A BIOPROCESS TO PRODUCE REOVIRUS: SERUM-FREE MEDIA DEVELOPMENT AND THE RELATIONSHIP BETWEEN INTRACELLULAR NUCLEOTIDE LEVELS AND VIRUS PRODUCTION

By

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A Bioprocess to Produce Reovirus: Serum-free Media Development and the Relationship Between Intracellular Nucleotide Levels and Virus Production

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FOREWORD

I would like to dedicate this thesis to my late aunt, Louise Kernatz, who recently lost a battle to cancer, and to my grandmother, Edna Kernatz. Their strong belief in the value of education inspired me to pursue my academic career to its fullest.

"I know nothing except the fact of my ignorance."

- Socrates

"Nothing shocks me. I am a scientist"

- Harrison Ford (Indiana Jones)

LIST OF ABBREVIATIONS

Abbreviations

ADP	adenosine diphosphate
AEC	adenylate energy charge (ATP + 0.5ADP)/(total adenylates))
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BHK	baby hamster kidney
bFGF	basic – Fibroblast growth factor
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
CHO	Chinese hamster ovary
CTP	cytidine triphosphate
DMEM	Dulbecco's modified Eagle's medium
EDTA	(ethylene dinitrilo)-tetraacetic acid
F12	Ham's F12 medium
FBS	fetal bovine serum
FMDV	foot and mouth disease virus
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
HSV	herpes simplex virus
IMDM	Iscove's modified Eagle's medium
IMP	inosine monophosphate
JMEM	Joklik's modified Eagle's medium
L-929	mouse L cells – 929
M199	Medium 199
M2-VSFM	modification 2 – Vero serum free medium
Mab	monoclonal antibody

MDBK	Madin-Darby bovine kidney
MDCK	Madin darby canine kidney
MEM	minimal essential medium
MRC-5	Medical Research Council clone 5 (human diploid lung
fiboblast)	
M-VSFM	modified-Vero serum free medium
NAD	nicotinamide dinucleotide
NTP	nucleotide triphosphate
PBS	phosphate buffered saline
PPi	pyrophosphate
RPMI	Roswell Park Memorial Institute medium
TCA cycle	tricarboxylic acid cycle
TCA	trichloroacetic acid
UDP	uridine diphosphate
UDP-glc	uridine diphosphate-glucose
v/v	volume/volume
VSFM	Vero serum free medium
VSV	bovine vesicular stomatitis virus

ABSTRACT

Mammalian cell culture has become a standard in the biotechnology industry for the production of therapeutics, including recombinant proteins, antibodies, and vaccines. New therapeutics are being discovered at a rapid rate, and some have unexpected applications. A recent study showed that reovirus, an enteric virus normally commonly found in the intestines of humans, can be used to treat cancer. It has shown promising results in the treatment of various forms of cancer and is now in phase I and II clinical trials for the treatment of brain, prostate, and breast cancer. These findings suggest the need to develop a cell culture process to produce the virus for human use. The requirements of such a process must include an acceptable cell line, a serum-free medium, and a scalable production process. We have successfully developed a culture process to produce reovirus that involves a regulated cell line, Vero; an animal-product free serum-free medium developed in our lab (M-VSFM); and microcarrier technology (Cytodex-1) to make it scalable. The microcarrier cultures produced high titers of both reovirus type 1-Lang and type 3-Dearing to > 10^9 PFU/ml and yielded comparable titers to cells in serum-supplemented media (10% FBS-DMEM). It was shown to be superior to the standard mouse L cell process used to produce reovirus in batch cultures with 260% higher specific productivity. The amount of virus produced from these cultures was found to be highly dependent on the intracellular ATP, total adenylate, and the adenylate energy charge (AEC) at the time of infection. A threshold limit of ATP and adenylate exists where a drop below this limit resulted in a 95% reduction in production titer compared to the control. A Km for ATP and total adenylate was calculated to be 3.2 and 3.3 fmoles/cell, respectively, for virus production. The productivity of the virus is not dependent upon the growth state of the cell but rather the ATP and adenylate levels in the cell at the time of infection. The AEC shared a negative sigmoidal relationship with virus productivity where cultures with an AEC higher than 0.97 had significantly lower virus titers. It was found that reovirus consumes as much as 28% of the ATP and GTP within the cell to fuel replication of its progeny. This information can be useful for optimizing a large-scale process to produce reovirus and may be applicable to other virus production systems.

The serum-free medium, M-VSFM, was further developed for the growth of Vero cells. Supplementation of the medium with 10 units/ml of EGF increased the final cell yield by ~ 45%. The medium was successfully altered to support the growth of other industrially important cell lines, including MRC-5, MDCK, and BHK. The new formulations performed better than other commercial serum-free formulations available on the market and promoted the growth of the cells to confluence in all cases. Since these cell lines are commonly used for the production of human and animal vaccines, including influenza, foot and mouth disease virus, and polio, the medium would be extremely useful for the pharmaceutical industry.

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CHAPTER 1

Introduction

1.1 Reovirus

1.1.1 Description of reovirus

The mammalian reoviruses are members of the *Orthoreovirus* genus of the family *Reoviridae*. The term "reo" refers to its nature, a **R**espiratory **E**nteric **O**rphan virus. There are more than 150 species in the family *Reoviridae* and they infect invertebrates, vertebrates and plants (Kapikian et al. 1996). This family also include a number of important human and animal pathogens such as rotavirus (Estes 2001; Kapikian et al. 2001) and orbivirus (Roy 1996) which infect commonly by the fecal-oral route.

The morphology of the virus is a non-enveloped symmetrical icosahedral which is approximately 70-85nm in diameter. The virus has a total of 8 structural proteins which encase the 10 strands of double-stranded RNA genome. The virus has an inner icosahedral capsid, termed the inner capsid particle (or ICP), that consists of 5 proteins, which is ~ 60 nm in diameter. The ICP, also known as the core, constitute a self-contained transcription unit which synthesizes and exports translatable mRNA's (Chandran et al. 2003). A diagram of the structure of mammalian reovirus with all of its structural proteins is shown in Figure 1.1.

An outline of reovirus replication is shown in Figure 1.2. The replication of the virus occurs in the cytoplasm. Uptake of the virus involves receptor-mediated endocytosis (via junction adhesion molecule, (Barton et al. 2001)) and the formation of clathrin-coated pits. The virus is engulfed in an endosome/lysosome where proteolytic cleavage of the outer capsid proceeds. After release from the endosome, the intact ICP initiates primary and secondary transcription of its 10-piece segmented genome (Nibert et al. 2001). The

particles assemble 6-7 hours after infection and are released from the cell upon cell lysis (Nibert et al. 2001).

1.1.2 Significance of reovirus to the health community

Reovirus causes, in most cases, mild gastrointestinal and upper respiratory tracts infections in humans. The infections are usually asymptomatic and rarely cause fatalities in adults. The majority of adults carry antibodies to this virus and are immune to its effects.

Rotavirus also belongs to the same family as reovirus. Rotavirus is a major cause of death in the 3rd world for children below 24 months of age. It can cause gastroenteritis and diarrhea by triggering the intestinal nervous system to secrete more water (Lundgren et al. 2000). The infections can be fatal if not detected and treated by oral re-hydration therapy. In developing countries rotavirus infections may cause up to 1 million deaths per year (Cook et al. 1990). In first world countries, such as the United States, Rotavirus causes almost 70,000 hospital admissions for diarrhea and as many as 100 deaths each year. Rotavirus is not a serious threat to adults and usually does not cause any side effects but some outbreaks have occurred (Yang et al. 1998).

Presently there is not a suitable vaccine for the prevention of diarrhea caused by Rotavirus. A vaccine (RotaShield by Wyeth-Ayerst laboratories) was developed and licensed in the United States in 1998 as an oral vaccine derived from rotavirus isolated from a rhesus monkey (Hochwald et al. 1999). However, this vaccine was withdrawn due to a high incidence of secondary infections caused in the recipients (Murphy et al. 2001). Development of a suitable and safe vaccine is still ongoing.

Reovirus, the prototype of this family, remains an effective model for studying the characteristics of the double stranded RNA viruses (Kapikian 1996). Due to its non-pathogenic nature it is commonly used in research for vaccine development.

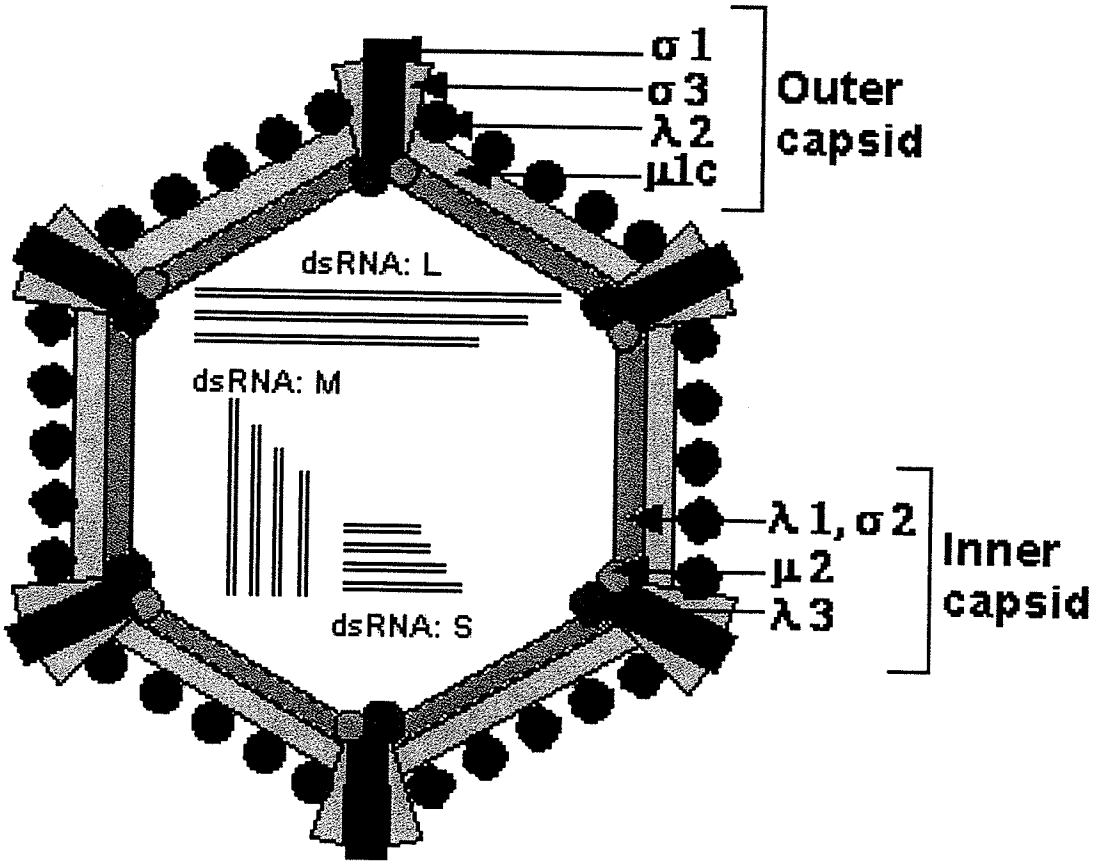


Figure 1.1. Diagrammatic representation of reovirus. λ3 – RNA-dependent RNA polymerase; λ2 – guanylyltransferase, methyltransferase; λ1 – binds RNA, Zn metalloprotein, possible NTPase, RNA helicase, RNA triphosphatase; μ2 – binds RNA, possible NTPase; μ1 – role in penetration, transcriptase activation; μNS – binds core, role in secondary transcription; σ1 – cell attachment protein, hemagglutinin, primary serotype determinant; σ2 – binds dsRNA; σ3 – binds ds RNA, zinc metalloprotein, involved in translation (Nibert et al. 2001).

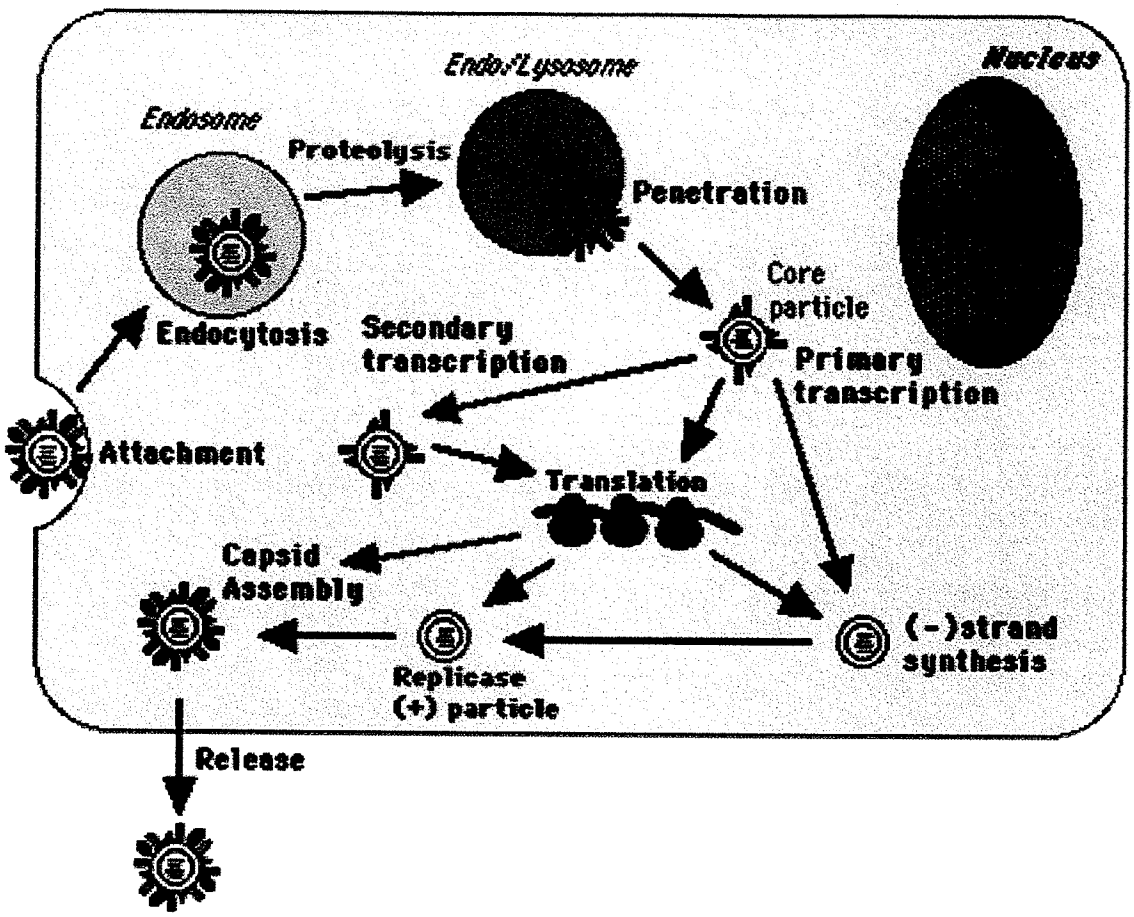


Figure 1.2. A representative diagram of reovirus replication in a mammalian cell. (Adapted from "Reoviruses and their replication", found in Fundamental Virology, 2001.)

1.1.3 Potential of reovirus as a cancer therapeutic

Recent work from the University of Calgary, which gained a significant amount of international publicity, was the discovery that reovirus could be used as a cancer cure through its property as an oncolytic agent (Coffey et al. 1998).

The history of its anti-cancerous potential is traced back to the 1970's. Hashiro et al. (Hashiro et al. 1977) documented the susceptibility of transformed cells to reovirus replication and also found that non-transformed cells were resistant to the virus. Soon after a group showed that WI-38 cells, which are normally not susceptible to reovirus, could be rendered infectible. This was following treatment with the SV-40 large T-antigen (Duncan et al. 1978). It was found that cells with high levels of Epidermal growth factor receptor (EGF-R) sustained viral replication, compared with those with low levels of the receptor (Strong et al. 1993). Cells which were transformed with the oncogene v-erbB were also infectible by the virus (Strong et al. 1996). This truncated gene encodes a constitutively activated receptor of a tyrosine kinase belonging to EGF-R, a gene involved in mitotic activity in cells.

Tyrosine kinase activity of the EGF-R stimulates a pathway, eventually leading to phosphorylation of a protein involved in mitosis, Ras. Activated Ras (Ras-GTP) can then activate myriad signaling pathways important in such cellular processes as differentiation and proliferation. Constitutive activation of signaling pathways downstream of Ras, such as the mitogen-activated protein kinase (MAPK) and the stress-activated, c-Jun NH_2-terminal protein kinase (SAPK/JNK) cascades, are implicated in cellular transformation and progression toward cancer.

As described, reovirus can take advantage of the constitutive tyrosine kinase activity of the truncated EGF-R. Transformation by oncogenes downstream of the EGF-R, such as *sos* or *ras*, has also been found to render a cell susceptible to reovirus infection. Ras signals may sensitize cells to reovirus by blocking cellular defenses against viral infection.

The discovery that an activated Ras pathway determined reovirus susceptibility and that this pathway is important in human cancer lead to the study of using reovirus as an oncolytic agent. Since this is a characteristic of tumour cells, they tested the selectivity of the reovirus to infect ras-transformed tumour cells in mice bearing tumours. So far more than 80% of human tumour cell lines have been proven to be infectible by the virus (Coffey et al. 1998). The experiments showed sufficient tumour regression to suggest the possibilities of the development of a new type of anti-cancer agent.

Reovirus is now undergoing clinical trials (Phase I and II) for the treatment of various cancers in human subjects (Norman et al. 2000). It is also being testing in the treatment of breast cancer showing early success (Norman et al. 2002; Hirasawa et al. 2003). These findings suggest the need to produce large quantities of reovirus for human therapeutic use.

1.2 Animal cell culture to produce vaccines as therapeutics

The concept of vaccines was developed over 200 years ago. A British physician, Edward Jenner, found that women who had been working on farms developed immunity to smallpox (serious disease) after having cowpox (not serious). He found that transferring some infected matter from a cowpox-infected woman to a cut on an uninfected boy gave him immunity to injections of smallpox. This was the first recorded incident of vaccination.

Vaccines have been proven to provide protection against many diseases which can be caused by certain types of bacteria and viruses. Presently there are vaccines for most common diseases such as influenza, cholera, polio, hepatitis, measles, mumps, yellow fever, rabies, to name just a few. The vaccines themselves are commonly preparations of killed whole bacterial cells or live attenuated or inactivation viruses. In some cases subunit vaccines are used which rely on the recognition of specific components by the

immune system to illicit a response. Examples of this include the capsid of *Streptococcus pneumoniae* or viral coat proteins.

The history of vaccine manufacturing dates back to Jenner, but the actual production of suitable vaccines starts just over 100 years ago. In fact, there was little knowledge of viruses until the 1930's at which point suitable methods of production were developed. The first production methods included the infection of embryonic hen's eggs and harvesting the resulting population of viruses. A common problem associated with chicken eggs is that they can cause an immunogenic reaction in the recipient of the vaccine as egg proteins can carry through purification processes. The process requires the individual inoculation of millions of eggs and an extensive purification process from the harvested fluids(Ghendon 1994). Also, the vaccine can differ antigenically and structurally from the original virus used to inoculate the eggs (Katz et al. 1990; Robertson et al. 1990).

The onset of tissue culture changed how vaccines were produced. Animal and human cells can be categorized into three groups. The first are those that are derived directly from animal tissue and do not undergo any subcultivations, thus are classified as primary. These are the closest to "normal" cells as they exhibit properties to those found in an intact healthy organism. The second are cells that can undergo subcultivation but have (a) a finite lifespan, (b) no tumourigenicity upon injection into an animal, (c) the karyology of that of its tissue of origin, and (d) anchorage-dependence, designated as cell strains. The last are those which are (a) immortal, (b) do not have the karyology of the original tissue from which they were derived, (c) can be tumourigenic (produce tumours) when injected into animals, (d) usually anchorage-independent and are called cell lines.

The first successful attempt at large scale production of vaccines in mammalian cells was in the 1950's using dissociated tissue (primary cells) from monkey kidneys. The tissue was isolated from the animal, physically and chemically dissociated with trypsin, and cultured in a liquid medium. This led to a large vaccination program that allowed

millions of people in developed and undeveloped counties to be immunized against diseases such as Polio.

Even though this technology was successful it was plagued with many problems. Some of the animal tissue contained adventitious agents (viruses, bacterial) that were carried over into the final purified vaccine. Also the use of large amounts of animal tissue put pressure on the animal supply. The fact that the cells were obtained from different animals caused difficulties in the standardization of the production.

This led to argument that human cell strains or continuous cell lines should be used in the manufacture of vaccines. Until the 1960's the only class of cultured cells used in the manufacture of human vaccines were those obtained from animal tissue without subcultivation. The 1960's saw the emergence of using non-primary cells for the production of vaccines. In 1964 the veterinary industry used a transformed cell line (BHK) for the production of foot and mouth disease (Shevitz et al. 1990). During this time the use of normal human diploid cells (WI-38) for the production of human vaccines was proposed (Hayflick et al. 1962). The advantages of using these cell lines is that they were well characterized, had a normal diploid karyotype and did not contain infectious agents. Therefore the process would be safer for human vaccines. Although it met with a great deal of resistance in the 1960's it was finally accepted in the 1970's for the production of human vaccines such as polio, adenovirus, rubella, rubeola, varidella, and rabies (Hayflick 1989).

This was a turning point in the vaccine industry. From this point on the use of cell strains (human diploid cells) and cell lines (Vero, MDCK, BHK, etc.) are commonly used in the manufacture of viruses for therapeutic use.

1.2.1 Cell types used in vaccine manufacturing: the suitability of the Vero cell line

There are many types of human and animal cell lines used in the manufacture of vaccines. Of the many animal cell lines used the most common are BHK (baby hamster kidney), MDCK (canine kidney), and Vero (green monkey kidney). Human cell lines are also used which include MRC-5 (human lung fibroblasts), Namalwa (human lymphoblastoid), RPMI (human B cells), and 293 cells (human embryonal kidney). Table 1.1 lists some of these cell lines and what types of vaccines they produce.

Due to its versatility and high growth rate, the BHK cell line is very popular in the production of therapeutics. It is an adherent cell line which is highly susceptible to infection by viruses. It is quite easy to culture and can be grown both in stationary or suspension culture (as cell aggregates). Since the 1960's it has been used to manufacture the foot-and-mouth disease virus in industrial-scale fermenters (Shevitz et al. 1990). They are also used in the production of rabies and some recombinant proteins, such as interleukins, erythropoietin, and antithrombin II. Other cell lines, such as MDCK, are particularly favored for vaccine production since they produce high titers of virus. They can be used in large-scale cultures and have been a successful substrate for the production of human vaccines (Palache et al. 1997; Govorkova et al. 1999; Kessler et al. 1999; Voeten et al. 1999; Voeten et al. 1999; Genzel et al. 2004). Human cells, such as WI-38, have been used to produce vaccines for viruses such as poliovirus (Hayflick 1989).

One of the most important animal cell lines, in regards to human vaccine production, is the Vero cell. These cells confer many advantages, including a high growth rate and a high susceptibility to viral infection. They have already been licensed by the World Health Organization (WHO) for the production of many vaccines (WHO 1987), such as polio (el-Karamany 1987; Shevitz et al. 1990); (Beale 1992) and influenza (Kistner et al. 1999). Vero cells have also been shown to be good substrates for the propagation of bovine vesicular stomatitis virus (Nikolai et al. 1992), herpes simplex virus (HSV) (Griffiths et al. 1981); (Totte et al. 1993) and rabies virus (Mendonca et al.). These

Table 1.1. Animal cells used in the manufacture of vaccines and their products

Cell line	Vaccine
MDBK (Madin-Darby bovine kidney)	BVD virus (bovine viral diarrhea)
NLST (swine testicular)	Aujeszky virus
Vero (Green monkey kidney fibroblast)	Aujeszky virus, Oral polio virus, bovine vesicular stomatitis virus, herpes simplex virus
MDCK (Madin-Darby canine kidney fibroblast)	Influenza
BHK (Baby hamster kidney fibroblast)	Foot and mouth disease virus, rabies
WI-38 (Human lung fibroblast)	Rubella, rubeola, varidella, rabies, polio

processes involve the culture of Vero cells on solid microcarriers (Cytodex-1) in large bioreactors up to 1000 litres (Montagnon et al. 1981; Montagnon et al. 1984). They have been indispensable to the pharmaceutical industry for many years.

1.3 Animal cell culture

1.3.1 Growth media

The mainstream use of animal cells in the biotechnology industry led to the inevitable development of culture media formulations. This has been an ongoing process for the past 50 years. The first attempts at culturing animal cells *in vitro* made use of biological fluids, such as serum and other blood or tissue extracts. This was followed by the attempt to culture animal cells in defined media through the analysis of the contents of biological fluids (Morgan et al. 1950). Another approach, developed by Eagle, consisted of finding the minimum ingredients that were essential for growth which lead to the development of Eagle's MEM (minimal essential media) (Eagle 1955). This media consisted of 13 amino acids, 8 vitamins, 6 ionic species and dialyzed serum to provide the necessary undefined components required for growth. As new cell lines became available in the scientific community, new formulations were developed. Many of these cell lines could be cultured in Eagle's MEM, while others required more complex formulations. These included DMEM (Dulbecco et al. 1959), F12 (Ham 1965), and RPMI (Moore et al. 1967).

Serum, which is a supernatant of clotted blood, is commonly used to provide animal cells with other components necessary for survival. These include attachment factors, micronutrients (trace elements, water insoluble nutrients), growth factors (hormones, proteases), and protective elements (antitoxins, antioxidants, antiproteases). In most cases the growth requirements of the cell are not met until serum is added to the culture medium at a concentration of 5 to 10%.

However with the use of serum come many problems including:

(a) Batch to batch variation which causes inconsistency in growth-promoting properties.
(b) The high protein content which can hinder product purification.
(c) The risk of contaminants – viruses, mycoplasma, prions.
(d) The risk of transmission of these contaminants to an end product used by humans – e.g. BSE (bovine spongiform encephalopathy or Mad Cow disease).
(e) Interference with hormones or growth factors which makes studying their interaction with cells difficult.
(f) Limited availability of fetal calf serum which has seen periods of world shortages.
(g) Expense – serum can account for up to 85% of the overall cost of medium when calculated for large-scale cultures.

For these reasons serum-containing culture systems are becoming undesirable for large-scale processes in industry (Merten 2002). The recent threat to human health caused by the undefined agents of bovine spongiform encephalopathy (BSE) is likely to limit the continued use of bovine serum in culture processes used for the synthesis of health-care products such as viral vaccines (Asher 1999).

1.3.2 Serum-free media for industrially important cell lines

Presently there are still limited types of serum-free formulations available from commercial suppliers and the selection still tends to be quite limited. Many claim to be "all-purpose" serum-free media to support a range of cell lines, but due to the variability of the growth factor requirement between cell lines, they remain quite limited in supporting cell growth (Ham et al. 1978; Ham 1981).

Since serum contains many undefined nutrients it has been difficult to reproduce the growth results associated with serum-supplemented media. Typical serum-free formulations have utilized a wide variety of components to mimic the action of serum.

These have included purified proteins (animal or recombinant origin), peptones, amino acids, inorganic salts, and animal cell or plant hydrolysates.

Serum-free media formulations have been developed for commonly used cell lines such as CHO (Gasser et al. 1985; Kurano et al. 1990; Castro et al. 1992; Yamauchi et al. 1992; Ogata et al. 1993; Zaworski et al. 1993; Amoureux et al. 1995; Chen et al. 1996; Haldankar et al. 1999; Lee et al. 1999; Liu et al. 2001; Takai et al. 2001; Meents et al. 2002; Derouazi et al. 2004; Kim et al. 2004) and Hybridoma (Murakami et al. 1982; Kovar et al. 1984; Schneider 1989; Federspiel et al. 1991; Mariani et al. 1991; Radford et al. 1991; Togami et al. 1991; Chen et al. 1993; Simonson et al. 1994; Butler et al. 1995). These cell lines are typically used to produce recombinant proteins (CHO) and monoclonal antibodies. Since these are immortalized cell lines they tend to lose their requirements for certain growth factors, thus making formulation development much simpler (Ham 1981).

However, cell lines that maintain anchorage-dependence and are thus more "normal" compared to primary cells have much more fastidious nutrient and growth factor requirements (Ham 1981). Thus the availability of serum-free formulations for these types of cells is not as widespread as they are for CHO and Hybridoma. These types of cells can include MDCK, BHK, and MRC-5. These types of cells are very important for industry as they are routinely used for the production of viral vaccines. Although there have been a few reports of a serum-free formulation for BHK (Neufeld et al. 1986; Kratje et al. 1994) and MDCK (Taub et al. 1979; Bashir et al. 1992) the others have remained proprietary and not published (Merten et al. 1994; Scharfenberg et al. 1995; Neermann et al. 1996). The MRC-5 cell strain, on the other hand, has yet to have any reports of a serum-free formulation.

1.3.3 Methods for culturing animal cells – the advantage of microcarriers

There is a wide range of techniques used in industry to culture animal cells. Depending on the cell line, stationary or agitated culture systems can be used. The stationary

systems include roller bottles, T-flasks, cluster-plates, and other similar vessels. These systems are typically used for small-scale production, or to grow cells to seed a larger bioreactor.

Agitated culture systems vary in design and composition. Cells can be grown in suspension, similar to bacteria, where they propagate in a free-floating state. This is a commonly used technique and has the advantage of easily being scaled-up for large systems (10,000 to 50,000 litres). Some cell lines require that they be anchored to a surface and cannot be grown in simple suspension. This problem is solved by the addition of microcarriers, where the cells can anchor themselves to the beads and be grown in a suspension culture. Other systems, rather than agitating the cells, immobilize the cells on solid supports and pass the culture medium over the cells. These perfusion systems confer the advantage of collecting the product downstream and can sustain cultures for long periods of time.

The concept of microcarrier technology was first reported by van Wezel in 1967 (van Wezel 1967) for the growth of human diploid fibroblasts. Several types of microcarriers exist, including solid and macroporous microcarriers. Macroporous microcarriers confer the advantage of providing protection for the cells from shear forces and have been applied successfully for the immobilization of adherent and suspension cells (Racher et al. 1990). Solid microcarriers have proven to be suitable in the production of vaccines, including influenza, polio, and rabies using human MRC-5 and Vero cell lines (Montagnon et al. 1981; Beale 1992; Kistner et al. 1999). Since then the development of microcarriers has been expanded and it has become a standard used in the biotechnology industry. The use of microcarriers confer many advantages, including providing a high surface-to-volume ratio, allowing manipulation during culture without interrupting the fermentation process, and facilitating scale-up possibilities (Reuveny 1990).

However, there are disadvantages to using microcarrier-based culture systems. These include cell destruction through bead to bead contact, bead aggregation (cell bridging),

and slow attachment rates. However, even with these concerns microcarriers are the best approach for large-scale cultivation of anchorage-dependent cell lines to date.

1.4 Glucose and glutamine metabolism in mammalian cells

In mammalian cells glucose and glutamine serve as the major carbon sources for metabolic energy. Glucose supplies energy primarily through aerobic glycolysis breaking it down to two equivalents of pyruvate, a C_3 (Fitzpatrick et al. 1993). However, mammalian cells tend to convert the majority (>90%) of pyruvate to lactate even in the presence of oxygen (Medina et al. 1990). Glucose can enter other pathways, such as the pentose phosphate pathway to generate pentoses for nucleic acids (Neermann et al. 1996). Glucose is converted to ribose-5-phosphate which can be used as the base for a purine or pyrimidine base (Nelson et al. 2000). It can also enter the TCA cycle to be respired for energy or stored as glycogen for later use. Figure 1.3 shows various routes for glucose metabolism.

However, that being said glucose is not required for the survival of many mammalian cell lines. Mammalian cells that are supplied with a nucleotide precursors, such as uridine or cytidine, can grow in the absence of glucose (Wice et al. 1981). This supported the hypothesis that the only purpose of glucose is to supply ribose-phosphate for nucleic acid synthesis. However, some cells cannot be grown without glucose as human fibroblasts stopped growing without it even with purine and pyrimideine bases and nucleosides (Zielke et al. 1976). This suggested that glucose is required for other processes, such as lipid and protein glycosylation (Zielke et al. 1978).

Glucose is not the only carbon source that supplies energy to the cell. Glutamine can generate as much as 50% of the cells aerobic metabolism in many cell lines (Butler et al. 1989; Nelson et al. 2000). Glutamine feeds directly into the TCA cycle which can be completely converted to CO_2 and reducing power (NADH and $FADH_2$) , or partially broken down to other metabolites, such as alanine, lactate, and aspartate (glutaminolysis) (McKeehan 1982). It is also required for purine biosynthesis as the major nitrogen donor

in the synthesis of inosinate (Nelson et al. 2000). Figure 1.4 outlines routes for glutamine metabolism in the cell.

Both glucose and glutamine serve to generate energy in the form of ATP. ATP is formed directly by substrate level phosphorylation during the oxidation of glucose and indirectly from glutamine metabolism via oxidative phosphorylation. Therefore the availability of these two carbon sources can directly affect the energy state of the cell.

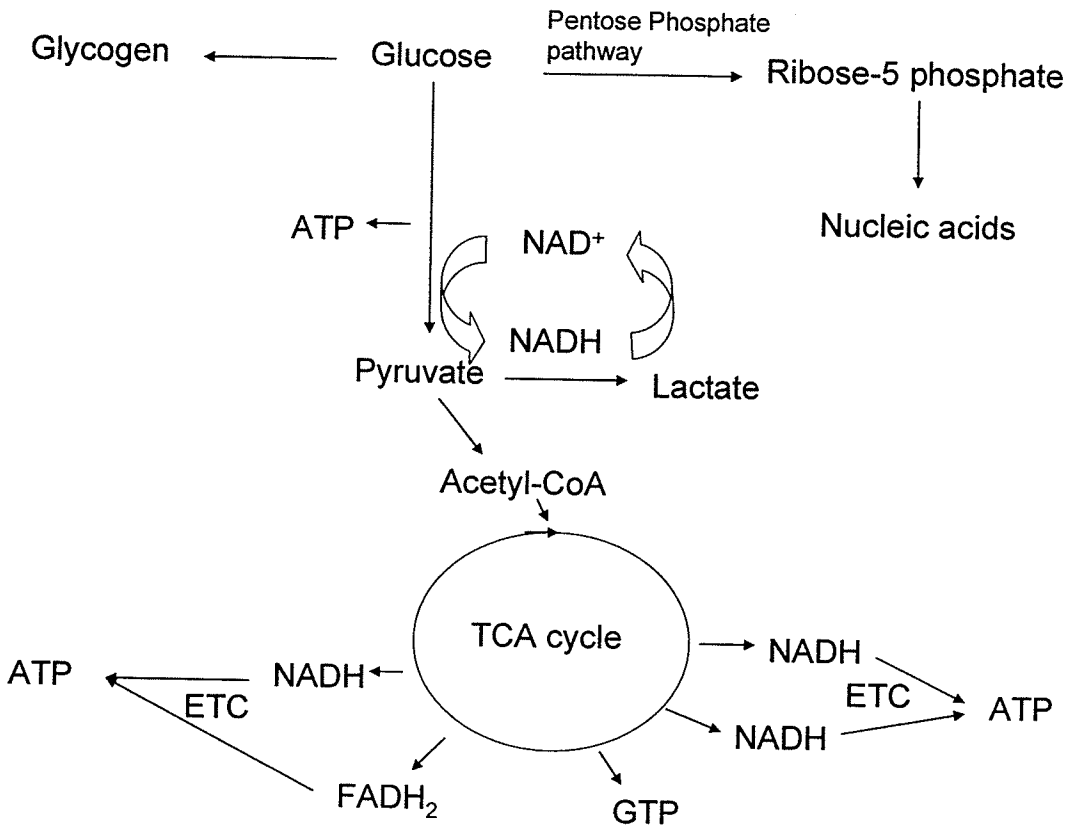


Figure 1.3. Overview of glucose metabolism in mammalian cells. ETC – Electron transport chain; TCA – tricarboxylic acid cycle.

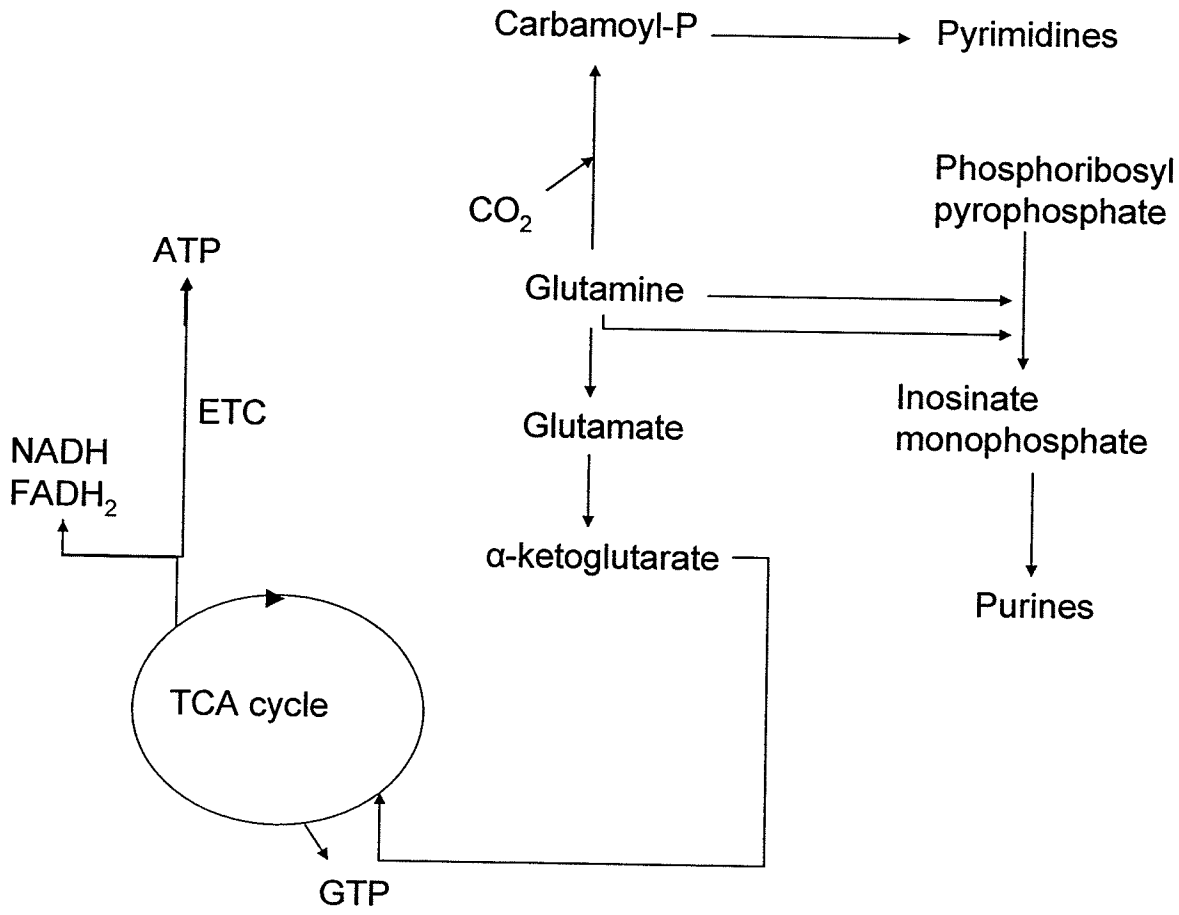


Figure 1.4. A general overview of glutamine metabolism in mammalian cells. ETC – Electron transport chain; TCA – tricarboxylic acid cycle.

1.5 Nucleotides as indicators of metabolic state of cells

Nucleotides are the driving forces for every process within the cell. They are the cellular "currency" and are involved in supplying energy for many processes, such as the manufacturing of macromolecules (proteins and membranes), act as intracellular signals in response to hormones, and are the substrates for deoxyribonucleic acid (DNA) and ribonucleic acids (RNA). For these reasons they are excellent indicators of the internal metabolic state of the cell.

1.5.1 Adenylates and metabolism

Intracellular adenylates serve as the major energy source for cells. Adenylate can exist in three different forms which carry a certain amount of energy depending on the amount of phosphorylation. In the order of increasing energy they are, AMP (adenosine monophosphate), ADP (adenosine diphosphate), and ATP (adenosine triphosphate). The conversion of ATP to ADP + Pi or AMP + PPi is highly exergonic and releases a significant amount of energy (-30.5 kJ/mol per phosphate bond) (Nelson et al. 2000). This energy is coupled to processes such as the synthesis of metabolic intermediates and macromolecules, the transport of substances, or even mechanical motion. Adenylates are also interchangeable with other nucleotides, (such as ATP + GDP -> ADP + GTP) which make them an excellent indicator of the total nucleotide charge.

An analogy can be drawn that the energy of the cell can be thought of as a battery. Thus the adenylate system, AMP + ADP + ATP can resemble electrochemical storage in its ability to receive, store, and donate energy. Reactions that interconvert ATP, ADP, and AMP are:

ATP → AMP + PPi (Equation 1.1)
ADP + Pi → ATP (Equation 1.2)
2 ADP <--> ATP + AMP (Equation 1.3)

Equation 1.1 represents the sum of the following two equations. Equation 1.2 represents reactions that regenerate ATP, such as oxidative phosphorylation or substrate-level phosphorylation. Equation 1.3 is carried out by adenylate kinase.

Catabolic pathways tend to charge the battery by producing ATP from ADP + Pi. Other processes which result in the breakdown of ATP can be considered to discharge the battery. Healthy cells keep them an order of magnitude away from their equilibrium ratios so that ATP hydrolysis can perform useful work when coupled to energy-demanding processes (Hardie et al. 2001).

Adenylates are consumed and produced based on the demands of the cell at any given time. The balance is maintained by regulation of energy producing and energy consuming pathways using the nucleotides themselves as signals. A series of enzymes (such as phosphofructokinase) have been shown to be allosterically regulated by adenine nucleotides (Ramaiah et al. 1964). When the cell has an abundance of energy, ATP will bind to phosphofructokinase and lower its affinity for its substrate, fructose6-phosphate:

PFK

Fructose 6-phosphate -----> Fructose-1,6-bisphosphate

The end result is lowered fructose 1,6-bisphosphate production and a slowdown of glycolysis. This enables the cell to slow down catabolic pathways in times of excess ATP. Lower energy signals, such as AMP, have the reverse effect on phosphofructokinase and increase its affinity for its substrate.

From these first examples a theory was extrapolated that proposed the adenine nucleotides would regulate the switch between anabolism and catabolism (Ramaiah et al. 1964). This was termed the "adenylate control hypothesis". This postulated that the ratios of ATP/ADP or ATP/AMP act as metabolic regulators rather than the actual adenylate concentrations. Such allosterically regulated enzymes can then "detect" the

relative concentrations of each as it has a high affinity for both nucleotides, but only one will cause a response. This type of control is not the only method of regulation, as enzymes can be regulated by feedback inhibition or regulation of gene expression (Nelson et al. 2000).

1.5.2 Adenylate energy charge

The adenylate energy charge is a mathematical relationship described first by Atkinson in the 1960's (Atkinson et al. 1967). This is a relationship between the adenylates that are phosphorylated (and thus have energy potential) over the total adenylate pool. The equation is:

$$\text{AEC (Adenylate energy charge)} = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$$

(Equation 1.4)

If each of the adenylates is represented in terms of its energy potential, ATP is considered to have 2 phosphates per adenylate moiety. ADP then will have 1 phosphate group per adenylate moiety and AMP 0 (completely discharged). Dividing by 2 gives us values between 0 and 1 and this defines the range of the adenylate energy charge (Atkinson 1968).

So how does the cell respond to decreases in adenylate energy charge? Any changes results in the cell switching on pathways that help in its stabilization (Chapman et al. 1971). Atkinson proposed that there are two types of enzymes that respond to energy charge and they are outlined in Figure 1.5 (Atkinson 1968). In conditions of low adenylate energy charge, the enzymes will tend to respond as indicated by curve R. On the other hand, in conditions of energy surplus (high adenylate energy charge), enzymes that utilize ATP will respond as outlined in curve U (Atkinson 1968). These types of responses have been shown with several enzymes, including phosphofructokinase, pyruvate dehydrogenase (Klungsoyr et al. 1968; Shen et al. 1968), citrate synthase and isocitrate dehydrogenase (Atkinson 1968) which respond as curve R. Enzymes which

respond as curve U are phosphoribosyl pyrophosphate synthetase, phosphoribosyl ATP synthetase, and aspartokinase (Atkinson et al. 1967; Klungsoyr et al. 1968). The intersection between the two curves represents a metabolic steady state where ATP-consuming and ATP-producing pathways are in equilibrium. Therefore, any decrease in the adenylate energy charge will result in an increase of ATP-producing pathways and a decrease in ATP-consuming pathways (Atkinson 1968). Similarly, increasing beyond this intersection would be met with resistance to return the AEC to its equilibrium.

The relative proportion of adenine nucleotides with respect to adenylate energy charge is shown in Figure 1.6. If an enzyme is regulated by adenylates it must respond to the concentrations of one or more of them at a given energy charge. Looking at the graph a high adenylate energy charge shows that ATP is the predominant species and ADP and AMP are in low amounts. Thus an enzyme such as phosphofructokinase (PFK) would be inhibited by high adenylate energy charge as ATP-binding to the enzyme lowers its affinity for its substrate. As the AEC decreases the AMP and ADP levels increase with a corresponding drop in ATP. This would result in AMP binding to PFK and thus increasing its affinity for its substrate.

There are other methods that the cell can use to increase energy charge. Under conditions of decreasing energy charge, and in the absence of a plentiful nutritional source (such as glucose), cells can maintain a high AEC by decreasing the AMP concentration. This is accomplished by AMP deaminase which catalyzes the deamination of AMP to IMP (inosine monophosphate) (Chapman et al. 1973). AMP deaminase activity increases with decreasing energy charge and is considered to protect against large variations of adenylate energy charge. The degradation of the adenylate pool concentration for energy charge stabilization has been shown in many tissues (Yoshino et al. 1981). However, this is not the only route for decreasing AMP as an alternate pathway exists. AMP can be dephosphorylated to adenine by 5^1-nucleosidase (Yoshino et al. 1981; Bontemps et al. 1986; van den Berghe et al. 1990). Deamination of AMP leads to an increase of equation 1.4; that is, bringing it closer to 1. Thus, as the energy charge decreases, [AMP] rises,

encouraging its own degradation, and thereby stabilizing the energy charge by reducing the adenylate pool size.

The adenylate energy charge varies between organisms and has a range that has been measured from 0.6 to 0.95. The energy charge is a result of the environment of the cell *in vivo* and represents the amount or lack of nutrients at any given time. Cells that require high amounts of glucose, brain cells for example, appropriately have a high adenylate energy charge of 0.95 (Siesjo 1978). Other cells in the body, such as the liver have a range of 0.6 to 0.8 (Kaminsky et al. 1984; Kaminsky Yu et al. 1985). For bacteria, actively growing cells usually maintain a level of 0.9 (Chapman et al. 1971; Ball et al. 1975; Swedes et al. 1975).

1.5.3 Adenylates and AEC as indicators of metabolic status

The relationship between AEC and regulatory control of enzymes involved in metabolism implies that AEC may influence areas such as cell viability and growth. It is commonly found that cells remain viable with AEC values higher than 0.30 (Chapman et al. 1971; Ball et al. 1975). In some cases, a drop to 0.15 in yeast does not affect the viability (Ball et al. 1975). In other cases, such as in *E. coli*, AEC has also been shown to be linked to cell growth, where a drop in ~ 10% of the AEC from the levels normally seen in actively growing cultures leads to arrested growth (Swedes et al. 1975). In addition, depletion of a carbon source leads to a decrease in AEC and subsequent decrease in cell growth (Walker-Simmons et al. 1977). The link between AEC and growth is not ironclad however, as some reports have shown that bacteria in stationary phase can have AEC of close to 1.0 in some cases (Leps et al. 1979). Interestingly, the absolute concentration of ATP and adenylate can drop to as much as 30% without affecting the viability of the bacteria (Swedes et al. 1975; Leps et al. 1979; Leps et al. 1979). Bacteria have even been grown in chemostat cultures at a normal rate with adenylate pools reduced by as much as 60% (Swedes et al. 1975).

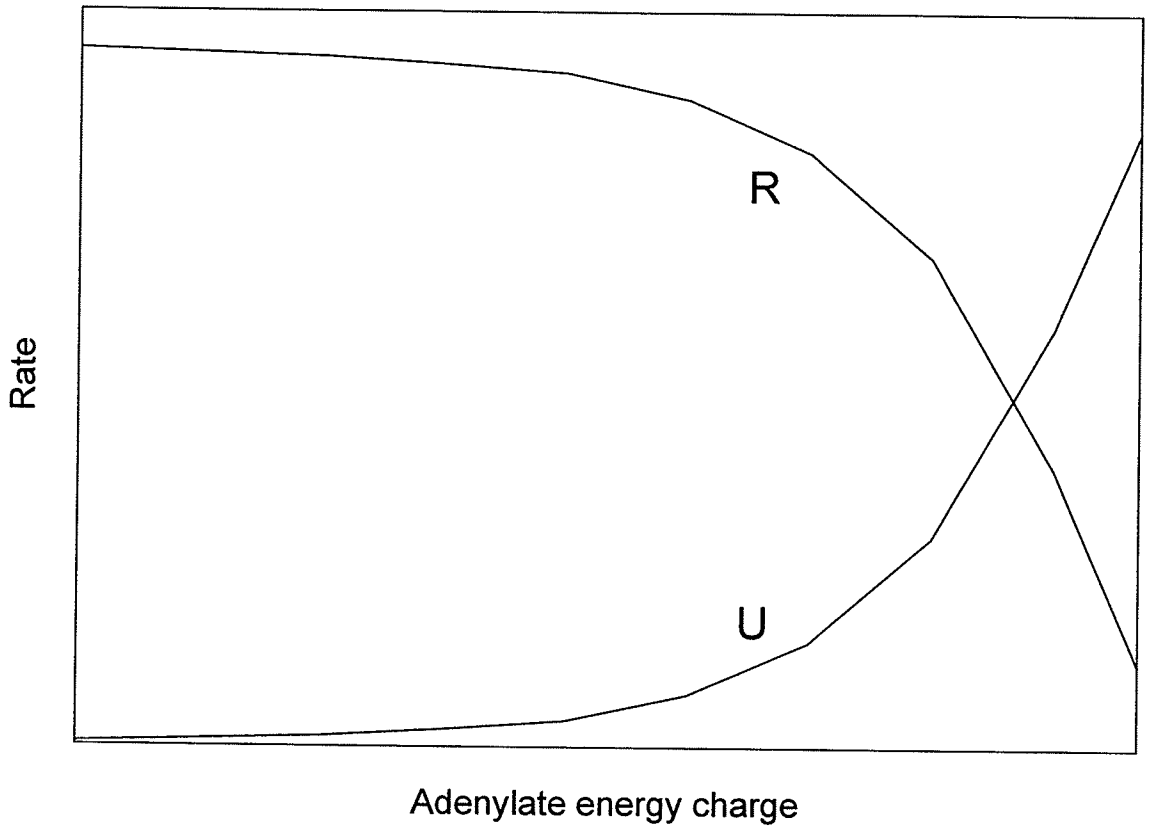


Figure 1.5. Activity rate of enzymes involved in ATP-regenerating (R) and ATP-utilizing (U) processes with respect to adenylate energy charge. (Adapted from (Atkinson 1968)).

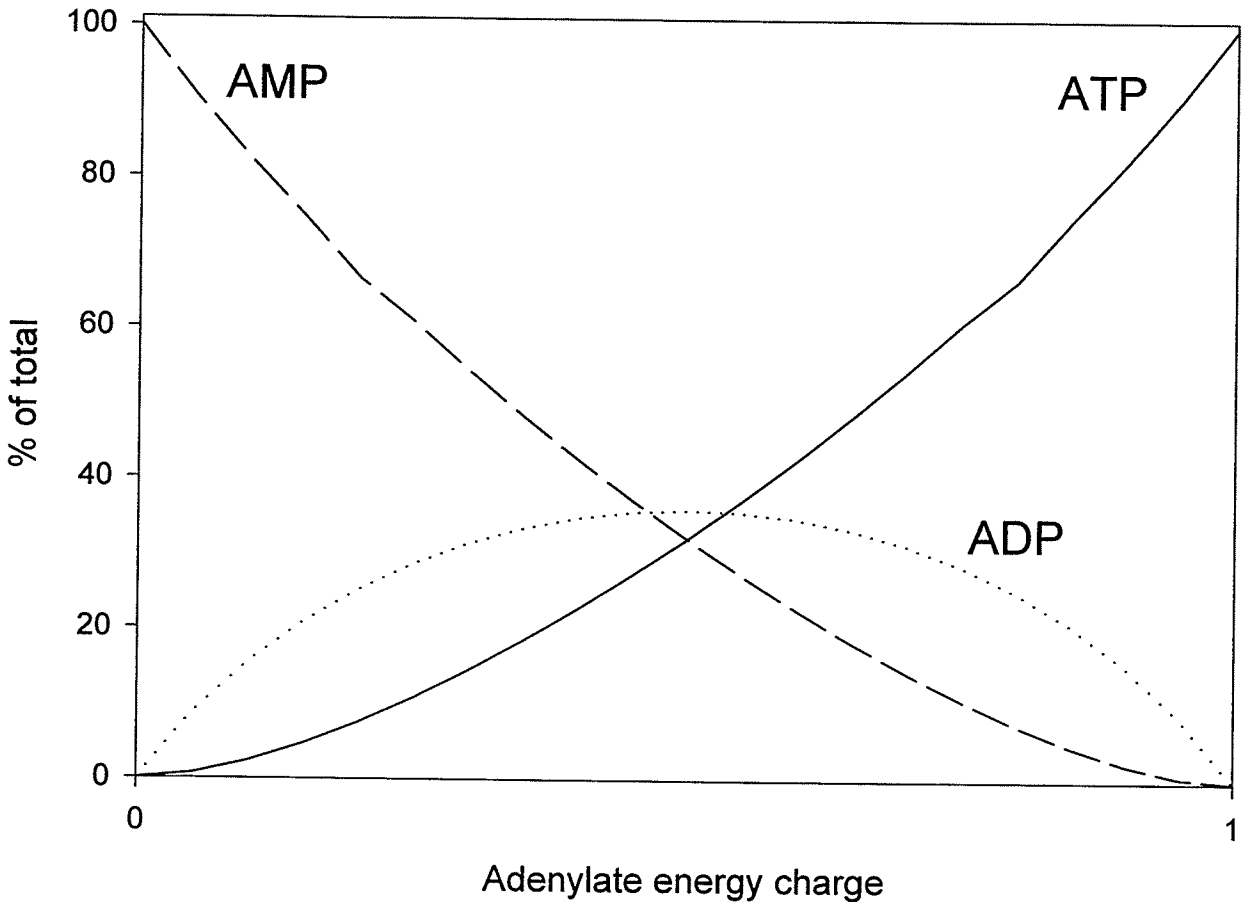


Figure 1.6. Relative concentrations of adenine nucleotides with respect to adenylate energy charge (adapted from (Atkinson 1968). Relative concentrations of ATP, ADP, and AMP according to the adenylate kinase reaction when at equilibrium (Keq=0.80).

Even though there is not a tight relationship between AEC and cell growth/viability there is evidence that AMP and cellular metabolism are closely related. It has been proposed that the ATP:AMP ratio is a more sensitive measure of the energy status of the cell (Hardie et al. 2001). Eukaryotic cells have very active adenylate kinase that interconvert ATP, ADP, and AMP (equation 1.3, above) and maintains the reaction close to equilibrium. If at equilibrium the AMP:ATP ratio will vary as the square of the ADP:ATP ratio (equation 1.6):

$$\frac{[ATP][AMP]}{[ADP]^2} = K \qquad \text{(Equation 1.5)}$$

$$[ATP][AMP] = K \times [ADP]^2$$

Divide both sides by $[ATP]^2$

$$\frac{[AMP]}{[ATP]} \alpha \frac{[ADP]^2}{[ATP]^2} \qquad \text{(Equation 1.6)}$$

Since healthy cells maintain an ATP:ADP ratio of 10:1, the ATP:AMP ratio will be 100:1. If the ADP:ATP ratio increases 5-fold, the AMP:ATP ratio will rise by 25-fold. Thus the ATP:AMP ratio is a more sensitive measure of the energy state of the cell. Elevations in AMP levels activate the AMP-activated protein kinase cascade (AMPK). This cascade that results in inactivation of ATP-consuming pathways, such as fatty acid and sterol synthesis (Hardie et al. 1997). It also activates ATP-producing pathways, such as fatty acid oxidation and glycolysis (Hardie et al. 1999). Given these results with AMPK it is not surprising that earlier research found that a drop in AEC results in lower or halt in protein production (Swedes et al. 1975; Walker-Simmons et al. 1977). This was most likely due to increased AMP concentrations activating AMPK.

