

Optimisation of Vero Cell Growth and Productivity in Microcarrier Cultures.

By

JOHN MICHAEL BERRY

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

DOCTOR OF PHILOSOPHY

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**OPTIMISATION OF VERO CELL GROWTH AND PRODUCTIVITY
IN MICROCARRIER CULTURES**

BY

JOHN MICHAEL BERRY

**A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial
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Abstract

John Michael BERRY. Ph.D., The University of Manitoba, May (1996). Optimisation of Vero Cell Growth and Productivity in Microcarrier Cultures. Major Professor: Dr. Michael Butler.

Mammalian cell fermentation for human therapeutics production is dominated by suspended cell lines due to the perceived difficulties in the culture of adherent lines. To date no one has reported the optimisation of adherent cell culture processes. This is unfortunate as adherent cells are preferable for producing virus vaccines and many secreted proteins. This study optimises the physical and medium parameters for adherent African green monkey kidney (Vero) cell growth in batch and refed cultures and assesses their application to virus vaccine production.

Adherent cells must attach to substrata such as microcarrier beads to grow and two microcarriers were directly compared: solid polydextran Cytodex-1 and macroporus gelatin Cultispher-G. Batch Vero cultures were used to optimise stirrer speed, pH, dissolved oxygen tension, temperature, microcarrier concentration, cell inoculum concentration, bead precoating with attachment factors, medium formulation, serum type and concentration and nutrient concentrations for growth. The effects of several growth inhibitors were assessed. A novel serum-free medium for microcarrier culture was defined. Cultures were grown in spinner flasks and a 1.3 L Celligen bioreactor. The optima were used in daily refeeding and perfusion experiments to optimise the methods for high density cultivation. The effects of optimised growth conditions were assessed on reovirus infections.

These studies showed adherent cells could be easily cultured to cell yields $>2.00 \times 10^7$ cells/mL on either microcarrier. The amino acid serine was found to be absolutely required for high density cultivation on either bead type. Macroporous Cultispher-G beads offered a preferable environment for growth by protecting the cells from physical and chemical inhibition and by inducing more efficient nutrient consumption compared to solid Cytodex-1. Cultispher-G beads also produced higher (up to nine-fold in refed cultures) maximum cell/bead loadings. There were differences in several parameter optima for growth including pH, stirrer speed and medium nutrient concentrations, although, most parameter optima were similar. The Vero cells could grow in serum-free medium but require several very expensive components. Vero cells could be infected with reovirus particles when grown on either microcarrier type, although, Cytodex-1 were capable of giving higher virus titres compared to Cultispher-G. Optimisation of the multiplicity of infection could improve virus yields in perfused cultures with high cell to bead numbers.

Foreword

Ph.D. (L) *Philosophiae Doctor*. Doctor of Philosophy.

Doctor (L) Teacher, learned man.

Philosophy (L) *philosophia*. (2) Theory or logical analysis of the principles underlying conduct, thought, knowledge, and the nature of the universe.
(Webster's New World Dictionary, Second College Edition. Nelson, Foster and Scott. Toronto, Canada. 1970)

Most scientific disciplines can be broken down into theorists and experimentalists. Theorists do theory; experimentalists do experiments. But theory is meaningless without experiments and vice versa. The modern theorist is in effect an observer of everything, as theory is from Greek *theoros* - to observe. What theorists observe may suggest a train of thought indicating that if something as yet unknown is observed in the right way it will be seen. So the train of thought makes a prediction. The experimentalist determines whether or not the prediction is true. But experimentalists are always on the look out for things which were not predicted.

If someone believes something that on the face of it is unbelievable, they are cranks. In our own way, however, we are all cranks. One of the main requirements of human life is distinguishing the signal of the true from the noise of the fake (from information theory), then any noise which interferes with the signal is a serious matter. Moreover, if tolerance of noise becomes a habit, it can be a very serious matter.

Populus vult decepti, ergo decipiatur - people want to be deceived, therefore let them be deceived. The whole point of the scientific enterprise is that what is true is to be decided not by the opinion, majority or otherwise, but by the reproducibility of the observation and experiment and by the usefulness of the theory in pointing the way to new observations and new experiments.

Flanagan, D., *Flanagans Version*. (1989)

Chapter 1. Introduction

1. Introduction

1.1 Historical Background

True microbial cells, bacteria, fungi and yeasts, are used for the production of many compounds in the food, beverages, cosmetics and therapeutics industry. These uses increased as more scientific knowledge and expertise was attained in culturing them. However, there were few uses for mammalian cells until the biotechnology revolution - "the multidisciplinary science of manipulating organisms for the common good" (Kennedy, 1991) - in the early 1980's. Mammalian cells are now used for diverse applications such as biochemical research, cellular physiology, production of therapeutic or diagnostic compounds (Thilly, 1989), drug screening, targeting and toxicity (Gillies et al., 1986), skin grafting (Langer and Vasanti, 1993) and for artificial organs (Cunningham and Hodgson, 1992).

Table 1.1 lists the major breakthroughs which have occurred in the last 100 years in the field of mammalian cell technology (MCT). For further historical information see Paul (1975) or Butler (1991).

The uses for mammalian cells arose from the needs to research major human diseases and to manufacture therapeutic or diagnostic proteins to combat such diseases. Until the early 1980's it was perceived that production of therapeutics could be achieved more easily and economically using existing microbial technologies (Broad et al., 1991). This led the push to develop novel genetic engineering methods to introduce the required product genes into bacteria and fungi. At the time this approach caused several problems:

1. engineering the product genes into the cells was difficult due to expression vector size problems. Insulin, a small mammalian gene, was a rare success (Johnson, 1983).
2. product mRNA's were incompletely spliced due to cell processing differences (Lewin, 1990) which led to inactive products.
3. the cells could not generate disulphide bridges in proteins (Broad et al., 1991).
4. the cells could not correctly glycosylate the final proteins leading to inactive products

Table 1.1 - Listing of some of the **major historical events** which have shaped **mammalian cell culture** during the **last 100 years**. A more complete listing is available in Butler (1991).

Year	Researcher/s	Description of Development
1897	Loeb	Grew isolated blood cells in blood and plasma
1907	Harrison	Grew frog nerve cells in 'hanging drop' lymph clot
1912	Carrel	Introduced surgical aseptic techniques
1923	Carrel	Developed first true cell culture flask
1949	Enders	Grew polio virus using human embryonic cells
1952	Gey	Isolated the first continuous cell line - HeLa human cervical carcinoma cell line from Henrietta Lach
1955	Eagle	Developed the first chemically defined basal medium
1961	Hayflick and Moorhead	Showed human fibroblast cells WI-38 grew for a finite lifetime in culture
1965	Ham	Developed the first serum-free medium
1967	van Wezel	Grew adherent fibroblast cells on DEAE-sephadex A-50 microcarriers
1975	Kohler and Milstein	Developed the hybridoma cell system for monoclonal antibodies
1981	Eli-Lilly Inc. (Refer to Johnson, 1983)	Obtain drug license for the first bacterially produced recombinant product - human insulin
1982	Wellcome Foundation Ltd. (Refer to Finter et al., 1984)	Obtain drug license for interferon produced in human carcinoma Namalwa cells
1987	Genentech Inc. (Refer to Lubiniecki et al., 1989)	Obtain drug license for tissue plasminogen activator produced by recombinant CHO cells

(Pennica et al., 1983, Berman and Laskey, 1985, Arathoon and Birch, 1986, Rhodes and Birch, 1988 and Parekh and Patel, 1992).

There is recent evidence which suggests that these problems can be overcome for certain products. The use of cDNA technology has allowed mammalian genes for granulocyte colony stimulating factor (G-CSF), human growth hormone (hGH) and α -interferon to be produced in *E.coli*. None of these proteins are glycosylated. These proteins have also been shown to be active *in vivo* without glycosylation providing they are correctly folded. However, the half lives of unglycosylated proteins is reduced and they cause an increased risk of adverse events following treatment (Dorr, 1993, Hovgaard et al., 1993, Hussein et al., 1993 and Grossmann et al., 1995). Other proteins such as erythropoietin (EPO) have been shown to have activity *in vitro* but not *in vivo* when in the unglycosylated form (Tsuda et al., 1990, Sytkowski et al., 1991 and Higuchi et al., 1992). The N-linked glycosylation profiles appear to be involved in this effect (Wasley et al., 1991). Hence, the problems in expressing proteins in bacterial and fungal systems have not been completely overcome. For this reason there is still a large research effort into the technology of using mammalian cells for production purposes. There are several good reviews of important topics in MCT such as Spier and Griffiths (1985-1992), Lubiniecki (1990), Butler and Dawson (1992) and Morgan and Darling (1993).

Several commercial products are now manufactured using MCT including Genentech® Activase™ (tissue plasminogen activator engineered into Chinese hamster ovary (CHO) cells) for the treatment of myocardial infarctions, Amgen® Epogen™ (erythropoietin engineered into CHO cells and the world's first \$1 Bn. US. selling biotechnology drug) for the treatment of anemia, Wellcome Foundation® Wellferon™ (α, β -interferon produced from sendai virus infected Namalwa carcinoma cells) for the treatment of hairy cell leukemia and various virus vaccines (human polio and rabies from human lung fibroblast MRC-5 and African green monkey kidney cells). Various therapeutic monoclonal antibodies from murine and rodent hybridoma cells have also been developed but these have yet to achieve their expected potential (Abrams, 1992). The success of these products increased the research emphasis in MCT. The following

Chapters should give an indication of what can be achieved when mammalian cells are used to their full potential.

1.2 Mammalian Cell Lines

Choosing the host cells and the genetic engineering system is fundamental to success of MCT projects. There are many cell lines isolated from lung, kidney, liver, skin, muscle, nerve, cartilage and blood. These have been cloned and stored in cell banks with the American Type Culture Collection (ATCC) and the European Collection of Animal Cell Culture (ECACC). The major cell lines have been listed by Butler and Dawson (1991) and complete listings of all stored cells available for use are found in the ATCC/ECACC catalogues.

There are many metabolic pathways involved in mammalian cell growth but the main ones are glycolysis, tri-carboxylic acid (TCA), pentose phosphate, gluconeogenesis and glutaminolysis. Diagrams of these pathways are not included in this Thesis but can be found in biochemistry texts (e.g. Voet and Voet, 1990). Cell growth nutrient requirements will be discussed below (1.2.3.1).

1.2.1 Hybridomas

Hybridomas are hybrids of myelomas (i.e. fully transformed) (e.g. SP2/0, NS1 or P3X63Ag8) and lymphatic B cells secreting antibodies of a specific, required type (hence, monoclonal antibody (MAb)), developed by Kohler and Milstein (1975). They are the most widely used mammalian cells, as they grow in suspension so are easy to culture. The MAb's are useful for diagnostic kits (ELISAs) and targeting of cancer cells.

The ease of hybridoma culture has lead to them being intensely researched for growth, productivity and for the development of novel production technologies (Murakami et al., 1982, Winther, 1987, Jager et al., 1988, Kovar and Franek, 1987, Schneider and Veithin, 1990, Bibila et al., 1991, Lang et al., 1991, Toyoda et al., 1991, Kessler et al., 1993, Mercille et al., 1993 and Xie and Wang, 1994^a and ^b).

1.2.2 Chinese Hamster Ovary Cells

CHO cells have also been widely used as hosts for engineering product genes into. Indeed, 12 of the 14 currently licensed human therapeutics from MCT are produced in CHO cells (Lubiniecki and Lupker, 1994). Most CHO cell systems utilise the dihydrofolate reductase (DHFR) cloning and gene amplification system (Egrie, 1990, Hayter et al., 1991, Gu et al., 1993 and Hansen and Emborg, 1994), described by Kaufman et al. (1985). DHFR systems have FDA and WHO licenses for tPA (Lubiniecki et al., 1989) and erythropoietin (Egrie, 1990). CHO have also been engineered to produce humanised MAb's (Page and Sydenham, 1991).

CHO cells are simian virus 40 (SV40) transformed and grow either adherently or in suspension culture according to the clone and medium formulation used. The suspension cell growth is the major reason for their commercial exploitation, as the technologies for suspension culture are well understood.

CHO have also been widely used in media, process and molecular biology research (Smiley et al., 1989, Hayter et al., 1991, Reiter et al., 1991, Tsao et al., 1992, Ogata et al., 1993 and Ohlson et al., 1994).

1.2.3 Adherent Cells

The majority of the isolated cell lines including most primary and many immortalised cells require adhesion to surfaces for growth. Commercially significant adherent cells include African green monkey kidney (Vero) and baby hamster kidney (BHK) cells for human and veterinary virus vaccine production (Spier and Whiteside, 1976, Mered et al., 1980 and 1981, Montagnon et al., 1981, Radlett et al., 1985, Beale, 1992 and Mendonca et al., 1993). These are SV40 transformed but the transformation did not completely remove anchorage dependent cell growth. The generation of Vero cells was described by Yasumara and Kawakita (1963). Vero have been licensed for human polio and rabies vaccine production, as the cells do not shed contaminating SV40 viral particles during growth (Heraud, 1992). BHK cells are commercially used for veterinary vaccines such as foot and mouth disease (Shevitz et al., 1990 and Beale, 1992).

Vero cell processes have been scaled-up to 1,000 L in bioreactors (Montagnon et al., 1981) using microcarrier technology (van Wezel, 1967) discussed below (1.3).

Adherent growth has caused culturing difficulties and resulted in less commercial and research exploitation of such cell lines. The culturing difficulties include how to increase the culture volume whilst providing a suitable surface for growth, how to transfer oxygen to the cultures without damaging the cells or surfaces and the formulation of growth media. The methods of culturing anchorage dependent cells are discussed below (1.2.3.2). Vero cells are mostly used as controls for testing novel growth supports and bioreactor technologies (Clark et al., 1980, Mered et al., 1980, van Oss et al., 1983, Billig et al., 1984, Nahapetian et al., 1986, Lindner et al., 1987 and Altankov et al., 1991). These will be discussed below (1.3).

BHK suspension cell clones have been isolated and used for the commercial production of certain FMDV strains incapable of infecting the adherent cells (Radlett et al., 1985). However, no one has isolated a suspension culture Vero cell but they can grow as cell aggregates (Litwin, 1992), using the methods of Tolbert et al. (1980) for SV40 transformed murine 3T3 cells.

1.2.3.1 Nutrient Requirements

Mammalian cells require complex basal medium formulations incorporating trace elements, mineral salts, D-vitamins, D-carbohydrate and L-amino acids dissolved in high purity water. Glucose is the normal carbohydrate, although it can be substituted by fructose, galactose, maltose and mannose for Madin-Darby canine kidney (MDCK), Vero, HeLa, rodent insulinoma, murine L and 3T3 cell growth (Eagle et al., 1958, Leibovitz, 1963, Rheinwald and Green, 1974, Crespi et al., 1981, Barngrover et al., 1985 and Kriat et al., 1992). There are three metabolic pathways for glucose (i.e. glycolysis, TCA and pentose phosphate pathways) which provide energy and anabolic substrates for cell growth (e.g. adenosine triphosphate (ATP), as an energy store, reduced nicotinamide adenine dinucleotide phosphate (NADPH), for endergonic reactions of membrane component formation or ribose for nucleic acids) (Wice et al., 1982).

Amino Acids. Mammalian cells can not synthesise all the amino acids independently without essential precursor amino acids in the medium. Glutamine is the most important nutrient for ATP generation and ascites tumour, human diploid and HeLa cell growth (Rabinowitz et al., 1956, Griffiths, 1972 and Reitzer et al., 1979). Amino acids can also yield glucose via gluconeogenesis, if the glucose concentration is limiting, to maintain cellular nucleotide pools (Ryll et al., 1994).

Mitogens. Many other components are required to stimulate cell proliferation such as hormones and growth factors. These components are referred to as mitogens. Basal medium is usually supplemented with animal serum of various types and origins to supply glycosylated proteins, carbohydrates and trace elements (Barnes et al., 1984). Each cell line requires different mitogens and the responses to the growth factors and hormones remain incompletely understood although they are believed to either increase the availability of nutrients (McKeehan and McKeehan, 1980) or cause protein kinase phosphorylation and activation (Cherington, 1984).

Serum has been described as the "black box" of cell culture (Thilly, 1989), as it is highly undefined, prone to batch to batch variations and its composition differs according to source animal and dietary habits. Serum supplementation also causes purification and viral contamination problems (Hodgson, 1991). More recently bovine spongiform encephalopathy (BSE) caused by a prion particle has created chaos with regulatory agencies and could contaminate serum (Prusina, 1991 and Carr, 1996). These problems have generated the research into defined serum-free media for mammalian cell culture.

Serum-Free Media. Since BSE the regulatory agencies are demanding that cells be grown in defined serum-free media. This has proved a challenge as defined serum-free media are cell-line dependent due to differences in mitogen requirements. Full viral or chemical transformation is known to produce cells which either do not require protein growth factors (Temin, 1967, Holley et al., 1978, Scher et al., 1979 and Lanks and Kasambalides, 1980) or have more stable

regulatory proteins for cell division (Pardee et al., 1980). Hybridomas (fully transformed) were found to be easily cultured in serum-free media and could grow in basal medium supplemented with insulin, transferrin, ethanolamine and trace elements (Murakami et al., 1982) which increased research with hybridomas. Hybridomas can also be grown in protein-free medium (Schneider and Veithin, 1990). However, adherent cells have very complex nutrient requirements which has caused a lack of research into serum-free media for these cell lines.

Vero cells are transformed and passage indefinitely but they retain the growth factor and adherent characteristics of non-transformed cells. Thus, they are normally grown in serum-containing medium to supply the unknown mitogens and adherence factors. The serum mitogens include platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), hydrocortisone, fibronectin and fetuin. Many researchers have tried to formulate serum-free media by elucidating the growth factors of different cells. Several growth factors individually produce similar cell growth effects (Fisher and Lam, 1974, Engelhardt et al., 1977, Cherington et al., 1979, Bettger et al., 1981, Crespi et al., 1981, Giguere, 1982, Bradshaw et al., 1983, Cherington, 1984, Taub, 1984 and Grant et al., 1992). However, none of the serum-free media can grow adherent cells as well as serum containing medium which suggests they are deficient in vital components.

There are now several commercial serum-free and protein-free media for hybridomas (Gibco, 1995) but there are few available for fibroblast-like or endothelial cell types. However, Vero cells have been grown in Celox[®] TCM[™] supplemented media by the ATCC and in another serum-free media (Merten et al., 1994). Indeed, the commercial media suppliers are also developing serum-free media for Vero cells.

Costs of Serum-Free Media. Many mitogens are expensive to purchase in purified forms (e.g. fibronectin, EGF and FGF etc.). Indeed, fibronectin costs \$68.20 Cdn. for 1 mg and FGF costs \$13.69 for 1 µg (Sigma Chemical Co., St. Louis, MO, U.S.A., 1995). Most media using these components add up to 10 mg/L of fibronectin (Bottenstein and Sato, 1980, Bradshaw et al., 1983

and Schmid et al., 1992) and up to 5 µg/L of EGF or FGF (Weinstein et al., 1982, Wharton et al., 1982, Bradshaw et al., 1983, Forestall et al., 1992 and Hedlund and Miller, 1994). The media also contain up to 10 mg/L insulin and transferrin (Bottenstein et al., 1980, Bettger et al., 1981, Giguere, 1982, Bradshaw et al., 1983, Ham, 1984, Jager et al., 1988) which are also expensive (\$0.67 and \$0.88 per mg, respectively from Sigma Chemical Co., 1995). Thus, serum-free media are more expensive compared to serum containing ones although product recovery and processing advantages of serum-free media can outweigh the purchase cost disadvantage. After choosing the medium it can be optimised for growth which will be discussed below (1.4.2).

1.2.3.2 Culturing Anchorage Dependent Cells

Many different methods exist for growing cells and most can be used for both suspension and anchorage dependent cells. They include tissue culture flasks, roller bottles and fermenters.

Tissue Culture Flasks and Roller Bottles. Classically, cells were grown in glass (Carrell, 1913) and now in charged polystyrene tissue culture (TC) flasks which provide a flat surface area of between 25-150 cm² using 10-50 mL of media, respectively. These are used for stock cell growth and preliminary medium studies. Early polio vaccine was grown in flat glass Thompson bottles of 500-1,000 cm² but more recently these were replaced by cylindrical polystyrene roller bottles of 850-1,700 cm² (Nardelli and Panina, 1976 and Mirchamsy et al., 1977). Roller bottles require special rigs to roll them at slow speeds (3-5 rpm) and several thousand bottles per batch of vaccine are required. The FDA has approved some products in roller bottle processes using aseptic, robotic chambers for subculturing which removes their labour intensive nature and reduces contaminations. Roller bottles are favoured over alternate technologies through their reliable growth reproduction and speed of process development (Spier, 1992).

This Thesis hopes to show how to overcome the previously perceived problems with novel technologies through a thorough knowledge of cellular metabolism and bioreactor engineering involved. Indeed, Royce (1992) suggests there is a need to refocus on using bioreactors for

production purposes, as the knowledge has expanded enough to overcome the previous limitations.

Spinner Flasks. More complex technologies compared to static flasks exist for culturing cells such as spinner flasks. The main advantage of spinner flasks compared to static flasks is the continual stirring which increases gas entrainment at the gas:liquid interface. Thus, whilst the dissolved oxygen tension (DOT) and pH level are not fully controlled they can be maintained at potentially more favourable levels compared to static flasks. Spinners also maintain culture homogeneity preventing concentration gradients forming which could allow preferable nutrient consumption. However, their main disadvantage is the lack of parameter control which allows large fluctuations in the values of DOT and pH. These fluctuations could cause sub-optimal DOT and pH and lead to reduced maximum cell yields compared to fully controlled systems.

Several spinner flask designs are available for cell culture ranging from Erlenmeyer flasks stirred on shaker bases to specially designed flasks with side-arms and magnetic driven stirrer systems such as Bellco® (Vineland, NJ, U.S.A.) and Techne® (Cambridge, U.K.). The flasks range from 50 mL up to 5 L working volumes.

Bioreactors. The most complex technology for cell culture allows full process control. This classically used unmodified bacterial fermenters (i.e. bacterial growth vessels). However, modifications were necessary for mammalian cell culture and mammalian systems do not now resemble the classical fermenters. Mammalian systems are now generally termed as "bioreactors"; and the work below exclusively refers to them as bioreactors.

The bacterial fermenter modifications required to culture mammalian cells included slower stirring (≤ 100 rpm) with marine type impellers and without sidewall baffles to remove cavitation and to prevent cell damage. Prokop and Rozenberg (1989), Bliem and Katinger (1991), Hu and Peshwa (1991), Butler and Dawson (1992) and Griffiths (1992) have reviewed bioreactors. Bioreactors can result in increased cell and product yields due to the higher degree of control,

although they have high purchase costs compared to roller bottle and flat systems. However, bioreactors are more cost effective based on yields per unit volume as roller bottles are used once for 3-4 d and then discarded.

Bioreactor types include standard stirred tanks (Hu and Peshwa, 1991) and air-lifts (Birch et al., 1987), used in suspended culture. More recently bioreactors for culturing suspended and adherent cells have been designed using spin filters (Avgerinos et al., 1990 and Mercille et al., 1993), combinations of spin filters and air-lifts such as New Brunswick Scientific's (NBS®) Celligen™ (Martin et al., 1987), hollow fibre cartridge systems such as Endotronics® Acusyst™ (Hu and Peshwa, 1991) and fluidised bed systems such as those of Verax®, (Young and Dean, 1987 and Vournakis and Runstadler, 1992). The latter bioreactors all appeared within the last 10 yrs as the need to culture more mammalian cell types increased. Many such cells are not fully transformed and require a surface on which to grow. The growth of adherent cells in single units ≥ 1 L volume was made possible by the concept of microcarrier technology (van Wezel, 1967).

1.3 Microcarrier Types

Since van Wezel (1967) first reported human diploid fibroblast cell growth on solid Sephadex A-50™ substrata, the use and types of microcarriers has grown rapidly. Most microcarriers or "beads" are solid and the cells grow on their outer surfaces. At a similar time, Leighton et al. (1967) realised the potential of using porous sponge matrices of collagen, the main basal lamina component of cells *in vivo* (Grinnell, 1982), to increase the surface area for Walker tumor 256 cell growth. However, the control of macroporous bead production processes was poor and they were little used until Nilsson et al. (1986). The advantages and disadvantages of beads are discussed below (1.3.3).

Both solid and macroporous microcarriers can be used in standard bioreactors but with less success compared to modified ones, as gas sparging collects the microcarriers at the gas:liquid interface in foams. The highest degree of cell damage occurs through bubble disengagement at this interface (Handa et al., 1987). Suspension cells can be protected from damage by Pluronic

F68TM polyols, polyethylene glycol and polyvinyl pyrrolidone (Papoutsakis, 1991 and Chattopadhyay et al., 1995). However, these agents affect cell adhesion processes and can not protect microcarriers from bubble damage. Therefore these agents have not aided microcarrier cultures.

The NBS Celligen spin filter-sedimentation column system is ideal for microcarrier culture, as it allows a single unit to grow suspension or microcarrier cultures. The design of the Celligen sparge system protects the cells from coming into contact with gas bubbles provided they are either grown on microcarriers or the system is stirred at an optimal speed so the filter cage acts as a spin filter. The sedimentation column can also be used to significantly increase cell yields through the process of medium perfusion which will be discussed with microcarrier scale-up to further increase cell yields below (1.4 and 1.5).

1.3.1 Solid Microcarriers

Several solid beads have been developed and used including glass beads (Spier and Whiteside, 1976), protein coated glass (Varani et al., 1988), SuperbeadsTM (Imamura et al., 1982, Crespi et al., 1981, Butler and Thilly, 1982 and Morandi et al., 1982), gelatin GelibeadsTM (Bodeker et al., 1984), polyhydroxyethylmethacrylate (Kiremitci et al., 1989), polystyrene (White and Ades, 1990 and Roder et al., 1993), polyvinyl formal resin (Yamazi et al., 1989) and protein coated gelatin microspheres (Altankov et al., 1991). The most successful are Pharmacia® improved Sephadex-A50 beads.

Pharmacia has improved the polydextran and made CytodexTM beads (Gebb et al., 1982). Three Cytodex beads are available:

- (a) Cytodex-1 which have a positive charge throughout the matrix.
- (b) Cytodex-2 which have a positive charge only at the bead surface.
- (c) Cytodex-3 which are collagen coated Cytodex-1.

Prior to these changes poor manufacturing control resulted in significant cell yield differences between consecutive batches of beads. Hence, microcarrier systems were little used between

1967 and 1982. Morandi et al. (1982) suggest inconsistent cell attachment to the beads caused low cell yields. Indeed, the beads inhibited the growth of some cell lines. However, Cytodex bead diameters are now highly uniform at 180-220 μm and they give highly reproducible growth which increased their usage. The Cytodex beads allow virtually all cell types to be easily cultured at >20 L (Pharmacia, 1981). Bioreactor cultures (1,000 L) of Cytodex-1 beads are used for commercial production of polio and rabies vaccines using human MRC-5 and Vero cell lines (Montagnon et al., 1981 and Beale, 1992).

Cytodex-1 beads are the most widely used substrata for adherent cell research (Clark et al., 1980, Clark and Hirtenstein, 1981, Billig et al., 1984, Nahapetian et al., 1986, Lindner et al., 1987, Lindskog et al., 1987, Varani et al., 1988, Smiley et al., 1989, Kiremitci et al., 1989, Mendonca et al., 1993 and Hawboldt et al., 1994). However, both Cytodex-2 (Kim et al., 1992, du Laney et al., 1992 and Mukhopadhyay et al., 1993), and Cytodex-3 (Gebb et al., 1984, Cherry and Papoutsakis, 1988, Croughan et al., 1988, Tao et al., 1988, Adema et al., 1990, Pons et al., 1992 and Roder et al., 1993) have also been used. Indeed, Cytodex are used as the control beads for testing other novel solid bead types described above.

1.3.2 Macroporous Microcarriers

Nilsson et al. (1986) determined how to control the nature and size characteristics of the macroporous beads during processing. This had previously been a major limitation in the manufacture of macroporous beads as the size characteristics and pore structures were difficult to control. These authors produced the most popular macroporous beads, CultispherTM which are composed of gelatin (denatured collagen). The process allows the bead pore sizes to be varied but they still have highly variable diameters of between 180-330 μm which could lead to culture problems such as homogeneity or cell/bead loading differences. Four types of Cultispher are available defined by the pore size:

(a) Cultispher-S have small pore diameters (10 μm) used by Ohlson et al. (1994) for bioreactor cultures of CHO.

(b) Cultispher-G are the main bead type with normal pore size (20 μm) used in spinner flasks by Nikolai and Hu (1992) for CHO and Vero cell cultures and Schrimpf and Friedl (1993) for vascular endothelial cell cultures.

(c) Cultispher-GL have large pores (30-50 μm) diameter used by Schrimpf and Friedl (1993) for spinner flask cultures of vascular endothelial cells.

(d) Cultispher-GLD are titanium sphere weighted Cultispher-GL as used by Reiter et al. (1990) for bioreactor CHO and Vero cell cultures.

The pore diameter differences offer preferable attachment characteristics according to the cell type being used (e.g. Cultispher-S are preferable for CHO cells. Thus, a great deal of preliminary testing could be necessary to determine the preferable bead for the chosen cell line.

Other macroporous microcarriers have been developed using different substrates including collagen (Doillon et al., 1987, Nakagawa et al., 1989, Adema et al., 1990 and Wada and Vidal, 1991), cellulose (Ogawa et al., 1992), microporous polystyrene Polyhipe™ and Polypor™ (Lee et al., 1991 and Schrimpf and Friedl, 1993) and charged polyethyleneimine (Ong et al., 1994). Verax beads are not macroporous beads but are solid collagen (Dean et al., 1987) and have been highly successful in contract production of many cell types.

Macroporous beads have had less commercial success at this time, excepting Verax, as a result of their recent development. There have also been few reports directly comparing cell growth, attachment and metabolism in cultures of solid and macroporous beads using the same culture systems. Only Adema et al. (1990) and Schrimpf and Friedl (1993) have tested the two in direct comparison but no mention is made of whether the processes or techniques used were optimised for the cells involved. As will become clear below (1.4) the optimisation of all the parameters for cell growth is vital to remove any bead dependent differences which could impact on the results.

1.3.3 Advantages and Disadvantages of Microcarriers

There are many advantageous and disadvantageous of microcarrier culture compared to

static flask or roller bottles. Most are shared by both types of beads whilst others are inherent to only one type. The important batch culture effects will be discussed here and those involved in scale-up will be discussed below (1.5).

1.3.3.1 Advantages

The success of vaccines against polio etc. in the 1950's significantly increased the annual production volumes of adherent cells required as hosts for virus production. Thomson flasks and roller bottles have several common problems including highly labour intensive culturing, require a lot of cleaning and preparation (removed by single use roller bottles), contamination losses, require large incubators for the required number and ease of damage being made of glass or polystyrene (Paul, 1975 and Shevitz et al., 1990). Seeding fresh cultures requires a series of contamination prone steps to detach the cells involving either trypsin or mechanical removal, stopping the trypsin action, concentration of the cells by centrifugation and reseedling of fresh flasks. Furthermore the flasks have a fixed surface area:medium volume ratio of approximately $2.0 \text{ cm}^2/\text{mL}$. This will be discussed below (1.5). Shevitz (1990) suggests the increased volume needs for vaccines fueled the development of microcarriers.

Microcarriers remove the problems above, as they can be used in 1,000 L single units. Turn around and preparation times are shortened between batches, as stock cells can be maintained in smaller (1/10 production volume) pre-production bioreactors ready for inoculation of the production vessels. The bioreactors are constructed of stainless steel and are less prone to damage. Bioreactors are less labour intensive to operate and do not require incubator rooms. Hence, their unit production cost is reduced compared to flask operations. Their use has significantly reduced vaccine dosage costs. Process optimisation is discussed below (1.4).

Microcarriers also could allow reseedling of new cultures through bead:bead cell transfers. Cytodex-1 beads are capable of bead:bead transfer (Borys and Papoutsakis, 1992) and Cultispher-G beads have been shown to transfer surface growing cells (Foran et al., 1991). This allows easier scale-up and will be discussed below (1.5). Solid Cytodex beads have a surface

area of 4,400 cm²/g dry weight and the area of macroporous Cultispher beads is unknown but will be significantly higher (an order of magnitude according to Leighton et al., 1967) compared to solid beads. Thus, cultures at 1.00 g/L Cytodex-1 have a surface area of 4.4 cm²/mL which is more than twice that of static culture flasks.

Most adherent cells are used for virus production which requires the spent culture medium to be replaced and the cells infected with virus. Static flask/roller systems have an increased risk of contamination or spoilage in this process. However, microcarrier systems remove this problem, as culture manipulations are carried out through steam sterilised process lines.

1.3.3.2 Disadvantages

Disadvantages to microcarrier culture mostly relate to cell growth on solid beads. The low surface area to volume ratio is the main problem (Leighton et al., 1967), as spheres have the lowest ratio of a 3-dimensional structure. So high surface areas require more beads to be added increasing the total culture volume occupied by bead matrix and reducing the media volume significantly (>10%) at above 2.00 g/L beads. This could be overcome by concentrating the media to compensate for the bead volume which will be discussed below (1.5). Macroporous bead void volumes reduce the culture volume occupied by bead matrix.

Stirring solid bead cultures exposes the cells on the solid bead surfaces to the external environment. Thus, the cells are prone to damage from impacts between beads and also vessel surfaces such as the walls, impeller blades or probes. This and the related problem of shear from the stirrer speed have been investigated by others (Cherry and Papoutsakis, 1988 and 1989, Croughan et al., 1988 and 1990, Avgerinos et al., 1990, Borys and Papoutsakis, 1992 and Lakhotia and Papoutsakis, 1992). As the bacterial cell yield increases so the stirrer speed is also increased to improve homogeneity, oxygenation and nutrient supply. However, in mammalian microcarrier cultures increasing the speed also increases bead contacts and shear forces at the impeller tips. These contacts and forces cause cells to detach from the beads or reduces their growth rates when cultures are stirred at 90-100 rpm (Croughan et al., 1987). Macroporous

beads have been shown to protect cells from these effects (Leighton et al., 1967 and Adema et al., 1990).

Cells grown on solid beads are morphologically similar to those in static flasks (i.e. flattened). However, this differs from the morphology *in vivo* where they are round and grow in dense masses in body organs (Doillon et al., 1987). This could affect cell yields and their responses to nutrients. Barngrover (1989) suggested flat cells have highly increased surface area:volume ratios which aids the uptake of mitogens. No such difference in shape appears in porous beads, as cells grow in dense masses more akin to body organs (Shahar et al., 1994).

Cell attachment is a common problem to either bead type. Mukhopadhyay et al. (1993) suggest cell attachment to Cytodex beads is affected by the "Vroman effect" when serum proteins are present, as there is the chance of competition between proteins based on their molecular weight. Rapid cell attachment and even cell/bead distribution is needed for successful microcarrier culture even though bead:bead cell transfer is possible. Butler and Thilly (1982) found there was a critical inoculation cell:bead ratio which could also affect cell yields.

Another common problem in cultures of either bead relates to stirrer speeds, as low speeds cause bead aggregation from cell bridges between beads (Clark and Hirtenstein, 1981 and Cherry and Papoutsakis, 1989). These authors suggest aggregation is detrimental to cell yields and growth rates. Hence, there must be a compromise between the stirrer speed and the acceptable amount of bead aggregation.

1.4 Process Parameter Optimisation

Process development requires the optimisation of physical and media parameters for high cell yields whilst preventing the problems raised above (1.3.3.2). Process development begins with batch cultures to define the important culture values which are then used in refed and perfused cultures to optimise cell yields. There have been few published reports on process development of microcarrier cultures. Much of the early process optimisation work can be carried out in spinner flasks, excepting pH, DOT and gas flow rate which require bioreactors.

1.4.1 Physical Parameters

Optimal cell growth involves:

1. Temperature (Tsao et al., 1992).
2. pH (Ceccarini and Eagle, 1971 and Ceccarini, 1975).
3. DOT (Graff and McCarty, 1957, Jenssen et al., 1974, van der Valk et al., 1985 and Gille et al., 1991).
4. Stirrer speed (1.3.3.2). This will be discussed below (1.5).
5. Gas flow rate (in bioreactor cultures). This will be discussed below (1.5).
6. Microcarrier concentration, cell attachment and cell to bead distribution. This will be discussed below (1.5).

The physical parameters should be optimised first, as media utilisation could be influenced by these parameters.

Physical parameters are optimised by defining acceptable control values for each parameter such as 37°C, pH 7.1, 50% of air saturation DOT, 40 rpm, 0.05 L/L culture volume/min (in standard sparged bioreactors) or 0.30 L/L/min (spin-filter or Celligen bioreactors). The bead concentration can be maintained low (1.00 g/L) to reduce process costs. Each parameter is tested over a wide range of values whilst the others are maintained at the control levels. Once each parameter is optimised new cultures are inoculated using this value for the subsequent parameters. Stirrer speed, gas flow rate and microcarrier parameters are also involved in scale-up and will be discussed below (1.5).

Optimal cell growth in microcarrier cultures require optimal cell attachment to the beads (Clark et al., 1980). These authors showed Vero cell attachment to Cytodex-1 beads was optimal with static conditions at 1/2 the final working volume and cell growth was optimal at 60 rpm, pH 7.4 and at ≥ 5.00 g/L microcarrier concentration. No other parameters were described. However, others have used intermittent or continuous stirring for cell attachment to Cytodex-1 beads with similar results to the previous authors suggesting neither method is superior (Pharmacia, 1981 and Cherry and Papoutsakis, 1988 and 1989). Intermittent stirring could pose

a challenge in bioreactor cultures due to the lack of controllers capable of running such protocols over long time periods (24 h).

Macroporous Cultispher beads produce higher yields and protect cells from shear effects (Hu and Wang, 1987, Reiter et al., 1990 and Ohlson et al., 1994). However, there has also been no optimisation of culture parameters and there are numerous conflicting reports on cell attachment such as continuous stirring at 1/2 final working volume (Hy-Clone Application Notes, 1988) or conversely intermittent stirring at final working volume (Ohlson et al., 1994). The conflicting reports could explain why there has previously been a fear of using microcarrier processes and adherent cell types in research and production processes.

1.4.2 Medium Parameters

1.4.2.1 Cell Nutrient Components

Mammalian cell culture medium is complex and must include all the nutrients for cell growth described above (1.2.3.1). However, these nutrients must be elucidated in both batch and perfusion cultures to determine any differences. The first step is to determine the optimal basal medium formulation and Vero cells are commonly grown in either Medium 199 (M199) (ATCC Cell Bank and Chotteau and Bastin, 1994) or Dulbecco's Modified Eagle's Medium (DMEM) (Clark and Hirtenstein, 1981, Polastri et al., 1984 and Reiter et al., 1990). The media were further supplemented with foetal bovine serum (FBS) at 10.0% v/v (Chotteau and Bastin, 1994) or calf serum (CS) at 5.0% v/v (Polastri et al., 1984).

The nutrient consumption of Vero cells has been reported (Polastri et al., 1984 and Chotteau and Bastin, 1994). The most important components were glucose and glutamine. Polastri et al. (1984) determined the amino acid consumption/production profiles of Vero cells grown in DMEM. Histidine, arginine, tryptophan and methionine as well as glutamine were consumed and only alanine and serine were produced. More recently Chotteau and Bastin (1994) showed more complete nutrient profiles for Vero cells grown in M199. The cells consumed pyruvate, aspartic acid, isoleucine, leucine, lysine and valine along with the amino acids described by Polastri et al.

(1984). The different basal media could explain the different consumptions seen. Yet other basal media have also been used in Vero cell research including Leibovitz's L15 (Barngrover et al., 1985, Nahapetian et al., 1986 and Mendonca et al., 1993) and DMEM/Ham's Nutrient Mixture F12 (1:1) (Hu et al., 1985 and Hawbøldt et al., 1994). These reports all show variations in cell yields and growth rates which could be explained by the basal media or serum used, although this was not discussed.

There has been no optimisation of nutrient concentrations for Vero cell yields. High performance liquid chromatography (HPLC) analysis is fundamental to successfully defining the media. The optimal nutrient concentrations can be determined in matrix experiments where each component is added at a range of concentrations whilst maintaining others at constant concentrations. Cell yields are then monitored to define the optima.

There have been few reports of complete nutrient optimisation for any cell line. Xie and Wang (1994^a and ^b) attempted to define and formulate an optimised serum-free medium for murine hybridoma cells based on consumption/production kinetics. However, there are often conflicting reports on important nutrients, as with Vero cells above. Kilberg et al. (1993) suggest nutrient requirements are cell line dependent relating to the cells physiological role. However, nutrient metabolism could vary between media formulations according to the uptake mechanism being expressed or the concentrations of individual components in the media. Successful high yield culture requires all these nutrients to be defined.

1.4.2.2 Serum-Free Medium

Previous literature suggests it is difficult to achieve similar fibroblastic cell yields in serum-free media as in serum containing media. Litwin (1992) used an undefined serum ultrafiltrate for Vero cell aggregate culture but this achieved low cell yields ($0.50\text{--}0.70 \times 10^6$ cells/mL). Cinatl et al. (1994) formulated a highly complex (94 components) protein-free medium for Vero cultures in static flasks but again to low cell yields ($<0.50 \times 10^6$ cells/mL). Their results suggest the modified polyvinyl formal substrata could have influenced cell growth. Merten et al. (1994) have

also described a serum-free medium for Vero cells. These authors show long lag phases (up to 72 h) for such cells when subcultured which infers the medium is lacking important nutrients. However, as Vero cells could grow in serum and protein-free medium it suggests they can be cultured on microcarriers in serum-free medium. None of these authors optimised their media before the serum-free additions were made which could explain their lower cell yields.

The attachment process, glycoproteins and ions involved for adherent cells are well known and include collagen (Grinnell, 1982 and Ruoslahti and Pierschbacher, 1987), vitronectin (Knox and Griffiths, 1980 and Hayman et al., 1985), glycosaminoglycan (Ruoslahti, 1991), fibronectin (Yamada et al., 1976, Orly and Sato, 1979 and Ito et al., 1987), laminin (Varani et al., 1989 and Akimoto et al., 1992), tenascin (Weller et al., 1991), fetuin (Engelhardt and Mao, 1977 and Nie, 1992), Ca^{2+} (Curtis and McMurray, 1986 and Lengheden, 1994), serum and serum albumin (Mukhopadhyay et al., 1993). The nutrient requirements of cells could be influenced by the age of the animal the cells were derived from. Cells from young animals (e.g. BHK cells) require fibronectin for attachment and growth whilst cells from adult animals (e.g. Vero cells) only require it for attachment (Hughes et al., 1979).

The attachment glycoproteins are attracted to cell surface receptors or the integrins (Hynes, 1987). The integrins recognise common polypeptide sequences (arginine, glycine, aspartate and serine) in the glycoproteins (Akiyama et al., 1990). However, the particular glycoproteins recognised are cell line specific (Hynes, 1987). Therefore, the formulation of serum-free media could require these factors to be added.

1.4.2.3 Cell Growth Inhibitors

Nutrient optimisation must prevent the production and accumulation of toxic or inhibitory components in the medium. The inhibition of cell growth by metabolic waste products such as ammonia from amino acids and lactate from glucose is well documented. Ammonia, formed from glutaminolysis, and lactate, formed from an excess of pyruvate from glycolysis, inhibit or are toxic to L, BHK, MDCK, Vero, hybridoma and human diploid fibroblast cells (Ryan and

Cardin, 1966, Butler and Spier, 1984, Glacken et al., 1986, Reuveny et al., 1986, Kimura et al., 1987, Hassell et al., 1991, Dahl et al., 1992, Duval et al., 1992, Martinelle and Haggstrom, 1993 and Rutz and Little, 1995). The inhibitory effect of both wastes appears to be cell line dependent (Ryan and Cardin, 1966, Glacken et al., 1986 and Hassell et al., 1991). Other cell growth inhibitors have also been found including glycine, formate and medium osmolality (Waymouth, 1970, Vincent et al., 1992 and Ihrig et al., 1994). These could all be important to the final cell yields in culture. Therefore, in order to completely optimise the growth environment of the cells it is necessary to examine the effects of growth inhibitors.

1.4.3 Perfusion Culture

Perfusion is the continuous medium exchange to remove inhibitor compounds and supply fresh nutrients to increase cell yields. Determining the optimal perfusion rates and times to initiate perfusion begin the definition of a final process and are the preliminary phases of culture scale-up for increased product yield. Perfusion processes use the optimised media and physical parameters defined in previous batch studies. The initial aim should be to determine maximum cell/bead loadings in the system using low bead concentrations. This defines the optimal perfusion rate per bead concentration for scale-up studies using increased bead concentrations. The nutrients must be analysed in detail to determine which, if any, limit cell yields to ensure cell surface area is the only limitation. Once the limiting nutrients are known the perfusion medium can be enriched or the limiting components can be pumped into the cultures from separate concentrate solutions. No one has previously shown whether spinner flasks can be used successfully in early perfusion studies or whether bioreactors offer superior conditions.

Perfusion removes the major problem of scale-up which is the culture volume occupied by the bead matrix. Perfusion makes it unnecessary to concentrate the medium, as the medium is being completely changed every few hours or days. Full optimisation of the perfusion process could require some physical parameters such as the stirrer speed and gas flow rate to be increased, as the cell yields increase. Gas flow rates and stirrer speeds could require ramping to

provide sufficient gassing to maintain or control the DOT and pH and to increase nutrient diffusion to lower cell layers in multi-layered cultures.

1.5 Scale-Up

The scale-up of roller bottle processes is easy as all it requires is a linear increase in the number of bottles used in the process. For example 100 L of product requires 200 x 850 cm² roller bottles (no small task to handle). Microcarrier culture systems of 0.19 g/L Cytodex-1 have the equivalent surface area to each 850 cm² roller bottle. Therefore, an 8 L culture at 5.00 g/L Cytodex-1 has a higher surface area compared to 100 L of roller bottle culture. Cultispher-G cultures significantly increase the surface area over those with Cytodex-1.

Process scale-up begins after physical and nutrient component optimisation and the maximum cell loadings at low microcarrier concentrations are known. Scale-up uses these optimised parameters to determine whether the values can be increased proportional to the difference in vessel volumes or bead concentrations used to give high cell yields in larger vessels (>10 L) at higher microcarrier concentrations. It is the stage where standard operating procedures and process operating instructions for product licenses are defined and written. Bead to bead transfer of cells could be advantageous to scale-up as it would allow cell laden beads from small volume cultures to be used as the inoculum for larger vessels. The larger vessel could also be inoculated at a low bead concentration and grown to monolayer prior to increasing the bead concentration by adding fresh beads.

Most researchers have used scaled-up systems with no validation or optimisation to support their methods. For example several microcarriers have been used at ≥ 3.00 g/L with Vero and CHO cells (Gebb et al., 1984, Nahapetian et al., 1986, Cherry and Papoutsakis, 1988, Varani et al., 1988, Adema et al., 1990, Forestall et al., 1992 and Qualitz et al., 1993), even in batch cultures. The use of high microcarrier concentrations appears to come from Clark and Hirtenstein (1981) who reported significantly higher (20%) cell yields at 3.00 g/L Cytodex-1 compared to 1.00 g/L. The authors suggested the initial number of cells/cm² was optimal in the

3.00 g/L cultures. However, Mered et al. (1980) found similar cell yields at up to 2.50 g/L and reduced yields at 5.00 g/L. The stirrer speed used for refeed Vero cell growth has also varied in the literature with some authors using 35 rpm throughout (Nikolai and Hu, 1992) whilst others used 60 rpm (Clark and Hirtenstein, 1981) and even up to 90 rpm (Nahapetian et al., 1986). Whilst this could be explained by spinner flask design differences the authors did not supply any evidence of the speeds used being optimal for their processes. Therefore, there are conflicting reports which could be explained by cell line dependent or system differences.

