rate (see section 5.4.6). However, the batch culture data demonstrate that this does not necessarily have a positive effect on antibody production (see Section 3.4.11).
3. Cells increase production of some proteins when they are subject to stress; the cells are likely to be very stressed at low dilution rates which may result in greater antibody production rates.

5.4.3 Glutamine Utilisation and Ammonia Production

Total glutamine decreased with decreasing dilution rate in stage one and any remaining from stage one was fully utilised in stage two (Figure 5.4). The concentration of glutamine in stage two was below the limits for accurate measurement by the assay used.

Figure 5.4. The steady state glutamine concentrations in both stages of a two-stage chemostat culture at dilution rates of 0.01 - 0.05h⁻¹.

Figure 5.5. The steady state ammonia concentrations in both stages of a two-stage chemostat culture at dilution rates of 0.01 - 0.05h⁻¹.

Very little extra ammonia was produced in stage two, reflecting the limited amount of glutamine left over from stage one (Figure 5.5). Because of the low concentrations involved, a small concentration change had a significant impact on the calculated ratio of glutamine:ammonia. No significant conclusions concerning the ratio of ammonia produced to glutamine utilisation could therefore be drawn (data shown in Appendix 8).

5.4.4 Glucose Utilisation and Lactate Production

The specific rate of glucose utilisation showed a general trend of a decreasing rate of utilisation with decreasing dilution rate, with a slight digression at the dilution rates where a negative growth rate was observed (Figure 5.6).

Figure 5.6. The specific rates of glucose utilisation in both stages of a two-stage chemostat culture at dilution rates of 0.01 - 0.05h⁻¹.

Lactate production was once again closely related to glucose utilisation

(Figure 5.7), and the same trend of decreasing lactate:glucose ratio with decreasing growth rate was observed as in stage one.

Figure 5.7. The specific rates of lactate production in both stages of a two-stage chemostat culture at dilution rates of 0.01 - 0.05h⁻¹.

Therefore, in stage two less glucose was utilised more efficiently than stage one. There did not appear to be an increased demand for glucose as an energy source due to glutamine depletion (data shown in Appendix 8). At a dilution rate of 0.03h⁻¹ lactate was consumed, this has frequently been observed particularly towards the end of a batch culture (Velez *et al.*, 1986).

This result is similar to the trend observed in batch culture supplemented with extra glutamine and essential amino acids. During the extended stationary phase observed in such a culture glucose is utilised at a greatly reduced rate but with very little increase in the lactate

concentration. Therefore, as in batch culture in chemostat culture at a low growth rate, less glucose is being utilised much more efficiently.

5.4.5 Amino Acid Utilisation

The specific rates of amino acid utilisation show a general trend towards decreasing rates of utilisation with decreasing growth rate in both stage one and stage two. However, at lower dilution rates several amino acids were totally exhausted from the medium, while others may have been at limiting concentrations. As in batch culture, after glutamine, methionine was the first amino acid to become depleted from stage one and two at a dilution rate of 0.02 h^{-1} (data shown in Appendix 9). Tryptophan was depleted from stage one and two at a dilution rate of 0.01 h^{-1} , while at this dilution rate leucine was depleted from stage two only. Histidine and isoleucine were also approaching very low concentrations in the second stage at a dilution rate of 0.01 h^{-1} . Although histidine was present at dilution rates of 0.01 and 0.02 h^{-1} none was utilised. Serine was the only amino acid which showed an increasing rate of utilisation with decreasing growth rate however this did decline at a dilution rate of 0.01 h^{-1} . The trends of amino acid utilisation for certain amino acids in stage two are shown in Figure 5.8.

Figure 5.8. The specific rates of amino acid utilisation in the second stage of a two-stage chemostat culture at dilution rates of 0.01 - 0.05h⁻¹, negative values indicate net production.

As in batch culture, in chemostat culture proline and alanine were produced by the cells, and glycine was produced and consumed. In addition arginine and threonine were also both produced and consumed in the second stage. The specific rates of utilisation of amino acids were less in the second stage than in the first stage of the chemostat culture. This is consistent with the reduced growth rate in stage two compared to stage one.

5.4.6 Cell Cycle Analysis

The percentages of the cell population in the various stages of the cell cycle was determined across the dilution rate range in the two stages of the two-stage chemostat. The results are shown in Table 5.1.

Table 5.1

Cell Cycle Distribution Of NB1 Hybridoma Cells In Two-Stage Chemostat Culture						
	Dilution Rate (h^{-1})	Growth Rate (h^{-1})	Viability (%)	Percentage Of Cells In Stage Of The Cell Cycle		
				G1/G0	S	G2/M
Stage 1	0.05	0.054	93	41.7	50.7	7.6
	0.04	0.051	79	44.1	42.9	12.9
	0.03	0.037	82	49.7	31.9	18.4
	0.02	0.041	50	47.9	36.9	9.3
	0.01	0.024	41	60.0	31.8	8.2
Stage 2	0.05	0.027	68	55.5	38.9	5.6
	0.04	0.017	59	49.0	44.7	6.3
	0.03	0.019	46	81.6	14.2	4.2
	0.02	-0.008	20			
	0.01	-0.007	16	61.4	35.3	3.3

In the first stage of a two-stage chemostat there was a general trend towards an increasing percentage of cells in the G1/G0 phases of the cell cycle with decreasing growth rate while the percentages of cells in the other phases of the cell cycle decreased. This is consistent with the decreasing viability observed. The same general trend was also observed in the second stage of the chemostat culture. This result is consistent with other work which has also shown an increase in the percentage of cells in the G1/G0 phase with decreasing growth rate in chemostat culture (Martens *et al.*, 1993). This suggests the possibility that the increase in specific rate of antibody production with decreasing growth rate could be the consequence of increased synthesis of antibody in the G1/G0 phase compared to the other phases. However, the increase in the percentage of cells in the G1/G0 phase of the cell cycle in the second stage of a two-stage chemostat is not reflected by an increase in the specific production rate compared to stage one. Therefore, with this cell line, either the association between cell cycle and specific production is

incidental or it is also dependent on other factors such as the availability of nutrients essential for production. This was also the case for this cell line in batch culture (Chapter 3), where cells accumulated in the G1/G0 phase of the cell cycle during the decline phase without any increase in specific antibody production. In batch and chemostat culture glutamine may have been limiting both growth and antibody production.

5.5 CONCLUSIONS

- Two-stage chemostat culture has been shown to be a practical possibility in the culture of animal cells, and is a technique which can be further used in the study of factors involved in negatively growth-associated monoclonal antibody production.
- Steady states in terms of cell concentration, antibody production and nutrient utilisation were observed in stage one and stage two of a two-stage chemostat at all dilution rates.
- Monoclonal antibody production in both stages of a two-stage chemostat culture was negatively growth-associated.
- In the second stage, total cell and viable cell concentration and viability decreased with decreasing dilution rate.
- A positive growth rate in the second stage was observed only at dilution rates above 0.03 h^{-1} . Below this a negative growth rate was observed.
- Cell growth appeared to be limited by glutamine in single-stage culture and glutamine and several other amino acids in the second

stage of a two-stage system.

- In contrast to batch culture, glutamine depletion in chemostat culture did not appear to result in a decline in monoclonal antibody production at low growth rates.
- The NB1 cell line appeared to have a minimum growth rate of around 0.02 h^{-1} in single-stage culture and in two-stage culture.
- Specific rates of synthesis for the energy catabolites ammonia and lactate reflect the rates of glutamine and glucose utilisation respectively.
- It is possible that other medium components besides glutamine are used as energy substrates at low growth rates resulting in increased monoclonal antibody production despite the depletion of glutamine.

**6.0 EFFECT OF INCREASING THE
CONCENTRATION OF POTENTIALLY
LIMITING AMINO ACIDS ON GROWTH AND
PRODUCTION IN BATCH CULTURE AND
TWO-STAGE CHEMOSTAT CULTURE**

6.1 SUMMARY

The concentrations of specific individual amino acids were increased in stirred batch culture of NB1 hybridoma cells. This tended to have a negative effect on cell growth, and only in some cases had a positive effect on antibody production, possibly due to the negative effect on growth. The same phenomenon was observed when specific amino acids were fed to the second stage of a two-stage chemostat, specific growth rate tended to increase while specific production rate tended to decrease. An attempt was made to decrease the growth rate of a fed second stage by increasing its volume relative to stage one. This also resulted in an increased specific growth rate and a consequential decreased specific production rate, however, volumetric production was substantially increased.

6.2 INTRODUCTION

During the course of a typical batch culture of NB1 hybridoma cells growth ceased when glutamine was exhausted from the medium shortly followed by cessation of monoclonal antibody production. When extra glutamine was fed to the culture an extended stationary phase was observed during which both growth and production continued. At the end of this extended batch culture both methionine and cystine along with glutamine were exhausted from the medium and the concentrations of several other amino acids were significantly reduced, in particular tryptophan, leucine, histidine and arginine. It was thought possible that one or other of these amino acids may also be limiting growth and/or production in batch culture. Therefore an experiment was set up in which batch cultures of NB1 hybridoma cells were supplemented with double the concentration of these particular amino acids. Duval *et al.* (1991) showed that for their particular cell line vitamins were a limiting

nutrient in batch culture. Therefore a batch culture of NB1 hybridoma cells was also set up in which the vitamin concentration was doubled.

In two-stage chemostat culture, besides glutamine depletion, cystine appeared to be depleted at all dilution rates, while at the lower dilution rates methionine, tryptophan and leucine were depleted.

It was hoped that by supplementing batch culture with the potentially limiting amino acids their relative importance in growth and production would be revealed by increasing or decreasing the viable cell concentration and/or final antibody titre. By feeding the second stage of a two-stage chemostat with the potentially limiting amino acids and observing the effects on growth and production their relative importance may be determined under more controlled steady state conditions.

6.3 AIM

At low dilution rates in chemostat culture and towards the end of batch culture certain amino acids were depleted from the medium, or present at very low concentrations. The reduced concentration of these amino acids may be having a negative or positive effect on growth and/or monoclonal antibody production. The effect on growth and production of supplementing the medium with these amino acids was studied in batch and two-stage chemostat culture.

6.4 RESULTS AND DISCUSSION

6.4.1 Batch Culture

The effect of increasing the concentration of a particular amino acid on growth and production in stirred batch culture was determined by calculating the maximum specific growth rate and the maximum specific production rate by the partial cubic spline method. The total viable cell hours (integral of the viable cell curve), and the amount of antibody produced per viable cell hour were also calculated. Calculation of $\mu_{\max}$ and maximum q_{antibody} indicates the effect of an amino acid on the interaction between growth and production. Calculation of viable cell hours demonstrates how effective the particular amino acid is at maintaining viability without necessarily effecting specific growth rate. Calculation of antibody produced per viable cell hour gives the overall specific production rate throughout the entire period of the batch culture without placing undue emphasis on the maximum specific rate of production.

Non-Fed Batch Culture

A compilation of the results for the non-fed batch culture is shown in Table 6.1. None of the amino acids added had a positive effect on either the maximum viable cell concentration obtained or on the total number of viable cell hours. In non-fed batch culture the maximum viable cell concentration observed was 1.53×10^6 cells/ml. The maximum viable cell concentration seen in the amino acid supplemented cultures was in the range of $1.11 - 1.34 \times 10^6$ cells/ml. This was also reflected in the total viable cell hours of the cultures. The total viable cell hours of the control culture was 95.54 hours, while the other cultures were in the range of 73.19 - 80.13 hours. The negative effect of adding double the

concentration of certain amino acids on cell growth was also reflected by the calculated $\mu_{\max}$, which for the control was 0.072 h^{-1} , while the other cultures were in the range of $0.046 - 0.065 \text{ h}^{-1}$. The maximum growth rate of the control culture was greater than that observed in earlier batch culture experiments. Many factors may have contributed to this variation including variability in media, serum and culture conditions. This fact highlights the need to design experiments which are complete within themselves and the care which must be taken in making comparisons between experiments performed at different times.

Table 6.1

Results of Unfed Batch Culture Supplemented With 2X Concentration of Specified Amino Acids						
Culture Supplement	Maximum Viable Cell Concentration ($\times 10^6/\text{ml}$)	Final Antibody Titre ($\mu\text{g}/\text{ml}$)	Viable Cell Hours/ml ($\times 10^{-6}$)	Antibody per 10^6 Viable Cell Hours ($\mu\text{g}/10^6 \text{ cell h}$)	$\mu_{\max}$ (h^{-1})	max q antibody ($\mu\text{g}/10^6 \text{ cells/h}$)
Control	1.53	29.29	95.54	0.307	0.072	0.46
Leucine	1.31	21.61	75.33	0.287	0.056	0.39
Arginine	1.23	19.37	77.80	0.249	0.065	0.33
Methionine	1.12	33.16	79.20	0.419	0.049	0.80
Tryptophan	1.11	41.31	80.13	0.516	0.063	0.58
Cystine	1.34	30.38	77.47	0.392	0.046	0.50
Histidine	1.20	25.66	73.19	0.351	0.047	0.35

See Appendix 11 for actual concentration of specified amino acid added. Although none of the amino acids added to batch culture had a positive effect on the total number of viable cell hours or on the maximum

growth rate, some of them did have a positive effect on production. The addition of 2X the concentration of methionine, tryptophan and cystine to the batch culture increased the final titre of antibody, the amount of antibody produced per viable cell hour, and the maximum q_{antibody} . The addition of 2X the concentration of histidine resulted in a decreased final antibody titre and a decreased q_{antibody} but an increase in the amount of antibody produced per viable cell hour. Therefore, increasing the concentration of methionine, tryptophan, cystine and histidine had a negative effect on cell growth but a positive effect on antibody production.

Glutamine-Fed Batch Culture

The results are shown in Table 6.2. In glutamine-fed batch culture once again the addition of extra amino acids did not increase the total viable cell hours. However, the addition of 2X the concentration of tryptophan increased the maximum viable cell concentration to 1.64×10^6 cells/ml compared to 1.43×10^6 cells/ml for the control. The addition of the other amino acids resulted in a lower maximum viable cell concentration. The increase in viable cell concentration of the tryptophan supplemented culture was reflected in the increased μ_{max} of 0.067 h^{-1} compared to 0.061 h^{-1} for the control. All other cultures showed a decreased μ_{max} relative to the control.