Protein synthesis seems more sensitive to AEC than it does to adenylate or ATP concentration. Cells can continue to carry out protein synthesis even when 70% of the

ATP concentration had been depleted. When the AEC dropped by < 0.10 it lead to a decrease of 90% in protein synthesis in *E coli* (Swedes et al. 1975). Given the fact that protein synthesis consumes a great deal of ATP in the cell (more than 60% of total), and utilizes it directly during the charging of aminoacyl t-RNA (Nelson et al. 2000), it is not surprising that a drop in AEC would lead to a slowdown of protein production.

1.5.4 Other nucleotides and metabolism

Adenylates are not the only nucleotides that are directly involved in cell metabolism. Other nucleotide triphosphates play important roles in synthesis of genetic material and many different metabolic pathways.

GTP plays a central role in regulating and stimulating anabolic processes involved in cell growth. GTP is required for protein synthesis as it plays a role in the initiation and elongation steps of the growing peptide chain (Nelson et al. 2000). It is also used in intracellular signal transduction (Pall et al. 1988) and acts as a regulator in lipid-mediated glycosylation reactions in animal cells (Bossuyt et al. 1993). In addition, trafficking of intracellular vesicles is mediated by GTP-binding proteins (Pall et al. 1988; De Matteis et al. 1991). Declines in the GTP pool have been met with decreased growth in mammalian cell lines and is not surprising given that GTP itself is involved in mitogenic pathways, such as the Ras pathway (Sokoloski et al. 1986; Ayllon et al. 2000).

Other nucleotide triphosphates play roles in anabolic pathways. CTP is used for the production of lipid membranes (Bishop et al. 1988) and UTP supplies precursors to protein glycosylation (Ryll et al. 1992). UTP is also a precursor for the synthesis of CTP (Nelson et al. 2000).

1.5.5 Nucleotides and virus production

Viruses are obligate parasites which mean that they are dependent upon the host cell system to survive. Replication of viral progeny requires the production of genetic

material and protein which requires significant amounts of energy from the host cell. Indeed, ATP has been shown to decrease with a corresponding increase in O_2 consumption directly after a virus infection (Bardeletti 1977). It is likely then that the physiological state of the cell can have a significant impact on virus production.

There are many examples of a direct relationship between NTP's and processes involved in virus replication. ATP is required for the initiation and elongation stages of adenovirus replication (De Jong et al. 1983). A drop in ATP concentration, from 8 mM to 0.15 microM, can slow its replication down by 30%. The binding of simian virus tumour T-antigen to the SV40 origin of replication to initiate viral DNA synthesis is also an ATP-dependent process (Murakami et al. 1993). Similarly, the initiation of DNA synthesis in herpes simplex virus by the formation of a nucleoprotein complex is ATP-dependent (Gustafsson et al. 1994). A few examples of viral cores utilizing the host's ATP to generate other NTP's to fuel virus replication by a nucleoside diphosphate kinase have been observed (Testa et al. 1979). Some have ATP-dependent helicases (Aslani et al. 2002). These observations show the specific requirements of viruses for the host's NTP pool.

Some RNA viruses use de novo synthesis to initiate RNA synthesis where the RDRP (RNA-dependent RNA polymerase) uses a NTP primer (Kao et al. 2001). Some of these RNA viruses include rotavirus (Chen et al. 2000), vesicular stomatitis virus , alphavirus (+ve ssRNA), and flaviviridae. There is usually a requirement for a specific NTP over the others, such as GTP (Lohmann et al. 1999).

The hypothesis that virus production is linked to the physiological state of the cell has been supported in the literature. A correlation of virus production to cellular growth state has been reported (Chillakuru et al. 1991). This was attributed to a higher [ATP] in actively growing cells compared to cells in a stationary growth state (Mukhopadhyay et al. 1994). The conclusion was that virus production was proportional to these ATP levels in the cells. In addition, cell cultures had higher virus output when grown in a protein-free medium (Nadeau et al. 2002). This again was attributed to a higher

intracellular [ATP] in the protein-free medium compared to cells grown in a protein-containing medium.

1.6 Thesis objectives

Research on reovirus has revealed its importance to the healthcare industry. An alternative bioprocess is needed to produce reovirus that would be suitable for human therapeutic use or for rotavirus vaccine development. The development of a novel serum-free formulation (designated M-VSFM) for the growth of Vero cells has recently been developed in our lab (Butler and Berry, 1996. Data unpublished). Since Vero cells have been shown to be infectible by reovirus (Taber et al. 1976), this has provided for the unique opportunity to evaluate a serum-free culture system to propagate the virus. Due to the complete absence of animal products, and the low protein content, it will provide a safe alternative to industry for the production of therapeutics.

M-VSFM has been developed to support the growth of the anchorage-dependent cell line, Vero. It is possible that the nutritional and growth factor requirements are similar to Vero clones and other adherent and anchorage-dependent cell lines/strains, such as BHK, MDCK and MRC-5. Therefore the formulation will be tested to determine if they can support these cell types.

The amount of virus that can be produced from a culture has been linked to cellular metabolism (Nadeau et al. 2002). It has also been affected by the growth state of the cell (Chillakuru et al. 1991). Due to the broad role of nucleotides in cellular metabolism and cell growth, it is possible that these levels may affect virus production.

The goals of the Ph.D. were to:

(1) Evaluate and improve the performance of M-VSFM to support the growth of Vero cells. (re: Chapter 3)

The first phase of the study was to evaluate the newly designed medium, M-VSFM, to support the growth of Vero cells. This was done by growing the cells in agitated culture (spinner flasks) using Cytodex-1 as a microcarrier. The growth of the cells was compared to other standard formulations used to grow Vero cells, such as serum-supplemented media and the few serum-free formulations available on the market. The second phase of the study was to improve the performance of the medium by using nutrients, growth factors, and supplements known to be stimulatory for growth. The final phase of the study was to determine how well M-VSFM could support the growth of Vero clones, Vero E6 and Vero-76, which are routinely used in the production of viruses. If the medium is unsuccessful at supporting the Vero clones supplements or growth factors known to be beneficial to growth will be added.

(2) Test the hypothesis that M-VSFM can support the growth of other industrially important cell lines/strains. (re: Chapter 4)

The purpose of this study was to evaluate the original and improved M-VSFM formulation on the cell lines BHK, MDCK, and MRC-5. They will be propagated in both stationary and agitated (microcarrier) cultures. The performance of the medium will be compared to control media routinely used to culture these cells (as recommended by the supplier). If the original formulation is not able to support the growth of these cells various growth factors and supplements known to be stimulatory for these cell lines will be added to the medium.

(3) Test the hypothesis that a serum-free culture system of Vero cells would be suitable for the propagation of reovirus. (re: Chapter 5)

The first phase of this study was to determine how well a serum-free culture system of Vero cells would propagate reovirus. This will first be tested in stationary (T-flask)

cultures and then moved up to microcarrier cultures. The performance of the culture will be compared to Vero cells in a serum-based medium as a control and the overall production and specific productivities will be calculated. The second phase of the experiment will look at how this serum-free culture system of Vero cells compares to the standard serum-based mouse L-929 system for producing reovirus. As this method is currently the production method of choice in the literature it is important to compare the two systems.

(4) To test the hypothesis that virus production is not linked to growth state and that reovirus infection affects the physiological state of the cell. (re: Chapter 6)

The growth state and energy state is commonly a synonymous term in cell culture. However, this is not the case and even cells in a non-growth state (stationary phase) can have high energy levels (i.e.: ATP, AEC). That being said it is possible that if virus production is linked the energy state of the cell it should not be affected by the growth state.

The first purpose of this study is to test the hypothesis that virus production is not linked to growth state. Virus productivity from cultures in different growth states will be examined. Cultures that are actively growing (mid-exponential) and cells that are not growing (stationary phase) will be infected with reovirus. The intracellular nucleotides, AEC, and specific productivities will be compared. The results will be compared to those found in chapter 8 to determine whether virus production follows energy state more closely than growth state. This will give useful information for optimizing virus production systems to know the best time to initiate an infection.

The second purpose of this study is to determine how much energy, in the form of nucleotides, is required by reovirus during an infection. Cultures of Vero cells will be infected with a known amount of virus and the intracellular nucleotides will be monitored. In addition, the drain upon the AEC will be examined. This will give

important information on how much energy is required to produce viral progeny and may be useful for optimization strategies.

(5) Test the hypothesis that intracellular adenylate levels are dependent upon extracellular nutrient concentration. (re: Chapter 7)

Glucose and glutamine are two major nutrients that are very important for the metabolism of mammalian cells. The glucose and glutamine concentrations in the medium will be compared to the intracellular adenylate levels in the cell to determine the contribution that each has to the metabolic state. The ATP, ADP, AMP, and adenylate energy charge will be determined at varying glucose and glutamine concentrations. Understanding what levels can affect the energy state of the cell will be useful for determining how this will affect virus production in later studies.

(6) To determine the relationship between virus production and intracellular nucleotides levels. (re: Chapter 8)

Two approaches were used to answer this question. The first approach examined the effect of a lowered energy state (i.e.: intracellular [ATP]) on virus production. A culture with a lowered energy state will be infected with reovirus and the virus production from this culture will be compared to a control. The glucose and glutamine levels in the medium will be lowered to establish this lowered energy state as determined by experiments in chapter 6. The second approach will look at a spread of energy states (varying ATP levels) and how well these cultures produce a virus. The nucleotides and AEC will be examined at every step of the infection. The relationship between these and virus production will be determined.

CHAPTER 2

Materials and Methods

2.1 Cell Lines

The majority of the cell lines were obtained from the American Type Culture Collection (ATCC). Those that were not obtained from the ATCC were supplied from other sources (see below). The descriptions are as follows:

a) Vero (CCL-81), African green monkey kidney fibroblast.
b) MRC-5 (CCL-171), human lung diploid fibroblast.
c) MDCK (CCL-34), canine kidney fibroblast.
d) BHK (CCL-10), hamster kidney fibroblast.
e) L-929 (CCL-1), mouse fibroblast. (Supplied by Dr. Kevin Coombs, Medical Microbiology, University of Manitoba, Canada)
f) Vero E-6 (CCL-1586), African green monkey kidney fibroblast, clone derived from CCL-81. (Supplied by National Microbiology lab, Winnipeg, Manitoba, Canada)
g) Vero-76 (CCL-1587), African green monkey kidney fibroblast, clone derived from CCL-81. (Supplied by National Microbiology lab, Winnipeg, Manitoba, Canada)

2.2. Stock Cell Maintenance

Each of the stock cells were initially maintained in T25, T75, or T150 culture flasks using 10, 25, or 50 ml of media. The medium that was used to establish primary banks of the cells was 10% fetal bovine serum-supplemented DMEM (5% FBS-DMEM for MRC-5 cells). The flasks were passaged every 72 to 96 hours by a 1:3 to 1:5 ratio split depending on the cell line:

- Vero – 1 in 4
- MRC-5 – 1 in 3

- MDCK – 1 in 4
- BHK – 1 in 5
- L-929 – 1 in 5

2.3 Trypsinization protocol

The majority of the cell lines, with the exception of L-929, are anchorage-dependent and were trypsinized during sub-culture. The materials used in this procedure are:

PBS-EDTA (pH 7.5):
Dulbecco's phosphate buffered saline (Ca^{2+} and Mg^{2+} free, Gibco- BRL # 21600-010) containing 0.70 mM EDTA.

Trypsin (0.25% w/v):
Porcine trypsin in Hank's balanced salts solution containing 1.0 mM EDTA (Gibco-BRL 25200-072).

Trypsin inhibitor (0.25% w/v):
Soybean trypsin inhibitor (Gibco-BRL 17075-029) in Dulbecco's phosphate buffered saline (filter sterilized).

The protocol for each of the cell lines were as follows:

2.3.1. Stationary Cultures (T-flasks)

2.3.1.1 Vero, Vero E-6, Vero-76, MRC-5, and BHK cell lines

1) Remove medium supernatant from the culture flask by pipette and discard
2) Wash the cells in 10ml pre-warmed PBS-EDTA (0.25%) and let sit for 1 minute.
3) Remove the PBS-EDTA.
4) Add pre-warmed (37°C) 0.25% Trypsin-EDTA to the culture flask.

- 3.0 ml for 150 cm^2 flask
- 1.0 ml for 75 cm^2 flask
- 0.5 ml for 25 cm^2 flask

5) Dislodge the cells by swirling flask back and forth. Gently tap the flask until the cells become displaced.
6) Add equal or greater amount of trypsin inhibitor and resuspend cells with 8 ml of PBS-EDTA. Centrifuge at 500 x g for 5 minutes. (In the case of using serum-based media add 9 ml of media to the trypsinized cells)
7) Remove the cells from the centrifuge and discard the supernatant.
8) Resuspend cells in an appropriate amount of media (5 to 10 ml) and perform a cell count.
9) Inoculate a new flask with 1.0 x 10^5 cells/ml.

2.3.1.2 MDCK cells

1) Remove media supernatant from the culture flask by pipette and discard
2) Wash the cells in 10ml pre-warmed PBS-EDTA (0.25%) and let sit for 3 minutes.
3) Remove the PBS-EDTA.
4) Add pre-warmed (37°C) 0.25% Trypsin-EDTA to the culture flask
   - 8.0 ml for 150 cm^2 flask
   - 5.0 ml for 75 cm^2 flask
   - 3.0 ml for 25 cm^2 flask

   Incubate at 37°C for 15 to 20 minutes, with occasional swirling, until most of cells have been dislodged from the surface.
5) Dislodge the cells by swirling flask back and forth. Gently tap the flask until the cells become displaced.
6) Add equal or greater amount of trypsin inhibitor and resuspend cells with 8 ml of PBS-EDTA. Centrifuge at 500 x g for 5 minutes. (In the case of using serum-based media add 9 ml of media to the trypsinized cells)
7) Remove the cells from the centrifuge and discard the supernatant.

8) Resuspend cells in an appropriate amount of media (5 to 10 ml) and perform a cell count (2.4).
9) Inoculate a new flask with 1.0 x 10^5 cells/ml.

2.3.2 Microcarrier cultures: Cell counting

2.3.2.1 Vero, MRC-5, MDCK, and BHK

1) Take a 1ml sample from the spinner flask and pipette into a 1.8 ml Eppendorf tube.
2) Allow the beads to settle and remove the supernatant. Wash the beads with 1 ml of PBS-EDTA.
3) Remove the PBS-EDTA.
4) Add 0.5 ml of 0.25% Trypsin-EDTA and gently swirl the tube to facilitate dislodging of the cells.
5) When the cells have detached add 0.5 ml of trypsin-inhibitor.
6) Take a 150 µl sample and mix with an equal volume of Trypan Blue to count using the Trypan blue exclusion method (2.4).

2.3.3 Microcarrier Cultures: Cell passaging

2.3.3.1 Vero, MRC-5, BHK

1) Allow the microcarriers in the spinner flask to settle
2) Aspirate off ~80% of the supernatant
3) Aspirate out the microcarriers and pipette into a 50 ml tube
4) Allow the microcarriers to settle and remove as much of the medium as possible
5) Wash the microcarriers with 20ml of PBS-EDTA (resuspend the microcarriers with gentle pipetting)
6) Allow the microcarriers to settle
7) Aspirate the PBS-EDTA supernatant
8) Repeat the wash procedure and aspirate off the excess PBS-EDTA

9) Add 10 ml of trypsin (0.25%) to the microcarriers and resuspend
10) Gently agitate the microcarriers for 15 seconds every minute for 10 minutes with gentle swirling
11) Inspect via microscopy to ensure that the cells have dislodged from the beads (until you have ~95% "bald heads")
12) Add equal volume of trypsin inhibitor to the tube
13) Add 20 ml of PBS-EDTA and gently pipette the microcarriers
14) Allow the beads to settle and aspirate off the supernatant
15) Centrifuge the supernatant (with the cells) for 5 min at 500 x g.
16) Resuspend the cells in pre-warmed fresh medium and count the cells using the Trypan Blue exclusion method (2.4)
17) Inoculate a new flask with an appropriate amount of cells

2.4 Trypan Blue Dye Exclusion Cell Count

Trypan Blue is dye that stains cellular proteins and measures cell membrane integrity. Dead cells will take up the dye and viable cells will exclude it. Samples of a cell suspension were diluted 1:1 with 0.2% (w/v) trypan blue in Dulbecco's phosphate buffered saline (Gibco®). Both sides of the grid of the Neubauer haemocytometer slide were loaded with the cell suspension (approximately 10 µl). The number of cells present in the four grids, N, were counted through a microscope and the cell number calculated as follows:

$$N/4 \times (1 \times 10^4) \times \text{dilution factor} = \text{Viable cell number}$$

The blue cells are considered non-viable and are therefore not included in the cell number. The total cell count was the number of viable cells plus non-viable cells and was used in culture viability calculations according to the formula:

$$\frac{\text{Viable cells}}{\text{Total cells}} \times 100\%$$

2.5. Microcarrier Cultures

2.5.1. Microcarrier preparation

Cytodex-1 (Pharmacia®, Uppsala, Sweden) beads were swollen overnight at 20 g/L in 500ml of PBS (phosphate buffered saline, Gibco®) at pH 7.2. The supernatant was then discarded and the microcarriers were washed twice with fresh PBS (300ml) and then autoclaved at 121°C for 25 minutes.

2.5.2. Growth in spinner flasks

Glass spinner flasks (Bellco®, Vineland, NJ, U.S.A.) with volumes of 100, 250 and 500ml were used in the experiments. The inside of the flasks were pre-treated by siliconization with SigmaCote (Sigma®), washed thoroughly with distilled water, and autoclaved at 121°C for 20 minutes before use. The prepared microcarriers were first washed 1x with pre-warmed media and then added to the spinner flask at a concentration of 2 g/L (unless otherwise stated). Cells were added to spinner flasks at 2.0 x 10^5 cells/ml to ensure a cell to bead ratio of 20 to 1. They were then placed at 37°C with a 10% CO_2 overlay with a stir rate of 40 rpm unless otherwise noted. Daily cell counts were taken and counted using the Trypan Blue exclusion method (2.4.).

2.6. Media preparation

2.6.1. Serum-based formulations

The standard serum formulation was 10% fetal bovine serum (Gibco BRL ®)-supplemented DMEM. The powdered DMEM (Dulbecco's modified Eagle's medium) was dissolved in ~80% of the final volume of water (Milli-Q™, 18Ω conductivity) with the following supplements added: was further supplemented with these dry ingredients: 4.5 g/L D-glucose (25 mM), 600 mg L-glutamine (4 mM), 170 mg L-serine (2 mM), 55

mg sodium pyruvate (0.5 mM), 3.0 g sodium bicarbonate (36 mM), and 15mg Phenol Red (4×10^{-2} mM). The medium was adjusted to a pH of 7.1 – 7.2 with HCl prior to increasing to the final volume. The media was then filtered sterilized by peristaltic pumping through a 0.2 μM Acrocap filter (Gelman Sciences) into sterile glass bottles. The fetal bovine serum was then added to a concentration of 5 to 10% v/v. The medium was stored at 4°C.

2.6.2. Serum-free formulations

M-VSFM (Modified-Vero Serum Free Medium)

M-VSFM consisted of a DMEM basal medium supplemented with recombinant peptide hormones, nutrients, trace elements, and dry components (listed in Table 2.1). The DMEM was dissolved in ~80% of the final volume of water (Milli-Q™, 18Ω conductivity) followed by the dry components. The medium was adjusted to a pH of 7.1 – 7.2 with HCl prior to increasing to the final volume. The media was then filter-sterilized by peristaltic pumping through a 0.2 μM Acrocap filter (Gelman Sciences) into sterile glass bottles. The nutrients, trace elements, and hormones were combined as a 10x supplement, filter sterilized, and added to the final medium at 10% v/v.

Table 2.1. Preparation of M-VSFM.

The following components make **1 litre** of the serum-free formulation, M-VSFM. The DMEM is first dissolved in ~ 80% of the final volume in Milli-Q grade water. The remainder of the dry components are then added before adjusting the pH to 7.1 – 7.2. The medium is then filter sterilized followed by the addition of the Biogro 10X supplement (Biogro Technologies).

Component	Amount (mg, or ml) per litre	Final concentration	Source or stock solution
Dry components			
DMEM	1 package		powder
Glucose	4500	25 mM	powder
Glutamine	600	4 mM	powder
Serine	170	2 mM	powder
Sodium pyruvate	55	0.5 mM	powder
$NaHCO_3$	3000	36 mM	powder
Phenol red	15	4×10^{-2} mM	powder
$FeSO_4 \cdot 7H_2O$	1.0	3.6 μM	powder
Liquid components			
Biogro 10X Supplement	100 ml	10% v/v	10x Concentrate

2.7. Nucleotide Analysis

2.7.1. Nucleotide Extraction

Samples of cell cultures (1-4 x 10^6) were transferred to a 15 ml sterile centrifuge tube and sedimented at 350 x g for 8 minutes. The cell pellet was resuspended in 120 – 150 µl ice-cold 6 % trichloroacetic acid, kept in an ice bath and sonicated for 15 seconds (Micron Ultrasonic cell Disruptor, Mandel®). The suspension was subsequently kept on ice for 15 – 20 minutes. The trichloroacetic acid/cell suspension was transferred to a 1.5 ml centrifuge tube and sedimented at 14 000 x g for 10 minutes at 4°C. Supernatants were collected and neutralized with 27 µl Tris 0.5 M, pH 9.0 and 11.5 µl NaOH 2 M per 100 µl of trichloroacetic acid extract. Neutralized extracts were stored at -20°C.

2.7.2. Quantification of nucleotides by ion pair reverse phase HPLC

The HPLC was controlled by Waters Breeze software. The system included Waters 1525 Binary pumps, Waters 717 plus autosampler, an Alltech 200 x 4.6 mm Adsorboshpere OPA HR5u reverse phase C_{18} column, and a Linear UVIS 200 UV detector. The samples were injected using an autoinjector to deliver 100 µl samples into the C_{18} column. Nucleotides were separated using a gradient of two buffers modified from Ryll and Wagner (Ryll et al. 1991). Two gradient profiles were used (i.e.: Gradient 1 and Gradient 2), both of which separated 9 nucleotides.

Nucleotide peaks were identified by comparing retention time of the samples with the standard mixtures and by spiking the samples with standards. Quantification was performed by comparing the sample peak area to a standard peak area curve at 254 nm. Good separation was achieved with a new column but as the column aged (1 to 2 runs, approximately 50 samples) the ADP and UTP peaks fused. Extensive washing of the column would not alleviate this effect. The UTP peak was determined then by subtracting the ADP peak from the total peak area after being determined by luminometry (section 2.7.3). A typical chromatogram is shown in Figure 2.1.

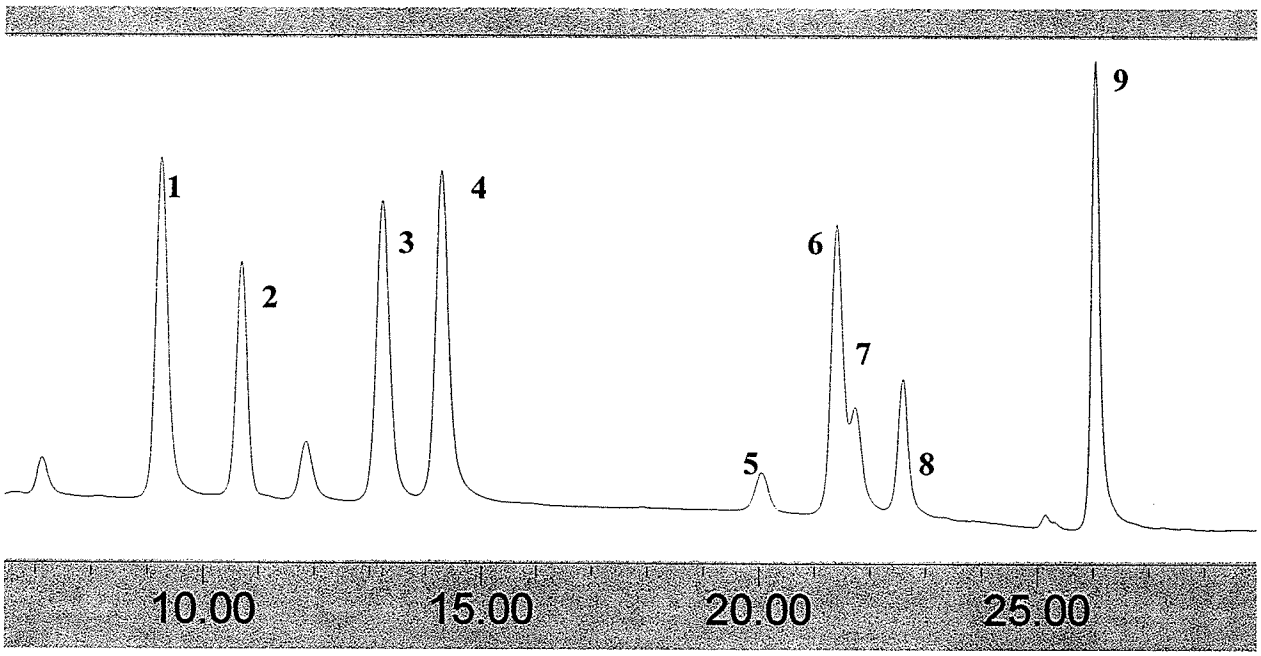


Figure 2.1. The gradient elution profile and chromatogram of a standard mix of nucleotides. The sample contains known amounts of nucleotides. They are, in order of elution, NAD (1), UDP-glucose (2), AMP (3), GDP (4), CTP (5), UTP (6), ADP (7), GTP (8), and ATP (9). As the column aged the UTP (6) and ADP (7) peaks would fuse. All other peaks are unknown components.

2.7.2.1. HPLC buffers and gradients

(1) Buffer A

48.6 g	KH_2PO_4
9.6 g	K_2HPO_4
10.88 g	tetrabutyl ammonium hydrogen sulfate

Dissolved in 3.8 L of water. The pH was adjusted to 5.3 with 85% (v/v) phosphoric acid. The solution was filled up to 4 litres with water and filtered through a 0.2 µm AcroCap filter.

(2) Buffer B

1050 ml	buffer A
450 ml	methanol (HPLC grade)

The pH was adjusted to 5.9 with 2.5 M KOH.

Gradient 1:

100% Buffer A for 2.5 minutes, 0 – 40% buffer B for 14 minutes, 40 – 100% buffer B for 8.5 minutes, 100% buffer B for 12 minutes, 100 – 0% buffer B for 4 minutes, 100% buffer A for 9 minutes. Total run time: 50 minutes. Flow rate 1.5 ml/min.

Gradient 2:

0 – 20% buffer B for 5 minutes, 20 – 24% buffer B for 10 minutes, 24 – 60% buffer B for 5 minutes, 60 – 100% buffer B for 8.5 minutes, 100% buffer B for 12 minutes, 100 – 0% buffer B for 4 minutes, 100% buffer A for 8 minutes. Total run time: 52.5 minutes. Flow rate 1.5 ml/min.

2.7.2.2. Preparation of samples and standards

Nucleotide extracts were diluted 1:1 with a 50% (v/v) solution of buffer A. Samples were vortexed and centrifuged at 14 000 x g for 10 minutes at 4°C. Standards were prepared as follows:

(1) Standard 1 (2.78×10^{-5}M)

40 µl each of 1×10^{-3} M NAD, UDP-Glc, AMP, GDP, CTP, and UTP.
+ 440 µl water
+ 720 µl 50% buffer A

The solution was mixed and centrifuged the same as the standards.

(2) Standard 2 (5.56×10^{-6}M)

100 µl standard 1 + 200 µl water + 200 µl 50% buffer A.

The solution was mixed and centrifuged the same as the standards.

(3) Standard 3 (2.78×10^{-5}M)

40 µl each of 1×10^{-3} M ADP, ATP, and GTP.
+ 560 µl water
+ 720 µl 50% buffer A

The solution was mixed and centrifuged the same as the standards.

(4) Standard 4 (5.56×10^{-6}M)

100 µl standard 3 + 200 µl water + 200 µl 50% buffer A.

The solution was mixed and centrifuged the same as the standards.

(5) Standard mix

Standard 1 and standard 3 were mixed in a 1:1 ratio. The standard mix was used at the beginning of each multiple run to monitor the retention time and the column resolution.

2.7.2.3. HPLC column and autoinjector set-up

To avoid precipitation in the HPLC pumps and lines, water was flushed through the system before the additions of buffer A and buffer B.

(1) The C-18 column was connected to the HPLC system and the methanol content reduced in the column from 70% to 0% by replacement with 0.2 μm filtered water (flow rate 1.5 ml/min). All lines were purged with water for 5 minutes.
(2) The 20% ethanol in the purging system of the autoinjector was replaced with 0.12 μm filtered water. Autoinjector lines were purged with water for 5 minutes.
(3) The water in the solvent conditioner was replaced with buffer A and buffer B. The HPLC lines were then purged with both buffers. The flow rate was set at 1.5 ml/min at 100% buffer A. The HPLC system and autoinjector were now ready for sample loading and initiation of the gradient protocol (2.7.2).

2.7.2.4 Column storage

To store the column, buffer A and buffer B in the solvent conditioner were replaced with 0.2 μm filtered water. The system was flushed at 1.5 ml/min for 20 minutes with water. Buffer B was then replaced with 100% methanol and a gradient was run from 0 100% buffer B at 1.5 ml/min. (i.e.: a step increase of 10% buffer B each 10 minutes was used). 100% buffer B was then run for 10 minutes and decreased to 70% buffer B for another 10 minutes. The column was removed and stored at room temperature.

2.7.3 Quantification of adenylate nucleotides by luminometry

2.7.3.1. Principle

Adenyl nucleotides were quantified by using a modified bioluminescent assay (Lundin *et al.*, 1986). This assay is based upon the quantitative measurement of the level of light produced from the reaction catalyzed by firefly luciferase in the presence of ATP. Light emission was measured with a luminescence photometer (Luminometer 1250, LKB-Wallace) linked to a chart recorder (Model SE 120, ABB Gorez, AG).

ATP + luciferin + O_2 ----------> oxyluciferin + AMP + PPi + CO_2 + light (Reaction #1)
Firefly luciferase

The intensity of light emission is directly proportional to the ATP concentration ranging from 10^{-6} M to 10^{-13} M. ADP and AMP were quantified by the bioluminescent method after enzymatic conversion to ATP.

(1) ADP conversion to ATP

2 ADP + 2 phosphoenolpyruvate ----------> 2 ATP + 2 pyruvate (Reaction #2)
pyruvate
kinase

(2) AMP conversion to ATP

AMP + CTP ----------> ADP + CDP (reaction #3)

ADP ----------> ATP (see Reaction #2)

2.7.3.2. Stock solutions

(1) Tris-acetate buffer (0.1M Tris, 6mM EDTA)

1.21g tris base
0.06g EDTA (free acid)
Dissolved in 80ml of water. The pH was adjusted to 7.75 with acetic acid (2 M). The solution was made up to 100ml and stored at 4°C.

(2) Luciferin-luciferase assay kit

The supplier is Roche Diagnostics GmbH (cat # 1 699 695). The kit contains 8 x 10 ml of a luciferin-luciferase reagent. The luciferin-luciferase reagent is dissolved in 10 ml of redistilled water without shaking or stirring. Aliquots (1.0 ml) were stored at -20°C for later use (storage of 6 months maximum).

(3) ATP standard (10^{-5} M)

1 vial of ATP standard was reconstituted with 10 ml of water. Aliquots (300 µl) were stored at -20°C.

(4) 0.2 M phosphoenolpyruvate (PEP)

125 mg tricyclohexylammonium salt of phosphoenolpyruvate was dissolved in 1375 µl Tris-acetate buffer. Aliquots (60µl) were stored at -20°C.

(5) Pyruvate kinase (2000 U/ml)

Pyruvate kinase (5000 U, Sigma P9136) was dissolved in 2.5 ml Tris-acetate buffer. Aliquots (60 µl) were stored at -20 ° C.

2.7.3.3. Luminometry reagents

(1) Luciferin-luciferase – 1 aliquot of 1050 µl kept on ice.
(2) PK-60µl + 60µl pyruvate kinase (2000 U/ml) kept on ice.
(3) ATP standard – 1 aliquot of 300 µl kept on ice.
(4) TA buffer – TRIS-ACETATE BUFFER (0.1m Tris, 6mM EDTA) kept at room temperature
(5) TCA blank – 100 µl 6% (v/v) TCA + 27 µl Tris (0.5 M, ph 9) neutralized with 2M NaOH.

2.7.3.4. Luminometry assays

The assays were performed in luminometer polystyrene cuvettes. The solutions were added to the cuvette and mixed at the lowest speed of the vortex. The light emissions were read after a stable line had been reached after the initial flash spike (~1 min).

The optimal luciferin-luciferase (L-L) concentration was determined empirically to obtain the best range at which to make a reading on the luminometer.

(a) Blank assay

1. Combine 430 µl TA buffer + 100 µl L-L + 5 µl TCA blank.
2. Add 5 µl PK

(b) Standard assay

1. Combine 430 µl TA buffer + 100 µl L-L + 5 µl TCA blank.
2. Add 10 µl ATP standard (ST1)
3. Add 5 µl PK
4. Add 10 µl ATP standard (ST2)

(c) Sample assay

1. Combine 430 µl TA buffer + 100 µl L-L + 5 µl TCA blank.
2. Add 10 µl sample (TCA nucleotide extract – section 2.7.1)
3. Add 5 µl PK

2.7.3.5 Calculation of ATP and ADP concentration

Refer to Figure 2.2:

$$\text{ATP in sample } (\mu M) = \frac{sATP\ (V)}{ST1\ (V)} \times \text{concentration of ATP standard.}$$

$$\text{ADP in sample } (\mu M) = \frac{sADP\ (V) - PK\ (V)}{ST2\ (V) - PK\ (V)} \times \text{concentration of ATP standard.}$$

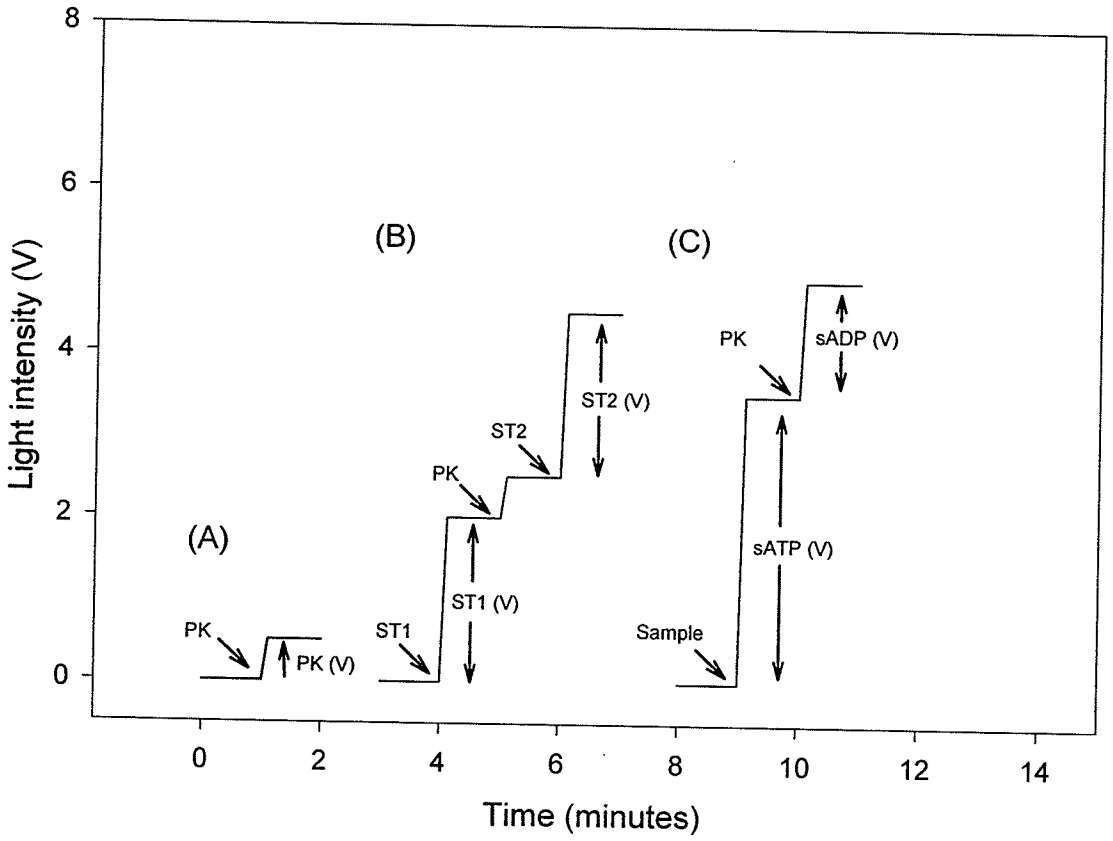


Figure 2.2. A representative graph of a bioluminescence assay used for determining ATP and ADP concentrations in a sample. (A) Blank assay; (B) Standard assay; (C) Sample assay. PK – pyruvate kinase and phosphoenolpyruvate; ST1 and ST2 – ATP standards.

2.8. Viral infection

2.8.1. Virus Infection

Multi-well plate cultures

L-929 cells were grown in 6-well cluster plates to near confluence (90%). Plates were infected by removing 90% of the medium and adding the virus sample at a multiplicity of infection (MOI) of 0.5 and 5.0 PFU/cell. The plates were gently rocked every 10min for 1 hour after infection. Fresh media was added to the flasks and incubated at 37°C for 10 days.

Microcarrier cultures

The cultures were infected by removing 90% of the medium and adding the virus sample at a multiplicity of infection (MOI) of 5.0 PFU/cell. The flasks were agitated intermittently at 40rpm for 1 min every 10 min for 1 hour. Fresh media was then added to the flasks and then placed at 37°C for 10 days. Flasks were then set at a stir rate of 40 rpm.

2.8.2 Virus sampling

Multi-well plate cultures

Sets of infected plates were removed from the 37°C incubator each day and frozen at -70°C for viral plaque assay. The samples were freeze-thawed 3x to ensure complete lysis of the cells to release the virus.

Microcarrier cultures

Samples (1 ml) of an evenly distributed culture were taken daily and stored at -70°C until required. The samples were then freeze-thawed 3x to ensure complete lysis of the cells.

2.9. Plaque Assay

2.9.1 Products used in the plaque assay

GIBCO products – GIBCO

Minimal Essential Medium (MEM) (S-MEM = Joklik's Suspension Modification)

- Cat# 22300-107

Medium 199

- Cat # 31100-084

Other products ("home-made")

L-glutamine (200mM) (= 100x)

Penicillin-Streptomycin (10,000 Units – 10mg/ml) (= 100x)

Amphotericin-B (1 mg/ml) (= 1000x)

Plasticware

Corning T25	Cat#	25100-25
Corning T75		25110-75
Corning 6-well cluster dish		430343
Corning P60 dish		430166
2dm vials (*7.4 ml*)	Wheaton Cat#	224884

Completed MEM:

(*Set-up media for direct use with L929 cells*)

940 ml S-MEM

25ml FCS

25ml VSP gammaglobulin serum

2mM L-glutamine (10ml of stock solution)

2 g/L $NaHCO_3$

Completed 2x Medium 199

(*plaque assay: i.e.: Agar/199 solution to overlay plaque assay*)

900 ml 2x 199 (made at double concentration)

25ml FCS

25ml VSP

20ml L-glutamine stock solution

20ml Pen-Strep stock solution (2x Pen/Strep)

2 g/L $NaHCO_3$

Agar: 2% (wt/vol) in dH_20; sterilized by autoclaving

Neutral Red Staining solution: 2% (wt/vol) in dH_20; filter sterilized

Plaque Assay Overlay (Agar/199)

Melt 2% agar (microwave 50% power) and place in 62°C water bath

Pour equal volume completed 2x 199 (@room temp) into 62°C agar; swirl to mix

Add Amphotericin-B (1000x → 1x desired)

Check temperature (wrist-test) before use.

Plaque Assay Staining:

Melt 2% agar as above

Add 2ml 2% Neutral Red per 50ml agar

Pour equal volume 2x PBS into agar

Add 2.5mls per well (6 well plate)

Gel Saline:

(For dilution of samples)

137mM	NaCl
0.2mM	$CaCl_2$
0.8mM	$MgCl_2$
19mM	Boric acid
0.1mM	NaB_4O_7 (Borax)
0.3% w/v	Gelatin – Type A 275 bloom (Fischer)

Add all ingredients to dH_20 except gelatin

Warm mixture

Add gelatin and dissolve

Aliquot to dilution tubes

Autoclave dilution tubes

2.9.2 Plaque assay procedure

Day 0: L-929 cells were passaged into an appropriate number of 6-well cluster plates at a cell density of 4.2 to 4.5 x 10^5 cells/ml in 2.5 ml of media. The plates were then incubated overnight. If there were many samples an appropriate number of 6 dilution tubes with gel saline diluent were also prepared and stored @ 4°C.

Day 1: A series of 6 serial 1:10 dilutions in gel saline were carried out on each of the samples to be titered. But, for high-titered samples, the samples were diluted 1:100 times, followed by 5 1:10 dilutions. 100 µl of each dilution is overlayed onto the cell monolayers in the wells. The inoculate are allowed to adsorb for 1 hr with periodic rocking. After adsorption, each well was overlayed with 3 ml completed Agar/199, allow agar to solidify, and placed in the incubator.

Day 4: The plates were "fed" by adding 2 ml fresh Agar/199 to each well and returned to the incubator.

Day 7: The plates are stained by adding 2ml Agar/PBS/Neutral Red to each well and returned to the incubator.

Day 8: The plaques are then counted. At least 20-200 plaques were counted to ensure statistical significance and the titer determined.

CHAPTER 3

Development of a Serum-free Medium for Vero Cells

3.1 Introduction

Serum-free media formulations have not been abundant for anchorage-dependent cells largely because of their complex nutrient and micronutrient requirements. This is unlike most transformed cell lines which typically lose their anchorage-dependence and are not as fastidious in their growth factor requirements (Temin 1967; Stiles et al. 1979); (Lanks et al. 1980). Even though Vero cells are a transformed cell line, they require growth factors similar to primary cell lines and thus makes developing a serum-free formulation challenging. They are typically grown in serum-supplemented media which contain known growth factors such as insulin, platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF), hydrocortisone, fibronectin and fetiun. Attempts at designing serum-free media have shown some results (Engelhardt et al. 1977; Cherington et al. 1979; Bettger et al. 1981; Crespi et al. 1981; Giguere et al. 1982; Bradshaw et al. 1983; Cherington et al. 1984; Taub et al. 1984; Grant et al. 1992). However none of these formulations have been able to grow anchorage-dependent cell lines as well as serum-supplemented media.

During the course of this study there were very few serum-free formulations available on the market. The companies that supply serum-free media for Vero cells were Biowhittaker, Clonetics, Life Technologies (Gibco), and JRH biosciences. Since they are owned by various biopharmaceutical companies the formulations are proprietary and are not published. This leaves the scientific community little choice but to develop their own formulation or purchase it from one of these suppliers.

A novel serum-free formulation (designated VSFM) for the growth of Vero cells has recently been developed in our lab (Butler and Berry, 1996. Data unpublished). The

medium (M-VSFM) described in this report was developed by incremental improvements over several years. Two important criteria in the development of this medium were the establishment of a minimal protein content and the use of non-animal derived components. The initial prototype medium contained a high amount of protein but this was gradually reduced as the cells were adapted to a medium containing a minimal content of non-animal derived proteins. Animal-derived components, such as insulin and transferrin, were replaced with Long R_3 IGF and iron sulfate respectively. This yielded the final formulation which is animal product-free with a low protein content (80 μg/L). The medium also contains a range of trace elements which are essential for the long-term growth and stability of the cells.

Since the development of M-VSFM the medium had not been well characterized for supporting Vero cell growth, therefore the initial purpose of this study was to evaluate the performance characteristics of the medium in stationary and agitated culture. In addition, improvements of the formulation using a variety of components that are known to enhance cell growth were studied.

3.2 Results

3.2.1 Growth profile of Vero cells in M-VSFM

Since the change of the formulation with the use of recombinant growth factors the growth characteristics of the medium had not been studied. Therefore this experiment was designed to determine how well the medium supported growth compared to a control serum-containing medium. Duplicate cultures (that had been passaged 3 times in each medium) were inoculated at a concentration of 2.0 x 10^5 cells/ml with 2.0 g/L Cytodex-1 microcarriers in 100 ml spinner flasks for both M-VSFM and 10% FBS-DMEM (Figure 3.1). Daily samples were taken and counted via the Trypan Blue exclusion method as described previously (2.3.3 and 2.4). There was an initial 24 hour lag phase after the start of the culture in both media types. The cells used this time to attach to the microcarriers and flatten out before they initiated exponential growth as observed by microscopy. After the lag the growth profile for each of the cultures was comparable (~24 hour doubling time) until the cells reached higher densities. The cells reached a maximum density of 1.0 x 10^6 cells/ml within 4 days in M-VSFM and 8.0 x 10^5 cells/ml in 10% FBS-DMEM. This growth profile was consistently observed in other microcarrier experiments where cells reached ~ 10^6 cells/ml (see Figure 5.2.a and 5.2.b). Cell-surface attachment was maintained throughout the cultures and there was no evidence of cell aggregation.

The higher density reached in the M-VSFM has been observed before in other cultures (data not shown). It is unknown why but it is possibly due to the fact that the cells are not producing as much ammonia or lactic acid in M-VSFM than in 10% FBS-DMEM. The medium yellows considerably faster in 10% FBS-DMEM than in M-VSFM which would suggest a buildup of acidic by-products in the medium.

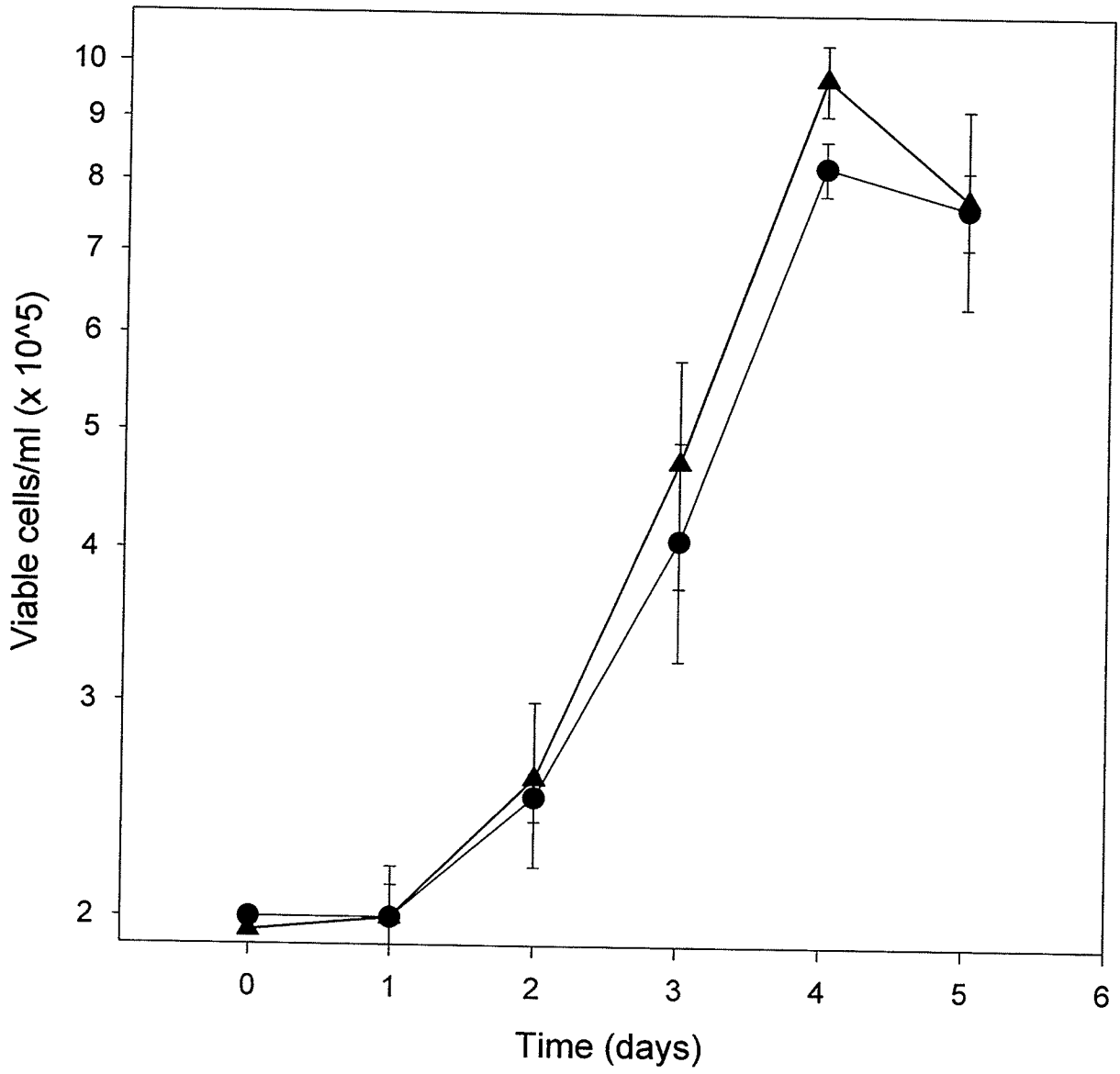


Figure 3.1. Growth of Vero cells in M-VSFM (▲) and 10% FBS-supplemented DMEM (●) in microcarrier culture. The cells were grown in 100ml spinner flasks incubated at 37°C with a 10% CO_2 overlay. Cytodex-1 microcarriers were added at a concentration of 2.0 g/L. The cells were added at a concentration of 2.0 x 10^5 cells/ml to initiate the culture. n=2.

3.2.2 Adaptation time of Vero cells to M-VSFM from serum-based media

One of the major concerns with serum-free media is the requirement for an adaptation period when the cells are coming from a serum-based formulation. Although serum-free formulations are designed to mimic the action of serum it is possible that there are some components or growth factors that may not be present (or in a limiting concentration). This can cause a marked reduced growth rate in the culture that can last for a long period of time while the cells adapt their metabolism to the new medium (can be as much as 5 – 10 passages). The specific changes in metabolism may include producing the components that are lacking in the serum-free formulation (such as attachment factors) or adjust their metabolism to suit the new medium (from a high protein to a low protein medium for example).

This study determines the amount of time required for adaptation from a serum-based formulation to M-VSFM (Figure 3.2). The cells used were pre-adapted to growing in serum-containing media (high passage in 10% FBS-DMEM -180 passages). T-flasks (T-75) were inoculated with 2.67 x 10^4 cells/cm^2 in 25 ml of M-VSFM and the final cell density determined after 5 days of growth using the Trypan Blue exclusion method (2.3.1 and 2.4). This was continued for a period of 8 passages.

The results show that there was significant variability in the growth of the cells during the first 4 passages. They reached a density of anywhere between 0.5 and 1.1 x 10^5 cells/cm^2 (which corresponds to 1.5 to 3.3 x 10^5 cells/ml in a 75 cm^2 T-flask, respectively). The first passage was quite high (1.0 x 10^5 cells/cm^2), presumably because the cells carried some factors with them from the serum-containing medium that enhanced the cell yield. It is possible that the cells reached stationary phase on the first passage which may have resulted in lower cell numbers in the 2^{nd}. By the 5^{th} passage the cells reached steady growth yields of ~ 9.0 x 10^4 cells/cm^2 (within the normal range for high passage Vero cells in culture, data not shown).

This type of variability was observed in other experiments during the first few passages in serum-free culture (both in M-VSFM and other commercial formulations).

3.2.3 Comparison of M-VSFM to other commercial serum-free formulations

At the time of this experiment there were four serum-free formulations available for Vero cells from commercial suppliers. In order to enhance the performance of the medium the M-VSFM formulation was compared to these to assess whether there was room for improvement. The cells were grown in 75 cm^2 T-flasks at 37°C with a 10% CO_2 overlay. Each of the flasks was inoculated with 2.67 x 10^4 cells/cm^2 and the final cell yield was determined after 4 days of growth. The cells were passaged 7 times in each medium, from a stock culture in 10% FBS-DMEM, and the average from the last 4 passages graphed (Figure 3.3).

There was considerable variation in most of the formulations during the first few passages (data not shown), with the exception of the Ultraculture medium. Cells in the FGM, VPSFM, M-VSFM, and Nephros medium saw a period of variability in final growth yield after 5 days of growth (observed previously in section 3.2.2). Upon the 4^{th} passage the final cell number was more consistent.

The highest cell yield was seen in the Ultraculture medium (Biowhittaker supplier). It showed consistent yields of > 2.1 x 10^5 cells/cm^2. The second highest performing medium was Gibco's VP-SFM, giving yields of 1.48 x 10^5 cells/cm^2. The other two media, FGM and Nephros, did not yield high numbers of cells, and were usually inferior to M-VSFM. FGM gave an average of 8.5 x 10^4 cells/cm^2 and Nephros had an average of 9.3 x 10^4 cells/cm^2. M-VSFM gave consistent numbers of ~ 1.2 x 10^5 cells/cm^2 with each passage.

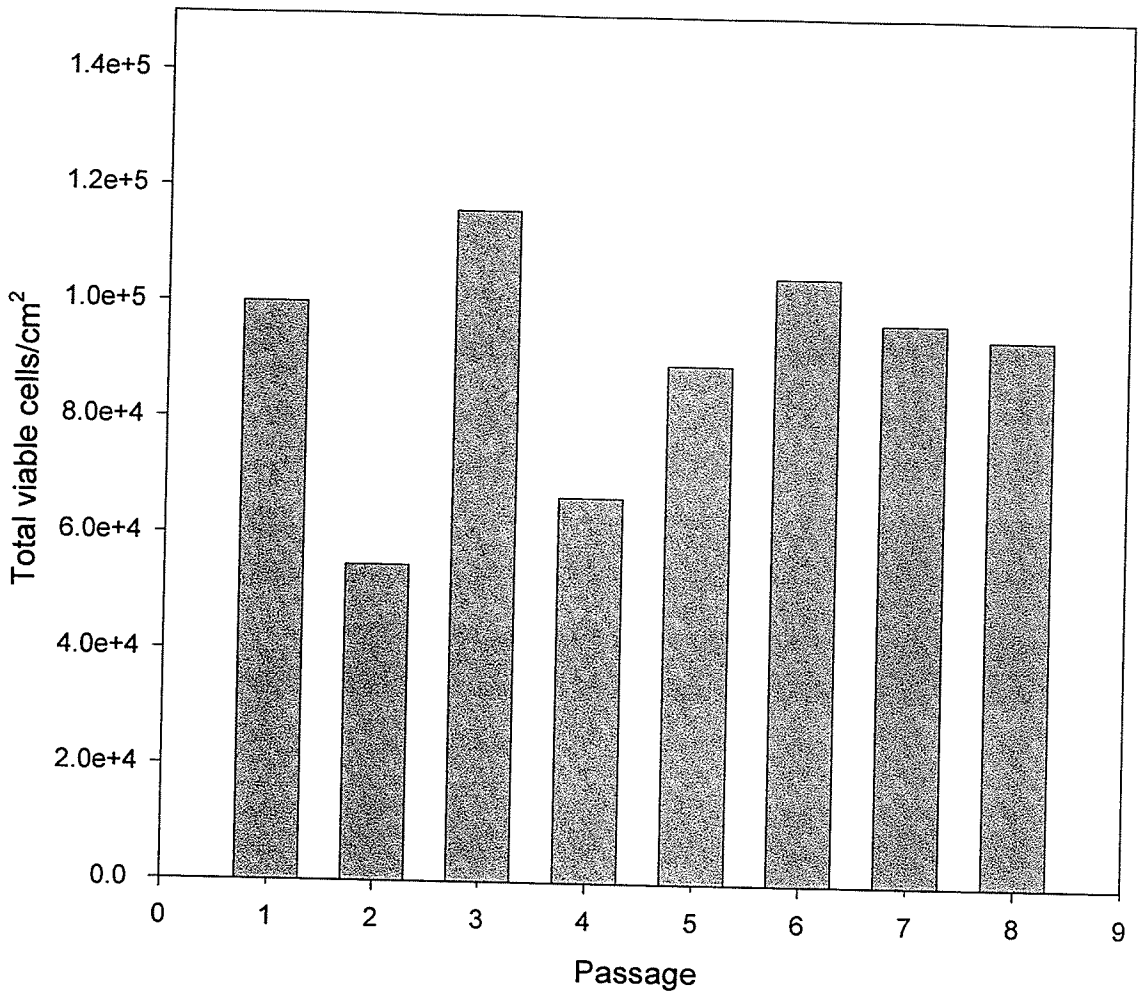


Figure 3.2. Adaptation period of Vero cells to M-VSFM from serum-based media. The cells were passaged in M-VSFM for a period of 8 passages. The starting cell inoculum was 2.67 x 10^4 cells/cm^2 in 25 ml of media. Cells were passaged every 5 days. This is representative of one experiment.