Undoubtedly, the scale-up of adherent cell culture systems requires high (>3.00 g/L) microcarrier concentrations but no-one has previously optimised the bead concentration or shown clear proof of the extra beads giving increased cell yields. Further, the refeed/perfusion rates used to achieve high cell yields vary greatly in previous reports. In Vero spinner flask cultures the rates range from one 50% medium exchange every 2 d (Clark and Hirtenstein, 1981), to a complete (100%) medium exchange every 2-3 d (Mered et al., 1981) up to as high as 8.0 medium exchanges every day (Nahapetian et al., 1986). In Vero, MDCK and CHO cell bioreactor cultures the rates range from 0.50-1.00 medium refeed volumes per vessel volume per day (v/v/d) (Martin et al., 1987 and Smiley et al., 1989), to 5.00-10.00 v/v/d (Ohlson et al., 1994) up to as high as 10.00-30.00 v/v/d (Reiter et al., 1990). Indeed, the Vero cell yields in many of these reports vary greatly with 3.00×10^6 cells/mL (Mered et al., 1980) up to 30.00×10^6 cells/mL (Nahapetian et al., 1986). The yield differences could be caused by the differences in refeed rates used or by the different media used between reports. No one has previously analysed their medium to determine the possible limiting nutrients for any cell line. Perfusion rates above 2.00 v/v/d of complete serum containing medium would overrule the increases in cell yields based on the process cost, as it would be cheaper to generate products from more batch cultures compared to perfused ones.

Scale-up requires a good knowledge of the cells needs and care must be taken to ensure there are no limitations. Hence, scale-up is the art of balancing all the important parameters in such a way as to linearly increase the cell yield.

1.6 Experimental Aims

The previous sections have attempted to clarify the depth of research which has been performed using Hybridomas and CHO cells in suspension culture systems. However, in comparison adherent microcarrier based systems have been little used for production process research. The perceived culturing problems of microcarriers are believed to be responsible for this. The previous research with microcarriers has mostly involved BHK and MDCK cells whilst African green monkey kidney (Vero) cells are little used although they are commercially significant. The choice of microcarriers was easy. Cytodex are the "industry standard" solid microcarrier and are well characterised. The most commonly used macroporous beads are Cultispher. As has been described earlier (1.3), there have been few previous reports of direct comparisons of cell growth between any solid or macroporous bead cultures, excepting those of Adema et al. (1990) and Schrimpf and Friedl (1993). Thus, it was decided to directly compare the process optimisation of Vero cell cultures on Cytodex-1 and Cultispher-G microcarriers. The processes were optimised using both spinner flasks and Celligen bioreactor cultures. The final process was then used to test whether these systems could be used for virus vaccine production using a reovirus.

The major aims of the Thesis research were as follows:

1. To determine the optimal protocols for cell harvesting and sampling and to define the limits in using them.
2. To evaluate the common methods of cell enumeration in microcarrier cultures in order to validate their use.
3. To optimise the physical parameters for the batch and high yield microcarrier culture of Vero cells.
4. To optimise the nutrient parameters for the batch and high yield microcarrier culture of Vero cells.
5. To determine the feasibility of scale-up using the optimised parameter values for high

yields of Vero cells.

6. To define the cell growth inhibitors which could influence the final cell yields in batch and high yield Vero cell cultures.

7. To directly compare and contrast the batch and high yield growth of Vero cells on solid and macroporous microcarriers.

8. To produce a chemically and hormonally defined serum-free medium for the growth of Vero cells on solid and macroporous microcarriers.

9. To determine the infectivity of Vero cells grown to high cell densities in perfusion and daily refed cultures for virus vaccine production.

Chapter 2. Materials and Methods

2. Materials and Methods

2.1 Cell Lines and Stock Cell Maintenance

African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection, Catalogue Number CCL 81. They were maintained in T25, T75 or T150 culture flasks using 10, 35 or 50 mL of original DMEM containing 25.00 mM D-glucose and 4.00 mM L-glutamine and supplemented with 5.0% v/v iron enriched, supplemented calf serum (sCS) (Gibco BRL[®], Grand Island, NY, U.S.A.). Flasks were passaged every 72 or 96 h by 1:10 dilution into fresh flasks following recovery by the standard trypsinisation protocol (2.2).

2.2 Standard Trypsinisation Cell Recovery Protocol

Stock cultures were taken and the supernatant discarded. The cell sheet was washed (x1) with Dulbecco's phosphate buffered saline (without Ca^{2+} and Mg^{2+}) containing EDTA (0.70 mM) (D-PBS⁻:EDTA) at pH 7.5 (10 mL). Porcine trypsin (0.25% w/v) in Hanks balanced salts solution (HBSS⁻) containing 1.00 mM EDTA (trypsin:EDTA) (Gibco BRL[®]) was added to culture flasks (1.00 mL to T25, 1.50 mL to T75 and 2.00 mL to T150 culture flasks). The solution was swirled (1 min every 2 min for a total time of 5-10 min) and the flasks were rapped against the palm of the hand (x10) once cells began to detach from the surface. DMEM + 5.0% v/v sCS (9 mL) was added and the cell suspension homogeneously resuspended and placed into a 15 mL centrifuge tube and centrifuged (Heraeus Sepatech[®] Megafuge[™], Frankfurt, Germany) at 300 x g for 5 min. The supernatant was discarded and the cell pellet resuspended in DMEM + 5.0% v/v sCS (10 mL). The cells were then either diluted into fresh culture flasks or counted by the trypan blue dye exclusion method by haemocytometer (2.5.2).

2.3 Basal Medium Preparation

Basal medium was obtained (where possible) as powder concentrate (10X) without glucose, glutamine, sodium pyruvate, phenol red or sodium bicarbonate from Gibco BRL[®]. Basal medium powder and supplements (Sigma[®] Chemical Co., St. Louis, MO, U.S.A.) were added to

0.9X the required volume of high purity water obtained after reverse osmosis (RO) followed by either distillation or Milli-Q™ (18MΩ conductivity) (Millipore®, Bedford, MA, U.S.A.) treatments. DMEM was enriched with glucose (25.00 mM), glutamine (4.00 mM) and bicarbonate (44.00 mM) unless otherwise stated. Phenol red was added at 15 mg/L from a 3 mg/mL stock solution in high purity water. The medium pH was adjusted to pH 7.1-7.2 with 6.00 M HCl or 10.00 M NaOH prior to increasing to the full volume and filter sterilisation.

Medium was sterilised by peristaltic pumping through Acrocap™ (Gelman®, Ann Arbor, MI, U.S.A.) or Millipak™ 60 (Millipore®) filters (0.20 µm) and was stored in 100 or 500 mL glass bottles at 4°C.

2.4 Experimental Methods

2.4.1 T25 Culture Flask Cell Growths

T25 culture flasks were inoculated at 0.10×10^6 cells/mL in medium (10 mL) and grown in incubators (Nu2700, NuAire®, Minneapolis, MN, U.S.A.) at 37°C with a 10% v/v CO₂ overlay.

2.4.2 Microcarrier Preparation

Cytodex-1 (Pharmacia®, Uppsala, Sweden) and Cultispher-G (Hy-Clone® Labs., Salt Lake City, UT, U.S.A.) beads (10 g) were swollen overnight in 500 mL of D-PBS⁻ at pH 7.2 and were swirled every few hours. The supernatant was discarded and the microcarriers were washed (x2) with 400 mL of D-PBS⁻ and autoclaved at 121°C for 20 min.

2.4.3 Growth in Spinner Flasks

Glass spinner flasks (100 mL working volume) (Bellco®, Vineland, NJ, U.S.A.) with paddle blades were used for experimental microcarrier cultures, unless otherwise stated. The paddle blade diameter was reduced to 5.6 mm and all openings on the stirrer shaft were silicon sealed. The flasks were thoroughly washed, siliconised, autoclaved at 121°C for 20 min and allowed to cool prior to use. Swollen microcarriers were added at 1.00 g/L Cytodex-1 and 1.70 g/L

Cultispher-G in growth medium (90 mL), prewarmed to 37°C. Stock cells were inoculated (0.05×10^6 cells/mL) and the volume made to 100 mL with growth medium prior to incubation. The flasks were sampled daily for cell counts by the trypan blue method (2.5.1-2). The flasks were stirred continuously at 40 rpm (Cytodex-1) or intermittently for 24 h then continuously and the speed raised to 60 rpm after 72 h (Cultispher-G) unless otherwise stated. Flasks were readjusted daily to pH 7.1 or 7.4 with 2.00 M NaOH after 72 h in culture unless otherwise indicated.

2.4.4 Culture Refeeding

Spinner flasks cultures were inoculated as above (2.4.3). Daily from 72 h onwards the beads were settled and one 50% v/v medium exchange with growth medium was made, unless otherwise stated. Daily samples were removed for cell counts by the trypan blue method (2.5.1-2).

2.4.5 Celligen Bioreactor Cultures

The 1.50 L Celligen™ (New Brunswick Scientific®, Edison, NJ, U.S.A.) glass vessel was siliconised. After drying the bioreactor was reassembled and filled with 1.30 L of D-PBS⁻. All tubing inlet lines were open and all harvest lines and the sparge filter line were closed. The sampling bottle was loosened and one blank port stopper was removed. The dissolved oxygen probe (Ingold®, Zurich, Switzerland) membrane was refilled with electrolyte (every 4-5 autoclavings). The pH probe (Ingold®) electrolyte was replaced with saturated KCl (every 10 autoclavings). The pH probe and amplifier were calibrated to pH 7.0 and 4.0 with standard buffers. The probes were inserted into their housings and the vessel was autoclaved at 121°C for 20 min.

Immediately after autoclaving all lines were closed and the sample bottle was tightened. The temperature probe was inserted into an RO water filled housing and the vessel allowed to cool to 37°C while stirring at 100 rpm. The condensor was connected to a running cold water supply and a drain. The bioreactor waste and overflow bottles were aseptically connected to the

harvest line and the gas exhaust line at the top of the condensor via flamed glass male:female connectors. Once at 37°C the dissolved oxygen probe and amplifier were calibrated to 0 and 100% using the dynamic gassing out technique with N₂ and compressed air.

Stock cells were inoculated (0.05×10^6 cells/mL) in growth medium (1.30 L) in a 2.00 L glass bottle containing 1.00 g/L Cytodex-1 or 1.70 g/L Cultispher-G, unless otherwise stated. The stirrer and temperature controllers were switched off and the D-PBS⁻ was pumped out of the vessel. The cell inoculum was added by positive pressure from the inoculum bottle with compressed air. As a safety precaution the pressure in the inoculum bottle was released every 1 min during inoculation. The control set-points were pH 7.1 (Cytodex-1) or 6.8 (Cultispher-G), 50% dissolved oxygen tension (DOT), 37°C and 0.50 L/min gas flow rate, unless otherwise stated. Cultures were stirred continuously at 40 rpm. The speed was manually ramped to 60rpm after 72 h in Cultispher-G cultures. Two 20 mL samples were removed daily and the second one was used for cell count analysis by the trypan blue method (2.5.1-2).

2.5 Sample Analysis

2.5.1 Cell Sampling

2.5.1.1 Suspension Cells

T25 culture flasks were rapped against the palm of the hand (x10). The flasks and spinner cultures were homogeneously suspended by swirling and samples (0.50 mL) were aseptically removed into graduated microfuge tubes (1.80 mL).

2.5.1.2 Adherent Cells

Spinner flasks were homogeneously suspended by swirling and samples (1.00 mL) were aseptically removed into graduated microfuge tubes. The beads were settled and the supernatant was removed (0.90 mL) and retained frozen at -20°C for assays.

The beads were washed (x1) with D-PBS⁻:EDTA (1.40 mL) at pH 7.5. The beads were settled and the wash was discarded. The cells were detached with trypsin:EDTA (1.00 mL of

0.25% or 0.50% w/v/1.00 mM, respectively) at 37°C for 5-10 min with tube inversion (x5-10) every 2 min. The trypsin action was stopped by addition of DMEM + 5.0% v/v sCS and the tube was centrifuged (300 x g for 5 min). The supernatant (0.50 mL) was discarded.

2.5.2 Trypan Blue Dye Exclusion Cell Count

Trypan blue solution (0.50 mL of 0.40% w/v trypan blue in D-PBS⁻ at pH 7.2) was added to the cell sample tubes (Amos et al., 1969). Sample tubes from microcarrier cultures were flicked against the index finger (x15). Suspension and microcarrier culture sample tubes were inverted (x10) and the cells pipetted onto an improved Neubaur haemocytometer slide and counted by microscope. The total number of blue and clear cells was determined in the sample (0.10 μ L). The cell viability was determined by the ratio of blue to total cells. The cell counts were adjusted for the dilution of the original samples. Each sample was counted at least twice.

2.5.3 Crystal Violet Nuclei Stain

The method was adapted from Lin et al. (1991) using a crystal violet reagent consisting of citric acid (0.50 mM) containing Triton X-100 detergent (2.00% w/v) and crystal violet dye (0.20% w/v). Cell samples (0.50 mL) were treated with crystal violet reagent (0.50 mL) and incubated at 37°C (1.50 h) with inversion (x5) every 15 to 20 min. Following incubation the tubes were flicked against the index finger (x10-15) and inverted (x10). The nuclei were pipetted onto an haemocytometer slide and counted by microscope, as with the trypan blue method.

2.5.4 Coulter Counter

The method was adapted from Coulter (1953). Each cell sample was diluted (1:200) in Isoton[™] buffer solution (20 mL) in a Coulter cuvette. The cuvette was inverted (x5) and placed onto the Coulter (Coulter[®] Electronics, Hialeah, FL, U.S.A.) counter. The channel was cleared and the count performed. The count obtained was adjusted for the sample dilution. A background blank was performed by diluting growth medium in Isoton.

2.5.5 Cell to Bead Distribution Patterns

The distribution of cells on the beads was determined by microscopic examination following staining. The beads (100) were observed and scored for cell content (0-25 cells/bead). Beads with >25 cells/bead were scored as having 25 cells/bead. Culture samples (250 μ L) were incubated at 37°C for 20 min with crystal violet (750 μ L of 0.20% w/v) (Sanford et al., 1951) or neutral red (750 μ L of 0.20% w/v) (Valdivieson-Garcia et al., 1993) in D-PBS⁻ in microfuge tubes. After incubation the beads were settled by centrifugation (300 x g for 20 s). The supernatant was discarded and the beads were washed (x2) with D-PBS⁻ (1.40 mL). The beads were finally resuspended in D-PBS⁻ (250 μ L) and placed on a 10 cm Petri-dish. The cell/bead distribution was recorded using an inverted microscope.

The theoretical Poisson cell to bead distribution was determined using Equation 2.1:

$$P(n) = ((e^{-\lambda} \times \lambda^n) / n!)$$

Equation 2.1

where $P(n)$ is the probability of observing a number of beads with a given cell to bead number.

λ is the cell to bead ratio added in the inoculum.

n is the observed cell to bead ratio.

A sample calculation is provided in Appendix 3.1.

2.5.6 Attachment Time, Lag Phase and Subsequent Growth

The attachment time was determined by inoculating cells (0.20×10^6 cells/mL) in growth medium (10 mL) in T25 culture flasks. Every 0.5 h for 8.0 h the flask supernatant was sampled and stained as for cell/bead distribution above (2.5.5). The attachment time was the time taken for $\geq 90\%$ of the inoculated cells to become attached to the beads. Subsequent growth was determined by sacrificing one flask daily for cell counts by the trypan blue method (2.5.2). The lag phase was determined using Equation 2.2:

$$L_p = (t_T - t_e)$$

Equation 2.2.

where L_p = lag phase time.

t_T = total time taken to highest cell count.

t_e = time that would have elapsed for the total number of cell generations at maximum cell generation time in the exponential phase.

A sample calculation is shown in Appendix 3.2.

2.5.7 D-Glucose and L-Lactate Analysis

Samples (25 μ L) were injected into a YSI 27 (YSI Instruments®, Yellow Springs, OH, U.S.A.) glucose/lactate analyser with the appropriate enzyme membrane installed. Each sample was analysed twice. The instrument was calibrated using standards (500 mg/dL glucose and 15.00 mM lactate) before each use and after every 6-10 samples. All results were calculated in mM.

2.5.8 Amino Acid Analysis

Amino acids were analysed by the method described by Christie and Butler (1994). Amino acid standards (500 mM) or samples (245 μ L) were mixed with L- α -amino-n-butyric acid (5 μ L of 25.00 mM) and trichloroacetic acid (TCA) (250 μ L of 10% w/v). The samples were centrifuged (11,000 $\times$ g for 5 min) at 4°C. The supernatant was removed and added to sodium borate buffer (500 μ L of 0.20 M) at pH 10.4 and centrifuged as above.

Samples of the supernatants (250 μ L) were placed into microfuge tubes (500 μ L). Orthophthalaldehyde (OPA) reagent (250 μ L) was added and the tubes were incubated (1.7 min). 5 μ L was autosampled by an SIL-9A autosampler (Shimadzu® Inc., Kyoto, Japan) and injected onto an C-18 (Alltech®, Deerfield, IL, U.S.A.) reverse phase column on an LKB (LKB-Pharmacia®, Uppsala, Sweden) 2100 series HPLC system. Amino acids were eluted from the column using gradients of acetonitrile and methanol. Results were analysed using Easychrom™ v.3.2 (Shimadzu®) computer software.

2.5.9 Ammonia

Culture samples (0.20 mL) were diluted with distilled water (0.80 mL) in test tubes with a magnetic microflea. NaOH (10 μ L at 10 M) was added and the solution stirred at 50 rpm. An Orion 9512 ammonia electrode (Orion[®] Inc., Boston, MA, U.S.A.) was placed into the solution, ensuring no air bubbles formed under the membrane. The mV reading was allowed to stabilise (2-3 min) and recorded. The ammonia concentration was determined against standard solutions of ammonium chloride (100.00-0.10 mM) and by multiplying by the sample dilution factor. A typical standard curve for ammonia is shown in Appendix 2.1.

2.5.10 Osmolarity Determination

Samples (25 μ L) of medium were placed into microfuge tubes and placed onto a Micro-Osmette (Precision Systems[®] Inc., Sudbury, MA, U.S.A.). The electrode was carefully lowered into the sample and the osmolarity determined by the freezing-point depression method. The instrument was standardised (100 and 500 mOsmol.L⁻¹) prior to use and after every 5 samples.

2.6 Statistical Analysis

All data was produced from at least two cultures inoculated from different cell stocks. The data was then averaged to give the means which were plotted or listed in the results. Standard errors of the mean were calculated using Excel v. 5.0a (Microsoft[®] Corp., Seattle, WA, U.S.A.) by dividing the standard deviation of the sample population by the square root of the number of data points in the population. A sample calculation is provided in Appendix 3.3.

Statistical analysis of differences between data series was performed using either two-way analysis of variance with replication (ANOVA) by the method of Sokal and Rohlf (1987) or by paired Student's t-test's. The paired Student's t-tests were performed using Sigmaplot[™] v. 1.02 (Jandel Scientific[®], San Rafael, CA, U.S.A.) for Windows[™] (Microsoft[®] Corp.) on the sample values. The t-test was two tailed with unequal sample variances and confidence intervals were set at 95%. The null hypothesis tested whether the sample values were likely to have come from

the same two populations with equal means. Sample calculations of paired Student's t-tests and ANOVA analyses are shown in Appendix 3.4 and 3.5.

2.7 Definition of Optimality

The term optimal, with reference to the research outlined below, was defined as the parameter value or nutrient component concentration which gave the significantly highest maximum cell yield of all the cultures in the experiment unless otherwise defined. Where the maximum cell yields were similar over a range of values the lowest component concentration was taken as the optimal. The optimal conditions defined refer exclusively to the flasks and bioreactors being used in the experiments and might not apply to other similar systems.

**Chapter 3. Optimisation of Adherent Vero Cell Detachment and Recovery Protocols from
T25 Culture Flasks**

3. Optimisation of Adherent Vero Cell Detachment and Recovery Protocols from T25 Culture Flasks.

3.1. Introduction

The techniques for cell growth and subculture vary between suspended and anchorage dependent cells. The subculture of suspended cells such as hybridomas, requires fewer process steps that could interfere with their viability or subsequent growth. Whereas, the subculture of adherent cells such as African green monkey kidney (Vero) or baby hamster kidney (BHK) cells, first requires them to be detached from the growth surface by enzymic methods such as trypsinisation or mechanical methods such as cell scraping.

Gebb et al. (1984) incorporated a wash step with a buffered saline:EDTA solution into their trypsin detachment procedures to increase the rate of recovery and the proportion of Vero and MRC-5 cells recovered. The EDTA concentration used was low (0.70-1.00 mM) and this step has been incorporated into trypsinisation protocols used by others (Lindner et al., 1987 and Lindskog et al., 1987). However, detachment with trypsin solutions has been shown to affect the recoveries, viabilities and subsequent growths of MRC-5 and Vero cells (Gebb et al., 1984, Lindner et al., 1987 and Lindskog et al., 1987). Lindner et al. (1987) claimed that the subsequent growth of MRC-5 and Vero cells was unaffected following trypsin detachment and recovery. However, much of their data showed pronounced lag phases (≥ 24 h). Once the adherent cells are detached they can be treated as suspended cells and recovered by centrifugation into pellets, which could also affect both the cell viability and recovery.

Other methods exist for detaching adherent cells from surfaces. These methods include enzymes such as collagenase or dispase (Cassiman et al., 1981) and chemicals such as ethylenediaminetetraacetic acid (EDTA) (Tao et al., 1988). Enzymic procedures function by proteolysis of proteins attached to the substrata and cell membranes (Hynes, 1987 and Ruoslahti and Pierschbacher, 1987). The EDTA procedure chelates Ca^{2+} ions resulting in the loss of cell adhesion. Tao et al. (1988) prevented the adverse effects of trypsin solutions by detaching

trypsin sensitive bovine embryonic kidney (BEK) and human foreskin fibroblast cells (FS-4) from surfaces using solutions with high concentrations of EDTA without trypsin (Tao et al., 1988). These authors used an Earle's balanced salts wash solution containing a high (3.00 mM) EDTA concentration at pH 7.5-7.8.

Trypsin, other enzymes and EDTA are all dissolved in balanced salts solutions without Ca^{2+} and Mg^{2+} ions e.g. Hanks balanced salts (HBSS⁻), to prevent osmotic or pH effects on cell recovery and viability. The enzyme solutions all have EDTA added to them to chelate any residual Ca^{2+} ions present, which prevents cell aggregation after detachment from the growth surface.

The purpose of this study was to optimise the harvesting procedures for Vero cells grown in static T25 culture flasks. The optimal EDTA and trypsin concentrations, centrifugation speed and exposure times for each step were determined. Cell recovery and viability indices from each treatment were normalised to a standard trypsin harvesting procedure. The subsequent cell attachment time and growth rate were also measured. The optimal parameters were defined in T25 culture flasks but should also be applicable to Vero cell microcarrier cultures.

3.2 Materials and Methods

The methods of culturing and counting the cells and the analytical procedures used have been described previously (2.1-2.5).

3.2.1 Experimental Culturing Procedure

Stock Vero cells were detached and recovered using the standard trypsinisation protocol (2.2). The cell pellet was resuspended and counted by the trypan blue dye exclusion method (2.5.1-2). The cells were inoculated at 0.20×10^6 cells/mL in T25 culture flasks with DMEM + 5.0% v/v iron enriched, supplemented calf serum (sCS) (10 mL). The flasks were incubated at 37°C for either 48 h or 96 h prior to use in the cell detachment and recovery experiments. Culture supernatants were discarded prior to each treatment.

3.3 Results and Discussion

3.3.1 Cell Detachment by EDTA Wash Solutions

Introduction. Cells could be recovered from culture flasks using wash solutions containing ethylenediaminetetraacetic acid (EDTA). Therefore, this study determined the effects of varied EDTA concentrations in the wash solution and the wash exposure time on Vero cell recovery to define the optimal wash conditions which would not cause any cell detachment.

Experimental. Stock Vero cells were inoculated and grown in T25 culture flasks, as described above (3.2.1). The cell sheets were washed with D-PBS⁻ containing EDTA at various concentrations (up to 3.40 mM) for various exposure times and any detached cells were recovered. Recovered cells were resuspended in DMEM + 5.0% v/v SCS and total cell recoveries from each EDTA treatment were normalised to the cell recovery from the standard trypsinisation procedure (2.2).

Results. Table 3.1 shows trypsin normalised Vero cell recoveries from T25 culture flasks using wash solutions containing various EDTA concentrations (up to 3.40 mM) and wash times. (1) High proportions of Vero cells could be recovered from T25 culture flasks using wash solutions containing EDTA. (2) Complete (100%) cell recovery from 48 and 96 h grown flasks was obtained with the standard trypsin solution (0.25% w/v trypsin:1.00 mM EDTA) after exposure times of 5-20 min. Complete cell recovery was also obtained with the wash solution at 3.40 mM EDTA after 20 min from 48 h (sub-confluent) flasks. (3) The analyses showed cell recoveries from 48 h flasks were increased proportional to the wash solution EDTA concentration used and were significantly higher ($P < 0.01$) at 3.40 mM after 20 min exposure compared to all other concentrations used. (4) Cell recoveries were increased proportional to the exposure time and were significantly higher ($P < 0.01$) after 20 min compared to all other times used.

(5) There were similar trends in cell recoveries from 96 h flasks compared to 48 h flasks at

Table 3.1 - The effect of the **EDTA concentration** in the D-PBS⁻:EDTA wash solution on **cell recovery** from **T25 culture flasks** grown for either **48 h** or **96 h**. The cells were harvested using the wash solution containing various EDTA concentrations (up to 3.40 mM). The cells were recovered by centrifugation (300 x g for 5 min) and the cell recovery was normalised to a standard trypsin detachment procedure (0.25% w/v porcine trypsin:1 mM EDTA for 5 min). Cell recoveries were determined after various exposure times (up to 20 min) at 20°C. The values are means of n=2.

Time (min)	Percentage Recovered Cells (% maximum count) ^a											
	EDTA Concentration (mM)										Standard Trypsin Solution	
	0.00		0.70		1.70		2.60		3.40			
	48 h	96 h	48 h	96 h	48 h	96 h	48 h	96 h	48 h	96 h	48 h	96 h
1	0	0	0	0	0	0	3	1	3	2	5	7
2	0	0	8	3	21	10	14	10	21	10	35	28
5	0	1	17	10	30	21	40	25	48	30	100	100
10	2	3	47	31	57	45	74	60	85	60	100	100
20	4	4	70	55	77	65	84	80	100	80	100	100

a - The normalised cell counts were obtained by comparison to the cell counts from 5 min trypsinisation of either 48 h or 96 h grown cells. The resulting cell concentrations obtained by the standard trypsinisation procedure were $0.81 \pm 0.06 \times 10^6$ cells/mL for 48 h and $1.85 \pm 0.11 \times 10^6$ cells/mL for 96 h grown cells.

the various exposure times used in this study. However, there was significantly higher ($P < 0.01$) cell recovery from 48 h flasks compared to 96 h (over-confluent) with respect to all wash solution EDTA concentrations and exposure times used. None of the EDTA concentrations (0.70-3.40 mM) used produced complete cell recovery from the 96 h flasks. Indeed, there were significantly fewer (20%, $P < 0.05$) cells recovered from the 96 h flasks at 3.40 mM EDTA with an exposure time of 20 min compared to the standard trypsin detached flask. (6) Few cells ($\leq 5\%$) were recovered in D-PBS⁻ in the absence of trypsin or EDTA from either 48 or 96 h flasks.

Cell Aggregates. EDTA wash solutions liberated the cells as aggregates (2-28 cells) at all wash solution EDTA concentrations and exposure times used. The cell aggregates were larger from the 96 h flasks (10-28 cells) compared to the 48 h flasks (5-10 cells). More of the recovered cells ($\geq 70\%$) were aggregated from 96 h flasks compared to 48 h ($\leq 40\%$) flasks. However, the viability of the aggregated cells was high ($> 90\%$) from all flasks. Vigorous pipetting could not singly suspend cells from the aggregates and resulted in reduced cell viabilities ($\leq 70\%$).

Discussion. Vero cells could be recovered using wash solutions containing EDTA (without trypsin) with long exposure times. This agrees with BEK and FS-4 cell recoveries of Tao et al. (1988). However, this method recovered the cells in large aggregates, as they detached from the surface in sheets (2-10 mm²). These aggregates could not be broken up even with vigorous pipetting which caused low cell viability ($< 75\%$). The cell sheeting or aggregation following removal with EDTA solutions also occurs with human fibroblasts (Cassiman et al., 1981). Thus, Vero could be recovered with EDTA wash solutions but this was not the optimal method to generate singly suspended cells with high viability for subsequent cultures.

3.3.2 Attachment Time and Cell Growth of EDTA Detached Cells

Introduction. The previous study (3.3.1) showed Vero cells could be recovered from T25

culture flasks with wash solutions containing EDTA. EDTA has no proteolytic function that could damage cell membranes, so should not interfere with subsequent attachment and growth of the recovered cells. No reports were found defining any effects of detachment with EDTA solutions. Therefore, this study determined the effects of Vero cell detachment with the various EDTA wash solutions without trypsin (3.3.1) on the attachment time and yields after 72 h in culture.

Experimental. The Vero cells detached with EDTA wash solutions above (3.3.1) were grown in T25 culture flasks the attachment time, lag phase and yield after 72 h in culture were determined (2.5.6).

Results. Table 3.2 shows the cell attachment time and yield after 72 h in culture following recovery with EDTA wash solutions. (1) There were differences in the cell attachment times in subsequent cultures following cell recovery in EDTA solutions. However, there was no significant difference in the cell yields after 72 h in culture with respect to any EDTA concentration used. (2) There was no significant difference in the attachment time of cells harvested using wash solutions containing 0.70 mM EDTA compared to the standard trypsin solution. There were significantly longer ($P < 0.01$) cell attachment times from cells recovered with wash solutions at >0.70 mM EDTA. (3) There was no significant difference in the lag phase times with respect to any wash solution EDTA concentration used.

Discussion. There has only been one report of any difference between cells harvested using EDTA solutions compared to trypsin (Tao et al., 1988). These authors suggested the BEK and FS-4 cells used were trypsin sensitive and thus, used EDTA solutions to detach them. No data was reported to show the trypsin sensitivity. Vero cell yields in this study were not significantly affected by detachment with solutions at any EDTA concentration used. However, subsequent cell attachment times were significantly longer from solutions at high EDTA concentrations which suggests the EDTA had interfered with the cell membranes. This could involve the removal of

Table 3.2 - The attachment time, lag phase and yield of Vero cells in T25 culture flasks detached with wash solutions containing various concentrations of EDTA for various exposure times. The cells were grown for 96 h in DMEM + 5.0% v/v SCS (10 mL) and were detached with EDTA (up to 3.40 mM) for 10 or 20 min. The detached cells were recovered by centrifugation (300 x g for 5 min). The cells were resuspended and diluted to 0.20×10^6 cells/mL in fresh flasks. The cell attachment time was measured (every 0.5 h for 8.0 h) and the cell concentration was determined after 72 h in culture. The values are means of n=2.

Wash Solution EDTA Concentration (mM)	Exposure Time (min)	Cell Attachment Time (h)	Lag Phase Time (h)	Cell Concentration After 72h ($\times 10^6$ /mL)
0.70	10	0.75 ± 0.20	6.00 ± 0.50	$1.41 \pm 0.10^*$
	20	1.50 ± 0.30	6.00 ± 0.50	1.33 ± 0.09
1.70	10	1.50 ± 0.30	6.00 ± 0.50	1.38 ± 0.09
	20	1.50 ± 0.30	6.00 ± 0.50	1.38 ± 0.09
3.40	10	1.50 ± 0.30	6.00 ± 0.50	1.31 ± 0.10
	20	1.50 ± 0.30	6.00 ± 0.50	1.37 ± 0.09

* - Highest cell yield.

Ca^{2+} from the membranes which could be required for cell attachment (Curtis and McMurray, 1986) but such increases did not affect cell yields after 72 h in culture.

3.3.3 Cell Detachment by Trypsin Solutions

Introduction. The use of EDTA solutions for cell recovery is rare, as trypsin solutions at concentrations up to 0.25% w/v are routinely used for recovery of adherent cells. However, trypsin detachment could damage the cell membranes and affect cell recoveries, viabilities and subsequent growth. Therefore, this study determined the optimal trypsin solution concentration and exposure time for Vero cell recovery with and without rapping of the flasks to improve cell detachment.

Experimental. The Vero cell sheets from flasks grown for 96 h were washed with D-PBS⁻ (6 mL) containing EDTA (0.70 mM) and the cells were detached using various trypsin solutions (1 mL of 0.25-1.00% w/v porcine trypsin:1 mM EDTA in HBSS⁻) with rocking and rolling for various exposure times (up to 30 min). The detached cells were recovered and counted by the trypan blue method (2.5.1-2). The cell detachment was performed with and without rapping the flask against the palm of the hand (x10-15) at each time point. The cell recoveries were normalised, as described above (3.3.1).

Results. Fig. 3.1A)-B) show Vero cell recoveries from T25 culture flasks with the various trypsin solutions and exposure times used. Table 3.3 shows the cell viability following recovery with the various trypsin protocols used. (1) Vero cells could be completely recovered with all the trypsin solutions used (up to 1.00% w/v) from flasks which were both rapped (5 min exposure) and unrapped (10 min exposure). (2) There were significantly higher ($P < 0.01$) cell recoveries from rapped flasks compared to unrapped flasks with respect to the trypsin concentrations and exposure times used. (3) The analyses showed cell recovery from rapped flasks was not

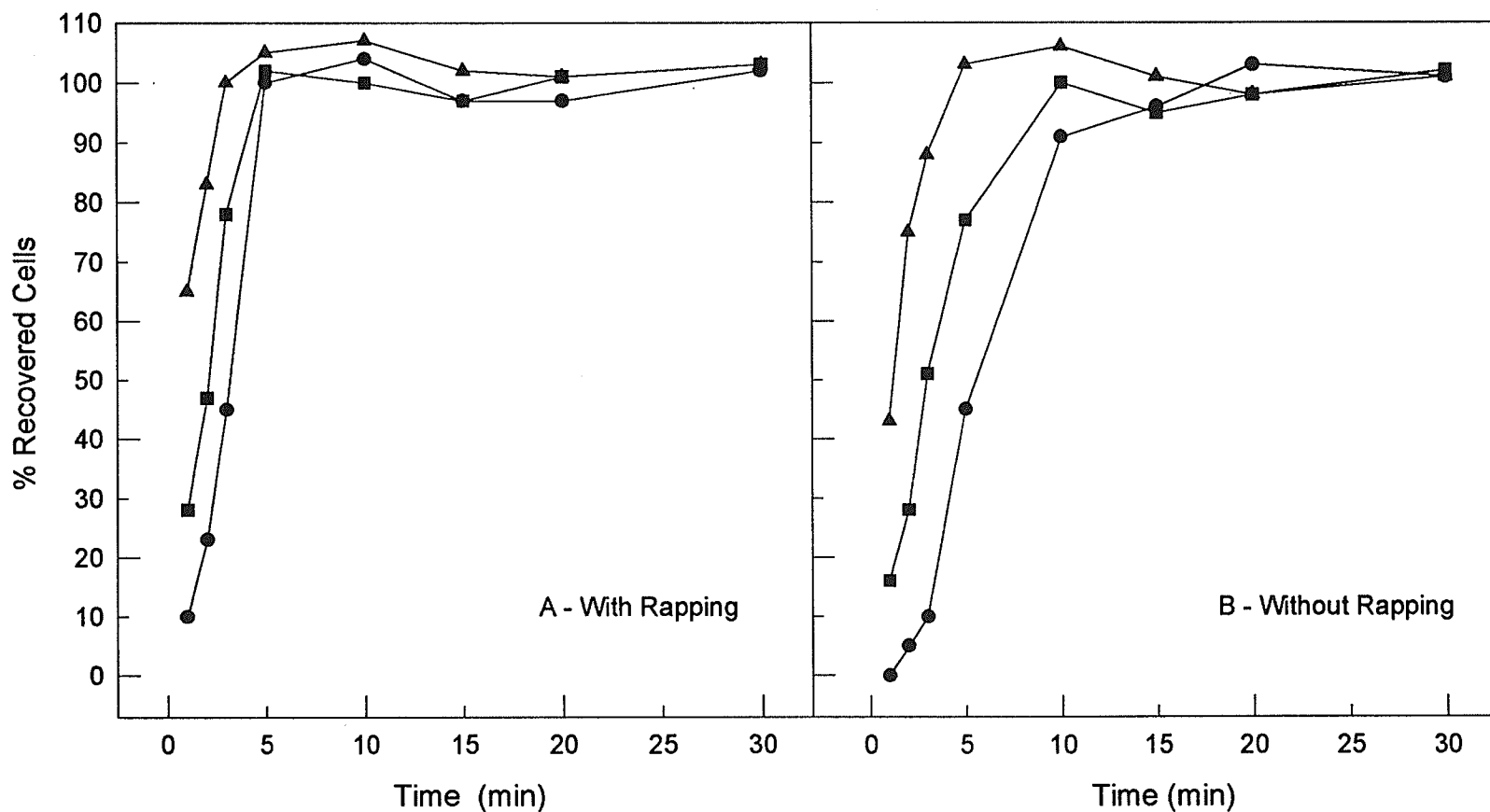


Fig.3.1 - The effect of the trypsin concentration for various exposure times (up to 30 min) on Vero cell recovery with rapping (A) or without rapping (B) from T25 culture flasks grown for 96 h. The cells were inoculated at 0.20×10^6 cells/mL in 10 mL of DMEM + 5.0% v/v SCS. Cells were detached from the flasks with solutions containing 1.00 mM EDTA and either ● 0.25% w/v, ■ 0.50 % w/v or ▲ 1.00% w/v porcine trypsin. The cells were recovered by centrifugation at $300 \times g$ for 5 min, and the cell recovery was normalised to the count from 5 min trypsin detachment with flask rapping, which was $1.85 \pm 0.11 \times 10^6$ cells/mL. The points are means of $n=2$.

Table 3.3 - The effect of the **trypsin solution concentration** and **exposure time** on **Vero cell viability** following recovery from T25 culture flasks **with** and **without rapping**. The cells were inoculated at 0.20×10^6 cells/mL in 10 mL of DMEM + 5.0% v/v sCS. Cells were detached from the flasks with solutions containing 1.00 mM EDTA and up to 1.00% w/v porcine trypsin. The cells were recovered by centrifugation at $300 \times g$ for 5 min, and the cell viability was determined by the trypan blue method. The values are means of $n=2$.

Trypsin Concentration and Exposure Time (min)	Vero Cell Viability by the Trypan Blue Method Following Recovery with Trypsin Solution (%)	
	Unrapped Flasks	Rapped Flasks
0.25% w/v Trypsin		
1	NC	100
2	100	97
3	100	97
5	100	100
10	97	97
15	97	95
20	93	95
30	96	95
0.50% w/v Trypsin		
1	95	100
2	95	95
3	97	95
5	97	95
10	95	95
15	97	97
20	97	92
30	97	96
1.00% w/v Trypsin		
1	100	97
2	100	100
3	95	100
5	95	95
10	95	96
15	97	95
20	95	95
30	95	94

NC - No cells detached from the flasks.

significantly different with exposure times of 5-30 min. However, there were significantly lower ($P < 0.01$) recoveries with exposure times of < 5 min. (4) There was no significant difference in the cell recoveries with respect to the trypsin concentrations or exposure times used.

(5) Visual observation of Vero cell detachment from the flasks showed there were differences between the trypsin concentrations used. Free cells were observed earlier (1 min) with 1.00% w/v trypsin solutions compared to 0.25% w/v trypsin (2 min). Indeed, a high proportion (50% of the total cells) of free cells were detached before the flasks were rapped at the first sample time (1 min) with the 1.00% w/v trypsin solution. Significantly fewer cells (85%, $P < 0.01$) were detached before the first sample time with the 0.25% w/v trypsin solution. The free cells were predominantly (90%) singles or doublets. (6) There was no significant difference in the cell viability ($> 90\%$) with respect to all trypsin solution concentrations and exposure times used.

(7) The use of the EDTA (0.70 mM) wash solution prior to trypsinisation lead to high cell recoveries without cell aggregate formation. Short wash times (1-2 min) were optimal for the subsequent cell recovery and viability. The use of an EDTA wash solution ensured complete Ca^{2+} chelation prior to trypsin detachment and prevented subsequent cell aggregation. Such short wash times prevented any cell detachment prior to trypsinisation.

Discussion. Others have reported exposure to trypsin solutions affecting Vero, MRC-5 and FS-4 cell viabilities and/or recoveries (Gebb et al., 1984, Hu et al., 1985 and Lindskog et al., 1987). However, trypsin solutions at 0.20-0.25% w/v were shown to have a sufficient concentration to detach most ($> 75\%$ of the total) Vero and MRC-5 cells from culture flasks (Lindner et al., 1987). Long exposure times (> 10 min) to trypsin solutions were found to decrease the cell viability (20%). Trypsin solutions with a high pH (pH 7.8) were shown previously to increase the FS-4 cell detachment rate (Hu et al., 1985) and all commercial trypsin solutions are now prepared at this pH (Gibco, 1992).

The results (Fig. 3.1A)-B)) show long exposure times with solutions of all trypsin

concentrations used had no effect on Vero cell recovery or viability. Therefore, the results in this study disagree with those of the previous authors. Flask rapping to detach the cells only affected cell recoveries from short trypsin exposures. There was no cell aggregation after recovery following any trypsin procedure.

3.3.4 Attachment Time and Cell Growth of Trypsin Detached Cells

Introduction. Trypsin detachment (3.3.3) did not affect cell recoveries or viabilities even after long (up to 30 min) exposure times but could affect the subsequent cell growth. Therefore, this study determined the effects of detachment with various trypsin solutions and exposure times on the Vero cell attachment time and yield after 72 h in culture.

Experimental. The Vero cells detached by the various trypsin procedures above (3.3.3) were inoculated in T25 culture flasks, as described previously (2.5.6). The culture flasks were analysed for the cell attachment time and subsequent yield after 72 h (3.3.2).

Results. Table 3.4 shows Vero cell attachment times and yields after 72 h in culture following detachment with the various trypsin solutions and exposure times used. (1) Trypsin solution detachment did affect Vero cell attachment times and yields after 72 h in culture. (2) There were significantly longer ($P < 0.01$) cell attachment times following detachment with trypsin solutions at $>0.25\%$ w/v with respect to all exposure times used. (3) Lag phases were increased proportional to the trypsin solution concentration and the exposure time used. Lag phases were significantly longer ($P < 0.01$) at $>0.25\%$ w/v trypsin solutions with respect to all exposure times used. (4) Cell yields after 72 h in culture were significantly higher ($P < 0.05$) at 0.25% w/v trypsin solutions compared to all other concentrations with respect to all exposure times. (5) Cell yields were significantly higher following a 5 min exposure times compared to all other times used with respect to all trypsin concentrations. (6) There was no significant difference in the cell growth

Table 3.4 - The attachment time, lag phase and yield after 72 h of Vero cells in T25 culture flasks following detachment using various trypsin solutions (up to 1.00% w/v) and exposure times (up to 15 min). The cells from 96 h culture flasks were detached and resuspended with DMEM $\pm$ 5.0% v/v sCS and recovered by centrifugation at 300 g for 5 min. The cells were resuspended and diluted to 0.20×10^6 cells/mL in 10 mL DMEM + 5.0% v/v sCS. Cell attachment was measured every 0.5 h for 8.0 h and the cell concentration was determined after 72 h in culture. The values are means of n=2.

Trypsin Concentration (% w/v)	Exposure Time (min)	Serum ^a (5.0% v/v sCS)	Cell Attachment Time (h)	Lag Phase (h)	Cell Concentration After 72 h ($\times 10^6$ /mL)
0.25	5	+	0.75 ± 0.20	6.00 ± 0.50	$1.44 \pm 0.09^*$
	10	+	1.50 ± 0.30	9.00 ± 1.00	1.21 ± 0.08
	15	+	3.50 ± 1.00	12.00 ± 1.00	1.01 ± 0.07
0.25	5	-	3.50 ± 1.00	12.00 ± 1.00	1.11 ± 0.07
0.50	5	+	2.50 ± 1.00	9.00 ± 1.00	1.18 ± 0.07
	10	+	3.50 ± 1.00	15.00 ± 1.00	1.01 ± 0.07
	15	+	5.00 ± 1.00	18.00 ± 1.50	0.89 ± 0.05
1.00	5	+	3.50 ± 1.00	15.00 ± 1.00	1.02 ± 0.07
	10	+	4.50 ± 1.00	18.00 ± 1.50	0.88 ± 0.05
	15	+	6.00 ± 1.00	24.00 ± 2.00	0.84 ± 0.05

a - Cells were resuspended in DMEM medium with (+) or without (-) 5.0% v/v sCS.

* - Highest cell yield.

rates ($0.037 \pm 0.003 \text{ h}^{-1}$) with respect to the trypsin concentrations and exposure times used.

Discussion. The (Table 3.4) trypsin concentration in the detachment solutions significantly affected Vero cell attachment times and yields after 72 h in this study. Long exposure times to high concentrations of trypsin significantly increased the values observed for all parameters. Lag phases of up to 24 h have been shown for MRC-5 and Vero cells detached using similar trypsin conditions (Lindskog et al., 1987), so the results in this study agree with the previous authors.

All lag phases in adherent cell cultures could be explained by either trypsin induced degradation of specific integrin adherence receptors (Hynes, 1987) or from fibronectin residues remaining on the membranes and interfering with subsequent reattachment (Kleinman et al., 1983). The trypsin concentration and exposure time could affect the amount of integrin and fibronectin degradation. Exogenous fibronectin and or vitronectin bind to flask surfaces and then attach to cell membrane integrins and are known to be required for BHK and bovine corneal endothelial (BCE-1) cells to attach, spread and divide on surfaces (Hughes et al., 1979, Bradshaw et al., 1983 and Underwood and Bennett, 1989). Hence, the cells could require up to 36 h to resynthesise the integrins to allow full attachment, spreading and initiation of cell division after exposure to high trypsin concentrations (1.00% w/v).

Cassiman et al. (1981) showed that dispase and trypsin could detach human diploid fibroblasts from culture flasks. The dispase detached cells had significantly enhanced cell plating and yields (x1.5 more cells after 168 h of growth) in subsequent cultures compared to trypsin detached cells. This could be explained by a reduced degradation of the integrin attachment receptors by dispase compared to trypsin and less biosynthesis would be required following subculture with dispase to produce a rapid cell attachment and therefore, good growth. However, dispase was not tested in this study.

3.3.5 Stopping the Action of the Trypsin Solution

Introduction. Vero cell attachment times, lag phases and cell yields after 72 h in culture were significantly affected (3.3.4) by the trypsin concentration and exposure time used to detach them. These effects probably result from the proteolytic action of trypsin which is usually blocked by the addition of either whole serum which contains protease inhibitors or serum containing medium to prevent further membrane damage during recovery. Therefore, this study determined the optimal resuspension conditions for Vero cell recoveries from T25 culture flasks.

Experimental. The Vero cultures were grown in T25 culture flasks for 96 h, as described above (3.3.1). The cell sheets were washed and detached with the standard trypsinisation procedure (2.2). The detached cells were resuspended in DMEM $\pm$ 5.0% v/v SCS prior to recovery by centrifugation (300 x g for 5 min). The cell recoveries were normalised and the subsequent cell attachment time and yield were determined (3.3.2).

Results. Table 3.4 shows the Vero cell attachment times and yields after 72 h in culture following recovery with serum containing and serum-free DMEM. (1) Vero cell attachment times and yields after 72 h in culture were affected by the resuspension medium following detachment with trypsin solutions. (2) The cell attachment time was significantly longer (79%, $P < 0.01$) for cells resuspended in serum-free DMEM compared to DMEM + 5.0% v/v SCS. (3) The cell yield after 72 h in culture was significantly higher (20%, $P < 0.05$) from cells recovered in DMEM + 5.0% v/v SCS compared to serum-free DMEM. (4) The lag phase was significantly longer (200%, $P < 0.01$) for cells resuspended in serum-free DMEM compared to DMEM + 5.0% v/v SCS. (5) There was no significant difference in the cell growth rates ($0.037 \pm 0.003 \text{ h}^{-1}$) following resuspension in either serum containing or serum-free DMEM.