The addition of 2X the concentration of methionine and cystine increased the final antibody titre compared to the control. Methionine, cystine and histidine increased the concentration of antibody produced per cell hour as well as the maximum q_{antibody} . All the other amino acids, apart from arginine, increased the maximum q_{antibody} relative to the control. However, this was not reflected by increased final antibody

titre due to their negative effect on total viable cell hours.

The addition of extra vitamins to glutamine-fed batch culture had a negative effect on cell growth and monoclonal antibody production.

Table 6.2

Results of Glutamine Fed Batch Culture Supplemented With 2X Concentration of Specified Amino Acids						
Culture Supplement	Maximum Viable Cell Concentration ($\times 10^6/\text{ml}$)	Final Antibody Titre ($\mu\text{g}/\text{ml}$)	Viable Cell Hours/ ml ($\times 10^{-6}$)	Antibody per 10^6 Viable Cell Hours ($\mu\text{g}/10^6 \text{ cell h}$)	$\mu \text{ max}$ (h^{-1})	max q antibody ($\mu\text{g}/10^6 \text{ cells/h}$)
Control	1.43	77.62	154.86	0.501	0.061	0.43
Leucine	1.44	64.20	133.70	0.480	0.053	0.60
Arginine	1.22	62.27	133.57	0.466	0.050	0.40
Methionine	1.06	80.80	132.82	0.608	0.050	0.57
Tryptophan	1.64	62.93	132.15	0.476	0.067	0.56
Cystine	1.18	80.35	138.64	0.580	0.057	0.52
Histidine	1.06	66.75	128.52	0.519	0.049	0.57
Vitamins	1.22	59.60	129.51	0.460	0.051	0.55

From the results of this experiment it would appear that supplementation of batch culture with 2X the concentration of an individual amino acid has a negative effect on cell growth and a negative or positive effect on monoclonal antibody production. It is difficult under these conditions to determine whether the extra amino acid affected antibody production directly or whether it was an indirect

effect due to its influence on growth rate. However, there was no apparent correlation between $\mu_{\max}$ and q_{antibody} to suggest that a decrease in $\mu_{\max}$ resulted in an increase in q_{antibody} . A possible reason for the observed negative effect of increased amino acid concentration on cell growth could have been the influence of competition between amino acids for access to amino acid transport systems into the cell. Therefore, by increasing the concentration of one amino acid another amino acid sharing the same transport mechanism may become limiting. The possibility of this occurring is highlighted by the fact that when all amino acids were added to the culture medium (Chapter 3) increased growth and production were observed, however, addition of individual amino acids had a negative effect on growth. Duval *et al.* (1991) also observed that addition of all consumed amino acids resulted in increased growth and antibody production, while selective addition of only four of the most rapidly consumed amino acids did not increase cell concentration or extend the life span of the culture. This will be discussed in Chapter 8.

6.4.2 TWO-STAGE CHEMOSTAT CULTURE

The positive effect of glutamine, cystine, methionine and tryptophan on monoclonal antibody production was further studied in two-stage chemostat culture.

The second stage of a two-stage chemostat was fed with additional glutamine to give an added concentration of 4 $\mu\text{moles/ml}$, i.e. equal to the concentration in the first stage feed. The dilution rate in stage one was 0.030h^{-1} and in stage two was 0.032h^{-1} . The difference in dilution rate was the equivalent of only 4ml/h so as to make these results comparable to a control with no second stage feed and therefore a

dilution rate of 0.030h^{-1} in stage one and two. Experiments were also run in which the second stage was fed with glutamine and cystine, glutamine and methionine, and glutamine and tryptophan all at concentrations equal to the first stage feed (i.e. glutamine: $4\mu\text{moles/ml}$; cystine: $0.26\mu\text{moles/ml}$; methionine: $0.18\mu\text{moles/ml}$; tryptophan: $0.06\mu\text{moles/ml}$). For each alteration of the second stage feed, the conditions in the first stage remained constant. The effects of a second stage feed on growth and production are shown in Table 6.3.

Table 6.3

Effect Of A Second Stage Amino Acid Feed On Growth And Monoclonal Antibody Production					
Amino Acid Feed	Viable Cell Concentration ($\times 10^6/\text{ml}$)	Specific Growth Rate (h^{-1})	Antibody Concentration ($\mu\text{g/ml}$)	Specific Production Rate ($\mu\text{g}/10^6$ viable cells/h)	Volumetric Production Rate (mg/l/h)
Control	1.22	0.0145	27.03	0.334	0.407
Glutamine	1.72	0.0213	28.78	0.252	0.433
Glutamine + Cystine	1.23	0.0217	24.69	0.246	0.302
Glutamine + Methionine	1.65	0.0364	26.79	0.224	0.370
Glutamine + Tryptophan	1.46	0.0254	32.61	0.366	0.535

A glutamine feed to the second stage of a two-stage chemostat resulted in an increase in the specific growth rate of the culture and a decrease in the specific antibody production rate. Such a result is in keeping with the negatively growth-associated antibody production kinetics observed in chemostat culture (Chapters 4 and 5). The increased growth rate also

adds to the evidence for glutamine being the growth-limiting substrate in the first stage of a two-stage chemostat. The specific rate of glutamine utilisation in the first stage of the chemostat was $0.0577 \mu\text{moles}/10^6 \text{ cells/h}$, while in the unfed second stage it was $0.0144 \mu\text{moles}/10^6 \text{ cells/h}$. When glutamine was fed to the second stage, the specific rate of utilisation increased to $0.0321 \mu\text{moles}/10^6 \text{ cells/h}$.

In the two-stage chemostat results presented in Chapter 5 cystine was indicated as a possible growth-limiting substrate due to its apparent depletion from the medium. However it is possible that its depletion was due to its instability in the HPLC assay used. When glutamine and cystine were fed to the second stage of the two-stage chemostat there was no increase or decrease in specific growth rate or specific antibody production rate compared to the glutamine only fed culture. However, the specific rate of cystine utilisation did increase from $0.0004 \mu\text{moles}/10^6 \text{ cells/h}$ to $0.0055 \mu\text{moles}/10^6 \text{ cells/h}$. This is most probably the result of an exaggerated depletion of cystine from the medium due to its instability in the HPLC assay. The fact that the cell and antibody concentration was unchanged compared to the glutamine only fed second stage suggests that cystine was not a limiting nutrient along with glutamine. This result also suggests that an increased cystine concentration does not have a positive or negative effect on growth or production in two-stage chemostat culture.

A glutamine and methionine feed to the second stage resulted in an increased specific growth rate and in a decreased specific production rate relative to the control and glutamine only fed second stage. This result may either indicate that besides glutamine, methionine was also at a growth-limiting concentration, or that an increased methionine

concentration had a positive effect on growth rate. Out of an initial concentration of 0.20 $\mu\text{moles/ml}$ of methionine in DMEM, 0.06 $\mu\text{moles/ml}$ remained for stage two at a dilution rate of 0.03 h^{-1} . In stage one methionine was utilised at a rate of $0.0025\text{ }\mu\text{moles}/10^6\text{ viable cells/h}$, but was being supplied to stage two at a rate of $0.0018\text{ }\mu\text{moles/h}$. Therefore it is possible that methionine could be a limiting nutrient in stage two. The specific rate of methionine utilisation was unchanged at $0.0006\text{ }\mu\text{moles}/10^6\text{ cells/h}$ in the non fed and the glutamine and methionine fed second stages, but the volumetric rate of methionine utilisation was increased from $0.0007\text{ millimoles/l/h}$ to $0.0010\text{ millimoles/l/h}$, reflecting the increased concentration of viable cells. The decrease in specific antibody production rate is in keeping with negatively growth-associated production.

These results suggest that the positive effect increased concentrations of glutamine, cystine and methionine had in batch culture was caused indirectly by effecting the growth rate of the cell.

A glutamine and tryptophan feed to the second stage of a two-stage chemostat resulted in an increased specific growth rate and an increased specific production rate relative to the control and the glutamine-fed culture. Compared to the glutamine and methionine fed culture the specific growth rate was less but the specific production rate was greater. This suggests that tryptophan is able to have a positive effect on antibody production as well as on cell growth. This result goes against the trend of decreasing production rate with increasing growth rate, and therefore suggests that in batch and chemostat culture tryptophan has a positive effect on production rate independently of growth rate.

Overall, these results suggest that feeding amino acids to the second stage of a chemostat had a negative effect on antibody production because it had a positive effect on cell growth. A more satisfactory method of increasing the specific production rate in stage two of a chemostat culture may therefore be to feed the second stage whilst maintaining a low growth rate. This is theoretically possible by increasing the volume of the second stage relative to the first stage so that the dilution rate in the second stage is decreased. The feasibility of achieving this with NB1 hybridoma cells was tested.

6.4.3 TWO-STAGE CHEMOSTAT CULTURE WITH UNEQUAL VOLUMES

A two-stage chemostat was set up in which the dilution rate of the first stage was 0.03h^{-1} feeding into a second stage with an increased volume and therefore a decreased dilution rate of 0.02h^{-1} . The effect of this 'shift down' in dilution rate on growth and monoclonal antibody production was observed when the second stage was not fed, fed with extra glutamine, and fed with all amino acids present in DMEM. The concentration of the amino acid feed into the second stage was equal to the amino acid feed into the first stage. The results are shown in Table 6.4.

The results were taken from one chemostat experiment after four volume changes had passed and a steady state was observed in the second stage. Glutamine was fed into the second stage of the glutamine fed culture at a rate of 17.04 mg/l/h to replace the 17.04 mg/l/h of glutamine utilised in stage-one, calculated from the results for stage-one given in Appendix 9. When all amino acids utilised in stage-one were fed into the second stage the rate at which they were fed (calculated from the results for stage-one in Appendix 9) is given in Appendix 12.

Table 6.4

Effect Of A Shift-Down In Dilution Rate On Second Stage Growth And Monoclonal Antibody Production					
Second Stage Feed	Viable Cell Concentration and Viability ($\times 10^6$ /ml)	Specific Growth Rate (h^{-1})	Antibody Titre ($\mu\text{g}/\text{ml}$)	Specific Production Rate ($\mu\text{g}/10^6$ viable cells/h)	Volumetric Production Rate ($\text{mg}/\text{l}/\text{h}$)
Control	0.99 24%	0.0180	38.93	0.271	0.268
Glutamine	1.66 44%	0.0210	39.78	0.198	0.329
Total Amino Acids	1.65 72%	-0.001	46.09	0.365	0.603

The specific growth rate of the second stage control culture was 0.018 h^{-1} compared to 0.015 h^{-1} in the equal volumed second stage. Having a shift down in dilution rate did not therefore decrease the specific growth rate. Once again the specific production rate decreased as the specific growth rate increased when the second stage was fed with glutamine. When total amino acids were fed, the viability of the culture was increased from 24% for the control to 72%, but the total cell concentration decreased relative to stage one giving a negative growth rate. Specific production rate increased, but the most dramatic increase was seen in the volumetric production rate which increased from $0.268 \text{ mg antibody/l/h}$ to $0.603 \text{ mg antibody/l/h}$.

The second stage of a two-stage chemostat culture therefore appeared to resist attempts to achieve a low specific growth rate and an increased specific production rate through feeding the second stage with extra nutrients. However, increases in growth and antibody productivity were seen in terms of the volumetric rates.

6.5 CONCLUSIONS

- The addition of increased concentrations of individual amino acids had a negative effect on cell growth in batch culture, but in some cases had a positive effect on antibody production. The positive effect on production was likely to be due to the negative effect on cell growth.
- Feeding the second stage of a two-stage chemostat with particular amino acids had a positive effect on cell growth and a negative effect on antibody production, except when glutamine and tryptophan were the feed in which case growth and production increased.
- Reducing the dilution rate in stage two compared to stage one did not decrease the specific growth rate compared to a two-stage chemostat with equal dilution rates in stage one and two. However, it did increase the volumetric production rate.
- Feeding the second stage of a two-stage chemostat decreased the specific production rate of antibody but increased the volumetric production rate, due to the increase in the viability of the second stage.

**7.0 THE EFFECT OF AMINO ACID
LIMITATION ON GROWTH AND ANTIBODY
PRODUCTION DURING BATCH CULTURE OF
NB1 HYBRIDOMA CELLS**

7.1 SUMMARY

Fifteen stirred batch cultures of NB1 hybridoma cells were set up. In each culture the concentration of a different amino acid was reduced in order to observe the effect, on growth and antibody production, of depletion of a specific amino acid. Depending on which amino acid was reduced in concentration in the medium, the percentage of antibody produced during the decline phase of the culture ranged from 2% to 69% of the total. Based on these observations in stirred batch culture, amino acids were classified into those whose depletion had a positive effect on antibody production and those whose depletion had a negative effect on antibody production. This classification was tested in batch and two-stage chemostat culture to see if depletion of all amino acids whose limitation had a positive effect on antibody production could be used to increase the productivity of batch and chemostat culture.

7.2 INTRODUCTION

The addition of extra amino acids in batch and two-stage chemostat culture appears in certain cases to be able to increase antibody production either by increasing the concentration of viable cells and therefore increasing volumetric production, or by increasing the specific production rate. In two-stage chemostat culture the supplementation of the second stage with various amino acids generally resulted in increased specific growth rates and decreased specific production rates. Attempts to maintain a low growth rate while feeding the second stage by decreasing the dilution rate relative to the first stage were unsuccessful, however an increased volumetric production rate was observed. An alternative method is therefore necessary in order to optimise specific antibody production in the second stage of a two-stage chemostat.