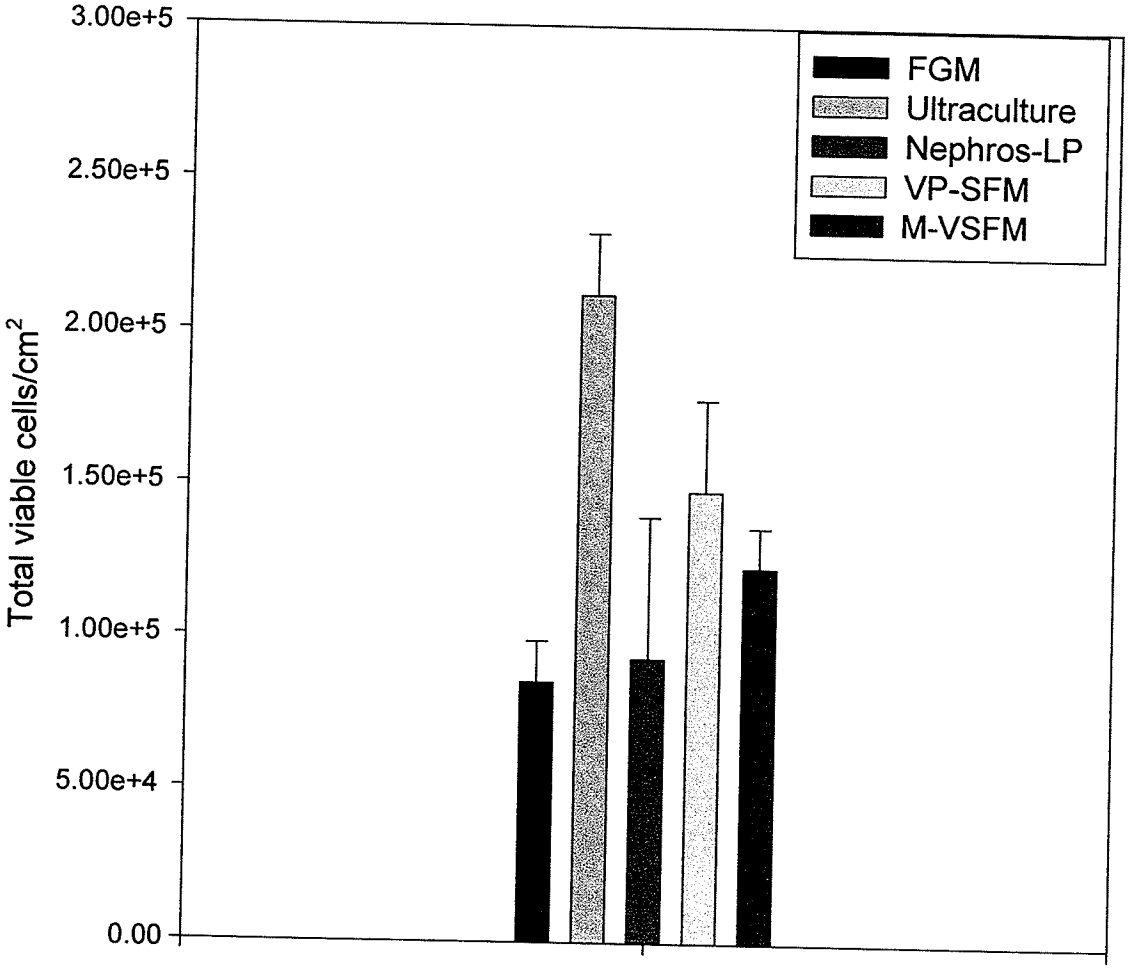


Figure 3.3. Comparison of commercially available serum-free media to M-VSFM to support the growth of Vero cells in stationary culture. The cells were grown in 75cm^2 T-flasks at 37°C with a 10% CO_2 overlay. The cells were inoculated with 2.67 x 10^4 cells/cm^2 over a total of 7 passages. The standard deviations are results from passages 4-7 (n=4).

The Nephros average growth data overlaps with the VP-SFM and M-VSFM. However, the Nephros medium gave consistently lower numbers of growth until the final passage (which had twice as many cells/cm^2), which may suggest it requires a longer time for adaptation to the medium.

3.2.4 Development and optimization of M-VSFM for Vero cell growth

3.2.4.1 Effect of epidermal growth factor on Vero cell growth

Examining the results from Figure 3.3 it is obvious that M-VSFM is satisfactory in its performance compared to other formulations. However, the Ultraculture medium outperformed M-VSFM indicating that there is room for improvement. Therefore, the second phase of the study was to improve the performance of the medium. The method used is a modified version of Sato's technique (Hayashi et al. 1976; Sato et al. 1976; Rizzino et al. 1979; Barnes et al. 1980). This involves testing previously defined components that have shown growth-promoting capabilities in similar cell lines. Starting with the "base" serum-free formulation, M-VSFM, specific components were added and the growth/viability of the cells measured. Some of these components that show growth–promoting abilities include recombinant growth factors, plant and animal hydrolysates, and various hormones.

The literature provides an extensive catalogue of various components which can be used to enhance cell growth. The most predominate of these are peptide growth-factors such as Insulin-like growth factor (IGF), epidermal growth factor (EGF), transforming growth factor (TGF), and fibroblast growth factor (FGF) to name a few. IGF and FGF are already present in the medium so the question is which of the listed growth factors would enhance cell growth and/or yield?

Epidermal growth factor (EGF) is a potent mitogen for a variety of cell lines including primary, mesenchymal, epidermal, and glial cells (Haley 1990). Its mitogenic activity is synergistic with other peptide growth factors such as IGF-1 and TGF (Krane et al. 1991;

Simmons et al. 1995). Since Vero cells are mesenchymal in origin and the medium contains IGF it is likely that EGF will have a growth-promoting effect. In addition, the presence of dexamethasone (from 5.5 to 390 ng/ml) with EGF has shown to act synergistically to enhance cell growth over that of EGF alone (Hosokawa et al. 1986; Ham et al. 1988).

The following experiment tested whether EGF alone or EGF with dexamethasone would improve cell growth of Vero cells in M-VSFM. Vero cells were inoculated into T-75 flasks with 2.67 x 10^4 cells/cm^2 in 25 ml of M-VSFM, M-VSFM + 1 unit/ml EGF, or M-VSFM + 1 unit/ml EGF and 100 units/ml dexamethasone (literature-recommended values). The cells numbers were determined after 5 days of growth using the Trypan Blue exclusion method (2.3.1 and 2.4) and were cultured in each medium for a total of 9 passages. The average final cell number of passage 4 to 9 were calculated and plotted on Figure 3.4 (n=5).

The first few passages in M2-VSFM had considerable variability in the final growth yield as observed previously with M-VSFM (3.2.2 and 3.2.3). Final cell density (with the same inoculum of 2.67 x 10^4 cells/cm^2 in 25 ml of media) ranged from 1.2 to 1.6 x 10^5 cells/cm^2 after 5 days of growth. Upon the 4th passage the growth yields became more consistent.

The average cell yield from passages 4 to 9 was much higher when the medium was supplemented with either EGF or EGF with dexamethasone. The medium with EGF reached an average of 1.97 x 10^5 cells/cm^2 which was much higher than that of the control, M-VSFM (1.36 x 10^5 cells/cm^2). When dexamethasone was added with EGF there was no significant difference in growth promotion to that of EGF alone (1.98 x 10^5 cells/cm^2).

The cells grown with EGF exhibited different morphological characteristics to those without. The cells formed ring-like patterns on the surface of the T-flask and this formation is more pronounced when dexamethasone is present. It is not known why this

occurs and there is nothing in the literature to explain this characteristic. However, the cells appeared healthy and there was no difference in cell viability between the cultures.

The dexamethasone did not exert an observable effect on cell growth with EGF present. However, it is possible that it may still be beneficial to the medium. A titration experiment of these two components may be warranted.

Since EGF significantly improved the overall performance of the medium the formulation was now supplemented with EGF. The new medium, containing 1 unit/ml EGF, was now designated M2-VSFM.

3.2.4.2 Effect of linoliec and oleic acid on cell growth

Lipids serve a variety of functions within the cell. They act as a structural component for cell membranes, as sensors for external signals, precursors to prostaglandin synthesis, and as a source of metabolic energy. Serum itself contains lipids, but basal media formulations do not normally contain any lipid components. Mammals themselves cannot synthesize *de novo* two classes of polyunsaturated fatty acids that are found in their tissues. These are the linoliec (*n*-6) and linolenic (*n*-3) families of fatty acids. Although saturated and some unsaturated fatty acids can be synthesized by mammalian cells (presence of desaturase enzymes) they cannot produce unsaturation points normally found in linoleic and linolenic acid (Δ6 and Δ3, respectively) (Needleman et al. 1986). Indeed, many transformed cell lines lose their ability to make linoleate (catalyzed by the Δ6-desaturase) (Spector et al. 1980). The CHO cell line cannot synthesize oleic acid due to the lack of the Δ9-desaturase (converts stearic acid, 18:0, to oleic acid, 18:1) (Chang et al. 1976). However, the culturing of cells *in vitro* has discovered that many cells can survive and propagate without the presence of these fatty acids. These include WI-38 (human diploid fibroblast), L-929 cells (mouse fibroblast), and monkey kidney (LLC-MK2) (Spector et al. 1980).

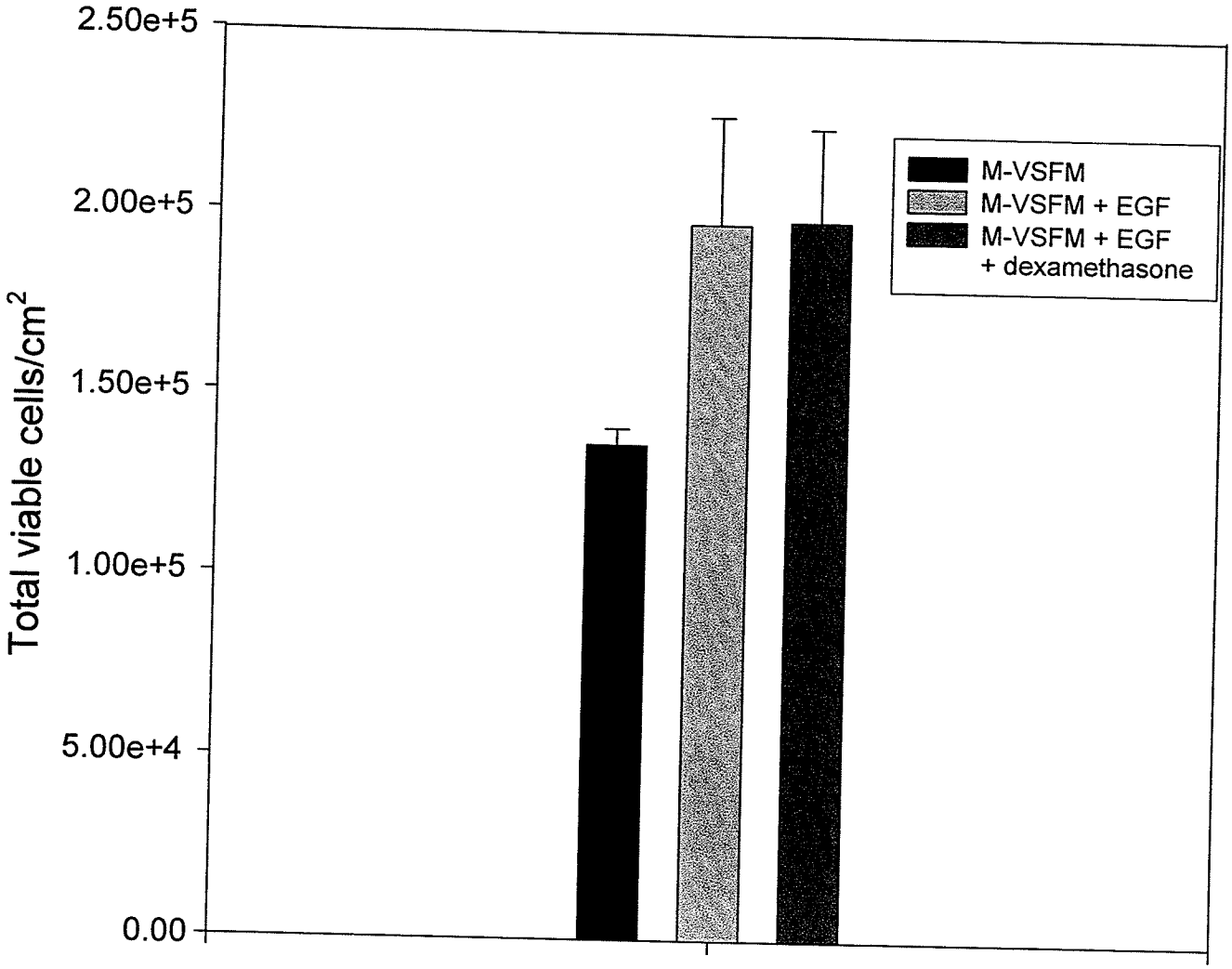


Figure 3.4. The effect EGF and EGF + dexamethasone on Vero cell growth in M-VSFM. The cells were grown in 75cm^2 T-flasks at 37°C with a 10% CO_2 overlay. The cells were inoculated with 2.67 x 10^4 cells/cm^2 and passaged every 5 days over a total of 9 passages. The averages from passages 4 to 9 are shown (n=5).

Certain lipids included as supplements to serum-free formulations and have been shown to enhance cell proliferation (Glassy et al. 1988) (Schmid et al. 1991) . Specific fatty acids, such as oleic and linoleic, will enhance both the growth and productivity of hybridomas grown in a serum-free medium over an extended number of passages (Butler et al. 1995; Butler et al. 1997). Due to growth-promoting effect of lipids in other cell lines in our lab it was presumed it would enhance Vero cell growth in M-VSFM.

M-VSFM was supplemented with a mixture of linoleic and oleic acid. Vero cells were inoculated into T-75 flasks with 2.67 x 10^4 cells/cm^2 in 25 ml of M-VSFM and M-VSFM + the fatty acids. The cell growth was determined after 5 days of growth using the Trypan Blue exclusion method (2.3.1 and 2.4) for a total of 7 passages. The results are shown in Figure 3.5.

Linoleic + oleic acid had no measurable effect on the growth yield over that of the control medium (1.36 x 10^5 cells/cm^2 for M-VSFM and 1.32 x 10^5 cells/cm^2 for M-VSFM + fatty acids). The initial passage saw an increase in growth but the latter passages saw a decrease in cell number in the presence of the fatty acids.

3.2.5 Supplementing M-VSFM with plant and tissue hydrolysates

Hydrolysates, which are enzymatic digests of proteins, are becoming wide-spread in cell culture. These are obtained from sources such as animal tissue, milk, and plants such as soy, wheat, or rice. The end product is a source of vitamins, lipids, minerals, and di- and tri-peptides that supply a rich energy source of basic amino acids for the cell.

Hydrolysates are typically used as a supplement to a basal medium, although it can not be used to replace it altogether. The types of cell lines that have been propagated with hydrolysates include CHO, hybridoma, BHK, Vero, lymphocytes, and others (Schlaeger 1996).

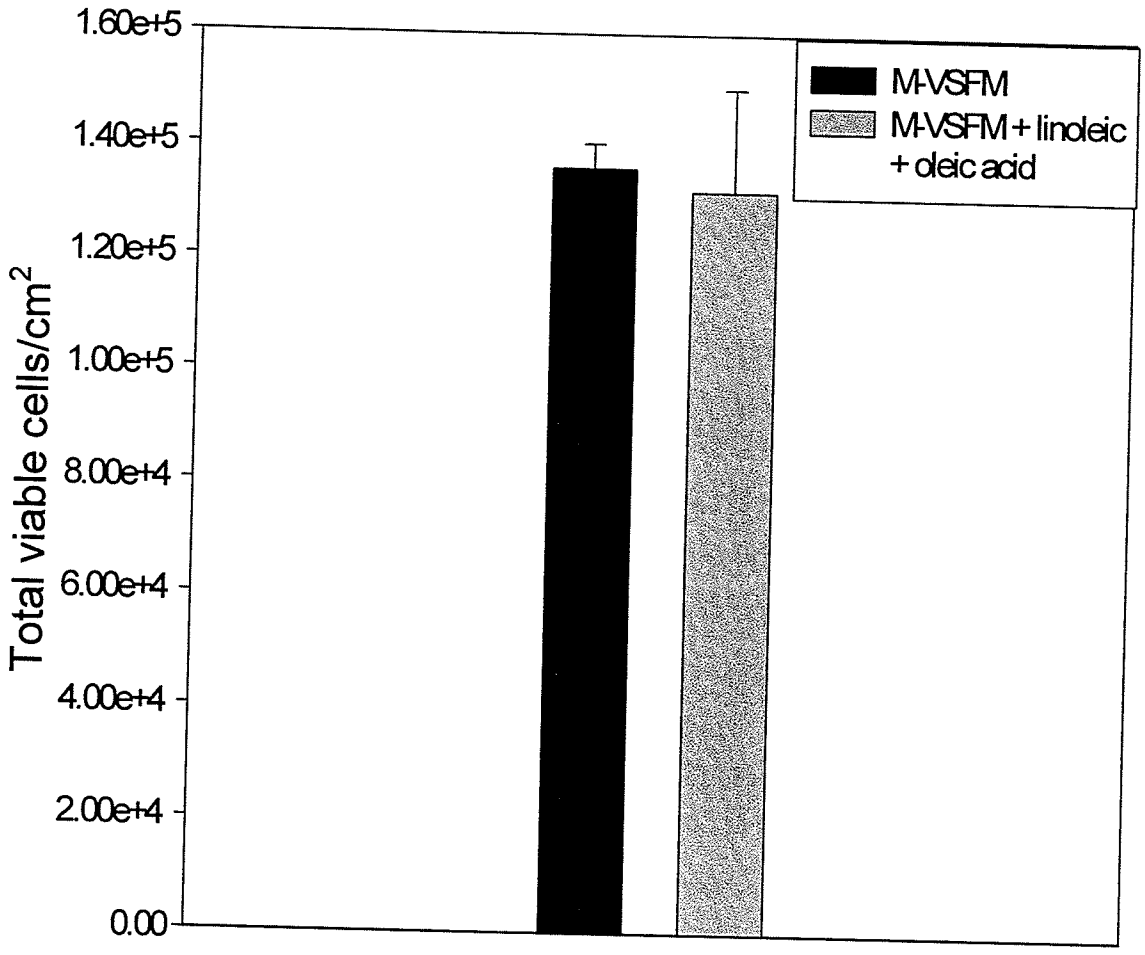


Figure 3.5. Growth of Vero cells in M-VSFM supplemented with fatty acids. The cells were grown in 75cm^2 T-flasks at 37°C with a 10% CO_2 overlay. The cells were inoculated with 2.67 x 10^4 cells/cm^2 and passaged every 5 days over a total of 7 passages. Each bar represents the average from passage 1 to 7 (n=7).

The one disadvantage to hydrolysates is that they are undefined. This may lead to the same problems that occur in serum-containing formulations such as batch to batch variation in the performance of the medium. Furthermore, hydrolysates derived from animal tissue can still carry the risk of introducing contaminants, such as viruses or prions, into a cell culture process used in the production of biologicals (Merten 1999; Merten 2002). This can limit their use in the large-scale production of human therapeutics due to health regulatory concerns.

However, the advantages of using hydrolysates include:

- No excess protein which can interfere with purification.
- A significant reduction in the cost of the media if growth factors in the medium could be reduced.

The growth promoting ability of hydrolysates has been well documented in the literature for the Vero and MDCK cell lines, including animal hydrolysates (casein peptone) (Merten et al. 1994), and plant hydrolysates (Merten et al. 1999; Sung et al. 2004). Therefore both were chosen as candidates for growth enhancement of M-VSFM. If successful these variants of the original formulation, although undefined, could serve as a less expensive alternative for industry (such as the animal vaccine industry). This study looks at the effect of supplementing M-VSFM with plant hydrolystates and tissue hydrolysates.

3.2.5.1 Plant hydrolysates: PH1 and PH2

Of the many plant hydrolysates available two were selected for their properties to support the growth of mammalian cell lines (Quest International). These are plant hydrolystates 1 and 2. These digests contain a rich source of di- and tri-peptides as well as amino acids.

M-VSFM was supplemented with 2.0 g/L (manufacturer recommended) of PH1 or PH2 to measure the effect on Vero cell growth. Cells were inoculated into T-75 flasks with a

starting cell density of 2.67 x 10^4 cells/cm^2 in 25 ml of media from a stock culture grown in M-VSFM (>5 passages). The cells were passaged every 5 days when the total cell number was determined by the Trypan Blue exclusion method (2.3.1 and 2.4). The results are show in Figure 3.6.

The growth of Vero cells was increased with the introduction of PH1 to the M-VSFM formulation. The first 3 passages saw only a slight increase in total cell number over that of the control, but the final 2 passages were more pronounced. The 4th and 5th passage reached a cell density of 1.95 and 2.03 x 10^5 cells/cm2, respectively. The control medium maintained a final cell density of 1.33 and 1.35 x 10^5 cells/cm^2 during the same time period.

The PH2, however, did not increase the growth yield. In fact the PH2 hydrolysate inhibited growth compared to that of the control during the first 3 passages (10-20%) and thus was discontinued. It is possible, however, that the first few passages were just random variation.

3.2.5.2 Protein hydrolysate supplement

AH1, a hydrolysate derived from an animal tissue enzymatic digest, was also tested to determine if it would improve the performance of M-VSFM for Vero cells. A range of recommended concentrations was used (0, 2, 5, and 10g/L) to supplement M2-VSFM. Cells were inoculated into T-75 flasks (from a stock culture pre-adapted to M2-VSFM, > 5 passages) with a starting cell density of 2.67 x 10^4 cells/cm^2 in 25 ml of media. The cells were passaged every 5 days when the total cell number was determined by the Trypan Blue exclusion method (2.3.1 and 2.4) over a total of 5 passages. The results are shown in Figure 3.7.

The growth results showed that the cells supplemented with AH1 reached higher cell densities, more than 50% to that of the control. The best results were observed with a 2.0 g/L (2.60 x 10^5 cells/cm^2) and 5.0 g/L (2.52 x 10^5 cells/cm^2), but high concentrations

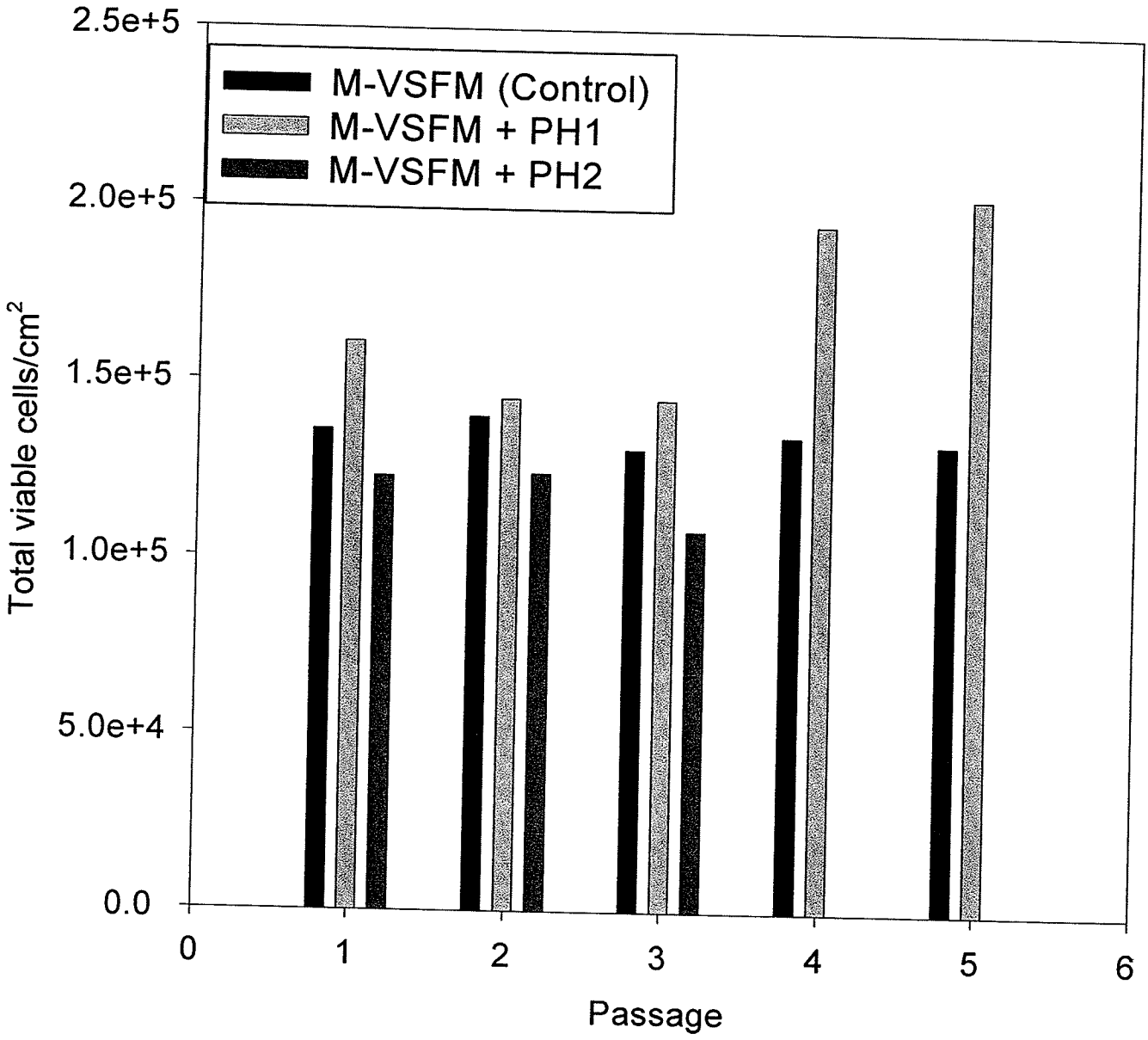


Figure 3.6. The effect of plant hydrolysates on Vero cell growth in M-VSFM in stationary culture. The cells were grown in 75cm^2 T-flasks at 37°C with a 10% CO_2 overlay. The cells were inoculated with 2.67 x 10^4 cells/cm^2 and passaged every 5 days over a total of 5 passages.

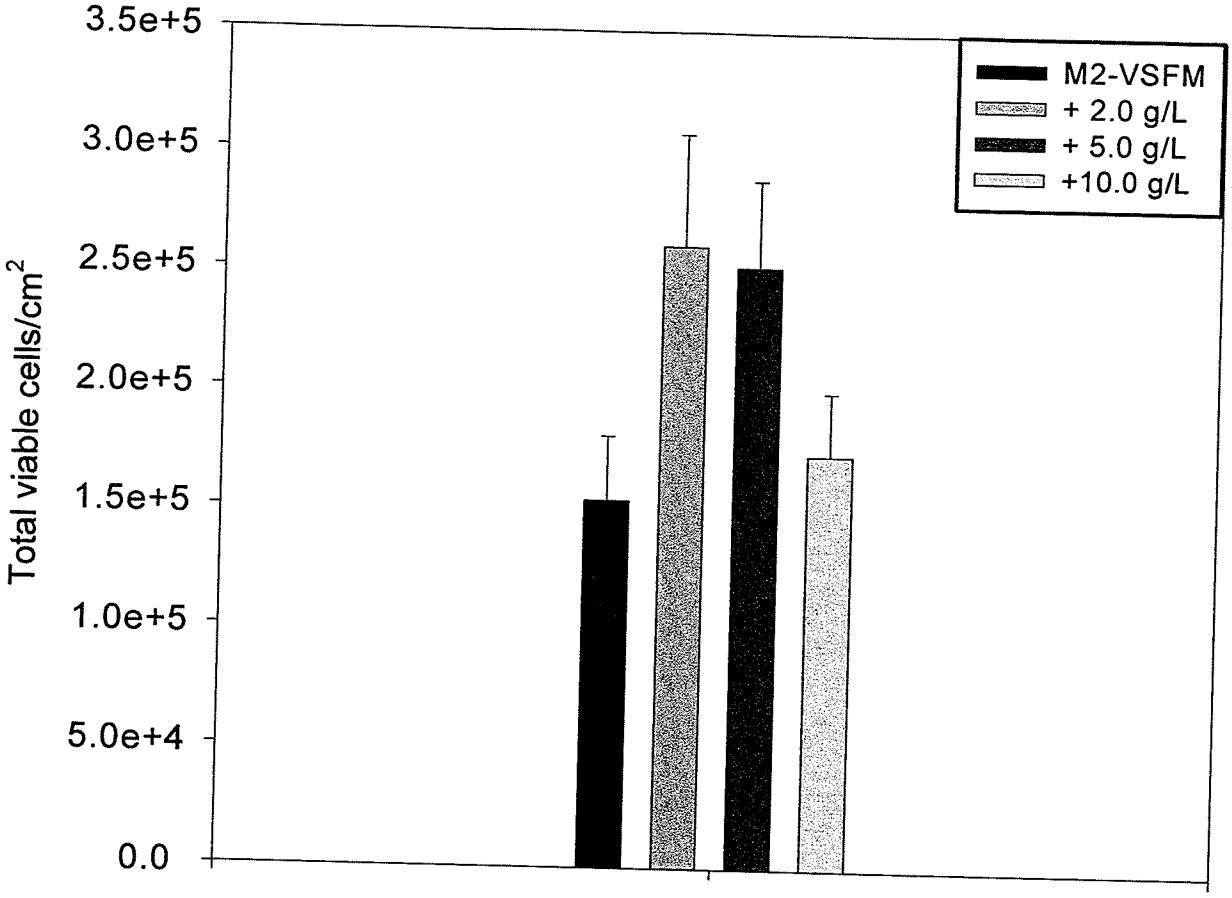


Figure 3.7. The effect of AH1 protein hydrolysate upon the growth of Vero cells in M2-VSFM in stationary culture. The cells were grown in 75 cm^2 T-flasks with a starting cell density of 2.67 x 10^4 cells/cm^2 in 25ml of media. The cells were counted after 5 days of growth by the Trypan Blue exclusion method. Each bar is the average final growth yield from passages 1 to 5 (n=5).

of 10.0 g/L (1.73 $\pm$ 0.24 x 10^5 cells/cm^2) seemed not to have any significant effect on cell growth compared to the control (1.54 $\pm$ 0.27 x 10^5 cells/cm^2).

3.2.6 Cell growth parameters: finding optimum starting cell density for Vero cells in M2-VSFM

The optimal starting density for Vero cells in M2-VSFM has never been explored. Growth rates were calculated from cells grown in T-flasks starting with varying cell densities. The densities tested were 8.0 x 10^4 cells/ml, 6.0 x 10^4 cells/ml, and 4.0 x 10^4 cells/ml. The growth rate was determined by the formula:

$$N = \frac{\log N_t - \log N_o}{0.301}$$

Where N is the number of generations in time t

N_o = the initial population number

N_t = the final cell number after time t

And the generation time is:

$$k = \frac{N}{t}$$

The results are shown in Table 3.1.

The cell growth rate was not severely affected by the decrease in starting cell density below that of 6 x 10^4 cells/ml. However, cells grown with a density of 4.0 x 10^4 cells/ml had the slowest doubling time of 37.4 hours. The cultures with the high starting cell density of 8.0 x 10^4 cells/ml resulted in near normal doubling times for Vero (30.4 hours) Therefore the best starting density for M2-VSFM was 8.0 x 10^4 cells/ml.

Table 3.1. Determining the optimal initial cell inoculation density for cell growth in M2-VSFM.

Vero cells were inoculated into T-75 flasks with either 8.0 x 10^4 cells/ml, 6.0 x 10^4 cells/ml, and 4.0 x 10^4 cells/ml. The final cell number was determined after 5 days of growth to calculate the generation time.

Starting density (cells/ml)	After 5 days of growth (cells/ml) n=3	Average growth rate	
		Gen/hr	Hr/gen
4.0 x 10^4	0.37 $\pm$ 0.06 x 10^6	0.0267	37.4
6.0 x 10^4	0.83 $\pm$ 0.24 x 10^6	0.0316	31.6
8.0 x 10^4	1.23 $\pm$ 0.125 x 10^6	0.0329	30.4

n represents the number of replicates

Gen = generation

Hr = hour

3.2.7 Adaptation of Vero cell clones E6 and 76 to M2-VSFM

Several clones derived from the original cell line have been cultured. They were chosen for their favorable properties to serve as a host for certain viruses. The first clone developed was the Vero-76 cell line in 1968 (Ref: ATCC). This line has a lower saturation density than the original Vero line. It was primarily used for the propagation of hemorrhagic fever viruses (Machupo, Junin, Lassa), Marburg or Ebola viruses, and more recently Rickettsia prowazekii (Policastro et al. 1996). When infected they exhibited cytopathic effects and the formation of plaques. The second clone is the Vero E6 cell line which was derived from the Vero 76 clone. It is used for the propagation of hemorrhagic fever viruses (Machupo, Junin, and Lassa), and Marburg or Ebola viruses. Vero E6 exhibit some degree of contact inhibition, more so than the original cell line which make it more useful in growing slow replicating viruses.

Due to the wide-spread use of these clones in virus production a suitable serum-free formulation would be desirable to the research community. Since these are clones of the original cell line it is reasonable to assume that they would have similar growth factor requirements and thus propagate in M-VSFM. Therefore the purpose of this study was to determine if M-VSFM can support the growth of these cell lines.

3.2.7.1 Vero-76

The ability of M2-VSFM to support the growth of the Vero-76 cell line (clone of the original Vero) was investigated. The Vero-76 cells were inoculated into T-75 flasks with 2.67 x 10^4 cells/cm^2 in 25 ml of either M2-VSFM or 10% FBS-DMEM as the control medium. The cells were grown for 5 days and the final cell number was determined by the Trypan Blue exclusion method (2.3.1 and 2.4). This was continued for a period of 6 passages. The results of this experiment are shown in Table 3.2.

The Vero76 cell line grew quite well in M2-VSFM with only a few passages for adaptation. The cells reached greater than 1.6 x 10^5 cells/cm^2 after 4 passages in M2-

VSFM. The control medium supported the cells much better with densities almost double that of those in M2-VSFM (2.09 to 3.83 x 10^5 cells/cm^2). Nevertheless M2-VSFM supports the growth of the Vero-76 cell line.

The Vero-76 cell line grows to higher cell densities than the original cell line. The 76 line does not exhibit contact inhibition to the same extent. Multi-layering can be observed in the T-flask where 2 to sometimes 3 layers of Vero cells form on the culture plate. This does not normally happen with the original cell line where the cells typically stop growing after forming a complete monolayer.

3.2.7.2 Vero E-6

The ability of M2-VSFM to support the growth of the Vero E6 clone cell line was also investigated. The cells were inoculated into T-75 flasks with 2.67 x 10^4 cells/cm^2 in 25 ml of either 10% FBS-DMEM (control medium), M2-VSFM, M2-VSFM + 2.0 g/L PH1, or M2-VSFM + 2.0 g/L AH1. The cells were grown for 5 days and the final cell number was determined by the Trypan Blue exclusion method (2.3.1 and 2.4). The results are shown in Table 3.2.

Unlike the Vero-76 cell line the E6 cells did not survive in M2-VSFM. They maintained their initial cell density of ~ 3.0 to 4.0 x 10^4 cells/cm^2 for 4 passages at which point the cells died.

Due to the success of hydrolysates with the original cell line as well as other cell lines (MDCK) the E6 cells were passaged in M2-VSFM with PH1 (2.0 g/L) to determine if this formulation could support the cells. The first 2 passages showed reasonable growth (1.44 x 10^5 cells/cm^2 on passage 1) almost 70% as high as that of the control (2.05 x 10^5 cells/cm^2). However, on the 3rd and subsequent passage the cells underwent apoptosis and were no longer viable in the medium.

Table 3.2. Growth of Vero-76 cells in M2-VSFM and 10% FBS-DMEM.

The Vero-76 cells were inoculated into T-75 flasks with 2.67 x 10^4 cells/cm^2 in 25 ml of either M2-VSFM or 10% FBS-DMEM as the control medium. The cells were grown for 5 days and the final cell number was determined.

	Cells/cm^2	
Passage number	M2-VSFM	10% FBS-DMEM
1	1.11E+05	2.32E+05
2	1.20E+05	2.89E+05
3	1.84E+05	2.72E+05
4	1.00E+05	2.09E+05
5	1.68E+05	3.83E+05
6	1.65E+05	3.60E+05

Another hydrolysate, AH1, was then supplemented into the medium due to its high growth promoting ability with the original cell line (3.2.5.2). The cells remained viable and had reasonable growth, reaching greater than 1.5 x 10^5 cells/cm^2 for 3 of the 4 passages. There was no indication of any loss of viability and the cells remained healthy throughout the 4 passages.

The original M2-VSFM formulation does not contain the components necessary to support the Vero E6 cell line alone. With the addition of AH1 the lacking component (s) were satisfied and the cells resumed growth. Therefore M2-VSFM supplemented with 2.0 g/L of AH1 is a suitable serum-free growth medium for the Vero E6 cell line.

Table 3.3. Growth of Vero-E6 cells in M2-VSFM and hydrolysate-supplemented M2-VSFM.

Vero E6 cells were inoculated into T-75 flasks with 2.67 x 10^4 cells/cm^2 in 25 ml of either 10% FBS-DMEM (control medium), M2-VSFM, M2-VSFM + 2.0 g/L PH1, or M2-VSFM + 2.0 g/L AH1. The cells were grown for 5 days and the final cell number was determined.

Passage #	Cells/cm^2			
	10% FBS-DMEM	M2-VSFM	M2-VSFM + 2.0 g/L PH1	M2-VSFM + 2.0 g/L AH1
1	1.40E+05	3.87E+04	-	-
2	2.07E+05	7.60E+04	-	-
3	1.53E+05	3.33E+04	-	-
4	1.83E+05	2.93E+04	-	-
5	2.05E+05	cd	1.44E+05	-
6	2.67E+05	-	9.33E+04	-
7	1.56E+05	-	1.87E+04	-
8	2.83E+05	-	cd	1.57E+05
9	2.60E+05	-	-	8.00E+04
10	3.05E+05	-	-	1.56E+05
11	2.63E+05	-	-	1.60E+05

(-) represents no culture conducted

(cd) represents cell death (culture had no viable cells)

3.3 Discussion

The initial purpose of this study was to evaluate the performance of M-VSFM. The original formulation was designed to meet the specific requirements of the pharmaceutical industry; namely not containing any products of animal origin and being chemically defined. M-VSFM met these requirements but had yet to be evaluated on the Vero cell line.

The new formulation was shown to support the growth of Vero cells comparable to that of serum-based media. In microcarrier cultures the Vero cells had a normal doubling time of ~ 25 hours reaching densities of ~1.0 x 10^6 cells/ml. This is consistent with what is seen in the literature with bench-top scale large-scale batch cultures of Vero cells used in vaccine production reach ~ 10^6 cells/ml (Montagnon et al. 1981; Montagnon et al. 1984) in serum-based formulations. Although some studies have shown much higher cell densities of 3-6 x 10^6 cells/ml (Merten et al. 1994) and 5 x 10^6 cells/ml (Kistner et al. 1999) these are typically perfusion systems with higher microcarrier concentrations. However, colleagues using M-VSFM with a higher microcarrier concentration (10 g/L) can reach higher cell densities of 2 x 10^6 cells/ml (Wu et al. 2004).

The higher densities reached in M-VSFM might be explained by the lower pH in the 10% FBS-DMEM cultures. This is most likely due to a buildup of toxic by-products, such as lactic acid, which can be inhibitory for cell growth (Butler et al. 1989). The 10% FBS-DMEM typically yellow (lowering of pH) more quickly than cultures in M-VSFM (data not shown) which could be the reason for the higher cell densities. In addition it has been shown that cultures in serum-free media have higher metabolic activity than in their serum counterparts (Neermann et al. 1996). However, this needs to be investigated further as M-VSFM has shown considerable variation in performance (especially early passages) and the differences may be due to random fluctuation.

The adaptation time for the Vero cell line coming from a serum-based formulation to M-VSFM was minimal. The initial 4 passages showed reduced growth of ~ 40% lower than what is normally observed when in 10% FBS-DMEM (~ 0.8 to 1.0 x 10^5 cells/cm^2). However, the cells maintained a high viability (>95%) and quickly adapted to close to their normal growth potential (~ 9.0 x 10^4 cells/cm^2). This is in contrast to other serum-free formulations published in the literature. Many can require much longer adaptation times and even have to employ a serum-weaning strategy for many passages before subculturing into the serum-free medium (Cruz et al. 1998; Kim et al. 1999). However, further passages in the medium may be required to reach higher cell densities often observed (~ 1.2 x 10^5 cells/cm^2) when Vero cells have been cultured for >10 passages in M-VSFM.

The formulation was compared to all available serum-free media from commercial suppliers at the time of the study. This included Biowhittaker, Life Technologies (Gibco), Clonetics, and JRH biosciences. The performance of M-VSFM was comparable to most of the other formulations in terms of final cell yield (1.2 x 10^5 cells/cm^2). However, the Ultraculture medium greatly outperformed M-VSFM and all of the others reaching cell densities almost twice as high (~2.1 x 10^5 cells/cm^2).

Since the formulations are proprietary it is difficult to determine which component(s) may be contributing to the increase in cell growth. It is highly likely that many of the components in M-VSFM are present in the other formulations and vice-versa. The cells in Ultraculture grew for a longer period of time (days 4-5) whereas the cells in the other media entered stationary phase at day 4. It may be that Ultraculture contains higher levels of glutamine or peptides that allow the cells to reach higher densities. In fact, the growth yields in Ultraculture were similar to that of M-VSFM supplemented with AH1 (Figure 3.7). Due to the viscous nature of the Ultraculture medium it is possible that the medium is supplemented with some sort of hydrolysate but no conclusion can be drawn without medium analysis.

The observation of the gap in performance between M-VSFM and Ultraculture led to the question that M-VSFM may lack certain stimulatory components (or have components that are inhibitory) for growth. Utilizing a well-documented growth factor for fibroblasts, EGF, the performance of M-VSFM was significantly improved. The improvement in cell growth was clearly seen as cells supplemented with EGF reached densities almost ~45% higher than those without EGF (1.96 x 10^5 cells/ml and 1.36 x 10^5 cells/ml after 5 days, respectively).

It was thought that the presence of dexamethasone with EGF would work synergistically to enhance cell growth more than EGF alone, but this was not the case in this study. The dose used (100 units, ml) was chosen from a mid-point from various literature values (Hosokawa et al. 1986; Ham et al. 1988). It is possible that EGF is masking the beneficial effect of dexamethasone or that it is not present at high enough a concentration. A titration experiment with varying amounts of dexamethasone with EGF could be explored to answer this question.

There was no improvement in cell growth with the lipid supplement of linoleic and oleic acid. In fact, the presence of the fatty acids decreased cell growth in later passages. The concentration used for the experiments was on the low end of literature values (normally 25 to 75 μM). Those studies showed a marked improvement in growth with concentrations from 10 μM to 25 μM linoleic acid, and 10 μM to 50 μM oleic acid (Butler et al. 1997). This is consistent with other studies with these fatty acids at concentrations less than 75 μM (Morisaki et al. 1984; Karsten et al. 1994). It is possible that the concentrations were too low to illicit an effect on the cells and higher concentrations of 10 to 100 μM should be attempted.

The plant hydrolysates had a mixed effect on cell growth in M-VSFM. Of the two hydrolysates tested, PH1 and PH2, only PH1 stimulated cell growth over that of the control by ~50%. The PH2 had a growth inhibitory and reduced cell growth over that of the control. This inhibitory effect has been observed with other cell lines using plant hydrolysates (Ikononou et al. 2001). It is not known why one would be stimulatory and

the other inhibitory other than the differences in their composition. Hydrolysates proved a rich source of amino acids as well as di and tri-peptides. The amino acid composition is provided by the manufacturer but the peptide composition is not. The PH1 supplement has higher glutamate, phenylalanine, tyrosine, and valine than the PH2 supplement by almost 2-fold. In addition, the PH2 hydrolysate does not contain glutamine (PH1 has 70 mg/g) which is a major nutrient source for mammalian cells (Butler et al. 1989). Elucidating which of the amino acids is being heavily consumed may provide an answer to which of them is growth-promoting in the culture. However, the effect may be due to one or more of the peptides as they have been documented to enhance cell growth (Franek et al. 2000; Franek et al. 2002). Indeed, significant uptake of peptide-bound amino acids has been observed in serum-free media with hydrolysates (Nyberg et al. 1999). Since the peptide composition is unknown only amino acid and/or peptide analysis of the medium during the growth stage would provide answers to those questions.

Even though PH1 increased the growth potential of the medium it was not included in the final formulation. M-VSFM supplemented with EGF gave similar growth profiles and no synergistic effect was observed with the two together (data not shown).

Supplementing M-VSFM with AH1 significantly improved the growth of Vero cells in stationary culture. The titration experiment from 2.0 to 10.0 g/L showed that 2.0g/L was enough to illicit an effect (manufacturer recommended dose is 1 – 5 g/L) with an increase more than 50% above that of the control medium. At the 10 g/L level the growth of the cells did not improve significantly. Other studies have shown a similar effect at 10 g/L (Sung et al. 2004). It is not surprising that AH1 enhanced the cell growth of Vero cells as its growth promoting effect is well documented in the literature (Schlaeger et al. 1992; Schlaeger 1996; Ikononou et al. 2001; Sung et al. 2004).

The purpose of testing the hydrolysates in the medium was to take the first step at designing a lower-cost medium for the animal vaccine industry. Presently, supplementing M-VSFM with any of the hydrolysates would exceed $60/L (US) with

present supplier prices. This is a problem since most animal vaccine producers use serum-based media that is less that $10 US/L (personal communication). The majority of the medium cost is with the recombinant peptide growth factors which contribute more than 95% of the total cost. If these growth factors could be removed from the formulation altogether, supplementing the medium with hydrolysates, the cost would be competitive with what companies currently use. Preliminary studies using a AH1-based DMEM formulation with 0.5% FBS has shown some promise in our laboratory growing cells to near that of 10% FBS-DMEM (data not shown). Similar results have been obtained for low serum media supplemented with AH1 (Velez et al. 1986; Schlaeger 1996).

The optimal inoculation density for M2-VSFM was never determined. The central dogma for cell culture is to start with a seed density of ~ 1.0 x 10^5 cells/ml for most cell lines (Butler 1997). However, if the inoculation density could be reduced to half without any loss of growth rate the speed of amplifying cultures could be increased. For example, inoculating two flasks with 0.5 x 10^5 cells/ml instead of one with 1.0 x 10^5 cells/ml would only require one extra day (6 day growth period instead of 5) to reach confluence. This could drastically reduce the time required to multiply enough cells to seed spinner flasks or bioreactor cultures.

The inoculation density typically used is 8.0 x 10^4 cells/ml for Vero cells in stationary culture. Therefore, lower seed densities were attempted, 4.0 and 6.0 x 10^4 cells/ml. However, the growth rate decreased with the lower seed densities from 31.6 hrs to 37.4 hours with 6.0 x 10^4 cells/ml and 4.0 x 10^4 cells/ml, respectively. The culture with 8.0 x 10^4 cells/ml as the starting inoculum had the quickest generation time of 30.4 hrs/gen. Starting at higher cell numbers has been shown to increase growth rate and reduce the lag phase in mammalian cells (Doverskog et al. 1997). This effect may be due to the fact that cells secrete autocrine growth factors into the medium to promote cell growth and at lower cell number these factors do not reach a high enough concentration to illicit an effect (Lauffenburger et al. 1989).

The Vero cell clones Vero-76 and Vero-E6 varied in their growth potential in M2-VSFM. The Vero-76 cell line quickly adapted to M2-VSFM after only a few passages, reaching densities comparable to the original cell line, ~ 1.6 x 10^5 cells/cm^2 after 5 days. The surprising finding was that the Vero-E6 cell clone did not grow well in M2-VSFM. It was thought that the E6 clone would have similar growth factor and nutrient requirements as the original Vero cell line, but this was not the case. The E6 clone could only be maintained in M2-VSFM for 4 passages with minimal growth. Due to the success of hydrolysates with the original Vero (3.2.5.2), MDCK (4.2.4), and MRC-5 (4.2.5.3) cell lines, PH1 and AH1 were added to the medium. The E6 cells did not respond to the PH1 but grew well with AH1 added to the medium at 2.0 g/L. Although the growth profile did not match that of the control medium, 10% FBS-DMEM (2.5 to 3.0 x 10^5 cells/cm^2), it was comparable to the original Vero cell line, reaching densities of ~1.5 x 10^6 cells/cm^2 in 5 days. The only major difference between AH1 and PH1 in terms of amino acid composition is that hydroxyproline is present in AH1 (which is not present in DMEM). However, the peptide component cannot be ruled out as the growth promoters.

3.4 Conclusion

The purpose of this study was to evaluate and improve the original M-VSFM serum-free medium for Vero cells. The formulation performed comparable to standard serum-containing media, such as 10% FBS-DMEM, giving normal doubling times of ~ 24 hours on microcarriers. The medium was improved with the addition of EGF to yield a new formulation, M2-VSFM. The new formulation, M2-VSFM, now stands as an excellent serum-free medium for the growth of Vero cells. It is free of any animal-derived components and has a low content of non-animal derived protein (90 μg/L). It is also suitable for the growth of Vero 76 clone and the Vero E6 clone, however the E6 clone requires the presence of AH1 in addition to the original formulation.

CHAPTER 4

The Modification of a Serum-free Medium to Support the Growth of MDCK, BHK, MRC-5, and L-929 Cell Lines

4.1 Introduction

The major aims of this paper were to adapt the original serum-free formulation, M-VSFM, to support the growth of industrially important mammalian cell lines. Mammalian cell lines vary significantly in their nutrient and growth factor requirements (Ham et al. 1978; Bottenstein et al. 1979; Barnes et al. 1980). Some cell lines, such as normal diploid cells, are quite fastidious with regard to specific growth factors. Alternatively, transformed cells can be propagated more easily in less complicated formulations. The question is then will M-VSFM support the growth of other cell lines?

There are many different mammalian cell lines commonly used in the biopharmaceutical industry. The most popular lines used for vaccine production are BHK (baby hamster kidney), MDCK (canine kidney), and MRC-5 (human lung fibroblasts).

The BHK cell line is highly susceptible to infection with virus and is used in the production of vaccines. Since the 1960's it has been used to manufacture the foot-and-mouth disease virus in industrial-scale fermenters (Shevitz et al. 1990). It has been used to supply hundreds of millions of doses to countries around the world (Radlett et al. 1985) They are also used in the production of other vaccines (rabies) and some recombinant proteins (interleukins, erythropoietin, antithrombin II, and others). Its versatility and high growth rate makes it very popular in the production of therapeutics.

MDCK cells are also used for the production of vaccines, both animal and human. They are particularly good for the production of influenza vaccines (Merten et al. 1999; Voeten et al. 1999; Tree et al. 2001) and can be grown in both stationary and agitated culture (microcarriers).

The human cell line MRC-5 is a normal diploid fibroblast (non-transformed) which is a valuable characteristic. Due to the risk of introducing unwanted cancerous agents into therapeutic products from immortalized cell lines, such as hybridomas (Petricciani 1985), MRC-5's are a desirable alternative for industry (Spier 1989). They are currently used for human vaccine production.

The hypothesis was that these cell lines may share the same growth factor and nutrient requirements to that of Vero. They are all fibroblasts that are anchorage-dependent or adherent in nature. Although some of them are derived from different tissues (MRC-5, human lung) the others were originally isolated from kidneys (BHK and MDCK). If this hypothesis holds true then the M-VSFM formulation should be sufficient to support them in culture.

The properties of the original medium were to be kept intact during the course of these experiments. That is, avoiding the use of animal-derived components, increasing the protein content, or other changes that would be undesirable to a large-scale industrial process (Froud 1999); (Merten 2002); the reasons of which were discussed previously in chapter 1.

The following study looked at the growth of these cell lines in M-VSFM. In the cases where M-VSFM did not support growth various growth factors, hormones, and other compounds known to be stimulatory will be added to the medium.

4.2 Results

4.2.1 Growth of BHK cells in M-VSFM

A few serum-free or low-serum formulations have been reported in the literature for BHK cells. Low serum formulations (0.1%) have been developed using IMDM as the basal medium and with a cocktail of hormones and animal-derived proteins (Schmid et al. 1991). More promising formulations that have eliminated serum include a protein-free medium using a DMEM/F12 mix for the production of human interleukin-2 (Kratje et al. 1994). This medium was supplemented with insulin, transferrin, sodium pyruvate, along with some amino acids. A similar medium using the same basal mix of DMEM/F12 employed FGF and EGF showing them to be stimulatory for growth (Neufeld et al. 1986). Other proprietary formulations have been reported in various scientific papers but without disclosure of the formulations (Scharfenberg et al. 1995; Neermann et al. 1996).

These formulations share many similarities with M-VSFM in terms of components and growth factors. The basal medium is similar (DMEM) and M-VSFM contains some of the same growth factors, including bFGF. Therefore it is likely that M-VSFM will support the growth. This study examines the M-VSFM formulation for the BHK cell line.

4.2.1.1 Growth of BHK cells in M-VSFM in stationary culture

This study was an attempt to culture the cells in M-VSFM. In an attempt to slowly adapt the cells from a serum-containing medium to a serum-free medium they were weaned off of serum slowly. By subculturing the cells in gradually lower amounts of serum the cells were given more time to adapt to serum-free conditions.

The cells were first passaged in the control medium, 10% FBS-DMEM, then subcultured into successively lower serum-concentrations by mixing it with M-VSFM (i.e.: 75% serum medium + 25% M-VSFM, 50% serum-medium + 50% M-VSFM, etc). T-75

flasks were inoculated with 25 mL of medium and the cells counted after 3 days of growth by the Trypan Blue exclusion method (2.3.1 and 2.4). The cells were weaned off the original formulation over a total of 4 passages. The results are shown in Table 4.1.

The BHK cells were incapable of growth in M-VSFM without the presence of at least 25% FBS-DMEM. As shown in Table 4.1 the doubling times of the BHK cells in T-flasks down to 50% FBS-DMEM is almost the same as that with 100% FBS-DMEM (15.8 hours in 100%, 18.4 hours in 50%). However, subsequent subculturing the cells into a lower serum-containing medium resulted in a dramatic increase in doubling time (33.3 hours with 25% FBS-DMEM). Below the 25% FBS-DMEM level the cells were unable to divide.

Observations of the cells by microscopy showed that the cells in M-VSFM did not adhere to the flask bottom (as was observed in the control medium). Subsequently, the cells clumped together in aggregates (~200 cells) and lifted off the flask surface to float in suspension. This clumping phenomenon has been observed before when BHK cells are passaged into serum-free media (Cruz et al. 1998).

4.2.1.2 Growth of BHK cells on microcarriers in M-VSFM

The BHK cells did not grow very well in M-VSFM in stationary culture. The problem of the lack of adherence to the flask could contribute to further cell death. The cells in the centre of the clump would not have access to nutrients as readily as the cells to the outside which could cause the cells to become necrotic.

Growing the cells on microcarriers might alleviate this problem. The greater available surface area of the microcarriers may provide a better substrate for the cells to propagate. This, with the agitation by stirring may help to prevent the cells from clumping. Therefore this experiment was designed to determine if M-VSFM could support the growth of BHK cells on microcarriers.

Table 4.1 Adaptation of BHK cells to M-VSFM from serum-based media

The cells were first passaged in the control medium, 10% FBS-DMEM, then subcultured into successively lower serum-concentrations by mixing it with M-VSFM. The cells were inoculated into T-75 in 25 mL of medium. The cells were enumerated after 3 days of growth by the Trypan Blue exclusion method.

Ratio of 10% FBS-DMEM (1^{st}) and M-VSFM (2^{nd})[a]	Initial cell count (Cells/cm^2)	Final cell count after 3 days (Cells/cm^2)	Doubling time (hours)
100%/0%	1.33×10^4	3.73×10^5	15.8
50%/50%	1.33×10^4	2.33×10^5	18.4
25%/75%	2.0×10^4	9.73×10^4	33.3
0%/100%	4.0×10^4	2.2×10^4	--[c]

[a] Where serum is 10%, no M-VSFM is added. At lower concentrations the 10% serum media mixture is combined with the M-VSFM to obtain 5% and 2.5% serum content. Note: The cells were passaged at least 3 times at each serum percentage to ensure adaptation to the medium.

[c] No viable cells observed after 3 days

The cells were inoculated in 100 ml spinner flasks with 2.0 g/L Cytodex-1 and 100 ml of media. The cells were incubated at 37°C with a 10% CO_2 overlay and set at a stir rate of 40 rpm. They were grown in either M-VSFM or 10% FBS-DMEM as a control (the stock culture was maintained in 10% FBS-DMEM). The results are shown in Figure 4.1.