Discussion. Vero cell attachment and lag phase times were affected by the resuspension

medium and were significantly increased by resuspension and recovery in serum-free DMEM following trypsin detachment. This agrees with previous results with MRC-5 and Vero cells (Lindner et al., 1987). This effect could be caused by the lack of serum in the DMEM resuspension medium to neutralise the trypsin action after cell detachment (Burger, 1970 and Sefton and Rubin, 1970). This could lead to further degradation of the cell membrane integrin receptors causing the effects above (3.3.4). However, Vero cell lag phases in this study were significantly reduced from those of Lindner et al. (1987) (40 h). Hence, cells should be resuspended in serum containing medium following trypsin detachment to prevent excessive proteolytic damage and improve growth after subculture.

3.3.6 Centrifugal Force and Time

Introduction. Complete cell recovery is achieved by centrifugation which creates shear forces dependent on the centrifuge rotor radius and the rotation speed used. The forces could affect the cell viability or recovery. No reports were found describing the effects of centrifugal forces or exposure times on cell recoveries and viabilities. Therefore, this study determined the optimal centrifugal force and exposure time for the recovery of Vero cells.

Experimental. The Vero cultures were grown in T25 flasks, as described above (3.3.1). The cells were detached by the standard trypsinisation procedure (2.2) and resuspended in DMEM + 5.0% v/v SCS. The detached cells were recovered by various centrifugal forces (30-1,100 x g) and exposure times (5-15 min) and recoveries were normalised, as described above (3.3.2).

Results. Fig. 3.2 shows Vero cell recoveries with the various centrifugal forces and times used. (1) Vero cell recoveries were dependent on the centrifugal force and exposure time used. (2) There were significantly higher ($P < 0.01$) cell recoveries at centrifugal forces $> 200 \times g$ compared to all other forces used and with respect to the exposure times (5-15 min) used. (3) Cell recoveries

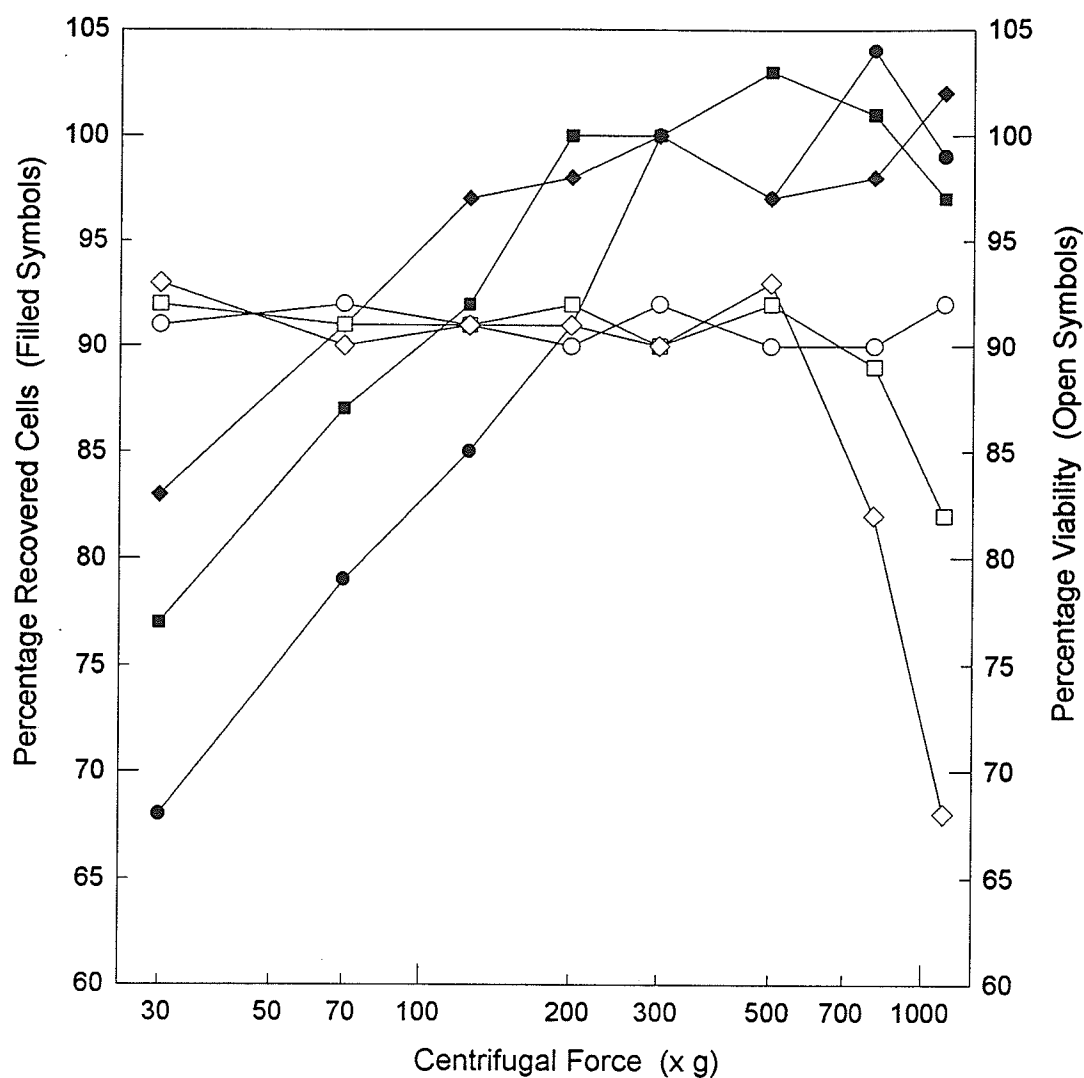


Fig.3.2 - The effect of various centrifugal forces (30-1,100 x g) and exposure times (5-15 min) on Vero cell recovery (Closed symbols) and viability (Open symbols) from T25 culture flasks grown for 96 h. The cells were inoculated at 0.20×10^6 cells/mL and detached with 0.25% w/v trypsin:1.00 mM EDTA for 5 min and resuspended in DMEM + 5.0% v/v SCS. Cells were recovered by centrifugation for ● 5 min, ■ 10 min or ♦ 15 min. The cell recovery was normalised to the recovery from centrifugation at 300 x g for 5 min, which was $1.85 \pm 0.11 \times 10^6$ cells/mL. The points are means of n=2.

were significantly higher ($P < 0.01$) at > 5 min centrifugations with forces of $< 200 \times g$. However, there were no significant differences between the exposure times at forces $> 200 \times g$.

Cell Aggregates. Cells recovered at high centrifugal forces formed cell aggregates during centrifugation which remained in the cell solution after gentle resuspension. Cell aggregate sizes were significantly higher (> 50 cells) at high forces ($800\text{--}1,100 \times g$) and long centrifugation times (15 min). The cell aggregates could be resuspended to single cells by vigorous pipetting but this significantly reduced (30%, $P < 0.01$) the cell viabilities. The cell viability was high (90%) from all centrifugal forces used at short (5 min) centrifugation times.

Discussion. Centrifugation is the best way to recover cells quickly from a suspension but creates shear forces and long (≥ 10 min) or high centrifugal forces ($\geq 500 g$) could reduce cell viability. No one has previously described this effect. Vero cell viability was not affected by the centrifugal force at short (5 min) times and the reduced viability seen after centrifugation at $800\text{--}1,100 g$ for 10–15 min was caused by the increased pipetting required to resuspend the cell pellet. The force was not responsible, as the aggregated cells showed high viabilities prior to such pipetting.

Conversely, low centrifugal forces for short times only loosely pelleted the cells and large cell losses occurred when discarding the supernatant. Longer centrifugation times at low forces formed tighter pellets and resulted in higher recoveries.

3.3.7 Attachment Time and Growth of Cells Recovered by Centrifugation

Introduction. Cell recovery and viability was affected by the centrifugal force and exposure time used. No reports were found defining the effects of centrifugation on cell attachment and growth in subsequent cultures. Therefore, this study determined the Vero cell attachment times and yields after 72 h in culture, following detachment with trypsin and recovery by various

centrifugation procedures.

Experimental. The Vero cells detached and recovered by centrifugation (3.3.6) were grown in T25 culture flasks (3.3.2). The attachment times and subsequent cell yield after 72 h in culture were determined (3.3.2).

Results. Table 3.5 shows the cell attachment times and yields from such centrifugations. (1) Vero cell attachment times and subsequent yields after 72 h in culture were affected by the centrifugal force and time used. (2) There were significantly longer (49%, $P < 0.01$) cell attachment times at $>500 \times g$ with respect to all exposure times used. (3) There were significantly longer (41%, $P < 0.01$) cell attachment times at >5 min exposures with respect to all centrifugal forces used. (4) Cell yields after 72 h in culture were significantly higher (21%, $P < 0.01$) at $300 \geq 500 > 1,100 \times g$ with respect to all the exposure times used (up to 15 min). (5) There were significantly longer (67%, $P < 0.01$) lag phases from high ($1,100 \times g$) centrifugal forces compared to low ($300 \times g$) forces with respect to all exposure times used. (6) There was no significant difference in the cell growth rates ($0.037 \pm 0.003 \text{ h}^{-1}$) with respect to all centrifugal forces and exposure times used.

Discussion. No previous reports were found describing the influence of centrifugation for cell recovery on subsequent cell growth. The centrifugal force and exposure time used for recovery significantly affected the subsequent growth and lag phases of Vero cells in T25 culture flasks. The low cell yields at high ($1,100 \times g$) centrifugal force could result from the lower cell viability of the singly suspended cells produced by vigorous pipetting.

3.4 Conclusions

The purpose of this study was to define the optimal protocols for the detachment and recovery of Vero cells from T25 culture flasks. The conclusions from the work above were that:

Table 3.5 - The attachment time, lag phase and yield after 72 h in culture of Vero cells in T25 culture flasks recovered using various centrifugal forces (300-1,100 x g) and exposure times (5-15 min). The cells were grown for 96 h in DMEM + 5.0% v/v sCS (10 mL) and were detached with 0.25% w/v trypsin in HBSS⁻:1.00 mM EDTA for 5 min. Cells were recovered by centrifugation and were resuspended and diluted to 0.20×10^6 cells/mL in T25 culture flasks. Cell attachment was measured every 0.5 h for 8.0 h and the cell concentration was determined after 72 h in culture. The values are means of n=2.

Centrifugal Force (x g)	Exposure Time (min)	Cell Attachment Time (h)	Lag Phase (h)	Cell Concentration After 72 h ($\times 10^6$ /mL)
300	5	0.75 ± 0.20	6.00 ± 0.50	$1.44 \pm 0.09^*$
	10	1.50 ± 0.30	9.00 ± 1.00	1.37 ± 0.09
	15	3.50 ± 1.00	12.00 ± 1.00	1.21 ± 0.08
500	5	0.75 ± 0.25	9.00 ± 1.00	1.33 ± 0.08
	10	1.50 ± 0.30	12.00 ± 1.00	1.23 ± 0.06
	15	3.50 ± 1.00	15.00 ± 1.00	1.15 ± 0.07
1,100	5	2.50 ± 1.00	12.00 ± 1.00	1.20 ± 0.07
	10	3.50 ± 1.00	15.00 ± 1.00	1.13 ± 0.06
	15	4.50 ± 1.00	18.00 ± 1.00	0.99 ± 0.06

* - Highest cell yield.

- **complete cell detachment** was possible with wash solutions containing 3.40 mM EDTA for 20 min in the absence of trypsin. However, this resulted in large cell aggregates (2-28 cells) or sheets which could not be singly suspended by vigorous pipetting.

- **cell viability and recovery** were unaffected by the trypsin concentration (up to 1.00% w/v), trypsinisation time (up to 30 min) or EDTA concentration used (up to 3.40 mM) in the detachment solutions.

- **the optimal EDTA concentration** in the wash solution was 0.70 mM with a contact time of ≤ 2 min.

- **the optimal cell detachment** was with a solution of 0.25% w/v trypsin with 1.00 mM EDTA with an exposure of 5 min. Long exposure times (15 min) or high trypsin concentrations (1.00% w/v) resulted in long cell attachment times (6 h) and lag phases (24 h) in subsequent growths.

- **the optimal resuspension medium** was DMEM + 5.0% v/v sCS prior to recovery.

- **the optimal cell recovery** was with a centrifugal force of 300 x g for 5 min. Similar cell recoveries and viabilities were found with centrifugation at 125-1,100 x g for ≤ 10 min. High centrifugal forces (1,100 x g) and long times (> 10 min) resulted in cell aggregates which required vigorous pipetting for resuspension. However, such pipetting produced low viabilities which reduced the cell yields of subsequent cultures. Low centrifugal forces (< 125 x g) for < 10 min resulted in reduced cell recoveries but with high viability.

**Chapter 4. Comparing Trypan Blue Dye Exclusion and Crystal Violet Nuclei Staining
Techniques for Counting Mammalian Cells**

4. Comparing Trypan Blue Dye Exclusion and Crystal Violet Nuclei Staining Techniques for Counting Mammalian Cells.

4.1 Introduction

Suspended cells e.g. hybridomas, are counted using the trypan blue dye exclusion method (Amos et al., 1969). This method involves dilution of cell samples with the dye and the solution is then placed on a haemocytometer slide and the cells are immediately counted under a microscope. This method is quick and easy and the cell viability can be estimated from the ratio of cells staining blue to total cells.

Adherent cells e.g. African monkey kidney (Vero) cells, have to be physically or enzymically detached from their growth surface before counting by the trypan blue method. This significantly increases the sample preparation time and could reduce the recovery and viability of Vero and human diploid lung fibroblast (MRC-5) cells (Gebb et al., 1984 and Lindskog et al., 1987).

Consequently, adherent cells are often counted by the crystal violet nuclei stain procedure (Sanford et al., 1951 and van Wezel, 1967), frequently used with microcarrier cultures (Morandi et al., 1982, Hu et al., 1985, White and Ades 1990 and Forestall et al., 1992). This method involves dilution of culture samples with a mixture of citric acid, Triton X-100 detergent and crystal violet dye. The mixture is incubated for a minimum of 1 h (Lin et al., 1991) for complete lysis of the cell membrane by the hypertonic citric acid solution and denaturation by the Triton X-100. The released nuclei become stained with the crystal violet dye and can be counted on a haemocytometer. This method is quick (excepting the incubation time), easy to perform and the cells can be treated without removal from the substrata. However, Sanford et al. (1951) proposed the nuclei staining method to show cell proliferation by an increase of nuclei and not as a method for estimating their true concentrations.

Other cell counting techniques have been developed for suspended and adherent cells including the Coulter counter, formazan dyes, enzyme assays and image analysis.

The Coulter counter (Coulter, 1953) was developed as an automatic counter and particle

sizer. The principle of the instrument is that a vacuum applied on the electrolyte at the top of an glass electrode draws a known volume of culture sample through an orifice at the base of the electrode. Particles or cells pass through an electric current applied across the orifice and disrupt the current proportional to their size. The instrument can be preset to ignore particles of a larger or smaller size than those of interest.

The formazan methods use dyes such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Green et al., 1984), which can only be metabolised by viable cells into fluorescing compounds. Cell samples are incubated and counted by haemocytometer under a microscope.

Cell death and lysis cause the release of the enzyme lactate dehydrogenase (LDH) and determining the LDH activity of a known sample volume, both with and without treatment (e.g. sonication) to disrupt the viable cells, can give the viable and total cell numbers (Tolman et al., 1978). The LDH activity is determined using a 96-well plate assay for the breakdown of lactate generating reduced β -nicotinamide adenine dinucleotide which can be monitored by light absorbance at 340 nm. Standard curves of LDH activity must be determined and compared to known cell concentrations for the enzyme method to be used effectively.

Image analysis (Foran et al., 1991) has been developed for counting cells on macroporous microcarriers. The method involves fixing the cells on the beads with formaldehyde and staining them with a series of histologic dyes. The beads are then photographed by scanning electron microscopy and the negatives scanned by a microdensitometer with and without filter processing to discriminate between cytoplasmic and nuclei staining. Scan results are then analysed by computer to determine the cell number.

Some of the methods above have been previously validated against the trypan blue counting method (Duval et al., 1990) with murine hybridoma cells. However, no direct comparison of trypan blue and crystal violet counting methods was found in the literature.

In this Chapter we report a direct comparison of the crystal violet, trypan blue and Coulter counter methods for counting suspended hybridomas (CC9C10) and adherent Vero and Chinese

hamster ovary cells (CHO). Data was generated in static flasks and with solid (Cytodex-1 and 3) and macroporous (Cultispher-G) microcarriers as substrata for the adherent Vero and CHO cells in spinner flask cultures. Cells were grown in batch, daily refed and nutrient limited cultures to assess whether the culture system influenced the results.

4.2 Materials and Methods

4.2.1 Cell Lines

Vero (2.1), murine hybridoma (CC9C10) and Chinese hamster ovary (CHO) cells were obtained from ATCC, catalogue numbers CCL-81, HB-121 and CCL-61 respectively. Stock cells were grown in T75 and T150 culture flasks (2.1). Cells for experiments were subcultured twice with a 1:4 dilution every 48 h for exponential phase cells or every 120 h for stationary phase cells. Cells were grown in DMEM + 5.0% v/v iron enriched, supplemented calf serum (sCS). The DMEM was prepared as described previously (2.3).

4.2.2 Cell Harvesting, Recovery and Subculture

CC9C10 culture flasks were rapped against the palm of the hand (15 times) and homogeneously resuspended. The required volume for the subculture was then removed and added to the new culture flask. Vero and CHO cells were detached and recovered by the standard trypsinisation procedure (2.2). Although most of the CHO cells were growing adherently on the culture flask surface, there was also a substantial proportion of suspension cells. Thus, CHO cell supernatants were retained to collect such suspended cells.

4.2.3 Cell Counting Protocols

Cell counting procedures have been described previously (2.5.1-4).

4.2.4 Fluorescence Activated Cell Sorting

Vero cells were fixed and prepared for fluorescence activated cell sorting (FACS) analysis by

the method of Shapiro (1988). The method was as follows:

I. A total of 3.00 to 6.00×10^6 cells were centrifuged at $300 \times g$ for 5 min. The supernatant was discarded.

II. The cell pellet was resuspended in of D-PBS⁻ (1.00 mL). Ice cold ethanol (1.70 mL) was slowly added whilst vortexing the tube. The cells were placed on ice and gently shaken to resuspend every 10 min for 1 h.

III. The cells were centrifuged at $300 \times g$ for 5 min. The supernatant was discarded.

IV. The cell pellet was washed (x2) with D-PBS⁻ (5.00 mL) and were centrifuged at $300 \times g$ for 5 min and the supernatant discarded.

V. The cells were resuspended with D-PBS⁻ (1.00 mL).

VI. RNase (1.00 mL of 1.0 mg/mL) was added and the tube incubated at 37°C for 30 to 45 min.

VII. The cells were centrifuged at $300 \times g$ for 5 min and the supernatant discarded.

VIII. The cell pellet was resuspended in D-PBS⁻ (5.00 mL) and was either stored at 4°C for up to 48 h or was immediately prepared for FACS analysis (IX to XIII).

IX. Cells were prepared for FACS analysis by centrifugation at $300 \times g$ for 5 min and the supernatant was discarded.

X. The cell pellet was resuspended in D-PBS⁻ (1.00 mL) and the tubes were wrapped in aluminium foil.

XI. Propidium iodide (1.00 mL at 50.0 $\mu\text{g/mL}$) in D-PBS⁻ was added and the tubes vortexed (20 s). The samples were incubated at 20°C for at least 1 h prior to FACS analysis.

XII. The cells were placed onto an EPICS-753 FACS instrument (Coulter[®] Electronics Inc., Hialeah, FL, U.S.A.). The cells were passed at a rate of 500 cells/s through an Argon ion laser beam at a wavelength of 488 nm. A total of $10,000$ cells were passed through the laser beam for each sample analysis.

XIII. The propidium iodide fluorescence of the cells was detected for wavelengths of >610 nm. The instrument analysed the forward angle (between 2 - 19°) and sideways (90°) light scatter

as the cells were passed through the laser beam.

4.3 Results and Discussion

4.3.1 Growth in Culture Flasks

Introduction. Trypan blue and Coulter counter methods have been directly compared and found to yield similar cell counts but the crystal violet method has never been directly compared to any of the whole cell count methods. Therefore, this study was performed to validate cell counting by the crystal violet nuclei staining method with the whole cell counting methods of trypan blue dye exclusion and Coulter counter.

Experimental. The suspension cell line was murine hybridoma CC9C10 and the adherent cells were Vero and CHO. The cells were inoculated and grown in T25 culture flasks (2.1). One flask of each cell line was sacrificed daily for cell counts by the trypan blue, crystal violet or Coulter counter methods.

Results. Fig. 4.1 shows crystal violet nuclei stain, trypan blue dye exclusion and Coulter counter method counts of the growth of suspension hybridoma (CC9C10), Vero and CHO cells in T25 culture flasks. (1) Counts of CC9C10, Vero and CHO cells in T25 culture flasks differed between the crystal violet nuclei stain, trypan blue dye exclusion and Coulter counter counting methods used. (2) There was no significant difference between cell counts by the trypan blue and Coulter counter methods during the exponential phase with respect to all cell lines used. (3) There were significantly higher (30%, $P<0.01$) Vero and CHO cell counts (Fig. 4.1B-C)) at 120 h (mid-stationary phase) by the trypan blue method compared to the Coulter counter method. Cell aggregates (2-15 cells) were found in the trypan blue count samples at this time but were not present when the counts were similar. (4) Cell counts of CC9C10 (Fig. 4.1A)) showed a significantly higher count (30%, $P<0.01$) after 48 and 72 h in culture by the crystal violet method

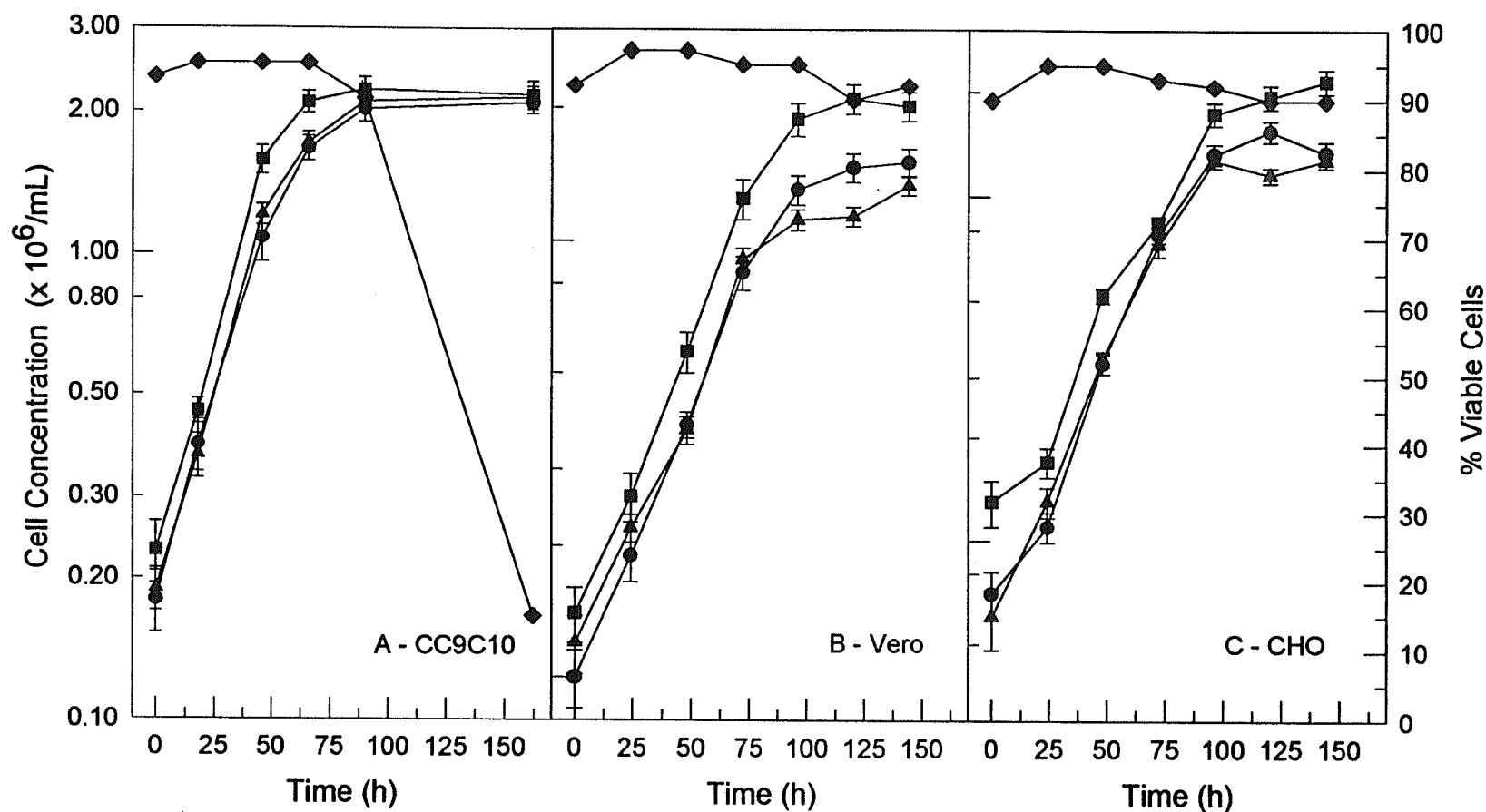


Fig. 4.1 - The effect of counting CC9C10 (A), Vero (B) and CHO (C) cells by the trypan blue, crystal violet nuclei staining or Coulter counter methods during batch growth in T25 culture flasks. The cells were inoculated at 0.10×10^6 cells/mL in 10 mL of DMEM + 5.0% v/v SCS. One culture flask was sacrificed daily for cell counts by either the ● trypan blue, ■ crystal violet nuclei staining or ▲ Coulter counter methods. Cell viability ◆ was also determined by the trypan blue method. The points are means of $n=2$.

compared to the other methods used. (5) Vero cell counts (Fig. 4.1B)) from 24 h onwards were always significantly higher (30-50%, $P < 0.01$) by the crystal violet method compared to the other methods used. (6) Cell counts of CHO (Fig. 4.1C)) showed significantly higher counts (30-50%, $P < 0.01$) at all time points, except 72 h in culture, by the crystal violet method compared to the other methods used. (7) There was no significant difference in the maximum CC9C10 cell count with respect to all the count methods used. This count remained constant through the decline phase as the viability by the trypan blue method was reduced (85%). (8) Vero and CHO cell viabilities remained high (>90%) throughout the stationary phase. (9) Vero and CHO cells had significantly higher (38%, $P < 0.01$) maximum counts at every sample time in the stationary phase by the crystal violet method compared to the other methods used. (10) There was no significant difference between the maximum counts of all cell lines by the crystal violet method.

Discussion. Duval et al. (1990) previously showed little difference between the trypan blue and Coulter counter methods with hybridoma cells. However, no reports were found comparing the crystal violet nuclei staining method against any other method for enumerating adherent cells. van Wezel (1985) suggested the crystal violet method gave more reproducible counts, though no evidence or reasoning for this statement was given. A report (Cherry and Papoutsakis, 1988) was found where bovine embryonic kidney (BEK) cells were counted by both trypan blue and crystal violet methods for the first three counts. The authors found no differences between the two methods and hence, used the crystal violet method thereafter.

The results (Fig. 4.1A-C)) in this study showed counts for all three cell lines during the exponential phase were similar by the trypan blue and Coulter counter methods. CC9C10 cells also had similar cell counts in the stationary phase by trypan blue and Coulter counter methods which agrees with the results of Duval et al. (1990). However, Vero and CHO cells were detached as small cell aggregates after 120 h which caused count differences between these methods. The aggregates could be counted by the trypan blue method but the Coulter counter either recorded each aggregate as one event or excluded them - a major limitation of the Coulter

counter method. Most CC9C10 cells were singly suspended, hence, counts by the trypan blue and Coulter counter methods were similar.

Cell line dependent count differences were found between the crystal violet and trypan blue or Coulter counter methods. Counts by the crystal violet method were consistently higher for the adherent cells (Vero and CHO). The CC9C10 suspension cells had few count differences between the methods and a similar maximum count by all methods. The maximum counts by the crystal violet method were similar in all cell lines.

4.3.2 Growth in Spinner Flasks

Introduction. Significant differences were found (4.3.1) between the nuclei staining and trypan blue counting methods for CC9C10, Vero and CHO cells grown in T25 culture flasks. However, the crystal violet counting method is primarily used in microcarrier research using spinner flasks, so the differences observed could be growth system dependent. Therefore, the above experiment was repeated with cells grown in spinner flasks, in batch and daily refed modes, to assess if the count differences between the methods were growth system dependent.

Experimental. CC9C10 (batch cultures only), Vero and CHO cultures were grown in 100 mL spinner flasks (2.4.3). Cytodex-1 and 3 (1.00 g/L) were used for Vero and CHO cells, respectively, and macroporous Cultispher-G (1.70 g/L) for Vero cells. Refed cultures were inoculated as above and were given one daily medium exchange (50% v/v) from 72 h onwards. The cells were counted by the crystal violet and trypan blue methods. Samples from Cytodex cultures were treated with the standard trypsin solution (0.25% w/v:1.00 mM EDTA) with and without dextranase (50 units/mL) to denature the beads. The Coulter counter method could not generate cell counts from solid microcarrier cultures.

Results. Fig. 4.2 shows batch CC9C10 cell growth in suspension culture and Vero and CHO cell

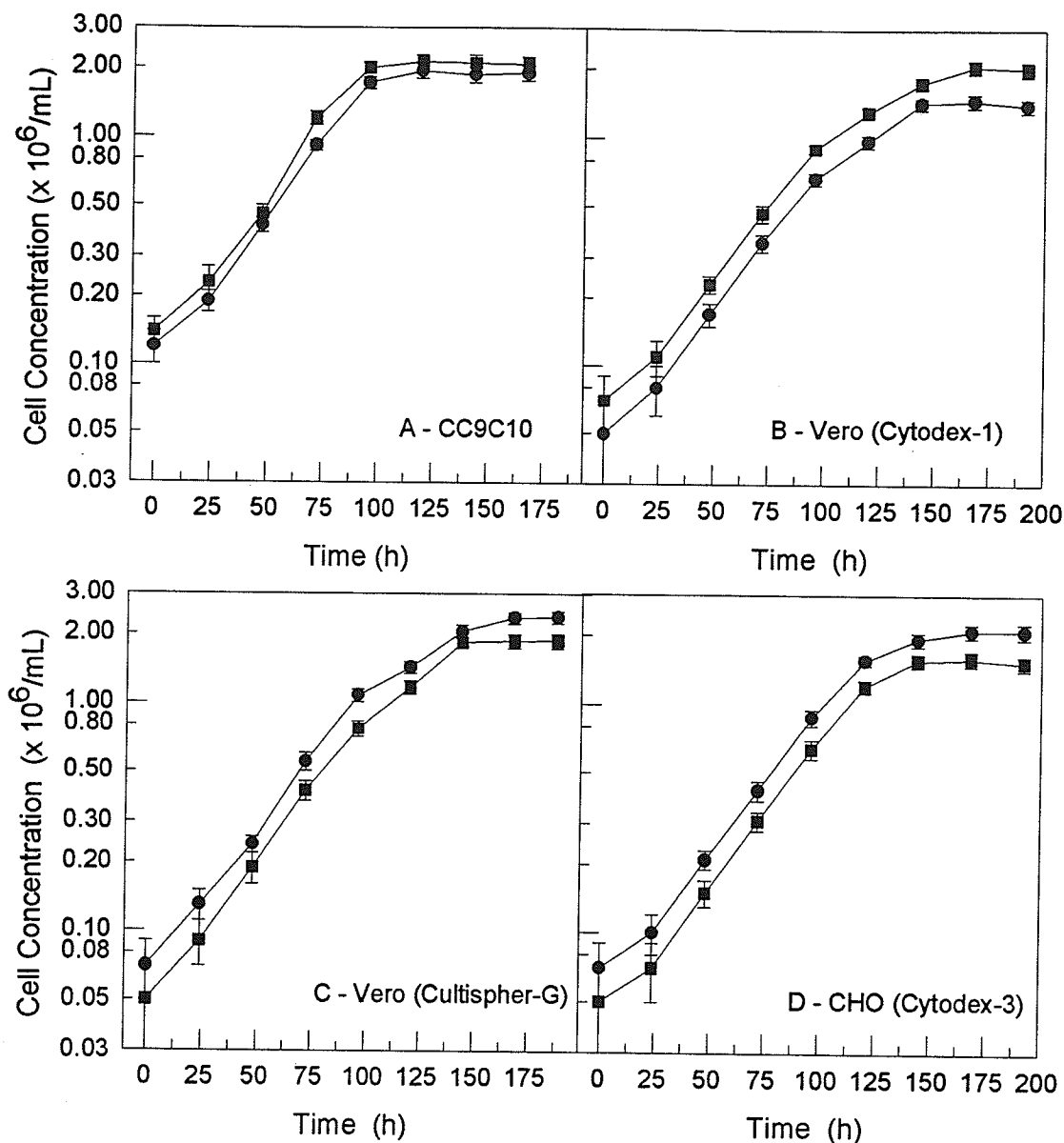


Fig. 4.2 - The effect of counting CC9C10 (A), Vero on Cytodex-1 (B), Vero on Cultispher-G (C) and CHO on Cytodex-3 (D) cells by trypan blue or crystal violet nuclei staining methods during batch growth in 100 mL spinner flasks. Cultures were inoculated at 0.15×10^6 CC9C10 cells/mL and 0.05×10^6 Vero and CHO cells/mL and stirred continuously at 100 rpm (CC9C10) or 40 rpm (Cytodex-1) or intermittently at 40 rpm for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G) in DMEM + 5.0% v/v SCS. Cells were counted by either the ● trypan blue or ■ crystal violet nuclei staining methods. The flasks were readjusted to pH 7.4 after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

growths in cultures of Cytodex-1 and 3 and Cultispher-G microcarriers counted by crystal violet and trypan blue methods. Fig. 4.3 shows daily refed growths of Vero and CHO cells on microcarriers. Table 4.1 shows the Vero cell viabilities by the trypan blue method following microcarrier sample treatments with trypsin solutions prepared with or without dextranase. (1) There were differences in the cell counts by crystal violet nuclei method compared to trypan blue in all the spinner cultures but the differences were cell line dependent. (2) There (Fig. 4.2A)) was only one significantly higher (32%, $P < 0.01$) CC9C10 cell count after 72 h in culture by the crystal violet method compared to trypan blue. (3) There was no significant difference in the maximum CC9C10 cell counts in the stationary phase with respect to either count method used. (4) There (Fig. 4.2B)-D) were significantly higher ($>30\%$, $P < 0.01$) Vero and CHO cell counts in both the stationary and exponential growth phases at every time point after 48 h by the crystal violet method compared to trypan blue. (5) There were some free CHO cells ($<15\%$ of the total count by the trypan blue method) present in the supernatant throughout the culture. However, few ($<1\%$) Vero cells were freely suspended at any time. (6) There was no significant difference between the maximum counts of Vero and CHO cells in batch spinner flasks by either count method used.

(7) There were (Table 4.1) differences in the Vero and CHO cell viabilities, following detachment with the standard trypsin solution (without dextranase), by the trypan blue method between the bead types. There were significantly lower (up to 30%, $P < 0.05$) cell viabilities in cultures of Cytodex-1 and 3 compared to Cultispher-G at all sample times used. The standard trypsin solution did not degrade Cytodex beads but did degrade Cultispher-G beads. However, when dextranase was added to the trypsin solution the solid beads were completely degraded and cell viability was not significantly different in cultures of either bead. Some ($\leq 10\%$ of the total cell count) free nuclei were always present in cell counts by the trypan blue method.

(8) The results (Fig. 4.3A)-C)) of daily refed (one 50% v/v medium exchange from 72 h onwards) growths of Vero and CHO cells showed similar trends to the batch cultures above. There was no significant difference in the general results in daily refed compared to batch

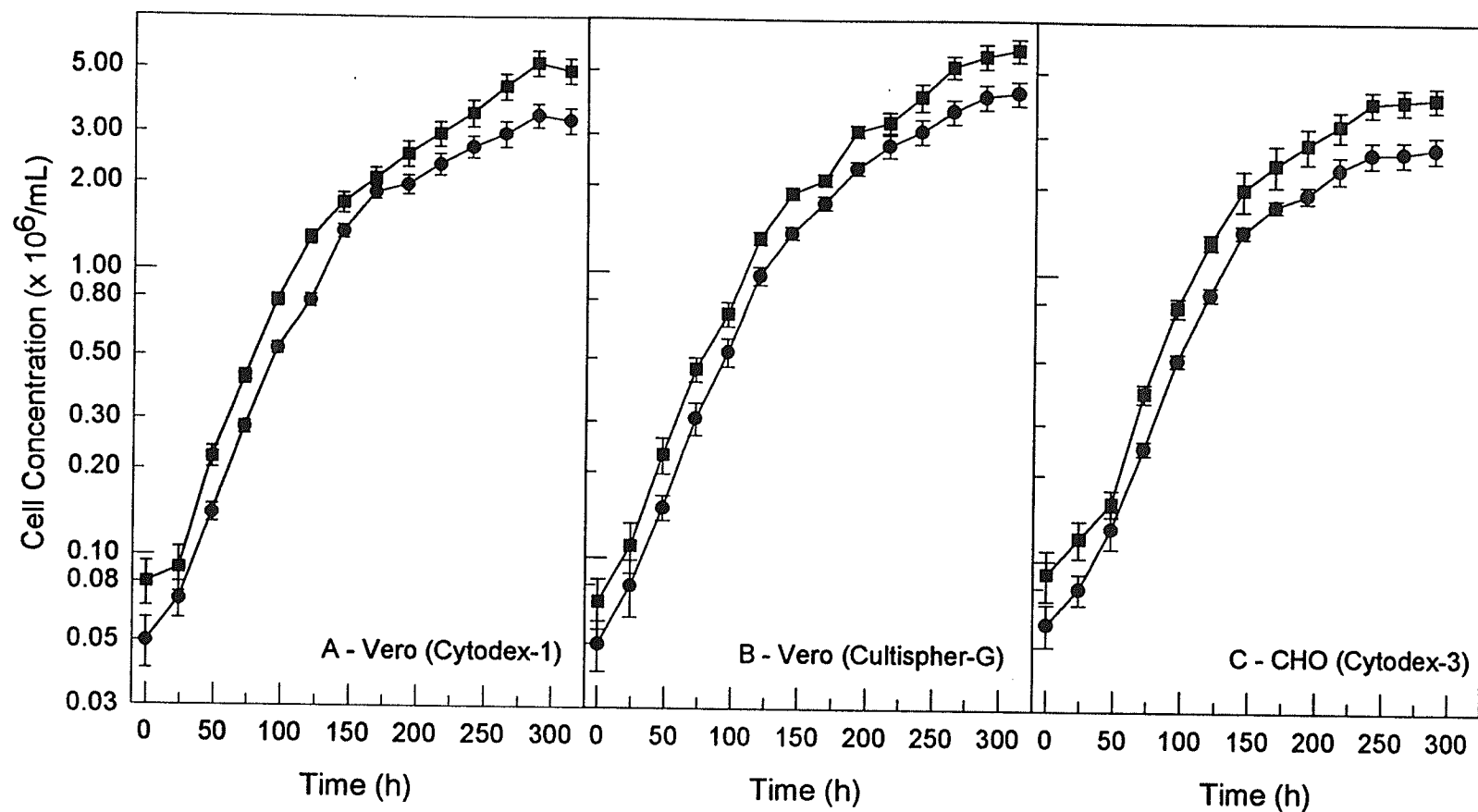


Fig. 4.3 - The effect of cell counting Vero on 1.00 g/L Cytodex-1 (A) or 1.70 g/L Cultispher-G (B) and CHO on 1.00 g/L Cytodex-3 (C) by trypan blue or crystal violet nuclei staining methods during daily refed growth in 100 mL spinner flasks. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS. Flasks were stirred continuously at 40 rpm. Daily refeeding by one 50% v/v medium exchange was initiated after 72 h in culture. Cells were counted by either ● trypan blue or ■ crystal violet nuclei staining. The flasks were readjusted to pH 7.4 after 72 h in culture with 2.00 M NaOH. The points are means of n=2.

Table 4.1 - The effect of the composition of the **enzymic detachment solution** on the **viability** of Vero cells in count samples from 100 mL spinner flask on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.15×10^6 cells/mL and stirred continuously at 40 rpm in DMEM + 5.0% v/v sCS. Flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). Cells were detached from the beads with solutions containing 1.00 mM EDTA and either porcine trypsin (0.25% w/v) or porcine trypsin:dextranase (0.25% w/v:50 units/mL). Cell viability was determined by the trypan blue method. The values are means of n=2.

Sample Time (h)	Cell Viability by the Trypan Blue Method Following Enzymic Detachment from Particular Bead Type (%)			
	Cytodex-1		Cultispher-G	
	Trypsin	Trypsin/Dextranase	Trypsin	Trypsin/Dextranase
0	70	95	97	100
24	73	95	100	100
48	70	95	95	97
72	70	92	95	92
96	72	92	92	95
120	71	94	95	95
144	70	94	93	92
168	73	95	94	93

spinner flask cultures with respect to either cell line used. (9) There were significantly higher counts for Vero (240%) and CHO (190%) cells in the daily refed cultures compared to batch with respect to either count method used. (10) There were significantly higher (>50%, $P<0.01$) stationary phase cell counts by the crystal violet method compared to the trypan blue method in daily refed cultures of either cells. (11) There were significantly higher (30%, $P<0.01$) numbers of freely suspended CHO cells (by the trypan blue count method) in daily refed compared to batch cultures at maximum counts.

Discussion. The crystal violet counting method has been used extensively to assess cell growth in microcarrier cultures since it was first proposed (van Wezel, 1967). However, the results above show trypan blue and crystal violet methods do not give the same counts for the adherent cells used and the differences were not growth system dependent. It was impossible to use the Coulter counter as Cytodex beads blocked the orifice of the electrode.

Trypsin detachment of cells was reported to reduce Vero and MRC-5 cell viabilities (Gebb et al., 1984) which could have affected counts by the trypan blue method through losses of any disrupted cells. Incomplete trypsin detachment could also have resulted in a reduced count by the trypan blue method. However, losses could also have occurred during the preparation for the crystal violet count, as nuclei also stick onto growth surfaces. The methods in this study eliminated errors from count sample preparation, as the cells were detached by previously optimised methods which do not affect cell viabilities by the trypan blue method (3.3.3) and only detached cells were counted.

The CHO and Vero cells (Table 4.2A)-D) and Fig. 4.3A)-C)) grown in spinner flasks showed high count differences between the crystal violet and trypan blue methods. Qualitz et al. (1993) previously reported maximum CHO cell counts by the trypan blue method (1.15×10^6 cells/mL) and the crystal violet method (1.85×10^6 cells/mL) which were lower compared to those in this study. However, the authors grew CHO cells in two different systems and used only one count method per system (crystal violet method with microcarriers and trypan blue with suspension

cells). These authors concluded microcarriers were the optimal culture system for CHO as maximum yields were significantly higher (38%) compared to suspension cultures. The data in this study suggests this could have resulted from the different count methods used between the growth systems. The previous authors count differences, even though they were not from the same flasks, were similar to the CHO cell count difference (32%) in this study. Vero cell counts by the crystal violet method also agree with previously reported maximum yields of approximately 2.00×10^6 cells/mL (Polastri et al., 1984 and Goetghebeur et al., 1991).

Cell Viabilities. Cell viabilities (Table 4.1), following detachment with trypsin solutions (without dextranase), by the trypan blue method were lower for Vero and CHO cells in cultures of solid Cytodex-1 and 3 compared to macroporous Cultispher-G beads. This could be explained by the solid beads being present in the culture samples throughout count processing. Bead collisions could have disrupted the detached cells during resuspension for the count. This mechanism would be similar to that proposed for BEK cell damage in highly agitated cultures (Cherry and Papoutsakis, 1988). Adding dextranase to the trypsin solution completely denatured the polydextran beads and gave high cell viabilities in the count samples agreeing with previous Vero and MRC-5 cell viabilities using dextranase to remove the Cytodex beads (Gebb et al., 1984). However, the count method differences could not be explained by the viability loss in the trypan blue method, as the blue stained cells were counted in the total counts. Some free nuclei (<10% of the total count by the trypan blue method) were observed in trypan blue method counts. However, there were still significant differences between the methods when these nuclei were added into the total count by the trypan blue method.

4.3.3 Sampling Time and Passage Number of Vero Cell Cultures

Introduction. The cell passage age could influence their growth and culture synchronicity. However, there have been few reports in the literature where cell counts were taken more than

once a day to assess culture synchronicity. Therefore, this study determined whether the Vero cell passage number (131-155) affected the count differences between the trypan blue and crystal violet methods.

Experimental. Stock Vero cells at passage numbers 131, 142 and 155 were grown in T25 culture flasks (4.3.1). The cells were subcultured 1:10 every 72 h by the standard trypsinisation protocol (2.2) prior to the experiment. Three flasks were harvested daily (every 6-10 h) for cell counts by the trypan blue and Crystal violet methods.

Results. Fig. 4.4 shows Vero cell growths from various passage ages in T25 culture flasks counted by the trypan blue and crystal violet nuclei stain methods. (1) Vero cell counts by crystal violet nuclei stain and trypan blue dye exclusion methods were not significantly affected by the passage numbers used (131-155). (2) The count differences between crystal violet nuclei stain and trypan blue methods had time-dependent cycling patterns with respect to all cell passage numbers used. The patterns were most pronounced from cells at high passage numbers (155). (3) There (Fig. 4.4C)) were no significant differences between the Vero cell counts by either method after 16, 40 or 90 h in cultures from passage 155 cells. However, there were significantly higher ($>30\%$, $P<0.01$) counts after 0, 24 and 122 h in culture by the crystal violet method compared to the trypan blue method. (4) The highest count differences (100%) between crystal violet and trypan blue methods in the exponential phase were found with cells at passage number 142 (Fig. 4.4B)). (5) The maximum counts in the stationary phase were significantly higher ($>30\%$, $P<0.01$) by the crystal violet method compared to the trypan blue method with respect to all passage number cells used. (6) There was no significant difference in the cell count differences between the crystal violet and trypan blue methods from passage number 131 and 155 cells. However, passage number 142 cells had significantly higher (300%, $P<0.01$) cell count differences between the methods during the exponential phase compared to the other passage ages used.