In batch culture glutamine has been shown to be an essential amino acid for growth and antibody production. However, it does not necessarily have to be the case that all amino acids are equally important for both growth and production. A concentration of a particular amino acid which is limiting in terms of growth may not be limiting for antibody production. Therefore when growth is limited on that particular amino acid, production may be able to continue in the absence of growth. If such an amino acid(s) was found, it may be possible to limit growth in the second stage of a two-stage chemostat on a nutrient which is more essential for growth than antibody production. Therefore growth will be inhibited but production will have a competitive advantage and will continue, hopefully at a greater specific production rate due to the slowed growth rate of the cell.

In a typical batch culture in serum supplemented DMEM growth and antibody production cease when glutamine is depleted at about 60 hours. Therefore DMEM media were made up in which the concentration of an amino acid intended to become limiting before glutamine was reduced so that under normal batch culture conditions it would have become limiting by 45 hours. Fifteen media were made up each with a different limiting amino acid and a control of normal strength DMEM was included in which glutamine was the limiting nutrient.

The concentrations of the limiting amino acids are given in Appendix 13.

7.3 AIM

The aim of this set of experiments was to reduce the concentration of an individual amino acid in the medium so that it would become the first nutrient to limit growth in batch culture. If production of antibody then continued into the decline phase of the culture it could be assumed that

the limiting amino acid was not so important in terms of production, whereas if growth and production ceased simultaneously this would suggest that the limiting amino acid was necessary for both.

7.4 RESULTS AND DISCUSSION

7.4.1 Batch Culture Limited on Individual Amino Acids

Batch culture experiments were performed in stirred spinner bottles. A compilation of the results are shown in Table 7.1 while specific utilisation rates of amino acids are given in Appendix 10.

The amino acid concentrations from which the utilisation rates in Appendix 10 are calculated are given in Appendix 14.

Control:

At 66 hours glutamine was depleted from the medium and the culture entered the decline phase. In order to make comparisons between cultures grown under different amino acid limitations, the effect of limitation on growth and production was determined by calculating total viable cell hours of the whole culture, antibody produced per viable cell hour, and the percentage of total antibody produced during the decline phase of growth. In the control culture 36% of the total antibody produced was produced during the decline phase and the antibody was produced at a rate of 0.197 $\mu\text{g}/\text{cell hour}$.

Table 7.1

Effect Of Amino Acid Limitation On Growth And Antibody Production In Batch Culture					
Limiting Amino Acid	Maximum Viable Cell Concentration ($\times 10^6/\text{ml}$)	Total Antibody ($\mu\text{g}/\text{ml}$)	Total Viable Cell Hours ($\times 10^{-6}/\text{ml}$)	% Antibody Produced in Decline Phase	Antibody/ 10^6 Cell Hours ($\mu\text{g}/10^6$ cell h)
Tryptophan	0.46	1.94	13.76	0	0.141
Serine	0.83	6.16	42.89	2	0.144
Lysine	1.24	11.03	56.95	18	0.194
Threonine	0.43	6.60	23.05	18	0.286
Methionine	1.04	10.40	53.71	24	0.194
Phenylalanine	1.09	9.48	57.29	25	0.165
Valine	1.17	13.22	55.34	28	0.239
Arginine	1.22	9.68	48.32	30	0.200
Tyrosine	0.97	10.57	54.72	33	0.193
Control	1.72	17.18	87.39	36	0.197
Glycine	1.20	18.66	73.82	37	0.253
Histidine	1.74	17.68	84.35	49	0.210
Cystine	1.15	20.48	66.80	54	0.307
Leucine	1.65	21.77	85.89	62	0.253
Isoleucine	1.62	26.15	102.94	69	0.254

Glycine Limited:

Glycine is both consumed and produced by this cell line in culture, therefore it was not surprising that at 48 hours glutamine and an increased concentration of glycine were present in the medium. Growth therefore continued until 62 hours when glutamine was depleted from the medium. This culture was therefore a reflection of glutamine rather than glycine limitation. 37% of the antibody was produced during the decline phase compared to 36% in the control culture. Glycine can therefore be classed as a non essential amino acid for this cell line. There was no significant change in the rates of amino acids utilised during the first 48 hours in terms of μmoles of amino acid utilised per

viable cell hour.

Lysine, Methionine, Phenylalanine, Valine and Tyrosine Limited:

The lysine, methionine, phenylalanine, valine and tyrosine limited cultures all responded in a similar manner to their limitation. The limiting amino acid and glutamine both reached limiting concentrations at 48 hours, therefore the effect of the limitation in the presence of glutamine was not observed during the decline phase. However, the percentage of antibody produced during the decline phase was less than the 37% observed in the glutamine only limited culture (see Table 7.1). Limitation on these five amino acids individually appeared to have a negative effect on both cell growth and antibody production, reflected by a decrease in total viable cell hours and a decrease in the percentage of antibody produced during the decline phase. However, the amount of antibody produced per viable cell hour was less than the control for lysine, methionine, phenylalanine and tyrosine limitation but greater than the control for valine limitation.

Threonine Limited:

At 48 hours of culture threonine and glutamine were still present in the medium therefore the growth phase continued until 62 hours. However, the growth observed in this medium was very poor with a maximum viable cell concentration of 0.43×10^6 cells/ml compared to 1.72×10^6 cells/ml in the control culture. The threonine concentration was therefore limiting growth. Although the percentage of antibody produced during the decline phase was less than the control at 18%, the concentration produced per viable cell hour was greater than the control at $0.286 \mu\text{g}/10^6$ viable cell hour compared to $0.197 \mu\text{g}/10^6$ viable cell hour. This suggests that threonine limitation has a negative effect on cell

growth but a direct or indirect positive effect on antibody production.

Serine Limited:

Glutamine reached a limiting concentrations at 48 hours at which point growth and production ceased. However serine was produced by the culture. In typical batch and chemostat culture net utilisation and production of serine have both been observed during the course of the same culture (see Chapters 3, 4, and 5). An enhanced capacity for serine synthesis in cancer cells has been reported as well as it being preferentially directed towards the provision of nucleotide precursors (Snell et al., 1987). However, this does not explain the net production of serine in a culture where its concentration was limiting growth unless the cells were unable to maintain a high enough internal concentration of serine. Glutamine showed an increased rate of utilisation during the first 48 hours. The amount of glutamine utilised per viable cell hour increased from 0.121 μ moles to 0.228 μ moles in the serine limited culture. The effect of serine limitation in the presence of glutamine was not observed during the decline phase. However, only 2% of the antibody was produced during the decline phase which is significantly less than the 37% in the glutamine only limited culture.

Tryptophan Limited:

At 48 hours of culture tryptophan and glutamine were still present in the medium, however the growth phase ceased at 16 hours. The growth observed in this medium was very poor with a maximum viable cell concentration of 0.46×10^6 cells/ml compared to 1.72×10^6 cells/ml in the control culture. The tryptophan concentration was having a limiting effect on growth even though it was being produced by the cell. As with serine limitation, glutamine was the only amino acid whose rate of

utilisation was increased significantly by the tryptophan limitation. Glutamine utilisation increased from 0.121 $\mu\text{moles}/10^6$ viable cell hour in the control to 0.348 $\mu\text{moles}/10^6$ viable cell hour in the tryptophan limited culture. Less than 2 $\mu\text{g}/\text{ml}$ of antibody was produced during the culture, and the production rate was the lowest seen at 0.071 $\mu\text{g}/10^6$ viable cell hour.

Arginine Limited:

At 48 hours the culture entered the decline phase even though arginine and glutamine were still present in the medium. However, arginine was produced by the culture rather than consumed. Although arginine was being produced by the cells its concentration in the medium had a negative effect on growth, total viable cell hours were decreased to just over half of the control. Although arginine limitation decreased the percentage of total antibody produced during the decline phase to 30%, it did not have a negative effect on the rate of antibody produced per cell hour. Arginine limitation therefore had a negative effect on cell growth without effecting antibody production except during the decline phase. All amino acids were utilised at a decreased rate in terms of $\mu\text{moles}/\text{viable cell hour}$.

Cystine Limited:

At 48 hours cystine and glutamine were still present in the medium at which point the culture entered a stationary followed by a decline phase. As the culture entered a stationary phase at 48 hours and the concentration of cystine in the medium changed very little from that point until the end of the culture it is likely that although cystine was present its concentration was limiting to growth. 54% of the antibody was produced during the decline phase which is significantly greater

than the 36% produced in the control. This suggests two possibilities: 1) cystine is essential for growth but not for production so that when growth stops production can continue, or 2) the concentration of cystine which is limiting for growth is not limiting for antibody production, therefore when growth stops production is able to continue. Not only was the percentage of antibody produced in the decline phase greater than the control, but also the final antibody titre was greater despite the total viable cell hours being less. The amount of antibody produced per viable cell hour was therefore greater than the control. There was no significant change in the rates of amino acids utilised during the first 48 hours in terms of μ moles of amino acid utilised per viable cell hour.

Histidine Limited:

At 48 hours histidine and glutamine were still present in the medium and growth continued until 66 hours when histidine was depleted but not glutamine. 49% of the total antibody was produced during the ensuing decline phase. Histidine limitation did not therefore appear to have a negative effect on antibody production.

Leucine Limited:

At 48 hours leucine and glutamine were still present in the medium. However, at 48 hours the culture entered the decline phase. At this point enough glutamine was still present in the medium for it to not be limiting to growth suggesting that growth was limited by the decreased leucine concentration. In terms of μ moles utilised/ 10^6 viable cell hour, all the amino acids were utilised to a lesser extent than in the control culture. Antibody production continued during the decline phase with 62% of the total production occurring after leucine was limiting growth. As with cystine limitation, this suggests that 1) leucine is

essential for growth but not for production so that when growth stops production can continue, or 2) the concentration of leucine which is limiting for growth is not limiting for antibody production, therefore when growth stops production is able to continue.

Isoleucine Limited:

At 48 hours isoleucine and glutamine were still present in the medium. However, at 48 hours the culture entered the decline phase. As glutamine was still present at a concentration known not to inhibit growth it was assumed that the concentration of isoleucine was growth-limiting. Decreased rates of utilisation were observed for all amino acids during the growth phase compared to the control culture. Antibody production continued during the decline phase with 69% of the total production occurring after isoleucine was limiting to growth.

Leucine and isoleucine limitation appears to cause an alteration in the kinetics of antibody production from growth-associated production to growth-dissociated production. A possible reason for the change in the kinetics of production may be due to the reported effect of isoleucine limitation on the cell cycle. Ley and Tobey (1970) reported that cultures of Chinese hamster cells (line CHO) when grown in isoleucine depleted medium were arrested in the G1 phase of the cell cycle. A normal cell cycle was reinitiated upon addition of isoleucine. The increased production of antibody during the decline phase is consistent with other observations of increased production coinciding with accumulation of cells in the G1/G0 phase of the cell cycle. This cell line did not demonstrate increased production with accumulation of cells in the G1/G0 phase of the cell cycle under glutamine limiting conditions but the possibility of this occurring under different limitations, such as

leucine and isoleucine, cannot be ruled out.

Based on this set of experiments four groups of amino acids have been identified:

1. Those which are consistently consumed and whose limitation has a negative effect on both growth and production.
i.e. Lysine, Methionine, Phenylalanine, Valine, Tyrosine, Threonine.
2. Those which are produced by the cells but which still have a negative effect at low concentrations on growth and production.
i.e. Serine, Tryptophan, Arginine.
3. Those whose limitation has no effect on growth and production.
i.e. Glycine.
4. Those amino acids whose limitation has a negative effect on growth but a positive effect on production.
i.e. Cystine, Histidine, Leucine, Isoleucine.

If this is the case then it should be possible to limit growth on the amino acids of group 4 in the second stage of a two-stage chemostat culture and observe continued production, or even increased specific production. Limitation on any other amino acids would be expected to decrease antibody production.

7.3.2 Limitation of Batch Culture on Groups of Amino Acids

The conclusions drawn from the previous experiment were tested in stirred batch culture. After 45 hours of culture amino acids were added to the culture at a concentration equivalent to the concentration utilised up to that point, i.e. after 45 hours all amino acids were returned to their original concentration in DMEM, apart from certain amino acids

which were left out. Four cultures were set up as follows: 1. extra glutamine added; 2. all amino acids added; 3. all amino acids added except for those whose limitation had a negative effect on growth but a positive effect on production (i.e. Group 4); 4. all amino acids added except for leucine and isoleucine.

It would be expected that when amino acids whose limitation is thought to have a positive effect on production are not added to a culture then antibody production should be greater than in the controls of glutamine and total amino acid fed cultures. As leucine and isoleucine limitation had the greatest positive effect on production they were jointly left out of one culture. A major assumption made in this experiment was that by not adding a particular amino acid whilst adding all others that particular amino acid would be at a limiting concentration in the culture, however this could not be guaranteed. The results of the experiment are shown in Table 7.2.

Table 7.2

Results of Supplementing Batch Culture With All DMEM Amino Acids Except For Those Whose Limitation Had A Positive Effect On Antibody Production			
Culture Supplementation	Final Antibody Titre (ug/ml)	Total Viable Cell Hours (x10⁻⁶/ml)	Antibody/10⁶ Viable Cell Hours (ug/10⁶cell h)
Glutamine	58.16	184.83	0.315
All DMEM Amino Acids	52.03	160.97	0.323
All DMEM Amino Acids Except Those In Group 4	52.27	165.99	0.315
All DMEM Amino Acids Except Leucine and Isoleucine	68.85	165.11	0.417

Adding extra amino acids to the batch cultures had a negative effect on cell growth compared to the glutamine only supplemented culture. It also had a negative effect on total antibody produced while having no significant effect on the rate of antibody production per viable cell hour. The exception was when leucine and isoleucine were omitted from the added amino acids. In this case the amount of antibody produced per viable cell hour was 25% greater than the other cultures. This is in keeping with the previous results showing that leucine and isoleucine limitation had a positive effect on antibody production in batch culture. This result can be more stringently tested under the steady state conditions experienced in chemostat culture.

7.4.3 Two-Stage Chemostat Culture

A two-stage chemostat culture was set up in which the first stage was fed with serum supplemented DMEM at a dilution rate of 0.03 h^{-1} , while the second stage was fed with all amino acids present in DMEM, all amino acids present in DMEM except for those whose limitation had a positive effect on antibody production in batch culture, and finally all amino acids except for isoleucine at a dilution rate of 0.02 h^{-1} . The results are shown in Table 7.3.