The cells grown in M-VSFM reached a cell density of 6 x 10^5 cells/ml after 5 days whereas the control medium saw the cells reach 1.5 x 10^6 cells/ml in the same time period. The growth rate of the cells in 10% FBS-DMEM was 0.056 h^{-1} (17.9 hr doubling time) between day 0 and 2 whereas cells in M-VSFM had a rate of 0.041 h^{-1}(24.4 hr doubling time).

The experiment showed that M-VSFM is able to support the growth of BHK cells in agitated culture. The problem of clumping did persist (clumps of 2-5 beads) but the cells spread out over the surface of the microcarriers and divide in spite of this. There was no loss of cell viability compared to that of the control.

4.2.2. Development of the M-VSFM formulation to support the growth of the BHK cell line

4.2.2.1 The effect of EGF on BHK cell growth in M-VSFM

The success of EGF on the growth of Vero cells in M-VSFM (3.5.1) prompted the use of the growth factor with BHK cells. They are both fibroblast cell lines and have been shown to respond to EGF in cell culture (Neufeld et al. 1986). Therefore EGF was supplemented to M-VSFM at varying concentrations to determine if the cells could be propagated with this formulation.

Figure 4.2 shows the growth of BHK cells in agitated culture. The BHK cells were grown in microcarrier culture (Cytodex-1, 2g/L) in 10%-FBS DMEM (same control as in Figure 4.1), M-VSFM, and M-VSFM + EGF. All of the cells were grown in 10% serum-

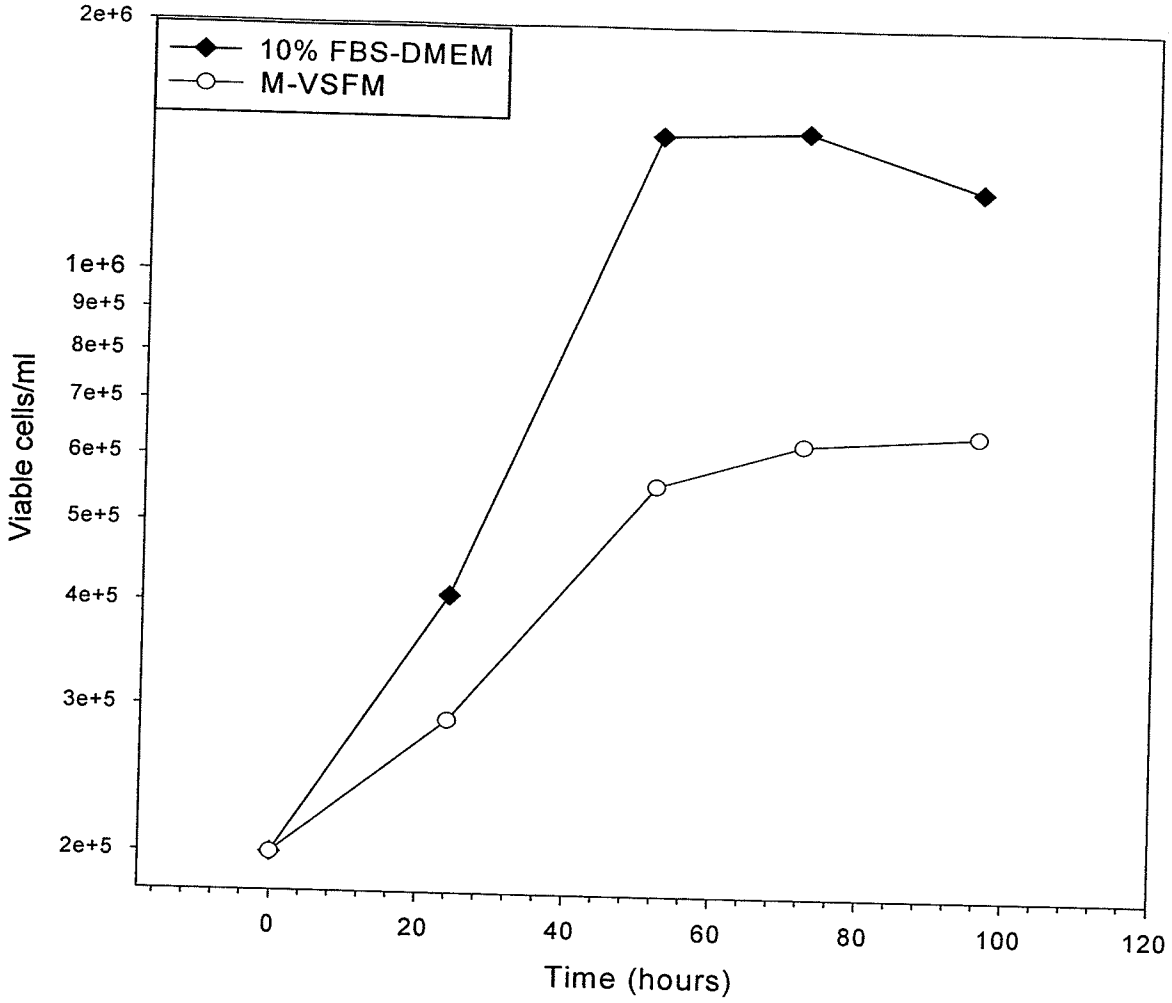


Figure 4.1. Growth of BHK cells on microcarriers (Cytodex-1) in M-VSFM. Spinner flasks (100ml) were inoculated with 2.0 x 10^5 cells/ml in 100 ml of either M-VSFM or 10% FBS-DMEM (control). Cytodex-1 microcarriers were added at a concentration of 2.0 g/L. The cells were grown in 100 ml spinner flasks incubated at 37°C with a 10% CO_2 overlay at a spin rate of 40 rpm.

DMEM and washed prior to being passaged into the spinner flasks. The initial inoculation density was 2.0 x 10^5 cells/ml.

The effect of EGF can clearly be seen. The cells grown without EGF in M-VSFM reached 6 x 10^5 cells/ml after 5 days, whereas cells grown with 4 units/ml EGF reached a final density of 1.0 x 10^6 cells/ml in 3 days. The growth rate of the cells grown with EGF was comparable to cells grown with serum. However, cells grown in serum reached a maximum density of 1.5 x 10^6 cells/ml, 150% higher than cells grown in M-VSFM + 4 units/ml EGF.

4.2.2.2 BHK cell growth in M-VSFM + EGF over continuous passage

It was important to establish that the new formulation supplemented with 4 units/ml of EGF could support the growth of BHK cells over continuous passage. After the cells reached confluence in the microcarrier cultures (Figure 4.2), the cells were trypsinized from the beads, and split 1/5 to a new 100 m spinner flask with 2 g/L Cytodex-1 (2.3.4). Figure 4.3 shows the growth of BHK cells over successive passages.

The cells maintained growth over 3 passages in the medium. The first passage reached 1.0 x 10^6 cells/ml within 4 days, but the second passage took 5 days to reach 7 x 10^5 cells/ml. This could be attributed to the fact that the cells were taken from stationary phase in the previous culture. The third passage was comparable to the first, reaching 9 x 10^5 cells/ml within 5 days. However, the 4^{th} passage (not shown) did not yield in successful growth. The cells showed successful bead attachment, but did not flatten out nor divide. The cells did remain viable according to the Trypan Blue stain, however, for a period of 2 weeks after inoculation when the culture was then discontinued.

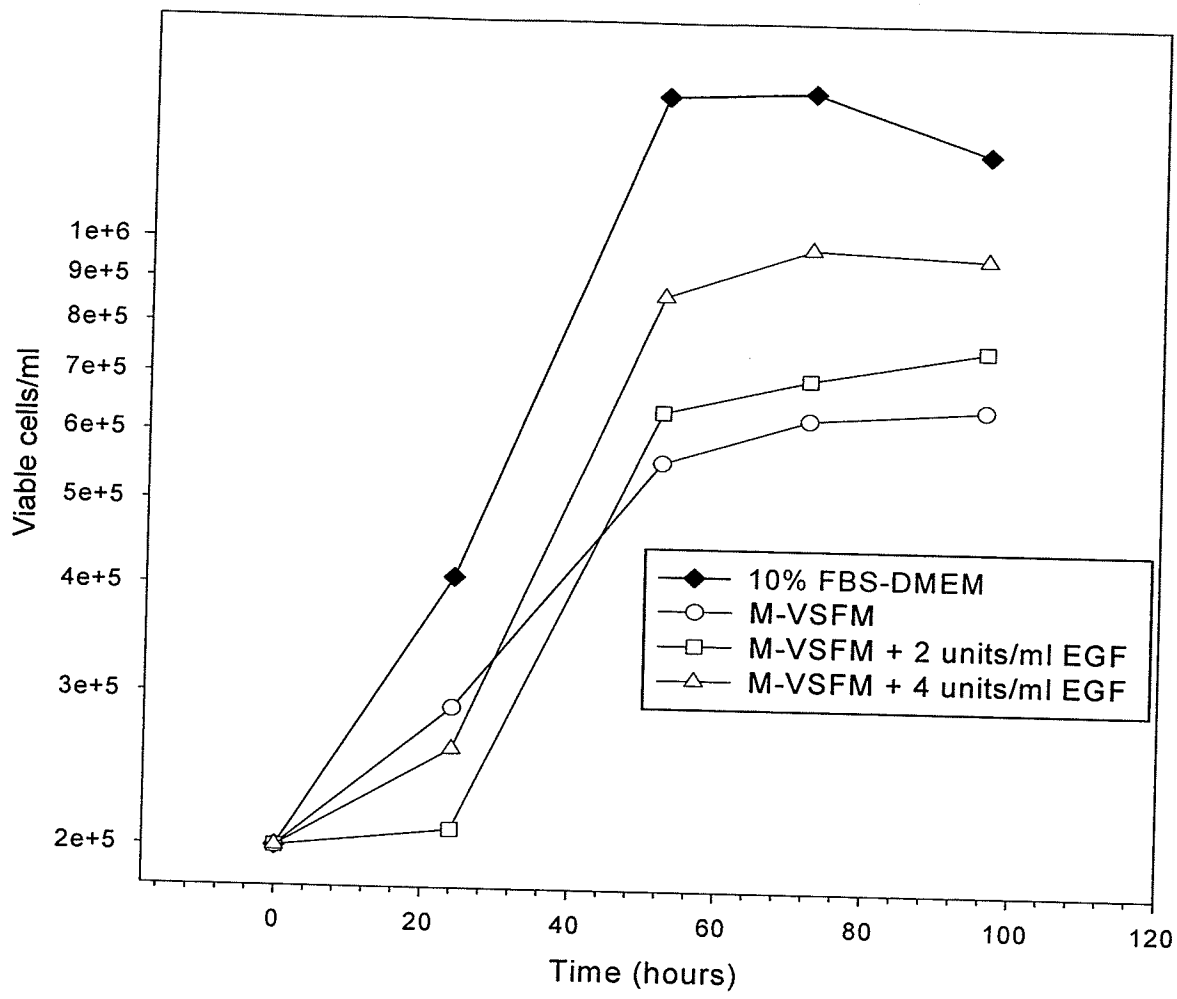


Figure 4.2. The effect of EGF on BHK cell growth in M-VSFM in agitated culture. Spinner flasks (100ml) were inoculated with 2.0 x 10^5 cells/ml in 100 ml of either M-VSFM, M-VSFM + 2 units/ml EGF, M-VSFM + 4 units/ml EGF, or 10% FBS-DMEM (control). Cytodex-1 microcarriers were added at a concentration of 2.0 g/L. The cells were incubated at 37°C with a 10% CO_2 overlay at a spin rate of 40 rpm. Statistical evaluation was performed with a tailed paired *t*-test. M-VSFM and M-VSFM + 4 units/ml EGF from day 2 to 5 are statistically different with a P=0.002.

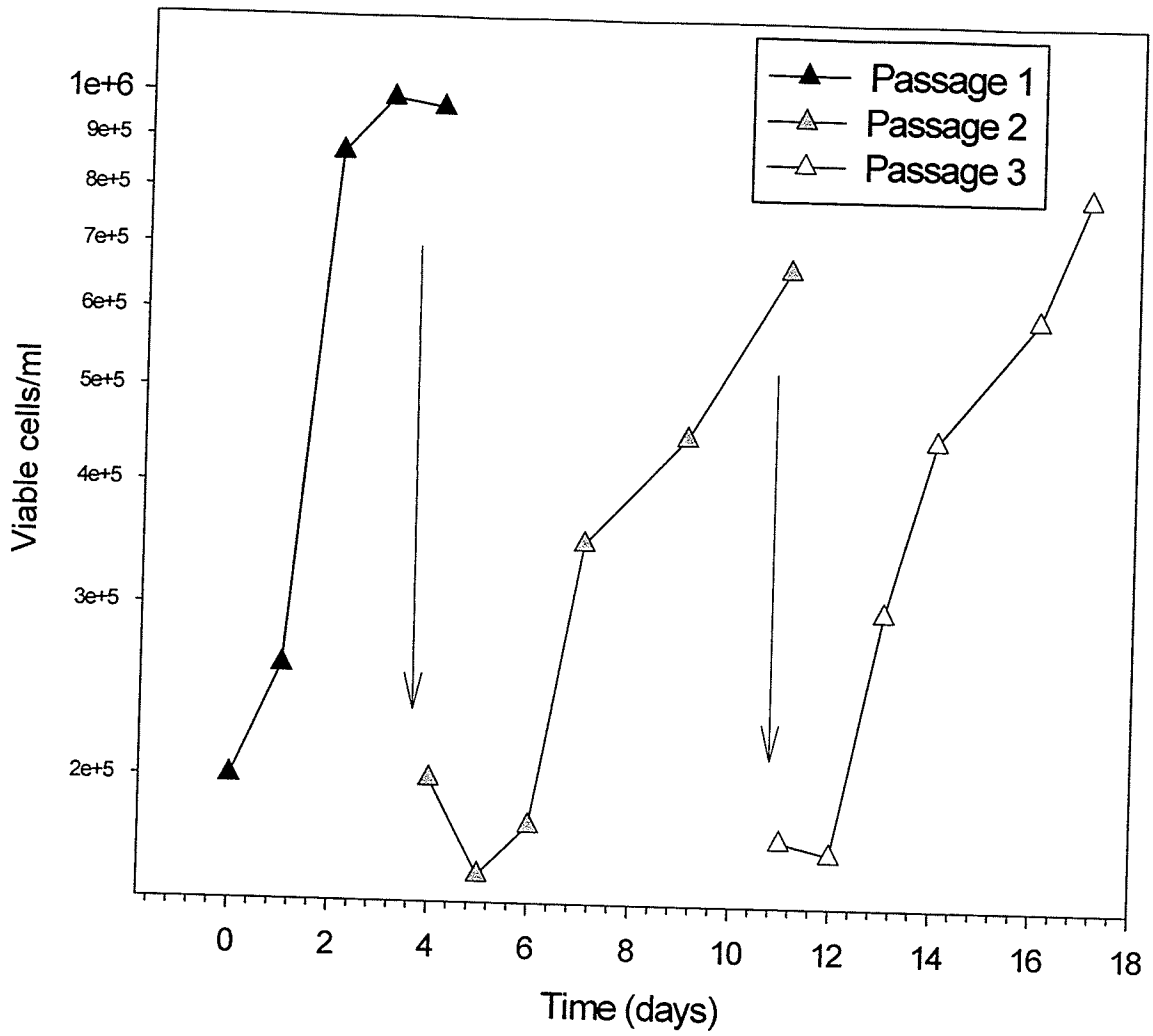


Figure 4.3. Growth of BHK cells in M-VSFM + 4 units/ml EGF over continuous passage in agitated culture. The cells were taken from the first passage in experiment 4.3.1.1 (M-VSFM + 4 units/ml EGF). They were trypsinized and inoculated into a new spinner flask (100 ml) at 2.0 x 10^5 cells/ml with 2.0 g/L Cytodex-1 as a microcarrier. The flasks were incubated at 37°C with a 10% CO_2 overlay at a stir rate of 40 rpm.

4.2.2.3 Titration of bFGF for BHK cells grown in M-VSFM

A titration of bFGF in the medium was carried out to determine if a higher concentration would enhance cell growth (which is a normal component). FGF has been reported to be stimulatory to BHK cells (Neufeld et al. 1986).

The cells were grown in 10% serum-DMEM before being passaged into M-VSFM. The cells were inoculated at a density of 2.0 x 10^5 cells/ml in 100ml spinner flasks with 2g/L Cyotdex-1. FGF was added at different concentrations, 0.5, 1.0 (control, normal M-VSFM), and 2.0 units/L. The results are shown in Figure 4.4.

Cell growth rate and final cell numbers were comparable for all concentrations of FGF tested. The final cell yields were not significantly different, with 0.5 units/L being the highest at 1.6 x 10^6 cells/ml after 7 days. Cells in 1.0 units/L FGF reached 1.24 x 10^6 cells/ml in the same time period, and cells in 2.0 units/L reached 1.33 x 10^6 cells/ml.

The titration range was chosen to maintain the original cost of the medium. For this reason, higher levels of FGF were not explored due the inevitable increase in cost per litre that would follow.

4.2.2.4 Growth of BHK cells with M-VSFM supplemented with AH1

The BHK cell line is one of the most common cell lines used for the production of veterinary vaccines including those for foot and mouth disease and rabies (Radlett et al. 1985; Sureau 1992). The unit cost for these vaccines has to be considerably low to be cost-feasible for the agriculture industry. The current cost of our serum-free medium, supplemented with EGF, is considerably high compared to those routinely used in the veterinary vaccine industry (personal communication). To be competitive the cost would have to be less than $10/L compared to where it stands now at > $60/L. It has been reported that AH1 can replace the action of serum and/or growth factors in the medium (Schlaeger 1996).

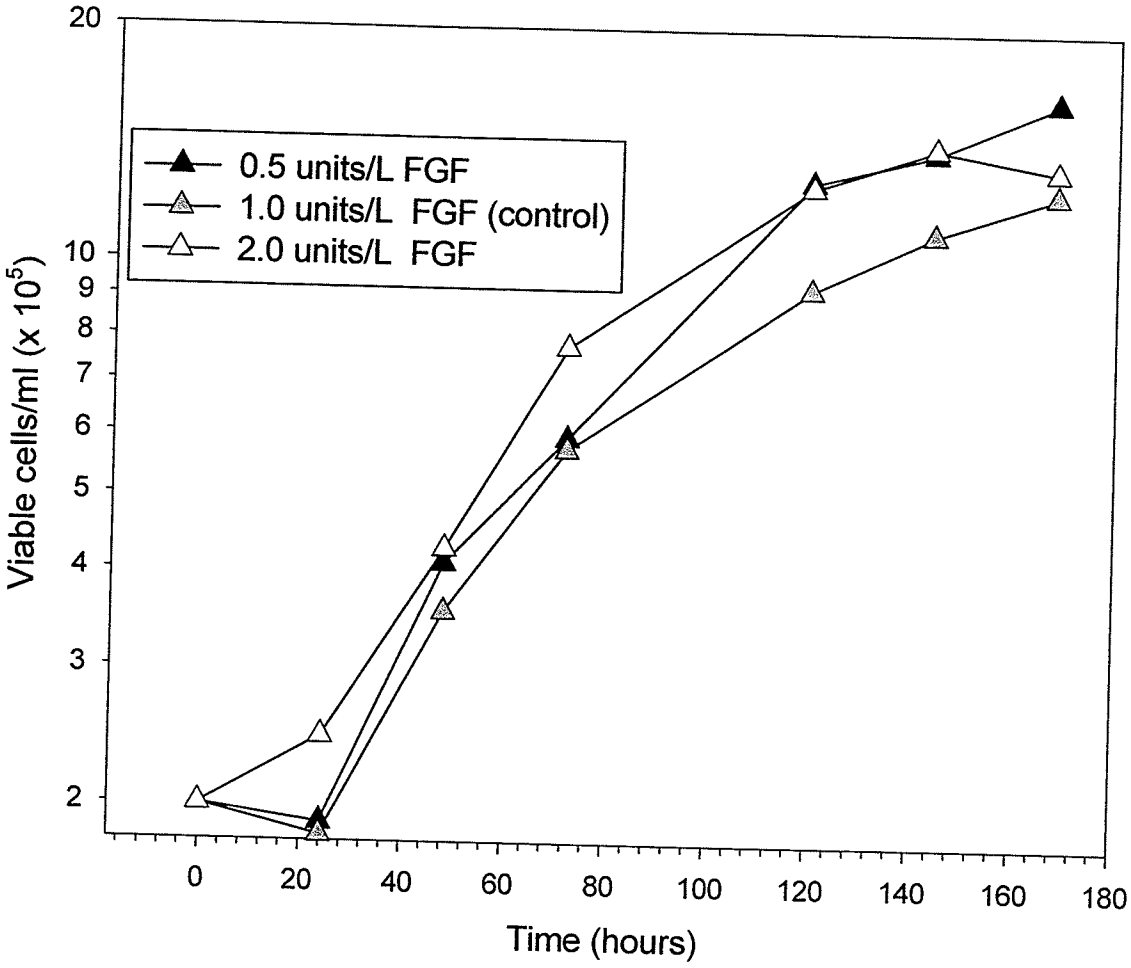


Figure 4.4. Titration of FGF in M-VSFM for BHK cell growth in agitated culture. Spinner flasks (100ml) were inoculated with 2.0 x 10^5 cells/ml in 100 ml of either M-VSFM, M-VSFM + 0.5 units/L bFGF, M-VSFM + 1.0 units/L bFGF (control), or M-VSFM + 2.0 units/L bFGF media. Cytodex-1 microcarriers were added at a concentration of 2.0 g/L. The cells were incubated at 37°C with a 10% CO_2 overlay at a stir rate of 40 rpm. Statistical analysis reveals that there is no difference between the 3 variables tested based on a paired *t*-test. The 0.5 units/L and 2.0 units/L cultures have a $P>0.10$. This is representative of one experiment.

Some serum-free formulations have used peptone or hydrolysates in the medium to grow BHK cells (Perrin et al. 1995; Heidemann et al. 2000). Other groups have used AH1 (animal tissue in origin) supplemented media in the propagation of BHK cells to produce a recombinant protein (Cruz et al. 1998). In all cases an increase in growth and/or productivity was observed in the peptone/hydrolystate-supplmented cultures.

Due to the success of AH1 in the literature and with Vero cells in M2-VSFM (3.2.5.2) it is likely that the addition of AH1 to M2-VSFM would improve the medium's performance for BHK cells. Furthermore, if the growth factors could be replaced with AH1 the medium cost would drop to less than $10/L making it attractive for industrial purposes. Therefore this initial study looked at the propagation of BHK cells in M2-VSFM supplemented with AH1 with the hope that the growth factors can later be removed.

BHK cells were inoculated into T-75 flasks at a starting cell density of 1.33 x 10^4 cells/cm^2 in 25 ml of media. The cells were passaged by trypsinization and counted by the Trypan Blue exclusion method (2.3.1 and 2.4). The cells were weaned from their maintenance medium (10% FBS-DMEM) by mixing the serum-medium with the new serum-free formulation (M2-VSFM + 5.0 g/L AH1). The ratio of serum-free to serum media was gradually increased in successive passages. The first passage was 100% serum-media, then 75% serum-free media/25% serum-media, 85% serum-free media/15% serum-media, until the medium was completely serum-free (6 passages). The cells doubling time increased gradually as the amount of serum-media was reduced (from 30 hours to 68 hours, data not shown). Once the cells had been weaned from serum they were passaged 5 times in the serum-free formulation. The results are shown in Table 4.2.

The cells were passaged once they had reached confluence (and had spread out on the flask like normal fibroblasts). The first passage had a longer doubling time of 75.2 hours and only reached 6.3 x 10^4 cells/cm^2. The second passage showed almost no growth reaching only 2.3 x 10^4 cells/cm^2 in 9 days. However, successive passages were better and the cells had reasonable doubling times between 40 and 58 hours.

Table 4.2. Growth of BHK cells in M2-VSFM supplemented with AH1 in stationary culture.

The cells were inoculated into 75 cm^2 T-flasks with a starting cell density of 1.3 x 10^4 cells/cm^2 in 25 ml of media. The cells were passaged once they reached confluence (time to reach confluence is indicated in parentheses beside the final cell number).

Medium: M2-VSFM +5.0 g/L AH1	Starting cell number (Cells/cm^2)	Final cell number (Cells/cm^2)	Doubling time Hr/gen
Passage 1	1.33 x 10^4	6.3 x 10^4 (7 days)	75.2
Passage 2	1.33 x 10^4	2.3 x 10^4 (9 days)	282
Passage 3	1.33 x 10^4	1.6 x 10^5 (6 days)	40.4
Passage 4	1.33 x 10^4	1.3 x 10^5 (8 days)	57.8
Passage 5	1.33 x 10^4	8.3 x 10^4 (5 days)	45.6

There was considerable clustering of the cells in the flask. The clumps would consist of greater than 200 cells. This was observed previously in M-VSFM (4.2.1). However, in the presence of AH1 a population of the cells anchored themselves to the flask and flatten out which was not observed in the M-VSFM medium.

4.2.3 Development of M-VSFM to grow MDCK cells

Serum-free media for these cells have been developed and published in the literature. Some of them were proprietary (Merten et al. 1994) whereas others were fully published with the formulations (Taub et al. 1979) (Bashir et al. 1992). The common components used in these formulations consisted of a mixture of DMEM or DMEM/Ham's F12 (1:1 ratio), supplemented with hydrolysates, sodium bicarbonate, hepes, insulin, transferrin, prostaglandin E1, triiodothyronine, hydrocortisone, epidermal growth factor, and fibroblast growth factor (EGF and FGF found to be growth stimulatory but not required). Due to the similarities to M2-VSFM in basal formulation and components it was hypothesized that it would support the growth of these cells.

4.2.3.1 Growth of MDCK cells in T-flasks in M-VSFM

The MDCK cell line had little difficulty adapting to the M-VSFM formulation. A gentle weaning process was performed to allow the cells to adapt to a serum and protein-free media. The cells needed only to be passaged to 5% serum, then 2.5%, and finally to M-VSFM to enable growth (data not shown). The cells were passaged into T-150 flasks at a starting cell density of 1.33×10^4 cells/cm^2 in 50 ml of media. Upon reaching confluence (4 days), the cells were counted by the Trypan Blue exclusion method (2.3.2 and 2.4). After 3 passages in M-VSFM the growth rate was determined. The results are shown in Table 4.3.

The doubling time for cells in M-VSFM was very close to that of media with 10% serum. The cells in M-VSFM had an average doubling time of 34.0 hours compared to 32.3

Table 4.3. Growth of MDCK cells in T-150 flasks in 10% serum and M-VSFM

The cells were passaged in T-150 flasks at a starting cell density of 1.33×10^4 cells/cm^2 in 50 ml of media. The cells were counted after 4 days of growth (upon reaching confluence) by the Trypan Blue exclusion method.

Medium	Initial cell count (cells/cm^2)	Final cell count after 4 days (cells/cm^2)	Doubling time (hours)
10% FBS-DMEM	1.33×10^4	1.75×10^5	32.3
M-VSFM [a]	1.33×10^4	1.53×10^5	34.0

[a] The M-VSFM was made from the 10X supplement.

hours in 10% FBS-DMEM. The cells grown in M-VSFM anchored themselves much more strongly than the cells grown with 10% FBS-DMEM. This may be due to the fact that anchorage proteins would have to be synthesized by the cells rather than be provided in serum-containing media. The cells required ~10 minutes longer to be dislodged from the flask during trypsinization compared to the cells in the 10% FBS medium.

Unfortunately with further passages the cell doubling time increased dramatically (>200 hours) (data not shown). Only during the initial few passages in M-VSFM did the cells have a doubling time close to that of serum-based media.

4.2.3.2 Growth of MDCK cells in microcarrier cultures

Simultaneously with the growth of the cells in stationary culture they were propagated in microcarrier cultures. MDCK cells were inoculated into 100ml spinner flasks at an initial cell density of 2.5 x 10^5 cells/ml. Microcarriers (Cytodex-1) were added to the flasks at a final concentration of 2.0 g/L. The cells were counted daily by the Trypan Blue exclusion method described previously (2.3.3 and 2.4). The results are shown in Figure 4.5.

In both types of media there was a long attachment time of the cells to the microcarriers. Approximately 50% of the cells were able to attach themselves to the beads after 24 hours. While the cells in 10% serum started dividing almost immediately afterward, reaching densities > 1.5 x 10^6 cells/ml, the cells in M-VSFM grew only slightly to 3.3 x 10^5 cells/ml after 4 days. The cells were unable to successfully divide in M-VSFM.

To eliminate the possibility that the initial cell density was too low and hindered cell attachment a second experiment was carried out. A higher inoculum was used (4.5×10^5 cells/ml) and the conditions were identical to the previous trial. The results are shown in Figure 4.6.

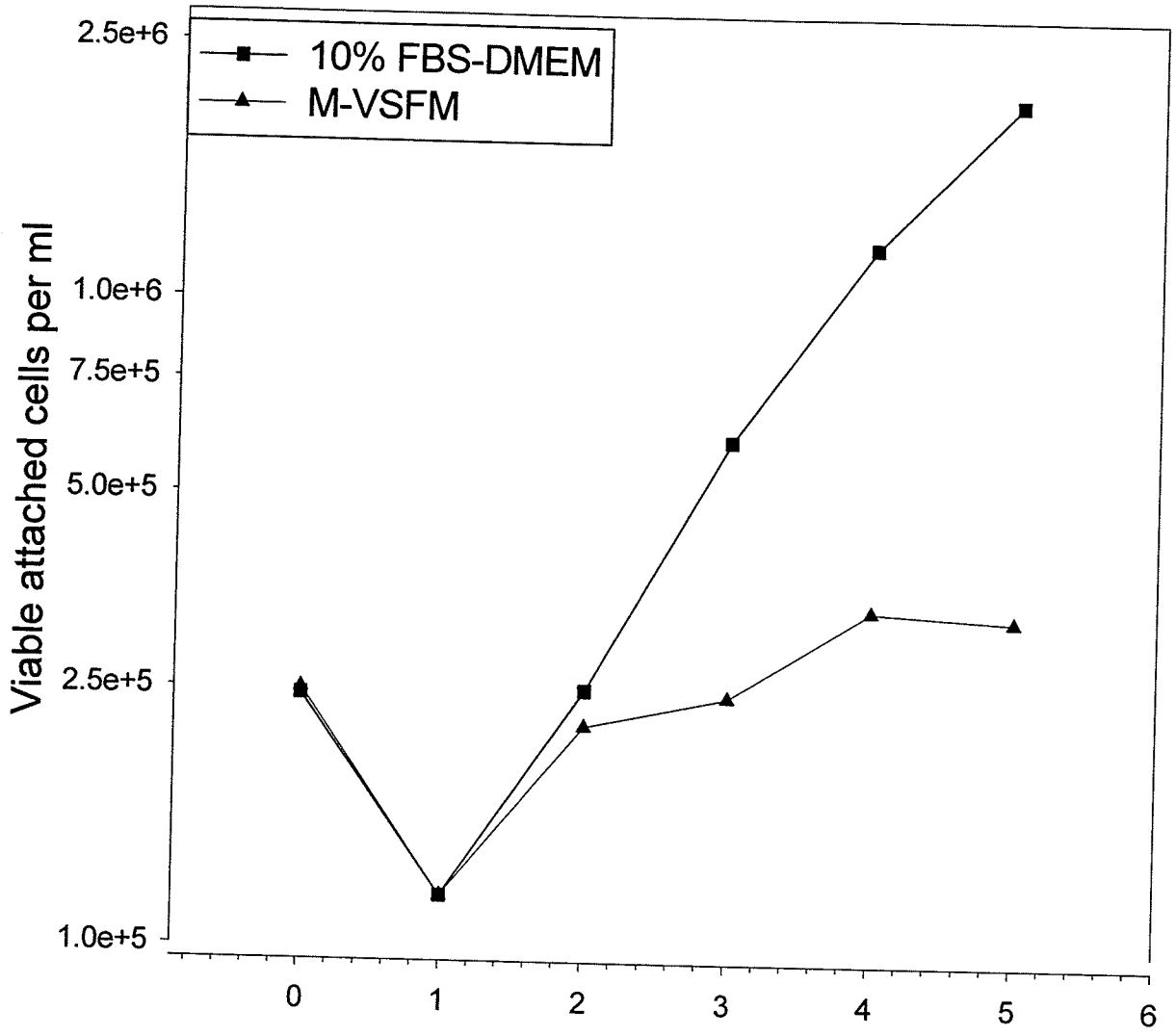


Figure 4.5. Growth profile of MDCK cells grown in M-VSFM and 10% FBS-DMEM on microcarrier (Cytodex-1). Spinner flasks (100ml) were inoculated with MDCK cells at a initial cell density of 2.5 x 10^5 cells/ml in either 10% FBS-DMEM or M-VSFM. Microcarriers (Cytodex-1) were added at a concentration of 2.0 g/L. The flasks were incubated at 37°C with an 10% CO_2 overlay and set at a stir rate of 40rpm. Each point is an average of two cultures (n=2) with the error being less than 10% of the mean.

The cells in 10% FBS-DMEM quickly attached to the beads and began to divide. The culture reached a density of 1.3 x 10^6 cells/ml, slightly lower than what was observed with the lower inoculum cultures (Figure 4.5). Again, the cells in M-VSFM did not divide and quickly died. The cells' affinity for the microcarriers was strong (most cells attached after an hour) but did not flatten out. It is likely that an attachment factor is required either in the medium or by synthesis by the cell.

4.2.4 An improved serum-free formulation for MDCK cells.

The original formulation could propagate the cells over a few passages with a comparable growth rate to serum-supplemented media (4.2.3). However, the growth rates decreased over successive passages until the cells had doubling times greater than 200 hours (data not shown). Therefore, a new formulation needed to be developed.

Previous studies in the literature have shown that hydrolysates can be stimulatory for MDCK cells in serum-free media (Merten et al. 1999). In particular, a soy-bean derived hydrolysate was used. Due to the success of these kinds of hydrolysates with other similar cell lines (Schlaeger 1996) it is likely that it would improve cell growth in M-VSFM.

The new formulation, M2-VSFM containing EGF, was not yet tested on the MDCK cell line since it had been developed (3.2.4.1) (only the original M-VSFM). Therefore, M2-VSFM, along with a hydrolysate-supplemented version using a plant hydrolysate (PH1) was evaluated with MDCK cells. Concurrently, commercially available serum-free formulations (available at the time of this study) were evaluated as well.

MDCK cells were taken from their maintenance medium, 10% FBS-DMEM and passaged into each of the test media. These included M2-VSFM, M2-VSFM + 2.0 g/L PH1, UltraMDCK (Biowhittaker), and 10% FBS-DMEM as a control. The cells were grown in each of the media types for a total of 10 passages (2.3.1.1) to ensure complete adaptation to the media. They were then inoculated into T-25 flasks with a starting cell

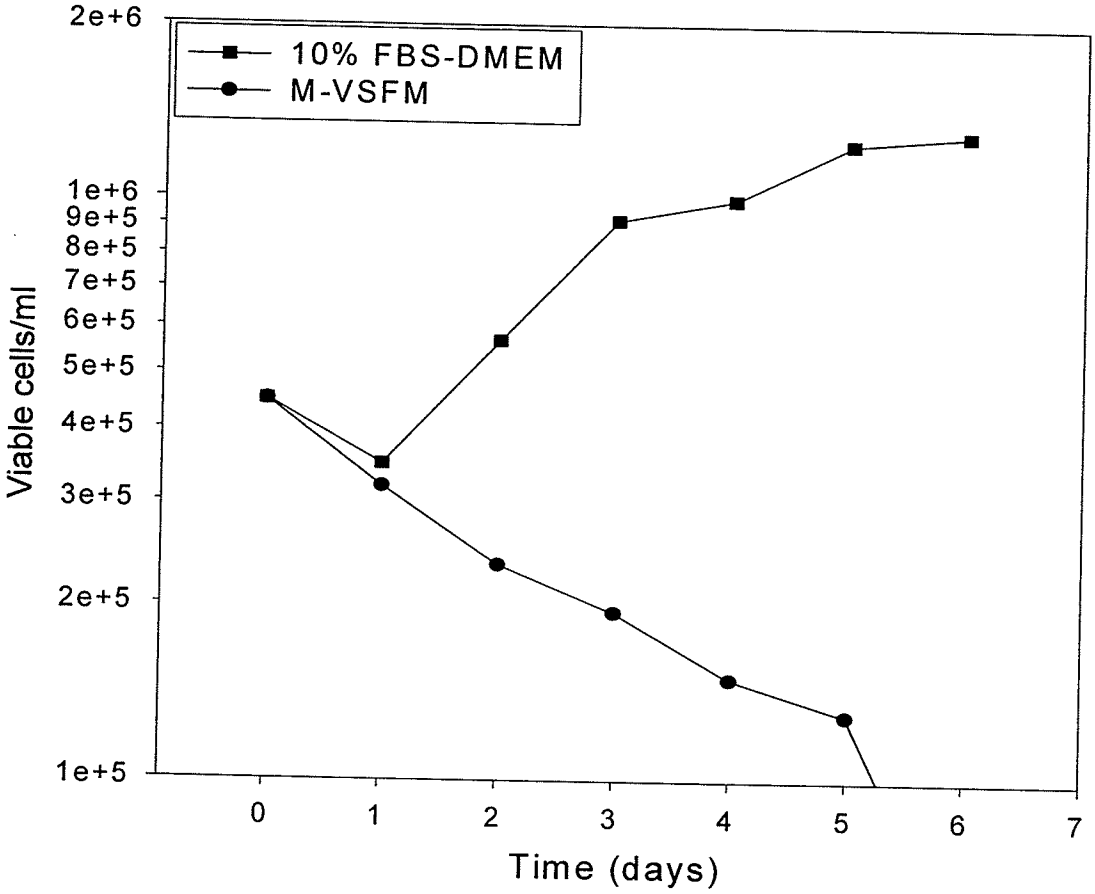


Figure 4.6. Growth of MDCK cells in M-VSFM and 10% FBS-DMEM on microcarriers (Cytodex-1) with a higher initial cell density. Spinner flasks (100ml) were inoculated with MDCK cells at a initial cell density of 4.5 x 10^5 cells/ml in either 10% FBS-DMEM or M-VSFM. Microcarriers (Cytodex-1) were added at a concentration of 2.0 g/L. The flasks were incubated at 37°C with an 10% CO_2 overlay and set at a stir rate of 40rpm. Each point is an average of two cultures (n=2) with error being less than 10% of the mean.

density of 2.0 x 10^4 cells/cm^2 in 8ml of media. Duplicate cultures were taken each day for cell counting by the Trypan Blue exclusion method (2.3.1.2 and 2.4). The growth curve is shown in Figure 4.7.

Although growth occurred in M2-VSFM, the growth rate was very low (0.0254 gen/hr from day 4 to 6). Supplementation with PH1 increased the growth rate to 0.0333 gen/hr (from day 2 to 5) as well as the final cell yield: 3.40 x 10^5 cells/cm^2 from 2.0 x 10^4 cells/cm^2 in 5 days. These growth rates were comparable to 10% FBS-DMEM (0.0335 gen/hr, day 2 to 4), but the highest cell yields were obtained in 10% FBS-DMEM (4.0 x 10^5 cells/cm^2). The M2-VSFM + PH1 formulation was superior to the only commercially available serum-free formulation, UltraMDCK, which had a growth rate of 0.0264 gen/hr or 37.9 hr doubling time (from day 2 to 5).

4.2.5 Development of M-VSFM for MRC-5 cell growth

MRC-5 cells are normal diploid fibroblasts (normal chromosome compliment) and therefore have a finite life span in culture. They cannot be cultured for more than 50 population doublings or ~10 passages. It is not surprising, then, that unlike their transformed counterparts they have complex nutrient and growth factor requirements (i.e.: CHO, BHK, etc) (Ham 1981).

Previous reports have provided formulations for the growth of MRC-5 cell line (Bettger et al. 1981). This formulation, designated MCDB 110, contains most of the same components in DMEM with the addition of a variety of growth hormones and vitamins. The formulation shares many similarities with M-VSFM. However, this medium was quite poor with respect to cell growth. More recent studies have reported the propagation of MRC-5 cells in serum-free culture but without full disclosure of the formulation (Candal et al. 1991; Maggetto et al. 1999).

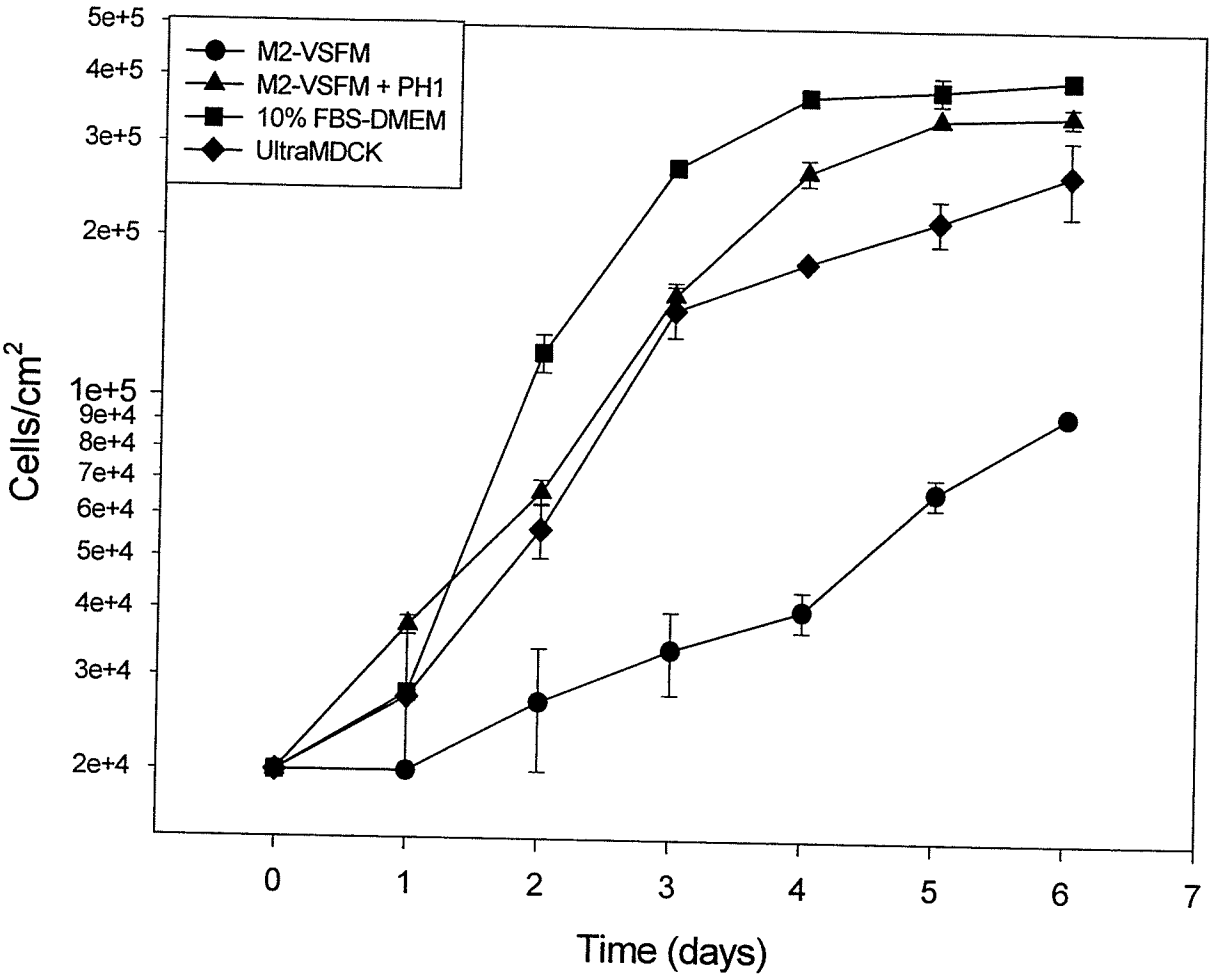


Figure 4.7. The growth of MDCK cells in M2-VSFM, M2-VSFM + PH1, 10% FBS-DMEM, and UltraMDCK.. The cells were grown in each media type for 10 passages before constructing the growth curve (passaged every 5 days). The cells were inoculated with 2.0 x 10^4 cells/cm^2 in 8ml of media, or 6.25 x 10^4 cells/ml, in 25 cm^2 T-flasks. The cells were counted each day by trypsinization and counted by the trypan blue exclusion method (N=2). Statistical evaluation was performed with a paired *t*-test. M2-VSFM + PH1 and UltraMDCK are statistically different with a P=0.05.

With the lack of literature on the growth of MRC-5 cells it is difficult to design a formulation without starting from scratch. However, even though there was limited success with the MCDB 110 medium it is possible that components present in M-VSFM that are not in MCDB 110 may have been limiting factors in the cell growth. Therefore, this study explored the performance of M-VSFM to propagate MRC-5 cells in culture.

4.2.5.1 Growth of MRC-5 cells in M-VSFM and MOD-VSFM

Prior to this experiment MRC-5 cells were attempted to be cultured in M-VSFM in T-flasks. The trials were unsuccessful and the cells would only remain viable for a short period of time (2-3 days) at which point they would undergo apoptosis (blebbing of the membrane, cell lysis).

It was speculated that perhaps the cells would perform better in agitated culture (microcarriers) as such a mode of growth can better provide nutrients to the cell. Cultures of MRC-5 cells were initiated in stationary culture, using 10% FBS-DMEM as the maintenance medium. The cells were harvested (2.3.1) by trypsinization and inoculated into 100ml spinner flasks at 3.5 x 10^5 cells/ml in two different media, M-VSFM and MOD-MVSFM (an altered VSFM formulation). The spinner flasks contained 4.0 g/L Cytodex-1 beads as the microcarrier. The cells were counted each day from the initiation of the cell culture using the Trypan Blue exclusion method (2.3.3 and 2.4). The results are shown in Figure 4.8.

There was no cell growth observed in either media-type after inoculation. However, cells in MOD-VSFM remained viable for 7 days after inoculation before it declined. This was not observed in the M-VSFM culture where the cells did not maintain viability for that length of time, decreasing after only 3 days. MOD-VSFM was also able to maintain good cell attachment throughout the entire culture period, whereas cells in VSFM unattached after 3 days.

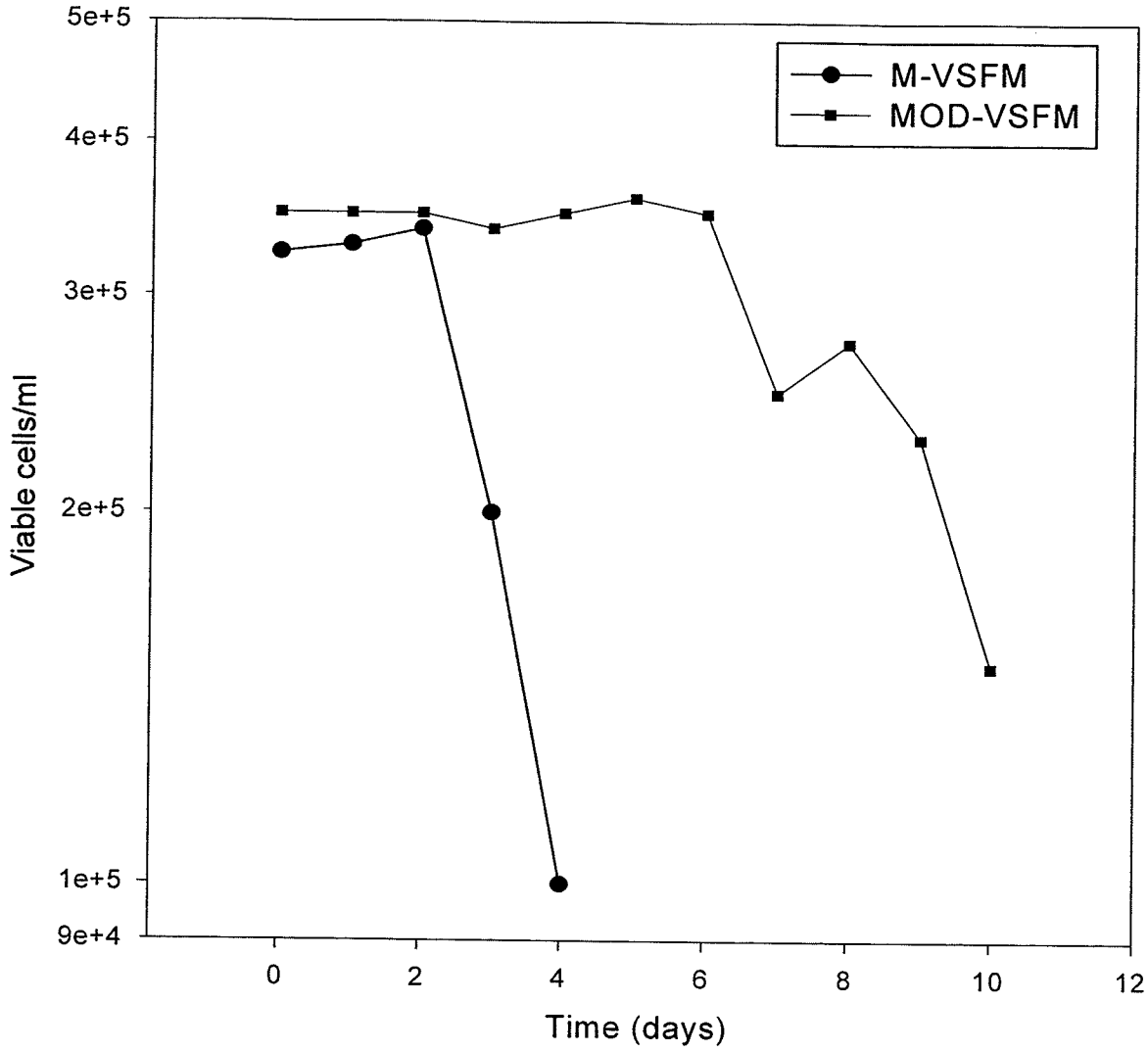


Figure 4.8. Growth of MRC-5 cells in Cytodex-1 microcarrier cultures in VSFM and MOD-VSFM. Spinner flasks (100ml) were inoculated with 4.5 x 10^5 cells/ml in 100 ml of either M-VSFM or MOD-VSFM media. Cytodex-1 microcarriers were added at a concentration of 4.0 g/L. The cells were incubated at 37°C with a 10% CO_2 overlay at a stir rate of 40 rpm.

4.2.5.2 The use of M-VSFM as a production medium for MRC-5 cells

Since growth of MRC-5 cells has not been successful in M-VSFM, the possibility that the media could be used to maintain cell viability in production was investigated. This could be used as a last step in virus production in large-scale cultures.

The possibility that DMEM was not rich enough in vitamins, amino acids, and other nutrients might have been the reason for the poor performance of the medium. In this study the DMEM formulation was replaced with medium 199, which is higher nutritionally than the DMEM formulation. It is based on Earle's salt solution containing an extensive range of amino acids, vitamins, nucleic acid derivatives, growth factors and lipids (Morgan et al. 1950; Morgan et al. 1955; Morton 1970). The components present in 199 that are not in DMEM are uracil, hydroxyproline, ascorbic acid (Vit C), alpha-tocopherol phosphate (Vit B), calciferol (Vit D_{12}), menadione (Vit K_3), nicotinic acid (Niacin), PABA, Vit A (acetate), cholesterol, and glutathione.

MRC-5 cells were grown in 100ml spinner flasks with 3g/L Cytodex-1 microcarriers in 10% serum-DMEM. The initial inoculation density was 1.2 x 10^5 cells/ml and the culture reached a maximum density of 7 x 10^5 cells/ml within 3 days. The media was then replaced with modified serum-free formulations of M-VSFM; M-VSFM-media 199; M-VSFM-199 + 2 units/ml EGF; and MOD-VSFM. The results are shown in Figure 4.9.

The cell viability declined the sharpest with M-VSFM-199. The viable cell number was maintained for 2 days after the media change followed by a sharp decline. MOD-VSFM seemed to maintain cell viability the longest, for 3 days after the media change. Similar results were seen with M-VSFM-199 + EGF which maintained viability for 3 days before declining. Cell growth was observed to some extent after 2 days of the media change, but this may have been due to growth factors carried over in the cells from the serum-based medium or random fluctuation.

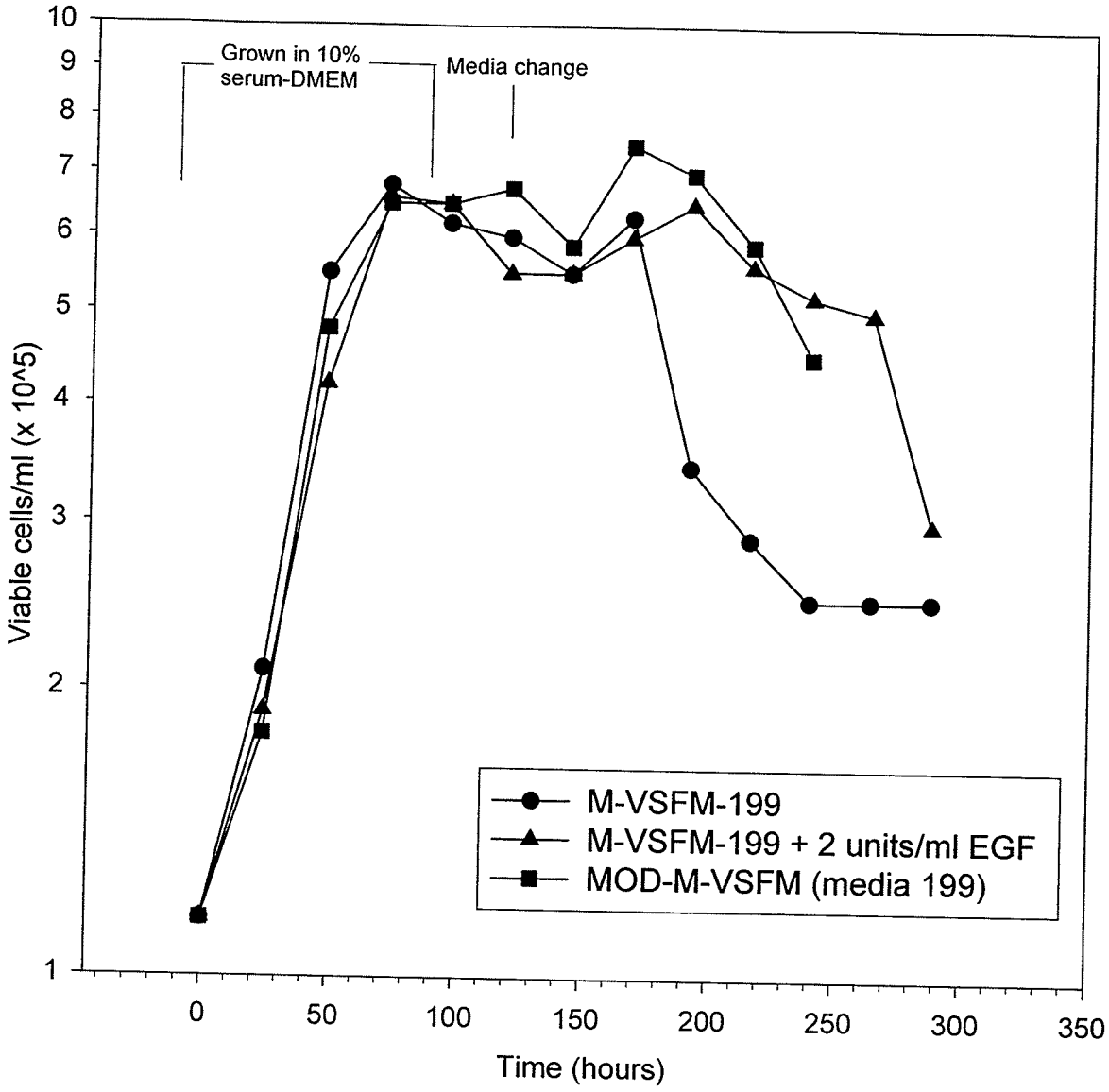


Figure 4.9. Variations of M-VSFM as a production media for MRC-5 cells. Spinner flasks (100ml) were inoculated with 2.0 x 10^5 cells/ml in 100 ml of either M-VSFM, M-VSFM-199 + 2 units/ml EGF, or MOD-M-VSFM. Cytodex-1 microcarriers were added at a concentration of 2.0 g/L. The cells were incubated at 37°C with a 10% CO_2 overlay at a stir rate of 40 rpm.

4.2.5.3 The effect of AH1 on the growth of MRC-5 cells in M2-VSFM.

M-VSFM was not able to support the growth of MRC-5 cells. Modifications of the formulation by the introduction of various hormones, growth factors, and vitamins would increase the longevity of the cells but would not stimulate growth (4.2.5.1 and 4.2.5.2).

The widespread success with hydrolysates in previous studies with the Vero, MDCK, and BHK cell lines prompted the study with MRC-5 cells. There is no literature examining the growth of MRC-5 cells with hydrolysate supplements, either animal or plant in origin. Since AH1 was the most stimulatory for growth in every cell line tested in our lab (data not shown) it was chosen.