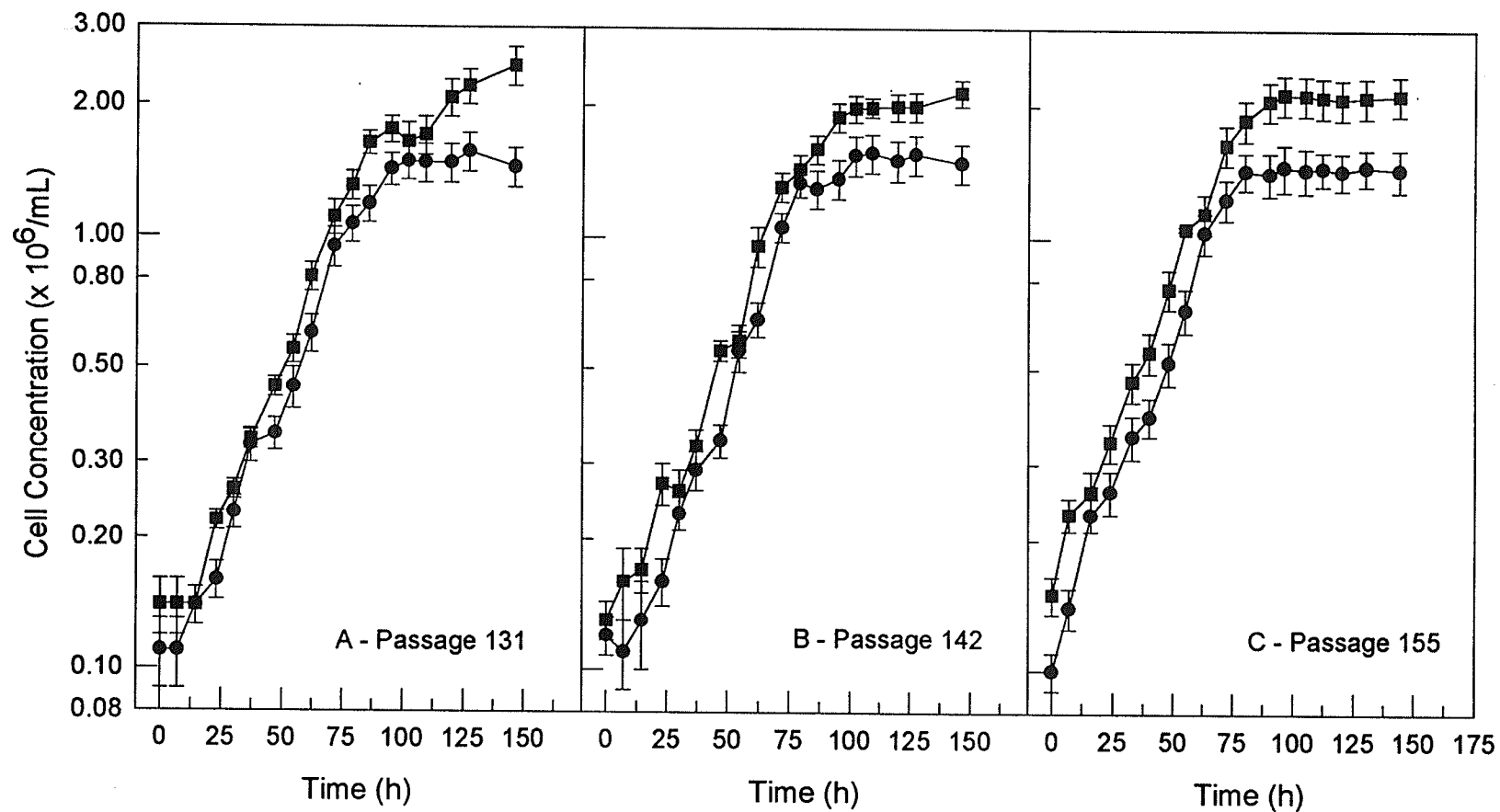


Fig. 4.4 - The effect of sampling time and passage number on the trypan blue and crystal violet nuclei staining counts of Vero cells at passage number 131 (A), 142 (B) and 155 (C) grown in T25 culture flasks. The cells were inoculated at 0.05×10^6 cells/mL in 10 mL of DMEM + 5.0% v/v SCS. Daily three flasks were sacrificed for cell counts by either the $\bullet$ trypan blue or $\blacksquare$ crystal violet cell counting methods. The points are means of $n=2$.

Discussion. Vero cells (Fig. 4.4A-C)) showed cyclic patterns in the cell count differences between the crystal violet and trypan blue methods. The cycling effect had the counts by both methods starting close together, moving apart and coming together again. The cycling periods were approximately 24 h or 1 Vero cell population doubling time. This phenomenon could be explained by a variable population of bi- or multi-nucleated cells. The cyclical effect also infers there was a higher degree of culture synchronicity in cells from higher passage numbers. Culture synchronicity after repeated trypsinisations and therefore, higher passage numbers has been shown with Vero and MRC-5 cells (Lindskog et al., 1987).

4.3.4 Origin of the Inoculum Vero Cells

Introduction. Surface (contact inhibition) or nutrient limitations could select for morphologically altered cells or could affect the progression of the cell cycle and both could affect the count differences between the methods. Therefore, an experiment was performed to determine whether the origin of the Vero cell inoculum affected the count differences between the crystal violet and trypan blue methods.

Experimental. Vero cell stock inoculum was produced by subculturing the cells (x5) from one of two different phases of the growth curve, (a) from late exponential phase which produced "normally passaged" cells or (b) after the culture had been in the stationary phase for 72 h which produced "nutrient limited" cells. The cultures were grown in T25 culture flasks (4.3.1) and one culture flask was harvested daily for cell counts by the trypan blue and crystal violet methods.

Results. Table 4.2 shows Vero cell growth of normally and nutrient limited cells in T25 culture flasks monitored by the crystal violet and trypan blue methods. (1) There were differences in the counts by the crystal violet and trypan blue methods between the cell passaging methods. (2) There were significantly higher (>30%, $P < 0.01$) counts from both normal and nutrient limited

Table 4.2 - The effect of growing Vero cells, either **passaged** during **late exponential phase** (normal) or from **72 h into the stationary phase** for 5 passages (nutrient limited), in T25 culture flasks. Cells were inoculated at 0.05×10^6 cells/mL in 10 mL of DMEM + 5.0% v/v SCS. One culture flask was sacrificed daily for cell counts by the trypan blue or crystal violet methods. The values are means of $n=2$.

Time (h)	Cell Concentration Obtained by Count Method ($\times 10^6$ /mL)			
	Normal Cells		Nutrient Limited Cells	
	Trypan blue	Crystal Violet	Trypan Blue	Crystal Violet
0	0.05 ± 0.02	0.07 ± 0.02	0.05 ± 0.02	0.07 ± 0.02
24	0.08 ± 0.02	0.11 ± 0.03	0.06 ± 0.02	0.09 ± 0.03
48	0.17 ± 0.03	0.23 ± 0.03	0.11 ± 0.02	0.16 ± 0.02
72	0.35 ± 0.04	0.49 ± 0.05	0.24 ± 0.03	0.31 ± 0.03
96	0.72 ± 0.06	0.93 ± 0.08	0.51 ± 0.05	0.71 ± 0.06
120	1.05 ± 0.10	1.54 ± 0.15	0.91 ± 0.08	1.36 ± 0.09
144	1.48 ± 0.12	2.05 ± 0.17	1.23 ± 0.08	2.03 ± 0.16
168	1.43 ± 0.14	2.07 ± 0.20	1.21 ± 0.07	2.00 ± 0.19

cells by the crystal violet method compared to the trypan blue method throughout the cultures. (3) The maximum counts by the trypan blue method were significantly higher (17%, $P < 0.05$) from normally passaged cells compared to nutrient limited ones. (4) There were no significant differences between the maximum counts by the crystal violet method with respect to cells from either growth phase. (5) There were significantly higher (60%, $P < 0.01$) count differences in the stationary phase between the methods in nutrient limited cells compared to normal cells (31%, $P < 0.01$). (6) Lag phases were significantly longer (360%, $P < 0.01$) for nutrient limited cells compared to normal cells.

Discussion. Amadori et al. (1994) showed there was a positive correlation between the passage age of the cells and the number of polyploid BHK cells in cultures. These authors also found that the older the cells the lower the maximum cell yield reached. Thus, nutrient or surface limitations could influence both the cell morphology and the cell cycle, as polyploid cells could result from a blockage during the mitosis phase which leads to cells with multiple copies of their DNA.

The results (Table 4.2) in this study suggest that if the cells are nutrient limited for multiple passages they lose their growth potential. This agrees with Amadori et al. (1994). However, previous results with passage 155 cells (Fig. 4.4C)) suggest the effect could be due to either a surface or nutrient limitation over multiple passages, as there was no count difference between passage number 131 and 155 cells when subcultured from late exponential phase.

Mossin et al. (1994) have shown that rat hepatocytes could produce polyploid and binucleated cells through repeated subculturing. The binucleated cells could explain the count differences in this study, as the crystal violet method counts nuclei and not whole cells.

4.3.5 Interaction of Surface and Nutrient Supply on Vero Cell Count Differences

Introduction. Surface and nutrient limitations affect cell growth, culture synchronicity and cell

morphology. However, which limitation has more importance to these effects has never been clearly determined. Therefore, this study determined whether fresh nutrients or surface for Vero cell growth had more effect on removing the count differences between the trypan blue and crystal violet methods.

Experimental. The Vero cell inoculum was prepared by method (b) above (4.3.4). The cells were grown in T25 culture flasks in either D-PBS⁻ or DMEM with or without 5.0% v/v sCS and supplemented with other nutrients. A residual sCS concentration (0.50% v/v sCS) was carried over with most of the cell inocula to promote attachment and spreading. The cell attachment time was also measured. One culture flask of each medium type was sacrificed daily for 72 h for cell counts by the crystal violet and trypan blue methods.

Results. Table 4.3 shows the cell yields after 72 h in culture from nutrient limited Vero cells in T25 culture flasks. Table 4.4 shows the attachment times and lag phases from these cells. (1) There were differences in the cell attachment times, lag phases and cell yields from nutrient limited Vero cells. (2) There was a significantly higher (41%, $P < 0.01$) cell count by the crystal violet method compared to the trypan blue method at time zero. (3) The cells did not attach to the flasks with D-PBS⁻ and DMEM solutions lacking serum. The count difference between the methods remained throughout the 72 h experiment for these cells. The free cells formed large aggregates (>50 cells) within 24 h in these cultures. Therefore, all other cultures were initiated with a residual serum carry-over (0.5% v/v) with the inoculum cells.

(4) In cultures with the serum carry-over there was no significant difference in the counts by both methods after 24 h in all media (including D-PBS⁻ without other nutrient additions). (5) However, cells inoculated in media without the major growth supplements (5.0% v/v sCS or fetuin, glucose, glutamine and serine) did not grow further and plateaued with no significant differences between counts by either method (up to 72 h in culture). These results showed that the available surface for growth was very important to the cell count differences.

Table 4.3 – The effect of **reattaching** and **subsequently growing stationary phase** Vero cells in T25 culture flasks with various **replating media**. The cells were inoculated at 0.20×10^6 cells/mL in 10 mL of each media. Daily one culture flask of each media was sacrificed for cell counts by the trypan blue and crystal violet methods. The values are means of $n=2$.

Replating Medium	Cell Concentration After Incubation Time ($\times 10^6/\text{mL}$)							
	0 h		24 h		48 h		72 h	
	Trypan Blue	Crystal Violet	Trypan Blue	Crystal Violet	Trypan Blue	Crystal Violet	Trypan Blue	Crystal Violet
D-PBS ⁻ or DMEM	0.18	0.23	0.17	0.22	0.19	0.27	0.19	0.25
D-PBS ⁻ + 0.5% v/v sCS	0.15	0.22	0.23	0.25	0.22	0.24	0.23	0.26
D-PBS ⁻ + 5.0% v/v sCS	0.17	0.25	0.26	0.27	0.29	0.34	0.28	0.37
D-PBS ⁻ + 50.0 mg/L Fetuin + 0.5% v/v sCS	0.15	0.21	0.22	0.23	0.23	0.23	0.24	0.30
D-PBS ⁻ + Gluc, Gln, Ser + 50.0 mg/L Fetuin + 0.5% v/v sCS	0.18	0.24	0.25	0.28	0.39	0.53	0.55	0.71
DMEM + 0.5% v/v sCS	0.17	0.23	0.22	0.25	0.32	0.40	0.32	0.43
DMEM + 5.0% v/v sCS	0.18	0.24	0.22	0.26	0.42	0.52	0.80*	1.13
DMEM + 50.0 mg/L Fetuin + 0.5% v/v sCS	0.16	0.21	0.22	0.24	0.38	0.50	0.68	0.89

Gluc - 25.00 mM glucose.

Gln - 4.00 mM glutamine.

Ser - 2.00 mM serine.

* - Highest cell yield.

Table 4.4 - The effect of the **reattachment solution** on the **attachment time** and **lag phase** of **stationary phase** Vero cells in T25 culture flasks. The cells were inoculated at 0.20×10^6 cells/mL in 10 mL of each media. Every 0.5 h for 8.0 h a sample (0.25 mL) of the supernatant was removed for cell counts by the trypan blue method. The lag phase was estimated from the subsequent growth rates by comparing the total number of cell population doublings to the number at the maximum growth rate. The results and standard errors of the mean are based on n=2.

Reattachment Solution	Cell Attachment Time (h)	Lag Phase (h)
D-PBS ⁻ or DMEM	NA	ND
D-PBS ⁻ + 0.5% v/v sCS	4.50 ± 0.50	ND
D-PBS ⁻ + 5.0% v/v sCS	1.50 ± 0.50	ND
D-PBS ⁻ + 50.0 mg/L Fetuin + 0.5% v/v sCS	4.50 ± 0.50	ND
D-PBS ⁻ + Gluc, Gln, Ser + 50.0 mg/L Fetuin + 0.5% v/v sCS	4.50 ± 0.50	27.00 ± 2.00
DMEM + 0.5% v/v sCS	4.50 ± 0.50	21.00 ± 2.00
DMEM + 5.0% v/v sCS	1.50 ± 0.50	12.00 ± 1.00
DMEM + 50.0 mg/L Fetuin + 0.5% v/v sCS	4.50 ± 0.50	21.00 ± 2.00

NA - No cell attachment.

ND - Not determined.

(6) In all other media at least one significantly higher (32%, $P < 0.01$) count by the crystal violet method compared to the trypan blue method was found after 72 h in culture. (7) Cell counts in cultures in DMEM + 5.0% v/v sCS had doubled twice by the trypan blue method after 72 h. However, there was a significantly higher (41%, $P < 0.01$) count by the crystal violet method at this time. (8) Cells inoculated in DMEM + fetuin had only doubled 1.5 times by the trypan blue method after 72 h in culture. There was also a significantly higher (31%, $P < 0.01$) count by the crystal violet method at this time. These results show that nutrient limitations were also important to growth and to the cell count differences.

(9) There (Table 4.4) were significantly shorter (67%, $P < 0.01$) cell attachment times in DMEM + 5.0% v/v sCS compared to all other media types used. (10) There were significantly shorter (48%, $P < 0.01$) lag phases in DMEM + 5.0% v/v sCS compared to all other media types used.

Discussion. One of the major serum proteins, fetuin, is an important nutrient for Vero cells to traverse the G_2/M transition point (Engelhardt et al., 1977) in the cell cycle. The fetuin binds growth factors such as FGF and facilitates transport to their site of action on the cell surface (Fisher and Lam, 1974 and Nie, 1992). The results (Table 4.4) agree with the previous authors as fetuin was important to Vero cell growth in this study. The lower cell growth rates with fetuin compared to sCS could be explained by a sub-optimal fetuin concentration (50.0 mg/L) as a higher optimal concentration (500.0 mg/L - Wang and Haslam, 1994) was previously shown for murine epithelial and stromal cells. The results indicated that the count difference between the methods was influenced by both available surface and/or nutrient limitations.

Cell Doming. Previous reports in the literature state that Vero cells are contact inhibited (Leighton et al., 1967 and Hawboldt et al., 1994) by the effects of Abercrombie and Ambrose (1962). However, there were Vero cell domes in all cultures similar to those shown with MDCK cells (Kersting et al., 1993). These could not form if contact inhibition occurred. The cells at the

dome surface, exposed to medium nutrients, are able to divide, whereas, the cells under the domes do not divide probably due to the limited surface area for growth (a sort of contact inhibition). However, the lack of growth under domes could also be explained by a nutrient diffusion limitation, as suggested previously for Vero and murine L-WRL10A cells (Werrlein and Glinos, 1974 and Nahapetian et al., 1986). The results show the surface limitations are more important to the cessation of cell growth which agrees with Abercrombie and Ambrose (1962) who suggested contact inhibition could be explained by this effect.

The cell domes could help to explain multi-nucleate cells, as cells under the domes could be trying to divide but due to a limitation become acytokinetic (can not complete their current round of cell division) and if cells under the domes were multi-nucleated, they could be trapped in this state. An estimation of the surface area occupied by cell domes in static culture flasks produced a value similar to the count differences (between 25-40%). This proposed cell doming theory agrees with data (Fig. 4.3A)-C)) from refed cultures where cell counts were increased 1.75-2.50 times over those in batch. This growth produced cell multi-layers (2-3 cells deep) on the Cytodex beads and the count differences were directly proportional to the number of cell layers below the active outer growing layer. Therefore, the cell doming or multi-layering was important to the final count differences.

4.3.6 Cell Cycle Analysis of Vero Cells

Introduction. The classical cell cycle (Fig. 4.5) has four phases. In the first (gap) phase (G_0/G_1), the cells synthesise RNA and ribosomes required for the biosynthesis of proteins involved in later phases of the cycle and their cytoplasm is organised. In the second (synthesis) phase (S), they replicate their nuclear DNA and have doubled both in total volume and nuclear DNA content at the end of the phase. In the third (gap) phase (G_2), the cells reorganise their cytoskeleton in preparation for cell division. In the fourth (division) phase (M), the replicated DNA is separated as chromosomes. The M phase has four sub-phases: prophase, metaphase,

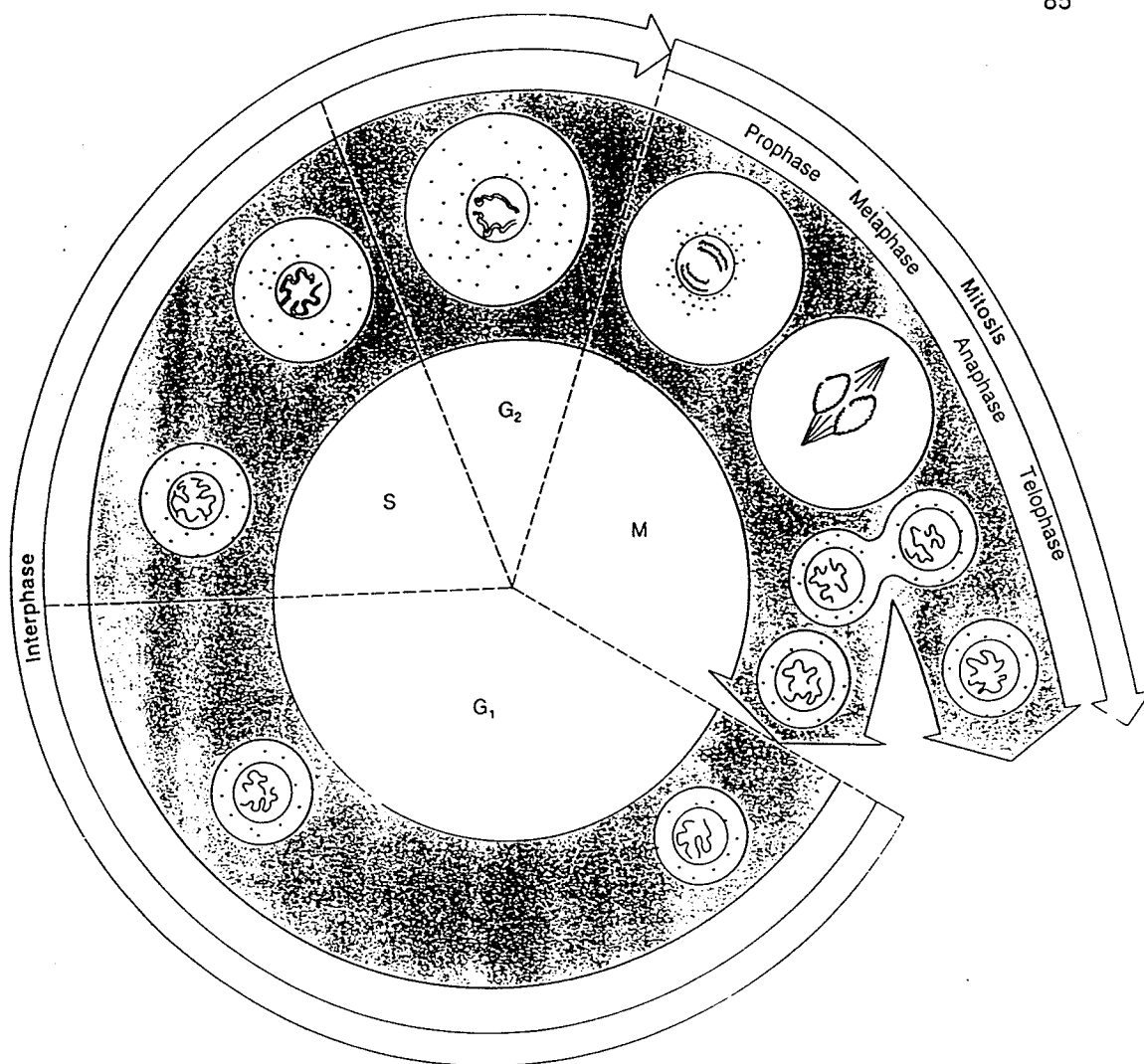


Fig. 4.5 - The classical cell cycle for eukaryotic cell lines. The average phase times are 8-12 h for G₀/G₁, 6-8 h for S, 3-4 h for G₂ and 1 h for M. Thus, the total cell cycle has an average time of 21-24 h. Modified from Prescott et al. (1990) and Lewin (1990).

anaphase and telophase. In prophase the centrioles move to the poles of the cells and spindles form between them. In metaphase the nuclear envelope breaks down and the DNA splits up into discrete chromosomes with centromeres. In anaphase the centromeres split and the chromosomes move to the centrioles and the cytokinesis begins as a constriction furrow. In telophase the nuclear envelope reforms around the separated chromosomes. The constriction furrow then narrows and the cells divide in late telophase by the cytokinesis forming two G_0/G_1 phase cells.

The previous results from this study inferred there could be multi-nucleate cells during the G_2/M phase of the cell cycle. These cells could account for the count method differences and could be induced by either a surface or nutrient limitation. If there were cells incapable of completing the M phase these should be seen as increased G_2/M peaks in cell cycle analyses. Therefore, Vero cells from the exponential and stationary phases were analysed by FACS to determine whether the count differences between the trypan blue and crystal violet methods could be related to the cell cycle.

Experimental. Vero cells were inoculated and grown in T75 culture flasks, as described above (4.3.1). The cells were grown to either (a) mid exponential or (b) late stationary phase prior to preparation for the analysis. The cells were detached and fixed with ice cold ethanol and prepared for FACS cell cycle analysis (4.2.4).

Results. Fig. 4.6 shows FACS analysis for mid-exponential phase Vero cells and Fig. 4.7 shows stationary phase cells. (1) There were differences in the FACS analysis data from mid-exponential compared to late stationary phases. The FACS cell cycle analyses generated three plots. The mid-exponential phase Vero cell plots (Fig. 4.6A-C)) are typical of FACS analysis with many cell lines. The first map (Fig. 4.6A)) shows a bit map (Map 1) of the cells' cytoplasmic granularity analysed by the sideways light scatter (y-axis). The sideways scatter was then plotted against the cell size analysed by the forward angle light scatter (x-axis). The results were then

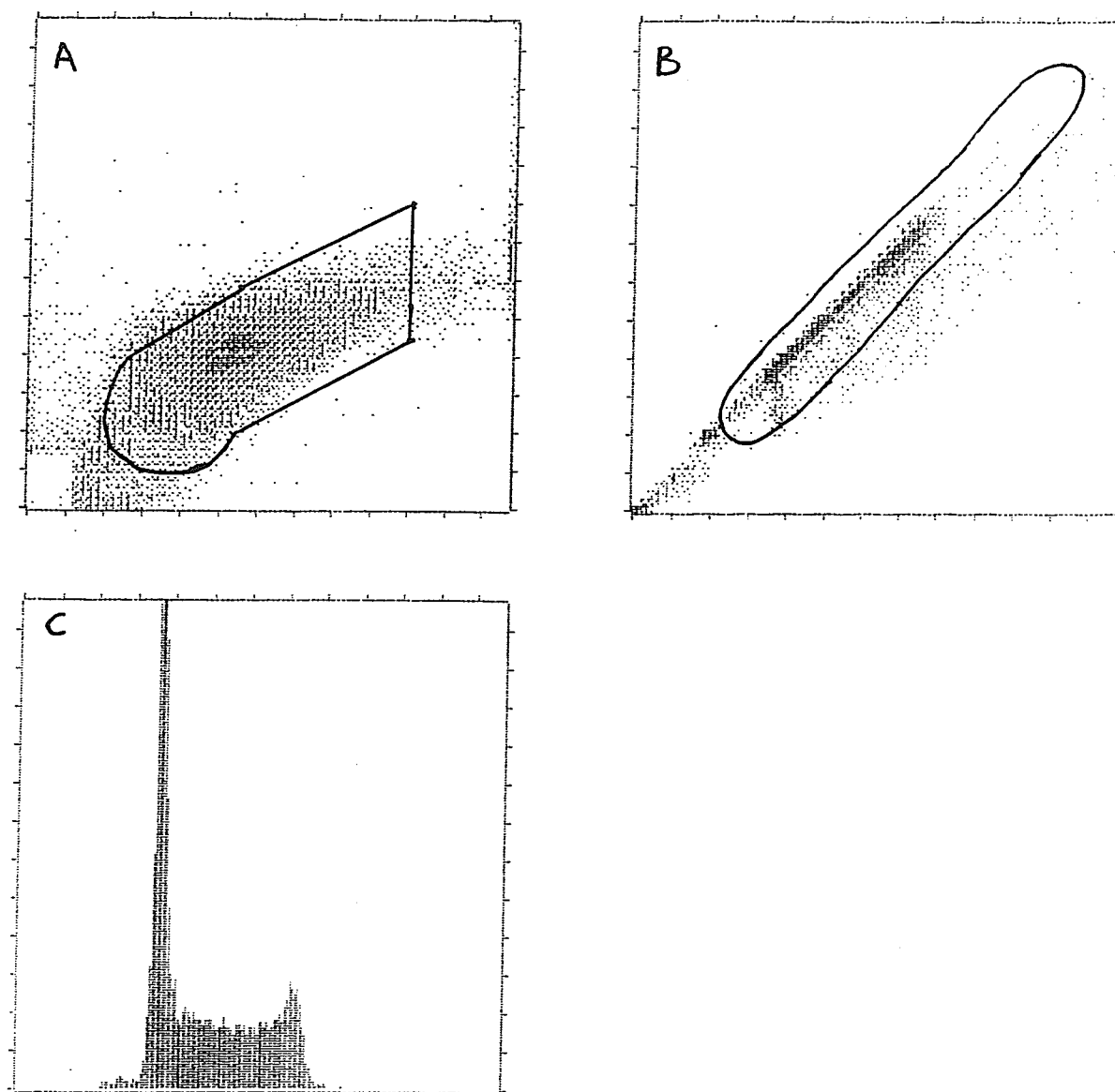


Fig. 4.6 - Typical FACS analyses for mid-exponential phase Vero cells showing Bit Map 1 (A), Map 2 (B) and Map 3 the cell cycle histogram (C). The cells were fixed with ethanol, treated with RNase (1 mg/mL) and stained with propidium iodide (50 μ g/mL). The analysis was performed by passing single cells through a laser beam with a wavelength of 488 nm. The fluorescence produced was detected for wavelengths ≥ 610 nm. The internal gating window of Map 1 and 2 is also shown from which Map 3 was then produced.

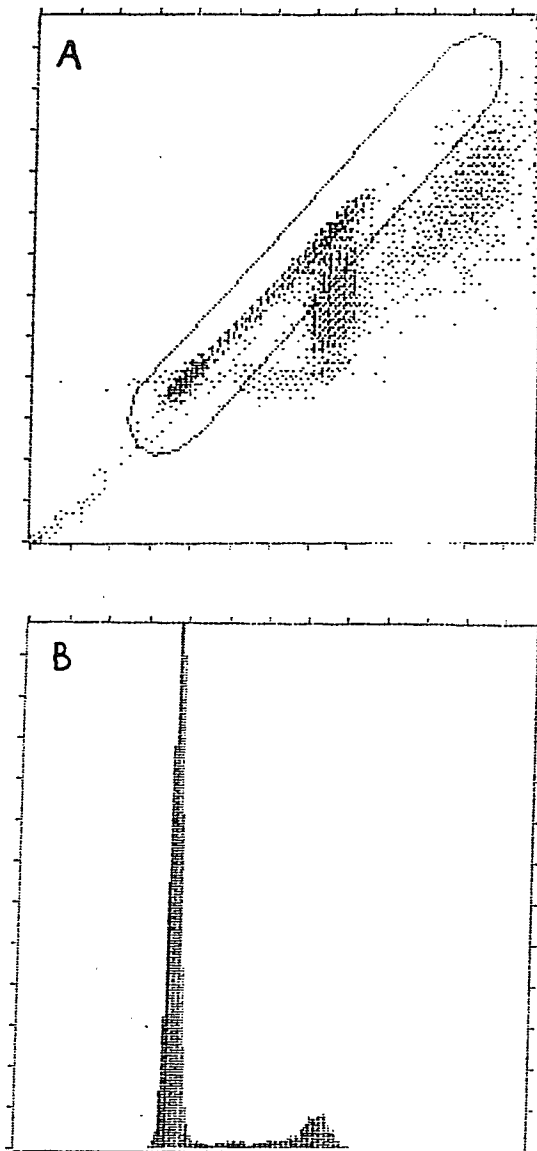


Fig. 4.7 - Typical FACS analyses for stationary phase Vero cells showing Map 2 (A) and Map 3 (B) the cell cycle histogram.

gated (using an internal FACS algorithm) to eliminate cell debris and aggregates.

Cells which were observed within the gating window of Map 1 were also analysed for both their peak (highest recorded) fluorescence and for their total integrated fluorescence, determined over the time taken for each cell to completely pass through the laser beam. The second map (Fig. 4.6B)) shows another bit map plot (Map 2) of the peak against the integrated fluorescence. This map showed that single cell fluorescences formed a linear diagonal according to the phase of the cell cycle each was in when fixed. Cells in G_0/G_1 appear at the left hand end of the diagonal, whereas cells in G_2/M appear at the right hand end of the diagonal. Cell doublets or cells with unusual ploidy would not fall on the diagonal.

The third plot (Fig. 4.6C)) shows the results of integrating Map 1 and 2 (using another internal FACS algorithm) to form the cell cycle histogram. This histogram plotted the number of cells from the total sample count (10,000 cells) recorded against each calculated linear fluorescence. The ranges employed to define the cell cycle phases within the histogram were set manually. The instrument then calculated the total number of cells within each defined range. This range total was compared to the sum of cells within the three defined ranges (i.e. the complete cell cycle) to give the proportions of cells within each cell cycle phase.

Cell Cycle Analysis - Mid-Exponential Phase Cells. (1) The mid-exponential phase cells (Fig. 4.6A)-C)) had no significant difference between the counts by the crystal violet and trypan blue methods prior to FACS analysis. (2) Fig. 4.6C) shows the cell cycle histogram for these cells with 56% in G_0/G_1 , 35% in S and 9% in G_2/M .

Cell Cycle Analysis - Stationary Phase Cells. (1) The stationary phase cells had a significantly higher count (38%, $P < 0.01$) by the crystal violet method compared to the trypan blue method prior to the FACS analysis. (2) Fig. 4.7A)-B) show the typical results of the fluorescence bit map (Map 2) and the cell cycle histogram for these cells. Map 1 for these cells was not significantly different from Fig. 4.6A) above. The peak vs. integrated fluorescence (Fig. 4.7A))

showed significant numbers (18% of the total count, $P < 0.05$) of cells outside the gating area. The plot also had a clear, right angled triangular appearance between the G_2/M and G_0/G_1 areas. (3) The (Fig. 4.7B)) results of the cell cycle analysis showed that there were 86% in G_0/G_1 , 7% in S and 7% in G_2/M .

Stationary phase cells from outside the diagonal (Fig. 4.7A)) were separated out by channelling onto a microscope slide. When observed these cell populations were found to contain a large proportion of incompletely divided doublets and binucleated cells.

FACS Sample Preparation Problems. (1) There were significant cell losses (up to 50% of the initial cell number) during preparation for the FACS analyses which occurred in the steps carried out at $4^{\circ}C$. The proportions of lost cells were the same for both mid-exponential and stationary phase cells. Cells were lost as large aggregates (>50 cells) which could not be singly suspended by vigorous pipetting and they became irreversibly attached to the pipettes during resuspension.

Discussion. Fluorescence activated cell sorting (FACS) analysis has been used previously to determine hybridoma and Vero cell cycle effects (Duval et al., 1990 and Hawboldt et al., 1994). One report was found showing problems with FACS analysis of hepatocyte cells (Mossin et al., 1994). These authors found that increased cell ploidy and binucleated cells affected the results. No reports were found describing cell losses during sample preparation. The significant losses from preparation steps at $4^{\circ}C$ could be typical for all anchorage dependent cells. The effect could be explained by cell membranes being attracted through the mediation of cell surface receptors normally attached to fibronectin or vitronectin (Holley and Kiernan, 1968). Suspension cells have no surface receptors which could explain why FACS analysis of such cells has had fewer problems. A similar explanation could be that cell membranes of G_2/M phase cells were more attractive compared to cells from other phases of the cycle. This could lead to preferential aggregation and removal of a large proportion of important cells from the analysis. Hence, the FACS results could be unrepresentative of the cell population prior to analysis.

Crystal Violet Nuclei Stain. The crystal violet method counts the number of nuclei present in a cell sample, as the nuclear envelope exists throughout most of the cell cycle up to anaphase (Fig. 4.5). The envelope then breaks down to allow chromosome separation and only reforms either at the end of telophase or during the cytokinesis into two discrete cells (Davis et al., 1990 and Prescott et al., 1990). Thus, cells in the anaphase-telophase transition can not be detected by the crystal violet method. However, the whole M phase is suggested to take ≤ 1 h (Lewin, 1990) and is a very small proportion of the total cell cycle time (e.g. $<5\%$ of one Vero cell population doubling time). Mammalian cell cultures are usually highly asynchronous and at any given sample time there should be a maximum of 40% of the total cell population in either the G_0/G_1 or S phases and 20% in the G_2/M phase, based on the accepted cell cycle times (Lewin, 1990 and Fig. 4.5). Hence, a very small proportion of G_2/M cells ($<10\%$) should have no nuclear envelope. This should give lower counts by crystal violet compared to the trypan blue method. However, this did not occur with Vero and CHO cells and no explanations could be found to reduce the count differences to $<20\%$.

G_1 Transition Point. The mitotic cell cycle has a transition point in the G_1 phase which, when passed, programmes the cells to complete that round of division. Following the transition the cells can not become arrested until the next G_1 phase (Darzynkiewicz et al., 1980). Therefore, there should be no significant difference in the stationary phase counts by either method. The results for the CC9C10 cells agree with this, as was shown previously with hybridoma cells (Duval et al., 1990). However, Vero and CHO cells disagree with this.

G_2 Transition Point. In fact, Vero cells have a second transition point in the G_2/M phase of the cell cycle, influenced by nutrient status (Engelhardt et al., 1977). This could explain the findings in this study, as cells in the stationary phase could be nutrient limited and unable to synthesise important components for cell division. Therefore, they become trapped in the G_2/M phase. If there were multi-nucleated cells trapped in the G_2/M phase this could explain the count method

differences.

FACS Analysis. The FACS results for mid-exponential phase cells show them mostly in the G_0/G_1 or S phases which agrees with there being no significant difference between the cell counts by either method prior to the analysis. Therefore, the FACS analysis plots could be representative of the mid-exponential phase cell populations prior to FACS analysis.

The stationary phase cells were also mostly in the G_0/G_1 phase suggesting there was no significant difference between the count methods prior to the analysis. These results agree with the classic cell cycle and those of previous authors with hybridomas and Vero cells (Duval et al., 1990 and Hawboldt et al., 1994). However, they do not agree with the hypothesis for multi-nucleated cells in the stationary phase, where increased cell numbers in the G_2/M phase were expected, or with the difference between the count methods prior to the analysis.

The stationary phase cell fluorescences (Fig. 4.7A)) did not form an absolutely linear diagonal and there were significant proportions outside the gating window for the cell cycle analysis. These results agree with those of Mossin et al. (1994), who showed hepatocyte cells with increased ploidy could not be FACS analysed. These authors cells were found with ploidies of 2, 4 and 8n chromosome content and there were also binucleated cells. Therefore, FACS analysis of adherent Vero cells from the stationary phase could be highly unrepresentative of the original cell population. These results contradict those of Hawboldt et al. (1994) who showed Vero cells became trapped in G_0/G_1 during the stationary phase.

4.3.7 Photographic Analysis

Introduction. Previous results (4.3.1-4.3.5) indicated multi-nucleate single Vero cells trapped in the G_2/M phase of the cell cycle were present in the stationary phase of cultures. No method to prove their existence had been found. Large sized ($\geq 15 \mu\text{m}$ diameter), single binucleate Vero cells were often observed, stained blue, in counts by the trypan blue method. This suggested

that photographic evidence of binucleated cells, which could be responsible for the count differences between the methods, could be obtained. Staining stationary phase cells with crystal violet and photographing them could show unusual morphologies and then resolve the cause of the count differences. Therefore, photographic analysis of stationary phase Vero cells was performed to determine if there were any unusual morphological features that could explain the crystal violet and trypan blue method count differences.

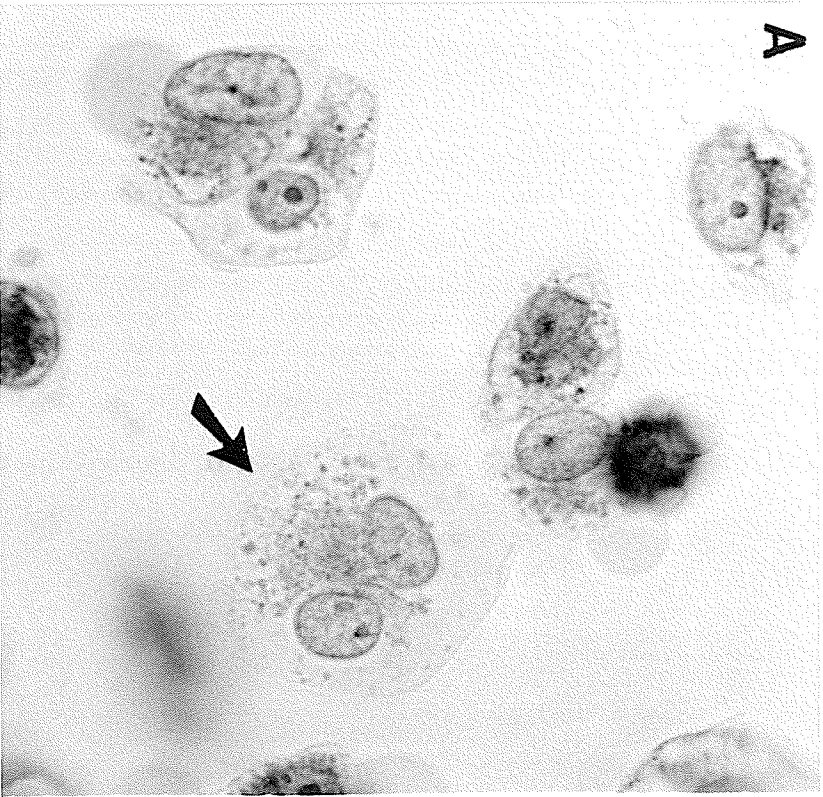
Experimental. Vero cells were recovered from cultures which had been in the stationary phase for 72 h. The cells were diluted to 0.20×10^6 cells/mL in DMEM + 5.0% v/v sCS and attached for 1.5 h to either poly-lysine coated (1 mg/mL) glass cover slips (1 mL of cells) or to T25 culture flasks (10 mL of cells). The attached cells were stained with crystal violet, washed with D-PBS⁻ and photographed with Kodak T_{MAX} 100 ASA black and white print film using X500 (normal) or X1,250 (oil-immersion) lenses with Normaski direct interference contrast microscopy.

Results. Plate 4.1 shows photographs of Vero cells attached to poly-lysine coated glass cover slips and polystyrene culture flasks stained with crystal violet dye. (1) Plate 4.1A) shows a photograph of a single Vero cell on a glass cover slip (X4,500 magnification under oil-immersion) which clearly had two complete but physically separated nuclei within one cell membrane; a binucleated Vero cell. Plate 4.1B) shows a photograph of cells attached to a T25 culture flask (X1,850 magnification under a normal lens) which had a number of binucleated cells. Hence, there was a sub-population of binucleate cells present in the cultures.

These binucleates could explain the count differences between the crystal violet nuclei stain and trypan blue methods. Binucleated single cells would count as two by crystal violet nuclei staining but as one by the trypan blue method. Hence, if approximately 33% of the total cell population were binucleated this could account for the differences between the two methods.

Cell Counts. The stock Vero cells used for the photographic analysis had a significantly higher

Plate 4.1 - The morphology of stationary phase Vero cells detached from T25 culture flasks and reattached to either a **T25 culture flask (A)** or a **poly-lysine coated glass cover slip (B)**. The cells were attached in DMEM + 5.0% v/v SCS for 1 h followed by staining with 0.20% w/v crystal violet in D-PBS for 20 min. The cells were washed with D-PBS⁻ and photographed on a Carl-Zeiss Jena microscope using Normaski Direct Interference Contrast techniques with a standard lens (X1,800) **(A)** or an oil immersion lens (X4,500 magnification) **(B)**.



(>30%, $P < 0.01$) count by the crystal violet method compared to trypan blue. However, there was no significant difference between the counts by either method after the cells were reattached to the new surfaces for photography. This could be due to the supply of the fresh surface or nutrient allowing cells trapped binucleated in G_2/M to divide, as described above (4.3.5). Such division must have occurred within the time taken (1.5-2.0 h) for reattaching to the surface and staining for photography. It was impossible to generate a preparation where the number of binucleated cells observed after reattaching to substrata agreed with the count difference from the original stock flask. It required a great deal of patience and observation of many cells (>200) to identify a few (12) binucleated cells for photographing.

Discussion. Binucleation has been described in rat hepatocytes (Wheatley, 1972 and Seglen, 1973). Significant numbers (35%) of these cells were binucleated after trypsin separation. The proportion of binucleated Vero cells by the count difference between the methods agreed with these authors. The authors also showed the cells to be acytokinetic, as they divided soon after replating, as with the Vero cells in this study. Rat follicular cells have also been shown to form binucleated cells when fibronectin depleted (Orly and Sato, 1979). However, as the cell count differences between the methods disappeared after reattaching to surfaces this suggests the binucleated cells divided to mononucleate progeny.

Therefore, differences between the common count methods could be caused by binucleated cells. Binucleation and the differences between the methods mean that cell counts from high density cultures obtained by the crystal violet method could be erroneous. Cell count overestimations by up to 70% could be possible. If such errors were removed, previously reported cell counts would be significantly reduced (e.g. Nahapetian et al., 1986 – 30.00×10^6 Vero cells/mL would be reduced to $18.00\text{--}21.00 \times 10^6$ cells/mL).

The count difference between the methods was only found in adherent cell lines (Vero and CHO cells). This suggests that primary cells, requiring adherence for growth, could also form binucleated cells. Therefore, adherent cell cycles and mitosis require further investigation, as

they differ from the phases defined with transformed or lower eukaryotic cells.

Maximum counts by the crystal violet method were shown above to be equal in all cell lines in the stationary phase (4.3.1). This suggests if the bi-nucleate Vero and possibly CHO cells had completed their last division it would have resulted in a similar whole cell count as CC9C10 cells. Thus, many cell lines could grow to similar maximum densities without nutrient or surface limitations. The available surface for the adherent cells could be important to this, as CC9C10 are a suspension cell line and therefore, have no surface constraint.

Binucleation Mechanism. The studies above have shown proof of both cell count differences between the crystal violet and trypan blue methods and also the existence of time dependent single binucleated Vero cells. Thus, the last question to answer is how do binucleated cells arise?

Vero cell mitoses must differ from those of fully transformed cells. Several mechanisms could explain how this occurs:

1. binucleation could be an intermediate step in fibroblast cell differentiation (Bayreuther et al., 1991). Cells progress from mononucleate to binucleate mitosis. The binucleate divisions could then progress to cells incapable of any cell division (i.e. terminally differentiated cells).
2. a mutation or modification in the synthesis of maturation promoting factor, S6 kinase II or lamin C would prevent nuclear envelope breakdown (Maller et al., 1989).
3. a failure in the transient intracellular free calcium concentration increase would block nuclear envelope breakdown (Patel et al., 1989).
4. lowered cell division factor nutrient pools could select for binucleated cells (fetuin - Engelhardt et al., 1977, or fibronectin - Hughes et al., 1979 and Orly and Sato, 1979, or actin - Sasger, 1975). Once these nutrients are depleted the cells become acytokinetic.
5. binucleated cells could progressively lose the reorganisation of the cellular cytoskeleton in the anaphase-telophase transition (Sasger, 1975 and Fujiwara and

Pollard, 1976). Hence, there would be no nuclear envelope breakdown and an aberrant chromosome separation could occur. Laskey et al., (1989) stated that leaky nuclear envelopes allow DNA synthesis without an M phase producing polyploid cells.

The intact nuclei from 2) or 3) above could then divide by an altered mechanism. Vero cell ploidy was not determined in this study but from the sizes of the nuclei in some photographs it would appear polyploid cells do exist, as were found previously with BHK and rat hepatocyte cells (Amadori et al., 1993 and Mossin et al., 1994). Indeed, the BHK cells were arrested in metaphase of the M phase resulting in polyploid cells. Amadori et al. (1993) suggested that this was caused by a failure of the ubiquitination mechanism of maturation promoting factor-cyclin B degradation. Hence, if this process generates polyploid cells, it could also produce binucleated cells if polyploid nuclei divided.

Amadori et al. (1993) suggested that nutrient effects could actively select for polyploid BHK cells. Ploidy and binucleation could be linked with the differentiation cascade, as cells losing nuclear envelope breakdown and progressively cell division could still replicate their DNA without nuclear division. This could lead to cells with 4 or 8 n chromosome content shown previously by Mossin et al. (1994).

4.4 Conclusions

The purpose of this study was to validate the use of the crystal violet nuclei staining method for counting adherent cell lines grown on microcarriers. The conclusions from the work were that:

- **the crystal violet nuclei staining method** gave significantly higher cell counts (>30%, $P < 0.01$) compared to either the trypan blue or Coulter counter methods with CC9C10 hybridoma, Vero and CHO cells. The count differences were cell line and time dependent. CC9C10 cells had fewer count differences between the methods compared to the adherent cells.

- **the trypan blue and Coulter counter methods** gave similar cell counts with all cell lines when the cells were singly suspended. Cell aggregates caused a significantly lower (up to 20%)

cell count by the Coulter counter method.

- **significant cell count differences** ($>30\%$, $P<0.01$) between the crystal violet and trypan blue methods were always present in the stationary phase of Vero and CHO cell growth.

- **the growth system** had no effects on the count differences between the crystal violet and trypan blue methods.

- **the cells nutrient status, passaging method and passage number** affected the count differences between the crystal violet and trypan blue methods.

- **binucleated Vero cells** were photographed in cultures where there were cell count differences between the methods. These binucleated cells could be responsible for the count differences between the crystal violet and trypan blue methods. The cycling of the count differences had a period equivalent to one Vero cell population doubling time (24 h). Therefore, binucleated cells must be a dividing sub-population of the culture.

- **nutrient or surface area limitation** could be responsible for producing the binucleated cells. Surface area was particularly important.

- **FACS analysis** was impossible with stationary phase adherent cell lines because of cell aggregation during sample preparation.

**Chapter 5. Optimisation of Physical Parameters for Vero Cell Growth in Microcarrier
Cultures**

5. Optimisation of Physical Parameters for Vero Cell Growth in Microcarrier Cultures

5.1 Introduction

The first microcarriers for adherent cell culture were solid spherical beads of SephadexTM A-50 (van Wezel, 1967) which were further developed into Cytodex-1 beads (Clark et al., 1980). These are the microcarriers most often used in research and production (Montagnon et al., 1981, Gebb et al., 1984, Radlett et al., 1985, Varani et al., 1988 and Borys and Papoutsakis, 1992). However, human foreskin (FS-4) and bovine embryonic kidney (BEK) cells grown in cultures of solid Cytodex-1 and 3 microcarriers have been shown previously to be vulnerable to damage (Croughan et al., 1987 and Lakhotia and Papoutsakis, 1992). This occurs due to the cells growing on the solid bead surfaces and bead to bead contacts cause cell lysis. High stirrer speeds increase the likelihood of bead contacts and increase the cell damage.

More recently, microcarriers with a macroporous sponge-like matrix have been produced (Nilsson et al., 1986) (Cultispher) based on those of Leighton et al. (1967). Few reports in the literature have used macroporous beads. However, they have been shown to protect monkey kidney (CV-1) cells from the high stirrer speed damage found in cultures of solid beads (Adema et al., 1990). Macroporous bead cultures have also produced higher Chinese hamster ovary (CHO) cell yields compared to solid beads (Reiter et al., 1991 and Ohlson et al., 1994).

Few direct comparisons between the two bead types have been conducted with the exceptions of Adema et al. (1990) and Schrimpf and Friedl (1993). Nikolai and Hu (1992) suggested macroporous beads produced significantly higher cell yields of CHO cells compared to solid beads. However, these authors did not conduct a direct comparison of the two bead types and made their conclusion by comparing their maximum cell yield in macroporous bead cultures to maximum yields in solid bead cultures previously reported by other authors in the literature.