For amino acid concentrations in second stage feed see Appendix 15.

Table 7.3

Results of Feeding Second Stage Chemostat Culture With All DMEM Amino Acids Except For Those Whose Limitation Had A Positive Effect On Antibody Production				
Second Stage Feed	Dilution Rate (h^{-1})	Specific Growth Rate (h^{-1})	Antibody Production Rate Volumetric (mg/l/h)	Specific ($\mu\text{g}/10^6$ cells/h)
All DMEM Amino Acids	0.02	-0.039	0.347	0.424
All DMEM Amino Acids Except Those In Group 4	0.02	0.005	0.172	0.144
All DMEM Amino Acids Except Isoleucine	0.02	-0.024	0.194	0.216

Feeding the second stage with anything other than all the amino acids present in DMEM had a negative effect on specific and volumetric rates of antibody production. However, leaving out only isoleucine from the feed resulted in higher productivity than leaving out all group 4 amino acids, as was observed in batch culture. The reason for this decrease in productivity is probably because the culture was not actually limited on

isoleucine or any of the other amino acids left out of the feed. A substantial amount of isoleucine is left over from stage one which is unlikely to be totally utilised in stage two. To overcome this, a medium needs to be made up with a reduced concentration of isoleucine so that less remains after the first stage. Time did not allow for this to be done.

7.5 CONCLUSIONS

- Amino acids can be classified into those whose limitation has a positive effect on antibody production, and those whose limitation has a negative effect on antibody production.
- The composition of the medium in which this cell line is grown can affect its production kinetics. Depending on which amino acid is first to limit growth, the majority of the antibody can be produced in the growth phase of the culture or in the stationary and decline phase. Therefore media can be designed to alter the kinetics of antibody production and to meet the requirements of particular production processes
- An amino acid may be at a concentration limiting to growth even if it is present in the culture medium.
- The way in which an amino acid limitation has a positive effect on antibody production is as yet unknown, but lends support to the theory suggesting competition between growth and production processes.

**8.0 EFFECT OF AMINO ACID
CONCENTRATION ON UTILISATION BY NB1
HYBRIDOMA CELLS**

8.1 SUMMARY

A linear relationship between the initial concentration of an amino acid and the extent to which it was depleted from the medium was observed in batch and chemostat culture. When the initial concentration of a particular amino acid was reduced the extent to which it was depleted from the medium was also reduced. Cells were grown in media in which all amino acids were present at the same initial concentration. The effect this had on growth and antibody production was small. The specific rate of amino acid utilisation was decreased when they were all present in the medium at an equal low concentration. Increasing the concentration of amino acids in the medium led to their wasteful utilisation without any benefit in terms of increased cell concentration or antibody titre.

8.2 INTRODUCTION

Amino acid utilisation and transport in animal cell cultures has been extensively reviewed by Patterson (1972), Wheatley *et al.* (1986) and Christensen (1990). Mammalian cells appear to make relatively few of their own amino acids and rely upon an exogenous supply. They also appear to use non-essential amino acids from the surrounding medium when these are available. There are three main pathways by which amino acids may enter cells: by simple diffusion, by facilitated diffusion, and by active transport. Although amino acids are charged molecules under physiological conditions, and therefore thought by some not to be freely diffusible across lipid membranes (Bender, 1985), there is evidence to suggest that amino acids can move rapidly and in large amounts across cell membranes in the absence of an energy source in a manner similar to other penetrating solutes (Wheatley *et al.*, 1986). Simple diffusion however is likely to be too slow to meet the demands of the cell unless assisted by some form of endocirculation. There is also

the problem of how cells retain and concentrate such molecules relative to the external environment. Facilitated diffusion is thought to offer another explanation for amino acid transfer, and it is generally assumed that there are protein molecules in the cell membrane which recognise amino acids, couple with them, and undergo a conformational change resulting in their flipping over to bring the attached amino acid to the internal side of the membrane. A further conformational change releases the amino acid and the carrying molecule returns to its original state. The process is believed to operate to drive amino acid molecules into the cell against a concentration gradient. Active transport has been the preferred method of explaining amino acid uptake for many years. It helps to explain the transfer of hydrophilic molecules across lipid membranes, it can account for the accumulation of amino acids against steep concentration gradients, and it offers a solution to the quasi-enzymic kinetics of uptake which some amino acids seem to follow.

Transporting systems have been characterised by Christensen (see Christensen, 1990). They are distinguished on the basis of competition between amino acids with one another for entry into the cell. Some show a preference for certain types of amino acids such as leucine, valine and phenylalanine, others for alanine, glycine and serine. Although they are relatively specific for each group of amino acids, they can also carry amino acids from other groups with lower affinity. In mammalian red blood cells, six transport systems have been identified which carry a discrete group of amino acids as well as two broad systems with wide specificities (Ellory, 1987). The main systems identified in mammalian cells are: Transport System A with preferred substrates of alanine, proline, serine, glycine and methionine; Transport System L with preferred substrates of leucine, isoleucine,

phenylalanine, methionine and valine; Transport System ASC with preferred substrates of alanine, serine and cystine; and Transport System Ly^+ with preferred substrates of lysine and arginine. As yet, none of these proposed transport systems have been characterised in molecular terms and their identification remains based on physiological studies, however, a number of mutant cell types have been identified which have difficulty in obtaining certain amino acids. Wheatley *et al.* (1986) have proposed that as amino acids are freely diffusible into the cell, there is a real possibility that the cell spends its energy holding molecules within its cytoplasm rather than in bringing them inside, transport into the cell depending on non-energy requiring diffusion systems.

It was noted in Chapter 6 that by adding double the concentration of particular amino acids to batch culture a decrease in the maximum viable cell concentration and in the total number of viable cell hours was observed. It was suggested that this might be due to competition between amino acids for transport into the cell, resulting in some essential amino acids limiting growth. Also, it has been observed that the specific rate of utilisation of a particular amino acid is greatest at the start of a batch culture, and steadily declines throughout the rest of the culture. This could either be due to a requirement by the cell for nutrients necessary for increased metabolic activity, or it may be due to the high concentration of an amino acid present at the start of a culture, reflecting an important role for diffusion as a method for transporting amino acids into the cell.

8.3 AIM

The aim of this set of experiments was to observe the effect of amino

acid concentration on its utilisation by the cell. This was achieved by assessing any correlation between amino acid concentration and utilisation in batch and chemostat culture, and by growing NB1 hybridoma cells in medium containing all the DMEM amino acids at equal concentrations, to see how this effected utilisation.

8.4 RESULTS AND DISCUSSION

8.4.1 Typical Batch and Two-Stage Chemostat Culture

The observed relationship between the initial concentration of an amino acid in DMEM and the extent to which it was utilised by the cell in a typical batch culture is shown in Figure 8.1.

Figure 8.1 The observed correlation between the initial concentration of an amino acid in DMEM and the extent to which it is utilised by a cell population during the course of a glutamine-fed batch culture.

In batch culture of NB1 hybridoma cells a high correlation between the

initial concentration of an amino acid and the extent to which it was utilised was consistently observed. In single-stage chemostat culture the correlation was equally high, as shown in Figure 8.2.

Figure 8.2 The observed correlation between the initial concentration of an amino acid in DMEM and the extent to which it is utilised by a cell population in single-stage chemostat culture at a dilution rate of 0.03h^{-1} .

In batch and chemostat culture the correlation between initial concentration of an amino acid in DMEM medium, and the amount which was utilised suggests that the greater the initial concentration, the more that particular amino acid was utilised. This correlation may either be a reflection of the actual demand for an amino acid by the cell, and therefore suggests a well designed medium, or it may be a reflection of the effect concentration has on transporting amino acids into the cell. If amino acid transport was a simple diffusion process than it would be expected that the more of an amino acid there was present in the

medium the greater would be its diffusion into the cell. Alternatively, if amino acid transport systems are shared between several amino acids, unless the transport system was very selective and only transported particular amino acids when they were in demand then concentration would play a part in deciding how much of a competing amino acid was transported into the cell.

When cells were grown in medium limiting in a particular amino acid other than glutamine, the decreased concentration of that amino acid was reflected by a decrease in its utilisation by the cell. Once again this suggests that the concentration of an amino acid in the medium plays a significant role in the extent to which it is utilised. This is demonstrated for isoleucine utilisation during an isoleucine limited batch culture in Figure 8.3. Data taken from Chapter 7 and Appendix 14.

Figure 8.3 The observed correlation between the initial concentration of an amino acid in DMEM and the extent to which it is utilised by a cell population during the course of an isoleucine limited batch culture.

Isoleucine utilisation in DMEM is usually similar to that of leucine, because their concentrations are similar (Figure 8.1), however its utilisation has been decreased in line with its concentration in an isoleucine limited medium.

In the second stage of a two-stage chemostat culture the correlation between concentration and utilisation of amino acids did not suggest any relationship between the two, as shown in Figure 8.4.

Figure 8.4 The observed correlation between the initial concentration of an amino acid in DMEM and the extent to which it is utilised by a cell population in the second stage of a two-stage chemostat culture at a dilution rate of 0.03h^{-1} .

The results for correlation between amino acid concentration and utilisation in two-stage chemostat culture are given in Table 8.1.

Table 8.1

Correlation In Two-Stage Chemostat Culture Between Initial Concentration and Utilisation Of Amino Acids		
Dilution Rate (h ⁻¹)	Stage 1 Correlation	Stage 2
0.05	0.97	0.47
0.04	0.97	0.75
0.03	0.97	0.65
0.02	0.98	0.56
0.01	0.98	0.39

These results suggests that the correlation observed in batch and single-stage chemostat culture may be incidental, or that when the concentration of amino acids in the medium is reduced then diffusion is not such an important method of entry into the cell, or that when glutamine and other amino acids become limiting then the relationship between concentration and utilisation breaks down, .ie. that the external concentration of the amino acid is approaching the value of the affinity constant for that nutrient.

The obvious way of finding out the relative importance of particular amino acids in the medium, and whether their utilisation is a reflection of demand by the cell or their concentration in the medium, would be to grow the cells in a medium in which all amino acids were at an equal initial concentration. Two media were made up, one in which all amino acids were at a concentration of 0.8 mM, and a second in which all amino acids were at a concentration of 321 mg/l. In each case the

glutamine concentration was equal to that of standard DMEM, 4.0 mM. However, two sets of cultures were performed in each medium, one was fed with an extra 2.0 $\mu\text{moles/ml}$ of glutamine at the end of the exponential phase, the other was not.

8.4.2 Batch Culture With Equal Concentrations of Amino Acids

In the control culture a strict correlation between initial amino acid concentration and $\mu\text{moles/ml}$ utilised was observed as before. In the culture in which all the amino acids were at an equal molarity an order of utilisation based on requirement by the cell rather than concentration was observed. The order of priority in terms of $\mu\text{moles/ml}$ of amino acid utilised by the cell was very similar for the two cultures as shown in Table 8.2, in which a negative value indicates production.

TABLE 8.2

Amino Acids In Order Of μ moles Utilised per Viable Cell Hour When Present At Equal Concentrations In The Medium					
Control (μ moles/ 10^6 cell h)		All Amino Acids At 0.8 mM (μ moles/ 10^6 cell h)		All Amino Acids At 321 mg/l (μ moles/ 10^6 cell h)	
GLN	0.0233	GLN	0.0222	GLN	0.0373
LEU	0.0020	LEU	0.0014	LEU	0.0048
THR	0.0016	TYR	0.0012	ILE	0.0043
VAL	0.0016	VAL	0.0012	VAL	0.0038
LYS	0.0015	THR	0.0012	THR	0.0033
ILE	0.0014	ILE	0.0010	LYS	0.0027
ARG	0.0011	LYS	0.0010	SER	0.0024
TYR	0.0008	ARG	0.0008	ARG	0.0020
PHE	0.0007	PHE	0.0004	CYS	0.0017
MET	0.0005	SER	0.0003	GLY	0.0016
HIS	0.0004	CYS	0.0002	PHE	0.0016
CYS	0.0004	MET	0.0001	HIS	0.0016
GLU	0.0003	GLU	0.0000	MET	0.0015
SER	0.0002	ASP	0.0000	TYR	0.0011
ASP	0.0000	HIS	-0.0000	TRP	0.0002
ASN	-0.0001	ASN	-0.0002	ASP	-0.0002
GLY	-0.0002	GLY	-0.0004	ASN	-0.0004
TRP	-0.0003	TRP	-0.0004	GLU	-0.0008
PRO	-0.0019	PRO	-0.0027	PRO	-0.0031
ALA	-0.0069	ALA	-0.0071	ALA	-0.0136

Although higher concentrations of most of the amino acids were present in the 0.8mM equal concentration culture, the μ moles utilised per viable cell hour tended to be less, except in the case of serine and tyrosine. When all amino acids were at an equal molarity the total number of viable cell hours was slightly less, as was the concentration of antibody produced per viable cell hour (Table 8.3). However, the fact that the difference was so small may be a reflection of the fact that the glutamine concentration was the same in both cultures, and growth and production was more dependent on the depletion of glutamine than on any of the

other amino acids which were present in excess.

Table 8.3

Cell Growth And Antibody Production When Amino Acids Were At Equal Concentrations In The Medium		
Medium	Total Viable Cell Hours ($\times 10^{-6}$ /ml)	Antibody Production ($\mu\text{g}/10^6$ cell h)
DMEM	164.65	0.185
DMEM GLN-Fed	240.38	0.200
8mM Amino Acids	131.57	0.204
8mM Amino Acids GLN-Fed	232.93	0.188
321mg/l Amino Acids	182.69	0.226
321mg/l Amino Acids GLN-Fed	163.89	0.222

Data for maximum viable cell concentration and concentration of antibody produced is given in Appendix 16.