The cells were grown in 100ml spinner flasks, with 2g/L Cytodex-1 as a microcarrier, in M2-VSFM supplemented with various concentrations of AH1 (1.0, 2.0, 3.0, and 5.0 g/L) and 5% FBS-DMEM as a control. The flasks were inoculated with an initial cell density of 2.0 x 10^5 cells/ml. The flasks were incubated at 37°C with a 10% CO_2 overlay at a spin rate of 40 rpm. The cells were counted every 24 hours by the Trypan Blue Exclusion method (2.3.3 and 2.4). The results are shown in Figure 4.10.a.

Supplementation of M2-VSFM with AH1 was successful at propagating MRC-5 cells in microcarrier culture. There is a concentration-dependent effect of AH1 on cell growth. This had a twofold effect – increasing the cellular growth rate as well as increasing the final cell density. The best growth results were observed with 5.0 g/L supplementation. The specific growth rate increased from 0.024 h^{-1} in 1.0 g/L of AH1 to 0.031 h^{-1} in 5.0 g/L. The 2.0 and 3.0 g/L cultures had growth rates of 0.022 h^{-1} and 0.019 h^{-1}, respectively. The maximum cell densities reached increased with AH1 concentration, being 3.4 x 10^5 cells/ml for 1.0 g/L, 8.8 x 10^5 cells/ml for 2.0 g/L, 8.8 x 10^5 cells/ml for 3.0 g/L, and 1.15 x 10^6 cells/ml for 5.0 g/L. The growth rate and maximum cell density reached for the control culture, 5% FBS-DMEM was 0.0295 h^{-1} and 6.6 x 10^5 cells/ml, respectively.

To determine how long the cells could be propagated in the medium the cells were harvested (2.3.3) and passaged into a new flask. The cells were maintained in the M2-VSFM + 5.0 g/L AH1 formulation. The results are shown in Figure 4.10.b.

The growth rate did not slow and the cells reached higher densities in the second passage (1.6 x 10^6 cells/ml within 4 days on passage 2, compared to 1.15 x 10^6 cells/ml within 4 days in passage 1). Successive passaging led to a decrease in cell viability at which point the cells would no longer divide. This was observed after 2 days at passage 3. The cells remained at the same density for this period before seeing a significant drop in viability on day 3.

4.2.6 Development of M-VSFM for L-929 cells

The L-929 cell line is a clone derived from one of the original cell strains to be established in cell culture, the L cell. The parent L strain was derived from alveolar and adipose tissue of an adult mouse (ATCC). It has lost its anchorage-dependence and is routinely grown in suspension.

This cell line is routinely used in the culturing of a wide variety of viruses. These include reovirus serotype 1, 2 and 3 (Nibert et al. 2001), herpes simplex virus (Garabedian et al. 1967), sindbis virus (Ito et al. 1989), and vaccinia virus (Hruby et al. 1980). Presently it is not used for the large-scale production of any therapeutic viruses.

These cells are typically propagated in a serum-supplemented MEM formulation which is recommended by commercial suppliers of the cell line (ATCC). Even though serum-free formulations have been developed (Evans et al. 1956; Waymouth 1959; Birch et al. 1971), they are not widely used.

This study evaluates how well the M2-VSFM formulation will support the growth of these cells compared to serum-supplemented media.

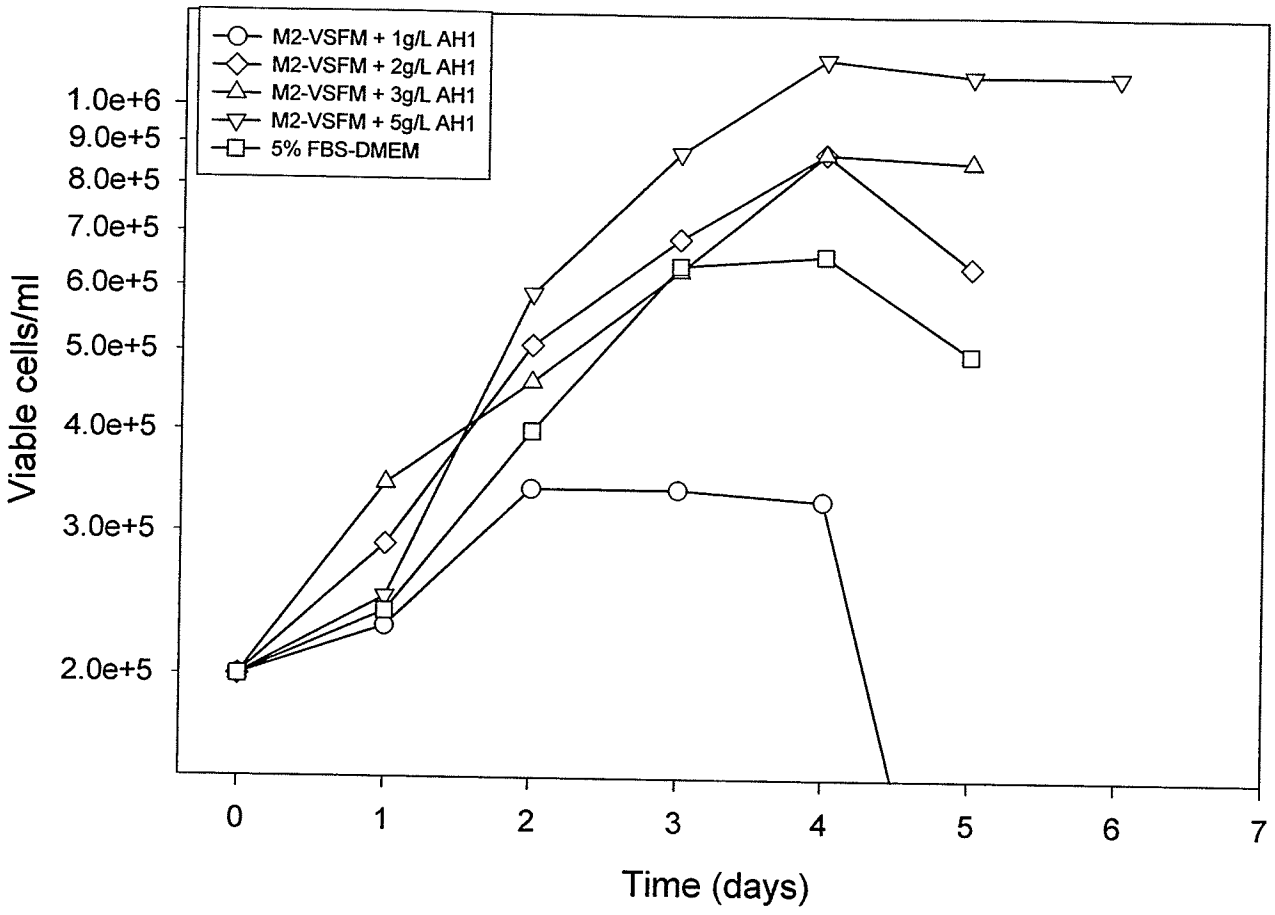


Figure 4.10.a. Growth of MRC-5 cells in M2-VSFM supplemented with AH1. The cells were grown in 100ml spinner flasks with 2g/L Cytodex-1 as microcarriers with an initial cell density of 2.0 x 10^5 cells/ml. The flasks were incubated at 37°C with a 10% CO_2 overlay at a spin rate of 40 rpm. Statistical evaluation was performed with a tailed paired *t*-test. The values between M2-VSFM 5.0 g/L AH1 and the rest of the cultures are statistically different with a $P<0.05$.

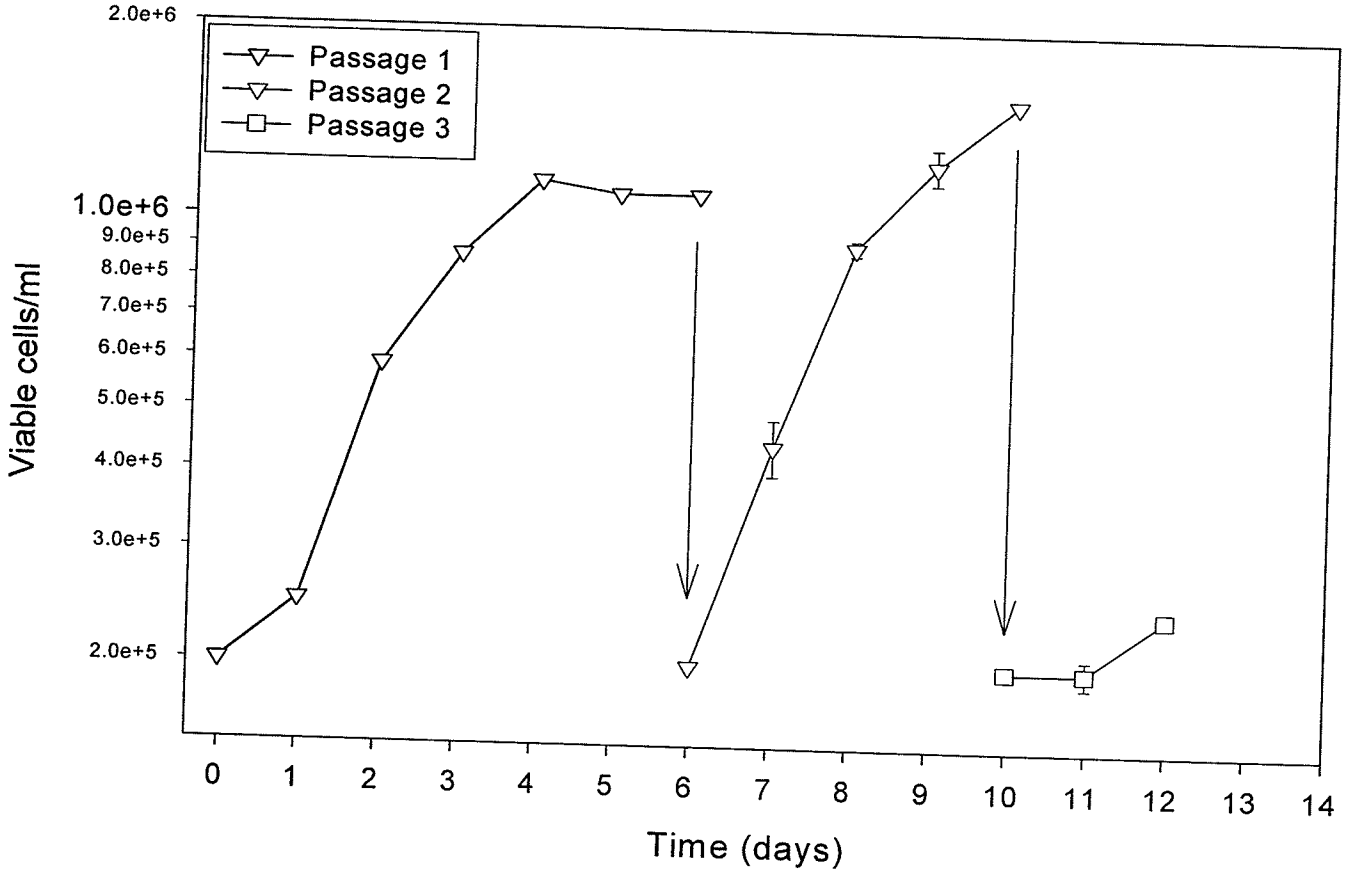


Figure 4.10.b. Growth of MRC-5 cells in M2-VSFM supplemented with 5 g/L AH1 over continuous passage. The cells were grown in 100ml spinner flasks supplemented with 2g/L Cytodex-1. The arrows indicate that the cells were harvested from the microcarriers and inoculated into a new flask with an initial cell density of 2.0 x 10^5 cells/ml. The error bars indicate the standard deviation of duplicate cultures (n=2).

4.2.6.1 Growth of L-929 cells in M2-VSFM in stationary culture

The initial experiment was designed to determine how long the L-929 cells required to adapt to M2-VSFM. The L-929 cells were maintained in a 5% FBS-MEM medium as recommended by the ATCC. They were then passaged into T-75 flasks with a starting cell density of 8.0 x 10^4 cells/ml in 25 ml of media. The media tested were either M2-VSFM or 5% FBS-MEM as the control. The final cell number after 5 days was determined by the Trypan Blue exclusion method described previously (2.4). The results of the first 4 passages are shown Table 4.4.

The L-929 did not require an adaptation period to M2-VSFM. The average doubling time for the cells in M2-VSFM was 27.1 $\pm$ 4.6 hours compared to the control medium, 5% FBS-MEM, with a doubling time of 25.1 $\pm$ 1.3 hours. The cells in M2-VSFM routinely reached high cell densities, as much as 4.0 to 5.0 x 10^7 cells/ml. The cells in M2-VSFM were typically larger (2x in diameter) to those that were grown in 5% FBS-MEM media (microscopy).

After 5 passages in each medium a growth experiment was conducted in the two formulations. The cells were inoculated into T-25 flasks with a starting density of 2.0 x 10^5 cells/ml in 10 ml of either M2-VSFM or 10% FBS-MEM media. The cells were counted each day using the Trypan Blue exclusion method described previously (2.4). The results are shown in Figure 4.11.

The cells in M2-VSFM had a longer lag time than those in 5% FBS-MEM (2 days instead compared to one). However, after 2 days the growth rates were comparable between both media with doubling times of 29.4 hours for M2-VSFM and 28.6 hours for 5% FBS-MEM. However, the cells in serum reached maximum cell density after only 4 days of growth (1.1 x 10^6 cells/ml) whereas in M2-VSFM the cells reached much higher numbers (2.27 x 10^6) by day 7. This may be attributed to the fact that DMEM is a much richer source of nutrients (amino acids, etc.) than MEM rather than the hormone/growth factor content.

Table 4.4. Growth of mouse L929 cells in M2-VSFM and 5% FBS-MEM

The L-929 cells were maintained in a 5% FBS-MEM medium. They were passaged into T-75 flasks with a starting cell density of 8.0 x 10^4 cells/ml in 25 ml of media. The final cell number after 5 days of growth was determined by the Trypan Blue exclusion method.

	Medium	
	M2-VSFM	5% FBS-MEM
Average doubling time (hours/gen)	27.1 ± 4.6[a]	25.1 ± 1.3

[a]Average doubling time taken from 4 passages out of serum

n=4

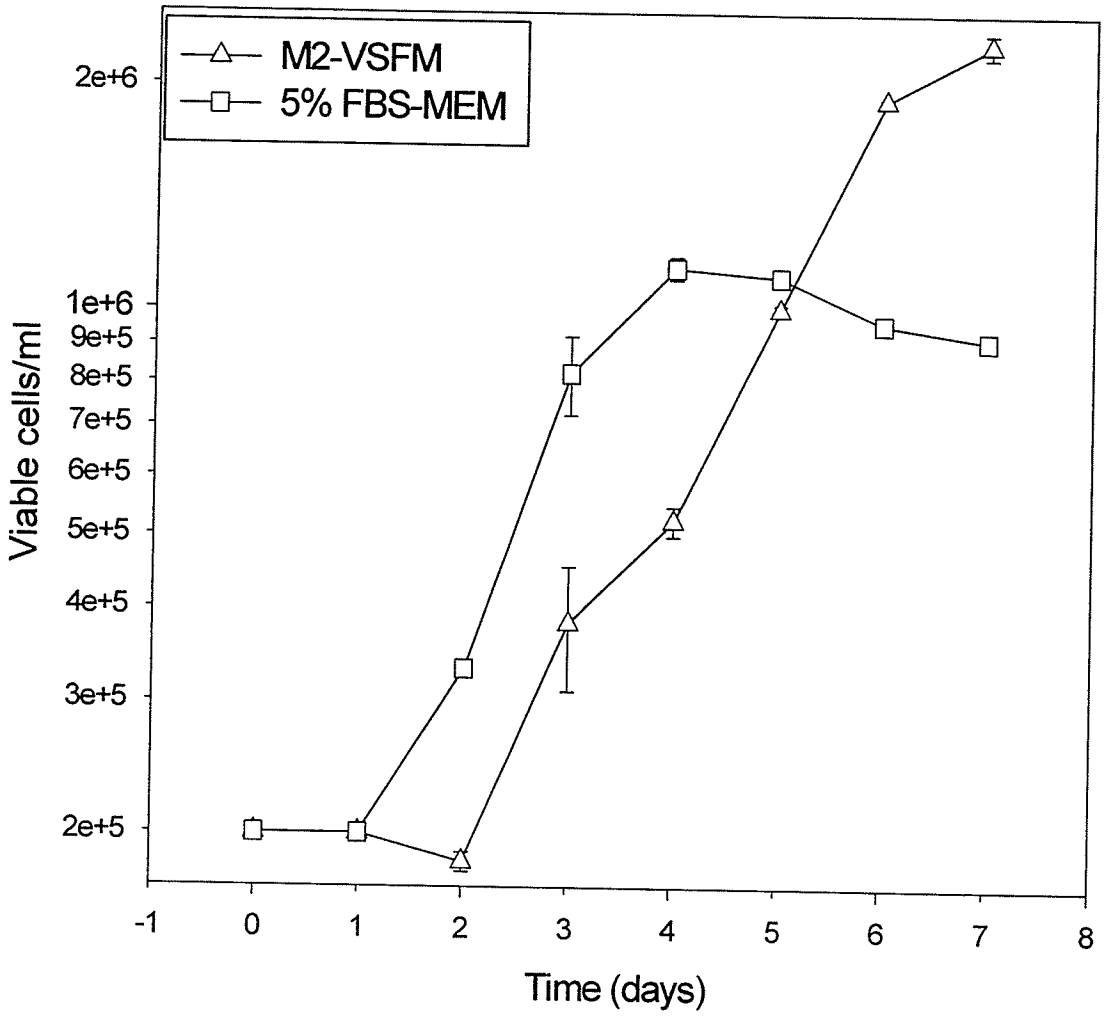


Figure 4.11. Growth of L-929 cells in M2-VSFM and 5% FBS-MEM in stationary culture. The cells were inoculated into T-25 flasks with a starting density of 2.0 x 10^5 cells/ml in 10 ml of either M2-VSFM or 10% FBS-MEM media. The cells were counted each day using the Trypan Blue exclusion method. Each data point represents duplicate cultures (N=2).

4.3 Discussion

The original formulation, M-VSFM, was not able to support the growth of BHK cells in stationary culture. The major problem with the medium is that the cells aggregated into large clusters (>200 cells). This resulted in very few cells attaching to the flask with the majority floating in the culture dish. The major concern with aggregation is the effect on nutrient uptake as the cells in the centre do not have as much access to nutrients as their counterparts on the surface. Indeed, the Trypan Blue stain revealed a significant percentage (>20%) of dead cells (data not shown). This phenomenon has been observed before in serum-free formulations (Cruz et al. 1998) but not with the degree of viability loss as in M-VSFM. Therefore this medium is not suitable for these cells in stationary culture.

To alleviate this problem the cells were grown on microcarriers. It was hypothesized that the microcarriers would provide a greater surface area for which the cells could attach. The culture was successful and the cells readily attached to the Cytodex-1 microcarriers, reaching densities > 6 x 10^5 cells/ml. There was no loss in viability as was seen with the stationary cultures (>95% viable). However, the final cell numbers were not very high and the control, serum-based medium, promoted the cells to densities almost three times as high (1.5 x 10^6 cells/ml).

Due to the poor performance of the medium an improved formulation was developed. M-VSFM was supplemented with EGF in an attempt to improve cell growth. This peptide growth factor had a significant impact on cell growth and yield, improving the final cell number by 66% with 4 units/ml of EGF. The response to EGF has been observed by other people in papers with BHK cells and is not unexpected as the EGF receptor is present on this cell line (Rozengurt et al. 1982; Neufeld et al. 1986). The medium with EGF was able to propagate the cells for successive passages with consistent growth rates and cell yields.

In a further attempt to increase cell growth a titration of the already present bFGF was carried out. However, neither doubling nor halving the FGF concentration (1.0 units/L control) had any effect on cell growth rate or final cell yield during the experiment. It must be noted, however, that a higher bFGF concentration may increase cell growth. An increase of that amount would result in a very expensive formulation (every μg costs ~$ 10 to $20 US) and thus makes it expensive for large-scale purposes.

AH1 had a significant impact on cell growth with the BHK cells. Where previously the cells would not culture well in T-flasks in M-VSFM, using M2-VSFM with AH1 allowed the cells to attach and divide successfully. Although there was still aggregation the cells maintained high viability (>95%). Furthermore the doubling times and final cell yields were reasonable, reaching >1.0 x 10^5 cells/cm^2 from 1.33 x 10^4 cells/cm^2 in 6 days. These results are promising and it is likely that the growth factors can be removed from the medium completely. However, further studies are needed to confirm this possibility.

The MDCK cell line was supported by M-VSFM. The cells would adapt quickly to the medium from serum-based formulations within only a few passages. They exhibited normal doubling times (~ 34 hours) during the first few passages. However, this success was short-lived and after ~ 4 passages the doubling time increased too much to be of any use (~200 hour doubling time). This was also the case in microcarrier cultures, where the cells remained viable (>95%) but had a doubling time of nearly 8 days, with normal or higher inoculum densities. Since the cells did not undergo apoptosis or cell death it is likely that the nutritional requirements are met for the cells. The lack of division is most likely due to the absence of a suitable mitogen.

The new formulation, M2-VSFM, was an improvement over the original. The addition of EGF had a profound effect on the cells' growth and acted as a potent mitogen. The doubling time increased to 39 hours but was still much lower than supplemented media (30 hours). There was also a long lag time of ~ 24 hours which was not seen in the control medium. However, the presence of EGF did dramatically effect the cell's growth and was maintained for greater than 10 passages. This is not surprising given the fact

that EGF has shown to be stimulatory for MDCK cells in culture (Bradshaw et al. 1983; Taub et al. 1984) since it expresses the EGF receptor (Rall et al. 1985).

The addition of the plant hydrolysate, PH1, dramatically increased the cellular growth rate and final cell yield with the MDCK cell line. With M2-VSFM + 2.0 g/L PH1, the doubling time and final cell yield were nearly identical to that of the control (serum-based) medium. This formulation was also superior to the only commercially available serum-free medium, UltraMDCK, in terms of final cell yield. Furthermore, the growth characteristics lasted for greater than 10 passages, much longer than the M-VSFM formula. Perhaps the hydrolysates meet the amino acid requirements of the cells that are not present in sufficient amounts in the DMEM. Indeed, many amino acids have shown to decrease rapidly in DMEM with this cell line, including methionine, glutamine, leucine, isoleucine, and valine (Butler et al. 1982).

The requirement of industry of an animal-component free serum-free medium is satisfied with this formulation (Merten 1999). Since it performs as well as serum-containing media it is more than suitable for large-scale production of human therapeutics, such as vaccines.

The MRC-5 cell line proved to be the most difficult of those tested to develop a serum-free formulation. The sheer lack of literature with regard to culture media formulations and stimulatory growth factors made the development an empirical exercise, for the most part. The original M-VSFM formulation could not propagate the cells in culture and cell death was quickly observed (data not shown). Microcarrier cultures had the same result where the cells would only remain viable for 2 days. A mixture of components known to promote the *survival* of MRC-5 cells in culture (Bettger et al. 1981), that were not present in M-VSFM, were added to make the MOD-VSFM formulation. This medium did enhance cell survival much longer than the original where cells remained <95% viable for 7 days.

It was hypothesized that the MOD-VSFM medium could be used as a last-step in a large-scale production process. The cells would be grown in a serum-medium on

microcarriers, allowed to grow to confluence, washed, and reconstituted into a serum-free formulation. This would only be a viable option if the medium could maintain the cells for at least 3 days as most viruses (being lytic or non-lytic) would require that amount of time to produce a suitable titer.

After growing the cells in a serum-based medium the MOD-VSFM maintained cell viability for ~3 days after the medium exchange. Similar results were seen with VSFM (199) + 2 units/ml EGF. Due to these results it is likely that the EGF alone is responsible for maintaining viability since it is a common component in both formulations.

Due to recent success with hydrolysates with the Vero, MDCK, and BHK cell lines a study with AH1 supplementation was attempted with the MRC-5 cells. The addition of 5.0 g/L in M2-VSFM successfully propagated the cells in microcarrier cultures. The final cell yield was dose-dependent and increased with higher AH1 concentrations (5.0 g/L being the highest). Furthermore, the medium outperformed the control medium (serum-based) with regard to final cell yield, reaching 1.15 x 10^6 cells/ml. The cells could be passaged 3 times in this medium before they began undergoing apoptosis. It is not known why the cells could not be passaged further but it is possibly due to the extensive trypsinization process required to remove the cells from microcarriers. Trypsin is damaging to cell membranes and this could have caused the decrease in viability.

The L-929 cells were much easier to culture than the anchorage-dependent cell lines. They did not require any adaptation time to M2-VSFM and had a normal doubling time of 27.1 ± 4.6 hours that fell within the range of the control medium, 5% FBS-MEM. The cells could be maintained in the medium for >10 passages without loss of growth rate. In addition, the cells reached much higher densities in M2-VSFM than in the control medium by over 200%. A possible explanation for the higher cell densities could be that the basal medium, DMEM, is richer in nutrients than MEM.

4.4 Conclusion

The major aim of this chapter was to adapt the M-VSFM formulation to suit other industrially-important cell lines. The cell lines tested were BHK, MDCK, and MRC-5. All of these cell lines are used in the manufacture of human or veterinary vaccines constituting a significant portion of the biotechnology industry. In all cases a serum-free formulation was developed for each cell line from the original M-VSFM formulation, although some only supported the growth for a finite number of passages. An adapted M2-VSFM formulation for the BHK (3 passages), MDCK (>10 passages), MRC-5 (2 passages), and L-929 (5 passages) cell lines were developed to support the growth of these cells, in some cases comparably to serum-supplemented controls. This makes it a potentially valuable formulation for the biopharmaceutical industry.

CHAPTER 5

Development of a Serum-free Culture System for the Production of Reovirus

5.1. Introduction

The mammalian reoviruses are members of the *Orthoreovirus* genus of the family *Reoviridae*. The *Reoviridae* include a number of important human and animal pathogens such as rotavirus (Kapikian et al. 1996) and orbivirus (Roy 1996). Rotavirus infection is estimated to be directly responsible for up to 2 million deaths each year, and despite intensive efforts (Lang et al. 1996), an effective vaccine for these agents remains elusive. Reovirus, the prototype of this family, remains an effective model for studying the characteristics of the double stranded RNA viruses (Tyler 2001).

Recent work from the University of Calgary, which gained a significant amount of international publicity, was the discovery that reovirus could be used as a cancer cure through its property as an oncolytic agent (Coffey et al. 1998). In the experiments the researchers showed sufficient tumour regression in animal models to suggest the possibilities of the development of a new type of anti-cancer agent which has been termed oncolytic. Presently reovirus is being tested (phase I and II clinical trials) to treat various forms of cancer including brain, prostate, and glioma (Oncolytics Biotech Inc.). It is also being tested in the treatment of breast cancer showing early success (Norman et al. 2002; Hirasawa et al. 2003). These findings suggest the need to produce large quantities of reovirus for human therapeutic use.

The virus is routinely grown in either monolayer or stirred cultures of mouse L929 fibroblasts (Nibert et al. 2001). However, it has also been reported that reovirus will grow in a number of other cell lines, including Vero and Chinese hamster ovary (Taber et al. 1976) (Davis et al., 1990).

Vero cells have been accepted for viral vaccine production under specified regulatory guidelines (WHO, 1987a,b) and are presently used in the commercial manufacture of human and animal rabies vaccine as well as human polio vaccine (el-Karamany 1987) (Shevitz et al. 1990; Beale 1992; Montagnon et al. 1999). Vero cells have been shown to be good substrates for the propagation of bovine vesicular stomatitis virus (Nikolai et al. 1992), 1992), herpes simplex virus (HSV) (Griffiths et al. 1981; Totte et al. 1993), and rabies virus (Mendonca et al.). These processes involve the culture of Vero cells on solid microcarriers (Cytodex-1) in large bioreactors up to 1,000 litres (Montagnon et al. 1981; Montagnon et al. 1984).

The production of Vero cells in a culture system for reovirus propagation has been studied previously in our laboratory (Berry et al. 1999). Microcarrier cultures (Cytodex-1 or Cultispher-G) in a serum-based medium were shown to successfully support the propagation of two serotypes of reovirus (type-1 Lang (T1L) and type-3 Dearing (T3D)).

However, serum-containing culture systems are becoming undesirable for the large-scale production of vaccines, as indicated in previous chapters. There are a number of disadvantages of serum supplementation including batch to batch variation in composition, the high protein content which hinders product purification and the potential for viral, mycoplasma or prion contamination. Furthermore, the recent threat to human health caused by the undefined agents of bovine spongiform encephalopathy (BSE) is likely to limit the continued use of bovine serum in culture processes used for the synthesis of health-care products such as viral vaccines.

We have recently developed a novel serum-free formulation (designated M-VSFM) for the growth of Vero and other anchorage-dependent mammalian cell lines. This formulation promoted the growth of Vero cells comparably to serum-supplemented media (Chapter 3). This provided an excellent opportunity to design a serum-free culture system to produce reovirus that would be suitable for therapeutic use. The characteristics of the medium (80 μg/L protein content, animal product free) would minimize the risk of introducing potentially harmful agents (such as prions or other viruses) into the final

product which is a concern with serum-supplemented cultures. This chapter investigates the characteristics of this serum-free medium in supporting the propagation of reovirus from Vero cells.

5.2 Results

5.2.1 Reovirus production in stationary cultures (data provided by Megan Patrick, 2000)

Reovirus strains type 1 Lang (T1L) and type 3 Dearing (T3D) was propagated in 6-well cluster dishes on monolayers of Vero cells grown in M-VSFM and 10% FBS-DMEM media (Fig 5.1.a, b). The Vero cells were grown to a density of 2.0 x 10^5 cells/cm^2 prior to infection with the virus. The propagation of virus was monitored in cultures infected with virus at different MOI's (multiplicities of infection of 0.5 and 5.0).

The production of the T1L strain is shown in Fig 5.1.a. In both media types an MOI of 5.0 PFU/cell gave a high yield of virus. Yields in M-VSFM reached a maximal titer of 1 x 10^9 PFU/ml after 5 days, and cells in 10% FBS-DMEM reached a maximal titer of 2.2 x 10^8 PFU/ml after 4 days. At an MOI of 0.5 PFU/cell M-VSFM supported a maximum yield of 1.6 x 10^8 PFU/ml (8 days), while 10% FBS-DMEM supported a maximum yield of 1.1 x 10^7 PFU/ml (3 days).

The propagation of the T3D strain is shown in Fig 5.1.b. Similar to the T1L strain, an MOI of 5.0 PFU/cell gave a high yield of virus. M-VSFM gave a maximum titer of 1.9 x 10^9 PFU/ml after 3 days, whereas 10% FBS-DMEM gave a maximum titer of 1.2 x 10^9 PFU/ml. At an MOI of 0.5 PFU/cell, M-VSFM supported a maximum yield of 1.5 x 10^9 PFU/ml after 4 days. At the same MOI, cells in 10% FBS-DMEM reached 0.9 x 10^9 PFU/ml in 2 days.

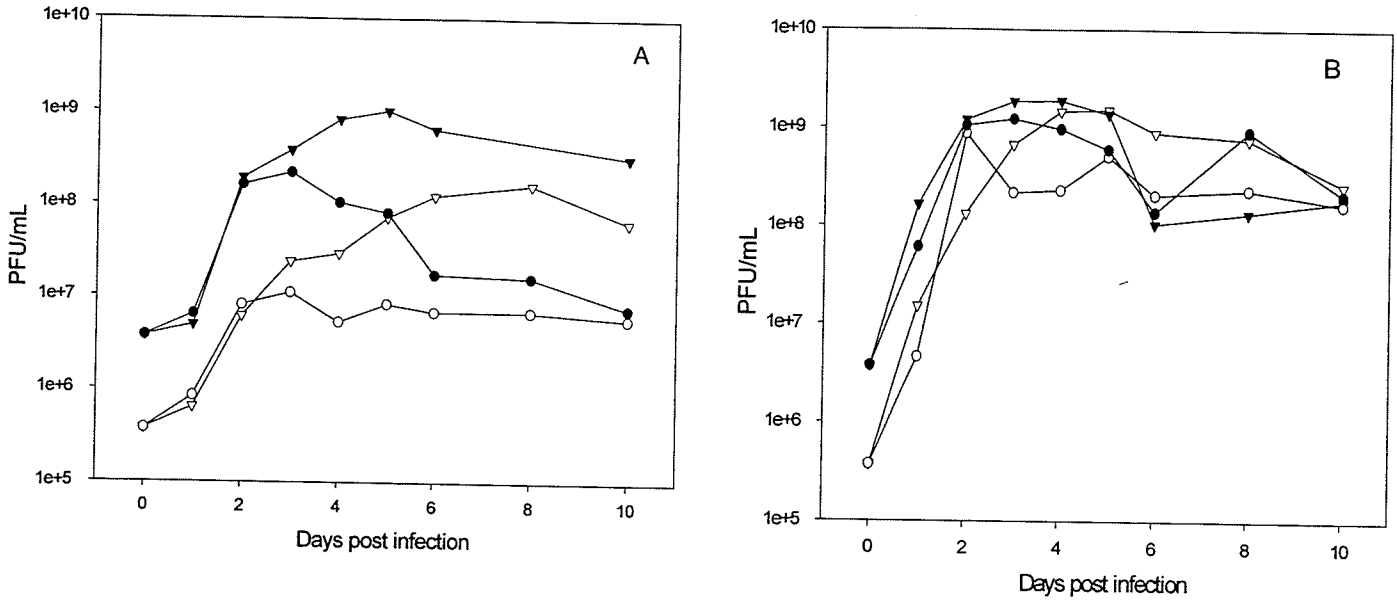


Figure 5.1. Reovirus production from Vero cells in M-VSFM and 10% FBS-DMEM in stationary culture. Reovirus strains (a) Type 1 Lang (T1L) virus or (b) Type 3 Dearing (T3D) virus were propagated in 6-well cluster dishes from monolayers of Vero cells grown in M-VSFM (▼,▽) or 10% FBS-DMEM media (●, ○). The cells were infected with an MOI of 0.5 PFU/cell (▽, ○) or 5.0 (▼, ●).

5.2.2 Reovirus production in agitated microcarrier cultures

The propagation of reovirus (T1L and T3D) is shown in Fig 5.2.a,b. Cells were grown in spinner flasks containing 2.0 g/L Cytodex-1 microcarriers. The flasks were inoculated with 2 x 10^5 cells/ml in both the M-VSFM and 10% FBS-DMEM cultures. The cells were infected with virus (Type 1 Lang and Type 3 Dearing) when the cells reached a density of 1.0 x 10^6 cells/ml (4 days). The cells were infected at an MOI of 5.0 PFU/cell with a stirring regime as outlined in the Materials and Methods section. The viral yield was monitored over a period of 9 days after infection.

Production of the T1L strain is shown in Fig 5.2.a. A maximum titer was reached within 2 days for cells grown in M-VSFM. The highest titer reached was 6.6 x 10^7 PFU/ml, and then steadily decreased with time. The cells grown in 10% FBS-DMEM gave a maximum titer of 3.8 x 10^7 PFU/ml, a little under one-half than that of M-VSFM.

A similar production pattern was observed for the T3D strain (Fig 5.2.b). Here, M-VSFM supported significantly higher titers of the virus, which reached a maximum yield of 1.1 x 10^9 PFU/ml after 2 days. Cells grown in 10% FBS-DMEM reached a titer of 6.1 x 10^8 PFU/ml in the same time period.

The final yield of the T3D strain (1.1 x 10^9 PFU/ml) was higher than the T1L strain (6.6 x 10^7 PFU/ml) from the cells grown in M-VSFM. This may have been due to the initial viability of the type 1 Lang sample used to infect the microcarrier cultures. This did not affect the growth profile, however, as the maximum titer for both strains was reached during the time when the cells were at their highest density of 1.0 x 10^6 cells/ml.

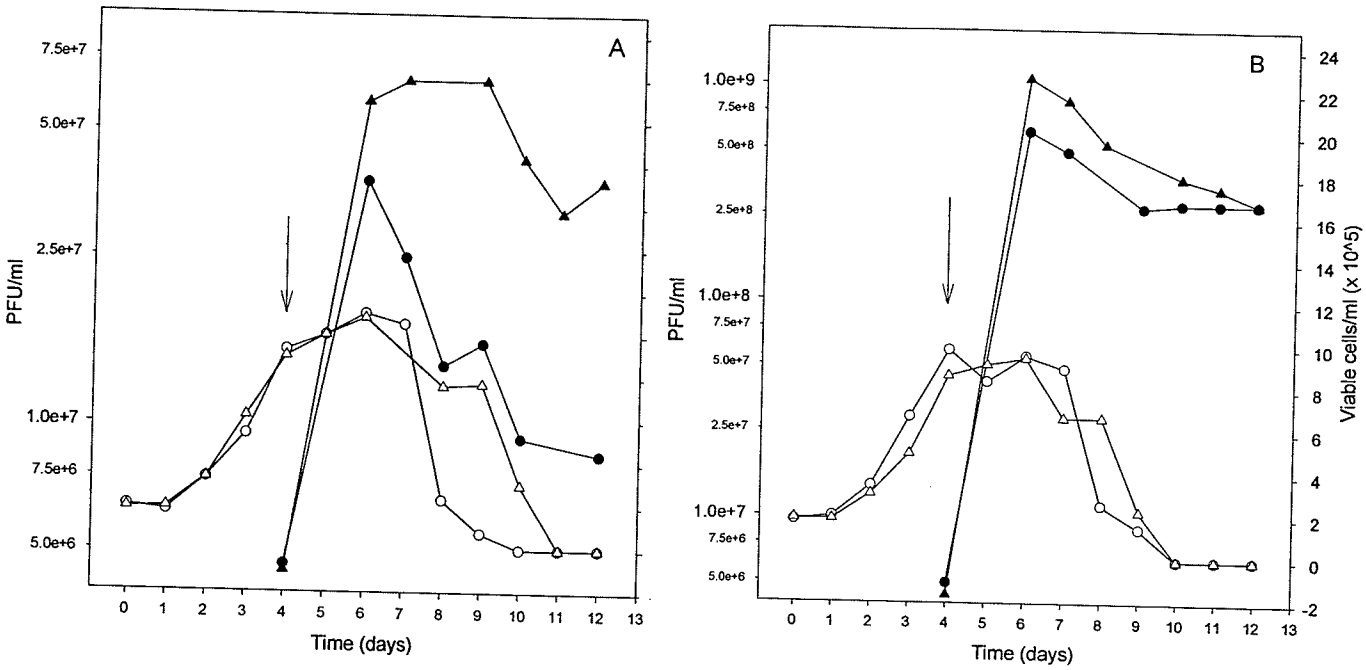


Figure 5.2. Reovirus production in microcarrier cultures of Vero cells in M-VSFM and 10% FBS-DMEM. The cells were grown in 100 ml spinner flasks incubated at 37°C with a 10% CO_2 overlay. Cytodex-1 microcarriers were added at a concentration of 2.0 g/L. The flasks were inoculated with 2 x 10^5 cells/ml in both the M-VSFM and 10% FBS-DMEM cultures. The cells were infected (indicated by arrow) with either (a) Type 1 Lang virus or (b) Type 3 Dearing virus when the cells reached confluence (4 days). The points show cell growth for M-VSFM (△) or 10% FBS-DMEM (○) and viral titer for M-VSFM (▲) or 10% FBS-DMEM (●).

5.2.3 Infectivity of reovirus in M-VSFM and FBS-DMEM cultures

The initial viral uptake by the cells was determined in both stationary and microcarrier cultures containing either M-VSFM or serum-supplemented medium (Table 5.1). After viral infection of the cultures, samples of culture supernatant were withdrawn after 1 hour and the extracellular virus titer was measured. This revealed significant differences between the serum-free and serum-supplemented cultures.

For stationary cultures containing M-VSFM and infected with the T1L strain, >97% of the extracellular virus decreased during the first hour in M-VSFM. However, cultures containing FBS-DMEM did not show that high of uptake rates and only reached an 85% decrease in the extracellular viral titer during this period. For the T3D strain, the extracellular viral titer decreased by 98% during this period in M-VSFM whereas only 68% was taken up by the cells in FBS-DMEM.

Similar results were seen in the microcarrier cultures. For the T1L strain the extracellular titer decreased by >50% in M-VSFM during the this post-infection period and only 14% in FBS-DMEM. For the T3D strain, the extracellular titer decreased by 50% during the first hour in M-VSFM, but only by 20% in FBS-DMEM.

5.2.4 Comparison of specific virus yields between M-VSFM and FBS-DMEM

Table 5.2 shows the specific productivity (PFU/ cell per day) of either strain of reovirus, T1L and T3D, in both types of media. The values were determined at the peak of productivity during the first 2 days post-infection and the total PFU/cell reached in this time was divided by time period. The viral productivity was significantly higher in cultures containing M-VSFM.. For the T1L strain, M-VSFM resulted in a 173% increase in viral productivity per cell compared to cells grown in FBS-DMEM. Similarly, for the T3D strain, cells in M-VSFM were 175% more productive in generating the virus.

Table 5.1. Comparison of virus uptake in M-VSFM and FBS-DMEM during the initial infection period.

Samples of the culture supernatant were taken after 1 hour of infection to determine the extracellular virus titer.

Reovirus Serotype	Culture type	MOI	M-VSFM Virus titer (PFU/ml)		FBS-DMEM Virus titer (PFU/ml)	
			Initial	After 1 hour	Initial	After 1 hour
T1L	Stationary	0.5	3.8 x 10^5	1.1 x 10^4	3.8 x 10^5	5.0 x 10^4
		5.0	3.8 x 10^6	9.8 x 10^4	3.8 x 10^6	6.3 x 10^5
	Microcarrier	5.0	5.0 x 10^6	2.3 x 10^6	5.0 x 10^6	4.3 x 10^6
T3D	Stationary	0.5	3.8 x 10^5	7.3 x 10^3	3.8 x 10^5	1.2 x 10^5
		5.0	3.8 x 10^6	7.5 x 10^4	3.8 x 10^6	1.2 x 10^6
	Microcarrier	5.0	5.0 x 10^6	2.5 x 10^6	5.0 x 10^6	4.0 x 10^6

MOI = Multiplicity of infection, PFU = Plaque forming units

Table 5.2. Comparison of specific productivity of Reovirus (T1L and T3D) from Vero cells grown in microcarrier cultures with M-VSFM or FBS-DMEM.

The values were taken from the first 2 days post infection, when viral productivity was at a maximum.

Media	T1L Specific productivity PFU/cell·day	T3D Specific productivity PFU/cell·day
M-VSFM	28.5	584
FBS-DMEM	16.1	364

5.2.5 A comparison between the production of reovirus from L-929 and Vero cells

The current method to produce reovirus in the research industry is to grow it from monolayer or stirred cultures of mouse L929 fibroblasts (Nibert et al. 1996) in a serum-based medium. A recent article has studied the production of reovirus in mouse L-929 cells as a prototype for a large-scale system for human therapeutic use (Jung et al. 2004). The cells were grown in a perfusion culture yielding >4.5 x 10^9 PFU/ml of the virus in a serum-based medium.

However there are many concerns surrounding this type of technology for production. The L929 cell line has not been accepted for production of health care therapeutics under specified regulatory guidelines. Furthermore, the continued use of serum in these processes poses a threat to humans by the possibility of transmitting contaminants or undefined agents (prions causing spongiform encephalopathies).

The purpose of this study was to compare the productivity between L-929 cells in a serum-based medium to Vero cells in M2-VSFM to produce reovirus (Type 3 Dearing).

5.2.5.1 Results

L929 cells were grown in suspension culture in 5% FBS-MEM in spinner flasks (500 ml). When the cells reached ~ 5.0 x 10^5 cells/ml the cells were pelleted. The pellet was resuspended in a small amount of medium (50 ml) and infected with reovirus type-3 Dearing at an MOI of 5.0 PFU/cell. The cells were gently swirled every 10 minutes over an hour period and then resuspended in 500ml of fresh media.

The Vero cells were grown in 500ml spinner flasks (500ml media volume) in M2-VSFM with 1.0 g/L Cytodex-1 microcarrier. When the cells reached confluence they were infected at an MOI described in materials in methods (2.8.1). The results are shown in Figure 5.3.

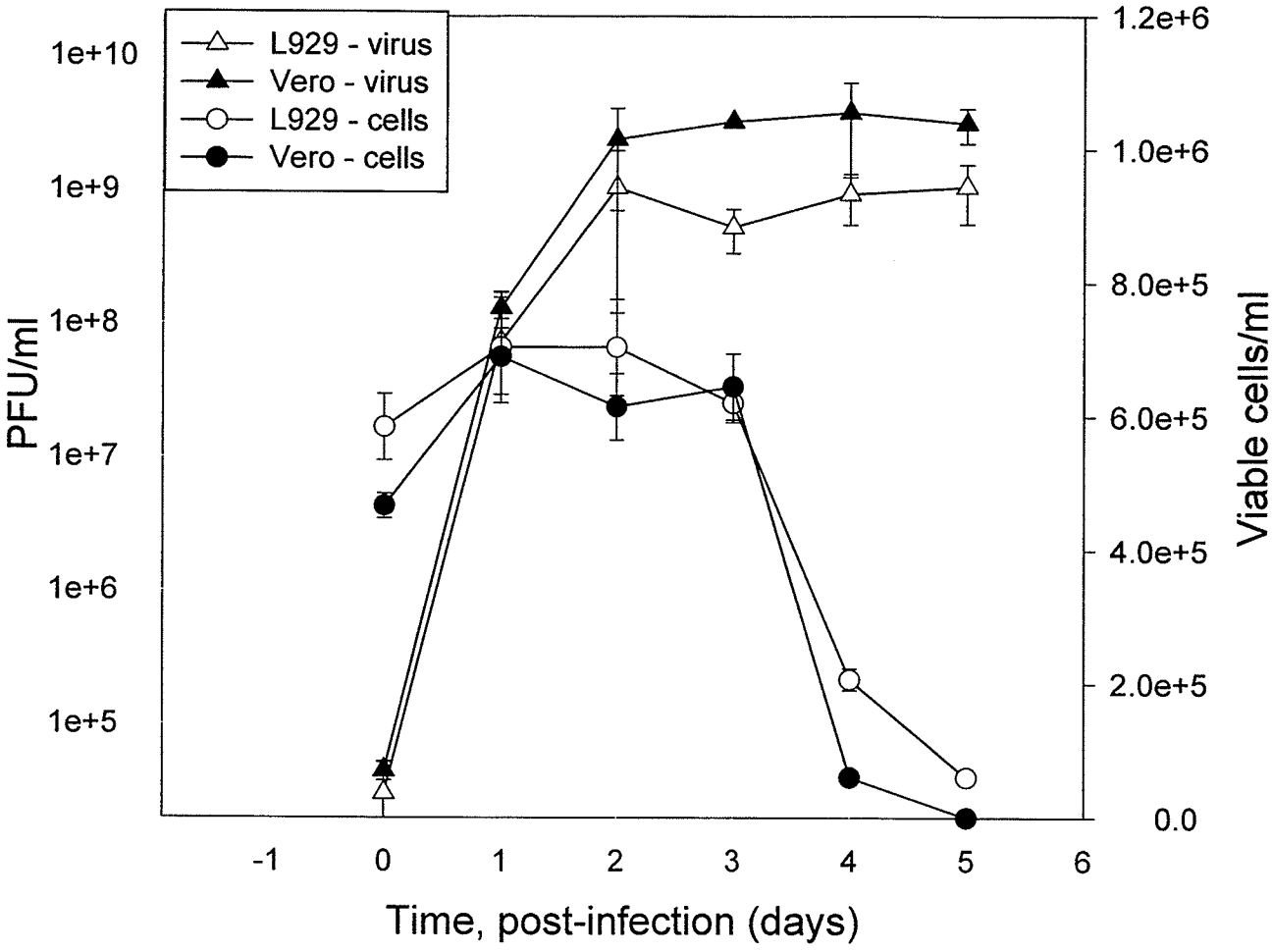


Figure 5.3. Production of reovirus Type-3 Dearing from two different agitated cell culture systems: mouse L929 vs. Vero cells. The L-929 cells were grown in spinner flasks (500ml) in 5% FBS-MEM. The Vero cells were grown in spinner flasks (500ml) supplemented with 2.0 g/L Cytodex-1 as a microcarrier. Once the cells reached ~ 5.0 x 10^5 cells/ml (mid-exponential) the cells were infected with reovirus type-3 Dearing at an MOI of 5.0 PFU/cell. Statistical analysis (paired *t*-test) reveals that the virus production profiles are significantly higher in the Vero system than in the L-929 system ($P<0.05$).

The Vero cell system produced reovirus with comparable yields to the standard L929 cell system (Table 5.3). The Vero cell model in M2-VSFM was shown to have a higher productivity than the L929 system. In the Vero system, titers of > 5.0 x 10^9 PFU (plaque forming units)/ml were reached, whereas the L929 system reached titers of only 1.5 x 10^9 PFU/ml. The specific productivity (Table 5.3) in the Vero system was 1938 PFU/cell•day compared to 740 PFU/cell•day in the L929 system.

5.3 The effect of serum of reovirus uptake in Vero cells

The results observed from earlier experiments (section 5.2.3) indicate that virus production and virus uptake is enhanced in M-VSFM over 10% FBS-DMEM. It was hypothesized that the serum-medium inhibited uptake and/or production of the virus in the culture. The presence of serum proteins has been shown to have an inhibitory effect on adsorption kinetics of other viruses, such as Vaccinia and respiratory syncytial virus (Chillakuru et al. 1991; Fassi Fihri et al. 1993). This study attempts to determine what effect the medium has on these two factors.

5.3.1 The effect of the culture media upon virus uptake

The viral uptake by the cells was monitored under different medium conditions. Vero cells (CCL-81) were grown in 6-well cluster plates. Each well was inoculated with 1.0 x 10^5 cells/ml (3 ml total) in either 10% FBS-DMEM or M2-VSFM to initiate the cultures. The cells were fed with 1.5 ml of fresh media on the 2nd day. On the 3rd day the medium was removed and the cells were washed with 1ml of fresh PBS. Fresh medium (1ml) was then added to each well and then virus was added at an MOI of 5.0 PFU/cell (reovirus type-3 Dearing). The cells were then placed at 37°C for 1 hour during the infection period. At each time point (T=0, 15, 30, 45, and 60 minutes) a plate was removed. The medium was aspirated from each well, the cells washed with 1 ml of PBS and then overlayed with 3 ml of fresh medium. The plate was then immediately stored at -60°C for virus titering.

Table 5.3. A comparison of the specific productivity of reovirus (T3D) from agitated cultures of Vero and mouse L-929 cells.

The Vero cells were grown in 500ml spinner flasks (duplicate) with 1 g/L Cytodex-1 microcarriers in M-VSFM media. The mouse-L929 cells were grown in 500ml spinner cultures (suspension) in 5% FBS-MEM media. The productivity was calculated during the first 2 days post-infection when virus production (per cell) was occurring at a maximum.

	Culture type	
	Vero	L-929
Specific productivity (PFU/cell·day)	1938	740

Figure 5.4 shows the virus uptake profile over the 1 hour time period under each of the media conditions (V=m2-VSFM, S=10% FBS-DMEM). The PFU/cell represents the intracellular titer. The first letter represents the medium of which the cells were grown and the second letter is the medium of which they were infected. The results are in triplicate (n=3).

When serum was present during the infection the uptake rate is increased. Cultures grown in serum-free media uptake virus more readily when serum is present during the infection (VS) than when it is not (VV). The opposite effect is observed (lower uptake) when cells grown in serum are infected under serum-free conditions (SS and SV). This would suggest that a physiological change has happened within the cell to facilitate the greater uptake rates.

It is possible that the serum is modifying the surface of the plastic wells. This may effectively "coat" the surface with non-specific protein which could prevent/reduce the amount of virus adsorption to the plastic. This may be a possible explanation for the increase in uptake rates with serum present.

5.3.2 The effect of the culture media upon the production of virus

Questions remained with respect to the medium type on virus production. Does it matter which medium the cells were grown in prior to the infection? Does this affect the overall virus titer or not at all? What effect does the medium have during the infection phase on the final viral titer? Is the type of medium important during the production phase and does this impact virus production? Therefore the purpose of this experiment was to determine what effect the medium has on virus production.

The time points of interest were pre-infection, during infection, and post-infection. A set of 6 conditions were used- VVV, VSS, VSV, SSS, SVV, SVS. The first letter is the

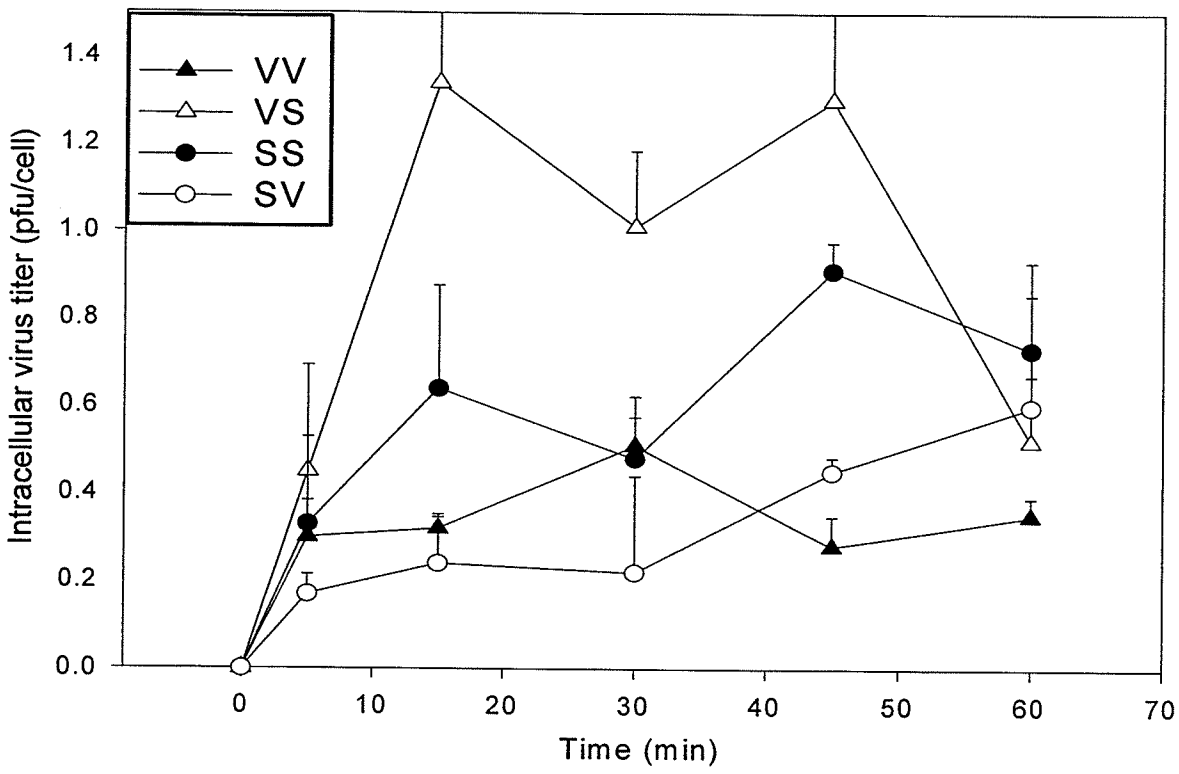


Figure 5.4. The influence of medium type on the specific uptake of reovirus (type-3 Dearing) into Vero cells. Cells were grown in 6-well cluster dishes and infected with reovirus at an MOI of 5.0 PFU/cell. The first letter represents the medium of which the cells were grown and the second letter is the medium under which the infection occurred (V = M2-VSFM, S = 10% FBS-DMEM). Statistical analysis reveals that that each set of data is significantly different according to a paired *t*-test ($P<0.05$). n=3.

medium the cells were cultured in, the second letter the medium in which the infection took place, and the third the medium of which the production phase was carried out.

The cultures were set up as described in 5.3.1. After the cells had reached near confluence (90%) the cells were infected with reovirus type 3-Dearing in the presence of the 2nd medium. After the 1 hour infection period the cells were overlayed with 4 ml of the 3rd medium and incubated for a 4 day period. Dishes were removed and frozen down at -60 °C for virus titering (2.9).

Figure 5.5 shows the production of virus in cells that were initially cultured in M2-VSFM. During the first 2 days of the infection there is no significant difference in the production profiles between the three conditions. However, by day 4 it is clear that cells *infected* in M2-VSFM had the highest PFU/cell (312 $\pm$ 11) over the culture that was infected in 10% FBS-DMEM (VSV – 117 $\pm$ 7.9). Using 10% FBS-DMEM as the production medium enhanced the production of the virus output over cells using M2-VSFM (VSS – 140 $\pm$ 12 PFU/cell compared to VSV – 117 $\pm$ 7.9 PFU/cell)

Figure 5.6 shows the production profile of cells that were initially cultured in 10% FBS-DMEM. The cells that were infected in M2-VSFM (SVS – 264 $\pm$ 19 PFU/cell) did not have higher titers than cells that were infected in 10% FBS-DMEM (SSS – 276 $\pm$ 7.6 PFU/cell) during the 4 day period. However, using M2-VSFM as the production medium did enhance the viral output from the cells (SVV - 310 $\pm$ 29 PFU/cell compared to SVS – 264 $\pm$ 19 PFU/cell).