Many physical culture parameters have been shown to affect yields of CHO, African green monkey kidney (Vero) and human fibroblast cells in static and stirred growth systems. These

include pH (Ceccarini, 1975), dissolved oxygen tension (DOT) (Krebs, 1950, and Graff and McCarty, 1957), temperature (Tsao et al., 1993), stirrer speed (Lakhotia and Papoutsakis, 1992), inoculum cell concentration (Hu et al., 1985) and microcarrier concentration (Clark and Hirtenstein, 1981). Few, if any, of these parameters are ever optimised by researchers prior to more detailed studies of their cell lines.

Some parameter optima such as the mode of stirring for cell attachment to microcarriers have been reported but there appears to be cell line dependent differences in the results. Optimal attachment to Cytodex beads has been suggested to be either static conditions for Vero cells (Clark and Hirtenstein, 1981) or continuous stirring for FS-4 cells (Croughan et al., 1990). However, such contradictions could be explained by system dependent differences in the spinner flask volume, paddle blade shape, stirrer speed, bead type or bead concentration used between the researchers. For instance Clark and Hirtenstein (1981) used 3.00 g/L Cytodex-1 in 100 mL spinner flasks and Croughan et al. (1990) used 5.00 g/L collagen coated Cytodex-3 beads in 250 mL spinner flasks.

Vero cells have been used previously for research into novel substrata, physical damage in microcarrier culture and for virus production (Gebb et al., 1984, Altankov et al., 1991, Goetghebeur and Hu, 1991, Cinatl et al., 1992, Nikolai and Hu, 1992 and Mendonca et al., 1993). Process optimisation has rarely been discussed for any cell line and no reports were found for Vero cells excepting those of Clark and Hirtenstein (1981) and Nahapetian et al. (1986). However, the only parameter optima which have been suggested for Vero cells are stirrer speed, inoculum cell concentration and mode of attachment in Cytodex-1 cultures (Clark and Hirtenstein, 1981). No parameter optima for Cultispher cultures have been reported in the literature.

Production processes in bioreactors using adherent cell lines are rare and their main uses are in the manufacture of rabies and polio virus vaccines from Vero and human diploid lung fibroblast (MRC-5) cells. These processes have been successfully scaled-up to 1,000 L (Montagnon et al., 1981 and Beale, 1992). Indeed, bioreactor production processes with

suspension cells are also rare, except for the production of human interferon from Namalwa cells at the 8,000 L scale (Finter et al., 1984) and the production of monoclonal antibodies from hybridoma cells at the 1,000 L scale (Birch et al., 1987). This could be explained by processes in roller bottles and static culture flasks being simpler to work with, give more reproducible results and acceptable processes can be developed faster in them. Hence, explaining why the primary biotechnology drug, erythropoietin (Amgen® - Epogen™), is produced in an aseptic, robotic roller bottle process (Egrie, 1990, Spier, 1992 and Lubiniecki and Lupker, 1994) in a serum-free production medium using CHO cells.

No one has previously optimised the major physical parameter values for Vero cell growth in microcarrier cultures. Hence, this study of Vero cell cultures on solid Cytodex-1 and macroporous Cultispher-G attempts to address the previously perceived problems and to define optimal values for the following physical parameters: mode of stirring for cell attachment, stirrer speed for cell growth, pH, DOT, temperature, bead concentration, inoculum cell concentration, precoating of the beads and spinner flask volume. Optimisation was performed in both semi-controlled spinner flasks and in a highly controlled Celligen bioreactor.

5.2 Materials and Methods

The methods for culturing and counting the cells and nutrient analyses have been described previously (2.1-2.5).

5.2.1 Inoculation and Growth of Cultures

Stock Vero cells were recovered by the standard trypsinisation protocol (2.2) and inoculated at 0.05×10^6 cells/mL in either 100 mL spinner flasks (2.4.3) or 1,300 mL Celligen bioreactor (2.4.5) on microcarriers (2.4.2), unless otherwise stated. The growth medium was DMEM + 5.0% v/v iron enriched, supplemented calf serum (sCS). Spinner flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH, unless otherwise stated. The control values for the Celligen bioreactor were described previously (2.4.5). Cultures were sampled daily for cell

counts by the trypan blue method (2.5.1-2).

5.3 Results and Discussion

5.3.1 Speed and Mode of Stirring for Cell Attachment

Introduction. Adherent cells such as Vero must be attached to substrata for growth and a quick attachment phase has been suggested to prevent lag phases and/or low maximum cell yields. The stirring mode and speed and the culture volume at inoculation could affect the attachment. Therefore, this study determined the optimal culture conditions for Vero cell attachment to microcarriers.

Experimental. Vero cells were inoculated in 100 mL spinner flasks at 10 cells/bead in Cytodex-1 or 30 cells/bead in Cultispher-G cultures. The cells were attached to the microcarriers in either (i) 50 or (ii) 100 mL of medium. The flasks were stirred for 24 h with various stirring speeds (up to 100 rpm) and modes (without stirring, intermittently with 3 min stirring every 30 min or continuously). The optimal attachment speed and mode were determined from the cell/bead distribution patterns of culture samples stained with crystal violet or neutral red dye. The optimal conditions following attachment were the stirrer speed and mode yielding the highest proportion of beads with a cell/bead number within 1 standard deviation (SD) of the inoculum ratio.

Results. Fig 5.1 shows the proportions of Cytodex-1 beads with a Vero cell/bead number within 1 SD of the inoculum ratio after 6 h of attachment with either intermittent or continuous stirring at various speeds in 50 or 100% medium volume. Fig. 5.2 shows cell/bead distribution patterns following cell attachment to Cytodex-1 either without stirring or with continuous stirring at 40, 75 or 100 rpm. Fig. 5.3 shows the proportions of Cultispher-G beads showing neutral red staining following Vero cell attachment with either intermittent or continuous stirring at various speeds with 50 or 100% medium volume. (1) Cell/bead distribution patterns (Fig. 5.1, Fig. 5.2A)-D)) and

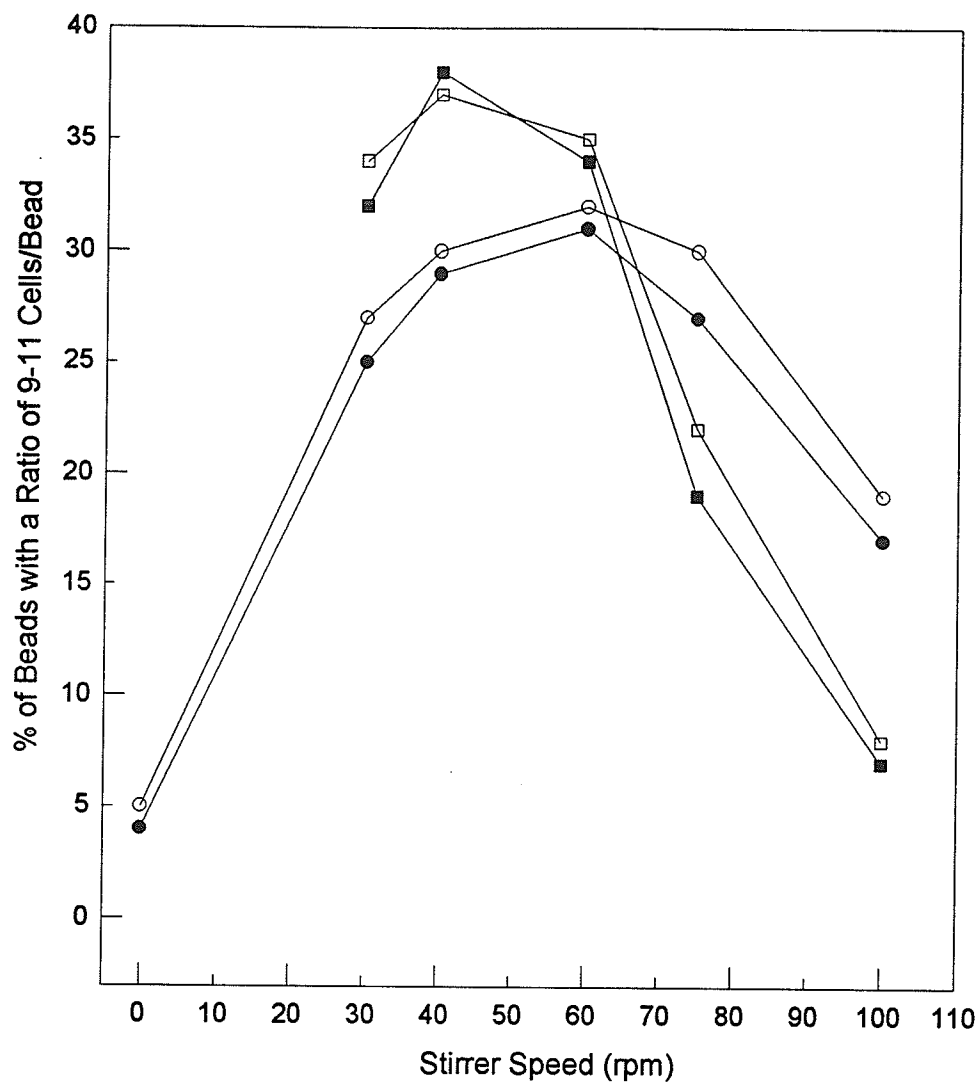


Fig. 5.1 - The effect of the **stirring speed** and **mode for attachment** on the proportions of **Cytodex-1** beads at the **optimal number of Vero cells/bead** (10 ± 1 SD) after 6 h in culture. The cells were inoculated at 10 cells/bead in 100 mL spinner flasks on 1.00 g/L Cytodex-1 in DMEM + 5.0% v/v sCS at either 50% (Open symbols) or 100% (Closed symbols) medium volume. The spinner flasks were stirred for 24 h either ● intermittently or ■ continuously at various speeds (up to 100 rpm). Bead samples were stained with 0.2% w/v crystal violet in D-PBS⁻ and counted under an inverted microscope. The points are means of $n=2$.

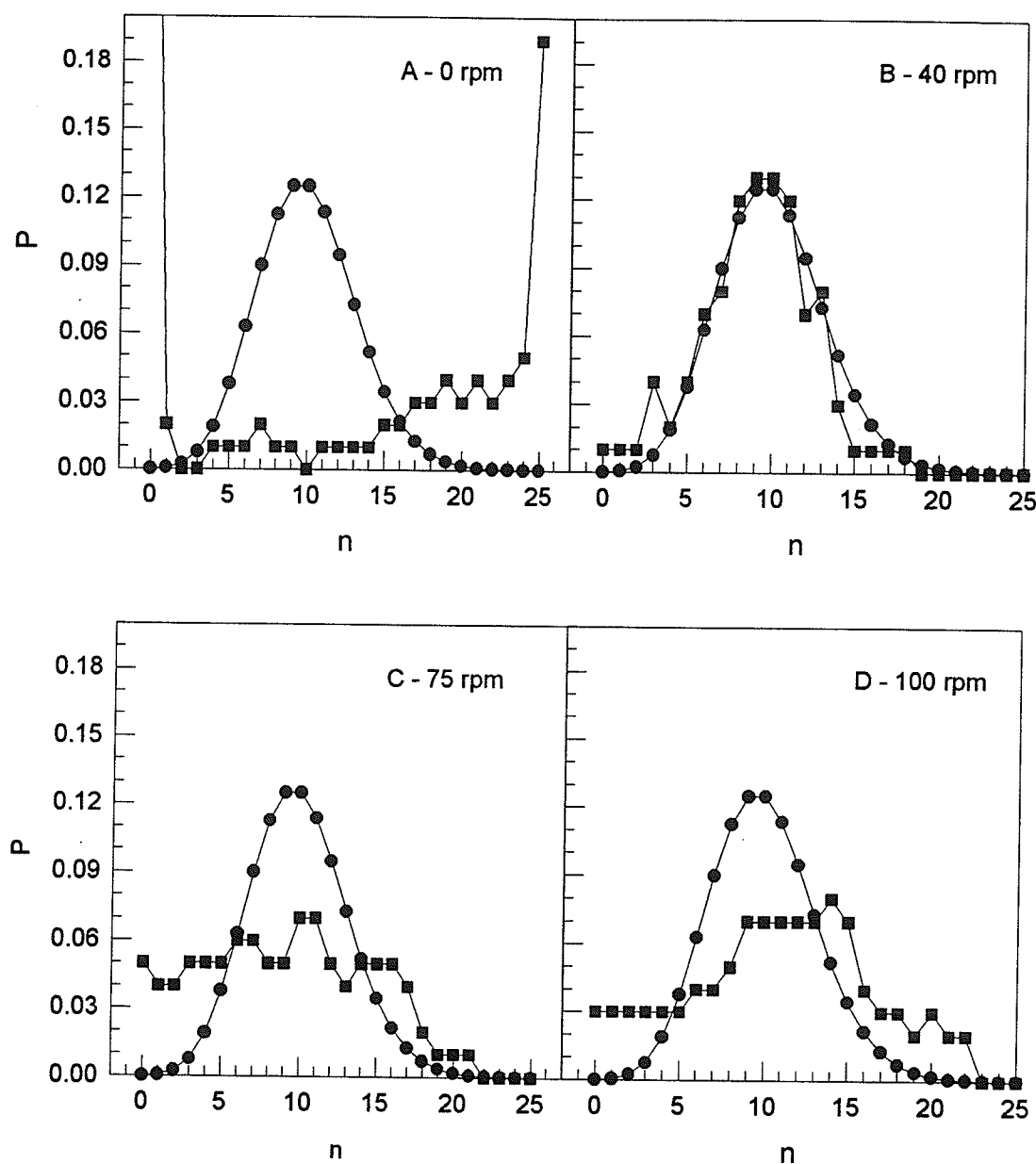


Fig. 5.2 - The effects of the stirring speed and mode on Vero cell/bead distribution patterns in 100 mL spinner flasks on 1.00 g/L Cytodex-1. The cells were inoculated at 10 cells/bead in DMEM + 5.0% v/v SCS and ■ stirred either without stirring (A), or continuously for 6 h at 40 (B), 75 (C) or 100 (D) rpm. The plot shows the probability (P), (i.e. the proportion of beads with a cell/bead number of n), against the given cell/bead number (n). ● The theoretical Poisson cell/bead distribution for an inoculum of 10 cells/bead is also plotted. The points are means of $n=2$.

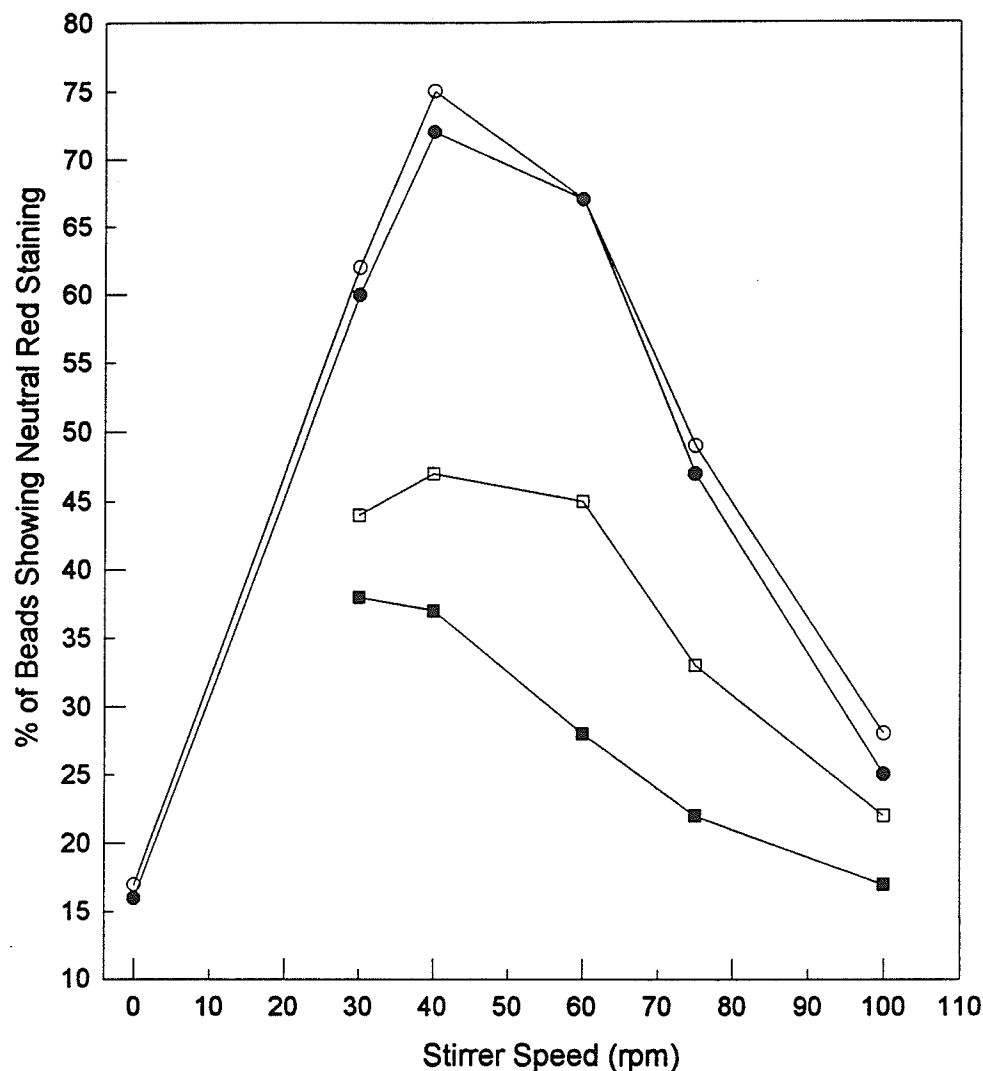


Fig. 5.3 - The effect of the stirring speed and mode for Vero cell attachment on the proportions of 1.70 g/L Cultispher-G beads staining with neutral red after 24 h. The cells were inoculated at 30 cells/bead in 100 mL spinner flasks in DMEM + 5.0% v/v SCS at either 50% (Open symbols) or 100% (Closed symbols) medium volume. The flasks were stirred for 24 h either ● intermittently or ■ continuously at various speeds (up to 100 rpm). Bead samples were stained with 0.2% w/v neutral red in D-PBS⁻ and the number of beads showing any red staining was counted under an inverted microscope. The points are means of n=2.

Fig. 5.3) in cultures of either bead were affected by the stirrer speed and mode for attachment.

Cytodex-1. (2) The analyses allowed the stirrer speeds to be ranked and showed there were significantly higher ($P < 0.05$) proportions of beads at 9-11 cells/bead (Fig. 5.1) with intermittent stirring at 40-75 rpm and with continuous stirring at 40-60 rpm compared to all other speeds used. (3) The analyses showed there were significantly higher (25%, $P < 0.01$) proportions of beads at the optimal cell/bead number with continuous stirring compared to intermittent at between 30-60 rpm. However, this was reversed at between 60-100 rpm. (4) There was no significant difference (Fig. 5.1) between cell attachment in 50 and 100% medium volume with respect to either stirrer mode used. (5) The plot of the percentage of beads against the given cell/bead ratio (Fig. 5.2A)) with continuous stirring was not significantly different from the theoretical Poisson distribution at 40 rpm. However, there were flat profiles with a significantly wider ($P < 0.01$) range of cell/bead ratios (between 0-50 cells/bead) at 75 and 100 rpm or without stirring compared to the theoretical distribution. The highest proportion of beads were cell-free in the culture without stirring. (6) The optimal stirring speed for cell attachment was 40 rpm.

Cultispher-G. It was impossible to determine the cell/bead distribution pattern of Cultispher-G cultures using the crystal violet stain, as it could not be washed out of the macroporous gelatin matrix and thus, the attached cells could not be identified. Neutral red stain also stained the gelatin but could be washed out with D-PBS⁻ washing (x3) leaving the nucleic acids stained red and permitting visualisation of beads with cells attached. An accurate count of the cell/bead ratio was impossible due to the random attachment of various sized cell aggregates. Thus, the optimal conditions were estimated after washing by scoring the beads with red staining (cell containing) as positives and the unstained beads as negatives.

(1) The analyses allowed the speeds to be ranked and showed there were significantly higher ($P < 0.01$) proportions of red staining beads with intermittent stirring at 30-60 rpm compared to all other speeds used. The results were similar with continuous stirring, except there were

significantly higher proportions ($P < 0.01$) of red staining beads at 30-40 rpm. (2) The staining was more intense and in highly localised areas of the beads following attachment with intermittent stirring at >60 rpm and with all continuously stirred cultures. (3) There were significantly higher proportions of red stained beads with intermittent stirring at 50 (32%, $P < 0.01$) and 100 (71%, $P < 0.01$) % medium volume compared to continuous stirring with respect to all the speeds used. (4) There was no significant difference between attachment at 50 and 100% volume with respect to the stirrer speeds and modes used in this study. (5) The optimal stirring speed for cell attachment was 40 rpm.

Cell Aggregates. Free cells in small aggregates (2-10 cells) were seen after 24 h in Cytodex-1 cultures following attachment with continuous stirring at 75-100 rpm. No aggregates were found in any other Cytodex-1 cultures. There were large aggregates after 24 h in all continuously stirred (30-100 rpm) Cultispher-G cultures. The aggregates were small (10-30 cells) at 30-40 rpm but were significantly larger (≥ 50 cells) at 100 rpm. However, there were few free cells ($< 5\%$ of the total) in any intermittently stirred (30-100 rpm) Cultispher-G cultures.

Discussion. Cytodex-1. The attachment conditions in Cytodex-1 cultures were shown to affect maximum yields of FS-4 cells (Hu et al., 1985) and lag phases of Vero cells (Lindner et al., 1987). Butler and Thilly (1982) and Butler et al. (1987) showed a critical minimum inoculum concentration (5 cells/bead) for Madin-Darby canine kidney (MDCK) cells in Superbead™ cultures to achieve a distribution profile closely matching the theoretical Poisson distribution. The inoculum ratio of 10 Vero cells/Cytodex-1 bead, used in most experiments in this study, was chosen as it was above this critical minimum concentration.

Clark and Hirtenstein (1981) showed the optimal Vero and MRC-5 cell attachment conditions, from high inoculum concentrations (0.70×10^6 cells/mL), were 50% volume without stirring for 24 h. Pharmacia® recommends either this method or 50% volume with intermittent stirring at 60 rpm for attachment of all cell lines to Cytodex beads. Conversely, others have

shown Vero and CHO cell attachment to Cytodex-1 with continuous stirring at 40 rpm (Nahapetian et al., 1986, Kim et al., 1992 and Ng, 1995).

The cell/bead distribution profile following the attachment phase helps to define the optimal parameters (Butler and Thilly, 1982). The optimal profile is a curve centered around the cell inoculum value and calculated using the Poisson random distribution equation (Equation 2.5.1 in 2.5.5). Optimal attachment conditions would result in an observed cell/bead distribution closely matching the Poisson profile. However, attachment in spinner cultures could be influenced by many uncontrollable parameters which prevent the optimal profile being achieved. Uneven cell/bead distributions after attachment could then result in low maximum cell yields through incomplete occupation of the available surface area (Clark and Hirtenstein, 1981). A lack of bead to bead cell transfer could affect this and will be discussed below (5.3.8).

The optimal attachment in Cytodex-1 cultures was obtained with continuous stirring at 40 rpm. Cell/bead distributions were significantly reduced with intermittent stirring and attachment without stirring gave the widest range of cell/bead distributions observed in Cytodex-1 cultures in this study. These results contradict those of Clark and Hirtenstein (1981) and also the Pharmacia guidelines but they agree with the other reports (Nahapetian et al., 1986, Kim et al., 1992 and Ng, 1995). The poor distributions of cultures without stirring were caused by the cells sedimenting at a slower rate compared to the beads and cells only attached to the upper beads in the sediment. Therefore, whilst Clark and Hirtenstein (1981) could have had higher attachment rates their cultures probably had poor cell/bead distributions.

Few free Vero cells were present following attachment in Cytodex-1 cultures. This suggests Vero cells have a high attachment affinity for Cytodex-1 beads which agrees with previously reported data (Ng, 1995).

Cultispher-G. There have been apparently contradictory results for cell attachment to macroporous beads. Hy-Clone® state the optimal cell attachment conditions for Cultispher cultures are 50% medium volume with continuous stirring at 40-60 rpm. However, intermittent

stirring at 60 rpm was shown to be optimal for CHO cell attachment to Biomat™ and Cultispher-G or S beads (Adema et al., 1990 and Ohlson et al., 1994).

Assessment of the cell/bead distribution patterns in Cultispher-G cultures using the same methods as Cytodex-1 was impossible due to staining of their matrix. Accurate assessment of cell/bead distributions in Cultispher-G cultures has been successful with fluorescent microscopy and image analysis (Foran et al., 1991). Neutral red staining was previously used for cytotoxicity assays of Vero cells in culture flasks (Valdievieson-Garcia et al., 1993) and was found in this study to reversibly stain the gelatin beads whilst permanently staining the cells. This allowed an assessment of the number of beads with cells attached.

The highest number of Cultispher-G beads with cells attached was with intermittent stirring at 30-40 rpm with 100% medium volume. Indeed, there were large cell aggregates in all the continuous stirred cultures at the end of the attachment phase which contradicts Hy-Clone application literature but agrees with previous results where intermittent stirring with a period of 5 min on/15 min off at 40 rpm over 5 cycles was found to be optimal for CHO cell attachment (Ohlson et al., 1994). The difference in the speed of attachment between Vero and CHO cells could be cell line dependent.

General. The results show Vero cell attachment differs between the bead types. Cells were attracted quickly (<2 h) to Cytodex-1 beads which could be explained by the positively charged polydextran beads attracting the negatively charged cell membranes. The cells could then firmly attach and flatten onto the beads through the mediation of serum proteins. However, Vero cells were attached at a much slower rate (by an order of magnitude) to Cultispher-G beads which agrees with previous results (Ng, 1995). This could be explained through Cultispher-G having no matrix charge to attract the cells. Thus, attachment requires proteins either from the serum or secreted by the cells in the initial phase of the culture.

5.3.2 Period of Intermittent Stirring for Cultispher-G

Introduction. Vero cell attachment in Cultispher-G cultures was shown above (5.3.1) to be optimal with intermittent stirring at 40 rpm in 100% medium volume. However, the intermittent stirring frequency (the on/off cycling times) could affect the attachment, as short off times could generate attachment profiles similar to continuous stirring. Therefore, the effect of the intermittent stirring off time on Vero cell attachment to Cultispher-G was determined.

Experimental. The Vero cells were inoculated in spinner flasks with intermittent stirring at 40 rpm in 100% medium volume, as described above (5.3.1). The cultures were intermittently stirred for 3 min after various off times (between 15 to 120 min). Cell attachment was assessed by neutral red staining (5.3.1) and the cell attachment time was also determined (2.5.6).

Results. Table 5.1 shows the proportion of Vero cells attached to the Cultispher-G beads and the cell aggregation with the various intermittent stirring periods. (1) There were no significant differences in the cell attachment times with intermittent stirring off times between 30-120 min. However, the cell attachment time was significantly longer (75%, $P < 0.01$) with off times of 15 min. (2) There were significant numbers ($P < 0.01$) of free cells after 6 h but few after 12 h of cell attachment with off-times of 30-120 min. However, the free cell number was not significantly different after 24 h compared to 12 h with respect to all cultures. (3) There were bead aggregates after 6 h in all cultures. Significantly larger (229%, $P < 0.01$) bead aggregates were found after 24 h with off times of 120 min compared to 30 min.

Cell/Bead Distribution. Neutral red staining showed there was no significant difference in the proportion of beads with cells attached (78%) with off times of 30-120 min. There were significantly fewer (27%, $P < 0.01$) beads with red staining with the 15 min off time. Thus, as could be expected, attachment at short off-times was approaching the continuous stirred cultures

Table 5.1 - The effect of the **intermittent stirring period** on the Vero cell **attachment times** and **bead aggregation** in **Cultispher-G** cultures. The cells were inoculated at 0.05×10^6 cells/mL in 100 mL spinner flasks in DMEM + 5.0% v/v sCS. The cells were attached to 1.70 g/L Cultispher-G stirring intermittently at 40 rpm with a 3 min stirring time and with various times (between 15-120 min) without stirring for 24 h. The cultures were sampled every 3 h for 12 h and then after 21 and 24 h in culture. The samples were counted by the trypan blue method to determine the proportions of the original cell counts which had attached to the beads and the bead aggregate size range was determined using an inverted microscope. The values are means of n=2.

Sample Time (h)	Proportion of Cells Attached to the Beads and The Bead Aggregate Size at the Intermittent Stirring Period							
	3 min on/15 min off		3 min on/30 min off		3 min on/60 min off		3 min on/120 min off	
	Cells Attached (%)	Aggregate Size Range (Beads)	Cells Attached (%)	Aggregate Size Range (Beads)	Cells Attached (%)	Aggregate Size Range (Beads)	Cells Attached (%)	Aggregate Size Range (Beads)
0	0	1	0	1	0	1	0	1
3	10	1-2	15	1-2	15	2	12	1-3
6	29	2-3	39	2-3	34	2-6	28	3-10
9	55	2-3	80	2-4	70	3-8	67	4-12
12	75	2-4	97	2-5	90	4-10	92	6-15
21	95	2-5	97	2-5	96	6-12	93	10-22
24	96	2-5	100	2-5	94	8-15	97	10-25
Proportion of Beads Showing Red Staining Following Attachment	27		79		77		77	

above (5.3.1). No cell aggregates were formed in any of these cultures.

Discussion. There has been little previous data on cell attachment to Cultispher-G beads, excepting those described above (5.3.1). The results show the optimal intermittent stirring period for attachment to Cultispher-G was 3 min on/30 min off for at least 12 h in this study. Ng (1995) previously reported the same intermittent stirring period as optimal for Vero cell attachment to Cultispher-G. However, the results also suggest the total attachment time with intermittent stirring could be shortened to approximately 12 h but this was not determined.

5.3.3 Maximum Cell Yields Following Cell Attachment

Introduction. The cell attachment to the beads could affect maximum cell yields, so the cells must be optimally attached prior to growth but the optimal stirring speed for attachment could differ from that for growth. Therefore, an experiment was performed to determine the optimal speed for Vero cell growth following optimal and sub-optimal attachment to the microcarriers.

Experimental. The Vero attachment cultures above (5.3.1-2) were retained and their growth to maximum cell yield was monitored daily by the trypan blue method (2.5.1-2). Cultures with cells attached without stirring were continuously stirred at 40 rpm after 24 h. Cultures initially at 50% medium volume were made to 100% after 24 h and continuously stirred at 40 rpm. Cultures with cells attached with intermittent stirring were continuously stirred at the same speeds after 24 h.

Results. Fig. 5.4 shows Vero cell growth in Cytodex-1 and Cultispher-G cultures following attachment (24 h) with intermittent stirring (for various off time periods) and Fig. 5.5 shows growth with continuous stirring at various speeds (30-100 rpm). Table 5.2 shows the cell growth rates and lag phases in these cultures. (1) Maximum Vero cell yields (Fig. 5.4A)-B) and Fig. 5.5A)-B)) in cultures of either bead were affected by the stirrer mode and speed for cell

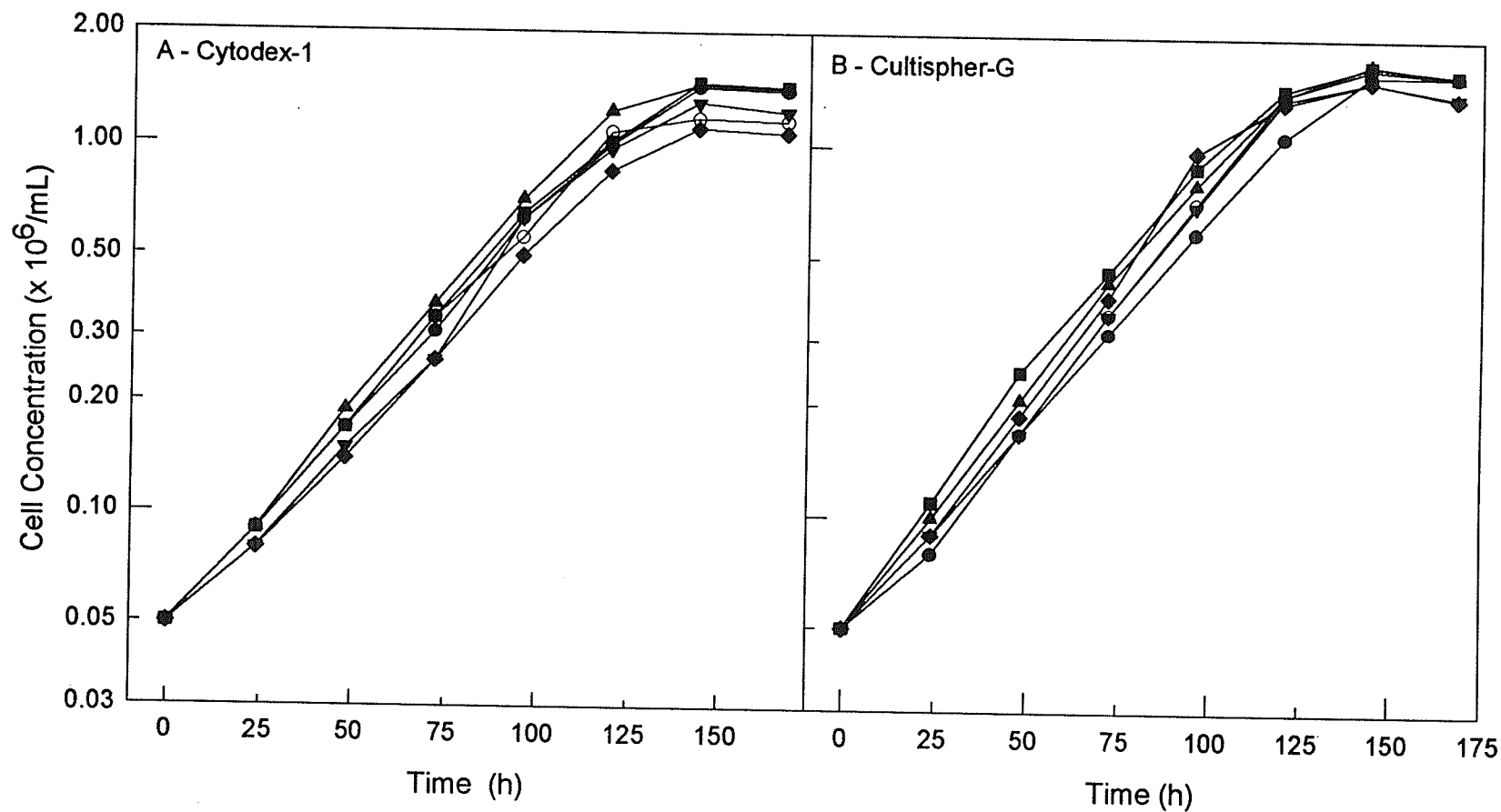


Fig. 5.4 - The effect of the intermittent stirring speed and mode for attachment on Vero cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A) and 1.70 g/L Cultispher-G (B) stirring at the same speeds continuously from 24 h onwards. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS and attached to the beads either $\circ$ without stirring or intermittently stirred (3 min on/30 min off) at $\blacksquare$ 30, $\blacktriangle$ 40 (100% volume), $\bullet$ 40 (50% volume), $\blacktriangledown$ 60 or $\blacklozenge$ 100 rpm. The flasks were readjusted to pH 7.4 daily after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

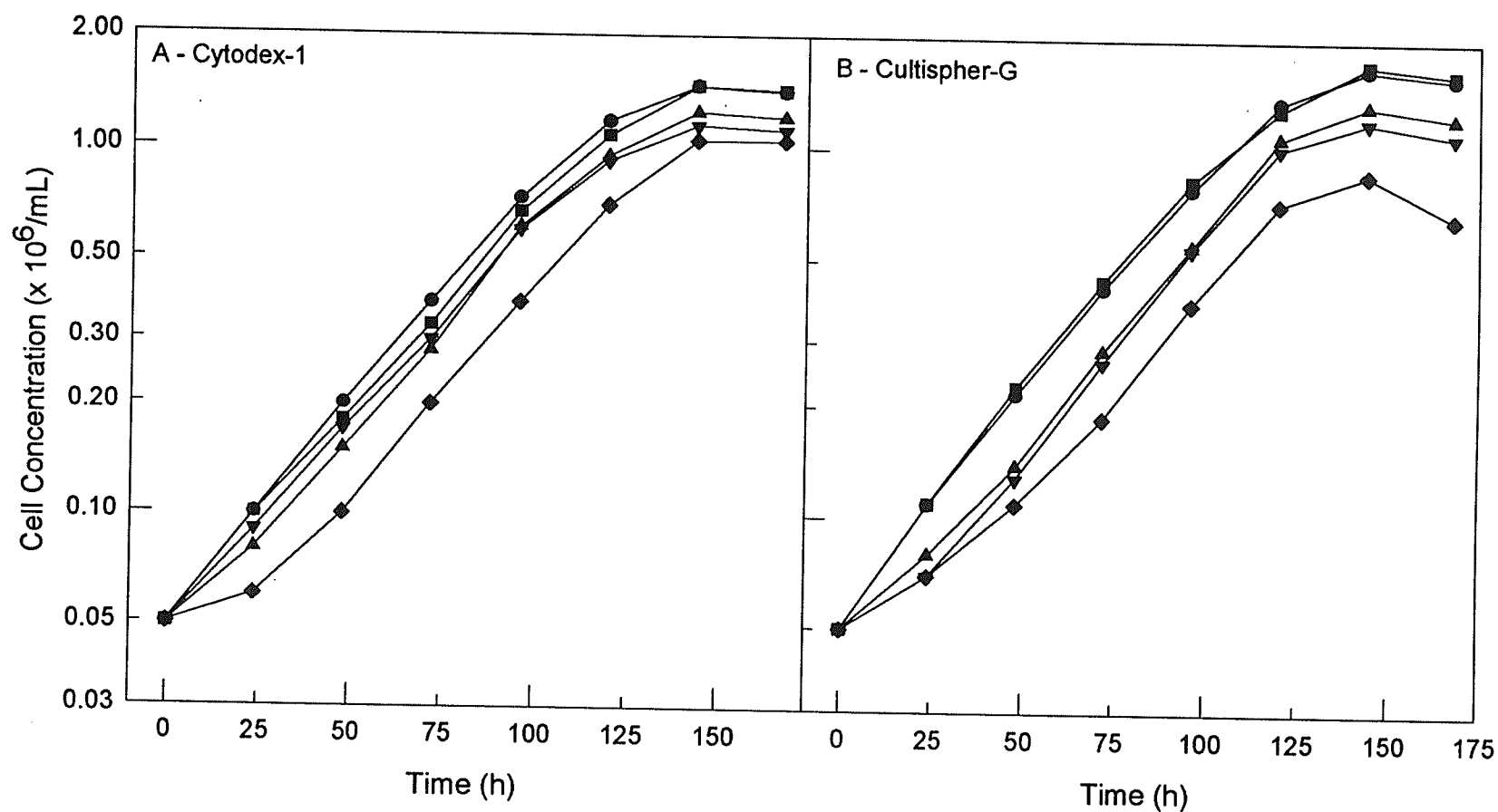


Fig. 5.5 - The effect of the continuous stirring speed on Vero cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A) and 1.70 g/L Cultispher-G (B) at the same speeds throughout. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS and continuously stirred at either ● 30, ■ 40, ▲ 60, ▼ 75 or ◆ 100 rpm. The flasks were readjusted to pH 7.4 daily after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

Table 5.2 - The effect of the **stirrer speed** and **mode** for Vero cell attachment on **cell growth rates** and **lag phases** in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS. The cultures were either intermittently stirred for 24 h then continuously at the same speed or continuously stirred throughout at various speeds (30-100 rpm). The flasks were readjusted to pH 7.4 daily after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Stirring Speed (rpm)	Cell Attachment Time and Lag Phase Following Attachment with Various Stirring Speeds and Modes							
	Cytodex-1				Cultispher-G			
	Intermittent Stirring		Continuous Stirring		Intermittent Stirring		Continuous Stirring	
	Specific Cell Growth Rate (h)	Lag Phase (h)	Specific Cell Growth Rate (h)	Lag Phase (h)	Specific Cell Growth Rate (h)	Lag Phase (h)	Specific Cell Growth Rate (h)	Lag Phase (h)
30	0.034 ± 0.003	3.00 ± 0.50	0.034 ± 0.003	3.00 ± 0.50	0.035 ± 0.003	3.00 ± 0.50	0.036 ± 0.003	3.00 ± 0.50
40	0.034 ± 0.003	3.00 ± 0.50	0.034 ± 0.003	3.00 ± 0.50	0.036 ± 0.003	3.00 ± 0.50	0.036 ± 0.003	3.00 ± 0.50
40*	0.034 ± 0.003	3.00 ± 0.50			0.036 ± 0.003	3.00 ± 0.50		
40#	0.034 ± 0.003	3.00 ± 0.50			0.036 ± 0.003	3.00 ± 0.50		
60	0.033 ± 0.003	6.00 ± 1.00	0.032 ± 0.003	3.00 ± 0.50	0.034 ± 0.003	3.00 ± 0.50	0.033 ± 0.003	6.00 ± 1.00
75			0.031 ± 0.003	6.00 ± 1.00			0.030 ± 0.003	12.00 ± 1.00
100	0.031 ± 0.003	6.00 ± 1.00	0.030 ± 0.003	18.00 ± 2.00	0.034 ± 0.003	6.00 ± 1.00	0.030 ± 0.003	12.00 ± 1.00

* - Culture inoculated at 50% medium volume.

- Culture inoculated at 100% medium volume with no stirring for 24 h.

attachment and growth. (2) There were significantly higher (17%, $P < 0.05$) cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the stirrer speeds and modes used. (3) There were significantly higher ($P < 0.05$) cell yields in cultures of either bead stirred at 30-40 rpm compared to all other speeds used and with respect to either stirrer mode for attachment. (4) There were significantly higher (19%, $P < 0.05$) cell yields in Cultispher-G cultures following cell attachment with intermittent stirring compared to continuous stirring with respect to all the speeds used. Cell yields in Cultispher-G cultures were not affected by any intermittent stirring period used for cell attachment. There was no significant difference in the cells yields in Cytodex-1 cultures with respect to the stirrer modes for attachment used. (5) The optimal stirrer speed for growth in cultures of either bead with no adjustment during the cultures was 40 rpm. (6) There was no significant difference (Table 5.2) in the cell growth rates in cultures of either bead with respect to all the stirrer speeds and modes used. (7) There was no significant difference in the lag phases in cultures of either bead following cell attachment with intermittent stirring at up to 60 rpm. However, there were significantly longer (250%, $P < 0.01$) lag phases in all Cytodex-1 cultures at 100 rpm and Cultispher-G cultures at 75 and 100 rpm compared to 40-60 rpm. (8) There were large cell aggregates (10-50 cells) in all the continuous stirred Cultispher-G cultures following the attachment phase. These aggregates disappeared within 48 h in culture at 30-60 rpm. The aggregate disappearance gave unusual neutral red staining patterns with intense red areas on a small proportion (25-30%) of the beads. Free cells in aggregates (up to 50 cells) were present after the attachment phase in cultures of either bead with continuous stirring at 75-100 rpm. These disappeared within 72 h in Cytodex-1 cultures but persisted throughout Cultispher-G cultures accounting for a large proportion (40%) of the total cells at maximum cell yield.

Bead Aggregates. Bead aggregates formed after 24 h in Cytodex-1 cultures with intermittent (5-20 beads) and continuous (2-5 beads) stirring for attachment at 30-60 rpm. The aggregates persisted without significantly increasing in size in intermittent stirred cultures. The aggregates

increased in size (5-10 beads) and accounted for most (72%) of the beads by the end of the continuous stirred cultures. Large bead aggregates (23 beads) formed after attachment without stirring and these persisted at this size throughout the cultures.

Bead aggregates formed after 24 h in Cultispher-G cultures following attachment without stirring (10-30 beads), with intermittent stirring (5-17 beads) at 30-60 rpm and with continuous stirring (2-5 beads) at 30-40 rpm. The aggregates persisted throughout in all the cultures and did not increase in size in intermittent stirred cultures but increased in size (5-15 beads at maximum cell yield) in continuous stirred cultures.

Discussion. The Vero cells reached the same maximum yield in Cytodex-1 cultures at 30-40 rpm following attachment with either stirring mode or medium volume used. The optimal continuous stirring speed (40 rpm) for growth was lower than that (60 rpm) previously proposed for Vero and MRC-5 cells (Clark and Hirtenstein, 1981). However, this could be explained by the different spinner flasks used between the studies or by the previous authors different attachment mode of static conditions in 50% medium volume. The authors maximum cell yields were significantly lower (40%, $P < 0.01$) compared to this report (using crystal violet data from 4.3.2).

The highest cell yields in this study were observed in cultures with Cytodex-1 bead aggregates present. This result contradicts Clark and Hirtenstein (1981) who suggested 60 rpm was optimal as it prevented cell bridging between the beads from causing lower cell yields. Cultures at 75-100 rpm had high proportions of singly suspended Cytodex-1 beads which agrees with the findings of the previous authors.

The lower cell yields in cultures at high stirrer speeds (100 rpm) in this study also agree with previous reports where CHO, BEK and FS-4 cells were damaged in cultures of solid Cytodex-1 at similar speeds (90-160 rpm) (Croughan et al., 1987 and 1988, Cherry and Papoutsakis, 1988 and Lakhotia and Papoutsakis, 1992).

The Cultispher-G cultures all grew to similar maximum cell yields following attachment with intermittent stirring. These yields show Cultispher-G beads protected the cells from shear forces

at high stirring speeds. This agrees with previous authors who showed CV-1 cells were protected in cultures of macroporous Biomat beads at high (100 rpm) stirrer speeds, as their yields were significantly higher (413%, $P < 0.01$) compared to Cytodex-3 cultures (Adema et al., 1990). However, maximum cell yields in Cultispher-G cultures in this study were also obtained when bead aggregates were present.

Maximum cell yields in Cultispher-G cultures were not significantly different following sub-optimal attachment with continuous stirring compared to intermittent stirring at 30-40 rpm. Cell aggregates were formed after 24 h in the continuous stirred cultures but these attached onto the beads within 48 h. This did not occur at 75-100 rpm. This attachment resulted in highly skewed proportions of beads showing red staining (20-30% of the beads contained $\geq 70\%$ of the cells) after 72-96 h in culture. This suggests that as long as the cells can become attached to the Cultispher-G beads there is no limitation to cell yield.

The disappearance of cell aggregates with continuous stirring at 30-40 rpm was indicative of entrapment in the Cultispher-G bead pores and not of a protein mediated attachment. Intense neutral red staining confirmed where cell aggregates had attached/entrapped in the beads. Similar cell yields following poor cell/bead distributions could be explained by the large interior surface area of Cultispher-G beads available for cell growth. However, poor distributions caused cell counting problems, especially at low cell concentrations ($\leq 0.20 \times 10^6$ cells/mL).

Maximum cell yields in Cultispher-G cultures were significantly lower with continuous stirring throughout at 100 rpm. This could be explained by the high proportions of aggregated cells at this speed which never attached to the beads. The aggregates made accurate sampling difficult, as they had to be included in total counts but were not growing on the microcarriers. The aggregate size did not increase which suggests these cells were either not actively growing or were growing at highly reduced growth rates compared to cells in the beads. This is indicative of a contact inhibition similar to cell domes in culture flasks discussed previously (4.3.5). Cells in the aggregates could also have consumed medium nutrients without growing reducing the amounts available for the actively growing cells.

Cell/Bead Distributions. The poor cell/bead distributions in Cytodex-1 cultures following attachment without stirring (5.3.1) could explain the severe bead aggregation and lower maximum cell yields observed in these cultures. Most cells were attached to a small proportion of the total beads and these then aggregated. Therefore, not all of the bead surface was available for cell growth. The low yields could also be explained by the cell-free beads adsorbing significant concentrations of serum factors (e.g. fibronectin, vitronectin etc. - Underwood and Bennett, 1989) resulting in decreased numbers of cell divisions on the cell-containing beads.