When the amino acids were present at an equal concentration in terms of mg/l, their concentration in mM was greater than 1mM, except for cystine and tryptophan. The order of amino acids in terms of μmoles utilised was very similar to the control and the equal molarity cultures (Table 8.2). However, the μmoles of amino acids utilised per viable cell hour was significantly greater when a much higher concentration of amino acid was present. The higher concentration of amino acids utilised was not reflected in a higher viable cell concentration, or a greater production of antibody (Table 8.3). This would suggest that at high concentrations amino acids enter the cell unnecessarily and are

wastefully metabolised resulting in increased rates of alanine production (Table 8.2).

8.5 CONCLUSIONS

- The initial concentration of an amino acid in the medium appears to affect the rate at which it is utilised, and can therefore lead to wasteful utilisation of certain amino acids.
- Increasing the concentration of amino acids in the medium can lead to their wasteful utilisation without any benefit to the cell.
- Increasing the concentration of one amino acid may limit the availability of another due to competition for entry into the cell.
- When amino acids are present in the medium at high concentrations, for example at the beginning of a batch culture, and in the first stage of ~~as~~ two-stage chemostat, diffusion into the cell appears to be the main mode of entry, as demonstrated by the concentration effect on utilisation. However, as amino acids become depleted from the medium, for example in the second stage of a two-stage chemostat, selective transport mechanisms may become more important enabling the cell to be more selective in the particular amino acids it takes in and the rates at which it takes them in.
- By adding all amino acids at an equal molar concentration, growth and production were not increased. This suggests that the differential in concentrations between amino acids in DMEM did not result in any particular amino acid being limiting. However, the specific rate of amino acid utilisation was decreased when they were present in the

medium at an equal molarity.

9.0 DISCUSSION

Monoclonal antibody production by hybridoma cells has been studied under a number of different culture conditions which have included batch culture, fed-batch culture, continuous culture, perfusion culture with and without total cell retention, single-stage chemostat culture, and now two-stage chemostat culture. Kinetics of growth and antibody production have been studied in these systems as well as the influence of various nutritional and physical conditions. A general consensus has been reached from these studies that factors which have a negative influence on cell growth will often have a positive influence on specific rates of antibody production, and that maintaining the viability of the cell culture under these stressful conditions, rather than continued growth, is the key to increased specific and volumetric production. This has led to studies on the manipulation of the cells physical environment to inhibit growth while increasing the specific antibody production rate by osmotic stress (Oyass *et al.*, 1989; Ozturk *et al.*, 1992; Oh *et al.*, 1993), temperature extremes (Merten *et al.*, 1985), low dissolved oxygen tension (Reuveny *et al.*, 1986), low or high pH (Miller *et al.*, 1988), and even the use of metabolic inhibitors to inhibit growth but not antibody production (Modha, 1990). Perfusion culture has been used to limit cell growth while maintaining high production rates. The unhelpful paradox of this situation is that factors which increase the specific production rate of antibody by the cell tend to reduce the viability of the culture, and therefore do not necessarily result in increased volumetric productivity.

The aims of this investigation were to explore the feasibility of growing animal cells in a two-stage chemostat system and to use such a system to study the growth-associated and growth-dissociated monoclonal antibody production kinetics displayed by hybridoma cell lines in batch culture.

The advantages of a two-stage chemostat culture system over batch are that it allows the controlled manipulation of culture conditions and cell growth rate during a constant and reproducible steady state, as opposed to the constantly changing physiological and cellular conditions experienced in batch culture. A two-stage chemostat system is the ideal medium in which to study growth-dissociated production kinetics. The feasibility of using single-stage chemostat culture is limited due to its essential requirement for cell growth, therefore studies on production by a non-growing population are not possible. Because the second stage of a two-stage system is continually inoculated by the first stage, growth is not a prerequisite of operation, therefore non growth-associated product formation can be studied under steady state conditions of low and zero growth rate.

Initially the kinetics of growth and antibody production by the hybridoma cell line NB1 were studied in batch culture. Cell growth and antibody production ceased when glutamine was depleted at the end of the exponential phase of growth. This cell line therefore exhibited the characteristics of growth-associated antibody production as classified by Pirt (1975) and Merten *et al.* (1987). However, when batch culture was supplemented with extra glutamine growth stabilised at a reduced rate during an extended stationary phase while specific production rate demonstrated a second peak in a partial cubic spline plot. The specific production rate during the stationary phase was equal to, but not greater than, the production rate during the exponential phase suggesting that production was still growth-associated, or partially growth-associated. Negatively growth-associated production would be expected to be demonstrated by an increase in specific production rate during the decreased growth rate of the stationary phase, instead it remained the

same. Supplementing batch culture with extra essential and non-essential amino acids resulted in increased growth rates and increased specific production rates, typical of growth-associated production. However, the possibility that the increased specific production rate was due to the extra amino acids rather than the increase in specific growth rate was not ruled out. The linear correlation between antibody concentration and viable cell hours, and the fact that the majority of the antibody was produced during the growth phase, provided evidence for antibody secretion from viable cells rather than release of an intracellular store by dead cells, as demonstrated by Renard *et al.* (1987).

In general, the kinetics of antibody production observed in batch culture have also been observed in chemostat culture, resulting in reports of positively growth-associated antibody production (Low *et al.*, 1987; MacMichael, 1989), inversely growth-associated production (Boraston *et al.*, 1984; Reuveny *et al.*, 1986; Miller *et al.*, 1988; and Frame and Hu, 1991), as well as growth-dissociated production (Ray *et al.*, 1989; Hiller *et al.*, 1991).

In this study single-stage chemostat culture demonstrated the negative association between growth and production which had been hinted at in batch culture. Increasing specific rates of antibody production with decreasing growth rate were observed, this is typical of negatively growth-associated antibody production. The fact that positively growth-associated and negatively growth-associated antibody production was observed by the same cell line but under different culture conditions suggested that characteristics of production may depend on the culture conditions rather than being absolutes determined by the cell. It is possible that when the growth rate of the cell population was decreased

by nutrient limitation, specific antibody production increased due to its increased competitive advantage. Factors, such as the accumulation of cells in the G1/G0 phase of the cell cycle are to the advantage of the production process. The growth demands on the cell are lessened during the G1/G0 phase of the cell cycle because all of its macromolecular synthesis pathways are known to cease, or be reduced, apart from protein synthesis, therefore the competition for common nutrients and pathways between growth and production is reduced.

In the second stage of a two-stage chemostat system increased specific production rates were observed with decreasing growth rate, as in the first stage. An increase in the specific production rate in the second stage compared to the first stage, even though the growth rates were less, was not observed except at the lowest dilution rates when the specific growth rate was negative. This again indicates that, as in the stationary phase of batch culture, decreased growth rate alone does not necessarily result in increased specific antibody production. An increase in the specific production rate of the cell would appear to depend on the nutrients available to the cell (therefore greater production in stage one compared to stage two), and a low growth rate (increased specific production rate with decreasing growth rate). However, an increased specific production rate is also observed when growth is negative in the second stage and nutrients are scarce, suggesting a change in the physiology of the cell.

Providing the correct nutritional environment for increased specific production while maintaining a low growth rate was attempted by feeding the second stage with amino acids. A second stage amino acid feed resulted in an increased growth rate and, consistent with negatively

growth-associated production, a decrease in specific production rate. Even when a shift down in growth rate was attempted, by decreasing the dilution rate in the second stage relative to the first, growth rate remained high and production rate low in a fed compared to unfed culture. Feeding the second stage with all DMEM amino acids did however have the effect of increasing the volumetric rate of production, due to the increased viability of the culture. An alternative method of achieving a low growth rate and high production rate in the second stage was therefore required.

The observation that in glutamine limited batch culture antibody production was positively growth-associated while in glutamine depleted chemostat culture production was negatively growth-associated suggested the possibility that production kinetics could be manipulated by altering the nutrients available to the cell. If limitation of growth on an amino acid other than glutamine, which appeared essential for both growth and antibody production, resulted in continued or increased production during the decline phase of batch culture, it may also be possible to limit growth in the second stage of a two-stage chemostat and observe increased specific production.

The observation that it was possible to manipulate the kinetics of antibody production in batch culture was demonstrated by designing media with different growth-limiting amino acids. Depending on the identity of the growth-limiting amino acid the kinetics of antibody production could be altered from strictly growth-associated, as with serine limitation, to growth-dissociated, as with isoleucine and leucine limitation. The change in cellular physiology which occurred under these different limitations, and which gave rise to the observed

alterations in production kinetics was not determined. The change may result directly from the antibody production process requiring a specific amino acid, or indirectly through a change in the growth and physiology of the cell, such as accumulation of Chinese hamster cells in the G1/G0 phase of the cell cycle under isoleucine limiting conditions (Ley and Tobey, 1970). The same result has yet to be demonstrated in two-stage chemostat culture possibly because the concentration of isoleucine was not low enough to be the growth-limiting nutrient.

These findings suggest that the classification of hybridoma cell line production kinetics into growth-associated and growth-dissociated may not be a true reflection of an inherent characteristic of the cell but merely a reflection of the cells response to its environment, in particular nutrient limitation of growth only or growth and antibody production. The fact that production is often observed during the decline phase of a cell population, and that production continues or is increased under conditions where cell growth is inhibited demonstrates that the requirements for antibody production are less stringent than for growth.

Hybridoma cells are selected primarily for their ability to grow under particular culture conditions. The cells ability to produce antibody, although essential, is secondary to its selection for growth. Growth is therefore the primary concern of the cell and antibody production processes have been selected to survive on 'scraps' in terms of nutrients and energy. That antibody production is not essential to the survival of hybridoma cells is demonstrated by the selection of growing non-producers originating from a population of producers. Antibody production has therefore been selected for on the basis that it can continue in the presence of the cells over-riding need to grow. Its

requirements must therefore be less stringent than those for growth. If the metabolic burden of antibody production was too great on the cell it would have a negative effect on growth, such a cell would be out competed by those with a less demanding production process because of their ability to grow faster. Therefore selection would favour non-producers or producers with an undemanding or less demanding antibody production process. For this reason selection would favour the development of hybridoma cell lines which grow when nutrients are available and environmental conditions ideal for growth with a minimum of antibody production. When conditions are no longer favourable for growth, production can continue at an increased rate because it is no longer competing with growth. Conditions which are detrimental to growth are not necessarily detrimental to production because production has been selected to survive on a lower nutrient and energy requirement, and when growth is inhibited by stressful conditions more nutrients and energy are available for antibody production. Therefore, as has been shown for this cell line, it is possible to selectively inhibit growth by nutrient limitation while not affecting or even increasing specific production in batch culture, so that a cell line which initially was thought to exhibit growth-associated production kinetics was shown to be capable of growth-dissociated production kinetics in a different environment.

In conclusion, it would appear that the kinetics of production observed in batch culture are the result of conditions imposed on the cell by its environment rather than an inherent characteristic of the cell, and that the growth and production kinetics observed can be explained by assuming that growth and production systems inside the cell are competing for a common pool of resources.

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APPENDICES

APPENDIX 1.

Dulbecco's Modified Eagle Medium Formulation

	Concentration	
	(mg/l)	(mM)
Inorganic Salts		
CaCl ₂	200.00	
Fe(NO ₃).9H ₂ O	0.10	
KCl	400.00	
MgSO ₄	97.67	
NaCl	6400.00	
NaH ₂ PO ₄ .H ₂ O	125.00	
Other Components		
D-Glucose	4500.00	
Phenol Red	15.00	
Amino Acids		
L-Arginine.HCl	84.00	
L-Arginine		0.40
L-Cystine.2HCl	62.57	
L-Cystine		0.20
L-Glutamine	584.00	4.00
Glycine	30.00	0.40
L-Histidine HCl.H ₂ O	42.00	
L-Histidine		0.20
L-Isoleucine	105.00	0.80
L-Leucine	105.00	0.80
L-Lysine HCl	146.00	
L-Lysine		0.80
L-Methionine	30.00	0.20
L-Phenylalanine	66.00	0.40
L-Serine	42.00	0.40
L-Threonine	95.00	0.80
L-Tryptophan	16.00	0.08
L-Tyrosine (disodium salt)	104.20	
L-Tyrosine		0.40
L-Valine	94.00	0.80
Vitamins		
D-Ca pantothenate	4.00	
Choline Chloride	4.00	
Folic Acid	4.00	
i-Inositol	7.20	
Nicotinamide	4.00	
Pyridoxal HCl	4.00	
Riboflavin	0.40	
Thiamine HCl	4.00	

APPENDIX 2.

MEM Amino Acid Solution - 50X

Amino Acids	Concentration (mg/l)
L-Arginine HCl	1050.00
L-Cystine	1050.00
L-Histidine	400.00
L-Isoleucine	1300.00
L-Leucine	1300.00
L-Lysine HCl	1823.50
L-Methionine	375.00
Phenylalanine	825.00
L-Threonine	1200.00
L-Tryptophan	200.00
L-Tyrosine	900.00
L-Valine	1175.00

APPENDIX 3.

MEM Non-Essential Amino Acids Solution - 100X

Amino Acids	Concentration (mg/l)
L-Alanine	890.00
L-Asparagine.H ₂ O	1500.00
L-Aspartic Acid	1330.00
L-Glutamic Acid	1470.00
Glycine	750.00
L-Proline	1150.00
L-Serine	1050.00

APPENDIX 4.

MEM Vitamins Solution - 100X

Components	Concentration (mg/l)
NaCl	8500.00
D-Ca Pantothenate	100.00
Choline Chloride	100.00
Folic Acid	100.00
i-Inositol	200.00
Nicotinamide	100.00
Pyridoxal HCl	100.00
Riboflavin	10.00
Thiamine HCl	100.00

APPENDIX 5.

Amino Acid Analysis by HPLC

1. Amino Acid Derivatisation

Solutions to be made up:

a) Drying Solution

200 μ l Methanol

200 μ l 1M Sodium Acetate

100 μ l Triethylamine (TEA)

b) Derivatising Agent:

Prepared just before derivitisation

50 μ l PITC

350 μ l Methanol

50 μ l TEA

50 μ l MQ Water

Procedure:

20 μ l of sample, standard (Food Hydrolysate Standard, Sigma) or blank (0.1M HCl) was pipetted into a labelled glass culture tube. This was loaded into a vacuum vial, a vacuum pulled and the sample dried for 30 - 45 minutes.