These results suggest that whenever the *infection medium* was changed for the infection process the virus output was lowered. Changing the medium thus has an impact on the physiological state of the cell which is affecting virus production. On the other hand, whenever the M2-VSFM was the *production medium* the virus output was higher.

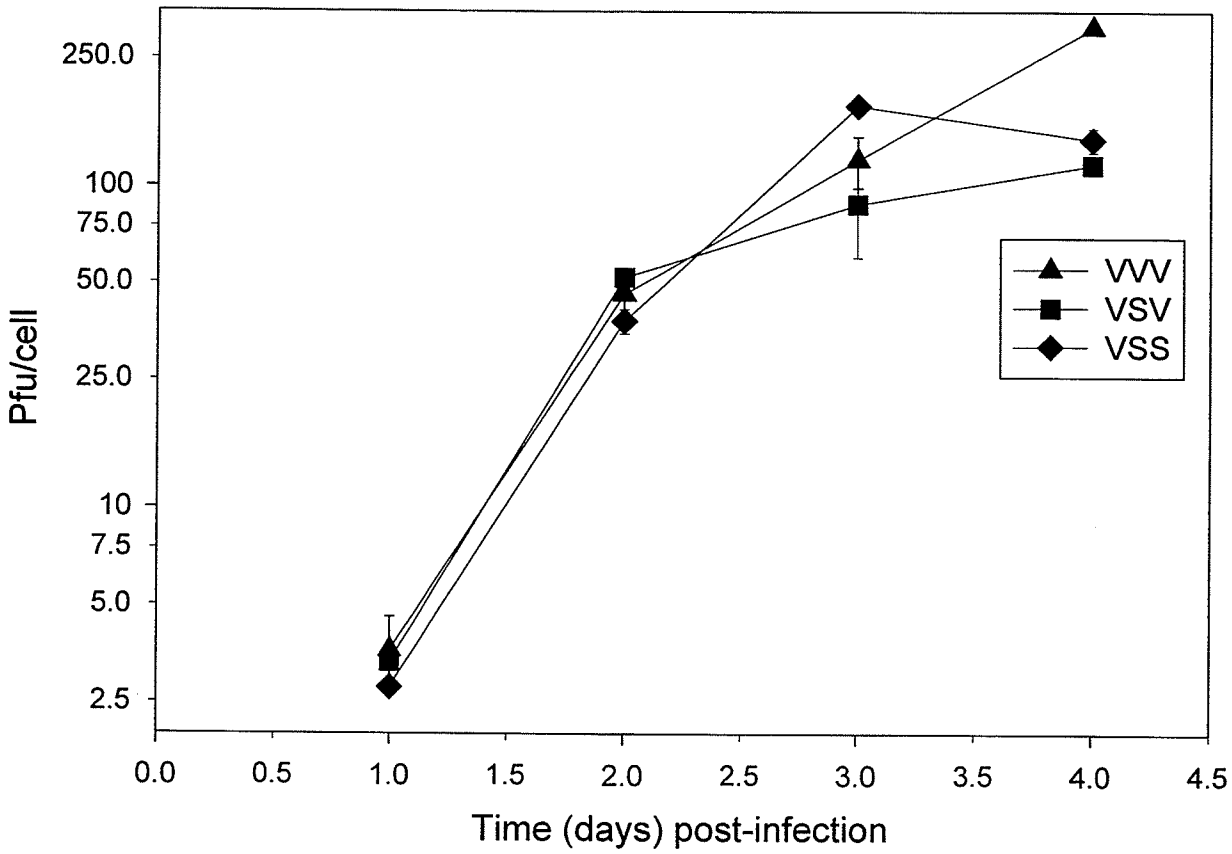


Figure 5.5. The effect of media upon the specific productivity of reovirus (type-3 Dearing) from Vero cells adapted to serum-free culture. Vero cells were grown in 6-well cluster plates. V=M-VSFM; S=10% FBS-DMEM. The first letter represents the growth medium; the second letter the medium used during infection; and the third the medium used during the virus multiplication state. n=3.

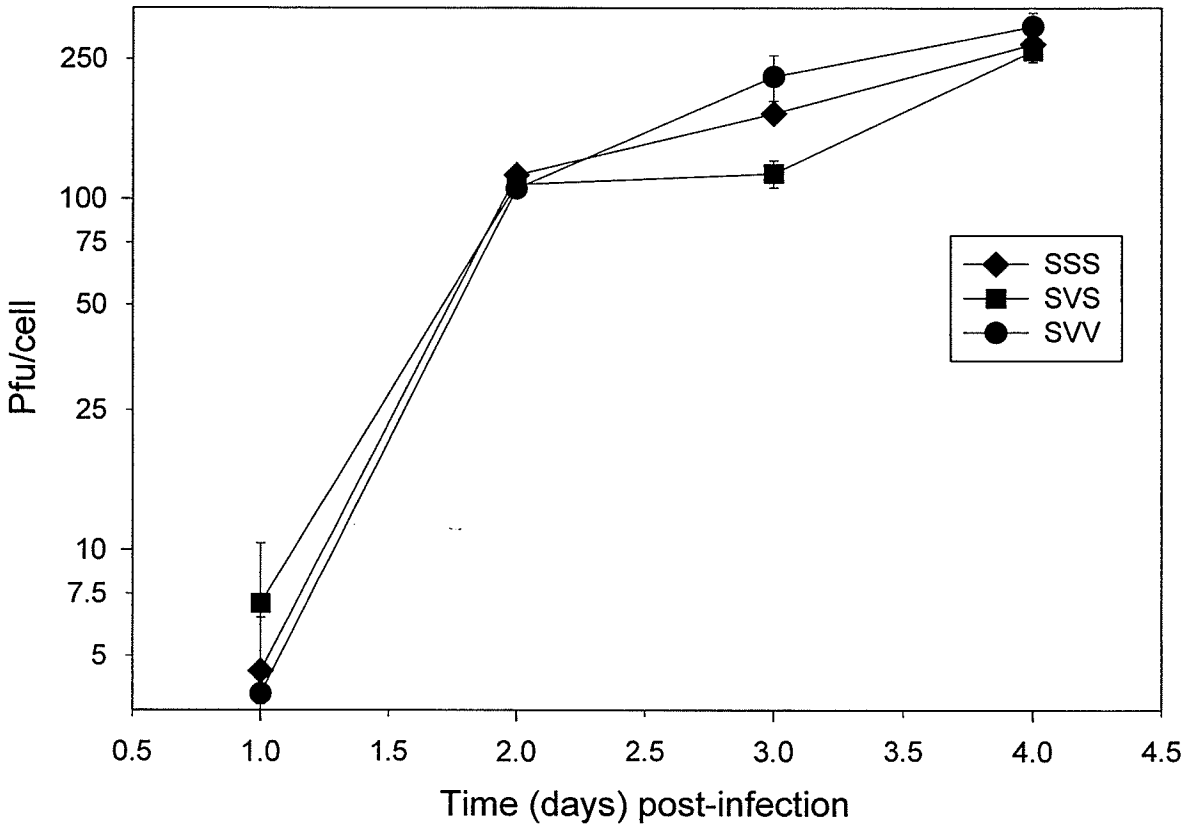


Figure 5.6. The effect of media upon the specific productivity of reovirus (type-3 Dearing) from Vero cells adapted to serum-based formulations. Vero cells were grown in 6-well cluster plates. V=M-VSFM; S=10% FBS-DMEM. The first letter represents the growth medium; the second letter the medium used during infection; and the third the medium used during the virus multiplication state. n=3

5.4 Discussion

There was a clear difference when M-VSFM was used in the specific productivity of the virus in agitated culture. For the two strains a substantial increase in viral productivity was found over the serum-containing system. Cells in M-VSFM produced >170% more virus over cells grown with serum. This suggests that the cells in M-VSFM may be in a metabolic state more favorable for viral infection than in the serum-supplemented medium. Indeed, serum contains many undefined components that could hinder the infectivity of the virus or possibly enhance the cytopathic effects mediated by the virus. This is consistent with our observations of a sharper decline in the cell viability in the serum-containing cultures.

Infectivity of reovirus seems to be hindered by the presence of serum. The rate of uptake of virus into the cells is higher in M-VSFM than in traditional serum media. The high protein content in serum may inhibit the infection of the virus, or by some other means. This further supports the advantage of serum-free formulations for these types of processes.

The stability of reovirus was higher in M-VSFM than in FBS-DMEM. In the microcarrier cultures, for both the T3D and T1L strain, a sharper decline in the viral titer was observed in FBS-DMEM. This would suggest that serum contains viral inhibitors, such as proteases, that decrease the stability of the virus. This further supports a conclusion that M-VSFM is better suited for a large-scale process.

The overall yield of the two strains was significantly different in the microcarrier cultures. The maximum yield of the type 1 Lang was only 6.6 x 10^7 PFU/ml, whereas the type 3 Dearing strain reached titers of 1.1 x 10^9 PFU/ml. It is not known why there is a discrepancy in the overall production yields. It is possible that the T1L virus sample used for this experiment was "weak" viruses and therefore was not as infectious. High viral titers have been achieved with the T1L strain in microcarrier cultures of Vero cells previously in our lab (Berry et al, 1999).

The comparison between the L929 cell system and the Vero system for the production of reovirus revealed that the Vero system was superior for the conditions tested. The specific productivity in the Vero cells was 260% higher than in the L929 cells. Other reports have shown that Vero cells routinely produce more virus than other cell lines (Wu et al. 2004). It is also likely that the medium has an effect on productivity as higher viral titers are seen in serum-free cultures, both with our experience (5.2.1 and 5.2.2) and in the literature (Merten et al. 1994).

The type of medium used for virus production revealed that virus uptake is enhanced in 10% FBS-DMEM over that in M2-VSFM. It is not known why virus uptake was enhanced in the serum-based medium. It is possible that the addition of serum to the serum-free culture contained growth factors not present in the medium, thus giving the cells a temporary "boost" to their metabolism, leading to increased ATP production. Even though there is a clear relationship between intracellular ATP concentration and virus production (Chapter 7) a correlation could not be reached since the nucleotide complements were not determined in these experiments.

The fact that virus uptake was enhanced in the presence of serum was contradictory to the findings with the microcarrier cultures (Table 5.1). In this case the cultures in serum had lower uptake of virus. It is possible that the mode of culture had an impact on the results and this may explain the apparent contradiction.

Virus production was enhanced when M2-VSFM was used during the virus multiplication stage. It is possible that the overall metabolism of the cells was higher in the M2-VSFM as cells generally do have higher nutrient uptake rates in serum-free culture (Neermann et al. 1996). The phenomenon of seeing increased viral titers when the production medium is changed to serum-free has been observed before (Frazzati-Gallina et al. 2001). In this case the cells were grown in the presence of low serum (1%) and then switched to serum-free during the virus multiplication phase. This resulted in higher titers than using the serum-medium throughout.

5.5 Conclusion

We have shown that two types of reovirus can be propagated from our serum-free medium M-VSFM in both stationary and agitated culture. The final viral yield was higher in both types of culture systems over the yield obtained from cells grown in serum-supplemented medium. Furthermore, the productivity in M-VSFM is superior to serum-supplemented cultures. This system would be suitable for the development of a bioprocess for the production of reovirus as a human therapeutic.

CHAPTER 6

Virus productivity in different growth states and the energy drain of virus infection upon the host cell

6.1. Introduction

The general practice when conducting in vitro viral infections is to ensure that the cells are infected when they are in a healthy metabolic state to ensure a maximum production of viral progeny. Cells that are actively growing (i.e.: exponential phase) are presumed to have a higher "metabolic state" than cells that are not growing (i.e.: stationary phase). It follows then that infecting cells during exponential phase will lead to more virus production. Indeed, this has been confirmed with infections of cells in different growth states (Mukhopadhyay et al. 1994). This has lead to a dogma that cells should be infected during the exponential phase as they are more "metabolically inclined" to produce virus.

However, is it possible that cells can be actively metabolizing but not actively dividing? Could cells in stationary growth phase have comparable energy levels to actively dividing cells? Stationary phase can be brought on by various events, such as nutrient depletion, toxic by-product accumulation, or contact inhibition. In the latter case the cells need not be metabolically inactive as long as there are sufficient nutrients in the medium. Would these cells then produce the same amount of virus if they had similar energy levels to actively growing cells?

There are many methods to assess energy levels of the cell. This can involve examining cell growth, glucose uptake, oxygen consumption, amino acid utilization, and lactate accumulation to name a few. However, these are the result of a pre-existing "energetic" condition that does not necessarily indicate the future potential of the cell. For example, cell growth is commonly associated with a 'high metabolic state". Although it may indicate conditions were optimal for cell growth preceeding the cell count it does not

necessarily mean that cell growth will continue in the future. Furthermore, these parameters do not necessarily indicate how well the system will produce a virus.

More accurate methods to determine the energy state involve monitoring intracellular nucleotides. As nucleotides are the driving forces, or "cellular currency" of the cell, they are excellent indicators of the overall energetic potential. Some commonly monitored nucleotide triphosphates include ATP, GTP, UTP and CTP. There are also nucleotide ratios which have been linked to physiological state. In the 1960's, Atkinson postulated that the adenylate energy charge (AEC) could be correlated to the physiological state of the cell according to the relationship [ATP] + 0.5 [ADP]/([ATP] + [ADP] + [AMP]) (Atkinson 1968). An AEC of > 0.8 has been correlated to actively growing cells, whereas an AEC of < 0.60 generally indicates the culture will enter stationary phase. This has been an accurate predictor of growth state and overall health of the cell.

The purpose of this chapter was to answer two major questions. The first is what the impact of growth state on virus productivity is and how does this correspond to the intracellular nucleotide levels during the infection? The second is how much energy does the virus require to fuel the production of progeny virus?

The intracellular nucleotide triphosphates (ATP, GTP, UTP, CTP), other nucleotides (ADP, AMP, UDP-glucose, NAD, and GDP), and the AEC were monitored during the course of these experiments as indicators of the cellular energy state.

6.2. Results

6.2.1. Growth state and virus production

Two sets of growth conditions were set up for an infection with reovirus. One set of Vero cells were grown until they had reached stationary phase while the other was cultured until mid-exponential phase. The cells were grown in 6-well cluster plates with 2.5 x 10^5 cells in 5ml of media (M2-VSFM) in duplicate. Each of the cultures was infected with reovirus type-3 Dearing at an MOI of 5.0 PFU/cell. The intracellular nucleotides, cell number, and virus titer were determined as described in the materials and methods (2.3.3, 2.4, 2.7.2, 2.9) and graphed. Figure 6.1.A shows the cell number; 6.1.B the intracellular [ATP], 6.1.C the total adenylate; 6.1.D the total PFU/ml; 8.1.E the specific virus production; 6.1.F the adenylate energy charge; and 6.1.G the adenylate profile during the course of the infection.

The first observation was that the ATP profiles are considerably different during the infection (Figure 6.1.B). The initial [ATP] for both cultures was similar, 4.71 $\pm$ 0.1 fmoles/cell for the exponential phase and 4.57 $\pm$ 0.36 fmoles/cell for the stationary phase culture. After this point, however, the cells in exponential phase had a lower [ATP] than the cells in stationary phase, likely because the actively growing cells were utilizing ATP to promote cell growth processes. The stationary phase culture maintained a high [ATP] throughout this time as it was presumably still actively metabolizing but not dividing (Figure 6.1.A). This was also true for the total adenylate profiles (Figure 6.1.C). The concentration of adenylate in both cultures were nearly identical (5.32 and 5.35 respectively) at the time of infection, but were significantly different afterwards. The exponential culture maintained a lower level of adenylate (3.1 to 6.2 fmoles/cell) whereas the stationary culture had a higher range (6.6 to 8.3).

The total virus production (Figure 6.1.D) was comparable for both systems reaching ~ 4 x 10^7 PFU/ml after 3 days. The specific virus production for both systems was also comparable (Figure 6.1.E). The exponential culture reached 72 $\pm$ 11 PFU/cell on day 4

whereas the stationary culture reached 94 ± 19 PFU/cell after 5 days. The specific productivity overlapped for each day except on day 3 with an average of 39 ± 15 PFU/cell per day and 48 ± 6.8 PFU/cell per day in the stationary and exponential culture, respectively. Statistical analysis of the results using a paired *t*-test indicate that the difference is not significant (P = 0.23).

The AEC profile for these cultures is shown in Figure 6.1.F. The energy charge for the exponentially growing culture was slightly higher than the cells from the stationary phase, 0.93 ± 0.02 and 0.88 ± 0.01, respectively. After the infection the AEC of the growing culture dropped considerably as it is using ATP to fuel growth (Figure 6.1.G), down to 0.5 at day 2 post-infection. After this point the cell growth slowed down and the energy charge recovered to > 0.70. The stationary culture maintained a high energy charge throughout the infection period at a level > 0.85 since it was not utilizing ATP as much as the exponential phase culture (Figure 6.1.G).

The profiles of the other intracellular nucleotides, including NAD, UDP-glucose, CTP, UTP, GTP, and GDP are shown in Table 6.1. The [GTP] of both cultures was nearly identical at the time of infection having 2.45 ± 0.29 and 2.36 ± 0.10 for the stationary and exponential phase culture, respectively. After this point the [GTP] was considerably higher in the stationary culture than in the actively growing cells, presumably since it was being used to fuel cell growth.

The [UTP] of the stationary culture was considerably lower at the time of infection than the growing culture (2.07 ± 0.51 and 4.77 ± 0.83, respectively). However, the [UTP] was then lower in the growing culture for the next 3 days presumably since it was being used to fuel cell growth. The levels after day 4 in the stationary culture could not be determined.

The [NAD] of both cultures was nearly identical at the time of infection having 2.05 ± 0.13 and 1.99 ± 0.02 for the stationary and exponential phase culture, respectively. The

[NAD], similar to the [GTP] and [ATP] was considerably higher in the stationary phase culture during the course of the infection by almost 2-fold.

The [CTP] was higher in the growing cells for the majority of the first 3 days of the infection, especially at the time of infection (0.90 ± 0.02 compared to 0.17 ± 0.02). The [UDP-glucose] was higher in the exponential phase culture during the first 3 days (except day 1) and almost 4-fold higher at the time of infection (0.54 ± 0.09 compared to 0.14 ± 0.01). However, the [UDP-glucose] was maintained at a higher level in the stationary phase culture from day 3 to 5 PI.

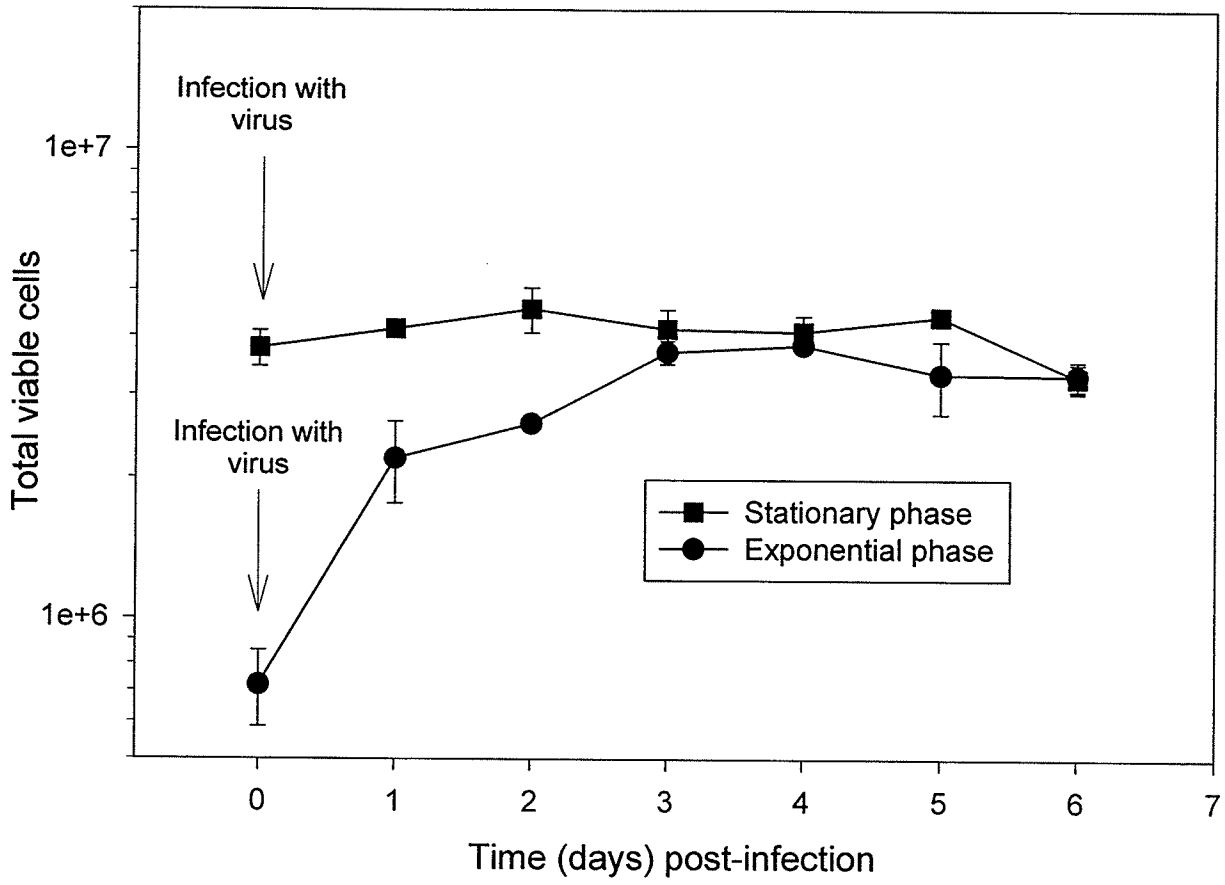
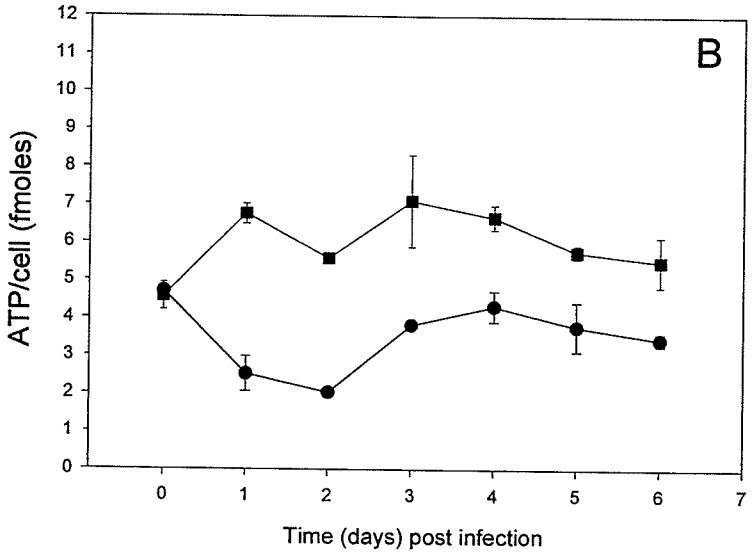


Figure 6.1.A. The cell growth profiles of the two infected cultures in different growth states. Vero cells were grown in 6-well cluster plates in a serum-free medium. The cultures were established and taken either from 5 days of growth (stationary phase) or 1 day of growth (mid-exponential phase) at which point they were infected (Time 0). They were then infected with reovirus type-3 Dearing at an MOI of 5.0 PFU/cell. Exponential phase, -●-; stationary phase, -■-. n=2.



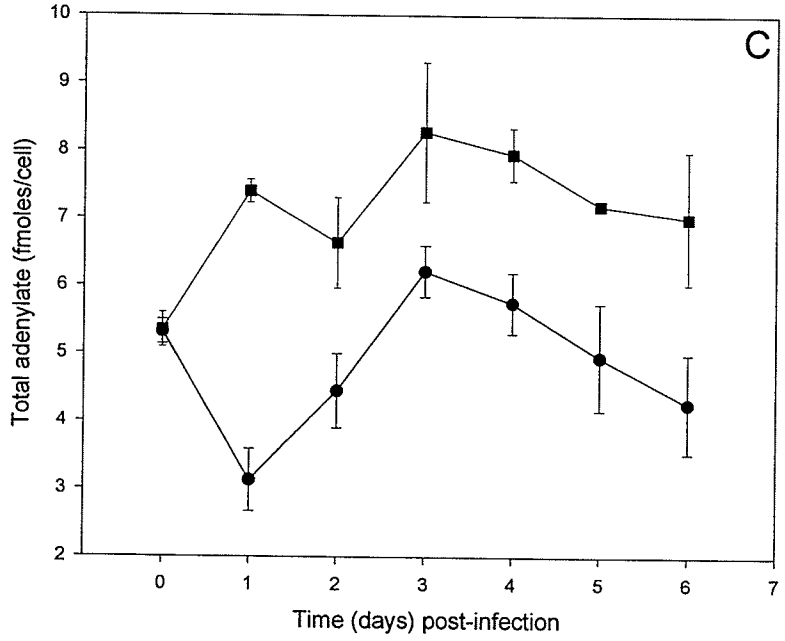


Figure 6.1.B and 6.1.C. The intracellular ATP (B) and total adenylate (C) concentrations of reovirus-infected cells in different growth phases. Vero cells were grown in 6-well cluster plates in a serum-free medium. The cells were then infected during either mid-exponential phase or stationary phase at an MOI = 5.0 PFU/cell with reovirus type 3-Dearing. Exponential phase, -●-; stationary phase, -■-. Statistical analysis was performed upon the above data sets using a paired *t*-test. The difference between the two data sets is significant ($P < 0.05$). n=2.

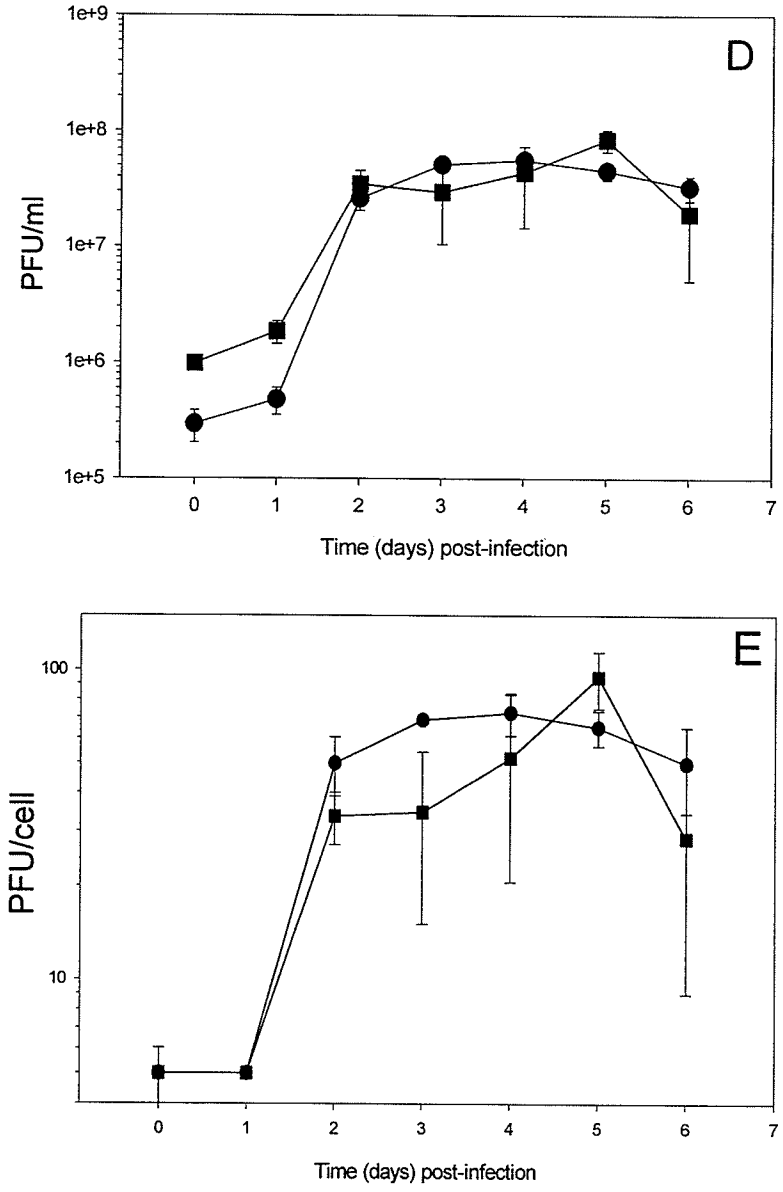


Figure 6.1.D and 6.1.E. The total (D) and specific (E) virus production profiles from cells in different growth phases. Vero cells were grown in 6-well cluster plates in a serum-free medium. The cells were then infected during either mid-exponential phase or stationary phase at an MOI = 5.0 PFU/cell with reovirus type 3-Dearing. Exponential phase, -●-; stationary phase, -■-. Statistical analysis was performed upon the above data sets using a paired *t*-test. The difference between the two data sets is not significant (P = 0.96) for total virus production and specific virus production (p=0.23). n=2.

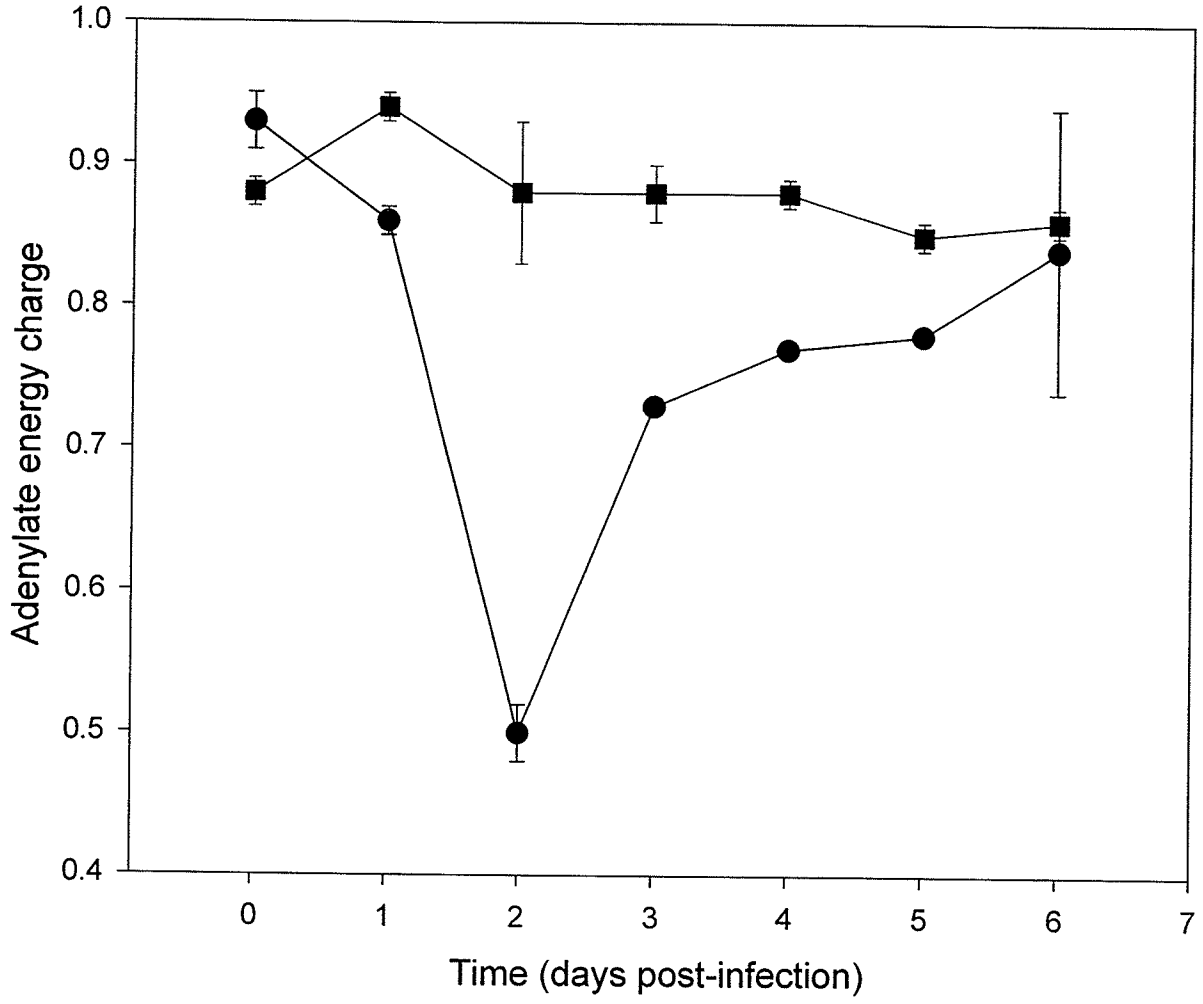


Figure 6.1.F. The adenylate energy charge profiles of infected cells in different growth states. Vero cells were grown in 6-well cluster plates in a serum-free medium. The cells were then infected during either mid-exponential phase or stationary phase at an MOI = 5.0 PFU/cell with reovirus type 3-Dearing. Exponential phase, -●-; stationary phase, -■-. n=2.

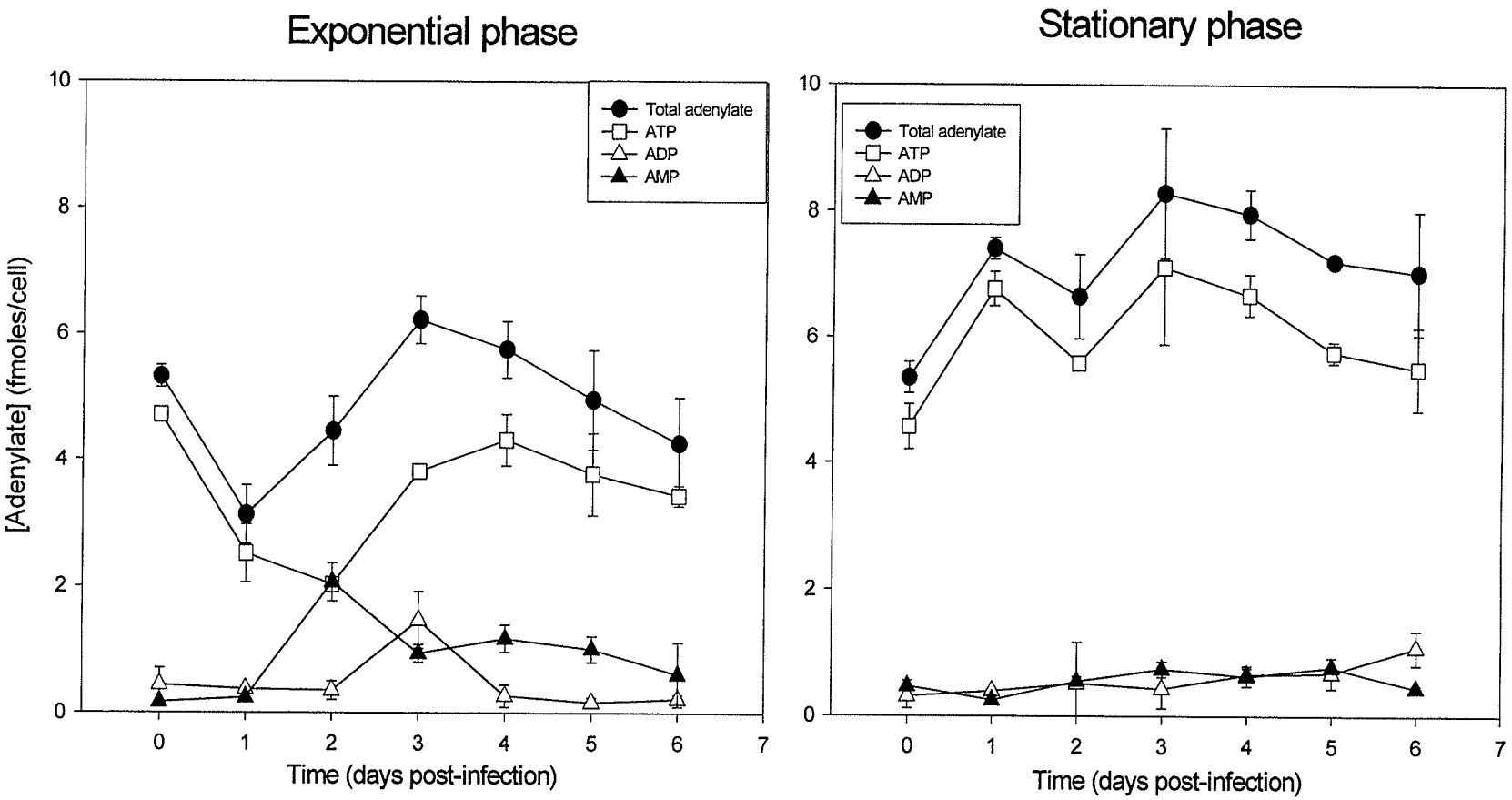


Figure 6.1.G. The adenylate profile of infected cells in different growth states. Vero cells were grown in 6-well cluster plates in a serum-free medium. The cells were then infected during either mid-exponential phase or stationary phase at an MOI = 5.0 PFU/cell with reovirus type 3-Dearing. n=2.

Table 6.1. Nucleotide levels of infected cells in different growth states

Stationary phase

Time (post-infection)	Intracellular nucleotide levels (fmoles/cell)					
	NAD	UDP-glucose	CTP	UTP	GTP	GDP
0	2.05 ± 0.13	0.14 ± 0.01	0.17 ± 0.02	2.07 ± 0.51	2.45 ± 0.29	0.13 ± 0.02
1	2.82 ± 0.02	0.60 ± 0.03	0.58 ± 0.04	3.76 ± 0.07	3.25 ± 0.12	0.17 ± 0.01
2	2.42 ± 0.05	0.17 ± 0.05	0.22 ± 0.00	3.23 ± 0.20	3.04 ± 0.06	0.16 ± 0.01
3	3.66 ± 1.04	0.22 ± 0.12	0.17 ± 0.02	3.49 ± 0.48	4.10 ± 0.80	0.25 ± 0.05
4	4.24 ± 0.01	0.29 ± 0.07	N/a	N/d	3.53 ± 0.12	0.23 ± 0.01
5	4.49 ± 0.04	0.45 ± 0.00	0.16 ± 0.00	N/d	2.84 ± 0.02	0.26 ± 0.04
6	4.53 ± 0.65	0.23 ± 0.00	0.24 ± 0.03	N/d	2.08 ± 0.17	0.27 ± 0.03

N/a – too low for detection
N/d – not determined
n=2

Exponential phase

Time (post-infection)	Intracellular nucleotide levels (fmoles/cell)					
	NAD	UDP-glucose	CTP	UTP	GTP	GDP
0	1.99 ± 0.02	0.54 ± 0.09	0.90 ± 0.02	4.77 ± 0.83	2.36 ± 0.10	0.07 ± 0.01
1	1.31 ± 0.21	0.60 ± 0.06	0.49 ± 0.10	3.20 ± 0.79	1.38 ± 0.29	0.13 ± 0.01
2	2.07 ± 0.26	0.44 ± 0.08	0.26 ± 0.00	3.11 ± 0.15	1.12 ± 0.05	0.27 ± 0.04
3	1.72 ± 0.06	0.17 ± 0.02	0.33 ± 0.02	0.80 ± 0.69	1.66 ± 0.08	0.17 ± 0.01
4	2.44 ± 0.25	0.09 ± 0.03	0.19 ± 0.03	1.88 ± 0.39	2.31 ± 0.24	0.22 ± 0.03
5	2.57 ± 0.43	0.17 ± 0.03	N/a	1.16 ± 0.67	2.19 ± 0.45	0.21 ± 0.03
6	2.54 ± 0.03	0.75 ± 0.83	0.16 ± 0.02	0.86 ± 0.67	1.48 ± 0.27	0.21 ± 0.01

N/a – too low for detection
n=2

6.2.2. The energy demand of a viral infection upon a cell

It has been reported that there are significant changes in the cellular metabolism upon a viral infection. Although the details can vary somewhat, the overall result is similar. Upon infection there is an initial drain on ATP in the cell as the virus quickly taps into the host protein producing machinery (Bardeletti 1977). This is followed by an increase in glucose uptake and oxidation which is presumably used to regenerate the amount of ATP to produce the virus (Bardell 1984; Chillakuru et al. 1991; Nadeau et al. 1996; Kim et al. 1999; Xie et al. 2002). In many of these cases an increase as much as 50 to 60% in glucose metabolism is seen in infected cells over that of uninfected cells. However, the effect that reovirus has upon the host cell's metabolism has not yet been studied. It is unclear how much of an impact it has upon the cells' system with respect to nucleotide levels and the overall growth state.

These experiments were designed to determine the energy drain that reovirus infection has on Vero cells. A mock-infected culture was performed alongside the infected culture while in exponential phase described in section 6.2.1. Both of the cultures of Vero cells were established in M2-VSFM and allowed to grow to mid-exponential phase in 6-well cluster plates (2.3.1). The medium was then aspirated and the cells infected with reovirus type-3 Dearing (2.8.1) or PBS for the mock-infected culture. The cell number, cell viability, and intracellular adenylate were measured during the course of the infection. For the purpose of direct comparison the data from section 6.2.1 of the exponentially growing culture and the mock-infected culture are shown together. The cell growth is shown in Figure 6.2.A, the AEC in Figure 6.2.B, the intracellular [ATP] in Figure 6.2.C, and the total adenylate in Figure 6.2.D.

The cell's viability remained comparable between the two cultures throughout the entire production period (Figure 6.2.A). Cell viability was >90% up until day 4 post-infection, then slowly decreased to 75% and 70% in the mock-infected and infected cultures, respectively, by day 6. The cell growth was comparable between the cultures until day 4

when the infected cultures showed an increase of non-viable cells due to the lytic nature of the virus.

During the course of the infection the demand of the virus upon the cell's metabolism could clearly be seen. The AEC remained at a slightly lower level in the infected cells compared to that of the mock-infected cells (Figure 6.2.B). The difference appeared 1 day post-infection and then became more pronounced with time. The mock-infected cells dropped to 0.6 after 2 days whereas the infected cells dropped to 0.5 in the same time period. The infected cells maintained an average AEC that is 0.09 lower (10%) than that of the mock-infected cells for the infection. This drop was most likely due to the demand on the ATP pool in the cell to fuel the production of viral progeny which was shown in Figure 6.2.C. The infected and mock-infected cells had an average of 3.42 fmoles/cell and 4.49 fmoles/cell, respectively. This corresponded to a 24% reduction in the total ATP in the cell when infected. The infected cells showed an increase in [ADP] and [AMP] which indicated that ATP was being used to fuel virus production (data not shown).

The total adenylate profiles are shown in Figure 6.2.D. The infected cells had a slightly lower amount of adenylate than the mock-infected cultures. The average total adenylate from day 0 to 6 was 4.88 fmoles/cell and 5.57 fmoles/cell for the infected and mock-infected cells, respectively, which corresponded to a 12% reduction in total adenylate. It is possible that either some of the adenylate was being degraded (salvage pathway) or *de novo* synthesis of adenylate was decreased. Since the products of the salvage pathway were not determined it is not possible to determine which the major contributor is. However, the results may not be statistically significant ($p=0.07$) and therefore should be repeated.

The levels of the other intracellular nucleotides are shown in Table 6.2. For the most part, the levels of the nucleotides measured remained comparable until 2 days post-infection. After this point all of the nucleotides in the infected culture decreased with respect to the mock-infected culture. The [NAD] was lower by ~ 30% during day 2 and

day 3 post-infection in the infected culture at which point they raised to levels similar to that of the mock-infected culture (days 4-6). The UDP-glucose levels saw a sharp increase (2-fold) in the infected culture 1 day post-infection, but then maintained a lower level throughout the rest of the infection. In contrast, the CTP concentrations were comparable between the two cultures and were not significantly different.

As with the [ATP], the [GTP] also considerably decreased in the infected culture. The [GTP] was 28% lower during the course of the infection but not with a corresponding increase in GDP. Since the GMP was not measured it is difficult to determine if the GTP was being utilized and converted to GMP.

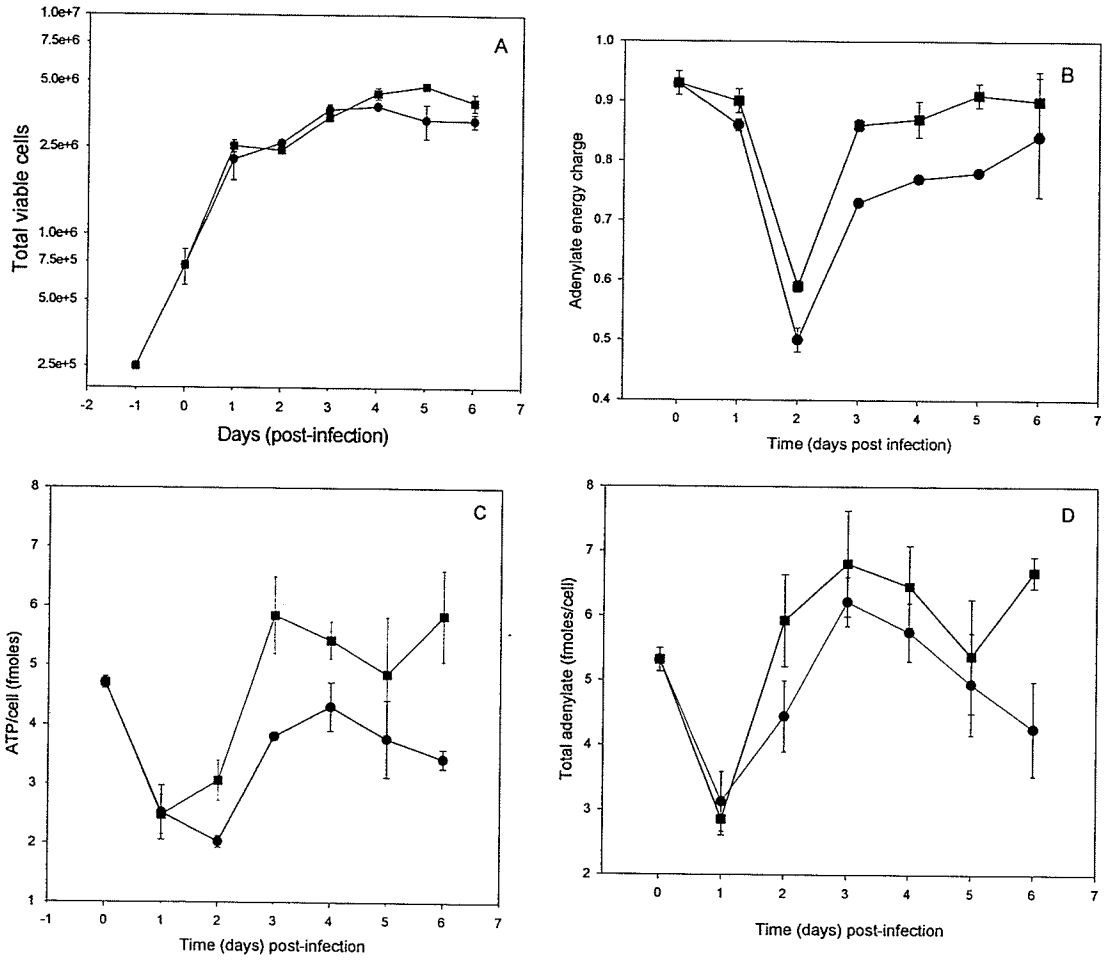


Figure 6.2. The cell growth (A), AEC (B), intracellular [ATP] (C), total adenylate (D) profiles of infected and mock-infected Vero cells. Vero cells were grown in 6-well cluster plates in M2-VSFM with an initial seed density of 2.5 x 10^5 cells/well in 5 ml of media. At day 2 the cells were infected with reovirus type-3 Dearing at an MOI = 5.0 PFU/cell using the infection procedure as outlined in the materials in methods. Mock infected cells were treated with an equivalent amount of PBS in place of the virus. Infected, -●-; mock-infected , -■-. Statistical analysis was performed upon the above data sets using a paired *t*-test. The difference between the AEC ($P < 0.05$) and ATP ($P < 0.05$) are significant. The difference between the cell growth ($P > 0.16$) and total adenylate ($p=0.07$) are not significant. n=2.

Table 6.2. Nucleotide profiles of infected and mock-infected cultures.

Mock-infected cells

Time (post-infection)	Intracellular nucleotide levels (fmoles/cell)				
	NAD	UDP-glucose	CTP	GTP	GDP
0	1.99 ± 0.02	0.54 ± 0.9	0.90 ± 0.02	2.36 ± 0.10	0.07 ± 0.01
1	1.26 ± 0.16	0.33 ± 0.26	0.40 ± 0.04	1.32 ± 0.17	0.12 ± 0.00
2	2.88 ± 0.46	0.48 ± 0.00	0.32 ± 0.00	1.73 ± 0.15	0.43 ± 0.10
3	2.39 ± 0.31	0.24 ± 0.08	0.37 ± 0.02	2.60 ± 0.34	0.19 ± 0.05
4	2.50 ± 0.24	0.15 ± 0.03	0.20 ± 0.02	2.83 ± 0.16	0.17 ± 0.03
5	2.56 ± 0.41	0.20 ± 0.02	N/a	3.07 ± 0.76	0.15 ± 0.02
6	3.29 ± 0.38	0.21 ± 0.06	N/a	3.89 ± 0.24	0.20 ± 0.02

N/a – too low for detection

Infected cells

Time (post-infection)	Intracellular nucleotide levels (fmoles/cell)				
	NAD	UDP-glucose	CTP	GTP	GDP
0	1.99 ± 0.02	0.54 ± 0.09	0.90 ± 0.02	2.36 ± 0.10	0.07 ± 0.01
1	1.31 ± 0.21	0.60 ± 0.06	0.49 ± 0.10	1.38 ± 0.29	0.13 ± 0.01
2	2.07 ± 0.26	0.44 ± 0.08	0.26 ± 0.00	1.12 ± 0.05	0.27 ± 0.04
3	1.72 ± 0.06	0.17 ± 0.02	0.33 ± 0.02	1.66 ± 0.08	0.17 ± 0.01
4	2.44 ± 0.25	0.09 ± 0.03	0.19 ± 0.03	2.31 ± 0.24	0.22 ± 0.03
5	2.57 ± 0.43	0.17 ± 0.03	N/a	2.19 ± 0.45	0.21 ± 0.03
6	2.54 ± 0.03	0.75 ± 0.83	0.16 ± 0.02	1.48 ± 0.27	0.21 ± 0.01

N/a – too low for detection

6.3. Discussion

The relationship between growth state and virus production has been studied previously in the literature. It was found that virus production during the stationary or lag phase was almost 60% lower than that of cells in the exponential phase, 40 PFU/cell compared to 100 PFU/cell respectively, over a 48 hour period (Chillakuru et al. 1991). This was not consistent with our findings where we observed productivities of 39.4 $\pm$ 14.5 and 48.1 $\pm$ 6.8 PFU/cell day for the stationary and exponential phases, respectively. The specific productivity overlapped during the course of the infection and there was no significant difference between the two cultures based on a paired *t*-test analysis (P=0.23). The apparent contradiction between these results and those reported in the literature may be due to the fact that the cells in stationary phase had run out of nutrients and/or accumulated a toxic amount of metabolic by-products (such as lactate, ammonia). These conditions are known to decrease cell viability and other cellular processes, such as virus and protein production (Jensen et al. 1961; Furusawa et al. 1962). Since the Vero cells had fresh medium added after the infection this would not be a factor and thus not have this variable to interfere with virus production. This resulted in a high metabolic state (indicated by high ATP levels) without any cell division. Furthermore, Chillakuru *et al* examined only 48 hours post-infection during the production period, whereas we examined the productivity over 5 days. It is possible that productivity may have overlapped if the culture was continued for more time in their case.

The [ATP] and total adenylate at the time of infection were identical between the growth phases. This was not pre-arranged and was actually fortuitous. The levels afterward were considerably different between the two growth states with the stationary culture having much higher ATP and total adenylate. The levels afterward did not lead to an increase or decrease in the virus output. If this were the case the culture during stationary phase would have had a higher specific productivity and total production. Since the productivities were comparable the [ATP] and [adenylate] at the time of infection appear to be the major contributing factor to productivity.

The AEC of the two growth phases did not appear to share any relationship with productivity. The AEC was significantly lower in the stationary culture at the time of infection than the exponential phase culture (0.88 and 0.93, respectively). This did not lead to a decrease in virus production or specific productivity. Furthermore, the higher AEC maintained in the stationary phase culture during the production phase of the virus did not lead to any increases in titer. Although the high AEC was unexpected it has been observed with other cultures in stationary phase (Ryll et al. 1991) and in infected cultures in stationary phase (Mukhopadhyay et al. 1994).

The [GTP] between the two cultures was nearly identical at the time of infection. As with the ATP, the GTP levels were considerably higher in the stationary phase culture for the duration of the production phase of the virus (after day 1 PI). Again, the [GTP] after the infection had no impact on virus production. This supports the finding that the energy level at the time of infection is the most important factor.

The profiles of the other intracellular nucleotides, including NAD, UDP-glucose, CTP, UTP, GTP, and GDP are shown in Table 6.1. The [GTP] of both cultures were nearly identical at the time of infection having 2.45 ± 0.29 and 2.36 ± 0.10 for the stationary and exponential phase culture, respectively. After this point the [GTP] was considerably higher in the stationary culture than in the actively growing cells ($p=0.008$), presumably since it was being used to fuel cell growth.

The higher metabolic activity in the exponential phase culture was further supported by the other nucleotide profiles. The lower [NAD] ($p=0.004$) suggests that the cells were utilizing NAD for oxidation reactions (glycolysis, TCA cycle, etc). Both the UDP-glucose and CTP levels were not significantly different ($p=0.47$ and $p=0.37$, respectively). The [UTP] were not determinable, so a comparison was not possible with respect to this nucleotide triphosphate.

The mock-infected culture revealed interesting information about how reovirus infection impacts the metabolic state of Vero cells. The first is that infection did not have any

impact on the growth of the cells at this MOI (5.0). In fact the infected cells grew to the similar cellular densities as the mock-infected culture. This suggests that reovirus does not disturb cellular growth processes in Vero cells which is supported by earlier findings in our lab (Berry et al. 1999).

The second is that infected cells had a significantly different nucleotide profile than uninfected cells. Although this was expected it was not known what degree these metabolic indicators would change. The infected cells showed signs of increased metabolism and usage of high energy nucleotides. The host cells' [ATP] was 28% lower that the uninfected cells with a corresponding decrease in AEC by 10%. This ATP was presumably being used to fuel protein production for the virus and has been observed in other viral infections (Rubella in BHK cells) (Bardeletti 1977). However, the total adenylate appeared to be lower in the infected culture. It is not known whether this was due to decreased *de novo* synthesis of adenylate or that it was being degraded via the salvage pathway. Statistical analysis revealed that the levels were *not* significantly different according to a paired *t*-test ($p=0.07$), so it is possible it might have been random fluctuation and not a real difference.

This decrease in adenylate and AEC has been observed with HIV-infected cells (Carlucci et al. 1996). In this case the AEC dropped from 0.82 to 0.80 with infected cultures. This was matched with a lower incorporation of formate (precursor for purine biosynthesis) indicating a decrease in *de novo* synthesis of adenylate in infected cultures. Since neither the formate uptake nor inosinate monophosphate levels were determined in the reovirus-infected cultures it is not possible to determine whether the infected cultures had lower *de novo* synthesis or increased salvage pathway activity, respectively.

The [GTP] between the cultures was similar to the ATP profiles. A 28% reduction in [GTP] was observed in the infected culture which was identical to the decrease in [ATP]. GTP is used in protein production (Nelson et al. 2000) so it follows that the production of virus is tapping into the GTP stores of the cell. This lead to a decrease in the GTP/GDP ratio (data not shown) which can indicate an increase in metabolic activity.

Further evidence of increased metabolism was the lower levels of [NAD] in the infected culture as it is presumably being used in glycolysis and the TCA cycle (reduced to NADH). However, *t*-test results indicate that this may have not been significant although not with high confidence levels ($p=0.08$). Both the [UDP-glucose] and [CTP] did not vary between the two cultures ($p=0.36$ and $p= 0.88$, respectively) and were comparable during the infection period.

From the above results the actual demand of ATP by the virus can be calculated. By integrating the curves the average difference is 1.07 fmoles/cell day between the mock-infected and the infected culture during the course of the infection. The average PFU/cell day is 48.1 for the infected culture giving an ATP usage of 0.022 fmoles ATP/PFU. However, it cannot be concluded that the ATP difference between the two cultures is solely due to the viral infection and for the production of viral progeny.