Bead Aggregates. Bead aggregate sizes increased (up to 35 beads) in Cultispher-G cultures which were at $\text{pH} \leq 6.7$ at each readjustment time. Initially the cultures were at pH 7.4-7.5 after equilibration with 10% v/v CO_2 overlay at 37°C . Cell respiration of glucose and amino acids (e.g. glutamine) could produce significant concentrations of lactate which lowers the culture pH (Duval et al., 1992). Cultispher-G cultures decreased to pH 6.5 after 72 h without readjustment and bead aggregation was increased under such conditions. Cytodex-1 cultures never decreased to $\text{pH} < 7.0$ after 72 h and thus, showed less bead aggregation.

The pH dependent bead aggregate size increase suggests the beads and or cells became more attractive to each other at low pH . This could be explained by serum protein interactions or cell surface charge effects which are enhanced at low pH (Hu et al., 1985 and Lengheden, 1994). The aggregates increased the bead number removed in some samples and constant observation was required to ensure cell count accuracy.

Free Cells. Freely suspended cells were found in Cultispher-G cultures 24 h after reaching maximum cell yield, following attachment with intermittent stirring at 75-100 rpm. Cultures attached at 30-60 rpm showed few detached cells until 48-72 h after maximum yield. This could be explained by the increased shear forces at high stirrer speeds and could relate to a nutrient depletion required for cell adherence such as glucose for sialylation of gangliosides (Kleinman et al., 1981). This will be discussed in Chapter 6.3.

5.3.4 Ramping the Stirring Speed During Growth

Introduction. The optimal stirring speed without adjustment for cell attachment and growth in cultures of either bead was 40 rpm (5.3.3). However, low maximum cell yields at 75-100 rpm could result from sub-optimal attachment conditions in these cultures. Higher speeds could benefit later stages of cultures through increased nutrient supply or by reducing bead aggregation. Therefore, an experiment was performed to determine whether speed ramping following optimal cell attachment could improve maximum Vero cell yields in microcarrier cultures.

Experimental. Vero cultures were grown in spinner flasks following optimal cell attachment, as described above (5.3.1-2). The cultures were continuously stirred at 40 rpm prior to speed ramping either (a) by two speed ramps, one after 48 h to 60 rpm and the second after 96 h to 100 rpm or (b) by one ramp after 72 h to 60, 75 or 100 rpm.

Results. Table 5.3 shows the maximum cell yields, growth rates and lag phases in the speed ramped cultures. (1) There was no significant difference in the maximum cell yields in cultures of either bead with respect to the speed ramps used (up to 100 rpm). (2) The cell growth rates and lag phases in cultures of either bead were not significantly different with respect to any speed ramp used. (3) The cells reached a monolayer in Cytodex-1 cultures at cell yields of $0.80\text{--}0.90 \times 10^6$ cells/mL and were forming two cell layers over much of the bead surface at maximum yield. It was impossible to determine the state of the beads in the Cultispher-G pores.

Bead Aggregates. There were significantly larger (257%, $P < 0.01$) bead aggregates in cultures of either bead at 40 rpm (5-20 beads) throughout compared to all the speed ramps (2-5 beads) used. Cultispher-G aggregate sizes were significantly increased (60%, $P < 0.01$) in cultures with pH < 6.7 at each readjustment time, as described above (5.3.3).

Table 5.3 - The effect of **speed ramping** on maximum Vero cell yields, growth rates and lag phases in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS and optimally attached to the microcarriers by stirring at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G). The speed was then at either 40 rpm throughout, or ramped after 72 h to 60 75 or 100 rpm or ramped after 48 to 60 rpm then after and 96 h to 100 rpm. The flasks were readjusted daily to pH 7.4 after 72 h with 2.00 M NaOH. The values are means of n=2.

Stirrer Speed Used (rpm)	Maximum Cell Yield, Growth Rate and Lag Phase at the Stirrer Speed Used					
	Cytodex-1			Cultispher-G		
	Maximum Cell Yield ($\times 10^6$ cells/mL)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Maximum Cell Yield ($\times 10^6$ cells/mL)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
40	1.48 ± 0.10	0.034 ± 0.003	3.00 ± 0.50	1.78 ± 0.12	0.036 ± 0.003	3.00 ± 0.50
40-60	$1.54 \pm 0.11^*$	0.034 ± 0.003	3.00 ± 0.50	$1.85 \pm 0.12^*$	0.036 ± 0.003	3.00 ± 0.50
40-75	1.49 ± 0.10	0.034 ± 0.003	3.00 ± 0.50	1.77 ± 0.12	0.036 ± 0.003	3.00 ± 0.50
40-100	1.38 ± 0.09	0.033 ± 0.003	4.50 ± 0.50	1.70 ± 0.12	0.035 ± 0.003	4.50 ± 0.50
40-60-100	1.48 ± 0.10	0.034 ± 0.003	4.50 ± 0.50	1.80 ± 0.12	0.036 ± 0.003	4.50 ± 0.50

* - Highest cell yield.

Discussion. Bead aggregates and cell multi-layers formed in Cytodex-1 cultures at low speeds (40 rpm) were suggested to limit maximum Vero and MRC-5 cell yields (Clark and Hirtenstein, 1981) by decreasing the surface area or cell division nutrient pools. However, there was no significant difference in the maximum cell yields in cultures of either bead with or without speed ramping to 100 rpm. The results of Cytodex-1 cultures ramped to 100 rpm disagreed with previous reports of reduced CHO, FS-4 and BEK cell yields in cultures at similar speeds (90 rpm) (Croughan et al., 1987 and 1988, Cherry and Papoutsakis, 1988 and Lakhotia and Papoutsakis, 1992). However, the cells were optimally attached and in mid-exponential growth phase prior to ramping in this study whilst the previous authors ramped the speed after 24 h in culture when their cells were not in the exponential growth phase. Therefore, previous reports of high speed cell damage or shear force detachment causing low cell yields could be exaggerated by the methods used.

Nutrient Supply. Poor nutrient diffusion was suggested to reduce yields in cultures with multi-layers of Vero and murine L-WRL10A cells (Werrlein and Glinos, 1974 and Nahapetian et al., 1986). The authors suggested increased stirrer speeds to improve nutrient supply to lower cell layers. However, the results in this study indicate there were no nutrient limitations in batch cultures even when grown at 40 rpm throughout. This could be explained by the lack of multilayers on the Cytodex-1 beads in batch growths and suggests the cells were not growing in dense masses in the Cultispher-G pores. One advantage of ramped stirrer speeds (to ≥ 60 rpm) in this study was the reduction of bead aggregate sizes which agreed with Clark and Hirtenstein (1981) who reported singly suspended Cytodex-1 cultures with stirring at 60 rpm.

5.3.5 Optimum pH

Introduction. The culture pH could affect the attachment and growth of the cells. Previous studies (5.3.3-4) showed wide fluctuations in the culture pH during growth. Therefore, the

optimal pH for Vero cell growth in microcarrier cultures in the Celligen bioreactor was determined.

Experimental. The Vero cultures were grown in the Celligen bioreactor on microcarriers with various controlled pH values (between pH 6.5-7.8), as described previously (2.4.5). At each pH set-point the other set-points were: temperature of 37°C, DOT of 50% of air saturation, stirring continuously at 40 rpm either throughout (Cytodex-1) or for 72 h then ramped to 60 rpm (Cultispher-G) and gas flow rate of 0.50 L/min. The pH was controlled at set-point by sparging CO₂ to lower the pH and by pumping 1.00 M Na₂CO₃ solution to raise the pH. The pH was maintained within 0.10 units of the set-point throughout in all cultures. Daily the culture pH was measured off-line and the pH amplifier was reset to the new value when it had drifted by more than 0.05 units.

Results. Fig. 5.6 shows maximum Vero cell yields in Cytodex-1 and Cultispher-G cultures at the pH set-points used (pH 6.5-7.8) and cell growth at the pH optima. Table 5.4 shows the cell growth rates and lag phases in these cultures. (1) Maximum cell yields (Fig. 5.6A)) in cultures of either bead were affected by the pH set-points used. There were significantly higher maximum cell yields in Cytodex-1 cultures ($P < 0.01$) at pH 7.1 and Cultispher-G cultures ($P < 0.05$) at pH 6.8 compared to all other pH set-points used. (2) The optima for cell growth were pH 7.1 in Cytodex-1 cultures and pH 6.8 in Cultispher-G. There were significantly higher (27%, $P < 0.01$) maximum cell yields (Fig. 5.6B)) at the optimal pH values in Cultispher-G cultures compared to Cytodex-1. (3) The cell growth rates (Table 5.4) were significantly affected by the pH set-points used. The cell growth rates in cultures of either bead were significantly higher ($P < 0.01$) in the range of pH 6.8-7.1 compared to all other set-points used. (4) There was no significant difference in the lag phases in cultures of either bead at pH 6.8-7.5. However, the lag phases were significantly longer (600%, $P < 0.01$) at pH 6.5 and 7.8.

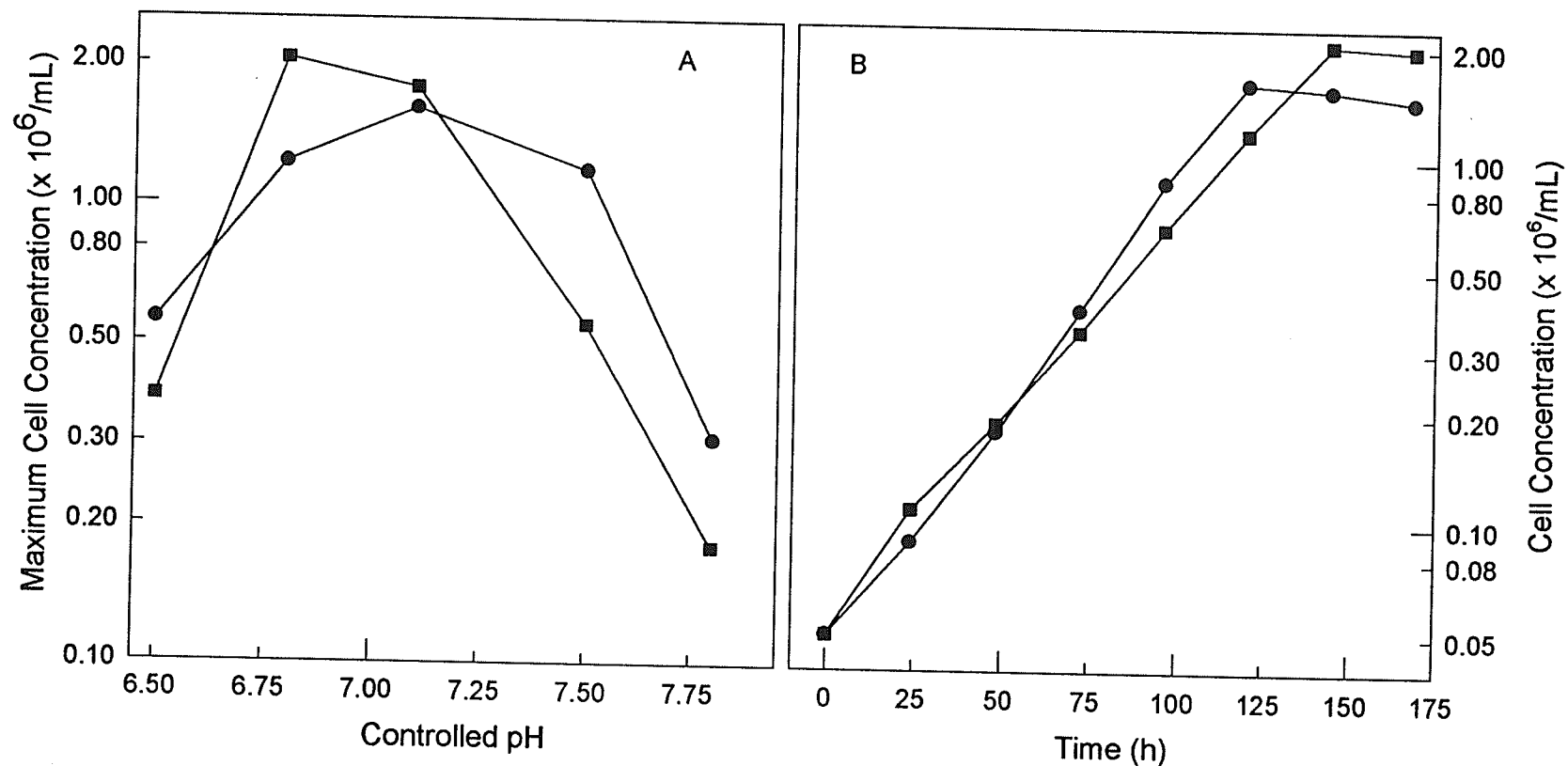


Fig. 5.6 - The effect of **controlled pH** on the maximum Vero cell yield (A) and growth at the optimal pH (B) in 1,300 mL Celligen bioreactor on microcarriers. The cells were inoculated at 0.05×10^6 cells/mL on either ● 1.00 g/L Cytodex-1 or ■ 1.70 g/L Cultispher-G in DMEM + 5.0% v/v SCS. The cultures were grown at pH's between 6.5-7.8. The other parameters were controlled at set-points of 37°C, 50% DOT, 40 rpm continuously (Cytodex-1) or 40 rpm up to 72 h followed by 60 rpm (Cultispher-G) and at 0.50 L/min gas flow rate. The values are means of n=2.

Table 5.4 - The effect of **controlled pH** on the Vero cell **growth rate** and **lag phase** in 1,300 mL Celligen bioreactor on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS. The other parameters were controlled at 37°C, 50% DOT, 40 rpm continuously (Cytodex-1) or ramped to 60 rpm after 72 h (Cultispher-G) and 0.50 L/min gas flow rate. The values are means of $n=2$.

Controlled pH	Vero Cell Growth Rate and Lag Phase at the Controlled pH			
	Cytodex-1		Cultispher-G	
	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
6.5	0.024 ± 0.002	3.00 ± 0.50	0.020 ± 0.002	3.00 ± 0.50
6.8	0.039 ± 0.003	3.00 ± 0.50	0.045 ± 0.004	3.00 ± 0.50
7.1	0.042 ± 0.003	3.00 ± 0.50	0.043 ± 0.003	3.00 ± 0.50
7.5	0.038 ± 0.003	18.00 ± 2.00	0.029 ± 0.003	18.00 ± 2.00
7.8	0.022 ± 0.002	18.00 ± 2.00	0.015 ± 0.002	18.00 ± 2.00

Bead Aggregates. Bead aggregates were formed in Cultispher-G cultures. These were small (3-4 beads) at pH 7.8 and were significantly larger (400%, $P < 0.01$) at pH 6.5 at maximum cell yield. The aggregates at pH 7.8 did not increase in size throughout the culture. However, the aggregates at pH 6.5 significantly increased (400%, $P < 0.01$) in size over the course of the culture.

Discussion. The growth medium used in these studies (DMEM + 5.0% v/v sCS at 44.0 mM sodium bicarbonate) was at pH 7.4 following equilibration with a 10% v/v CO₂ overlay. However, previous studies (5.3.3-4) showed Cultispher-G cultures rapidly decreased to pH <6.7 and Cytodex-1 cultures to pH <7.0 after 72 h of Vero cell growth, probably as a result of lactate production. Ceccarini and Eagle (1971) and Ceccarini (1975) showed similar pH optima for plating and growth but the optima varied greatly between cell lines. The authors showed optima of pH 7.0-7.1 for HeLa and WI38 cells and pH 7.6-7.8 for human KL2 and Penny D fibroblast cells. The optimal pH for Vero cells had not been previously reported.

This study found the optimal pH was lower (pH 6.8) in Cultispher-G cultures compared to Cytodex-1 (pH 7.1). No one has previously shown differences in parameter optima between the beads. However, the cells grew poorly at pH 6.5 and 7.8 in cultures of either bead. The poor growth could be explained by effects on medium components. For example, Rabinowitz et al. (1956), Griffiths (1972) and Reitzer et al. (1979) suggested glutamine was the major carbon source for Ehrlich ascites, human fibroblast and HeLa cell growth. However, glutamine naturally decomposes (Lin and Agrawal, 1988) in solutions and high pH increases the decomposition rate. Hence, if glutamine was important for Vero cell growth, a high pH could result in a lower maximum cell yield. This will be discussed in Chapter 6.3.

Cellular glutamine metabolism produces ammonia and glutamate and physiological levels of ammonia (3 mM) inhibit baby hamster kidney (BHK) but not Vero or MDCK cell growth (Hassell and Butler, 1991). Murine hybridoma cell inhibition or toxicity by ammonia has been shown to increase with increased culture pH (Doyle and Butler, 1990). This will be discussed in Chapter

8.3.

Cellular metabolism of glucose produces lactate and CO₂. However, lactate also inhibits hybridoma cell growth (Duval et al., 1992 and Omasa, 1992). Lactate and CO₂ also lower the medium pH which could result in growth inhibition (Ceccarini and Eagle, 1971) and will be discussed in Chapter 8.3.

High pH (pH >7.5) reduces FS-4 cell adhesion to substrata (Hu et al., 1985) and trypsin solutions are prepared at an alkaline pH to enhance the detachment. Therefore, high pH could have affected cell adhesion resulting in reduced growth as was observed in this study. This also agrees with Lengheden (1994) who showed reduced adherent human fibroblast cell growth at pH >7.5.

The low growth at pH 6.5 could be explained by enzyme inhibition, as low pH reduces the phosphofructokinase activity of hybridoma cells (Duval et al., 1992). This enzyme regulates glycolysis, which could reduce the availability of substrates from glucose for processes such as glycoprotein sialylation (e.g. of gangliosides) resulting in reduced cell adhesion or division on substrata (Kleinman et al., 1983 and Ruoslahti et al., 1982).

Bead Aggregation. Severe bead aggregation occurred in the Celligen bioreactor following attachment to Cultispher-G with the optimal intermittent stirring protocol. These cultures were lost within 72 h, as the aggregates formed a single mass in the stirrer shaft draft tube. Therefore, attachment to Cultispher-G was performed with continuous stirring which was shown above (5.3.3) not to reduce cell yields in spinner flasks. However, optimal attachment could be performed in spinner flasks prior to inoculation of the bioreactor. All bioreactor cultures in this Chapter were continuously stirred.

5.3.6 pH Control in Spinner Flasks

Introduction. The culture pH affects cell growth (5.3.5) and the pH of spinner flask cultures is

impossible to control. Hence, growth media are often supplemented with HEPES buffer to improve pH control. However, cell growth produces lactate from glucose metabolism and this lactate could reduce the pH of spinner flask cultures to sub-optimal levels for growth. No one has previously described pH readjustment in spinner flask cultures to enhance cell yields. Therefore, this study determined the effects of daily pH readjustment on maximum Vero cell yields in microcarrier cultures.

Experimental. Preadapted stock Vero cells were inoculated in original DMEM + 5.0% v/v sCS buffered with either (a) sodium bicarbonate (44.00 mM) or (b) with sodium bicarbonate (44.00 mM) and supplemented with HEPES buffer (25.00 mM). The cells were grown in spinner flasks, as described above (5.3.3-4).

Results. Table 5.5 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G with the various pH readjustment protocols. Table 5.6 shows cell growth rates and lag phases in these cultures. (1) Vero cell growth was affected by the pH readjustment method. (2) There were significantly higher (23%, $P < 0.05$) maximum cell yields in cultures of Cultispher-G compared to Cytodex-1 in either DMEM and with respect to all pH readjustment methods used. (3) There was no significant difference in the maximum cell yields in Cytodex-1 cultures with readjustment to pH 7.1, pH 7.5 or with unadjusted pH. (4) There was no significant difference in maximum cell yields in Cytodex-1 cultures with or without HEPES buffer in the medium with respect to all the pH readjustments made. (5) The maximum cell yield in Cultispher-G cultures was significantly higher ($P < 0.05$) with daily readjustment to pH 7.1 compared to the other readjustments used. (6) Maximum cell yields in pH readjusted Cultispher-G cultures were not significantly different with or without HEPES buffer in the medium. However, maximum cell yields were significantly higher (24%, $P = 0.01$) in pH unadjusted Cultispher-G cultures with HEPES buffer compared to without HEPES. (7) The cell growth rates (Table 5.6) were not significantly different with respect to the pH readjustment method in cultures of either bead. (8) Lag phases (Table 5.6) were significantly

Table 5.5 - The effect of pH readjustment to high and low pH on the growth of Vero cells in 100 mL spinner flasks on **1.00 g/L Cytodex-1 (A)** and **1.70 g/L Cultispher-G (B)**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS prepared with or without 25.00 mM HEPES buffer at pH 7.1 or pH 7.5. Cultures were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). Culture pH was either adjusted daily after 72 h to the original values of pH 7.1 or pH 7.5 with 2.00 M NaOH or was unadjusted. The flasks were at pH 6.7, 6.8 and 7.3 in Cytodex-1 cultures and at pH 6.5, 6.8 and 6.8 in Cultispher-G at the end of the experiment from unadjusted, adjusted pH 7.1 and adjusted pH 7.5, respectively. The values are means of n=2.

Table 5.5A) - Cytodex-1.

Time (h)	Cell Concentration in Cytodex-1 Cultures ($\times 10^6$ /mL)					
	DMEM Without HEPES Buffer			DMEM With HEPES Buffer		
	Unadjusted	Adjusted (pH 7.1)	Adjusted (pH 7.5)	Unadjusted	Adjusted (pH 7.1)	Adjusted (pH 7.5)
0	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02
24	0.09 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.09 ± 0.02	0.10 ± 0.02	0.09 ± 0.02
48	0.18 ± 0.03	0.19 ± 0.03	0.20 ± 0.02	0.18 ± 0.03	0.19 ± 0.02	0.19 ± 0.03
72	0.42 ± 0.04	0.41 ± 0.03	0.39 ± 0.03	0.38 ± 0.03	0.37 ± 0.03	0.36 ± 0.02
96	0.70 ± 0.05	0.80 ± 0.04	0.77 ± 0.05	0.75 ± 0.06	0.76 ± 0.07	0.71 ± 0.06
120	1.12 ± 0.06	1.35 ± 0.07	1.25 ± 0.09	1.38 ± 0.09	1.31 ± 0.08	1.24 ± 0.07
144	$1.27 \pm 0.08^*$	$1.45 \pm 0.10^*$	1.48 ± 0.10	$1.48 \pm 0.10^*$	$1.45 \pm 0.10^*$	$1.35 \pm 0.10^*$
168	1.25 ± 0.11	1.44 ± 0.11	$1.51 \pm 0.11^*$	1.45 ± 0.09	1.42 ± 0.11	1.27 ± 0.07
192	1.17 ± 0.08	1.39 ± 0.09	1.46 ± 0.10	1.44 ± 0.10	1.37 ± 0.09	1.21 ± 0.06

* - Highest cell yield.

Table 5.5B) - Cultispher-G.

Time (h)	Cell Concentration in Cultispher-G Cultures (x 10 ⁶ /mL)					
	DMEM Without HEPES Buffer			DMEM With HEPES Buffer		
	Unadjusted	Adjusted (pH 7.1)	Adjusted (pH 7.5)	Unadjusted	Adjusted (pH 7.1)	Adjusted (pH 7.5)
0	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02
24	0.10 ± 0.02	0.10 ± 0.02	0.09 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.09 ± 0.02
48	0.20 ± 0.03	0.20 ± 0.03	0.19 ± 0.03	0.19 ± 0.03	0.20 ± 0.03	0.17 ± 0.03
72	0.42 ± 0.03	0.43 ± 0.02	0.41 ± 0.02	0.37 ± 0.03	0.40 ± 0.03	0.35 ± 0.03
96	0.81 ± 0.07	0.87 ± 0.07	0.75 ± 0.07	0.78 ± 0.06	0.80 ± 0.06	0.71 ± 0.06
120	1.16 ± 0.09	1.54 ± 0.09	1.34 ± 0.09	1.36 ± 0.09	1.42 ± 0.09	1.30 ± 0.08
144	1.27 ± 0.10*	2.05 ± 0.13*	1.76 ± 0.12*	1.57 ± 0.12*	2.02 ± 0.13*	1.73 ± 0.12*
168	1.20 ± 0.08	2.02 ± 0.13	1.47 ± 0.09	1.40 ± 0.09	2.00 ± 0.13	1.67 ± 0.11
192	1.01 ± 0.07	1.87 ± 0.12	1.11 ± 0.07	1.21 ± 0.07	1.81 ± 0.12	1.61 ± 0.11

* - Highest cell yield.

Table 5.6 - The effects of the **pH readjustment** protocol on the Vero cell growth rates and lag phases in 100 mL spinner flasks of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS prepared with or without HEPES buffer (25.00 mM). The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). The flasks were readjusted to pH 7.1 daily after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

pH Readjustment Protocol	The Vero Cell Growth Rate and Lag Phase in Cultures of Either Bead Type			
	Cytodex-1		Cultispher-G	
	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
No HEPES				
pH 7.1 Adjusted	0.033 ± 0.003	3.00 ± 0.50	0.035 ± 0.003	3.00 ± 0.50
pH 7.5 Adjusted	0.033 ± 0.003	6.00 ± 1.00	0.035 ± 0.003	6.00 ± 1.00
pH 7.5 Unadjusted	0.033 ± 0.003	6.00 ± 1.00	0.035 ± 0.003	6.00 ± 1.00
25.00 mM HEPES				
pH 7.1 Adjusted	0.033 ± 0.003	3.00 ± 0.50	0.035 ± 0.003	3.00 ± 0.50
pH 7.5 Adjusted	0.033 ± 0.003	6.00 ± 1.00	0.035 ± 0.003	6.00 ± 1.00
pH 7.5 Unadjusted	0.033 ± 0.003	6.00 ± 1.00	0.032 ± 0.003	6.00 ± 1.00

shorter (50%, $P < 0.01$) in cultures of either bead with an initial pH of 7.1 compared to pH 7.5 in cultures of either bead.

pH. The pH fluctuated during cell growth in all cultures. After 72 h Cytodex-1 cultures inoculated at pH 7.1 and 7.5 had decreased to pH 6.7 and 7.0, respectively. The pH was then readjusted daily to the values at inoculation but was decreased again at each daily readjustment time to pH 6.8 and 7.3 following readjustment to pH 7.1 and 7.5, respectively. The pH unadjusted Cytodex-1 culture was inoculated at pH 7.5 and decreased to pH 6.6 at the end of the cultures. After 72 h Cultispher-G cultures inoculated at pH 7.1 and 7.5 had decreased to pH 6.7. The pH then decreased to pH 6.6 and 6.8 following readjustment to pH 7.1 and 7.5, respectively. The unadjusted Cultispher-G culture had decreased from pH 7.5 to 6.5 at the end of the culture.

Discussion. Maximum Vero cell yields in spinner flasks were higher from pH 7.1 at inoculation with daily readjustment to the same pH from 72 h onwards in cultures of either bead. However, cell growth in Cytodex-1 cultures was not dependent on pH readjustment which suggests the previous reports of cell yields in Cytodex-1 cultures initiated at pH 7.4-7.5 would not have been affected by a lack of pH readjustment (Clark and Hirtenstein, 1981, Gebb et al., 1984, Polastri et al., 1984 and Reiter et al., 1990). None of the previous reports with Vero cell growth discuss pH readjustment during the cultures. However, maximum cell yields were dependent on pH readjustment to pH 7.1 in Cultispher-G cultures. None of the previous reports in the literature using Cultispher-G cultures describe pH readjustment in spinner flasks which could mean the reported cell yields were under estimated.

Others have used HEPES buffer (15.00-25.00 mM) supplemented medium to improve pH control in Vero cell cultures (Mered et al., 1980, Clark and Hirtenstein, 1981, Lindskog et al., 1987 and Cinatl et al., 1992). However, the addition of HEPES buffer had no significant effects on Vero cell growth in most cultures in this study. Indeed, pH readjustment was still required in all cultures, especially Cultispher-G. These results show there was no advantage in using

HEPES buffer which also significantly increases the basal medium cost (>200%).

5.3.7 Dissolved Oxygen Tension

Introduction. The dissolved oxygen tension (DOT) could affect cell yields and growth rates.

Therefore, the optimal DOT for Vero cell growth in microcarrier cultures was determined in the Celligen bioreactor.

Experimental. The Vero cultures were grown in the Celligen bioreactor with various controlled DOT levels (10-100% of air saturation), as described above (5.3.5). Other parameters were controlled at the set-points above (5.3.5) with pH 7.1 (Cytodex-1) and 6.8 (Cultispher-G). The DOT was controlled at set-point by sparging compressed air, blended with pure O₂ (when required), to raise the DOT or by sparging N₂ to lower the DOT.

Results. Fig. 5.7 shows the maximum Vero cell yields in cultures of either bead at the various DOT set-points used (10-100%). Table 5.7 shows the cell growth rates and lag phases in cultures of either bead at the DOTs used. (1) Maximum cell yields (Fig. 5.7) in cultures of either bead were affected by the DOT set-points used. There were significantly higher (37%, $P < 0.01$) cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the DOTs used. (2) There were significantly higher ($P < 0.01$) maximum cell yields in Cytodex-1 cultures at 10-50% DOT compared to all the other values used. There was no significant difference in the ranking in Cultispher-G cultures, except the optimal range was wider (10%-75% DOT). The optimal range of DOT was 25-50%. (3) There were significantly higher cell growth rates (Table 5.7) in cultures of either bead at 10-75% DOT compared 100% DOT. (4) There was no significant difference in the lag phases (Table 5.7) in cultures of either bead with respect to any DOT used.

Discussion. Cells *in vivo* are exposed to oxygen concentrations ranging between 0-100% of the

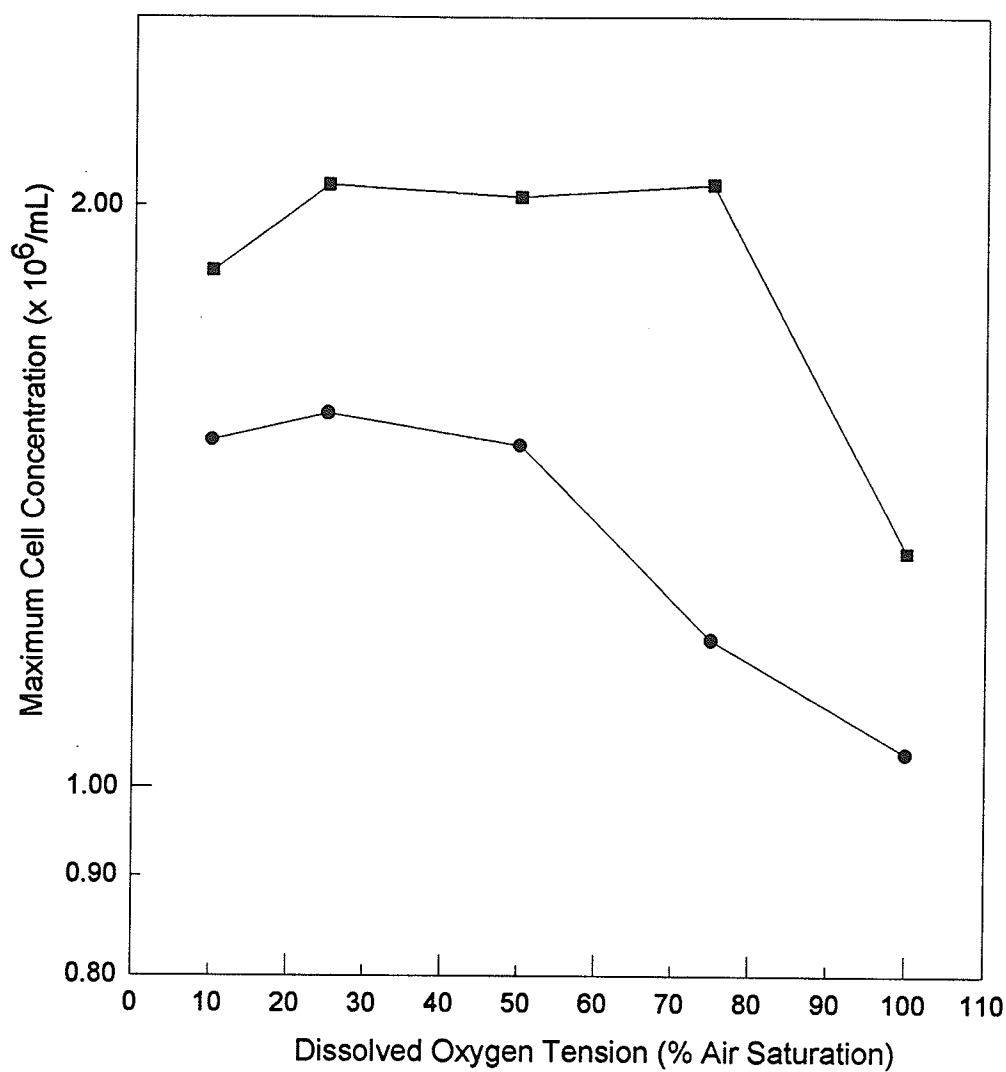


Fig. 5.7 - The effect of the **dissolved oxygen tension** on the maximum Vero cell yield in 1,300 mL Celligen bioreactor on microcarriers. The cells were inoculated at 0.05×10^6 cells/mL on either ● 1.00 g/L Cytodex-1 or ■ 1.70 g/L Cultispher-G in DMEM + 5.0% v/v sCS. The cells were grown at various DOT levels from 10–100% of air saturation. The other controls were at 37°C, pH 7.1 (Cytodex-1) and pH 6.8 (Cultispher-G), 40 rpm (Cytodex-1) or 40 rpm up to 72 h followed by 60 rpm (Cultispher-G) and 0.50 L/min gas flow rate. The points are means of $n=2$.

Table 5.7 - The effect of the **dissolved oxygen tension** on the **Vero cell growth rate and lag phase** in 1,300 mL Celligen bioreactor on **1.00 g/L Cytodex-1** or **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS and grown at various DOT levels from 10–100% of air saturation. The other controls were at 37°C, pH 7.1 (Cytodex-1) and pH 6.8 (Cultispher-G), 40 rpm (Cytodex-1) or 40 rpm up to 72 h followed by 60 rpm (Cultispher-G) and 0.50 L/min gas flow rate. The values are means of n=2.

Controlled DOT (% air saturation)	Vero Cell Growth Rate and Lag Phase at the Controlled DOT			
	Cytodex-1		Cultispher-G	
	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)
10	0.041 ± 0.003	3.00 ± 0.50	0.043 ± 0.003	3.00 ± 0.50
25	0.042 ± 0.003	3.00 ± 0.50	0.045 ± 0.004	3.00 ± 0.50
50	0.041 ± 0.003	3.00 ± 0.50	0.045 ± 0.004	3.00 ± 0.50
75	0.038 ± 0.003	4.50 ± 0.50	0.045 ± 0.004	4.50 ± 0.50
100	0.037 ± 0.003	4.50 ± 0.50	0.039 ± 0.003	4.50 ± 0.50

concentration in air. However, few reports in the literature have discussed the effects of varying oxygen concentrations (as DOT) on cell growth. Nahapetian et al. (1986) discussed the importance of DOT for Vero cell growth in refed spinner flasks and Qualitz et al. (1993) discussed the oxygen uptake rate of CHO cells in spinner flasks. Neither of these authors proved their DOT level as optimal. Previous reports have shown significantly lower (>50%) maximum yields of human fibroblast cells at <2% DOT (Graff and McCarty, 1957) and human diploid fibroblasts, CHO and human endothelial cells at >90% DOT (Krebs, 1950, van der Valk et al., 1985 and Grant et al., 1992).

The results in this study agree with lower cell yields at high DOTs. However, the maximum cell yield at 100% DOT was significantly higher (24%, $P<0.05$) in the Cultispher-G cultures suggesting the cells were protected from the adverse DOT in these beads compared to Cytodex-1. This could be explained by the cells growing in aggregates inside the Cultispher-G bead pores whereas they grow on the outer surface of Cytodex-1 as monolayers. Therefore, cell aggregates could limit the diffusion of high oxygen concentrations to lower cell layers. Another possible explanation is cell morphology differences between the beads, as the cells are flattened in Cytodex-1 cultures and have a high surface area to volume ratio whereas the cells could be more rounded in Cultispher-G cultures with a low surface area to volume ratio. This could affect the diffusion of oxygen into the cells.

It was impossible to preadapt the cells to the DOT levels used. This could explain why the growth rates at 100% DOT were high for 48 h before significantly reducing. Grant et al. (1992) previously showed a similar effect and found it was due to the growth stimulation of fibroblast growth factor (FGF). The FGF was unaffected after 24 h exposure to high DOTs retaining its stimulating effect. However, growth stimulation was completely removed after ≥ 48 h exposure to >90% DOT. The results in this study could be explained by this effect.

5.3.8 Temperature

Introduction. High or low temperature could reduce maximum cell yields and growth rates by affecting optimal enzyme activities. No one has previously determined the optimal temperature for Vero cell growth. Therefore, the optimal temperature for Vero cell growth in microcarrier cultures was determined in the Celligen bioreactor.

Experimental. The Vero cultures were grown in the Celligen bioreactor at various temperatures (between 35-39°C), as described above (5.3.5). All other parameters were controlled at the set-point values described above (5.3.7) and DOT was controlled at 50% of air saturation.

Results. Table 5.8 shows the maximum Vero cell yields, growth rates and lag phases at the various temperatures (35-39°C) used. (1) Maximum cell yields in cultures of either bead were affected by the temperatures used. (2) There were significantly higher maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the temperatures used. (3) There were significantly higher (23%, $P < 0.05$) maximum cell yields in cultures of either bead at 37°C and 39°C compared to 35°C. The optimal temperature for Vero cell growth was 37°C. (4) There were significantly higher cell growth rates (22%, $P < 0.05$) in cultures of either bead at 37-39°C. (5) There were significantly longer (100%, $P < 0.01$) lag phases (Table 5.8) in cultures of either bead at 35°C compared to 37 or 39°C.

Discussion. Mammalian cells are routinely cultured at the average human body temperature (37°C). However, previous CHO cell research showed maximum cell yields were reduced, the specific productivity rates were increased and the growth rate decreased with temperature stress at up to 42°C (Tsao et al., 1992). No one has previously shown the optimal temperature for Vero cell growth.

The maximum Vero cell yields were similar in cultures of either bead at 37-39°C but were

Table 5.8 - The effect of **controlled temperature** on the maximum Vero cell yields, growth rates and lag phases in 1,300 mL Celligen Bioreactor on **1.00 g/L Cytodex-1** or **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/bead in DMEM + 5.0% v/v sCS. The culture was controlled at temperatures of 35, 37 or 39°C. The cultures were stirred at 40 rpm continuously (Cytodex-1) and ramped to 60 rpm after 72 h in culture and controlled at pH 7.1 (Cytodex-1) or 6.8 (Cultispher-G), DOT at 50% of air saturation and gas flow rate at 0.50 L/min. The values are means of n=2.

Temperature (°C)	Maximum Cell Yield, Growth Rate and Lag Phase at the Stirrer Speed Used					
	Cytodex-1			Cultispher-G		
	Maximum Cell Yield ($\times 10^6$ cells/mL)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Maximum Cell Yield ($\times 10^6$ cells/mL)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
35	1.16 ± 0.07	0.024 ± 0.002	6.00 ± 1.00	1.17 ± 0.07	0.024 ± 0.002	6.00 ± 1.00
37	$1.41 \pm 0.10^*$	0.031 ± 0.003	3.00 ± 0.50	$2.05 \pm 0.14^*$	0.037 ± 0.003	3.00 ± 0.50
39	1.32 ± 0.09	0.030 ± 0.003	3.00 ± 0.50	2.00 ± 0.14	0.037 ± 0.003	3.00 ± 0.50

* - Highest cell yields.

reduced at 35°C. This suggested the cells could grow with no effects at higher temperatures compared to the normal. Vero cells are of simian origin and could be expected to have similar characteristics to human cells so it was not surprising the optimal growth temperature was 37°C. However, the equivalent cell growth at 39°C was surprising. This could be explained by the physiology of animal kidneys where there is an insulating fat layer to help maintain the organ temperature for detoxification functions.

5.3.9 Microcarrier Concentration

Introduction. Microcarriers offer highly increased surface areas compared to static culture flasks for cell growth. However, no-one has previously determined the optimal microcarrier concentrations for batch culture. Therefore, this study determined the effects of using various microcarrier concentrations on maximum Vero cell yields in batch cultures.

Experimental. The Vero cultures were inoculated with either (a) fixed cell concentration of 0.05×10^6 cells/mL or (b) fixed inoculum ratio of 10 cells/bead (Cytodex-1) and 30 cells/bead (Cultispher-G) on various concentrations of Cytodex-1 (0.50-5.00 g/L) or Cultispher-G (0.85-8.50 g/L) and grown in spinner flasks (5.3.3).

Results. Table 5.9 shows maximum Vero cell yields, time taken to reach maximum yield, cell growth rate and lag phases in cultures of either bead with either a fixed cell inoculum concentration (0.05×10^6 cells/mL) or a fixed cell/bead ratio (10 cells/bead in Cytodex-1 and 30 cells/bead in Cultispher-G). Table 5.10 shows maximum cell/bead loadings in these cultures. (1) Maximum cell yields in cultures of either bead were not affected by the microcarrier concentration or the cell inoculum used. (2) There were significantly higher (21%, $P < 0.05$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the microcarrier concentrations and cell inocula used. (3) There was no significant difference in

Table 5.9 - The effect of the **microcarrier concentration** on the maximum yield, time taken to maximum yield, growth rate and lag phase from either a **fixed inoculum Vero cell concentration (A)** or a **fixed cell/bead ratio (B)** in 100 mL spinner flasks. The cells were inoculated at either 0.05×10^6 cells/mL (either bead) or 10 cells/bead in Cytodex-1 (0.50-5.00 g/L) and 30 cells/bead in Cultispher-G (0.85-8.50 g/L) in DMEM + 5.0% v/v sCS. The cultures were stirred at 40 rpm either continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G) and readjusted daily to pH 7.4 with 2.00 M NaOH after 72 h. The values are means of $n=2$.

Table 5.9A) - Fixed cell inoculum concentration.

Conc. (g/L)	Maximum Cell Yield, Time Taken to Reach Maximum Yield, Growth Rate and Lag Phases at the Microcarrier Concentration Used			
Cytodex-1	Maximum Cell Yield ($\times 10^6$ /mL)	Time To Max. Yield (h)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
0.50	1.33 ± 0.09	144	0.033 ± 0.003	3.00 ± 0.50
1.00	$1.47 \pm 0.11^*$	144	0.034 ± 0.003	3.00 ± 0.50
2.00	1.42 ± 0.10	144	0.034 ± 0.003	6.00 ± 1.00
3.00	1.36 ± 0.09	144	0.033 ± 0.003	6.00 ± 1.00
5.00	1.26 ± 0.08	144	0.032 ± 0.003	6.00 ± 1.00
Cultispher-G				
0.85	1.67 ± 0.12	144	0.035 ± 0.003	3.00 ± 0.50
1.70	$1.77 \pm 0.13^*$	144	0.036 ± 0.003	3.00 ± 0.50
3.40	1.71 ± 0.12	144	0.036 ± 0.003	3.00 ± 0.50
5.10	1.68 ± 0.12	144	0.035 ± 0.003	6.00 ± 1.00
8.50	1.63 ± 0.12	144	0.035 ± 0.003	6.00 ± 1.00

Table 5.9B) - Fixed cell/bead inoculum ratio.

Conc. (g/L)	Maximum Cell Yield, Time Taken to Reach Maximum Yield, Growth Rate and Lag Phases at the Microcarrier Concentration Used			
Cytodex-1	Maximum Cell Yield ($\times 10^6$ /mL)	Time To Max. Yield (h)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
0.50	1.24 ± 0.08	168	0.034 ± 0.003	6.00 ± 1.00
1.00	1.47 ± 0.11	144	0.034 ± 0.003	3.00 ± 0.50
2.00	$1.49 \pm 0.10^*$	120	0.033 ± 0.003	3.00 ± 0.50
3.00	1.46 ± 0.10	120	0.023 ± 0.002	3.00 ± 0.50
5.00	1.32 ± 0.09	96	0.025 ± 0.002	3.00 ± 0.50
Cultispher-G				
0.85	1.50 ± 0.11	168	0.035 ± 0.003	6.00 ± 1.00
1.70	$1.71 \pm 0.12^*$	144	0.036 ± 0.003	3.00 ± 0.50
3.40	1.69 ± 0.12	120	0.034 ± 0.003	3.00 ± 0.50
5.10	1.66 ± 0.12	120	0.029 ± 0.003	4.50 ± 0.50
8.50	1.70 ± 0.12	72	0.040 ± 0.003	4.50 ± 0.50

* - Highest cell yield.

Table 5.10 - The effect of the **microcarrier concentration** and **Vero cell inoculum concentration** on maximum Vero cell/bead loadings in 100 mL spinner flasks on **Cytodex-1** and **Cultispher-G**. The cells were inoculated at either 0.05×10^6 cells/mL or at 10 (Cytodex-1) and 30 (Cultispher-G) cells/bead in DMEM + 5.0% v/v SCS. The cultures were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted to pH 7.4 daily after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Cytodex-1			Cultispher-G		
Conc. (g/L)	Maximum Cell/Bead Loading		Conc. (g/L)	Maximum Cell/Bead Loading	
	Fixed Cell Concentration	Fixed Cell/Bead Ratio		Fixed Cell Concentration	Fixed Cell/Bead Ratio
0.50	591	551	0.85	2,183	1,961
1.00	327	327	1.70	1,086	1,084
2.00	158	166	3.40	532	552
3.00	102	108	5.10	366	362
5.00	55	59	8.50	216	222

maximum cell yields (Table 5.9A-B)) in cultures of Cytodex-1 at 0.50-5.00 g/L and Cultispher-G at 0.85-8.50 g/L with respect to either cell inoculum concentration used. (4) There was no significant difference in the cell growth rates in cultures of either bead from fixed cell inoculum concentrations (Table 5.9A)) with respect to the microcarrier concentrations used. However, there were significantly slower growth rates from fixed cell/bead inoculum ratios (Table 5.9B)) in Cytodex-1 cultures at 3.00 and 5.00 g/L (42%, $P<0.01$) and in Cultispher-G cultures at 5.10 g/L (17%, $P<0.05$) compared to all other cultures. (5) There was no significant difference in the time taken to reach maximum cell yield in cultures of either bead from a fixed cell inoculum concentration (Table 5.9A)) with respect to all the microcarrier concentrations used. However, maximum cell yields from fixed cell/bead inoculum ratios (Table 5.9B)) were reached significantly earlier in Cytodex-1 cultures at 5.00 g/L (33%, $P<0.01$) and Cultispher-G cultures at 8.50 g/L (100%, $P<0.01$) compared to the controls (1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G). (6) There were significantly longer (100%, $P<0.01$) lag phases from fixed cell inoculum concentrations in cultures of Cytodex-1 at 3.00-5.00 g/L and Cultispher-G at 5.10-8.50 g/L. There were significantly longer (100%, $P<0.01$) lag phases from fixed cell/bead ratios (Table 5.10) in cultures at 0.50 g/L Cytodex-1 and 0.85 g/L Cultispher-G. (7) The optimal microcarrier concentrations for batch Vero cell growth were 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G.

Cell/Bead Loadings. (1) The maximum cell/bead loadings (Table 5.10) in cultures of either bead were affected by the microcarrier concentration used. (2) The maximum loadings were 591 cells/bead in 0.50 g/L Cytodex-1 cultures and 2,183 in 0.85 g/L Cultispher-G from a fixed cell inoculum concentration. (3) The maximum loadings were proportionally reduced as the bead concentration was increased and were significantly lower (90%, $P<0.01$) in cultures of Cytodex-1 at 5.00 g/L and Cultispher-G at 8.50 g/L with respect to either cell inoculum used. (4) Maximum cell/bead loadings were significantly higher (251%, $P<0.01$) in Cultispher-G cultures compared to Cytodex-1 with respect to all cell inoculum and microcarrier concentrations used.

Bead Occupancy. At maximum cell yield the whole bead surface was covered and the cells were multi-layered (> 2 cell depths) in 0.50 and 1.00 g/L Cytodex-1 cultures. At 2.00 and 3.00 g/L Cytodex-1 the cells covered all of the bead surface and were mono-layered. However, at 5.00 g/L the beads were not completely covered to a cell mono-layer. There were large cell-free areas (30% of the total surface area) on many beads and a significant proportion of empty beads (21%) in cultures from the fixed inoculum concentration (0.05×10^6 cells/mL).