10 μ l of drying solution was added to each tube, vortexed and dried again (30 mins).

Derivatisation was achieved by adding 20 μ l of derivitising agent, leaving for 20 mins at room temperature and then drying again (30 - 45 mins).

10 μ l of methanol was then added, vortexed and finally dried thoroughly (45 - 60 mins).

Derivitised samples could be stored at -20 C for up to two weeks.

2. HPLC Analysis

Reagents:

Eluent A

19.0 g of sodium acetate trihydrate.

1 litre of water

0.5 ml TEA

Titrated to pH 6.40 with glacial acetic acid and filtered through a 0.2 μ m filter. 940 ml of the resulting solution was added to 60 ml acetonitrile.

Eluent B

600ml of acetonitrile was added to 400 ml of MQ water, after which it was degassed by sonicating under vacuum for 20 seconds.

Sample Diluent

710 mg of disodium hydrogen phosphate (Na_2HPO_4)

1 litre of water

Titrated to pH 7.40 with 10% phosphoric acid (H_3PO_4)

The resulting solution was mixed with acetonitrile so that acetonitrile equalled 5% by volume.

Procedure:

The samples were resuspended in 100 μ l sample diluent by sonicating and vortexing. Analysis was achieved using a "Nova-Pak" C18 (Waters) HPLC column and the concentration of amino acids determined by comparison of peak area and retention times with standards.

APPENDIX 6

Double-Antibody Sandwich ELISA for Estimation of Mouse IgM

REAGENTS

a) Coating Buffer (0.05M carbonate, pH 9.6)

Na_2CO_3	0.159g
NaHCO_3	0.293g
NaN_3	0.02g
distilled H_2O to	100ml

Stable 7 days at 4°C.

b) Wash Buffer (PBS-Tween, pH 7.4)

Stored as 10x concentrate at 4°C.

NaCl	80.0g
KH_2PO_4	2.0g
Na_2HPO_4 (anhydrous)	19.2g
KCl	2.0g
distilled water to	1000ml

Concentrate diluted 1:10 and 0.5ml/litre Tween 20 added before use.

c) Sample/conjugate buffer (0.1M Tris, pH 7.0).

Tris (hydroxymethyl) aminomethane	6.05g
NaCl	2.922g
Tween 20	0.1ml
BSA Fraction V (Sigma 4503)	1.0g

Distilled water to 450ml, pH adjusted to 7.0 with concentrated

HCl, and made up to final volume of 500ml with distilled water.
Made up fresh weekly.

d) Substrate Buffer (0.1M Sodium acetate/citrate, pH6.0)

Sodium Acetate 2.1g

Distilled water to 90ml. pH adjusted to pH 6.0 with 1M citric acid (21g citric acid in 100ml distilled water) and made up to final volume of 100ml with distilled water. Stored 1 month at 4°C.

e) Substrate Stock

Tetramethylbenzidine 0.01g

DMSO 1.0ml

Stored ambient in dark for 2 weeks.

f) Hydrogen Peroxide

1 part 30% H₂O₂ diluted with 68 parts 0.1M acetate/citrate buffer (10µl + 680µl). Stored ambient for up to 2 weeks.

g) Coating Antisera

Mouse IgM (µ chain specific) from goat (Sigma, code no. M8644). Reconstituted in 0.135M NaCl and diluted to a working concentration of 2µg/ml (100µl + 49,900µl) in coating buffer prior to use.

h) Standards

Mouse IgM, Kappa (Sigma, code no. M2770). Stored in 25µl aliquots at -20°C at a concentration of 1mg/ml. Diluted 1:100 (10µl + 990µl sample/conjugate buffer); and by a further 1:100

(10 μ l + 990 μ l) to give a 1,000ng/ml working stock. This working stock was diluted further to give a range of standards between 10 - 100ng/ml.

i) Conjugate

Goat-anti-mouse (μ chain specific) horse radish peroxidase conjugated (Sigma, code no. A8786). Stored at 4°C. Used diluted in sample/conjugate buffer 1:4,000 (12.5 μ l in 50ml buffer).

PROTOCOL

1. Controls

- a) no coating
- b) no coating/ no sample or standard
- c) no sample or standard
- d) no conjugate
- e) quality control

to check for non-specific binding (a, b, c), peroxidase in reagents (d) and for consistency of ELISA (e).

- 2. Nunc Immunoplate I (2-39454) were coated with 100 μ l per well coating antiserum (or control coating buffer) overnight at 43C with plates wrapped in cling film (also for all subsequent incubation steps), after which they were washed 4x with PBS-Tween buffer and tapped dry.
- 3. Standards or samples were added, 100 μ l per well, and left for 2 hours at room temperature on a plate shaker, then washed and dried as above.

4. 100µl per well of conjugate was then added and the plates were incubated for 1 hour at room temperature on the plate shaker. The plates were then washed as above, the last wash being left on for 10 minutes while the substrate was prepared.
5. Substrate Preparation.
 - a) 1 part TMB stock was diluted in 100 parts acetate/citrate buffer.
 - b) 100µl H₂O₂ solution was added to every 10ml of diluted TMB.
6. 100 µl substrate was added per well after which the plates were wrapped in tin foil and incubated in the dark at room temperature for 1 hour. The reaction was stopped with 50µl 2.5M H₂SO₄ per well and read at 450nm on a plate reader, blanking on an empty well.
7. A standard curve was plotted and concentrations of the samples read from the linear area of the curve.

APPENDIX 7

Amino Acid Concentrations in Non Glutamine-Fed and Glutamine Fed Batch Culture

Amino Acid Concentrations in Batch Culture of NB1 Hybridoma Cells										
Amino Acid	Amino Acid Concentration (mM)									
Time (hrs)	0.0	22.5	34.5	47.5	58.5	71.5	83.0	98.5	123.0	146.0
ASP	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GLU	0.06	0.05	0.03	0.02	0.00	0.00	0.00	0.00	0.00	0.02
ASN	0.00	0.04	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00
SER	0.34	0.32	0.29	0.27	0.23	0.26	0.26	0.24	0.23	0.25
GLN	3.22	2.44	1.71	0.61	0.09	0.04	0.03	0.03	0.02	0.03
GLY	0.26	0.26	0.25	0.26	0.26	0.24	0.30	0.33	0.34	0.38
HIS	0.16	0.13	0.12	0.10	0.09	0.08	0.09	0.09	0.09	0.09
ARG	0.34	0.33	0.29	0.23	0.18	0.18	0.19	0.22	0.23	0.24
THR	0.63	0.47	0.43	0.42	0.35	0.35	0.36	0.35	0.36	0.39
ALA	0.26	0.47	0.63	0.92	0.99	0.98	1.06	1.08	1.06	1.14
PRO	0.05	0.09	0.12	0.18	0.21	0.23	0.25	0.26	0.25	0.27
TYR	0.31	0.23	0.25	0.30	0.22	0.22	0.24	0.23	0.23	0.25
VAL	0.69	0.62	0.54	0.50	0.43	0.40	0.41	0.41	0.40	0.42
MET	0.17	0.14	0.12	0.10	0.08	0.07	0.08	0.08	0.07	0.08
CYS	0.09	0.09	0.07	0.07	0.05	0.04	0.04	0.04	0.04	0.05
ILE	0.71	0.63	0.57	0.53	0.46	0.41	0.41	0.40	0.39	0.41
LEU	0.70	0.61	0.52	0.46	0.37	0.33	0.34	0.33	0.32	0.35
PHE	0.34	0.31	0.28	0.27	0.23	0.22	0.23	0.22	0.22	0.24
TRP	0.09	0.08	0.07	0.07	0.06	0.06	0.06	0.06	0.06	0.06
LYS	0.67	0.59	0.52	0.48	0.41	0.38	0.41	0.41	0.40	0.43

Amino Acid Concentrations in Glutamine-Fed Batch Culture of NB1 Hybridoma Cells										
Amino Acid	Amino Acid Concentration (mM)									
Time (hrs)	0.0	22.5	34.5	47.5	58.5	71.5	83.0	98.5	123.0	146.0
ASP	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
GLU	0.06	0.04	0.03	0.02	0.01	0.00	0.00	0.00	0.00	0.01
ASN	0.00	0.03	0.04	0.04	0.08	0.10	0.11	0.11	0.08	0.07
SER	0.34	0.28	0.29	0.27	0.25	0.28	0.27	0.28	0.22	0.21
GLN	3.22	1.66	0.95	0.64	3.97	2.69	2.52	1.76	0.39	0.22
GLY	0.26	0.22	0.24	0.26	0.15	0.13	0.13	0.17	0.17	0.26
HIS	0.16	0.13	0.13	0.11	0.09	0.09	0.08	0.07	0.04	0.04
ARG	0.34	0.34	0.33	0.30	0.24	0.22	0.19	0.12	0.16	0.04
THR	0.63	0.46	0.45	0.41	0.34	0.37	0.33	0.30	0.24	0.23
ALA	0.26	0.43	0.68	0.95	1.24	1.50	1.73	1.95	2.02	2.33
PRO	0.05	0.08	0.12	0.18	0.25	0.34	0.45	0.55	0.70	0.84
TYR	0.31	0.24	0.19	0.19	0.19	0.18	0.21	0.19	0.14	0.14
VAL	0.69	0.55	0.54	0.50	0.44	0.42	0.39	0.34	0.26	0.26
MET	0.17	0.13	0.12	0.10	0.08	0.08	0.06	0.05	0.00	0.00
CYS	0.09	0.07	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.00
ILE	0.71	0.57	0.57	0.53	0.48	0.48	0.46	0.42	0.34	0.34
LEU	0.70	0.54	0.52	0.46	0.40	0.38	0.33	0.28	0.17	0.16
PHE	0.34	0.28	0.28	0.26	0.23	0.22	0.21	0.18	0.13	0.13
TRP	0.09	0.07	0.08	0.07	0.06	0.07	0.06	0.05	0.04	0.04
LYS	0.67	0.54	0.53	0.48	0.42	0.40	0.36	0.31	0.22	0.21

APPENDIX 8

Glutamine, Ammonia, Glucose and Lactate Steady State Concentrations
in Two-Stage Chemostat Culture

Steady-State Glucose And Lactate Concentrations In Two-Stage Chemostat Culture				
Dilution Rate (1/h)	Glucose (mM)		Lactate (mM)	
	Stage 1	Stage 2	Stage 1	Stage 2
0.05	6.64	1.76	30.00	32.95
0.04	6.12	2.88	29.69	34.02
0.03	2.30	0.63	38.30	36.00
0.02	8.81	7.16	16.27	23.27
0.01	10.74	9.32	14.99	15.27

Steady-State Glutamine And Ammonia Concentrations In Two-Stage Chemostat Culture				
Dilution Rate (1/h)	Glutamine (mM)		Ammonia (mM)	
	Stage 1	Stage 2	Stage 1	Stage 2
0.05	0.18	0.00	2.09	2.16
0.04	0.11	0.00	2.17	2.58
0.03	0.11	0.00	2.29	2.30
0.02	0.06	0.00	2.17	2.27
0.01	0.00	0.00	2.16	2.21

APPENDIX 9

Two-Stage Chemostat Culture: Amino Acid Steady-State Concentrations

Steady-State Amino Acid Concentrations in Two-Stage Chemostat Culture										
Dilution Rates (h ⁻¹)	Stage One					Stage Two				
	0.05	0.04	0.03	0.02	0.01	0.05	0.04	0.03	0.02	0.01
Amino Acid	Amino Acid Concentration (mM)									
ASP	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GLU		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ASN	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SER	0.20	0.13	0.17	0.17	0.17	0.13	0.12	0.13	0.13	0.15
GLN	0.18	0.12	0.11	0.06	0.00	0.00	0.00	0.00	0.00	0.00
GLY	0.35	0.30	0.32	0.28	0.58	0.30	0.33	0.30	0.43	0.68
HIS	0.10	0.09	0.09	0.05	0.05	0.07	0.07	0.07	0.05	0.05
ARG	0.13	0.17	0.19	0.11	0.09	0.14	0.12	0.14	0.10	0.10
THR	0.30	0.30	0.31	0.20	0.20	0.31	0.32	0.31	0.21	0.22
ALA	1.01	0.84	0.86	0.72	0.57	0.90	0.88	0.90	0.75	0.59
PRO	0.29	0.36	0.28	0.12	0.07	0.30	0.38	0.30	0.16	0.10
TYR	0.26	0.24	0.23	0.18	0.17	0.21	0.19	0.21	0.16	0.16
VAL	0.41	0.37	0.42	0.22	0.14	0.33	0.26	0.33	0.17	0.10
MET	0.07	0.06	0.07	0.00	0.00	0.04	0.03	0.04	0.00	0.00
CYS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ILE	0.42	0.37	0.46	0.19	0.09	0.35	0.22	0.35	0.13	0.06
LEU	0.32	0.25	0.33	0.10	0.04	0.21	0.12	0.21	0.05	0.00
PHE	0.24	0.21	0.23	0.14	0.13	0.18	0.18	0.18	0.13	0.11
TRP	0.11	0.08	0.07	0.05	0.00	0.06	0.07	0.06	0.04	0.00
LYS	0.41	0.36	0.39	0.23	0.23	0.32	0.31	0.32	0.22	0.23

APPENDIX 10

Specific Rates of Amino Acid Utilisation in Amino Acid Limited Batch Culture

The Specific Rates Of Amino Acid Utilisation During The First 48 Hrs Of Batch Culture Limited On A Particular Amino Acid