The theoretical amount of ATP required to produce one virus can be calculated based on the protein and genetic content of the virus. The amount of protein in the virus is ~ 109 MDa. Therefore, 109 MDa protein/(120 Da/amino acid) = 9.08 x 10^5 amino acids. The energy to produce protein can be calculated based on the amount of amino acids (Nelson et al. 2000):

Addition of each amino acid to peptide:
4 ATP/amino acid added x 9.08 x 10^5 amino acids = 3.6 x 10^6 ATP

Activation/termination of protein synthesis:
3 ATP/initiation x 1598 proteins = 4794 ATP
1 ATP/termination x 1598 proteins = 1598 ATP

Total = 3.61 x 10^6 ATP for reovirus protein.

The genomic content of reovirus is ~ 23,500 bp (47,000 bases). To manufacture the genome it would require:

2 ATP to charge each base + ~ 10 ATP to manufacture each purine or pyrimidine = 12 ATP base.

12 ATP/base x 47,000 bases = 5.64 x 10^5 ATP.

5.64 x 10^5 ATP + 3.61 x 10^6 ATP = **4.2 x 10^6 ATP/virus.**

((4.2 x 10^6 ATP/virus) * (1 x 10^15 fmoles/mol))/(6.02 x 10^23 molecules/mol) = **0.0070 fmoles ATP/virus.** This is >3-fold lower of the measured value of 0.022 fmoles ATP/virus, but this discrepancy could be due to the particle to PFU ratio. This has been reported to range anywhere from 1:1 (rare cases), but more commonly in the range of 50:1 to 500:1 (Nibert et al. 2001). Since the total particles were not determined in this experiment it is not possible to estimate the actual ratio.

6.4. Conclusion

These results indicate that cells produced the same amount of virus when their energy states were comparable, independent of growth state. The dogma that cells that have arrested growth will not produce as much virus as actively dividing cells is untrue. As long as the cell is actively metabolizing and can supply the proper substrates for the virus to replicate there will not be a loss in productivity. Therefore using growth state as an indicator of a healthy metabolic state is misleading and should not be used to determine the optimal time for a virus infection.

Reovirus infection leads to an overall energy drain upon the cell. High energy nucleotide triphosphates are utilized at a greater rate in infected cultures and show a 1/3 decrease in the available ATP and GTP. The result of this is a lowering of the adenylate energy charge in the infected cells. However, reovirus infection does not significantly impact

the growth of Vero cells. Since reovirus infection requires such a significant amount of energy to make progeny virus it is possible that energy levels of the cell may impact the overall titer.

CHAPTER 7

The Relationship between Glucose and Glutamine Concentrations on Intracellular ATP

7.1. Introduction

The results from Chapter 6 suggested that the energy state of the cell may impact virus productivity. If this is true then cultures in a lower energy state would have lower virus output. In order to test this hypothesis conditions must be set up to lower the energy state of the cell (with respect to ATP) while maintaining high cell viability. This could be accomplished by altering the carbon sources in the medium.

The nutritional requirement of mammalian cells (in vitro) is supplied by a medium that contains a variety of carbon and nitrogen sources. The compositions of these carbon sources can affect the metabolism and thus change the energy levels in the cell. The most commonly used carbohydrate in culture media is glucose as it can feed various pathways for energy production. It is usually present at concentrations between 5 - 25 mM and supplies energy to the cell through aerobic glycolysis (Fitzpatrick et al. 1993). The tendency for mammalian cell lines is to convert the majority of the generated pyruvate to lactate rather than entering the TCA cycle, even with oxygen present (Medina et al. 1990). Part of the glucose enters other pathways, such as the pentose phosphate pathway which is used to generate nucleic acids (Neermann et al. 1996).

Although glucose provides a great deal of energy it is not the only carbon source to be utilized by cells. Glutamine serves as the other major source of energy for the cell. It has been shown to supply as much as 50% of the cells aerobic metabolism and is the major energy source for many cell lines (Butler et al. 1989; Nelson et al. 2000). Glutamine feeds directly into the TCA cycle which can be completely converted to CO_2, or partially broken down to other metabolites, such as alanine, lactate, and aspartate (glutaminolysis) (McKeehan 1982). It is also required for purine biosynthesis as the major nitrogen donor

in the synthesis of inosinate. Glutamine is usually supplemented in culture media at 1 – 6 mM.

Since glucose and glutamine are the major energy sources lowering these two components in the medium should affect the cell's ability to generate ATP. Various concentrations were explored to generate low energy conditions without affecting the viability of the cell. These formulations would then be used to determine its effect on virus production (Chapter 8).

7.2. Results

7.2.1. The effect of glucose concentration upon intracellular ATP levels in Vero cells

Previous studies with hybridoma cells (Barnabe et al. 1998) showed that the intracellular ATP levels decreased at the lower glucose in the medium. The levels tested in that paper were from 0, 1, 3, 4, 5, 12, and 25 mM (control) of glucose. Therefore this study looked at the ATP levels in Vero cells under the same conditions.

Vero cells that had been pre-adapted to growth in M2-VSFM were used for this experiment. Actively growing cells were inoculated into T-25 flasks with 2.0 x 10^6 cells/flask (near confluence) in an altered M2-VSFM formulation with lowered glucose levels (0, 1, 3, 4, 5, and 25 mM (control)). The cells were incubated for 24 hours in the specific medium and were then harvested for nucleotide extraction (2.7.1). The cell intracellular ATP concentration was then determined by luminometry (2.7.3). The results are shown in Figure 7.1.

The ATP levels for most of the conditions (glucose at 1 mM, 3 mM, and 4 mM) did not vary significantly from the control with 6.7 fmoles/cell. 1 mM glucose had 6.0 fmoles/cell; 3 mM had 7.5 fmoles/cell; and 4 mM had 7.8 fmoles/cell. Surprisingly, the

cells in 5 mM glucose had a lower ATP level than most of the others, 4.5 fmoles/cell. The cells that had no glucose at all had expectedly dropped to quite low levels of ATP (1.7 fmoles/cell).

The expected result was to have a gradual lowering of ATP concomitantly with lowering glucose levels. This was not observed and only when glucose was removed completely did the ATP levels drop significantly. However, at 0 mM glucose the cells cannot survive for any period of time and therefore make that condition useless for the purposes of a virus production experiment.

7.2.2. The intracellular ATP concentration in Vero cells under varying amounts of glucose and glutamine

The previous study showed that Vero cells must rely on other sources for the generation of ATP. This is not surprising since glycolysis can be fed from a variety of sources (such as amino acids). A search in the literature showed that the majority of mammalian cells derive their energy from glutamine (including Vero) and thus lowering glutamine in the medium should yield lower ATP levels. The objective of this experiment was to determine what combination of glucose and glutamine levels would give a ~50% reduction in ATP levels without killing the cell.

Vero cells that were pre-adapted to growth in M2-VSFM were inoculated into T-25 T-flasks with a starting cell density of 2.0 x 10^6 cells/flask in 5 ml of media. A matrix of different glucose and glutamine concentrations was examined (the control medium contains 25 mM glucose and 4 mM glutamine). After the cells grew for 24 hours they were harvested and prepared for ATP analysis (2.7.1 and 2.7.3). The results are shown in Table 7.1.

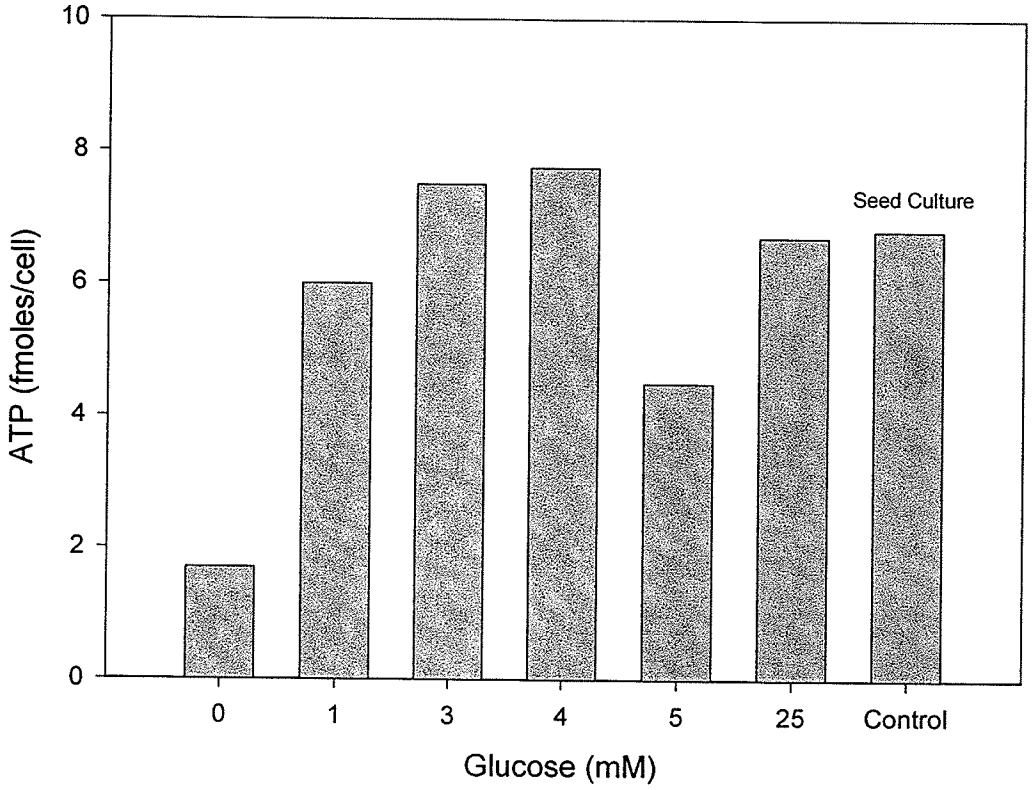


Figure 7.1. The effect of glucose levels on the intracellular [ATP] within Vero cells. Actively growing Vero cells were inoculated into T-25 flasks with 2.0 x 10^6 cells/flask (near confluence) in a medium (5 ml) with the specified glucose levels. The cells were harvested after 24 hrs and the intracellular ATP concentration was determined. The ATP level of the seed culture is shown on the far right (which contained 25mM glucose).

A definite trend can be seen within some of the columns (varying glutamine). When glutamine is lowered a decrease in ATP can be seen in at specific glucose concentrations (12.5, 5, and 1 mM glucose). There is no trend observed when the rows are examined (varying glucose) except when 0 mM glucose is finally reached. This observation suggests that Vero cells rely more on glutamine as an energy source than glucose and matched those observed in the literature (Butler et al. 1989). However, there are a few cases where the ATP levels did not give expected results. A few of the samples gave unusually high ATP levels at low nutrient concentrations (ex: 3 mM glc/0 mM gln with 5.94 fmoles/cell) and some with very low ATP levels where nutrient levels were high (lower right of table). It is not known whether this is a real phenomenon or just experimental error. It is possible that the nucleotide extraction procedure was not uniform for those samples which have been known to cause variation in ATP levels.

The viability for all of the cultures was >95% for the majority of the cultures after the 24 hour incubation period. The only decrease in viability was observed with those cells in cultured in 0 mM glc/0mM gln and 0 mM glc/0.5 mM gln. The carbon sources were obviously too low in these conditions to support the cells.

7.2.3. The intracellular ATP concentration of Vero cells in agitated culture with lowered glucose and glutamine levels

The most compelling data from the matrix experiment (Table 7.1) was that in the 0 mM glutamine row where the ATP levels decreased significantly in 24 hours. The ATP levels would give a range that would be suitable in measuring the effect of ATP concentration on virus production. However, before this could be attempted it was necessary to ensure that the cells would remain viable for a prolonged period of time (i.e.: longer than 24 hours) during the virus production phase (typically 2 days post-infection). Therefore, this experiment determined how long the cells would remain viable at those nutrient levels. The cell viability was monitored using the Trypan Blue exclusion method which measures membrane integrity. Cells that are dead will readily take up the dye and stain

Table 7.1 The intracellular ATP concentration (fmoles/cell) in vero cells after 24 hours when grown under reduced glucose and glutamine concentrations

[Glutamine] (mM)	[Glucose] (mM) 0	1	3	5	12.5	25
0	2.89	3.59	5.94	2.37	4.10	3.59
0.5	-	2.98	4.63	3.22	4.40	4.67
1	4.20	5.59	3.59	5.42	5.76	5.80
2	2.67	5.47	5.97	5.76	6.10	2.32
4	4.20	5.01	5.11	5.96	5.31	3.72

Control cells (at T=0): **5.35 fmoles/cell**

blue whereas viable cells will exclude the dye. For the purposes of measuring viability over a long period of time microcarrier cultures were used.

Vero cells were inoculated (2.0 x 10^5 cells/ml) into 100ml spinner flasks with 2.0 g/L of Cytodex-1 in M2-VSFM. The cell numbers were determined daily by the Trypan Blue exclusion method (2.3.3 and 2.4) as well as extracting the nucleotides for luminometric analysis (2.7.1 and 2.7.3). After the cells had reached confluence (day 6) the medium was removed from the flasks and the cells washed 1x with a non-glucose/glutamine containing M2-VSFM. It was then replaced with a lowered nutrient medium containing 0 mM glutamine with 1, 2, or 5 mM glucose. The ATP levels are shown in Table 7.2.

Once the medium was changed to the starvation formulations there was a sharp decrease in intracellular ATP in the cultures. After 1 day of starvation the ATP dropped to 2.4 fmoles/cell in the 1 mM and 5 mM glucose cultures whereas the 3 mM culture maintained a level close to that of the control (4.42 and 4.89 fmoles/cell respectively). On the 2nd day of starvation, however, the ATP levels all dropped to ~ 2.0 fmoles/cell in all of the nutrient limited cultures. On the 3rd day the ATP levels finally dropped to near 0 fmoles/cell for all of the cultures except the control.

The viability of the cells remained high after 1 day of starvation for the 1, 3, and 5 mM glucose cultures (>95%). After the 2nd day the viability remained high only in the 3 and 5 mM cultures (88% and 84% respectively) whereas the 1 mM glucose culture had only 53% viability. On the 3rd day the only culture to maintain a reasonable viability were the cells in the 5 mM glucose (59%). Thus the 5 mM glucose/0 mM glutamine formulation was able to lower the intracellular ATP concentration by half within 2 days while maintaining a high viability during this period.

Table 7.2. Intracellular [ATP] in Vero cells under low glucose/glutamine conditions.

The cells were grown in 100ml spinner flasks with 2g/L cytodex-1 in normal M2-VSFM. Once the cells had reached near confluence they were washed 1x in a non glucose/glutamine medium and then exchanged for lowered glucose media (no glutamine).

Time (days)	[ATP] (fmoles/cell)				Viability index[a] (max 100%)			
	Glucose concentration				Glucose concentration			
	1mM	3mM	5mM	Control[b]	1mM	3mM	5mM	Control
1	6.35	6.35	6.35	6.35	99	99	99	99
2	5.83	5.83	5.83	5.83	99	99	99	99
3	8.73	8.73	8.73	8.73	99	99	99	99
4	7.93	7.93	7.93	7.93	99	99	99	99
5	4.23	4.23	4.23	4.23	99	99	99	99
6	5.47	5.47	5.47	5.47	99	99	99	99
7	2.36	4.42	2.42	4.89	99	99	99	99
8	2.19	1.67	1.59	6.79	53	88	84	99
9	0	0	0	4.78	7.5	23	59	99
10	0	0	0	4.21	0	0	2.5	99
11	0	0	0	5.21	0	0	0	99
12	0	0	0	5.46	0	0	0	99
13	0	0	0	6.04	0	0	0	99
14	0	0	0	6.94	0	0	0	99

[a] Viability index: calculated by Trypan Blue Exclusion method
[b] Control: 25 mM glucose

7.3. Discussion

The aim of this study was to develop a nutrient-poor medium that would lower the energy levels of the cell without causing cell death. Since glucose and glutamine are the major sources of energy for mammalian cells they were chosen as the most likely components to affect ATP levels. To determine what levels of glucose and glutamine levels had on cellular energy levels, cells were initially subjected to lowered glucose concentrations. The results indicated that Vero cells are more reliant on glutamine than glucose for energy. Lowered levels of glucose (1 to 5 mM) did not have any significant impact on the intracellular ATP after 24 hours. Only when glucose was completely removed from the medium did the energy level decrease. But without glucose the cells quickly became non-viable which would not suit the objective.

It is well documented that mammalian cells utilize glutamine to generate ATP. Just how much glutamine a cell uses varies from cell line to cell line, but it is generally accepted that glutamine provides for the majority of the energy in the cell (Butler et al. 1989) (Neermann et al. 1996). This was supported by the initial experiment with lowered glucose levels. Even with very low levels of glucose (1 mM) the cells maintained intracellular [ATP] to that of the control (25 mM). Thus the second experiment was designed to determine what combination of glucose and glutamine would lower the energy levels in the cell. The results showed that when glutamine was completely removed from the medium the cells had lower levels of ATP, even at high glucose concentrations (25 mM), to less than 50% of the control.

Although removal of glutamine altogether allowed for lowered ATP levels in the cell it was unclear how long cell viability could be maintained at such levels. This was an important issue as the medium must maintain cell viability long enough for the virus to reach its peak titer (usually 2 days post-infection, see Chapter 5). Therefore the cells were subjected to various nutrient-poor formulations in microcarrier cultures over a longer period. Of the formulations tested, the one with 5 mM glucose and 0 mM glutamine maintained cell viability over a 3 day period. At glucose concentrations lower

than this (1, 3 mM) the cells would die sooner and thus would not remain alive for a virus production experiment. The cells in 5 mM glucose and 0 mM glutamine had a >50% reduction in intracellular ATP compared to that of the control after 24 hours.

7.4. Conclusion

Vero cells can be induced into a lower metabolic state by lowering the glucose and glutamine concentration in the medium. The cells can maintain high viability for 3 days while in a medium containing 5 mM glucose and 0 mM glutamine with a loss of >50% of the intracellular ATP. These conditions will be suitable to establish cultures with lowered energy states to determine the impact it has on virus productivity.

CHAPTER 8

The Relationship Between Intracellular Nucleotide Levels and Virus Production

8.1. Introduction

Viruses are obligate parasites and are therefore dependent upon the host cell system to survive. Replication of viral progeny requires the production of genetic material and protein which requires significant amounts of energy from the host cell. It is likely then that the physiological state of the cell can have a significant impact on virus production. Indeed, the results from the previous Chapter 6 indicated that the energy demand by reovirus is significant (28% of total ATP and a 10% of the total AEC) which further supports this hypothesis. It is likely then that ATP levels may affect virus productivity. Could this be used as a "productivity gauge" of virus output from mammalian cell culture?

Recent studies in the literature have shown that higher virus titers are produced in cells with higher intracellular [ATP]. One group showed that cells grown in a protein-free medium had higher virus production than cells in a protein-containing medium. This was attributed to higher ATP levels (Nadeau et al. 2002). Furthermore, cells in an active growing state have shown to produce more virus than non-growing cells. Again, this has been attributed to a higher [ATP] in the actively growing cells (Mukhopadhyay et al. 1994).

However, a clearly defined relationship between energy state and virus production has yet to be established. The studies in the literature have generally looked at two conditions to study the effect on virus production. In addition, these studies have been restricted to the measurement of intracellular [ATP] and/or [ADP] as an indicator of cellular energy state. It is still not clear what relationship [ATP] has to virus production. Is this a linear relationship? Will virus production increase directly with an increase in [ATP]? Or is there a minimum level of [ATP] that must be maintained before virus

production stops altogether? Is [ATP] the only nucleotide that might correlate to virus production? What other nucleotide triphosphates, such as GTP, might play a role? Since the AEC has been an accurate predictor of the metabolic state of the cell (Atkinson 1968) could it be used to predict how much virus will be produced from a cell?

The purpose of this study was to examine the relationship between cellular energy state and virus production. This may be relative to a combination of many factors, including AEC, [ATP], total adenylate, and other nucleotides such as GTP. Three major questions are to be answered. First, what effect do these factors have on the amount of virus produced? Is it a linear relationship or is there a minimum energy level that must be maintained before there is a loss of productivity? Second, is there a relationship between AEC and virus production? And lastly, which other nucleotides might correlate to the amount of virus produced from a culture? The conditions established in Chapter 7 were used to generate cultures with a variety of energy states to answer these questions.

8.2. Results

8.2.2. Virus production in microcarrier cultures of Vero cells under nutrient limiting conditions

Studies in the literature revealed viruses did not replicate to as high a titer in cells with lowered ATP levels (Nadeau et al. 2002). However, the two cultures which were compared were grown in different media formulations; one contained protein whereas the other did not. This was proposed to be the reason for the lowered ATP levels as cells had an altered metabolism with protein present. It is not clear, however, if the results observed were an artifact of the medium itself. The previous experiments with Vero cells in microcarrier culture (7.2.3) showed that the ATP levels can be safely lowered by 50% by reducing the glucose and glutamine levels in the medium without a loss in cell viability. Using these same conditions this experiment examined how virus production would proceed under lowered intracellular ATP levels without significantly changing the medium composition.

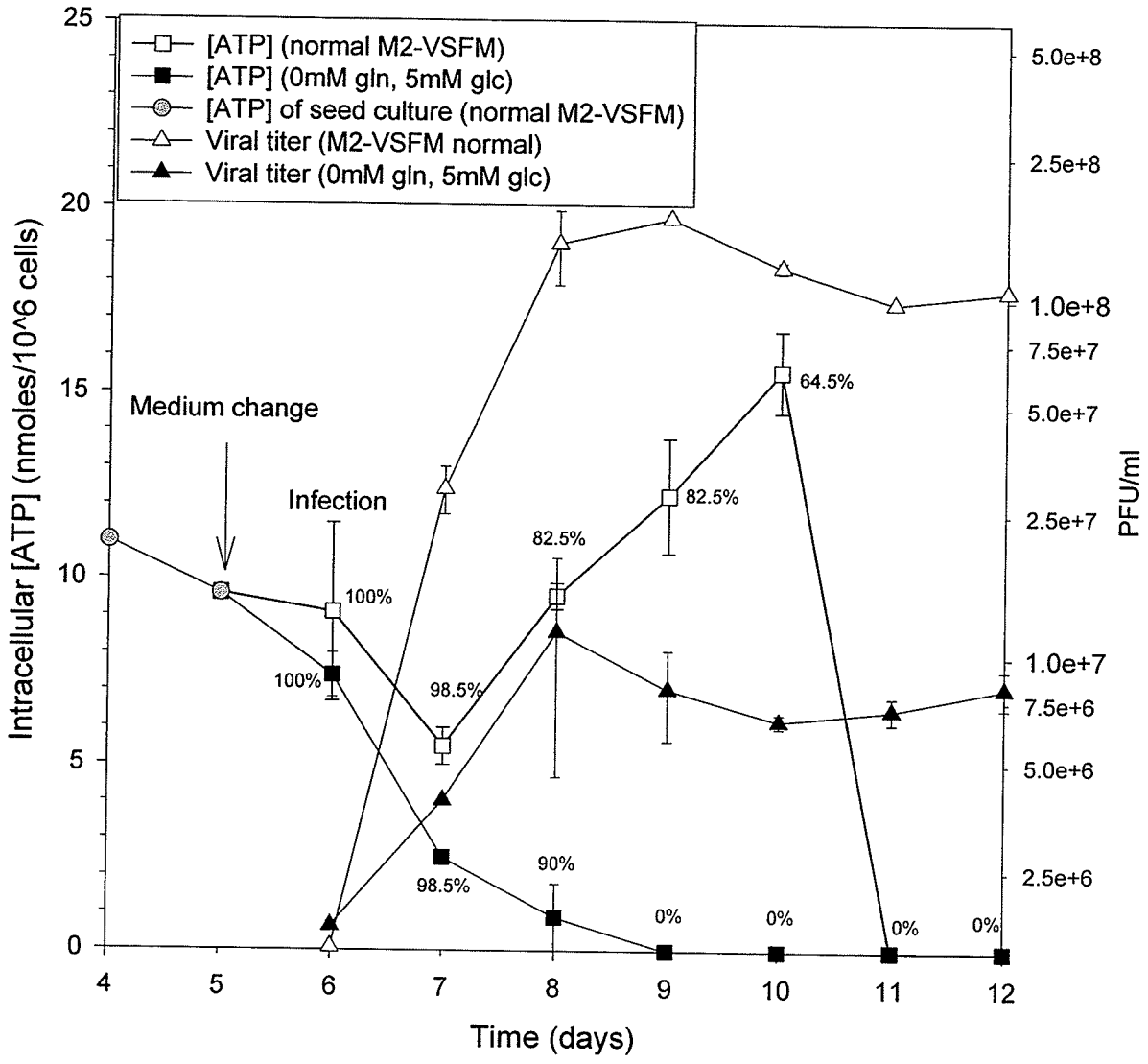


Figure 8.1. The effect of intracellular [ATP] upon virus production. The cells were initially grown in a 500ml spinner flask in normal M2-VSFM with 2 g/L cytodex-1. After the cells had reached near confluence the cells were transferred to a 100ml spinner flask and the medium was exchanged for the lowered glucose/no glutamine formulation. After 24 hours the cells were infected with reovirus type-3 Dearing at an MOI of 5.0 PFU/cell. Percentages indicate the cell viability determined by the Trypan Blue exclusion method (2.4). A statistical analysis was performed on the virus production profiles between the two conditions. The paired t-test gives a $P < 0.05$ indicating that they are significantly different. n=2.

Vero cells were inoculated into a 500ml spinner flask with 2.0 g/L of Cytodex-1 in M2-VSFM (2.5.2). After the cells had reached near confluence they were transferred to 100ml spinner flasks and the medium was exchanged for either the control medium (normal M2-VSFM) or a lowered glucose/glutamine formulation (M2-VSFM with 5.0 mM glucose, 0 mM glutamine). The cells were maintained in this medium for 24 hours at which point they were infected with reovirus type-3 Dearing at an MOI of 5.0 PFU/cell (2.8.1). The viable cell number (2.3.3 and 2.4) and viral PFU (2.8.2 and 2.9) were determined every 24 hours. The results are shown in Figure 8.1.

During the first 2 days post-infection there was a significant difference in specific productivity between the two conditions. The culture with the lowered ATP levels (2.5 fmoles/cell) at the time of infection had a specific productivity of 28.6 PFU/ml cell during this period. The control culture which had higher ATP levels (5.5 fmoles/cell) had a specific productivity of 467 PFU/ml cell. Therefore the culture with the lowered ATP at the time of infection had a 94% reduction in productivity. The viability of the two cultures were both high (>80%) during this time period. In both cases the maximum titer was reached 2 days post-infection.

8.2.3. Virus production under different nutrient conditions

The microcarrier experiment (8.2.1) lead to the hypothesis that lowered intracellular ATP levels had a significant impact on virus production. However, it was not determined what the exact relationship is between ATP levels and virus production. Is this a linear relationship or is there a minimum energy level that must be maintained before there is a loss of productivity?

To answer this question this experiment was designed to obtain a series of cultures with a spread of ATP levels. Cultures of Vero cells were subjected to varying nutrient levels to achieve this. Cultures of Vero cells were grown in the control medium for 1 day (M2-VSFM -normal glucose and glutamine levels) in a 24 multi-well plate with an initial cell

density of 5.0 x 10^4 cells/ml in 1 ml of media. The media was then removed and the cells were washed with 1.0 ml of PBS. The PBS was aspirated and the cells overlayed with the following media formulations: (a) Control medium - 25 mM glucose and 4 mM glutamine; (b) Medium 1 - 0 mM glutamine and 5.0 mM glucose; (c) Medium 2 - 0.5mM glutamine and 5.0mM glucose; (d) Medium 3 - 2.0 mM glutamine and 5.0 mM glucose; (e) Medium 4 - 4.0 mM glutamine and 5.0 mM glucose. After a 24 hour incubation in the new medium the cells were infected with reovirus type-3 Dearing at an MOI of 5.0 PFU/cell (2.8.1). The cell numbers and viability were determined by the Trypan Blue exclusion method (2.3.1 and 2.4) and the virus titer by a standard plaque assay described previously (2.9). Virus production, specific virus production, and cell growth are shown in Figure 8.2.A, 8.2.B, and 8.2.C respectively.

The cultures incubated in the control medium had a normal virus production profile (Figure 8.2.A), reaching a peak titer of 5.7 x 10^7 PFU/ml after 5 days post-infection. A similar production profile was observed for medium 3 reaching a peak titer of 6.0 x 10^7 PFU/ml after 5 days. The other media had lowered titers to varying degrees. Medium 4 saw a peak of 3.1 x 10^7 PFU/ml (day 4), medium 2 reached only 3.7 x 10^6 PFU/ml and medium 1 had 8.6 x 10^5 PFU/ml (only slightly higher than the infection titer).

The specific production profile shows a similar pattern to that of the total production (Figure 8.2.A). The control medium reached 58 PFU/cell; medium 4 reached 59 PFU/cell; medium 3 reached 81 PFU/cell; medium 2 reached 6 PFU/cell; and medium 1 reached 4 PFU/cell.

All of the cultures, except for cells in medium 1, continued to grow up to 2 days post-infection (Figure 8.2.C). The control medium was the exception and continued to grow until 4 days post-infection. There was no noticeable difference in the cell viability between any of the cultures and they maintained a viability of >90% during the course of the infection. However, after day 4 the viability began to drop as the virus began to lyse the remaining cells.

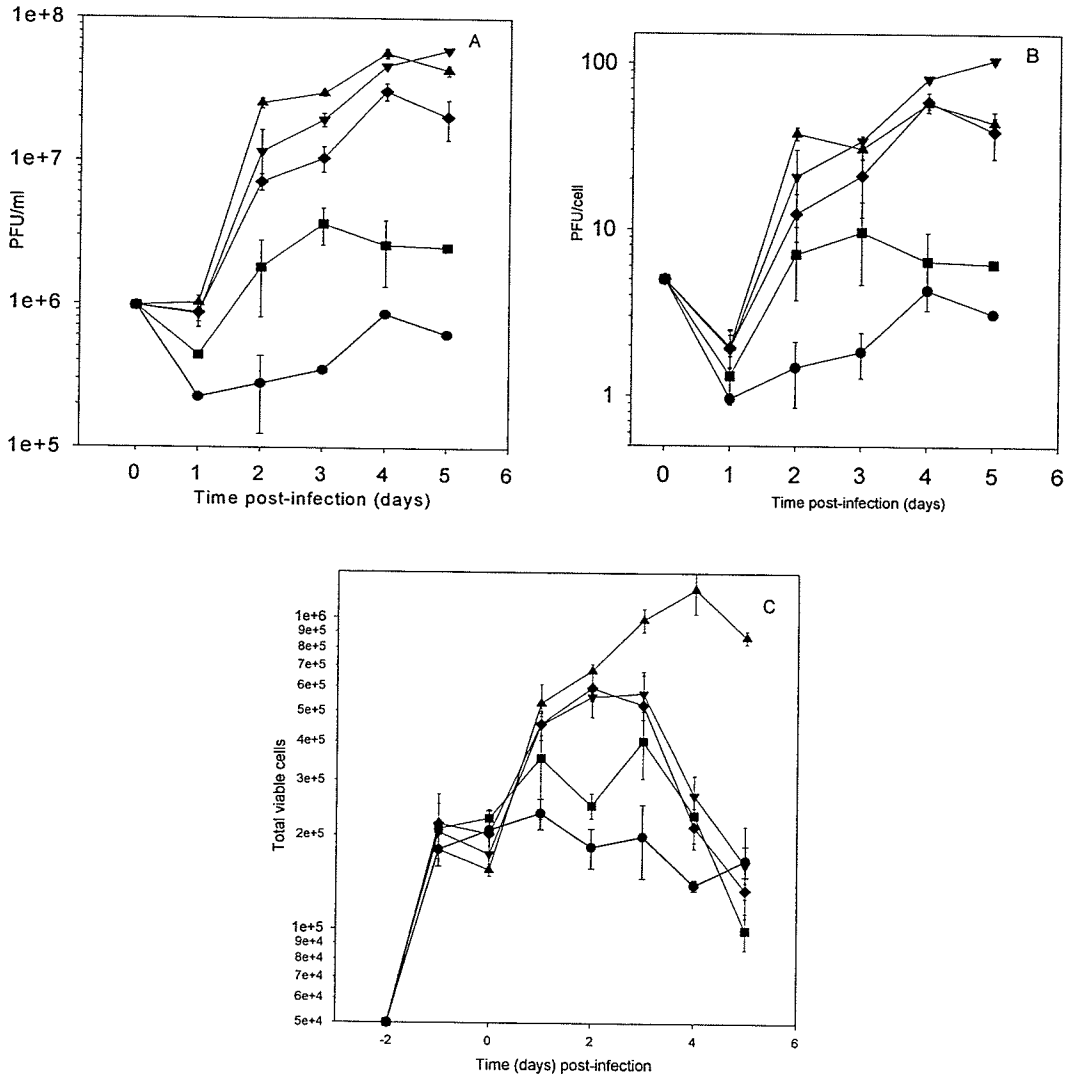


Figure 8.2. The total virus production (A), specific reovirus production (B), and cell growth (C) of Vero cells under varying nutrient conditions. Vero cells were grown in 24-well cluster plates in the control medium for 24 hours and then replaced with a nutrient depleted medium (Medium 1, 2, 3, and 4). After 1 day in the new medium the cells were then infected with reovirus type 3-Dearing at an MOI of 5.0 PFU/cell. The viral titer was determined by the plaque assay as described in the materials in methods. Medium 1, -●- (0 mM gln and 5.0 mM glc); Medium 2, -■- (0.5mM gln and 5.0mM glc) ; Medium 3, -▼- (2.0 mM gln and 5.0 mM glc); Medium 4, -◆- (4.0 mM gln and 5.0 mM glc); Control, -▲- (25 mM glc and 4 mM gln). n=2. Statistical analysis was performed upon the above data sets. The difference between the control medium and each of the others is significant ($P < 0.05$ in each case).

8.2.3.1. ATP levels and virus production

The intracellular ATP concentration was monitored during the course of the infection in section 8.2.3. The infected cells were harvested daily and prepared for nucleotide analysis (2.7.1). The ATP levels were then determined by the luciferase-luciferin assay described previously (2.7.3). The ATP profile is shown in Figure 8.3.A.

Depending on the medium the cells had intracellular [ATP] ranging from 1.9 to 6.0 fmoles/cell. The ATP profile of the cells varied significantly at day 0 post-infection (1 day after the cells were treated with the nutrient-depleted media). The control medium had 6.0 fmoles/cell; medium 3 had 4.2 fmoles/cell; medium 4 had 3.2 fmoles/cell; medium 2 had 2.7 fmoles/cell; and medium 1 had 1.9 fmoles/cell. The ATP profiles after this point did not vary significantly during the course of the infection with the exception of the control medium at day 3.

The cultures with an intracellular [ATP] < 3.0 fmoles/cell at the time of infection lead to a >95% reduction in overall viral titer compared to the control. However, cells with intracellular [ATP] > 3.0 fmoles/cell at the time of infection had limited effects on productivity compared to the control. This suggests that there is an ATP cutoff, or threshold, that must be maintained for high productivity.

The ATP levels after day 1 post-infection did not have any impact on overall virus production. The levels of the control medium was the only exception where it reached ~ 2.0 fmoles/cell during this period where the others maintained levels of ~ 0.9 fmoles/cell. Since there was no increase in titer observed between the control culture and any of the high producing cultures (such as media 3 and 4) this lead to the hypothesis that ATP levels 1 day post-infection is not contributing to overall productivity.

8.2.3.2. Total adenylate levels and virus production

The total adenylate levels (ATP + ADP + AMP) of the infected cultures from section 8.2.3 were measured using a combination of the luciferin-luciferase assay and HPLC analysis (2.7.2 and 2.7.3). The luciferin-luciferase assay was used to determine the ATP and ADP levels and HPLC analysis for the AMP levels. The adenylate profiles are shown in Figure 8.3.B.

The total adenylate profile mirrors that of the ATP (Figure 8.3.A) during the course of the infection. The greatest spread of intracellular concentration is seen at the time of infection at day 0. The control medium had 6.2 fmoles/cell; medium 3 had 4.3 fmoles/cell; medium 4 had 3.4 fmoles/cell; medium 2 had 2.8 fmoles/cell; and medium 1 had 2.0 fmoles/cell.

This, as with the [ATP], has a positive correlation with virus production. The cultures with < 3.0 fmoles/cell had significantly lower virus titers that those with > 3.0 fmoles/cell at the time of infection. Medium 2 had 2.8 fmoles/cell of total adenylate at the time of infection and had a maximum virus titer of 6 PFU/cell. This was considerably lower than cultures with > 3.0 fmoles/cell at the time of infection, such as medium 3 with 4.3 fmoles/cell and a maximum titer of 81 PFU/cell.

Again, the adenylate levels after the infection did not vary significantly after the infection with the exception of the control medium at day 3. The levels of the control reached ~ 3.0 fmoles/cell during this period where the others maintained levels of ~ 1.0 fmoles/cell. Since there was no increase in titer observed between the control culture and any of the high producing cultures (such as medium 3 and 4) this was concluded not to be a contributing factor to overall productivity.

8.2.3.3. Adenylate energy charge profile

The AEC profile of the infected cultures from section 8.2.3, according to the relationship [ATP] + 0.5 [ADP]/([ATP] + [ADP] + [AMP]), is shown in Figure 8.3.C.

The AEC of medium 2, 3, 4 and the control were similar from the time of infection at day 0 until day 2 post-infection. During this time the AEC was >0.90 at day 0 and then showed a decrease to just under 0.5 (at which point the cells stopped growing, Figure 8.2.C). After day 2 the cultures in the nutrient depleted formulations tended toward a higher AEC much more quickly that the control medium. The control medium maintained a lower AEC between 0.5 and 0.7 between day 2 and 4 with a corresponding increase in total adenylate (Figure 8.3.B). This is presumably because the control culture still maintained a low level of growth between day 2 and 4 and were therefore using ATP to fuel cell growth processes. The other cultures during the same time period had a range of 0.79 (medium 3) to 0.95 (medium 2) and had stopped growing at day 2 post-infection. The AEC value of cells maintained in Medium 1 did not change significantly from a high value >0.90 and may be explained by the fact that there was no growth in the culture.

Figure 8.3.D shows the total adenylate profile of the cells in each of the media. In response to nutritional stress the cells maintain a higher energy charge by breaking down AMP (thus maintaining a high ATP/AMP ratio). The cells in medium 1 and 2 saw a drop in total adenylate levels 1 day post-infection with a steady decrease downward. Once the [ATP] began to fall the [AMP] increased, presumably because ATP was being utilized to fuel virus replication. However, at day 4 the [AMP] dropped to near zero with a concurrent decrease in total adenylate. This suggested that the cells had run out of a nutrient source and were maintaining a high energy charge (0.7 at day 4 and 0.95 at day 5) by degrading AMP. Similar profiles were observed in medium 3 and 4 although they maintained low levels of AMP and ADP on day 3 and 4 (i.e.: did not completely degrade their AMP).

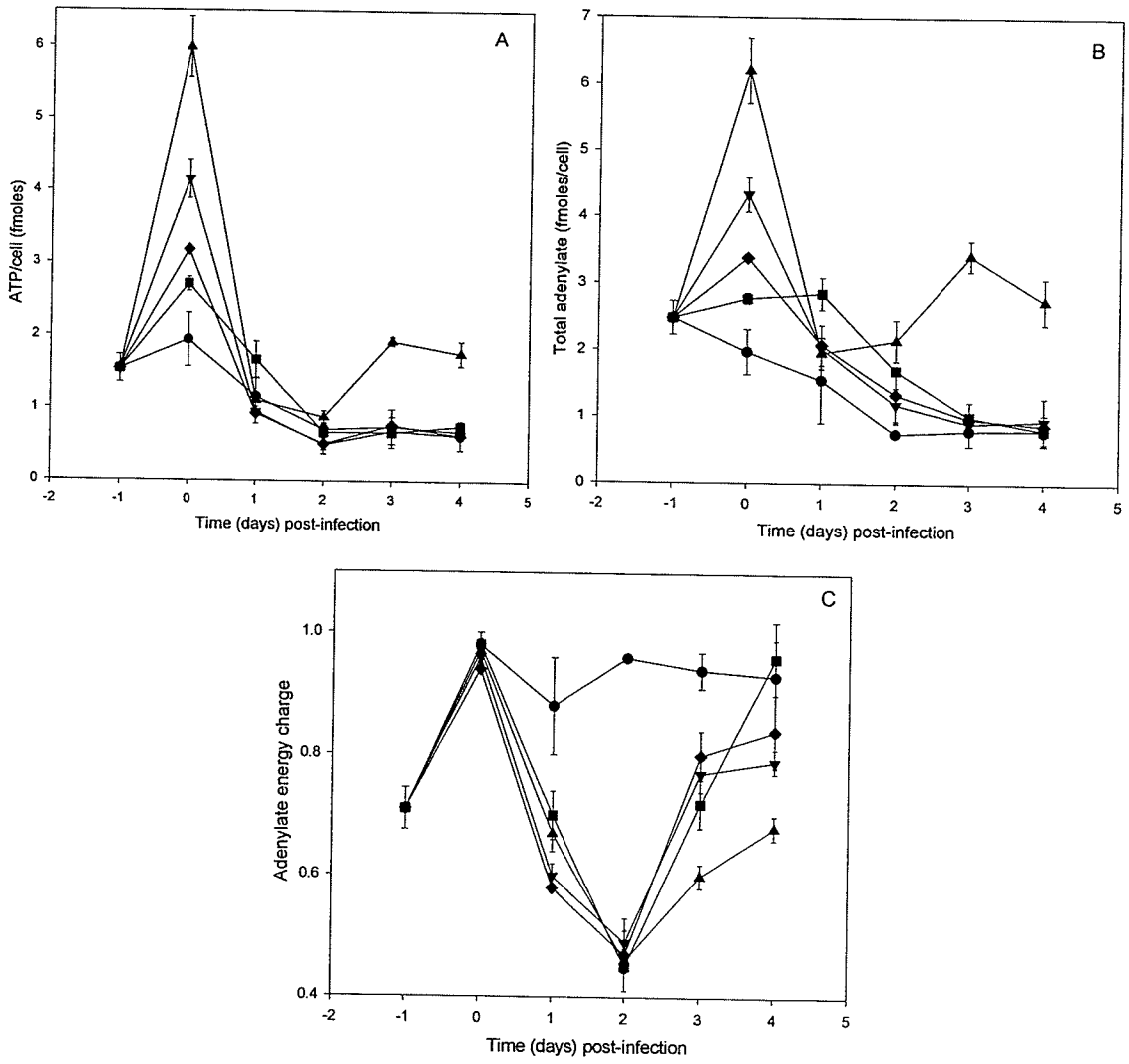


Figure 8.3. The intracellular [ATP] (A), total adenylate (B), and AEC (C) of infected cells under varying nutrient conditions. Vero cells were grown in 24-well cluster plates in the control medium for 24 hours and then replaced with a nutrient depleted medium (Medium 1, 2, 3, and 4). After 1 day in the new medium the cells were then infected with reovirus type 3-Dearing at an MOI of 5.0 PFU/cell. The nucleotides were extracted from the Medium 1, -●- (0 mM gln and 5.0 mM glc); Medium 2, -■- (0.5mM gln and 5.0mM glc) ; Medium 3, -▼- (2.0 mM gln and 5.0 mM glc); Medium 4, -◆- (4.0 mM gln and 5.0 mM glc); Control, -▲- (25 mM glc and 4 mM gln). n=2.

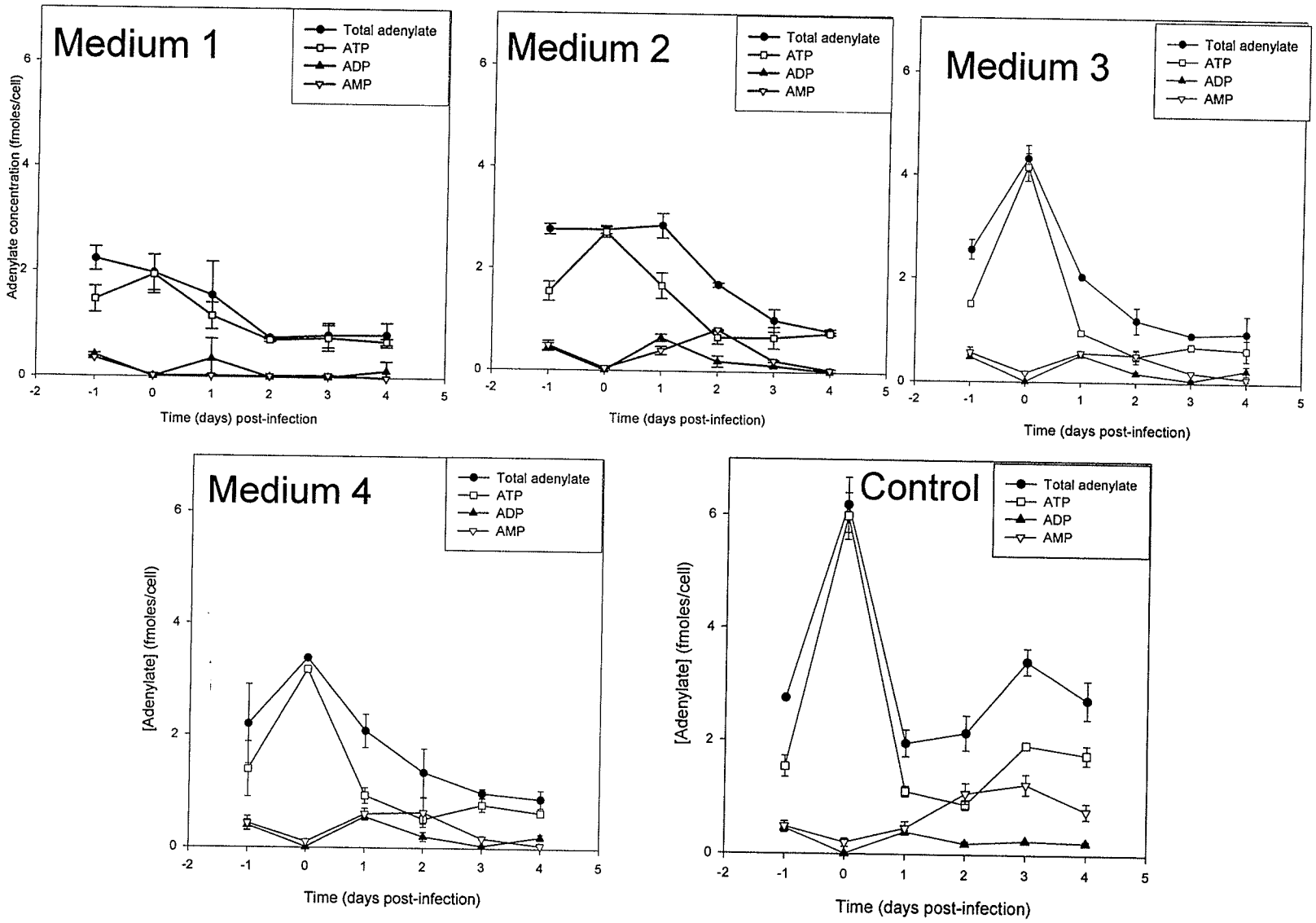

Medium 1
Total adenylate
ATP
ADP
AMP
Adenylate concentration (fmoles/cell)
Time (days) post-infection
Medium 2
Time (days post-infection)
Medium 3
Time (days post-infection)
Medium 4
[Adenylate] (fmoles/cell)
Time (days post-infection)
Control
[Adenylate] (fmoles/cell)
Time (days post-infection)

Figure 8.3.D. Adenylate profile of reovirus-infected cultures of Vero cells under varying nutrient conditions. These graphs display the relative concentrations of ATP, ADP, and AMP during the infection. Cells in medium 1, 2, 3, 4 and the control are shown. Vero cells were grown in 24-well cluster plates in the control medium for 24 hours and then replaced with a nutrient depleted medium. After 1 day in the new medium the cells were then infected with reovirus type 3-Dearing at an MOI of 5.0 PFU/cell. The nucleotides were extracted from the infected cells and the levels determined as described in the materials and methods. n=2.

8.2.3.4. GTP levels and virus production

GTP is an important nucleotide for the production of proteins. As with ATP, GTP is used in specific steps during protein synthesis, specifically the elongation and translocation steps during peptide chain elongation. It is likely then that the levels of GTP in the cell could have an impact on virus production since the virus is dependent on the host system to replicate progeny virions.

The intracellular GTP levels of the infected cultures in section 8.2.3 were determined by ion pair reverse phase HPLC described previously in the materials and methods (2.7.2). The intracellular GTP profiles during the infection period are shown in Figure 8.3.E.

The GTP levels during the infection shared a clear relationship with virus production. The levels were similar to the ATP profile seen in Figure 8.3.A. Examining the day 0 levels, Medium 1 had the lowest GTP with 3.7 fmoles/cell; medium 2 had 4.23 fmoles/cell; medium 3 had 5.38 fmoles/cell; medium 4 had 4.73 fmoles/cell; and the control had 8.0 fmoles/cell. The GTP levels at the time of infection shared a trend with virus production. The cells with the lowest [GTP] (medium 1 and 2) < 4.2 fmoles/cell had a significantly reduced virus output (95% reduction in overall production). The cells with a [GTP] over 4.2 fmoles/cell maintained productivity compared to that of the control over a 4 day production period.

The GTP concentration of the cells in medium 1 remained high relative to that of the control cells after infection (almost 5-fold). This was also observed with the cells in medium 2 which had a 2-fold higher GTP concentration to that of the control after infection (except day 3). The high levels of GTP in the cultures under nutritional stress suggest that GTP-utilizing processes, such as protein production, were not operating to the same extent as that of the control. This was supported by a high GTP/GDP ratio (Table 8.1) in the nutrient limited cells.

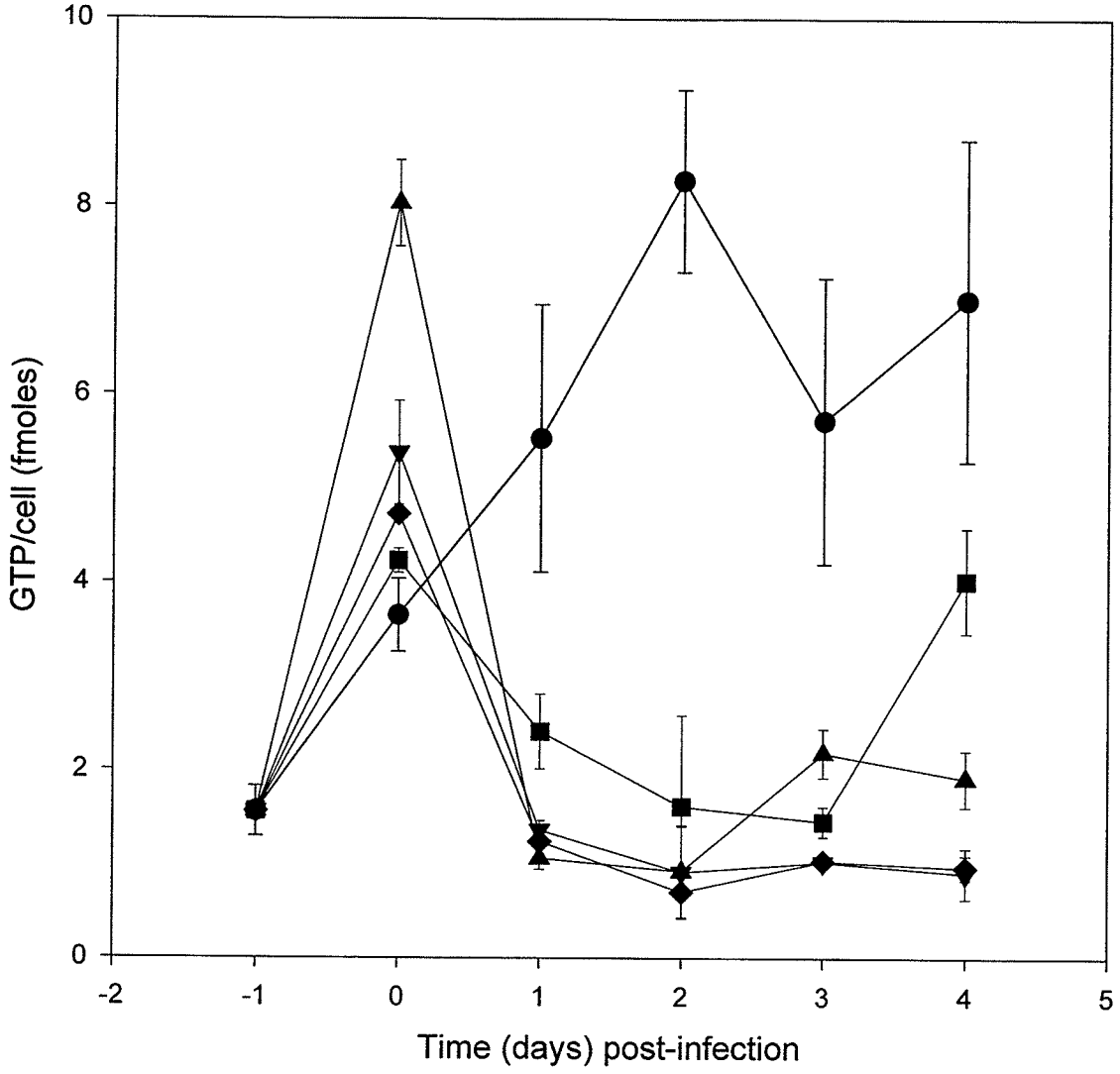


Figure 8.3.E. The intracellular [GTP] of infected cells under varying nutrient conditions. Vero cells were grown in 24-well cluster plates in the control medium for 24 hours and then replaced with a nutrient depleted medium (Medium 1, 2, 3, and 4). After 1 day in the new medium the cells were then infected with reovirus type 3-Dearing at an MOI of 5.0 PFU/cell. The nucleotides were extracted from the infected cells and the levels determined as described in the materials and methods. Medium 1, -●- (0 mM gln and 5.0 mM glc); Medium 2, -■- (0.5mM gln and 5.0mM glc) ; Medium 3, -▼- (2.0 mM gln and 5.0 mM glc); Medium 4, -◆- (4.0 mM gln and 5.0 mM glc); Control, -▲- (25 mM glc and 4 mM gln). n=2.

Table 8.1. GTP/GDP ratio of infected cells under varying nutrient conditions.

Time (days) post-infection	GTP/GDP ratio				
	Medium 1	Medium 2	Medium 3	Medium 4	Control
-1	22.3 ± 7.5	22.3 ± 7.5	22.3 ± 7.5	22.3 ± 7.5	22.3 ± 7.5
0	112 ± 97.1	64.2 ± 9.74	37.6 ± 4.62	26.2 ± 0*	41.0 ± 20.8
1	57.7 ± 32.3	12.8 ± 2.94	8.55 ± 2.77	6.93 ± 1.30	9.59 ± 0.17
2	56.2 ± 8.53	7.10 ± 5.41	6.2 ± 2.73	5.61 ± 1.74	6.14 ± 2.22
3	233 ± 213	13.6 ± 1.40	14.1 ± 0.83	14.2 ± 0.49	13.9 ± 0.98
4	N/d	59.5 ± 46.9	14.0 ± 5.46	41.7 ± 31.6	15.5 ± 1.11

N=2

N/d – Not determinable

* - only one value

8.2.3.5. NAD and UDP-glucose levels during the infection

The intracellular NAD and UDP-glucose levels of the infected cultures in section 8.2.3 were determined by ion pair reverse phase HPLC described previously in the materials and methods (2.7.2). The profiles are shown in Table 8.2.

NAD levels are excellent indicators of metabolism. NAD is an electron carrier and is directly involved in glycolysis (oxidation of glucose), the TCA cycle, and the breakdown of fatty acids to name a few pathways. Therefore the production of NADH from NAD indicates a shift towards catabolism and the breakdown of macromolecules to supply energy to the cell. Conversely, an increase in UDP-glucose levels is an indicator of a shift to anabolism. UDP-glucose is a precursor for glycogen synthesis which signals a cell is storing excess energy.

The NAD levels of Medium 1 and 2 were considerably lower at the time of infection than the others. Medium 1 had an intracellular [NAD] of 1.39 $\pm$ 0.05 fmoles/cell; medium 2 had 2.17 $\pm$ 0.11 fmoles/cell; medium 3 had 3.65 $\pm$ 0.35 fmoles/cell; medium 4 had 4.06 $\pm$ 0.00* fmoles/cell, and the control 4.40 $\pm$ 0.20 fmoles/cell. The cultures in medium 3, 4 and the control had similar NAD levels from 1 day PI to 4 days PI. The cells in medium 2 had a sharp decrease in [NAD] at 3 days PI to 0.47 fmoles/cell which was approximately 25% to that of day 2. Medium 1 saw an overall decrease in [NAD] to ~ 0.80 fmoles/cell at 1 day PI and remained at this level until the culture was no longer viable (day 4 PI).