At maximum cell yield in the 0.85 g/L Cultispher-G cultures there were a large proportion (40%) of beads showing red staining throughout their matrix. However, as the bead concentration was increased the proportions of such beads was reduced.

Discussion. Low Cytodex-1 microcarrier concentrations (1.00 g/L) have been shown to yield significantly (25%) lower maximum Vero cell yields compared to 3.00-5.00 g/L (Clark and Hirtenstein, 1981). Therefore, increasing the surface area by increasing the concentrations of Cytodex-1 was suggested to lead to improved cell yields. Hence, this could explain why others have used 3.00-5.00 g/L Cytodex-1 for FS-4, BEK and CHO cell growth (Hu et al., 1985, Cherry and Papoutsakis, 1988, Pons et al., 1992 and Qualitz et al., 1993) without any optimisation or further rationale. This approach could lead to exaggerated results without optimisation of some cell growth parameters. The macroporous microcarriers have a sponge-like matrix which significantly increases the surface area per bead for cell growth. Therefore, using these beads high concentrations could be unnecessary.

A T25 culture flask with 10 mL medium has a surface area of $2.5 \text{ cm}^2/\text{mL}$ of medium. These flasks produce maximum Vero cell yields of 1.90×10^6 cells/mL (4.3.1). Cytodex-1 beads at 1.00 g/L have a surface area of $4.4 \text{ cm}^2/\text{mL}$ of medium. These cultures produced 1.47×10^6 cells/mL in this study. Therefore, the lower yields in Cytodex-1 cultures could agree with Clark and Hirtenstein's (1981) previous conclusions. However, considering the significantly higher (76%, $P < 0.01$) surface area/mL in Cytodex-1 cultures compared to T25 flasks it is hard to believe low cell yields resulted from a surface area limitation. Furthermore, higher bead

concentrations could increase the bead to bead collisions ultimately leading to decreased cell yields, as shown previously with BEK cells (Cherry and Papoutsakis, 1988).

The results in this study show maximum Vero cell yields were unaffected at up to 5.00 g/L Cytodex-1 or 8.50 g/L Cultispher-G. The only advantage of the higher bead concentrations was in significantly reducing the time taken to reach the maximum cell yield when the cultures were inoculated at fixed cell/bead ratios. Therefore, the Cytodex-1 results disagree with those of Clark and Hirtenstein (1981). This could be explained by the semi-optimised conditions in the cultures in this study.

Significantly higher (20-40%, $P < 0.05$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 were consistently found in these studies. Indeed, the maximum cell yields in Cultispher-G cultures were similar to those in T25 culture flasks. The reduced cell yields in Cytodex-1 cultures could result from differences in nutrient utilisation or growth inhibition from the excreted products which were not analysed in this study. However, there have been previous reports of reduced murine 3T3 cell yields in Cytodex-1 cultures and this was found to result from a reduced secretion of an important adhesion protein, laminin (Bober et al., 1987 and Varani et al., 1990).

5.3.10 Bead to Bead Transfer of Cells

Introduction. The optimal inoculation of microcarrier cultures should lead to even cell/bead distributions with each cell attaching to one microcarrier and then growing to colonise that bead. Bead to bead cell transfer could allow complete utilisation of the bead surface area if the attachment phase resulted in sub-optimal distribution profiles or could remove the need for cell detachment from the beads prior to inoculation of larger cultures. Therefore, this study determined whether bead to bead transfer of Vero cells could occur in microcarrier cultures.

Experimental. The Vero cultures were grown in 100 mL spinner flasks, as described above

(5.3.3). The cultures were grown for 72 h prior to the addition of an extra 1.00 g/L Cytodex-1 or 1.70 g/L Cultispher-G. Every 24 h after this addition the cell attachment and bead aggregation were determined by removing 0.5 mL of the culture into a microfuge tube and staining the beads with neutral red dye (5.3.1). The beads were washed (x4) with D-PBS⁻ prior to viewing under an inverted microscope to assess bead aggregation and cell colonisation of empty beads.

Results. Fig. 5.8 shows Vero cell growth in cultures of either bead with and without the addition of fresh microcarriers after 72 h. (1) Vero cells were transferred between Cytodex-1 beads in growing cultures. Fresh (cell-free) beads were quickly attracted (≤ 24 h) to the originals and the cells then gradually colonised them. The transfer of cells between Cultispher-G beads was difficult to visually assess by the methods used due to the macroporous matrix. Hence, it was unclear whether any cell transfer had occurred. (2) There was no significant difference in the maximum bead aggregate size in Cytodex-1 (8 beads) or Cultispher-G (10-15 beads) cultures with or without the addition of fresh beads. (3) There was a significant increase (100%, $P < 0.01$) in the proportion of Cytodex-1 bead aggregates containing high numbers of beads (5-8 beads) in the cultures with the fresh beads added. These cultures also had a significant proportion (25%, $P < 0.01$) of cell-free beads at maximum cell yield. At maximum cell yield most (79%) of the cell containing beads had a complete monolayer. Neutral red staining showed that, prior to the addition of fresh beads, there was a significant proportion (26%, $P < 0.01$) of cell-free Cultispher-G beads. However, the proportion of such beads significantly increased (300%, $P < 0.01$) following the bead addition. This proportion persisted throughout the remainder of the culture and suggested there was little or no association of the fresh and original beads. Hence, there was possibly no Cultispher-G bead to bead cell transfer. (4) There was no significant difference in the maximum cell yields (Fig. 5.8) in cultures of either bead with or without fresh beads added.

Discussion. Previous results in the literature have reported the formation of Cytodex-1 and 3 bead aggregates in BEK and CHO cell cultures (Cherry and Papoutsakis, 1989 and Borys and

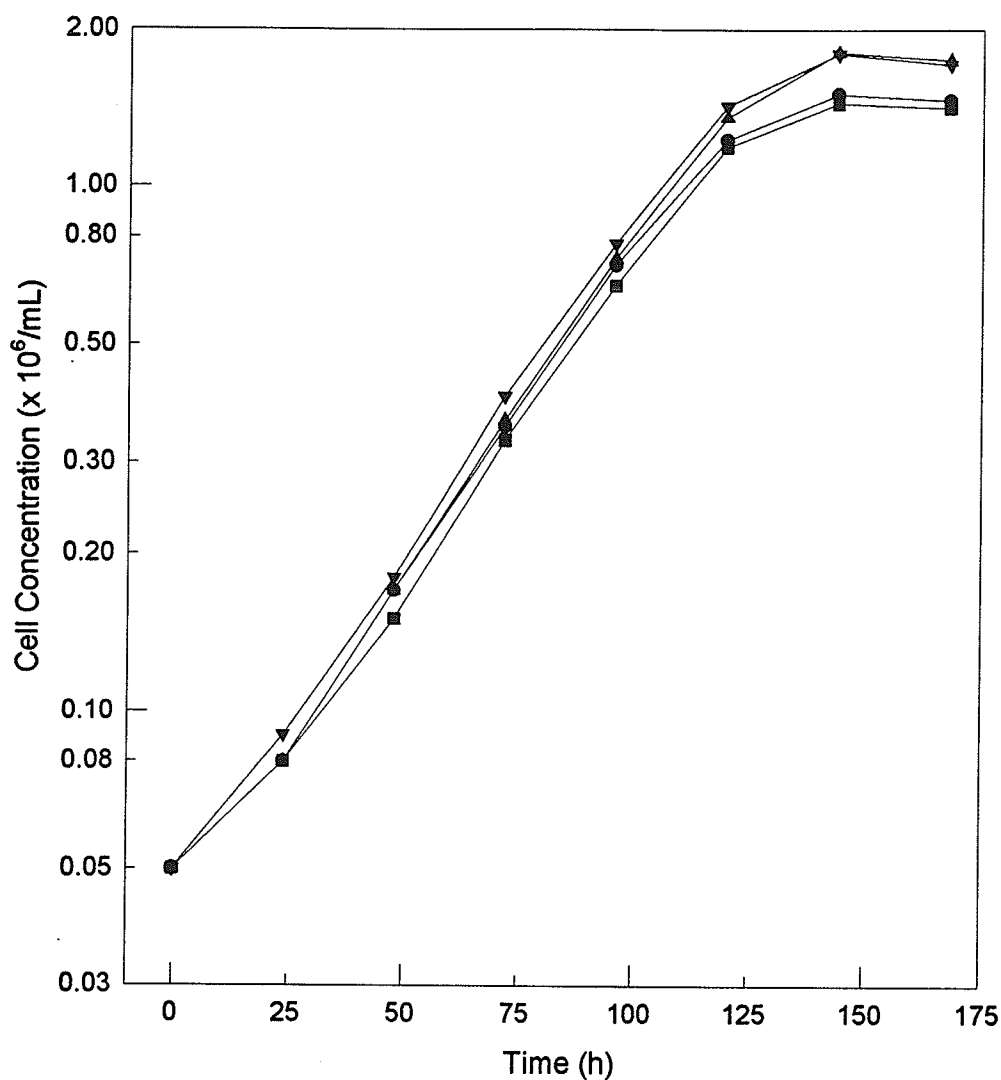


Fig. 5.8 - The effect of adding fresh microcarriers to growing cultures of Vero cells in 100 mL spinner flasks. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS. The cultures were stirred at 40 rpm either continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The cultures were at either ● 1.00 g/L Cytodex-1 throughout, ■ 2.00 g/L Cytodex-1 from 72 h, ▲ 1.70 g/L Cultispher-G throughout or ▼ 3.40 g/L Cultispher-G from 72 h. The flasks were readjusted to pH 7.4 after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

Papoutsakis, 1992). This could occur from either serum protein interactions on the bead surfaces or more likely from cell to cell and cell to bead attractions between beads. The Cytodex-1 aggregation was also reported to allow the transfer of CHO cells between beads (Borys and Papoutsakis, 1992). The bead to bead transfer of CHO cells was also reported between Cultispher-G beads (Foran et al., 1991). However, Cytodex-1 bead aggregation was shown to yield significantly lower maximum Vero and MRC-5 cell yields (Clark and Hirtenstein, 1981). Thus, bead to bead transfer of cells is another process with apparently contradictory reports in the literature although, this could be explained by cell line dependent differences. Clark and Hirtenstein (1981) reported bead aggregates in their cultures, probably from cell to cell and cell to bead attractions suggesting cells could have been transferred which would contradict their own statements.

Vero cells could transfer between Cytodex-1 beads. Indeed, bead aggregation occurred in all cultures during growth and cells transferred between the aggregated beads, filling the void spaces at maximum cell yield. In cultures with fresh beads added most beads were covered with a monolayer at maximum cell yield but this transfer did not increase maximum cell yields.

The bead to bead transfer process in Cultispher-G beads could not be assessed by the methods used in this study. If cells were transferred they did not increase maximum cell yields. The fact the Cultispher-G bead aggregate sizes did not increase after the fresh bead addition indicates there was little or no further bead aggregation around the originals. Hence, there was probably no cell transfer. This would disagree with the CHO transfer described by Foran et al. (1991). This could also be explained by a cell line dependent difference. The failure of cell transfer in Cultispher-G beads could be expected as the cells were growing into the inner pores of the beads and not on their outer surfaces.

The transfer process could be important to increasing cell yields in daily refed and perfusion cultures or larger process vessels, as the addition of fresh beads would be a good method of increasing the surface area for cell growth without having to detach the cells from the original beads. This would remove a process step which could result in either reduced growth or viability

of the cells after detachment, as discussed previously (3.4).

5.3.11 Cell/Bead Inoculum Ratio

Introduction. High cell inoculum concentrations (therefore, high cell/bead ratios) could result in increased maximum yields through improved nutrient and/or surface utilisation. Therefore, this study determined the effects of the Vero cell/bead inoculum ratio on maximum cell yields in microcarrier cultures.

Experimental. The Vero cultures were inoculated at various cell/bead ratios in cultures of 1.00 g/L Cytodex-1 (between 5-200 cells/bead) and 1.70 g/L Cultispher-G (between 15-600 cells/bead) and grown in spinner flasks (5.3.3). The multiplication indices were calculated as the number of cell population doublings (cell generations) which occurred between the initial and maximum cell yields in the cultures.

Results. Table 5.11 shows maximum Vero cell yields, growth rates, lag phases and multiplication indices in cultures of either bead from the various inoculum ratios (5-200 in Cytodex-1 and 15-600 cells/bead in Cultispher-G) used. (1) Maximum cell yields in cultures of either bead were affected by the cell inoculum ratio used. (2) There were significantly higher (15%, $P=0.05$) cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the cell inoculum ratios used. (3) The maximum cell yields (Table 5.11A-B)) were significantly higher ($P<0.05$) in Cytodex-1 cultures from 200 cells/bead and in Cultispher-G cultures from 600 cells/bead compared to all other inoculum concentrations used. (4) The multiplication index (Table 5.11A-B)) in cultures of either bead was proportionally reduced with the increasing cell/bead ratio used at inoculation. There were significantly higher ($P<0.01$) multiplication indices from the lowest inoculum ratios in cultures of either bead. (5) The growth rates were proportionally reduced with the increased inoculum ratio. There were significantly lower (45%,

Table 5.11 - The effect of the inoculum cell/bead ratio on the maximum Vero cell yield, growth rate, lag phase and cell multiplication index in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A) and 1.70 g/L Cultispher-G (B). The cells were inoculated at various cell/bead ratios (5-200 cells/bead in Cytodex-1 and 15-600 cells/bead in Cultispher-G) in DMEM + 5.0% v/v SCS. Cultures were stirred at 40 rpm either continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily after 72 h in culture to pH 7.4 with 2.00 M NaOH. The values are means of n=2.

Table 5.11A) - Cytodex-1.

Inoculum Cell to Bead Ratio (Cells/bead)	Maximum Cell Concentration ($\times 10^6/\text{mL}$)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Cell Multiplication Index (Generations)
5	1.09 ± 0.08	0.033 ± 0.003	4.50 ± 0.50	5.18
10	1.44 ± 0.10	0.034 ± 0.003	4.50 ± 0.50	4.85
15	1.56 ± 0.10	0.034 ± 0.003	4.50 ± 0.50	3.95
30	1.48 ± 0.11	0.031 ± 0.003	6.00 ± 1.00	3.31
50	1.52 ± 0.12	0.029 ± 0.003	6.00 ± 1.00	2.61
100	1.62 ± 0.11	0.028 ± 0.002	9.00 ± 1.00	1.70
160	2.07 ± 0.14	0.026 ± 0.002	12.00 ± 1.00	1.37
200	$2.27 \pm 0.15^*$	0.022 ± 0.002	12.00 ± 1.00	1.18

Table 5.11B) - Cultispher-G.

Inoculum Cell to Bead Ratio (Cells/bead)	Maximum Cell Concentration ($\times 10^6/\text{mL}$)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Cell Multiplication Index (Generations)
15	1.74 ± 0.09	0.036 ± 0.003	4.50 ± 0.50	5.86
30	1.68 ± 0.10	0.035 ± 0.003	4.50 ± 0.50	5.07
60	1.71 ± 0.11	0.035 ± 0.003	6.00 ± 0.50	4.10
90	1.75 ± 0.09	0.033 ± 0.003	6.00 ± 1.00	3.55
150	1.80 ± 0.12	0.031 ± 0.003	9.00 ± 1.00	2.85
300	1.79 ± 0.10	0.030 ± 0.002	9.00 ± 1.00	1.84
480	2.08 ± 0.12	0.026 ± 0.002	12.00 ± 1.00	1.38
600	$2.49 \pm 0.12^*$	0.022 ± 0.002	12.00 ± 1.00	1.23

* - Highest cell yield.

$P < 0.01$) cell growth rates in cultures of Cytodex-1 from 200 cells/bead and Cultispher-G from 600 cells/bead compared to the controls (10 cells/bead in Cytodex-1 and 30 cells/bead in Cultispher-G). (6) Lag phases were proportionally lengthened with the increased cell inoculum ratio used. There were significantly longer (300%, $P < 0.01$) lag phases in cultures of Cytodex-1 from 200 cells/bead and Cultispher-G from 600 cells/bead compared to the controls.

Free Cells. Free cells (25% of the total cells) were found in the supernatants after 24 h in cultures of either bead from high inoculum ratios. These cells disappeared within 48 h in culture. Free cells (15%) were also found 24 h after the maximum cell yield was reached in these cultures. However, the time taken for free cells to appear after maximum cell yield was proportionally shorter with the increased inoculum ratio (48 h from 10-100 and 30-300 cells/bead and 96 h from 5 and 15 cells/bead in Cytodex-1 and Cultispher-G cultures, respectively).

Discussion. High inoculum ratios (>100 cells/bead) were shown to produce significantly increased (27%) maximum Vero and FS-4 cell yields in Cytodex-1 cultures (Clark et al., 1980 and Hu et al., 1985). The maximum Vero cell yields in this study were also significantly higher in Cytodex-1 cultures from high inoculum ratios which agrees with the previous authors. Indeed, high ratios also produced significantly higher cell yields in Cultispher-G cultures as well. However, although the maximum cell yields were increased this led to significantly reduced multiplication indices which showed there were fewer cell generations in these cultures. This effect could result from the acidic pH produced within 48 h in culture which could not be controlled by one daily readjustment. This pH effect was discussed above (5.3.4-5). The effects of controlled pH on high inoculum cell ratio cultures were not tested in the bioreactor.

Cell Detachment. Vero cells were detached earlier in cultures of either microcarrier from the higher inoculum ratios. No one has previously reported this effect. The early cell loss from the substrata could be disadvantageous to virus infection processes, as the point of infection would

become critical to prevent reduced virus titres.

Cell Growth Rates. The significantly reduced cell growth rates from high inoculum ratios were a major disadvantage. These could result from the number of cells/bead at inoculation affecting the attachment process, as free cells were present in these cultures after the 24 h attachment period. The high inoculum cultures could also consume the nutrients faster resulting in earlier nutrient limitation and hence, fewer cell population doublings.

5.3.12 Precoated Microcarriers

Introduction. Adherent cell attachment to substrata requires the mediation of serum proteins and metal ions which attach to the substrata and cell surface receptors. The proteins could also be required for cell division. Thus, precoating the microcarriers could affect the rate of cell attachment. Therefore, this study determined the effects of precoating the microcarriers with various compounds on Vero cell attachment and growth.

Experimental. The microcarriers were precoated (18 h) at 20°C with one of the following, either (i) sCS, (ii) DMEM + 5.0% v/v sCS, (iii) DMEM + 10 g/L bovine serum albumin (BSA) (Fraction V) or (iv) were uncoated. The cells were inoculated in either (a) DMEM + 5.0% v/v sCS (control) or (b) serum-free DMEM. The Vero cultures were grown in spinner flasks, as described above (5.3.1). Cultures attached with serum-free DMEM were made to 5.0% v/v sCS after 12 or 24 h of attachment.

Results. Table 5.12 shows maximum Vero cell yields in cultures of either bead with and without precoating. (1) There were significantly higher (16%, $P < 0.05$) cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the precoatings and media types used. (2) There was no significant difference in the maximum cell yields in cultures of either precoated or uncoated

Table 5.12 - The effect of **precoating the microcarriers** prior to use on the maximum Vero **cell yield** and **growth rate** in 100 mL spinner flasks on **1.00 g/L Cytodex-1** or **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS or in serum-free DMEM for 12-24 h then with 5.0% v/v sCS. The beads were either uncoated or were precoated with sCS, DMEM + 5.0% v/v sCS or DMEM + 10 g/L BSA for 18 h. Cultures were stirred at 40 rpm continuously for Cytodex-1 and intermittently for the first 24 h then continuously and ramped to 60 rpm after 72 h for Cultispher-G. The flasks were readjusted daily after 72 h in culture to pH 7.4 with 2.00 M NaOH. The values are means of n=2.

Precoating	Maximum Cell Concentration ($\times 10^6$ /mL)					
	5.0% v/v sCS At 0 h		5.0% v/v sCS After 12 h		5.0% v/v sCS After 24 h	
	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G
None	1.51 ± 0.11	1.65 ± 0.12	1.25 ± 0.08	1.45 ± 0.11	1.21 ± 0.07	1.35 ± 0.09
sCS	1.53 ± 0.13	1.73 ± 0.11	1.43 ± 0.11	1.67 ± 0.12	$1.38 \pm 0.08^*$	1.64 ± 0.12
DMEM + 5.0% v/v sCS	$1.58 \pm 0.12^*$	$1.74 \pm 0.12^*$	$1.48 \pm 0.10^*$	$1.70 \pm 0.11^*$	$1.38 \pm 0.08^*$	$1.73 \pm 0.11^*$
DMEM + 10 g/L BSA	1.53 ± 0.11	1.68 ± 0.12	1.33 ± 0.09	1.57 ± 0.11	1.12 ± 0.07	1.47 ± 0.10
	Specific Cell Growth Rate (h^{-1})					
	5.0% v/v sCS At 0 h		5.0% v/v sCS After 12 h		5.0% v/v sCS After 24 h	
	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G
None	0.033 ± 0.003	0.034 ± 0.003	0.031 ± 0.003	0.034 ± 0.003	0.033 ± 0.003	0.034 ± 0.003
sCS	0.031 ± 0.003	0.040 ± 0.003	0.033 ± 0.003	0.037 ± 0.003	0.035 ± 0.003	0.036 ± 0.003
DMEM + 5.0% v/v sCS	0.032 ± 0.003	0.039 ± 0.003	0.032 ± 0.003	0.036 ± 0.003	0.035 ± 0.003	0.037 ± 0.003
DMEM + 10 g/L BSA	0.034 ± 0.003	0.042 ± 0.003	0.030 ± 0.003	0.032 ± 0.003	0.035 ± 0.003	0.034 ± 0.003
	Lag Phase (h)					
	5.0% v/v sCS At 0 h		5.0% v/v sCS After 12 h		5.0% v/v sCS After 24 h	
	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G
None	6.00 ± 1.00	6.00 ± 1.00	24.00 ± 2.00	21.00 ± 2.00	24.00 ± 2.00	24.00 ± 2.00
sCS	4.50 ± 0.50	4.50 ± 0.50	15.00 ± 1.50	18.00 ± 2.00	21.00 ± 2.00	18.00 ± 2.00
DMEM + 5.0% v/v sCS	4.50 ± 0.50	4.50 ± 0.50	15.00 ± 1.50	15.00 ± 1.50	15.00 ± 1.50	18.00 ± 2.00
DMEM + 10 g/L BSA	9.00 ± 1.00	6.00 ± 1.00	21.00 ± 2.00	24.00 ± 2.00	24.00 ± 0.00	24.00 ± 0.00

* - Highest cell yield.

beads following attachment in DMEM + 5.0% v/v sCS. (3) There were significantly higher (16%, $P=0.05$) cell yields in cultures of serum precoated beads of either type compared to uncoated or BSA precoated beads following attachment in serum-free DMEM for 12 or 24 h. (4) There were significantly higher (17% and 23%, $P<0.05$) cell yields in cultures uncoated beads following attachment in DMEM + 5.0% v/v sCS compared to serum-free DMEM for 12 or 24 h, respectively. (5) There was no significant difference in the cell growth rates following cell attachment with respect to either precoated or uncoated beads and in all media used. (6) There were significantly longer (39%, $P<0.01$) lag phases in cultures of either uncoated or BSA coated beads compared to sCS or DMEM + 5.0% v/v sCS coated beads following cell attachment in all the media used. There were significantly longer (256%, $P<0.01$) lag phases in cultures of precoated and uncoated beads following attachment in serum-free DMEM (12 or 24 h) compared to DMEM + 5.0% v/v sCS.

Discussion. Adherent cell attachment to substrata is mediated by the serum proteins fibronectin and vitronectin (Knox and Griffiths, 1980, Hayman et al., 1985, Underwood and Bennett, 1989, Ruoslahti and Pierschbacher, 1987 and Ruoslahti, 1991) and metal ions such as Ca^{2+} (Curtis and McMurray, 1986). The serum proteins also mediate cell division (Ruoslahti and Pierschbacher, 1987). Previous studies showed precoating surfaces with serum proteins improved neuroblastoma cell attachment and growth (Bottenstein and Sato, 1980). The plating efficiency has been shown to be important for Vero, MRC-5 (Gebb et al., 1984) and FS-4 (Hu et al., 1985) cell attachment and growth in Cytodex-1 cultures. Others have investigated the CHO and Vero cell attachment kinetics and cell yields in cultures of Cytodex-1 and 2 precoated with serum and BSA (Kim et al., 1992 and Mukhopadhyay et al., 1994). The attachment factors (e.g. fibronectin and vitronectin) are known (Knox and Griffiths, 1980, Hayman et al., 1985, Underwood and Bennett, 1989, Ruoslahti and Pierschbacher, 1987 and Ruoslahti, 1991) to attach to microcarriers and other substrata rapidly (<1 h). However, these factors can be competitively replaced or blocked by larger serum proteins such as BSA (the Vroman effect)

over a longer time (>6 h). If or when such blocking occurs, cell attachment could be blocked or the attachment rate slowed significantly (>100%), as was shown with Vero cells (Mukhopadhyay et al., 1993). Kiremitci et al. (1989) suggested the serum concentration in the medium governed the number of attachment sites present on the beads.

The results in this study showed no differences in maximum Vero cell yields in cultures of precoated or uncoated beads when attached in DMEM + 5.0% v/v sCS. Thus, the plating efficiency did not affect maximum cell yields. Indeed, neither bead type required precoating for Vero cell growth after attachment in DMEM + 5.0% v/v sCS. Hence, these results contradict the Vroman effect.

Ng (1995) previously showed Vero cells attached significantly faster (100%) to uncoated Cytodex-1 and to serum precoated Cultispher-G beads in serum-free medium. Therefore, this authors Cytodex-1 results also disagree with the Vroman effect. This authors Cultispher-G bead results suggest serum factors were required for cell attachment which agrees with the Vroman effect. However, an alternative explanation of this attachment in DMEM + 5.0% v/v sCS could be that serum proteins increased cell aggregation rates which directly competed with attachment to the microcarriers. This would contradict the Vroman effect, as high molecular weight serum proteins would then have no effect on attachment.

Lag Phases. There were significantly longer lag phases with uncoated and BSA coated beads compared to sCS and DMEM + 5.0% v/v sCS precoated beads in all Vero cultures. However, maximum cell yields in cultures of uncoated and BSA coated beads were unaffected following attachment in DMEM + 5.0% v/v sCS but were significantly lower in serum-free DMEM. Hence, the initial cell attachment was only important to the subsequent initiation of growth. The similar cell yields previously observed (5.3.1) in sub-optimally and optimally attached Cultispher-G cultures support this statement. Therefore, the serum factors were required more for cell growth rather than for cell attachment.

The long lag phases in cultures of uncoated and BSA precoated beads attached in serum-

free DMEM suggests the cells could have exhausted their internal and/or external pools of serum dependent growth nutrients during the serum-free attachment period. These nutrients were only replaced after 12 or 24 h, hence, the lag phases of these cultures. The cultures with serum precoated beads had significantly shorter lag phases suggesting the nutrients were attached to the microcarriers and therefore, available to the cells following attachment. These components could include iron saturated transferrin, growth factors such as epidermal, fibroblastic or platelet derived growth factor or the adhesion proteins (Sasger, 1975, Barnes et al., 1984, Fujiwara and Pollard, 1976 and Voet and Voet, 1990). However, bead precoating was unnecessary as the maximum cell yields were similar in all cultures.

5.3.13 Spinner Flask Volume

Introduction. Process scale-up for any cell line requires the use of larger culture vessels. Therefore, this study determined whether the working volume of the spinner flasks used in the experiments above affected maximum Vero cell yields in microcarrier cultures.

Experimental. The Vero cultures were grown in spinner flasks of various working volumes (between 100-500 mL), as described above (5.3.1). Cultures of either bead were stirred at (i) 40 rpm throughout (following intermittent stirring for 24 h for cell attachment to Cultispher-G) or (ii) at 40 rpm ramped to 60 rpm after 72 h.

Results. Table 5.13 shows maximum Vero cell yields in the various sized spinner flasks (100-500 mL) used. (1) Maximum cell yields in cultures of either bead were affected by the spinner flask volume and stirrer speed used. (2) There were significantly higher (23%, $P < 0.05$) cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the flask sizes and stirrer speeds used. (3) There were significantly higher ($P = 0.05$) cell yields in 100 mL working volume spinner flasks compared to all other volumes with respect to either stirrer speed used.

Table 5.13 - The effect of the spinner flask working volume on maximum Vero cell yield, growth rate and lag phase on 1.00 g/L Cytodex-1 or 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL and grown in 100, 250 and 500 mL working volume spinner flasks in DMEM + 5.0% v/v SCS. Cultures were stirred at 40 rpm continuously or intermittently for 24 h and were then stirred at either 40 rpm throughout or ramped to 60 rpm from 72 h. The flasks were readjusted daily to pH 7.4 after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Spinner Flask Volume (mL)	Maximum Cell Concentration			
	(x 10^6 /mL)			
	Cytodex-1 (40 rpm)	Cytodex-1 (40-60 rpm)	Cultispher-G (40 rpm)	Cultispher-G (40-60 rpm)
100	$1.46 \pm 0.11^*$	$1.44 \pm 0.11^*$	$1.75 \pm 0.12^*$	$1.78 \pm 0.12^*$
250	1.17 ± 0.08	1.38 ± 0.09	1.45 ± 0.11	1.72 ± 0.12
500	1.00 ± 0.06	1.22 ± 0.08	1.22 ± 0.08	1.57 ± 0.13
	Specific Cell Growth Rate			
	(h ⁻¹)			
	Cytodex-1 (40 rpm)	Cytodex-1 (40-60 rpm)	Cultispher-G (40 rpm)	Cultispher-G (40-60 rpm)
100	0.033 ± 0.003	0.033 ± 0.003	0.034 ± 0.003	0.034 ± 0.003
250	0.032 ± 0.003	0.033 ± 0.003	0.034 ± 0.003	0.034 ± 0.003
500	0.033 ± 0.003	0.032 ± 0.003	0.033 ± 0.003	0.034 ± 0.003
	Lag Phase			
	(h)			
	Cytodex-1 (40 rpm)	Cytodex-1 (40-60 rpm)	Cultispher-G (40 rpm)	Cultispher-G (40-60 rpm)
100	6.00 ± 1.00	6.00 ± 1.00	6.00 ± 1.00	6.00 ± 1.00
250	6.00 ± 1.00	6.00 ± 1.00	6.00 ± 1.00	6.00 ± 1.00
500	6.00 ± 1.00	6.00 ± 1.00	6.00 ± 1.00	6.00 ± 1.00

* - Highest cell yields.

(4) There was no significant difference in the maximum cell yields in cultures of either bead in 100 mL spinner flasks with respect to either stirrer speed used. However, maximum cell yields were significantly higher (22%, $P < 0.05$) in cultures of either bead in 250 and 500 mL spinner flasks at 40 rpm ramped to 60 rpm compared to 40 rpm throughout. Therefore, the optimal spinner flask working volume was 100 mL. (5) There was no significant difference in the cell growth rates or lag phases with respect to any spinner flask volume used.

Discussion. The 100 mL working volume spinner flasks have been the most widely used in previous microcarrier research (Mered et al., 1980, Butler and Thilly, 1982, Nahapetian et al., 1986, Altankov et al., 1991 and Kim et al., 1992). Others have used larger volume flasks such as 150 mL (du Laney et al., 1992), 500 mL (Clark et al., 1980) and 500-5,000 mL (Clark and Hirtenstein, 1981). However, only Clark and Hirtenstein (1981) showed similar Vero and MRC-5 cell yields from all the spinner flask volumes they used.

The results in this study agree with 100 mL working volume spinner flasks being optimal. Maximum cell yields were significantly lower in cultures of either bead in 250 and 500 mL flasks compared to 100 mL flasks but these yields could be significantly increased by using the ramped stirring speeds (40-60 rpm). These results suggest the larger flasks failed to supply the cells with an important nutrient for growth which could be explained by the oxygen supply, as the surface movement was greatly reduced in the 500 mL compared to the 100 mL flasks. The scale-up of spinner flasks is more complex compared to bioreactors due to the lack of parameter control and through gas supply problems. Indeed, gas is only supplied by surface movement and the entrainment of gases could be affected by several spinner flask design ratio differences such as the depth to volume of the liquid, the paddle blade size and the surface area to volume ratio.

Table 5.14 shows the differences in dimensions between the Bellco 100, 250 and 500 mL flasks used in these experiments. The major differences between the flasks were the liquid depth to volume, the paddle blade sizes, the paddle blade to vessel diameter and the surface area to volume ratio. The important parameters are suggested to be differences in the paddle

Table 5.14 - Differences in the major dimensions between the 100, 250 and 500 mL working volume Belco spinner flasks.

Spinner Flask Dimension	Spinner Flask Working Volume (mL)		
	100	250	500
Liquid Depth	4.70 cm	5.50 cm	6.70 cm
Liquid Depth to Flask Volume Ratio	0.047 cm/mL	0.022 cm/mL	0.013 cm/mL
Paddle Blade Height	2.60 cm	2.60 cm	2.60 cm
Paddle Blade Diameter	4.50 cm	5.20 cm	6.50 cm
Flask Diameter	6.20 cm	8.00 cm	10.50 cm
Paddle Blade to Flask Diameter Ratio	0.740	0.650	0.620
Surface Area to Flask Volume Ratio	0.302	0.201	0.173

blade dimensions and the surface area to volume ratios. The 100 and 250 mL flasks used the same sized paddle blades. However, the blade diameter came within 0.5 cm of the 100 mL vessel inner wall and successful culturing was impossible without a size reduction, as the cells could not attach to the beads. These blades were reduced in diameter (0.6-0.8 cm) to remove this problem. The paddle to vessel diameter was also reduced as the flask volume increased, explaining the reduced surface movement in the 500 mL flasks. Thus, the 500 mL flasks could require increased stirrer speeds to achieve similar oxygen transfers. The increased maximum cell yields in speed ramped flasks confirm this statement. The liquid surface area to volume ratio also decreased as the flask volume increased which could give reduced oxygen entrainment. These differences could reduce the flask $k_L a$ by 20-30% resulting in low maximum cell yields.

5.4 Conclusions

The purpose of this study was to define the optimal physical conditions for the batch growth of Vero cells in cultures of Cytodex-1 and Cultispher-G in spinner flasks and Celligen bioreactors. Many of the results contradict those of previous reports in the literature and showed many differences between the optimal parameters for each bead type and specifically that:

- **no precoating** of the microcarriers was required for Vero cell attachment and growth to high concentrations.
- **the optimum microcarrier concentrations** were 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. Higher bead concentrations did not increase the maximum cell yields.
- **the optimum stirrer mode for cell attachment** was continuous stirring for Cytodex-1 and intermittent stirring, with a period of 3 min on/30 min off for 24 h, for Cultispher-G.
- **the optimum stirring speed for cell attachment** on either bead was 40 rpm.
- **the optimum stirring speed for cell growth** on either bead was 40 rpm until 72 h then ramped to 60 rpm. This decreased the bead aggregate sizes (25%) without reducing the maximum cell yields. Vero cell aggregates, produced with 40 rpm continuous stirring, attached

by entrapment in Cultispher-G cultures and produced similar maximum yields as optimally attached cells. The cells were protected in Cultispher-G cultures from the shear effects of high speeds, following optimal cell attachment.

- **the optimum pH** for cell growth was pH 7.1 in Cytodex-1 and pH 6.8 in Cultispher-G cultures.

- **the optimum DOT** was 50% of air saturation in cultures of either bead. However, similar maximum cell yields were produced in the DOT range of 10-50%. Ramping the stirrer speed above 60 rpm in spinner flasks, to increase surface entrainment of oxygen, was only possible with cultures of Cultispher-G beads without reducing the maximum cell yield.

- **the optimum temperature** for cell growth was 37°C for cultures of either bead type. A similar maximum cell yield was produced at 39°C. However, 35°C reduced the maximum cell yields and growth rates in cultures of either bead.

- **the optimum inoculum ratio** was in the range of 30-50 (Cytodex-1) and 90-150 (Cultispher-G) cells/bead or at a cell concentration of $0.15-0.25 \times 10^6$ cells/mL in cultures of either bead. This was based on total growth time, multiplication index and maximum cell yield. The maximum cell yields were significantly increased from high inoculum ratios (38% from 200 cells/bead on Cytodex-1 and 50% from 600 cells/bead on Cultispher-G). However, such increased maximum cell yields were outweighed by the time taken to generate the inoculum, the volume of media required and the reduced multiplication index.

- **the optimal spinner flask volume** for cell growth was 100 mL working volume for cultures of either bead. The maximum cell yield in Cytodex-1 cultures was significantly lower (24%) in larger working volume spinner flasks (500 mL) continuously stirred at 40 rpm. The maximum cell yield could be increased by ramping the stirrer speed to 60 rpm after 72 h in culture but was still significantly lower compared to the 100 mL flasks (17%).

- **the maximum batch cell yields** were significantly higher (26%) in Cultispher-G cultures. This could reflect a more efficient nutrient utilisation or increased surface area for growth in Cultispher-G beads or an altered cell laminin secretion in Cytodex-1 cultures.

Chapter 6. Optimisation of Growth Medium for Vero Cell Microcarrier Cultures.

1. Major Nutrients.

6. Optimisation of Growth Medium for Vero Cell Microcarrier Cultures. 1. Major Nutrients.

6.1 Introduction

Eagle (1955) formulated the first complete minimal basal medium, Eagle's Minimal Essential Medium (EMEM). Others have modified this medium to produce the common basal media such as Dulbecco's Modified Eagle's Medium (DMEM) (Dulbecco and Freeman, 1959) or Roswell Park Memorial Institute medium (RPMI 1640) (Moore et al., 1967). However, the effects of using different basal media on the growth of cell lines has rarely been reported in the literature.

The African green monkey kidney (Vero) cell bank in the American Type Culture Collection (ATCC) is cultured in basal Medium 199 (M199) (Morgan et al., 1950) supplemented with 10.0% v/v foetal bovine serum (FBS). M199 has been used in studies on Vero cell growth kinetics (Chotteau and Bastin, 1994). However, many other basal medium formulations have also been used, supplemented with many different bovine serum types and concentrations for Vero cell growth. Table 6.1 shows the media and serum concentrations used for Vero cell research in the previous literature. The media differences mean the concentrations of the major nutrients (glucose and glutamine) have also varied. For example Gebb et al. (1984) and Polastri et al. (1984) used DMEM (25.00 mM glucose and 4.00 mM glutamine), Hassell and Butler (1990) used Glasgow's Modified Eagle's Medium (GMEM) (25.00 mM glucose and with either glutamine or glutamate at 4.00 mM), Lindskog et al. (1987) used DMEM (25.00 mM glucose and 2.00 mM glutamine), Reiter et al. (1990) used DMEM (5.60 mM glucose and 2.50 mM glutamine) and Mendonca et al. (1993) used L-15 (with 5.00 mM galactose and 2.00 mM glutamine and without glucose) medium. The maximum cell yields in these media have varied greatly from 0.39×10^6 cells/mL in MEM (White and Ades, 1990) up to 2.00×10^6 cells/mL in DMEM (Polastri et al., 1984 and Reiter et al., 1990). The lower cell yields produced with MEM could have been caused by either nutrient limitations or inhibition of uptake mechanisms by components only present in this medium. Clark and Hirtenstein (1981) showed DMEM was the preferable basal medium for Vero cells.

Table 6.1 - The basal medium formulations, serum types and serum concentrations previously used for Vero cell culture.

Serum Type and Concentration	Basal Medium Formulation			
	DMEM/(GMEM)	DMEM/F12	L-15	MEM
5.0% v/v FBS	Nikolai and Hu (1992) and Reiter et al. (1990)		Nahapetian et al. (1986) and Mendonca et al. (1993)	
10.0% v/v FBS	Gebb et al. (1984)	Hu et al. (1985), Cinatl et al. (1992) and Hawboldt et al. (1994)		Chotteau and Bastin (1994)
5.0% v/v CS	Polastri et al. (1984)			
10.0% v/v CS	Hassell and Butler (1990) (GMEM)			
5.0% v/v NBCS	Lindskog et al. (1987)			
7.0% v/v CS + 2.0% v/v FBS + 1.5% v/v SUP				White and Ades (1990)

SUP - Undefined serum ultrafiltrate solution.

The source and concentration of supplemented bovine sera for Vero cell growth has varied (Table 6.1) from FBS at 10.0% v/v (Chotteau and Bastin, 1994) to calf serum (CS) at 5.0% v/v (Polastri et al., 1986). No previous reports were found determining whether one serum type has advantages over the others. However, maximum yields of murine 3T3 and Vero cells were previously shown to be reduced proportional to the foetal bovine serum (up to 55% at 1.0% v/v FBS) (Holley and Kiernan, 1968) and calf serum (up to 77% at 1.0% v/v CS) (Engelhardt and Mao, 1977) concentration used at <5.0% v/v. These authors reported no significant differences in cell yields at >5.0% v/v serum.

There have been a number of reports in the literature on the important growth nutrients for mammalian cell lines. Rabinowitz et al. (1956), Griffiths (1972) and Reitzer et al. (1979) showed glutamine and not glucose was the primary growth nutrient of Ehrlich ascites, human diploid fibroblast and HeLa cells. Wice et al. (1974) showed that glucose was utilised for anabolic substrates and not as a cellular fuel in HeLa cells, as glucose could be replaced by uridine and cytosine nucleosides. Giguere et al. (1982) showed the substrata matrix affected bovine corneal endothelial (BCE-1) cell nutrient consumption rates. Vero cell nutrient consumption has been reported previously (Polastri et al., 1984). However, the authors only monitored the amino acids. The authors found the only amino acids consumed during growth in DMEM + 5.0% v/v CS were arginine, glutamine, histidine, methionine and tryptophan. The cells produced alanine and serine. No other nutrients were analysed during growth. A later and more complete study by Chotteau and Bastin (1994) showed that Vero cells grown in M199 + 10.0% v/v FBS consumed the same amino acids as the previous authors but also aspartate, isoleucine, leucine, lysine, phenylalanine and valine. The cells were also shown to consume glucose, oxygen and pyruvate from the medium. These cells were found to produce lactate and ammonia but were not shown to produce any amino acids.

Despite the nutrient balances of Polastri et al. (1984) and Chotteau and Bastin (1994), the Vero cell culture medium has never been optimised. Further to this, no reports were found in the literature describing any differences in either the nutrient consumption or optimal nutrient

concentrations for Vero cell growth in cultures of solid or macroporous microcarriers.

This paper presents a nutrient evaluation and optimisation for the batch growth of Vero cells in cultures of solid Cytodex-1 and macroporous Cultispher-G microcarriers. Cell growth was performed in both tissue culture and spinner flasks to identify the important nutrients. Amino acid analysis was performed by high performance liquid chromatography (HPLC). The concentrations of the major nutrients were then optimised using matrix experiments.

6.2 Materials and Methods

The methods used for culturing and counting the Vero cells and subsequent nutrient analyses have been described previously (2.1-2.5).

6.2.1 Basal Medium Formulation

Stock Vero cells were preadapted (2.1) to DMEM, DMEM/Ham's nutrient mixture formulation 12 (F12) (1:1), Iscove's Modified Dulbecco's Medium (IMDM), L-15, M199, Optimem-1 and RPMI 1640. The media were prepared (2.3) and supplemented with iron enriched, supplemented calf serum (sCS) at either 2.0 or 5.0% v/v. Stock cells were detached and recovered by the standard trypsinisation protocol (2.2) and were counted by the trypan blue method (2.5.1-2).

6.3 Results and Discussion

6.3.1 Basal Medium Formulation

Introduction. Many different basal media formulations have been used for Vero cell growth. Therefore, this study determined (1) the preferable basal medium for the growth of Vero cells at either (a) high or (b) low serum concentrations and (2) whether medium enrichment of glucose and glutamine to the same levels in all media affected cell yields in T25 culture flasks.

Experimental Setup. Stock Vero cells were preadapted (>5 passages) to various basal medium

formulations which were prepared (2.3) with either (a) the "original" formulation or (b) "enriched" to the same glucose (25.00 mM) and glutamine (4.00 mM) concentrations found in DMEM and IMDM, excepting the proprietary formulated Optimem-1 (Gibco, 1992). The media were completed by supplementation with iron enriched, supplemented calf serum (sCS) at either (i) "low" - 2.0% v/v or (ii) "high" - 5.0% v/v concentrations. Cultures were grown in T25 culture flasks (2.1) and one flask of each media type was sacrificed daily for cell counts by the trypan blue method (2.5.1-2).

Results. Table 6.2 shows the maximum Vero cell yields and growth rates in cultures with the various media used. (1) Maximum Vero cell yields and growth rates in cultures of either bead were affected by the basal media formulation used. (2) There were significantly higher (29%, $P < 0.01$) maximum cell yields with supplementation at 5.0% v/v sCS compared to 2.0% v/v with respect to all the basal media used. (3) There were significantly higher (36%, $P < 0.01$) maximum cell yields in enriched compared to original medium formulations with respect to either sCS concentration used, excluding DMEM, IMDM and Optimem-1. (4) There was no significant difference in the maximum cell yields in DMEM and IMDM with respect to either sCS concentration used. (5) The analyses allowed the media to be ranked and showed maximum cell yields were significantly higher ($P < 0.05$) in $\text{DMEM} = \text{IMDM} > \text{Optimem-1} \geq \text{RPMI 1640} \geq \text{DMEM/F12} \geq \text{L-15} \geq \text{M199}$ at 5.0% v/v sCS. (6) There were no significant differences in the cell growth rates in most of the media. The cell growth rates were significantly higher (93%, $P < 0.01$) in enriched compared to original formulations of L-15 and M199, respectively, with either sCS concentration used. (7) There were no interactions between the serum concentrations and medium formulations used in this study.

Discussion. DMEM and IMDM produced the highest maximum Vero cell yields, even when the glucose and glutamine concentrations in all the media were equal. Clark and Hirtenstein (1981) showed higher Vero cell yields in DMEM compared to MEM and M199 and DMEM (Table

Table 6.2 - The effect of the **basal medium formulation** on the **maximum Vero cell yield** and **growth rates** in T25 culture flasks. Cells were preadapted to various basal media with 2.0 or 5.0% v/v sCS for 6 passages. The cells were then inoculated at 0.10×10^6 cells/mL in 10 mL of the various media. Daily one culture flask of each medium was sacrificed for cell count by the trypan blue method. The media were prepared with either the original formulation containing 25.00, 25.00, 17.50, 0.00, 5.60 or 11.00 mM glucose and 4.00, 4.00, 2.50, 2.00, 0.70 or 2.00 mM glutamine in DMEM, IMDM, DMEM/F12, Leibovitz L-15, M199, RPMI 1640 respectively or supplemented with glucose and glutamine. The values are means of n=2.

Basal Medium Formulation	Medium Supplementation	Maximum Cell Concentration ($\times 10^6$ /mL)		Specific Cell Growth Rate (h^{-1})	
		5.0% v/v sCS	2.0% v/v sCS	5.0% v/v sCS	2.0% v/v sCS
DMEM	-	$1.89 \pm 0.14^*$	1.42 ± 0.10	0.032 ± 0.003	0.030 ± 0.002
DMEM/F12 (1:1)	+	1.31 ± 0.10	1.27 ± 0.08	0.028 ± 0.002	0.028 ± 0.002
DMEM/F12 (1:1)	-	1.21 ± 0.09	1.14 ± 0.07	0.030 ± 0.003	0.031 ± 0.003
IMDM	-	1.85 ± 0.12	$1.46 \pm 0.11^*$	0.032 ± 0.002	0.032 ± 0.002
L-15	+	1.15 ± 0.09	0.75 ± 0.06	0.028 ± 0.002	0.029 ± 0.002
L-15	-	0.35 ± 0.04	0.22 ± 0.03	0.019 ± 0.001	0.011 ± 0.001
M199	+	1.12 ± 0.09	0.75 ± 0.06	0.029 ± 0.002	0.029 ± 0.002
M199	-	0.89 ± 0.06	0.67 ± 0.04	0.025 ± 0.002	0.026 ± 0.002
Optimem-1	-	1.44 ± 0.11	1.41 ± 0.11	0.031 ± 0.002	0.031 ± 0.002
RPMI 1640	+	1.36 ± 0.10	0.79 ± 0.06	0.030 ± 0.002	0.031 ± 0.002
RPMI 1640	-	1.09 ± 0.08	0.63 ± 0.05	0.028 ± 0.002	0.028 ± 0.002

- + - Medium supplemented to a total of 25.00 mM glucose and 4.00 mM glutamine.
- - Original medium.
- * - Highest cell yield.