Limiting Amino Acid	Control	Tryptophan	Serine	Lysine	Threonine	Methionine
Amino Acid	Specific Utilisation Rate (u moles/viable cell hour)					
ASP	0	0	0	0	0	0
GLU	0.0009	-0.0037	0.0012	0.0008	-0.0028	0.0009
ASN	-0.0019	-0.0042	0	0	0	0
SER	0.0031	0.0039	-0.0041	0.0039	0.0113	0.0049
GLN	0.1213	0.3481	0.2276	0.1559	0.4339	0.1893
GLY	0.0071	0.0069	0.0082	0.0054	0.0149	0.0072
HIS	0.0024	0.0011	0.0013	0.0014	0.0035	0.0019
ARG	0.0057	0.0026	0.0037	0.0036	0.0114	0.0045
THR	0.0110	0.0143	0.0155	0.0084	0.0140	0.0124
ALA	-0.0240	-0.0432	-0.0283	-0.0178	-0.0632	-0.0207
PRO	-0.0041	-0.0089	-0.0037	-0.0030	-0.0081	-0.0036
TYR	0.0051	0.0005	0.0022	0.0024	0.0056	0.0033
VAL	0.0071	0.0087	0.0066	0.0064	0.0163	0.0074
MET	0.0025	0.0019	0.0020	0.0019	0.0037	0.0021
CYS	0.0027	0.0020	0.0016	0.0013	0.0035	0.0017
ILE	0.0064	0.0076	0.0086	0.0085	0.0223	0.0085
LEU	0.0110	0.0096	0.0100	0.0089	0.0239	0.0119
PHE	0.0034	0.0022	0.0019	0.0021	0.0059	0.0028
TRP	0.0007	-0.0084	0.0006	0.0007	0.0017	0.0009
LYS	0.0087	0.0021	0.0033	0.0059	0.0188	0.0068

The Specific Rates Of Amino Acid Utilisation During The First 48 Hrs Of Batch Culture Limited On A Particular Amino Acid (Cont'd)

Limiting Amino Acid	Control	Phenylalanine	Valine	Arginine	Tyrosine	Glycine
Amino Acid	Specific Utilisation Rate (m moles/viable cell hour)					
ASP	0	0	0	0	0	0
GLU	0.0009	0.0009	0.0010	0.0009	0.0009	0.0003
ASN	-0.0019	0	0	-0.0029	0	-0.0022
SER	0.0031	0.0066	0.0034	-0.0004	0.0047	0.0035
GLN	0.1213	0.1799	0.1887	0.0919	0.1787	0.1536
GLY	0.0071	0.0080	0.0053	0.0025	0.0063	-0.0003
HIS	0.0024	0.0023	0.0009	0.0005	0.0016	0.0019
ARG	0.0057	0.0048	0.0027	-0.0014	-0.0031	0.0055
THR	0.0110	0.0133	0.0095	0.0059	0.0107	0.0120
ALA	-0.0242	-0.0196	-0.0195	-0.0284	-0.0200	-0.0358
PRO	-0.0041	-0.0033	-0.0036	-0.0055	-0.0036	-0.0064
TYR	0.0051	0.0039	0.0013	0.0006	0.0027	0.0019
VAL	0.0071	0.0085	0.0018	0.0029	0.0071	0.0064
MET	0.0025	0.0028	0.0019	0.0010	0.0017	0.0023
CYS	0.0027	0.0016	0.0015	0.0008	0.0022	0.0012
ILE	0.0064	0.0099	0.0049	0.0029	0.0089	0.0062
LEU	0.0110	0.0128	0.0076	0.0042	0.0102	0.0099
PHE	0.0034	0.0023	0.0014	0.0008	0.0021	0.0018
TRP	0.0007	0.0011	0.0004	3.3E-05	0.0004	0.0009
LYS	0.0087	0.0087	0.0052	0.0029	0.0069	0.0083

The Specific Rates Of Amino Acid Utilisation During The First 48 Hrs Of Batch Culture Limited On A Particular Amino Acid (Cont'd)

Limiting Amino Acid	Control	Histidine	Cystine	Leucine	Isoleucine
Amino Acid	Specific Utilisation Rate (u moles/viable cell hour)				
ASP	0	0	0	0	0
GLU	0.0009	0.0009	0.0005	0.0006	0.0008
ASN	-0.0020	-0.0025	-0.0017	-0.0019	-0.0017
SER	0.0031	0.0013	-0.0001	2.3E-05	0.0022
GLN	0.1213	0.1104	0.1631	0.1000	0.0854
GLY	0.0071	0.0029	-0.0085	0.0019	0.0039
HIS	0.0024	0.0013	0.0021	0.0004	0.0006
ARG	0.0057	0.0040	0.0057	0.0031	0.0034
THR	0.0110	0.0065	0.0113	0.0028	0.0040
ALA	-0.0241	-0.0281	-0.0497	-0.0331	-0.0185
PRO	-0.0041	-0.0043	-0.0070	-0.0068	-0.0031
TYR	0.0051	0.0018	0.0035	-4.6E-05	0.0019
VAL	0.0071	0.0033	0.0072	-0.0011	0.0014
MET	0.0025	0.0014	0.0023	0.0009	0.0016
CYS	0.0027	0.0016	0.0024	0.0004	0.0017
ILE	0.0064	0.0037	0.0063	-0.0013	0.0028
LEU	0.0110	0.0073	0.0100	0.0054	0.0069
PHE	0.0034	0.0014	0.0029	-2.3E-05	0.0012
TRP	0.0007	0.0002	0.0009	0.0004	0.0007
LYS	0.0087	0.0056	0.0068	0.0025	0.0038

APPENDICES

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Appendix 11

Concentration of Amino Acid in Culture Supplemented With 2X Specified Amino Acid (all other amino acids as in DMEM, Appendix 1)	
Culture Supplement	Amino Acid Concentration (mg/l)
Control	As for DMEM in Appendix 1
Leucine	210
Arginine	168
Methionine	60
Tryptophan	32
Cystine	96
Histidine	84

Referred to on page 125

Rate of Amino Acid Feed to Stage-Two of an Unequal Volume Two-Stage Chemostat (pg. 132)	
Amino Acid	Amino Acid Feed Rate (mg/l/h)
SER	0.73
GLN	17.04
GLY	0.17
HIS	0.52
ARG	1.09
THR	1.75
TYR	1.25
VAL	1.35
MET	0.59
CYS	1.44
ILE	1.85
LEU	0.86
PHE	0.02
TRP	1.79
LYS	

Referred to on page 132

Concentration of the Limiting Amino Acid in Batch Culture (pg. 137)	
Limiting Amino Acid	Amino Concentration (mM)
SER	0.10
GLY	0.09
HIS	0.08
ARG	0.14
THR	0.34
TYR	0.14
VAL	0.26
MET	0.08
CYS	0.10
ILE	0.23
LEU	0.31
PHE	0.11
TRP	0.00
LYS	0.31

Referred to on page 137

Appendix 14

Concentration of Amino Acids During Culture Under Conditions Where the Specified Amino Acid was Designed to be Limiting to Growth. Referred to on page 138

Control

Time (hours)	0	48	119
Amino Acid	Concentration (mM)		
ASP	0.00	0.00	0
GLU	0.03	0.00	0
ASN	0.00	0.06	0.55
SER	0.42	0.33	0.451
GLN	5.23	1.70	0.084
GLY	0.53	0.33	0.429
HIS	0.21	0.14	0.158
ARG	0.51	0.35	0.315
THR	0.84	0.52	0.561
ALA	0.08	0.78	1.763
PRO	0.02	0.14	0.348
TYR	0.42	0.28	0.333
VAL	0.83	0.62	0.597
MET	0.21	0.14	0.116
CYS	0.18	0.10	0.035
ILE	0.87	0.68	0.605
LEU	0.88	0.56	0.484
PHE	0.42	0.32	0.341
TRP	0.09	0.07	0.058
LYS	0.83	0.58	0.618

Phenylalanine Limited

Time (hours)	0	48	110
Amino Acid	Concentration (mM)		
ASP	0.00	0.00	0.00
GLU	0.03	0.00	0.00
ASN	0.00	0.00	0.00
SER	0.42	0.23	0.36
GLN	5.23	0.00	0.00
GLY	0.53	0.30	0.40
HIS	0.21	0.14	0.16
ARG	0.51	0.38	0.39
THR	0.84	0.45	0.55
ALA	0.08	0.65	1.00
PRO	0.02	0.12	0.15
TYR	0.42	0.31	0.35
VAL	0.83	0.58	0.63
MET	0.21	0.13	0.14
CYS	0.18	0.13	0.14
ILE	0.87	0.58	0.56
LEU	0.88	0.51	0.52
PHE	0.11	0.04	0.06
TRP	0.09	0.06	0.07
LYS	0.83	0.58	0.66

Serine Limited

Time (hours)	0	48	86
Amino Acid	Concentration (mM)		
ASP	0.00	0.00	0.00
GLU	0.03	0.00	0.01
ASN	0.00	0.00	0.00
SER	0.10	0.19	0.26
GLN	5.23	0.00	0.00
GLY	0.53	0.34	0.38
HIS	0.21	0.18	0.18
ARG	0.51	0.43	0.41
THR	0.84	0.48	0.54
ALA	0.08	0.73	0.89
PRO	0.02	0.11	0.11
TYR	0.42	0.37	0.36
VAL	0.83	0.68	0.67
MET	0.21	0.16	0.16
CYS	0.18	0.14	0.15
ILE	0.87	0.67	0.63
LEU	0.88	0.65	0.61
PHE	0.42	0.37	0.36
TRP	0.09	0.08	0.07
LYS	0.83	0.75	0.75

Threonine Limited

Time (hours)	0	48	86
Amino Acid	Concentration (mM)		
ASP	0.00	0.00	0.00
GLU	0.03	0.06	0.03
ASN	0.00	0.00	0.00
SER	0.42	0.29	0.32
GLN	5.23	0.12	0.00
GLY	0.53	0.36	0.36
HIS	0.21	0.17	0.17
ARG	0.51	0.38	0.36
THR	0.34	0.17	0.17
ALA	0.08	0.83	1.02
PRO	0.02	0.12	0.12
TYR	0.42	0.36	0.34
VAL	0.83	0.64	0.61
MET	0.21	0.17	0.16
CYS	0.18	0.14	0.14
ILE	0.87	0.61	0.55
LEU	0.88	0.60	0.54
PHE	0.42	0.35	0.34
TRP	0.09	0.07	0.07
LYS	0.83	0.61	0.59

Tryptophan Limited

Time (hours)	0	48	86
Amino Acid	Concentration (mM)		
ASP	0.00	0.00	0.00
GLU	0.03	0.08	0.09
ASN	0.00	0.06	0.06
SER	0.42	0.37	0.41
GLN	5.23	0.71	0.62
GLY	0.53	0.44	0.47
HIS	0.21	0.19	0.21
ARG	0.51	0.48	0.48
THR	0.84	0.65	0.68
ALA	0.08	0.64	0.73
PRO	0.02	0.14	0.15
TYR	0.42	0.42	0.44
VAL	0.83	0.72	0.76
MET	0.21	0.19	0.20
CYS	0.18	0.15	0.17
ILE	0.87	0.77	0.82
LEU	0.88	0.76	0.81
PHE	0.42	0.39	0.41
TRP	0.00	0.11	0.15
LYS	0.83	0.80	0.84

Tyrosine Limited

Time (hours)	0	48	86
Amino Acid	Concentration (mM)		
ASP	0.00	0.00	0.00
GLU	0.03	0.00	0.00
ASN	0.00	0.00	0.00
SER	0.42	0.29	0.36
GLN	5.23	0.00	0.00
GLY	0.53	0.35	0.39
HIS	0.21	0.16	0.17
ARG	0.51	0.60	0.60
THR	0.84	0.52	0.55
ALA	0.08	0.67	0.88
PRO	0.02	0.13	0.13
TYR	0.14	0.06	0.06
VAL	0.83	0.62	0.60
MET	0.21	0.16	0.16
CYS	0.18	0.12	0.09
ILE	0.87	0.61	0.54
LEU	0.88	0.58	0.53
PHE	0.42	0.36	0.36
TRP	0.09	0.08	0.07
LYS	0.83	0.63	0.64

Valine Limited

Time (hours)	0	48	86
Amino Acid	Concentration (mM)		
ASP	0.00	0.00	0.00
GLU	0.03	0.00	0.00
ASN	0.00	0.00	0.00
SER	0.42	0.33	0.42
GLN	5.23	0.00	0.00
GLY	0.53	0.38	0.43
HIS	0.21	0.18	0.18
ARG	0.51	0.44	0.43
THR	0.84	0.57	0.62
ALA	0.08	0.62	0.90
PRO	0.02	0.12	0.14
TYR	0.42	0.39	0.42
VAL	0.26	0.21	0.21
MET	0.21	0.16	0.16
CYS	0.18	0.14	0.13
ILE	0.87	0.73	0.66
LEU	0.88	0.67	0.63
PHE	0.42	0.38	0.39
TRP	0.09	0.08	0.08
LYS	0.83	0.69	0.70

Arginine Limited

Time (hours)	0	48	79
Amino Acid	Concentration (mM)		
ASP	0.00	0.00	0.01
GLU	0.03	0.00	0.10
ASN	0.00	0.09	0.11
SER	0.42	0.44	0.47
GLN	5.23	2.47	1.00
GLY	0.53	0.45	0.47
HIS	0.21	0.19	0.19
ARG	0.14	0.19	0.18
THR	0.84	0.66	0.64
ALA	0.08	0.93	1.77
PRO	0.02	0.19	0.30
TYR	0.42	0.41	0.40
VAL	0.83	0.74	0.71
MET	0.21	0.18	0.17
CYS	0.18	0.16	0.09
ILE	0.87	0.78	0.72
LEU	0.88	0.76	0.70
PHE	0.42	0.39	0.38
TRP	0.09	0.09	0.08
LYS	0.83	0.74	0.74

Histidine Limited

Time (hours)	0	48	99
Amino Acid	Concentration (mM)		
ASP	0.00	0.00	0.00
GLU	0.03	0.00	0.00
ASN	0.00	0.07	0.05
SER	0.42	0.39	0.38
GLN	5.23	1.99	0.00
GLY	0.53	0.44	0.43
HIS	0.08	0.04	0.00
ARG	0.51	0.40	0.30
THR	0.84	0.65	0.55
ALA	0.08	0.90	1.66
PRO	0.02	0.15	0.37
TYR	0.42	0.37	0.31
VAL	0.83	0.73	0.59
MET	0.21	0.17	0.12
CYS	0.18	0.13	0.03
ILE	0.87	0.76	0.58
LEU	0.88	0.67	0.47
PHE	0.42	0.37	0.32
TRP	0.09	0.08	0.07
LYS	0.83	0.66	0.57