The UDP-glucose levels increased in medium 1 and 2 compared to the other conditions after the cells were infected with virus (day 1 PI). The UDP-glucose concentration in medium 1 reached 1.41 fmoles/cell and medium 2 reached 0.72 fmoles cell 1 day after they were infected. The other cultures maintained levels between 0.42 and 0.62 fmoles cell on the same day. From day 2 PI onward the levels were almost 3 to 4 times as high in medium 1 and 2 than the other conditions.

Table 8.2. NAD and UDP-glucose levels of infected cells under varying nutrient conditions

NAD

Time (days) post-infection	Fmoles/cell				
	Medium 1	Medium 2	Medium 3	Medium 4	Control
-1	1.67 ± 0.24	1.67 ± 0.24	1.67 ± 0.24	1.67 ± 0.24	1.67 ± 0.24
0	1.39 ± 0.05	2.17 ± 0.11	3.65 ± 0.35	4.06 ± 0.00*	4.40 ± 0.20
1	0.80 ± 0.15	1.26 ± 0.53	1.40 ± 0.00	1.44 ± 0.13	1.14 ± 0.10
2	0.80 ± 0.08	1.84 ± 0.19	1.44 ± 0.12	1.42 ± 0.43	1.31 ± 0.16
3	0.85 ± 0.21	0.47 ± 0.26	1.21 ± 0.07	1.29 ± 0.13	2.05 ± 0.09
4	N/d	0.41 ± 0.02	0.59 ± 0.13	0.55 ± 0.13	2.06 ± 0.11

N=2
N/d – Not determinable
* - only one value

UDP-glucose

Time (days) post-infection	Fmoles/cell				
	Medium 1	Medium 2	Medium 3	Medium 4	Control
-1	0.70 ± 0.14	0.70 ± 0.14	0.70 ± 0.14	0.70 ± 0.14	0.70 ± 0.14
0	1.29 ± 0.50	0.87 ± 0.24	1.33 ± 0.22	1.06 ± 0.00*	1.34 ± 0.40
1	1.41 ± 0.02	0.72 ± 0.34	0.62 ± 0.14	0.42 ± 0.02	0.49 ± 0.22
2	0.83 ± 0.10	1.07 ± 0.40	0.24 ± 0.01	0.10 ± 0.2	0.29 ± 0.07
3	0.32 ± 0.03	0.10 ± 0.09	0.08 ± 0.01	0.09 ± 0.05	0.17 ± 0.12
4	N/d	0.15 ± 0.01	0.45 ± 0.03	0.32 ± 0.15	0.19 ± 0.03

N=2
N/d – Not determinable
* - only one value

8.2.3.6. Virus production as a function of ATP and total adenylate at the time of infection

The intracellular nucleotides at the time of infection were plotted against specific virus production to determine which of them shared a correlation. The data points were taken from three different pools of infected cells. Cultures 5 and 6 were grown in a nutrient depleted formulation with the same protocol as those in section 8.2.3 (5 mM glucose, 0.75 mM glutamine for 24 hours prior to infection). Data for cultures 11-14 were taken from infected cells in different growth states (Chapter 6). The remainders were the infected cultures from section 8.2.3. Correlation R values near 1 or -1 indicate that the two data sets share a strong relationship whereas 0 indicates that they do not. A critical r value of 0.623 is required to reject the null hypothesis that the two data sets are not related. Included with the nucleotides measured were certain nucleotide ratios such as the AEC and the NTP ratio ((ATP + GTP/(UTP + CTP)), which is a measure of metabolic energy (Ryll et al. 1992). The data is shown in Table 8.3.

Only total adenylate, ATP, and AEC had correlation values greater than 0.623 (considered to be significant) with r values of 0.78, 0.74, and -0.69, respectively. The others either had no correlation to virus productivity (r close to 0) or a very weak probability ($r < 0.6$). It was surprising that GTP could not be correlated to virus production as it showed a visible trend in Figure 7.3.E. But when combined with data from other experiments it showed that it was not a good predictor of productivity.

The ATP, total adenylate levels, and AEC were plotted against the specific virus production (max PFU/cell reached after 4 days). Figures 8.4.A and 8.4.B show the specific virus production as a function of ATP during the first 2 days post-infection and 4 days post-infection, respectively. Looking at 2 days post-infection, there is a hyperbolic relationship between intracellular [ATP] and specific production rate. When this is observed at 4 days post-infection a sigmoidal relationship emerges and a plateau effect can be seen. The curve equation is a 4 parameter sigmoidal plot according to the formula:

$$y = y0 + \frac{a}{1 + e^{-\left(\frac{x-x0}{b}\right)}}$$ (Equation 8.1)

Where, for Figure 7.4.B, the constants (x0 and y0) and coefficients (a and b) are: y0 = 4.595; x0 = 3.197; a = 67.94; and b = 0.08. The coefficient of determination (R squared) for Figure 8.4.B was 0.88 suggesting that it is a good model to fit the data. The graph indicated that a minimum [ATP] must be maintained at the time of infection to prevent loss of productivity. Cultures with an [ATP] lower than ~ 4.0 fmoles/cell resulted in a reduction in the production rate of viruses during the first 2 days of the infection. The higher [ATP] contributes to both an increased rate and total virus production during the first 2 days. Looking over a 4 day period a sharper drop-off effect can be seen with cells having an [ATP] lower than ~ 3.1 fmoles/cell at the time of infection. A Km of 3.2 fmoles/cell was determined as the amount of ATP required for half of the maximum output of virus (Vmax of 72 PFU/cell). From a total production standpoint a further increase in [ATP] above 3.4 fmoles/cell did not result in any increase in final productivity.

A similar profile was observed with the virus production after 4 days as a function of total adenylate at the time of infection (Figure 8.4.C). The same relationship was observed according to equation 8.1. The constants (x0 and y0) and coefficients (a and b) are as follows: y0 = 5.020; x0 = 3.28; a = 67.37; and b = 0.08. The curve fit the data well, having an R squared of 0.94. Here as well a critical adenylate concentration is reached where virus production is significantly affected. The Km with respect to [adenylate] is 3.3 fmoles/cell. Cells with levels > 3.5 fmoles/cell all had similar levels of virus output.

The AEC of the cultures shared a negative sigmoidal relationship (Equation 8.1) with virus productivity and is shown in Figure 8.4.D. The constants (x0 and y0) and coefficients (a and b) are as follows: y0 = 9.507; x0 = 0.9699; a = 63.10; and b = -0.0009.

In this case a critical AEC level of 0.97 is reached where virus productivity drops off. AEC levels above this value lead to a significant decrease in virus productivity. This is most likely due to the fact that these cultures are depleting their AMP to maintain a high energy charge, which is supported by the adenylate data in Table 8.3. The cultures with a high AEC tend to have lower total adenylate. This is not surprising since those are the cultures that have low glucose and glutamine in the medium.

Table 8.3. Correlation coefficients between intracellular nucleotides at the time of infection and specific virus production.

The correlation coefficients between the data sets were performed using the Pearson statistical analysis to determine the "r values". The calculated values were compared to the critical value of r which is required to accept the null hypothesis that there is no correlation between the data sets. R values are between -1 and 1 where values close to -1 or 1 indicate that there is a strong correlation between the two sets of data. The values were determined in Microsoft Excel. Data is from three independent experiments from different pools of cells (Culture 1-4, 7-10, 15-16 are from 1 experiment and one pool, 11-14 from second, and 5-6 from a third).

	Intracellular nucleotide level (fmoles/cell) or nucleotide ratio									
Culture	Total adenylate	ATP	AEC	GTP	UTP	CTP	UDP-glucose	NAD	NTP ratio	Max PFU/cell
1	1.72	1.68	0.97	3.37	12.4	3.39	0.93	1.43	0.36	4.43
2	2.2	2.2	0.99	3.92	11.6	3.1	1.64	1.36	0.37	4.23
3	2.7	2.64	0.98	4.32	12.3	2.1	1.03	2.09	0.48	8.7
4	2.81	2.78	0.99	4.14	12.9	2.43	0.7	2.25	0.45	6.13
5	3.15	3.15	0.99	3.77	0.32	0.26	0.91	1.48	21.6	12
6	3.15	3.15	0.99	3.52	0.34	0.31	0.82	1.33	19.9	16.1
7	3.37	3.18	0.94	4.73	15.0	1.75	1.06	4.06	0.47	59
8	3.38	3.28	0.97	5.53	15.6	2.18	0.76	2.88	0.5	59
9	4.14	3.96	0.96	5.77	20.1	1.91	1.48	3.4	0.44	78
10	4.51	4.35	0.96	4.99	15.4	2.08	1.17	3.9	0.53	84
11	5.17	4.64	0.91	2.29	4.18	0.88	0.61	1.97	1.37	64
12	5.19	4.83	0.89	2.66	2.43	0.19	0.13	2.14	2.86	81
13	5.44	4.32	0.88	2.24	1.71	0.16	0.15	1.96	3.52	74

14	5.53	4.78	0.95	2.44	5.35	0.91	0.48	2	1.15	80
15	5.85	5.69	0.97	8.35	26.6	2.46	1.06	4.55	0.70	55
16	6.54	6.29	0.96	7.70	14.9	2.23	1.62	4.26	0.68	61
[a]R value	**0.78**	**0.74**	**-0.69**	**0.08**	**0.10**	**-0.35**	**-0.25**	**0.53**	**-0.34**	
Correlation	**Yes**	**Yes**	**Yes**	**No**	**No**	**No**	**No**	**No**	**No**	

[a]Critical r value for 14 degrees of freedom (n-2) with a p = 0.01 is 0.623.

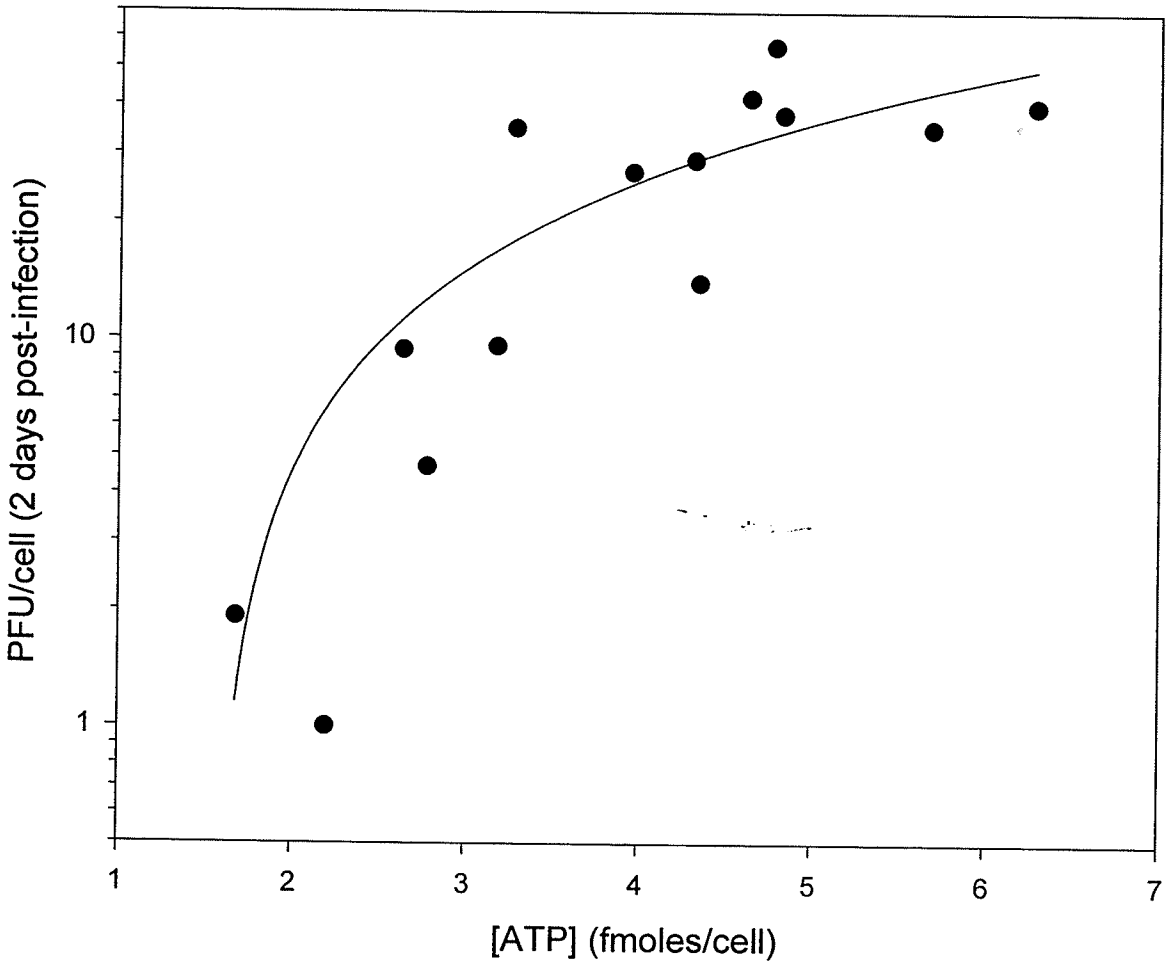


Figure 8.4.A. Virus production at 2 days post-infection as a function of intracellular ATP. The virus production profile of infected cells is plotted as a function of the [ATP] at the time of infection. This shows the PFU/cell after 2 days of the infection. The data points are taken from two independent experiments of reovirus-infected Vero cells.

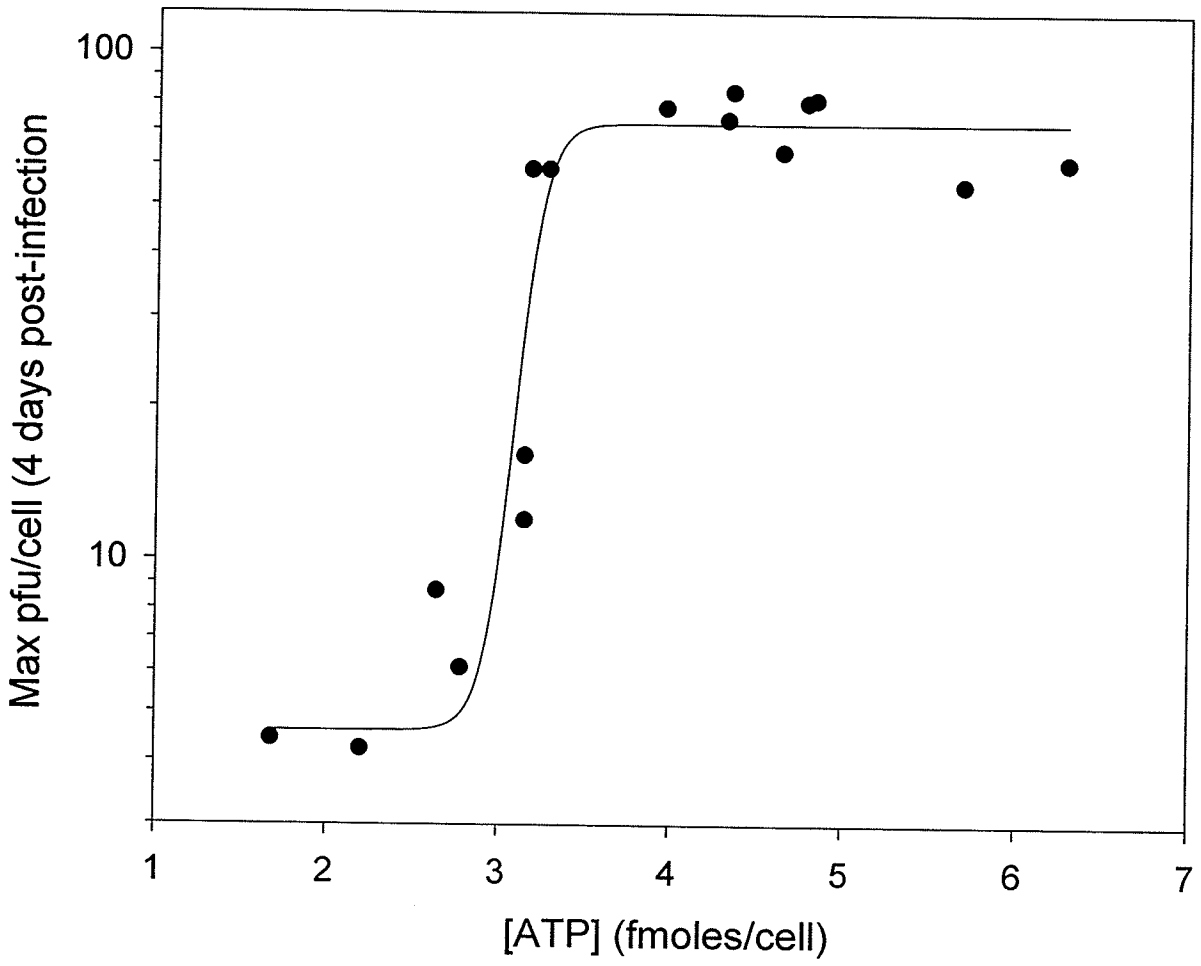


Figure 8.4.B. Virus production at 4 days post-infection as a function of intracellular ATP. The virus production profile of infected cells is plotted as a function of the [ATP at the time of infection using a 4 parameter sigmoidal curve. This shows the PFU/cell after 4 days of the infection. The data points are taken from three independent experiments of reovirus-infected Vero cells. R squared = 0.88.

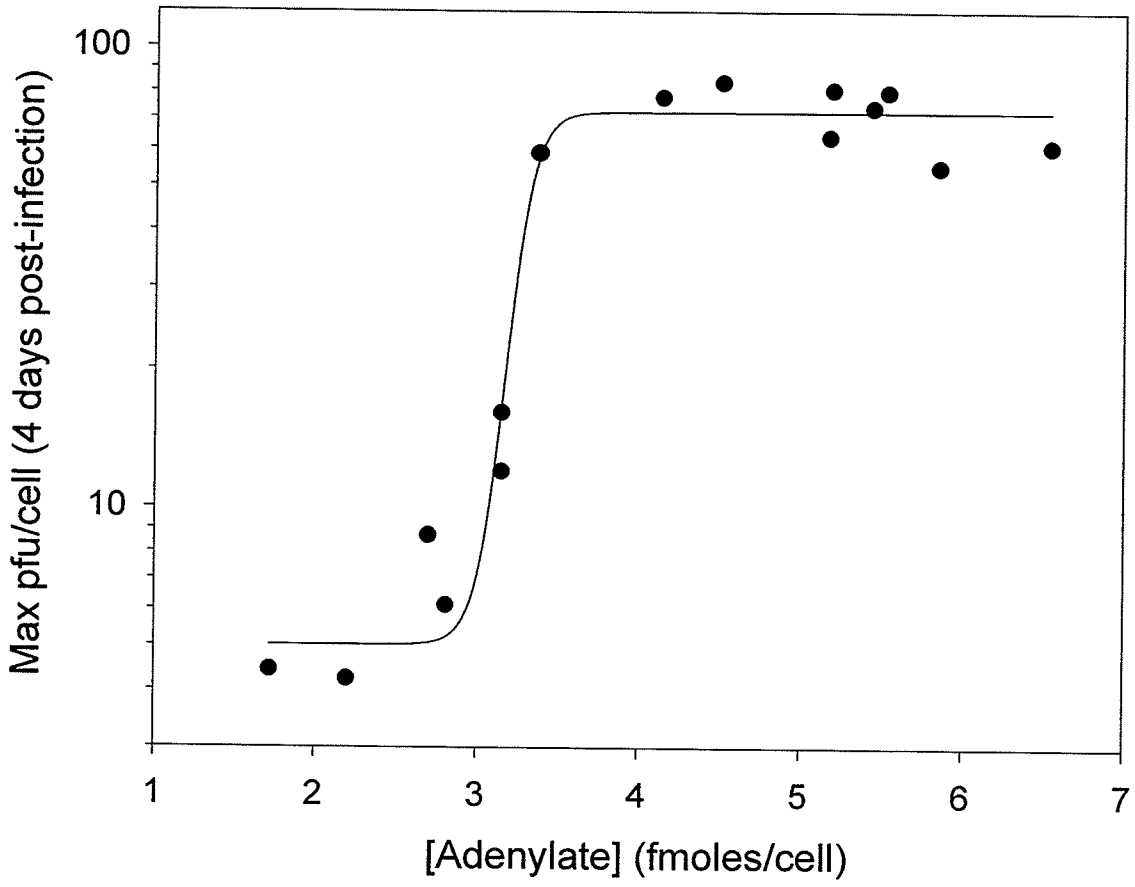


Figure 8.4.C. Virus production at 4 days post-infection as a function of total intracellular adenylate. The virus production profile of infected cells is plotted as a function of the total adenylate at the time of infection. This shows the virus production profile after 4 days. The data points are taken from three independent experiments of reovirus-infected Vero cells. R squared = 0.94.

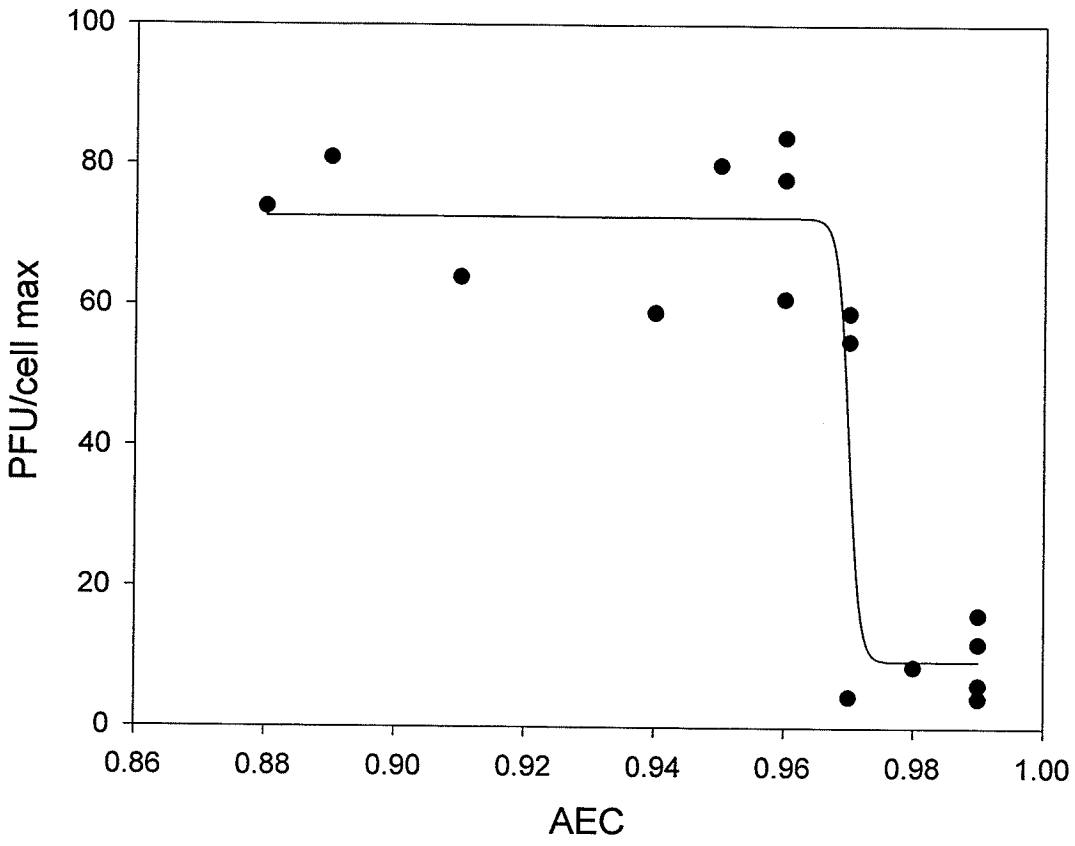


Figure 8.4.D. Virus production at 4 days post-infection as a function of adenylate energy charge. The virus production profile of infected cells is plotted as a function of the AEC at the time of infection. This shows the virus production profile after 4 days. The data points are taken from three independent experiments of reovirus-infected Vero cells. R squared = 0.83.

8.3. Discussion

There have been some studies reported in the literature looking at the relationship between cellular energy state and virus production. The central dogma is that the best productivity will be attained when cells are infected just prior to stationary phase. The presumption is that cells coming from a growth phase are actively metabolizing; therefore supplying an ample amount of energy and molecular precursors to fuel virus replication. This relationship has been tested looking at virus production during different growth states (Chillakuru et al. 1991) and more detailed studies examining the ATP levels during these different states (Mukhopadhyay et al. 1994). But the exact relationship between ATP and virus production has not been fully defined. In particular how AEC, total adenylate, or other nucleotides may play a role.

We succeeded in obtaining a spread of cellular energy states in cells by lowering the glucose and glutamine concentration in the medium and then examined how this would affect virus production. Virus production was significantly altered depending on the [ATP] and total adenylate at the time of infection. The levels 1 day post-infection did not have any impact on the total virus output. If this were the case then we would have seen a continued increase in specific virus production in the control culture as it reached a higher [ATP] and total adenylate concentration 3 days post-infection compared to the rest of the cultures (Figure 8.3.D). Considering that reovirus makes its primary transcripts in 2 hours and the majority of its proteins ~12 to 24 hours post-infection this stands to reason that the [ATP] is extremely important during that time period (Shatkin et al. 1983; Coombs 1996; Nibert et al. 2001). Indeed, some of the processes involved in reovirus replication, such as the proposed helicase activity of the λ1 core protein and post-translational folding of the cell attachment protein, are ATP-dependent (Leone et al. 1996; Noble et al. 1997; Chandran et al. 2003). However, since the K_m of these processes has not been determined the effect the ATP levels have on these processes cannot be elucidated. Furthermore, it may be possible that reovirus uptake was affected. Since the specific uptake of virus was not determined it cannot be ruled out as a possible reason for lowered titers.

Examining other nucleotide triphosphates intracellular [GTP] appeared to share a relationship with virus productivity. As with ATP, GTP is involved in protein synthesis and is used specifically for the elongation and translocation steps during peptide chain formation. Again, the time of infection was the crucial point where the levels of this nucleotide were important. The GTP levels after the infection played a little role in furthering productivity. In fact the cultures under the highest nutritional stress (medium1 and 2) had the highest GTP levels post-infection (days 1-4) but the lowest corresponding virus output. This can be attributed to the productive cells having a higher metabolic state and thus were utilizing GTP. The low GTP/GDP ratio in the productive cultures (medium 3, 4, and control) compared to the nutrient limiting conditions (medium 1 and 2) supported this. Other studies have shown similar findings of a lower GTP/GDP ratio in infected cells (Carlucci et al. 1996). However, when GTP data from other infected cultures were taken into account it could not be correlated to predict virus productivity (Table 8.3).

The UDP-glucose levels indicated that the cells in medium 1 and 2 had switched from catabolic to anabolic metabolism. Indeed they had stopped growing at this point. The UDP-glucose levels were higher in medium 1 and 2 from 1 day PI onward, almost 4 times as high as were found in medium 3, 4 and the control. An increase in [UDP-glucose] indicates that the cells are committing glucose to glycogen synthesis by condensing glucose-1-phosphate with UTP. UDP-glucose is the immediate donor of glucose residues to glycogen and acts as the substrate for glycogen synthase (Nelson et al. 2000). This indicates a switch to anabolic metabolism.

The NAD concentration in the nutrient-depleted cultures (medium 1 and 2) were significantly lower than the others (medium 3, 4, and control) at the time of infection. Since the NADH levels were not determined it is not possible to conclude whether this decrease was the result of the NAD being converted to NADH in oxidation pathways (such as glycolysis) or that de-novo synthesis was decreased. However, since ATP is a

precursor for NAD biosynthesis it is more likely that the lower ATP levels contributed to this.

We originally predicted to have a lower AEC under the starvation conditions, as glucose and glutamine are the major sources for ATP production, but this did not happen. It has previously been shown that glucose starvation will lower the AEC of cells within a matter of minutes (Ball et al. 1975; Live et al. 1975; Swedes et al. 1975). Since we did not measure the AEC during the 24 hour period of glucose depletion it is likely that a lower AEC was reached and then reversed. This reversal has been attributed to the action of AMP deaminase to maintain a high AEC in the cell under nutritional stress (Yoshino et al. 1981; Hardie et al. 2001). Indeed, the adenylate profile showed a decrease in ATP but without an increase in ADP or AMP levels. This indicates that once the cells had depleted their glucose they degraded their AMP to maintain the high adenylate energy charge. This has been observed before in glucose-starved cultures in other types of cells (Chapman et al. 1973; Yoshino et al. 1981; Ryll et al. 1991).

The intracellular nucleotides were compared to virus productivity to determine which of them shared a relationship. Only total adenylate, ATP, and AEC had a significant r value and could be correlated to virus productivity. In addition, the NTP ratio, according to the formula ([ATP] + [GTP])/([UTP] + [CTP]), has been shown to be a measure of metabolic energy (ATP and GTP) versus the growth potential (UTP and CTP) (Ryll et al. 1992). However, this ratio did not share any relationship with virus production.

The plot of [ATP] against virus production revealed a distinct relationship between the two. The [ATP] at the time of infection and specific productivity of virus was *hyperbolic* for 2 days post-infection and *sigmoidal* at 4 days post-infection. This shows that the [ATP] at the time of infection alters not only the total virus production of the system but also the production *rate* during the period where contributions by secondary infections do not play as great a role. Since ATP is an excellent indicator of cellular metabolism it is reasonable to assume that this is due to an altered physiological state of the cell. Glucose depletion has been shown to have a severe impact on protein synthesis (Swedes et al.

1975; Walker-Simmons et al. 1977; Edwards et al. 1979) by activation of AMP-activated protein kinase which inhibits protein synthesis (Hardie et al. 2001). Therefore it is likely that the lower viral output in the ATP-depleted cells can be attributed in part by lowered protein production in the cell.

The sigmoidal relationship between [ATP] and total virus production supports the hypothesis that there is an ATP threshold. A drop below this threshold (Km of 3.2 fmoles/cell and 3.3 fmoles/cell for ATP and total adenylate, respectively) results in a significant loss in productivity. This Km value is supported by other studies that have shown a similar loss in virus productivity in cultures with ATP levels below this Km (Nadeau et al. 2002). In that case, 293 cells that had intracellular ATP levels of 1-2 fmoles/cell during the infection had a 4-fold reduction in viral titer whereas cells with >4 fmoles/cell had optimal productivity. The plateau effect also indicates that increasing ATP levels by feeding would not necessarily result in an increase in virus production. This has been supported in other studies showing that feeding during the infection process with glucose and glutamine does not result in an increase in virus titer (Wu et al. 2004). Furthermore, increasing glucose and concentrations higher than 25mM in the medium would be toxic due to oxidative stress on the cells (Kannan et al. 1994), or lead to the crabtree effect. The crabtree effect results in the formation of lactic acid as the cell responds to the increase demand of NAD for the oxidation of glucose when glucose levels are elevated.

The AEC profiles of the cultures revealed a negative correlation to virus production. The AEC of the most distressed culture (medium 1) maintained an AEC of > 0.90 for the majority of the infection, whereas the other cultures had a sharp decrease to ~ 0.5 2 days post-infection. Thus a higher AEC during the infection period did not result in an increase in virus production but rather a *decrease*. The plot of AEC against virus productivity showed a negative sigmoidal relationship. A critical value of AEC is reached (0.97) where productivity is significantly decreased. This was the complete opposite of what was expected as high AEC is usually correlated to a healthy metabolic state (Atkinson 1968). However, given that cells under nutritional stress will degrade

their adenylate pool (confirmed in our data with the nutrient deprived cultures) this suggested that these cultures had high activity of AMP deaminase to maintain the high AEC. However, data points were not present on the declining portion of the curve, so it is not clear if there is a level of AEC (between 0.96 and 0.97) that leads to an intermediate degree of virus productivity. This needs to be explored with further experiments.

These results suggest that using AEC as an indicator of a healthy metabolic state would be misleading. We have found that the AEC can be high when the cell is under nutritional stress or during stationary phase. Furthermore, a *lower* adenylate energy charge does not necessarily mean the cell is under arrested growth or nutritional stress. We found the AEC for actively growing cells to be low because they were utilizing ATP to fuel cell growth (data not shown). Therefore, with respect to virus productivity, the AEC could be used as a *negative* indicator of the culture's potential.

8.4. Conclusion

The results show that the internal [ATP], total [adenylate], and AEC at the time of infection had a significant effect on reovirus production from Vero cells. A correlation between virus production and [ATP] and total [adenylate] has been determined as a sigmoidal relationship, indicating that a threshold level exists. AEC had a negative sigmoidal relationship with respect to virus productivity. A Km of 3.2 fmoles/cell ATP and 3.3 fmoles/cell adenylate must be maintained to prevent a loss in productivity. AEC levels above 0.97 appear to lead to a decrease in virus productivity.

These are useful parameters to monitor for optimization strategies. They can be used to avoid infecting cultures with lowered physiological states which would lead to a decrease in overall productivity. In addition, monitoring these levels can be used to predict virus output from a culture. Such parameters are very important for large-scale production of viruses, such as in vaccine production.

CHAPTER 9

Conclusions, Comments and Further Work

9.1 The aims of this thesis were to:

i. Evaluate and improve the performance of M-VSFM to support the growth of Vero cells. (re: Chapter 3)
ii. Test the hypothesis that M-VSFM can support the growth of other industrially important cell lines/strains. (re: Chapter 4)
iii. Test the hypothesis that a serum-free culture system of Vero cells would be suitable for the propagation of reovirus. (re: Chapter 5)
iv. To test the hypothesis that virus production is not linked to growth state and that reovirus has an impact on the physiological state of the cell. (re: Chapter 6)
v. To determine the relationship between virus production and intracellular nucleotides levels. (re: Chapter 7 and 8)

9.2 Evaluate and improve the performance of M-VSFM to support the growth of Vero cells. (re: Chapter 3)

The purpose of this study was to evaluate and improve the original M-VSFM serum-free medium for Vero cells. The formulation supported growth comparably to standard serum-containing media, such as 10% FBS-DMEM, giving normal doubling times of ~ 24 hours.

Improvement of the medium was achieved by testing various components known to be stimulatory to mammalian cells. Of the components tested in this study EGF, PH1, and AH1 were shown to enhance the growth of Vero cells in M-VSFM. Since the medium was to be maintained chemically defined (thus alleviating batch to batch variation) and animal product free (contamination threat) only the recombinant EGF was added to the

final formulation, M2-VSFM. The other two components, even though they enhanced the final cell yield, would not suit the requirements of the medium. The PH1 and AH1 are not chemically defined and may add variation in the performance of the medium. Although the plant hydrolysate PH1 does not pose any significant problem with regards to contaminating agents the same cannot be said for AH1. Being an animal tissue lysate it raises concern that it could potentially contain adventitious agents, such as viruses and prions. This would make the medium unsuitable for the production of human therapeutics.

However, that being said it may serve the animal vaccine industry. Many of these pharmaceuticals produce animal vaccines in serum-based formulations (usually inexpensive low-grade serum). It may not be economically feasible for such companies to use a medium such as M2-VSFM due to the increase in the end cost of the product (animal vaccines are usually quite inexpensive compared to human vaccines). However, the discovery that AH1 significantly improves cell growth may suggest pursuing the development of an alternate formulation for this purpose. A medium of low serum (<1.0%) to provide growth factors, supplemented with AH1 (adds only cents/L), would serve to be a suitable and inexpensive alternative to the high serum formulations that are presently used. In most cases the serum accounts for over 90% of the final cost of the medium. Preliminary studies (not shown) have shown success with a low serum (0.5%)-DMEM with 2.0 g/L of AH1.

The fact that AH1 alone had significantly increased the final cell yield warrants further study of its components. Hydrolysates are a rich source of amino acids as well as di and tri-peptides. Elucidating which of the amino acids is being heavily consumed may provide an answer to which of them is growth-promoting in the culture. However, the effect may be due to one or more of the peptides as they have been documented to enhance cell growth (Franek et al. 2000; Franek et al. 2002). A fractionation of the AH1 to determine which of the components is growth promoting would be useful. Once determined a synthetic source of the peptides or amino acids could be supplemented into the medium and thus maintain its chemically-defined nature.

The Vero 76 cell clone was easily propagated in the M2-VSFM formulation. However, the Vero E6 cell clone is unable to grow without supplementing the medium with AH1. Since this cell line is not routinely used in industry for the large-scale production of human therapeutics it may be a suitable medium. These cells are primarily used at bench-top scale to produce viruses for research and thus the presence of animal products is not of great concern.

The new formulation, M2-VSFM, now stands as an excellent serum-free medium for the growth of Vero cells. It is free of any animal-derived components and has a low content of non-animal derived protein (90 μg/L). Vero cells quickly adapt to the medium from serum-based formulations and the growth profile can be maintained for many passages (data not shown). The growth performance is comparable to commercially available formulations from reputable suppliers, such as Gibco and JRH. For all of these reasons this medium meets the needs of the pharmaceutical industry for the production of human therapeutics. It has already shown to be suitable for the production of viruses that are candidates for cancer therapies (reovirus) and future vaccines (Enterovirus 71 and Japanese Encephalitis virus) (Butler et al. 2000; Wu et al. 2002; Wu et al. 2004).

9.3 Test the hypothesis that M-VSFM can support the growth of other industrially important cell lines/strains. (re: Chapter 4)

The major aim of this paper was to adapt the M-VSFM formulation to suit other industrially-important cell lines. The cell lines tested were BHK, MDCK, and MRC-5. All of these cell lines are used in the manufacture of human or veterinary vaccines constituting a significant portion of the biotechnology industry.

In all cases a serum-free formulation that could support growth was developed for each cell line, some more successful than others. The BHK cell medium propagated the cells with comparable growth rates as the control for 3 passages. The MDCK medium, with

modifications, supported growth of the cells for >10 passages and was comparable to serum-supplemented media. The MRC-5 serum- free medium actually outperformed the control during the first 2 passages, reaching much higher cell numbers. However the cultures could not be maintained for further passages. The M2-VSFM formulation did not have to be altered for L-929 cells and had both higher cell growth rates and final cell densities than the control for the 5 passages that it was tested.

However, there remain some concerns with these formulations. The presence of animal-derived products in the manufacturing processes of human therapeutics is foremost in the minds of most biotechnology companies. This is of great concern as it opens the door to possibilities of transmitting contaminating agents, such as viruses or prions, to the final product (Merten 1999). Of the formulations developed in this study, the MRC-5 medium had to be supplemented with AH1. Since AH1 is an animal-derived product it is unlikely it would be commissioned for use in the production of a human vaccine. This would necessitate its removal from the formulation. Since the performance of the medium is solely dependent on AH1 it would be necessary to determine which components are growth promoting. Once they are determined, alternate sources (such as synthetic) could be used in its place.

The MDCK formulation was supplemented with a plant hydrolysate, PH1. Although plant-derived supplements are not of great concern with health regulatory bodies when used in manufacturing pharmaceuticals, they do pose a problem in terms of batch to batch variation. The very nature of the hydrolysates makes it near impossible to ensure homogenous batches which could cause a culture to perform differently in the future (lower growth rate, etc). Determining which amino acids/peptides are responsible for the enhancement of cell growth would be worthwhile so that the medium could be chemically-defined.

Overall these results show that variants of the M-VSFM formulation can support the growth of the BHK, MDCK, MRC-5, and L-929 cell lines, although with varying success. Many of these formulations could support the growth for many passages (>10

for MDCK, > 5 for L-929), but others could only be propagated for a limited amount of time (MRC-5 for 2 passages, BHK for 3). These formulations may be useful for the biopharmaceutical industry as production media (last-stage production step) or as growth media. Since the nutritional requirements of these cell lines were satisfied (for the most part) by M-VSFM it is likely that other cell lines could be propagated in this medium as well. It would be worthwhile to test other industrially important cell lines in M-VSFM, such as WI-38 and 293 cells, to expand its usefulness.

9.4 Test the hypothesis that a serum-free culture system of Vero cells would be suitable for the propagation of reovirus. (re: Chapter 5)

This study examined how a serum-free culture system would support the production of reovirus. A previous study showed that Vero cells were a suitable cell line and that microcarrier cultures supported high titers (Berry et al. 1999). Utilizing a serum-free medium was the next logical step in developing a process suitable for industry. In this study two types of reovirus were propagated successfully in M-VSFM, a serum-free medium designed in our lab. In both stationary and microcarrier cultures (Cytodex-1) of Vero cells high titers of virus were attained. The titers were comparable to standard serum-supplemented media. Furthermore, this system was superior to the standard cell culture system used for producing reovirus using the mouse L-929 cell line. The productivity in the serum-free Vero system was significantly higher (> 260%) than those reached in agitated cultures of L-929 cells.

This culture system is best suited for the large-scale production of reovirus. First of all, the Vero cell line has been licensed for the production of many vaccines and would therefore not encounter any significant regulatory blockades (Montagnon et al. 1984; Montagnon 1989; Montagnon et al. 1998). Secondly, the serum-free formulation used in this experiment does not contain any animal products and is completely chemically defined. This would alleviate the concerns of using serum in the production of human therapeutics due the possible threat of introducing contaminants (Asher 1999; Merten

1999). In addition, since it is chemically defined there would not be any batch to batch variation in performance of the medium, which is a common problem with serum-supplemented cultures. Lastly, microcarrier cultures have been shown to be suitable for large-scale vaccine production, greater than 1000 litres (Montagnon et al. 1984; Montagnon et al. 1999). This is convincing evidence that this culture system would work well in large-scale operations.

9.5 To test the hypothesis that virus production is not linked to growth state and that reovirus has an impact on the physiological state of the cell. (re: Chapter 6)

This study revealed a contradictory finding to what is generally accepted. The common view is that cells in exponential growth phase will have an elevated "metabolic state" and therefore produce more virus than cells in a stationary growth phase. This seemed logical if one assumes two things; (a) that virus production will increase incrementally with energy state; and (b) energy state is always lower in cells that are not dividing. The first assumption was refuted in the study in Chapter 8 which showed a sigmoidal relationship between metabolic energy (ATP and total adenylate) and virus production. The second assumption was refuted in this study where cells were shown to produce the same amount of virus given their energy states are comparable, independent of growth state.

This study examined the production of viruses in cells in two different growth states; stationary and exponential. The cultures had comparable virus production profiles and this was attributed to having identical ATP levels at the time of infection. The energy levels after the day of infection did not have any impact on virus production as the cells in stationary phase had considerably higher AEC and [ATP] than the exponentially growing culture. These results supported the previous conclusion that energy levels at the time of infection are the major contributing factor to overall productivity.

Although this data is compelling it should be tested on other growth states (such as lag phase) and at different points during the growth phase (i.e.: 1, 2, 3 days into exponential growth). Also, it would be interesting to monitor protein production in the stationary phase culture (under the same conditions as the previous experiment) to determine if its machinery as active as that in exponential phase.

The dogma that cells that have arrested growth do not have sufficient energy stores or active machinery to produce virus is misleading. In the case of reovirus, as long as the cell is actively metabolizing and can supply the proper substrates for the virus to replicate there will not be a loss in productivity. Therefore growth state should not be used as an indicator of a healthy metabolic state and should not be used to determine the optimal conditions for a virus infection.

It was also important to determine how much of the host cell's energy is tapped by reovirus upon an infection. This experiment was designed to monitor the NTP's in an infected and mock-infected culture. The results showed what was expected – a drain on the host's energy stores. There was a higher utilization of NTP's in the infected cultures and the cells had an average drop of 28% of both ATP and GTP compared to a mock-infected culture. The total adenylate levels also decreased by 10% but it is not known whether this was due to decreased *de novo* synthesis of adenylate or degradation of AMP to maintain a higher AEC (Hardie et al. 1999). The most surprising finding is that neither the infection nor the drain on the cells energy had any effect on the growth of the culture. However, this may be affected if the cells were infected with a higher MOI than 5.0.

These findings allowed for an empirically derived calculation of the amount of ATP used to produce a viable virion (plaque forming unit). Assuming that the difference in energy between the two cultures is strictly due to the requirements to produce the virus, the average ATP usage is 0.022 fmoles ATP/PFU. There has not yet been a report on the experimental amount of ATP used by a virus to replicate itself so it cannot be compared to other studies. It comes close to the theoretical amount of ATP to generate a virus

(0.0070 fmoles ATP) but without the particle to PFU ratio it is not possible to make an exact comparison.

It is interesting to observe that reovirus, at this MOI, does not completely drain the host's metabolism for its own purposes. It may be that at higher MOI the metabolism of the cells will become so overwhelmed that it will affect cell growth. Indeed, using higher MOI's does not lead to increased virus output and in some cases leads to lower virus production (Berry et al. 1999). It would be worthwhile to know at what MOI the virus infection affects the metabolism of the cell significantly enough to slow down growth and whether this leads to a decrease in productivity. It may be that lower MOI's lead to increase productivity because their effect on the host's metabolism is minimal. Indeed, increased productivity has been found with MOI's less than 0.5 with reovirus compared to higher MOI's (Berry et al. 1999; Jung et al. 2004). In these cases the drain on the host's energy supply may not have been significant, or even noticeable since 60% of the cells would be uninfected (compared to 99.4% at MOI=5.0 PFU/cell) (Condit 2001). A study examining the relationship between MOI and the drain on the cells metabolism may be worthwhile and may point to clues for optimization.

9.6 To determine the relationship between virus production and intracellular nucleotides levels. (re: Chapter 7 and 8)

A direct relationship between intracellular nucleotides and virus production was determined in this study. The method used was to lower the energy state of the cells by lowering the glucose and glutamine concentration in the medium. This allowed for a spread of energy states while maintaining viability of the cultures. Infection of these cells in different energy states revealed interesting information. The first was that the total intracellular adenylate and ATP at the time of infection was the greatest contributing factor to the overall productivity of the system. Secondly, a threshold level of these nucleotides exists with respect to virus production. Lastly, the AEC of the cells had a negative sigmoidal relationship to virus productivity.

The results show that the internal [ATP] and total [adenylate] at the time of infection had a significant effect on reovirus production. The levels of these nucleotides after the infection did not impact the virus output. From 24 hours post-infection on the [adenylate] and [ATP] were comparable between cultures that had different productivity (ex: medium 2 and medium 3). This agreed with the results from another study that had found increased virus productivity (adenovirus) in cultures with higher ATP levels (Nadeau et al. 2002). This study examined different medium formulations to assess the impact on energy metabolism and virus production. The cultures with the higher intracellular [ATP] (> 4 fmoles/cell) had almost an 8-fold increase in virus productivity compared to the cultures with lower intracellular [ATP] (< 2 fmoles/cell). The levels of ATP were not reported for the first 20h of the infection period, however, but the ATP levels after this time were considerably different up to 60h post-infection. The findings in this thesis suggest that the adenylate and ATP levels at the time of infection are the greatest influence on the overall productivity of the system. Even though the Nadeau paper did not report ATP levels preceding 20h post-infection it is possible they were already considerably different earlier on (i.e.: 1 hour post-infection). In any case the ATP levels were different in the cultures during the first 24 hours of the infection and this lead to different virus productivity, which agrees with the findings in Chapter 8.

Comparing results between different virus production systems is difficult as the viruses are inherently different in how they affect the host cell. Adenovirus is known to inactivate certain apoptotic pathways, such as P53 (Shenk 2001), whereas present knowledge of reovirus function indicates it does not have any such effect on the cell (Nibert et al. 2001). This may contribute to a longer viability period of the infected cells with adenovirus than reovirus, thus enabling for a longer production time. However, the similar findings suggest that the host cell's [ATP] will govern productivity regardless of the type of virus studied.

The plot of virus production as a function of intracellular [ATP] or [adenylate] showed a clear sigmoidal relationship between the two. A threshold level of these nucleotides

exists with a Km of 3.2 fmoles/cell and 3.3 fmoles/cell for ATP and total adenylate, respectively. These levels must be maintained to prevent a loss in productivity. It was originally expected to be a linear relationship but this was not the case. These results indicate that it is not simply a "more is better" type of relationship but an "on/off" relationship. If the cells are under nutritional stress they halt protein production and thus virus production (Swedes et al. 1975; Walker-Simmons et al. 1977; Edwards et al. 1979). This will not apply in every case and only those circumstances where the cells may encounter such conditions. They are routinely encountered in production systems where cells are grown to high densities just prior to infection. Nutrients are quickly depleted, and if not monitored, leads to a decrease in productivity. This is most likely where this adenylate threshold is reached where virus production is affected. The Km data was supported by results of other studies of virus productivity where cells with ATP levels of 1-2 fmoles/cell had a 4-fold reduction in specific viral titer (Nadeau et al. 2002).

There is a negative sigmoidal relationship between AEC and virus production. This has been a useful parameter to indicate physiological state (Atkinson 1968) and higher AEC usually correlates to a actively growing/metabolizing cell. However, it does not hold true with respect to virus production. High AEC can be maintained in cells with low levels of adenylate or under nutritional stress (Yoshino et al. 1981; Hardie et al. 2001) and even those in a healthy metabolic state (Atkinson 1968). Virus production was not higher in cells with higher AEC but rather reached a threshold of 0.97 where virus productivity was affected. Therefore, a high AEC (>0.97) is not necessarily a good indicator of a healthy state of the culture and can actually indicate it will not be productive with respect to virus production. This can be a useful parameter to monitor in virus production systems.

These findings can be useful for optimization studies in reovirus production. It can be used to avoid infecting cultures with lowered physiological states which would lead to a decrease in overall productivity. In addition, monitoring these levels can be used to predict to virus output from a culture.

9.7 Most significant findings in my thesis

I would have to say that the most important contributions of my thesis include: (a) the development of serum-free formulations for MDCK (> 10 passages), BHK (3 passages), and MRC-5 cell lines (2 passages); (b) the development of a serum-free culture system to produce reovirus that is suitable for industrial-scale production; and (c) the link between the host cell's energy state and virus production. The first two projects were more process development based research. This type of research is very important for industry. The development of new serum-free formulations can help to make existing or new therapeutic production processes safer and more reliable than they are today. The second project was an application of these formulations to produce a virus that will have significant therapeutic relevance. This research is absolutely necessary if we, as researchers, want to provide the best and highest quality therapeutics for human use. It was satisfying to provide serum-free media for others in the field to produce other clinically relevant viruses (Wu et al. 2002; Wu et al. 2004). Although these first two studies were very important from an industrial standpoint it was the latter which was the most scientifically interesting. The first two projects gave me the skills and groundwork to answer a deeper scientific question. What was the relationship between energy state and virus production? Although it was commonly thought that there was a link between the two it was never fully defined and was often just assumed, which is never good science. The study revealed a governing principle of how much a virus relies on the energy supplies of the host cell to produce itself. A threshold limit is reached where the cell either shuts down protein production machinery or the viruses own replication machinery is affected by the energy levels. The energy threshold limit (Km of ATP and adenylate), along with decoupling growth state and energy state with respect to virus production, were important discoveries. Perhaps manipulating these adenylate levels through media formulation may prove to be useful in optimizing virus production from cell culture systems. It is important to understand these underlying fundamentals if we are ever to improve cell culture processes.

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APPENDIX

A1: Sample calculation of a paired *t*-test

A *t*-test determines if the mean values of two data columns are significantly different by testing the hypothesis that the means of the two columns are equal. A paired *t*-test requires columns of equal length, since the data is assumed to be before-and-after data on the same subjects. The tests were calculated using SigmaPlot 2000 software.

Example:

The growth profiles of MDCK cells in M2-VSFM + Hy-Pep was significantly different (higher) than cells grown in UltraMDCK.

Days	M2-VSFM + Hy-Pep	UltraMDCK	d	d^2
0	20000.0000	20000.0000	0	0
1	37520.0000	27520.0000	10000	1.00E+08
2	66800.0000	56800.0000	10000	1.00E+08
3	157200.0000	147200.0000	10000	1.00E+08
4	269200.0000	181600.0000	87600	7.67E+09
5	339200.0000	218800.0000	120400	1.45E+10
6	346000.0000	268000.0000	78000	6.08E+09
Sum			316000 (S1)	2.86E+10 (S2)

Where d = difference between paired samples and n = number of paired groups = 7

S1 = sum of d S2 = sum of d^2 $\bar{d}=d/n = 45142.857$

$$(sd)^2 = \frac{(n)(S2) - (S1)^2}{(n)(n-1)} = \frac{(7)(2.86 \times 10\text{^}10) - (316000)^2}{(7)(6)} = 2.3891 \times 10\text{^}9$$

$$T = \frac{\bar{d} - d0}{\sqrt{sd}/\sqrt{n}} = \frac{45142.857 - 0}{\sqrt{2.3891 \times 10\text{^}9}/\sqrt{7}} = 2.444$$

T critical for 95% confidence level ($P < 0.05$) = 2.447 (Value obtained from software package)

T < T critical, therefore the samples are different. The growth profile of MDCK cells in M2-VSFM + Hy-Pep is higher than in UltraMDCK.

A2: Sample calculation of Pearson's correlation coefficient, r.

Pearson correlation calculations are based on the assumption that both X and Y values are sampled from populations that follow a normal (Gaussian) distribution. The analysis tests the null hypothesis that the two data sets are not correlated to one another. The "r value" is a number between -1 and 1 where values close to these numbers indicate a perfect positive or negative correlation between the data sets. A value of 0 indicates there is no correlation between the two. An example is shown below:

Ex: Chapter 7, relationship between intracellular ATP and virus productivity:

ATP concentration	Max PFU/cell
1.68	4.43
2.2	4.23
2.64	8.7
2.78	6.13
3.15	12
3.15	16.1
3.18	59
3.28	59
3.96	78
4.35	84
4.64	64
4.83	81
4.32	74
4.78	80
5.69	55

The r value was calculated using Pearson's equation according to the formula:

$$r = \frac{\sum XY - \frac{(\sum X)(\sum Y)}{n}}{\sqrt{\left(\sum X^2 - \frac{(\sum X)^2}{n}\right)\left(\sum Y^2 - \frac{(\sum Y)^2}{n}\right)}}$$

where X = ATP concentration

and Y = Max PFU/cell

and n = number of data points

The r values were calculated using Microsoft Excel spreadsheet to give 0.74. The critical r value needed to refute the null hypothesis for 12 degrees of freedom (n-2) is 0.623 with a p value of 0.01 (99% confidence).

Since $r > 0.623$ at 0.74 the null hypothesis is **rejected** with 99% probability that the two data sets are not correlated (ie: they are correlated).

List of publications from Ph.D. thesis:

Papers published or in progress:

(1) A. Burgener, K. Coombs, and M. Butler. *Intracellular ATP and total adenylate concentrations are critical predictors of reovirus productivity from Vero cells.* Submitted. April 2005.

(2) A. Burgener and M. Butler. *"Cell Culture Media".* Book chapter in "Cell Culture Technology For Pharmaceutical And Cell-based Therapies". Sadettin S. Ozturk, Wei-Shou Hu, editors. Marcel Dekker publisher. 2005. In press.

(3) Burgener, A., Patrick, M., Coombs, K., Moffatt, D., Huzel, N. and Butler, M 2001. *The modification of a serum-free media formulation for the production of reovirus and the growth of Vero, MRC-5, MDCK and BHK cells.* In "Animal Cell Technology: From target to market" (Lindner-Olsson, E, Chatzissavidou, N.and Lullau, E. eds.) European Society for Animal Cell Technology 17, 200-203. 2001.

(4) M. Butler, A. Burgener, M. Patrick, M. Berry, D. Moffatt, N. Huzel, N. Barnabe, and K. Coombs, University of Manitoba. *Applications of a serum-free media developed for the growth of Vero cells and the production of Reovirus.* Biotechnology Progress. 16: p854-858. 2000.

Abstracts:

(1) A. Bugener. *Growth factor requirements for specific animal cell lines.* Cell Culture and Upstream Processing. IBC Life Science conference. San Diego. December 2003.

(2) A. Burgener, K. Coombs, and M. Butler. *The significance of intracellular [ATP] to reovirus production.* 6TH Annual "Protein Expression in Animal Cells" conference. Sept 11th, 2003. Mont Tremblant.

(3) A. Burgener. *The application of a novel serum and protein-free media formulations for the growth of mammalian cell lines and the production of biologicals.* 6TH Annual "Protein Expression in Animal Cells" conference. Sept 11th, 2003. Mont Tremblant. Industrial workshop session.

(4) M. Butler, N. Huzel, N. Barnabe, M. Berry, A. Burgener, D. Moffatt, M. Patrick and K. Coombs, University of Manitoba. *Application of a serum-free medium developed for the growth of Vero cells.* Poster abstract. Cell Culture Engineering VII Conference, Santa Fe, NM. February 2000.

(5) A. Burgener, M. Patrick, N. Huzel, D. Moffatt, K. Coombs, M. Butler. *Reovirus production from microcarrier cultures of Vero cells.* Canadian Society of Microbiology meeting, June 2000. Abstract. University of Manitoba.

(6) M. Butler, M. Berry, A. Burgener, M. Patrick, and K. Coombs, University of Manitoba. *Production of reovirus from microcarrier cultures of Vero cells.* American Chemical Society National Meeting, March 2000. Abstract