Isoleucine Limited

Time (hours)	0	24	48	66	79	92	99
Amino Acid	Concentration (mM)						
ASP	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GLU	0.03	0.03	0.00	0.00	0.00	0.00	0.00
ASN	0.00	0.03	0.06	0.07	0.06	0.04	0.04
SER	0.42	0.41	0.35	0.33	0.33	0.29	0.30
GLN	5.23	4.31	2.19	0.79	0.15	0.00	0.00
GLY	0.53	0.46	0.39	0.35	0.33	0.31	0.32
HIS	0.21	0.23	0.19	0.17	0.16	0.14	0.15
ARG	0.51	0.49	0.39	0.33	0.28	0.28	0.27
THR	0.84	0.82	0.70	0.63	0.58	0.55	0.56
ALA	0.08	0.28	0.74	1.01	1.23	1.18	1.20
PRO	0.02	0.06	0.13	0.24	0.34	0.35	0.36
TYR	0.42	0.42	0.35	0.31	0.30	0.26	0.27
VAL	0.83	0.91	0.78	0.70	0.68	0.62	0.60
MET	0.21	0.21	0.15	0.12	0.11	0.09	0.09
CYS	0.18	0.15	0.12	0.08	0.05	0.03	0.03
ILE	0.23	0.22	0.13	0.09	0.06	0.04	0.04
LEU	0.88	0.81	0.63	0.52	0.46	0.40	0.41
PHE	0.42	0.44	0.37	0.33	0.31	0.29	0.30
TRP	0.09	0.09	0.06	0.05	0.05	0.04	0.04
LYS	0.83	0.86	0.69	0.60	0.55	0.53	0.54

Glycine Limited

Time (hours): Amino Acid	0	48	110
	Concentration (mM)		
ASP	0.00	0.00	0.00
GLU	0.03	0.02	0.00
ASN	0.00	0.05	0.00
SER	0.42	0.34	0.40
GLN	5.23	1.51	0.00
GLY	0.09	0.10	0.12
HIS	0.21	0.16	0.15
ARG	0.51	0.38	0.31
THR	0.84	0.55	0.50
ALA	0.08	0.95	1.62
PRO	0.02	0.18	0.30
TYR	0.42	0.38	0.33
VAL	0.83	0.67	0.59
MET	0.21	0.16	0.12
CYS	0.18	0.15	0.10
ILE	0.87	0.72	0.61
LEU	0.88	0.64	0.49
PHE	0.42	0.37	0.34
TRP	0.09	0.07	0.05
LYS	0.83	0.63	0.56

Cystine Limited

Time (hours): Amino Acid	0	48	110
	Concentration (mM)		
ASP	0.00	0.00	0.00
GLU	0.03	0.01	0.00
ASN	0.00	0.05	0.00
SER	0.42	0.43	0.55
GLN	5.23	0.76	0.00
GLY	0.53	0.76	0.80
HIS	0.21	0.15	0.14
ARG	0.51	0.36	0.31
THR	0.84	0.53	0.53
ALA	0.08	1.44	1.90
PRO	0.02	0.21	0.26
TYR	0.42	0.33	0.30
VAL	0.83	0.63	0.57
MET	0.21	0.15	0.12
CYS	0.10	0.04	0.01
ILE	0.87	0.70	0.58
LEU	0.88	0.61	0.48
PHE	0.42	0.33	0.31
TRP	0.09	0.06	0.05
LYS	0.83	0.64	0.61

Leucine Limited

Time (hours)	0	16	36	48	62	86	110
Amino Acid	Concentration (mM)						
ASP	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GLU	0.03	0.03	0.00	0.00	0.00	0.00	0.00
ASN	0.00	0.04	0.05	0.08	0.04	0.03	0.03
SER	0.42	0.32	0.28	0.42	0.31	0.34	0.33
GLN	5.23	3.02	1.47	0.89	0.11	0.00	0.00
GLY	0.53	0.36	0.32	0.45	0.29	0.33	0.34
HIS	0.21	0.17	0.15	0.19	0.12	0.13	0.13
ARG	0.51	0.43	0.36	0.38	0.30	0.31	0.31
THR	0.84	0.58	0.52	0.72	0.48	0.55	0.52
ALA	0.08	0.30	0.72	1.52	1.16	1.30	1.30
PRO	0.02	0.07	0.15	0.32	0.27	0.31	0.30
TYR	0.42	0.35	0.32	0.43	0.27	0.29	0.28
VAL	0.83	0.76	0.74	0.88	0.62	0.65	0.63
MET	0.21	0.16	0.13	0.17	0.10	0.10	0.10
CYS	0.18	0.13	0.11	0.16	0.09	0.08	0.08
ILE	0.87	0.68	0.66	0.93	0.59	0.62	0.58
LEU	0.31	0.22	0.11	0.07	0.02	0.04	0.04
PHE	0.42	0.33	0.30	0.42	0.26	0.28	0.27
TRP	0.09	0.08	0.06	0.07	0.05	0.05	0.05
LYS	0.83	0.61	0.53	0.72	0.45	0.49	0.48

Lysine Limited

Time (hours)	0	48	86
Amino Acid	Concentration (mM)		
ASP	0.00	0.00	0.00
GLU	0.03	0.00	0.00
ASN	0.00	0.00	0.00
SER	0.42	0.29	0.36
GLN	5.23	0.00	0.00
GLY	0.53	0.35	0.41
HIS	0.21	0.16	0.17
ARG	0.51	0.39	0.39
THR	0.84	0.56	0.62
ALA	0.08	0.68	0.88
PRO	0.02	0.12	0.14
TYR	0.42	0.34	0.37
VAL	0.83	0.62	0.65
MET	0.21	0.15	0.16
CYS	0.18	0.14	0.15
ILE	0.87	0.59	0.57
LEU	0.88	0.58	0.58
PHE	0.42	0.35	0.36
TRP	0.09	0.07	0.07
LYS	0.31	0.11	0.13

Methionine Limited

Time (hours) Amino Acid	0	48	86
	Concentration (mM)		
ASP	0.00	0.00	0.00
GLU	0.03	0.00	0.00
ASN	0.00	0.00	0.00
SER	0.42	0.29	0.36
GLN	5.23	0.00	0.00
GLY	0.53	0.33	0.39
HIS	0.21	0.16	0.17
ARG	0.51	0.39	0.39
THR	0.84	0.50	0.57
ALA	0.08	0.65	0.91
PRO	0.02	0.12	0.14
TYR	0.42	0.33	0.36
VAL	0.83	0.62	0.67
MET	0.08	0.02	0.03
CYS	0.18	0.13	0.14
ILE	0.87	0.64	0.64
LEU	0.88	0.55	0.57
PHE	0.42	0.34	0.37
TRP	0.09	0.07	0.07
LYS	0.83	0.64	0.71

Rate of Amino Acid Feed to Stage-Two of an Unequal Volume Two-Stage Chemostat (pg. 148)			
Amino Acid	Amino Acid Feed Rate (mg/l/h)		
	All Amino Acids	All Amino Acids Except Group 4	All Amino Acids Except Ile
SER	0.73	0.73	0.73
GLN	17.04	17.04	17.04
GLY	0.17	0.17	0.17
HIS	0.52	0.00	0.52
ARG	1.09	1.09	1.09
THR	1.75	1.75	1.75
TYR	1.25	1.25	1.25
VAL	1.35	1.35	1.35
MET	0.59	0.00	0.59
CYS	1.44	0.00	0.00
ILE	1.85	0.00	1.85
LEU	0.86	0.86	0.86
PHE	0.02	0.02	0.02
TRP	1.79	1.79	1.79
LYS			

Maximum Viable Cell Concentration and Antibody When Amino Acids Were At Equal Concentrations In The Medium (pg. 161)		
Medium	Max Viable Cell Concentration ($\times 10^{-6}/\text{ml}$)	Max Antibody Concentration ($\mu\text{g}/\text{ml}$)
DMEM	1.28	30.5
DMEM GLN-Fed	1.34	47.8
8mM Amino Acids	1.19	26.8
8mM Amino Acids GLN-Fed	1.16	43.7
321mg/l Amino Acids	0.99	41.3
321mg/l Amino Acids GLN-Fed	1.35	36.3

Appendix 17

Amino Acid Concentration During Glutamine Fed Batch Culture In Home-Made Dmem And When All Amino Acids Were At An Equal Concentration, mM And mg/L. Referred to on Page 159

Amino Acid Concentration During Control Culture in Home-Made DMEM

Time (hours)	Initial	0	24	46	70	91	124	165
Amino Acid	Amino Acid Concentration (mM)							
ASP	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
GLU	0.00	0.08	0.09	0.00	0.00	0.00	0.00	0.01
ASN	0.00	0.00	0.05	0.07	0.12	0.08	0.04	0.03
SER	0.40	0.31	0.31	0.35	0.37	0.25	0.19	0.26
GLN	4.00	3.61	2.53	3.14	2.80	0.99	0.00	0.00
GLY	0.40	0.33	0.38	0.33	0.32	0.26	0.28	0.36
HIS	0.20	0.21	0.20	0.19	0.18	0.12	0.10	0.11
ARG	0.40	0.43	0.37	0.27	0.23	0.18	0.14	0.16
THR	0.80	0.67	0.55	0.50	0.49	0.33	0.26	0.28
ALA	0.00	0.17	0.55	0.91	1.19	1.24	1.55	1.83
PRO	0.00	0.05	0.10	0.19	0.35	0.41	0.46	0.49
TYR	0.46	0.30	0.28	0.24	0.21	0.13	0.09	0.09
VAL	0.80	0.66	0.59	0.55	0.50	0.36	0.27	0.28
MET	0.20	0.15	0.13	0.10	0.08	0.03	0.04	0.04
CYS	0.20	0.10	0.10	0.09	0.08	0.04	0.00	0.00
ILE	0.80	0.62	0.56	0.52	0.51	0.38	0.28	0.28
LEU	0.80	0.66	0.58	0.50	0.46	0.28	0.16	0.17
PHE	0.40	0.31	0.30	0.27	0.25	0.17	0.13	0.13
TRP	0.08	0.07	0.06	0.04	0.20	0.17	0.13	0.13
LYS	0.80	0.60	0.57	0.49	0.45	0.29	0.23	0.25

Amino Acid Concentration, All Amino Acids Initially At Equal mM

Time (hours)	Initial	0	24	46	70	91	124	165
Amino Acid	Amino Acid Concentration (mM)							
GLU	0.03	0.05	0.00	0.00	0.00	0.00	0.02	0.02
ASN	0.00	0.00	0.06	0.09	0.08	0.04	0.04	0.04
SER	0.56	0.51	0.61	0.59	0.59	0.54	0.55	0.50
GLN	3.18	2.08	0.74	1.90	0.68	0.00	0.00	0.00
GLY	0.44	0.48	0.54	0.50	0.49	0.51	0.57	0.52
HIS	0.56	0.56	0.68	0.61	0.64	0.63	0.65	0.57
ARG	0.58	0.54	0.56	0.51	0.45	0.42	0.43	0.39
THR	0.66	0.56	0.56	0.51	0.44	0.42	0.42	0.38
ALA	0.07	0.34	1.01	1.30	1.63	1.80	1.90	1.72
PRO	0.02	0.07	0.18	0.32	0.51	0.68	0.72	0.65
TYR	0.66	0.48	0.59	0.40	0.43	0.41	0.38	0.37
VAL	0.64	0.59	0.59	0.51	0.45	0.39	0.40	0.36
MET	0.56	0.57	0.66	0.60	0.60	0.58	0.59	0.53
CYS	0.31	0.32	0.36	0.31	0.27	0.25	0.27	0.25
ILE	0.58	0.52	0.55	0.49	0.44	0.38	0.37	0.34
LEU	0.61	0.54	0.53	0.45	0.37	0.31	0.31	0.28
PHE	0.56	0.54	0.62	0.55	0.54	0.51	0.52	0.47
TRP	0.55	0.59	0.73	0.69	0.73	0.71	0.72	0.65
LYS	0.55	0.51	0.51	0.43	0.37	0.33	0.34	0.32

**Amino Acid Concentration, Amino Acids Initially At Equal
mg/l**

Time (hours)	Initial	0	24	46	70	91	124
Amino Acid	Amino Acid Concentration (mM)						
GLU	0.04	0.05	0.05	0.00	0.00	0.01	0.02
ASN	0.00	0.00	0.04	0.05	0.04	0.00	0.00
SER	2.60	2.51	2.19	1.99	2.55	2.42	2.24
GLN	4.03	2.83	0.81	0.43	0.00	0.00	0.00
GLY	3.42	3.64	3.27	2.98	3.95	3.88	3.66
HIS	1.68	1.69	1.50	1.32	1.75	1.73	1.62
ARG	1.40	1.37	1.18	1.06	1.30	1.25	1.17
THR	1.80	1.60	1.33	1.20	1.51	1.47	1.39
ALA	0.09	0.35	0.99	0.97	1.37	1.48	1.40
PRO	0.03	0.08	0.15	0.19	0.31	0.39	0.38
TYR	0.95	0.91	1.06	0.75	1.01	0.79	0.80
VAL	2.35	2.21	1.83	1.63	2.11	1.99	1.82
MET	1.83	1.82	1.60	1.41	1.89	1.84	1.70
CYS	0.54	0.58	0.45	0.34	0.49	0.48	0.45
ILE	2.22	2.06	1.69	1.49	1.93	1.75	1.58
LEU	2.26	2.09	1.69	1.46	1.89	1.72	1.56
PHE	1.57	1.55	1.36	1.18	1.58	1.52	1.41
TRP	0.59	0.59	0.52	0.47	0.63	0.61	0.56
LYS	1.63	1.57	1.27	1.13	1.48	1.44	1.33