6.1) is the most commonly used medium for these cells (Gebb et al., 1984, Polastri et al., 1984, Goetghebeur and Hu 1991 and Lee et al., 1991). The results in this study agree with this. However, cell yields were not significantly different in DMEM or IMDM. However, based on the purchase cost of 10 x 1 L packets of powdered media (Gibco BRL®, 1995) the DMEM (\$2.62 Cdn./L) would be the preferable medium as IMDM (\$8.13 Cdn./L) costs significantly more (210%). This price difference is mostly due to DMEM not containing (Table 6.3) HEPES buffer which significantly increases the medium cost at the concentration normally used (\$3.17 Cdn./L at 25.00 mM HEPES based on the price of 1.0 kg from Sigma® Chemical Co., 1995).

The higher yields in DMEM and IMDM could be explained by:- (1) DMEM and IMDM could have higher concentrations of essential nutrients for cell growth. Table 6.3 shows the formulations of the media used in this study and several components such as many amino acids were at higher concentrations in these media compared to original formulations of all other media used. The amino acids at higher concentrations in DMEM and IMDM were all shown to be consumed during Vero cell growth by Polastri et al. (1984) and Chotteau and Bastin (1994), except cystine. (2) There could be components at higher concentrations in DMEM/F12, L-15, M199 or RPMI 1640 competitively inhibiting the uptake and metabolism of the essential nutrients. There were several components at higher concentrations in these media such as glycine which was significantly higher (67 and 667%, respectively) in M199 and L-15 compared to the other media used. Vincent et al. (1994) showed glycine inhibits hepatocyte growth by increasing the specific glutamine consumption rate. (3) There were components only present in DMEM/F12, L-15, M199 or RPMI 1640 which could directly inhibit Vero cell growth. There were some components such as cysteine only present in these media. Harraki et al. (1994) showed cysteine inhibits Zn^{2+} uptake in human diploid lung fibroblast (MRC-5) cells which could result in reduced DNA synthesis (Epstein, 1982) which could reduce cell yields.

There were also (Table 6.3) numerous extra components in M199 and many components at significantly higher concentrations in L-15 compared to the other media used. These differences could have many effects on cell metabolism through increased medium osmolarity (Waymouth,

Table 6.3 - The common basal medium formulations and their component concentrations used for Vero cell growth in the previous literature. Sourced from Gibco BRL (1992).

Component	Component Concentration in Medium Formulation (mM)					
	IMDM	DMEM	M199	RPMI 1640	DMEM/F12	L-15
CaCl ₂ (Anhy.)	1.50	1.80	1.80		0.30	1.20
Ca(NO ₃) ₂ .4H ₂ O				0.42		
CuSO ₄ .5H ₂ O					0.00001	
Fe(NO ₃) ₃ .9H ₂ O		0.00025	0.002			
FeSO ₄ .7H ₂ O					0.0015	
KCl	5.44	5.40	5.40	5.40	3.00	5.40
KH ₂ PO ₄			0.44			0.44
MgCl ₂					0.60	0.98
MgSO ₄ (Anhy.)	0.81	0.81	0.81	0.41		0.81
NaCl	77.00	116.25	116.25	102.50	130.00	136.75
NaHCO ₃	36.00	44.00	26.20	23.80	29.00	
NaH ₂ PO ₄ .H ₂ O	0.91		1.00			
Na ₂ HPO ₄ (Anhy.)				5.60	1.00	1.34
Na ₂ HPO ₄ .7H ₂ O		0.50				1.34
Na ₂ SeO ₃	0.001					
ZnSO ₄ .7H ₂ O					0.003	
Adenine.SO ₄			0.055			
Adenosine(PO ₄) ₃			0.003			
Adenylic Acid			0.0006			
Cholesterol			0.0005			
Deoxyribose			0.004			
Galactose						5.00
D-Glucose	25.00	25.00	5.60	11.11	10.00	
Glutathione (Red.)			0.0002	0.0033		
Guanine.HCl			0.002			
HEPES	25.00					
Hypoxanthine.Na			0.002		0.03	
Linoleic Acid					0.00015	
Lipoic Acid					0.001	
Phenol Red	0.045	0.045	0.06	0.014	0.0035	0.028
Putrescine.2HCl					0.00065	
Ribose			0.0035			
Sodium Acetate			0.40			
Sodium Pyruvate	1.00				0.50	5.00
Thymidine					0.003	
Thymine			0.0025			
Uracil			0.003			
Xanthine.Na			0.002			

DL-Alanine	0.28		0.60		0.10	5.05
L-Arginine.HCl	0.40	0.40	0.35	0.95	1.00	2.40
L-Asparagine	0.17			0.35	0.10	1.67
L-Aspartic Acid	0.23		0.50	0.15	0.10	
L- Cysteine.HCl.H ₂ O			0.0006		0.20	0.68
L-Cystine.2HCl	0.29	0.20	0.085	0.20		
L-Glutamic Acid	0.50		1.00	0.15	0.10	
L-Glutamine	4.00	4.00	0.70	2.00	1.00	2.00
Glycine	0.40	0.40	0.70	0.15	0.10	2.70
L-Histidine.HCl.H ₂ O	0.28	0.28	0.15	0.10	0.150	1.60
L-Hydroxyproline			0.08	0.150		
L-Isoleucine	0.80	0.80	0.30	0.40	0.030	1.90
L-Leucine	0.80	0.80	0.90	0.40	0.10	0.95
L-Lysine.HCl	0.80	0.80	0.40	0.20	0.20	0.40
L-Methionine	0.20	0.20	0.20	0.10	0.03	1.00
L-Phenylalanine	0.40	0.40	0.30	0.10	0.03	1.50
L-Proline	0.35		0.35	0.17	0.30	
L-Serine	0.40	0.40	0.50	0.30	0.10	1.90
L-Threonine	0.80	0.80	0.50	0.20	0.10	5.04
L-Tryptophan	0.08	0.08	0.10	0.025	0.01	0.10
L- Tyrosine.2Na.2H ₂ O	0.40	0.40	0.20	0.10	0.03	1.15
L-Valine	0.80	0.80	0.40	0.17	0.10	1.70
Ascorbic Acid			0.0003			
α-Tocopherol			0.00003			
Biotin	0.00005		0.00004	0.0008	0.00003	
Calciferol			0.00025			
D-Ca Pantothenate	0.00002	0.02	0.00004	0.001	0.002	0.00420
Choline Chloride	0.03	0.03	0.004	0.02	0.065	0.0070
Folic Acid	0.009	0.009	0.00003	0.0025	0.003	0.00230
i-Inositol	0.04	0.04	0.0003	0.20	0.10	0.0110
Menadione			0.00006			
Niacin			0.0002			
Niacinamide	0.033	0.035	0.0002	0.008	0.0003	0.00820
p-NH ₂ Benzoic Acid			0.0004	0.0075		
Pyridoxal.HCl	0.02	0.02	0.0001			
Pyridoxine.HCl			0.0001	0.005	0.0003	0.00490
Riboflavin	0.001	0.001	0.00003	0.0005	0.0001	0.00030
Thiamine.HCl	0.019	0.012	0.00003	0.003	0.001	0.0030
Thymidine						
Vitamin A (Acetate)			0.0005			
Vitamin B ₁₂	0.00001			0.000004	0.001	

1970) or competitive inhibition (Voet and Voet, 1990) leading to reduced maximum cell yields in these media. Medium osmolarity will be discussed in Chapter 8.3.

The nutrient concentration differences between the media used in previous reports (6.1) could lead to exaggerated effects if the cells were nutrient limited during growth. None of these authors proved their media formulations were not affecting cell growth. The media differences and also cell count method differences, described previously (4.3) make it impossible to compare data between previous reports and this study.

The trends in Vero cell growth were similar at 2.0 and 5.0% v/v sCS, although, maximum cell yields were significantly lower at 2.0% v/v in all media used, except Optimem-1 and DMEM/F12. This effect could be expected, as Optimem-1 was formulated (Gibco, 1992) as a reduced serum requiring medium and DMEM/F12 was used previously as the base in serum-free media for baby hamster kidney (BHK), Vero, MRC-5 and Chinese hamster ovary (CHO) cells (Bradshaw et al., 1983, Cinatl et al., 1992, Forestall et al., 1992 and Watson et al., 1994). DMEM/F12 contains extra trace elements (Fe^{2+} , Zn^{2+} and Cu^{2+}) and fatty acids (linoleic and lipoic acids) which allow the serum to be reduced or completely removed. Indeed, Hu et al. (1985) found higher human foreskin fibroblast (FS-4) cell yields in DMEM/F12 compared to several other media. These authors suggested the trace elements in DMEM/F12 could be responsible for this effect. However, the sCS also enhanced the concentrations of iron, transferrin, insulin and other trace elements in the media used in the current study. The accumulation of trace elements to an inhibitory concentration in DMEM/F12 at 5.0% but not at 2.0% v/v sCS could explain why maximum cell yields were not significantly different at these concentrations.

6.3.2 Optimal Bovine Serum Type and Concentration

Introduction. The study above (6.3.1) showed the preferable basal medium for Vero cell growth was DMEM. However, maximum cell yields were affected by the iron enriched, supplemented

calf serum (sCS) concentration used and no one has previously suggested the optimal serum type or concentration for Vero or any other cell line. Therefore, this study determined the optimal bovine serum type and concentration for Vero cell growth in microcarrier cultures.

Experimental. Stock Vero cultures were grown in 100 mL spinner flasks (2.4.3) in DMEM containing either FBS, sCS or CS at 1.0-10.0% v/v. The cultures were grown on 1.00 g/L Cytodex-1 or 1.70 g/L Cultispher-G (2.4.3). Some Cultispher-G cultures were attached with continuous stirring at 40 rpm throughout with FBS and sCS.

Results. Table 6.4 shows maximum Vero cell yields and growth rates of cultures in DMEM supplemented with FBS, sCS or CS at various concentrations. Table 6.5 shows cell growth in Cultispher-G cultures supplemented with FBS and sCS following cell attachment with different stirring modes. Table 6.6 shows the purchase costs of the serum used in this study. (1) Maximum cell yields and growth rates in cultures of either bead were affected by the serum types and concentrations used. (2) Maximum cell yields were significantly higher (40%, $P=0.01$) in Cultispher-G compared to Cytodex-1 cultures with respect to all the serum types and concentrations used. (3) Maximum cell yields were proportionally and significantly lowered in Cytodex-1 cultures with the serum concentration with respect to all the serum types used. Maximum cell yields in Cultispher-G cultures were only proportionally and significantly lowered with the CS concentration. (4) There was no significant difference in the maximum cell yields in Cytodex-1 cultures supplemented with FBS and sCS with respect to any concentration used. There was no significant difference in maximum cell yields in Cultispher-G cultures supplemented at 2.5-10.0% v/v FBS and sCS. However, maximum cell yields in Cultispher-G cultures were significantly lower (20%, $P<0.05$) supplemented with 1.0% v/v sCS compared to 1.0% v/v FBS. Maximum cell yields were significantly lower in cultures of Cytodex-1 and Cultispher-G (14% and 22%, respectively, $P<0.05$) with CS compared to the other serum types used. (5) The analysis allowed the serum concentrations to be ranked based on the maximum

Table 6.4 - The effect of different serum types and concentrations on the maximum Vero cell yield and cell growth rates in 100 mL spinner flasks on 1.00 g/L Cytodex-1 or 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 1.0-10.0% v/v foetal bovine serum (FBS), calf serum (CS) or iron enriched, supplemented calf serum (sCS). Spinner flasks were stirred continuously at 40 rpm (Cytodex-1) or intermittently for the first 24 h, then continuously at 40 rpm and ramped after 72 h in culture to 60 rpm (Cultispher-G). Spinner flasks were sampled daily for trypan blue cell count. The flasks were readjusted to pH 7.4 after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Serum Concentration (v/v)	Maximum Cell Concentration					
	(x 10 ⁶ /mL)					
	FBS		sCS		CS	
	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G
1.0%	0.82 ± 0.06	1.88 ± 0.12	0.87 ± 0.06	1.51 ± 0.11	0.49 ± 0.03	0.91 ± 0.08
2.5%	1.22 ± 0.08	1.89 ± 0.12	1.20 ± 0.08	1.67 ± 0.12	0.95 ± 0.07	1.46 ± 0.11
5.0%	1.57 ± 0.10*	2.07 ± 0.14*	1.48 ± 0.11*	1.72 ± 0.11	1.47 ± 0.10	1.70 ± 0.11
10.0%	1.48 ± 0.10	2.00 ± 0.13	1.45 ± 0.10	1.80 ± 0.13*	1.53 ± 0.11*	1.74 ± 0.12*
	Specific Cell Growth Rate (h ⁻¹)					
1.0%	0.028 ± 0.002	0.032 ± 0.002	0.025 ± 0.002	0.030 ± 0.003	0.020 ± 0.001	0.022 ± 0.001
2.5%	0.033 ± 0.002	0.033 ± 0.002	0.030 ± 0.002	0.029 ± 0.002	0.026 ± 0.002	0.029 ± 0.002
5.0%	0.035 ± 0.003	0.038 ± 0.003	0.035 ± 0.003	0.036 ± 0.003	0.034 ± 0.002	0.029 ± 0.002
10.0%	0.034 ± 0.003	0.037 ± 0.003	0.029 ± 0.002	0.036 ± 0.003	0.034 ± 0.003	0.030 ± 0.003

ND - Value not determined.

* - Highest cell yield.

Table 6.5 - The effects of the **stirrer mode for attachment** on maximum Vero cell yields in 100 mL spinner flasks on **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM supplemented with various concentrations (up to 10.0% v/v) of FBS and sCS. The flasks were stirred either at 40 rpm continuously or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture. The flasks were readjusted to pH 7.4 daily after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Serum Concentration (% v/v)	Cell Concentration ($\times 10^6$ /mL)			
	Intermittent Stirring for 24 h		Continuous Stirring	
	FBS	sCS	FBS	sCS
1.0	1.88 ± 0.12	1.51 ± 0.11	0.10 ± 0.03	1.48 ± 0.10
2.5	1.89 ± 0.12	1.67 ± 0.12	0.18 ± 0.02	1.60 ± 0.11
5.0	$2.07 \pm 0.14^*$	1.72 ± 0.11	0.67 ± 0.04	1.75 ± 0.13
10.0	2.00 ± 0.13	1.80 ± 0.13	1.05 ± 0.08	1.75 ± 0.13

* - Highest cell yield.

Table 6.6 - The **purchase costs** of the commercial sera and the **cost/L** of **supplemented medium** used for Vero cell culture. The costs are quoted for 500 mL volumes of qualified foetal bovine serum and iron enriched, supplemented calf serum. The prices are taken from Gibco BRL Price List (1995).

Serum Volume	Purchase Cost of 1 x 500 mL volume of each Serum Type and Serum Cost/L of Supplemented Medium at the Concentration Used (\$ Cdn.)		
	FBS	sCS	CS
500 mL Volume	c. 350.00 ⁺	79.15	61.40
Supplemented Medium Concentration (% v/v)			
1.0	7.00	1.58	1.23
2.5	17.50	3.96	3.07
5.0	35.00	7.92	6.14
10.0	70.00	15.84	12.28

+ - Average price, as the purchase price varies between batches depending on availability.

cell yields in cultures of either bead produced with each concentration. Maximum cell yields were significantly higher ($P<0.05$) in cultures supplemented at ≥ 5.0 % v/v serum compared to all other concentrations used. (6) Cell growth rates were reduced proportional to the serum concentration and were significantly lower in cultures of either bead supplemented with 2.5% (14%, $P=0.05$) or 1.0% (23%, $P<0.05$) v/v serum with respect to all sera types used.

Stirring Mode for Cell Attachment. (1) Maximum cell yields (Table 6.5) in Cultispher-G cultures supplemented with FBS were affected by the stirrer mode for cell attachment. (2) There were significantly lower (74%, $P<0.01$) maximum cell yields in cultures supplemented with FBS following cell attachment with continuous stirring at 40 rpm compared to intermittent stirring at 40 rpm with respect to all concentrations used. (3) There were no significant differences in maximum cell yields with sCS at any concentration used with respect to either stirrer mode for attachment.

Free Cells. There were freely suspended cells in Cultispher-G cultures with FBS and sCS following the 24 h continuous stirring attachment phase. These cells formed aggregates (up to 50 cells) which did not attach to the beads in FBS supplemented cultures and caused the reduced cell yields. However, the aggregates in sCS supplemented cultures completely attached to the beads within 48 h in culture.

Serum Cost. The costs (Table 6.6) of the serum were highly varied but FBS was the most expensive and made the costs of supplementing the basal medium significantly higher (398%) compared to the other serum types at all the concentrations used. The sCS supplemented media was also significantly more (29%) expensive compared to CS at all the concentrations used. However, sCS was the most cost effective serum as cell yields were significantly higher (15%, $P<0.05$) compared to CS.

Discussion. Maximum Vero cell yields (Table 6.4) were affected by the serum type and concentration but the effects differed between the bead types. The maximum cell yields in Cytodex-1 cultures were not significantly different with FBS or sCS supplementation but were significantly lower in CS. Maximum cell yields were also proportional to the serum concentration in cultures supplemented at <5.0% v/v serum and were not increased when supplemented at >5.0% v/v with respect to any serum type used. This agreed with the results of Holley and Kiernan (1968), Hassell (1975) and Engelhardt and Mao (1977) who showed proportionally reduced cell yields at <5.0% v/v FBS or CS with 3T3 and Vero cells in T25 culture flasks.

There were few differences in maximum cell yields in Cultispher-G cultures supplemented with FBS or sCS at any concentration, except at 1.0% v/v sCS. However, the maximum cell yields were proportionally reduced in Cultispher-G cultures supplemented with CS at <5.0% v/v. No previous reports in the literature were found showing serum dependent cell yields in cultures of Cytodex-1 or Cultispher-G.

The Vero cell yields suggest there could be higher concentrations of growth promoting components in FBS or sCS compared to CS. The results also suggest the cells are less dependent on serum growth factors in Cultispher-G cultures compared to Cytodex-1. Most cell lines grow well in FBS supplemented media due to its increased growth factor concentrations compared to other sera (Barnes et al., 1984). However, the sCS was enriched with iron and supplemented with high concentrations of trace elements, insulin and transferrin (Gibco, 1992). Any of these components could explain the improved cell growth in cultures of either bead with sCS compared to CS. Iron could be important for cellular cytochrome electron transfer pathways (Voet and Voet, 1990). Transferrin could be important for iron or other trace element transport to the cells (Voet and Voet, 1990). Trace elements could be required as enzyme cofactors such as manganese for protein glycosylation (Mookerjee et al., 1972) and zinc for DNA synthesis (Epstein, 1982 and Lewin, 1990). However, all these components could be expected to affect cell growth in Cytodex-1 cultures as well.

There were no significant differences in cell yields between sCS and FBS. However, the

purchase cost/L (Table 6.6) was a major difference between these sera and FBS was significantly more (342%) expensive. Therefore, sCS was the preferable serum for Vero cells.

Cell Attachment to the Beads. Others have determined fibronectin and vitronectin are involved in fibroblastic cell attachment and spreading and their effects are well documented (Grinnell and Phan, 1983 and Underwood and Bennett, 1989). Vero cells growing in Cytodex-1 cultures could also require these proteins for adherence to the beads rather than for stimulation or regulation of growth. Commercial sera all contain fibronectin but the concentration present is significantly lower (90%) compared to that *in vivo* (Hayman et al., 1985) due to losses during serum clotting and cold precipitation processing. If the cells could not biosynthesise these factors (once the serum concentrations were depleted), then Cytodex-1 cultures could plateau at lower cell yields compared to Cultispher-G. Others have speculated that serum proteins were required for attachment and growth of Vero cells in cultures of Cytodex-1 (Mukhopadhyay et al., 1994). However, Ng (1995) and a previous study (5.3.11) found the rate of Vero cell attachment to Cytodex-1 increased (up to 100%) in serum-free DMEM. The results suggest serum factors were not required for cell attachment but were required for cell flattening and growth.

Bober et al. (1987) and Varani et al. (1988) previously reported significantly lower laminin secretion (an adherence factor) by rat LIS and chick fibroblast cells in Cytodex-1 cultures and suggested this explained the lower yields compared to T25 flasks. The results above (6.3.1-2) agree with lower cell yields in cultures of Cytodex-1 compared to T25 flasks or Cultispher-G beads. However, maximum cell yields were not significantly different in cultures of Cultispher-G and T25 flasks in DMEM + 5.0% v/v sCS ($1.80\text{--}1.90 \times 10^6$ cells/mL).

Cell attachment to or entrapment in Cultispher-G beads could also require proteins on the cell membranes to enhance cell aggregate formation or attachment. Collagen or other cell adhesion factors could be important to this process (Pearlstein, 1978, Couchman et al., 1983, Shaffer et al., 1984 and Akimoto et al., 1992) and surface deposition could require several hours, explaining attachment time differences (an order of magnitude) between the beads shown by Ng

(1995). Cultispher-G beads are manufactured from gelatin (denatured collagen) which could enhance cell yields by forming a basement lamina similar to that *in vivo* (Doillon et al., 1987).

The results also suggest the Cytodex-1 bead charge was important for cell attachment and could involve Ca^{2+} (Curtis and McMurray, 1986). The slower attachment rates in cultures of Cultispher-G could be explained by their lack of bead charge. Pearlstein (1978) showed fibroblast cells attached to surfaces and microcarriers in the absence of fibronectin and vitronectin but they did not flatten or grow, as with the Vero cells described previously (4.3.5). However, Vero cells flattened quickly (<6 h) in cultures of Cytodex-1 in DMEM + 5.0% v/v SCS. Vitronectin could explain this, as it is known to enhance CHO cell flattening (Danilov and Juliano, 1989).

Bead Surface Area. Lower maximum cell yields in 1.00 g/L Cytodex-1 cultures could be explained by low surface area for growth (Clark and Hirtenstein, 1981). These authors showed significantly higher (20%) cell yields in cultures with 3.00 and 5.00 g/L beads. However, previous data (5.3.9) contradicted this and showed the culture medium was more important, agreeing with the FS-4 cell growth results of Hu et al. (1985).

Macroporous Cultispher-G beads have large internal void spaces in which the cells can grow, whereas, the cells only grow on the bead surface of Cytodex-1. At maximum cell yield the Cytodex-1 bead surface was completely covered with 1-2 cell layers. Cultispher-G beads showed light neutral red staining suggesting there was still a large surface area available for growth even at maximum cell yield in batch cultures.

Cell Growth Rates. The Vero cell growth rates (Table 6.4) in cultures of either bead were significantly reduced at 1.0-2.5% v/v compared to higher levels with all serum types used. This suggests serum stimulated cell growth and the effect was maximal at 5.0% v/v SCS. However, the cell growth rates were not bead dependent. Doillon et al. (1987) showed stimulated collagen synthesis and also a reduced rate of DNA synthesis from chick fibroblast cell growth in collagen

beads. Vero cell growth rates were not significantly different in cultures of either microcarrier but were reduced (10-20%) compared to T25 culture flasks described above (6.3.1). The lower cell growth rates in microcarrier cultures could be explained by either a reduced DNA synthesis rate, by differences in the culture methods or adherence factor secretions (Bober et al., 1987). The similar cell yields in Cultispher-G and T25 culture flasks disagrees with there being a reduced cell DNA synthesis in these beads, as suggested by Doillon et al. (1987).

Cell Attachment Mode. Vero cell yields (Table 6.5) in Cultispher-G cultures supplemented with FBS were affected by the attachment stirring mode. The cells formed large aggregates and there was only a significant aggregate attachment at 10.0% v/v FBS which lead to significantly lower maximum cell yields at all FBS concentrations following attachment with continuous stirring compared to intermittent. No one has previously described this effect in the literature.

Cell aggregates also formed in similar sCS supplemented cultures but they completely attached to the beads within 48 h and did not affect maximum cell yields. Thus, sCS could be a preferable source of extracellular serum derived attachment factors compared to FBS.

6.3.3 Basal Medium Nutrient Consumption

Introduction. The studies above (6.3.1-2) showed the preferable medium for Vero cell growth was DMEM supplemented with 5.0% v/v iron enriched, supplemented calf serum (sCS). However, the studies did not determine the important Vero cell growth nutrients in the DMEM. Few, if any, reports in the literature have described the important nutrients or optimised them for any cell line. Therefore, this study determined the major nutrient consumption and production of Vero cells grown in microcarrier cultures.

Experimental. The Vero cultures were grown in original DMEM + 5.0% v/v sCS in 100 mL spinner flasks, as described above (6.3.2). The cultures were sampled daily for medium nutrient

analyses, as described previously (2.5.7-2.5.9).

Results. Fig. 6.1A) shows the normal Vero cell growth in DMEM + 5.0% v/v sCS in spinner flask cultures of Cytodex-1 and Cultispher-G microcarriers. Fig. 6.1B) and C) show the glucose consumption and lactate production and the glutamine consumption and ammonia production in the normal cultures, respectively. Table 6.7 shows the results of the cell growth rates and the specific consumption and production rates for glucose, glutamine, serine, lactate, ammonia and glycine and the conversion rates of lactate to glucose, glutamine to ammonia and serine to glycine. Table 6.8 shows the complete high performance liquid chromatography (HPLC) amino acid analysis of the DMEM sampled at maximum cell yield in normal cultures. Fig. 6.2A)-C) show the amino acid consumption profiles of other amino acids and Fig. 6.2D) shows the produced amino acids in these cultures. (1) There were differences in Vero cell growth and nutrient effects in cultures of either bead in DMEM + 5.0% v/v sCS. (2) Maximum cell yields (Fig. 6.1A)) were significantly higher (32%, $P < 0.01$) in Cultispher-G cultures compared to Cytodex-1. (3) There was no significant difference in the cell growth rates (Table 6.8) in cultures of either bead. (4) There were significantly higher concentrations (Fig. 6.1B) and Table 6.7) of glucose consumed (25%, $P < 0.01$) and lactate produced (44%, $P < 0.01$) in Cultispher-G cultures compared to Cytodex-1. (5) The lactate to glucose ratio (Table 6.7) was significantly higher (14%, $P = 0.05$) in cultures of Cultispher-G compared to Cytodex-1. (6) There (Table 6.7) was no significant difference in the specific glucose consumption rates or specific lactate production rates with respect to either bead type. (7) There (Fig. 6.1C) and Table 6.7) was no significant difference in the concentration of glutamine consumed in cultures of either bead. Indeed, almost all the glutamine was consumed prior to maximum cell yield. (8) There (Fig. 6.1C) and Table 6.7) was no significant difference in the ammonia concentration produced or the ammonia to glutamine ratios (0.81) at maximum cell yield with respect to either bead type. (9) There (Table 6.7) were significantly higher (34%, $P < 0.01$) specific glutamine consumption rates and ammonia production rates in cultures of Cytodex-1 compared to Cultispher-G. (10) There was no

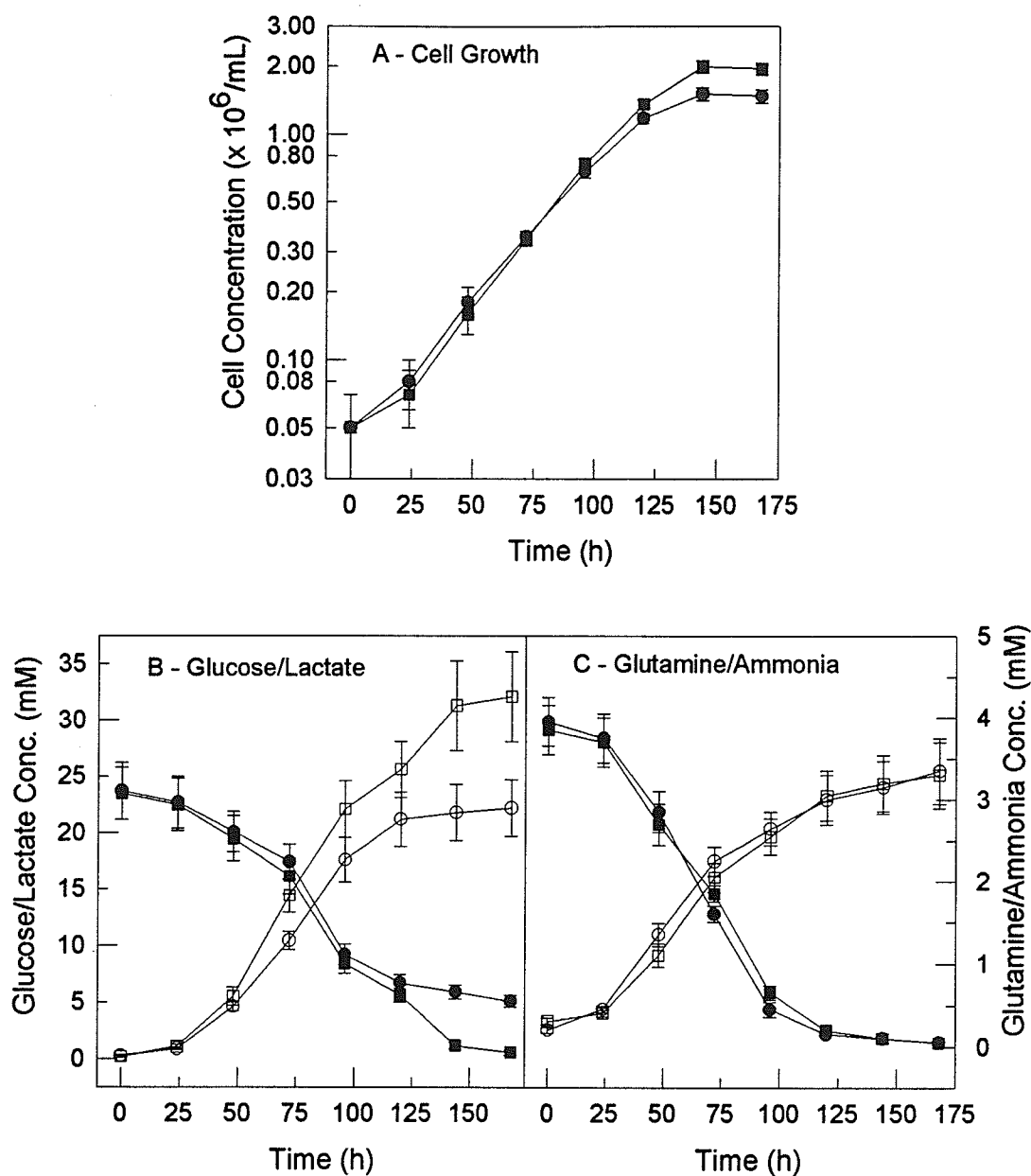


Fig. 6.1 - The normal growth (A), glucose/lactate (B) and glutamine/ammonia concentrations (C) of Vero cells in DMEM + 5.0% v/v sCS in 100 mL spinner flasks on ● 1.00 g/L Cytodex-1 and ■ 1.70 g/L Cultispher-G. Cultures were inoculated at 0.05×10^6 cells/mL and were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. Glucose/glutamine are open and lactate/ammonia are closed symbols. The points are means of $n=2$.

Table 6.7 - The cell growth rate, concentrations consumed or produced, specific consumption and production rates and conversion rates of glucose, lactate, glutamine, ammonia, serine and glycine at maximum Vero cell yield in 100 mL spinner flask cultures on 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS. The glucose and lactate were analysed by immobilised enzyme probes, the ammonia was analysed by a specific ammonia electrode and the amino acids were analysed by HPLC. The values are means of $n=2$.

Component Effect	Component Value Determined at Maximum Cell Yield	
	Cytodex-1	Cultispher-G
Specific Cell Growth Rate	$0.034 \pm 0.003 \text{ h}^{-1}$	$0.037 \pm 0.003 \text{ h}^{-1}$
Glucose Concentration Consumed	$17.80 \pm 0.30 \text{ mM}$	$22.30 \pm 0.50 \text{ mM}$
Specific Glucose Consumption Rate	$73.25 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$69.04 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$
Lactate Concentration Produced	$21.80 \pm 2.20 \text{ mM}$	$31.30 \pm 3.10 \text{ mM}$
Specific Lactate Production Rate	$89.71 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$96.60 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$
Lactate to Glucose Conversion Ratio	1.23	1.40
Glutamine Concentration Consumed	$3.85 \pm 0.40 \text{ mM}$	$3.75 \pm 0.40 \text{ mM}$
Specific Glutamine Consumption Rate	$15.82 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$11.60 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$
Ammonia Concentration Produced	$3.15 \pm 0.40 \text{ mM}$	$3.20 \pm 0.40 \text{ mM}$
Specific Ammonia Production Rate	$12.96 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$9.91 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$
Ammonia to Glutamine Conversion Ratio	0.82	0.85
Serine Concentration Consumed	$0.31 \pm 0.05 \text{ mM}$	$0.32 \pm 0.05 \text{ mM}$
Specific Serine Consumption Rate	$1.28 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$0.99 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$
Glycine Concentration Produced	$0.08 \pm 0.02 \text{ mM}$	$0.09 \pm 0.02 \text{ mM}$
Specific Glycine Production Rate	$0.033 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$0.28 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$
Glycine to Serine Conversion Ratio	0.26	0.28

Table 6.8 - The normal amino acid consumption and production of Vero cells in DMEM + 5.0% v/v sCS in 100 mL spinner flasks on 1.00 g/L Cytodex-1 or 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL and stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The amino acid concentrations were determined by HPLC in the supernatants after 144 h (i.e. maximum cell yield) in cultures of either bead. The flasks were readjusted to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Amino Acid	Amino Acid Consumption/Production and the Specific Consumption Rate			
	Cytodex-1		Cultispher-G	
	Concentration Consumed or Produced (mM)	Specific Consumption/Production Rate ($\mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$)	Concentration Consumed or Produced (mM)	Specific Consumption/Production Rate ($\mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$)
Alanine	+0.81	+3.33	+0.96	+2.97
Arginine	-0.14	-0.58	-0.13	-0.40
Asparagine	+ 0.03	+0.12	+0.03	+0.09
Aspartate	NP		NP	
Cysteine	NP		NP	
Cystine	UND		UND	
Glutamate	+1.11	+4.57	+1.13	+3.50
Glutamine	-3.85	-15.84	-3.75	-11.61
Glycine	+0.08	+0.33	+0.09	+0.28
Histidine	-0.11	-0.45	-0.13	-0.40
Isoleucine	-0.23	-0.95	-0.23	-0.71
Leucine	-0.28	-1.15	-0.29	-0.90
Lysine	-0.18	-0.74	-0.21	-0.65
Methionine	-0.05	-0.21	-0.08	-0.25
Phenylalanine	-0.08	-0.33	-0.10	-0.31
Proline	NP		NP	
Serine	-0.31	-1.28	-0.32	-0.99
Threonine	-0.12	-0.49	-0.13	-0.40
Tryptophan	-0.02	-0.08	-0.04	-0.12
Tyrosine	-0.06	-0.25	-0.08	-0.25
Valine	-0.11	-0.45	-0.10	-0.31
Total Consumed Amino Acids	-5.54	-22.70	-5.59	-17.20
Total Produced Amino Acids	+2.13	+8.35	+2.31	+6.84

NP - None present.

UND - Undetectable by OPA method.

-

- Consumed amino acid.

+

+ Produced amino acid.

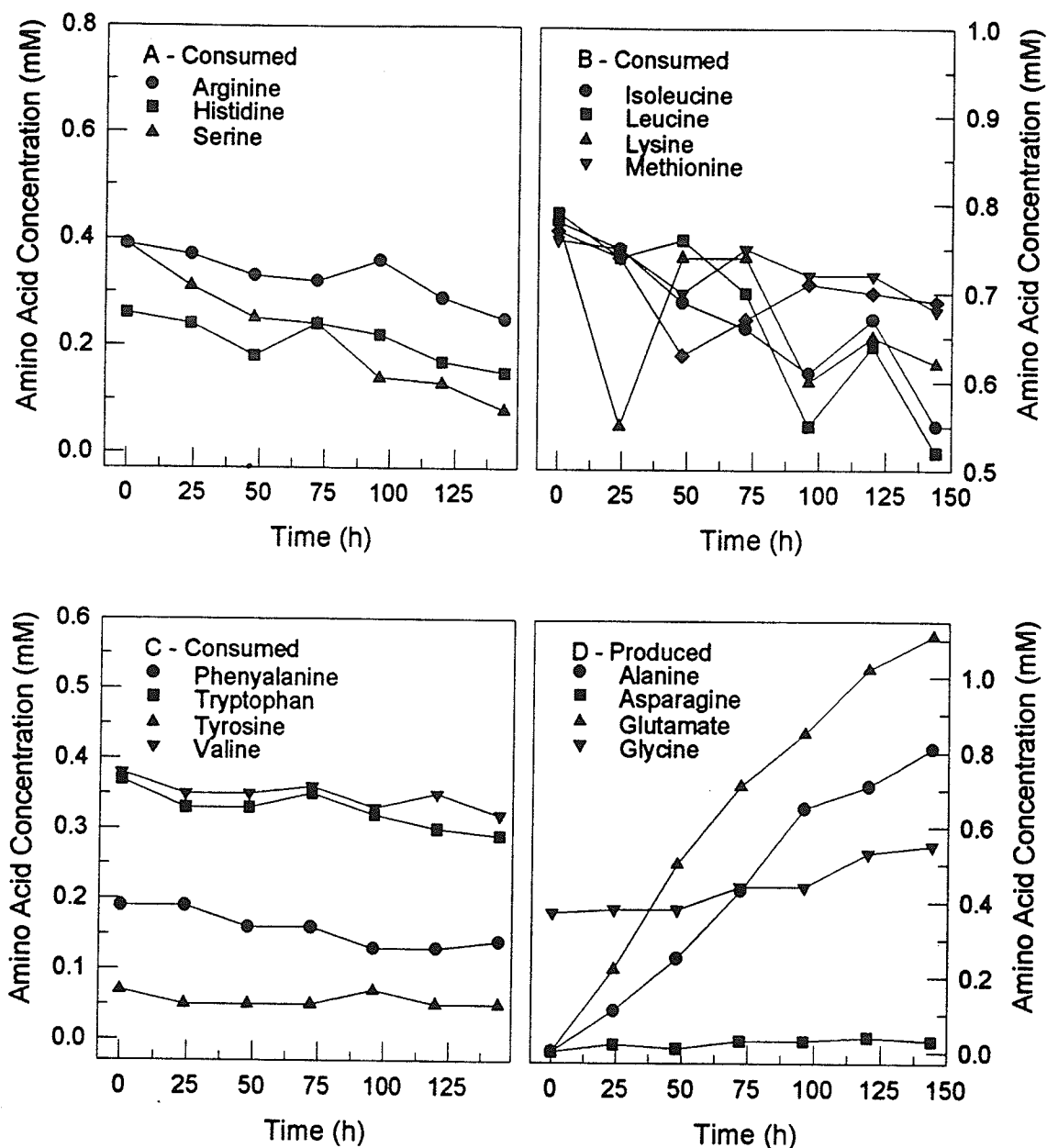


Fig. 6.2 - The consumption of: (A) arginine, histidine, serine, (B) isoleucine, leucine, lysine, methionine and (C) phenylalanine, tryptophan, tyrosine and valine and the production of: (D) alanine, asparagine, glutamate and glycine by normal Vero cultures in DMEM + 5.0% v/v SCS in 100 mL spinner flasks on 1.00 g/L Cytodex-1 or 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL. The amino acids were analysed after 144 h in culture by HPLC. The points are means of Cytodex-1 and Cultispher-G cultures and are of $n=4$.

significant difference in the concentration of serine, the second highest consumed amino acid, consumed (79%) at maximum cell yield. (11) There (Table 6.7) was a significantly higher (29%, $P<0.01$) specific serine consumption rate in Cytodex-1 cultures compared to Cultispher-G cultures. (12) There (Table 6.8) was no significant difference in the concentrations of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine consumed during Vero cell growth in cultures of either bead. However, only the initial histidine concentration was reduced by $>40\%$ at maximum cell yield. Arginine, isoleucine, leucine and tryptophan concentrations were reduced by 30-40%, lysine, methionine and phenylalanine were reduced by 20-30% and tyrosine and valine were reduced by 10-20%. (13) There (Table 6.8) were significant increases ($P<0.01$) in the concentrations of alanine, glutamate, glycine and asparagine during normal Vero cell growth in cultures of either bead. (14) There (Table 6.8) were no significant differences in the total concentrations of amino acids consumed or produced with respect to either bead type. However, there were significantly higher total specific amino acid consumption (32%, $P<0.01$) and production (22%, $P<0.05$) rates in cultures of Cytodex-1 compared to Cultispher-G.

Culture pH. The pH fluctuations in the cultures varied between the bead types used. Cultispher-G cultures after 72 h had decreased to pH <6.7 from pH 7.4. However, Cytodex-1 cultures had decreased to pH 6.8 after 120 h from pH 7.4. Thus, Cultispher-G cultures required more pH readjustment (x2 volume of NaOH) to maintain the optimal pH range (pH 6.8-7.1).

Discussion. Previous results (5.3 and Fig.6.1A)) have shown consistently higher maximum Vero cell yields in cultures of Cultispher-G compared to Cytodex-1. However, the cell growth rates were not significantly different in cultures of either bead. A major difference in cell growth between the beads was the culture pH. Cultispher-G cultures were always at pH <6.7 after 72 h compared to pH 7.0 in Cytodex-1 cultures from an initial pH of pH 7.4. This suggested cell metabolism could differ between cultures of either bead. Giguere et al. (1982) previously

suggested BCE-1 cell metabolism was different for growth on different substrata.

Nutrient Profiles - Glucose/Lactate. Nutrient analyses showed bead dependent differences in cellular metabolism of medium nutrients agreeing with Giguere et al. (1982). The glucose/lactate profiles (Fig. 6.1B)) showed Vero cells in Cultispher-G cultures consumed almost all of the glucose, whereas, there was a residual glucose concentration remaining at the end of Cytodex-1 cultures. The concomitant lactate production reflected the glucose consumption in cultures of either bead and was significantly higher in Cultispher-G cultures. The higher concentrations in Cultispher-G cultures could explain their lower pH as lactate acidifies the medium (Duval et al., 1992). However, there were no significant differences in specific glucose consumption or lactate production rates in cultures of either bead. Chotteau and Bastin (1994) previously reported similar nutrient effects for Vero cells in microcarrier cultures.

Glutamine. Glutamine (Fig. 6.1c)) was totally consumed prior to maximum cell yields in cultures of either bead. This suggests glutamine was the major growth nutrient, as was shown for Ehrlich ascites, human diploid fibroblast and HeLa cells by Rabinowitz et al. (1956), Griffiths (1972) and Reitzer et al. (1979). The specific glutamine consumption rates were also significantly higher in Cytodex-1 cultures compared to Cultispher-G reflecting the reduced cell yields in these cultures.

Glutamine could be metabolised through deamination by glutaminase into ammonia and glutamate. The glutamate could be utilised by deamination to α -ketoglutarate which is a precursor in the biosynthesis of other amino acids or enters mitochondrial ATP synthesis (McKeehan, 1982). Glutamate could be used for glutathione production to protect the cell membranes from peroxide and free radical damage (Meister and Anderson, 1983).

Ammonia. Vero cells produced ammonia in cultures of either bead, probably from the consumption of glutamine and other amino acids. Hassell et al. (1991) previously showed

similar ammonia concentrations (2.90 mM) from Vero cultures from 4.00 mM glutamine in the inoculum medium. However, Nahapetian et al. (1986) showed significantly higher ammonia (4.50 mM) concentrations in Vero cultures with 4.00 mM glutamine. This difference could be explained by the nutrient refeeding or different basal medium (L-15) used by the latter authors compared to the batch cultures of Hassell et al. (1991) and this study (GMEM and DMEM, respectively).

The specific ammonia production rate was significantly higher in Cytodex-1 compared to Cultispher-G, reflecting the low cell yields. However, the ammonia to glutamine ratios above (Table 6.7) were not significantly different in cultures of either bead and agree with the results (0.73 ammonia/glutamine) of Hassell et al. (1991). These ratios were both significantly lower (28%, $P < 0.01$) compared to Nahapetian et al. (1986) (1.13-1.30 ammonia/glutamine). Chotteau and Bastin (1994) also showed ammonia production by Vero cell microcarrier cultures.

The ammonia could either be utilised in the biosynthesis of purine and pyrimidine nucleotides or be excreted. Alanine could be produced from an excess of ammonia, liberated from the glutamate conversion to α -ketoglutarate. This ammonia could amidate pyruvate into alanine, which is excreted (Voet and Voet, 1990).

Serine. Serine (Table 6.3) was the second highest consumed amino acid and was almost totally consumed at maximum cell yield in cultures of either bead. Hence, serine was important to Vero cell growth. The specific serine consumption rate was significantly higher in Cytodex-1 cultures compared to Cultispher-G which again reflects the low cell yields. Serine was shown to be important for rabbit fibroblast RM 3-56, HeLa and human fibroblast (U-12) cell growth (Haff and Swim, 1957). However, the serine requirement of Vero cells contradicts the reports of Polastri et al. (1984), who showed serine production, and Chotteau and Bastin (1994), who had no serine consumption or production. Polastri et al. (1984) used a similar growth medium (DMEM with 10.0% v/v FBS) which suggests the nutrient results above should have been similar. However, Chotteau and Bastin (1994) used a different basal medium (M199) which could explain the

difference in serine consumption. The differences in maximum cell yields above (6.3.1) between DMEM, IMDM, DMEM/F12 and RPMI 1640 could be explained by differences in their initial serine concentrations (0.40, 0.40, 0.25, 0.28 mM, respectively). However, serine could not explain the low cell yields in L-15 or M-199 which both had significantly higher (478% and 20%, respectively) serine concentrations compared to DMEM.

Serine could be metabolised via transfer of its C_1 to tetrahydrofolate, producing N^5,N^{10} -methylene-tetrahydrofolate and glycine. This could explain the increased glycine concentration which was produced at a constant ratio to the serine concentration consumed. The N^5,N^{10} -methylene-tetrahydrofolate could be utilised for synthesis of either purine nucleotides or membrane precursors (Voet and Voet, 1990).

All other detectable amino acids which were consumed remained at above 50% of their initial concentrations at maximum cell yield in cultures of either bead. These included arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine. All these amino acids were previously reported to be consumed by Vero cells (Polastri et al., 1984 and Chotteau and Bastin, 1994), except tyrosine and they also agree with those consumed by rabbit RM3-56, HeLa and U-12 cells (Haff and Swim, 1957), except cysteine, which could not be analysed by the HPLC method used. Therefore, cysteine could also have been consumed by Vero cells but was probably not at a limiting concentration in DMEM.

Produced Amino Acids. Alanine, asparagine, glutamate and glycine were produced during Vero cell growth in cultures of either bead. Alanine and glutamate were the major produced amino acids. The production of alanine was shown for Vero cells by Polastri et al. (1984). The production of glutamate by Vero cell growth has not been described in the literature. Glutamate would result from glutamine metabolism (McKeehan, 1982). Alanine could be produced from an excess of pyruvate from glycolysis (Voet and Voet, 1990).

6.3.4 Optimisation of the Major Growth Nutrient Concentrations

Introduction. The results above (6.3.3) showed glucose, glutamine and serine were the major nutrients of DMEM for Vero cell growth in cultures of either bead. However, few, if any, reports in the literature have showed optimal nutrient concentrations for any cell line. An effective approach to defining optimal nutrient concentrations is by a matrix experiment where the concentration of one nutrient can be varied whilst holding others at constant concentrations. Therefore, a matrix experiment was performed to determine the optimal concentrations of glucose, glutamine and serine for maximum Vero cell yields in cultures of either bead.

Experimental. The Vero cultures were grown in spinner flasks, as described above (6.3.3) in DMEM containing various concentrations of glucose (10.00-32.50 mM), glutamine (2.00-8.00 mM) and serine (0.40-4.00 mM) to form a matrix.

Results. Table 6.9 shows maximum Vero cell yields in the matrix experiment cultures and Fig. 6.3 shows cell growth in cultures of either bead with constant 25.00 mM glucose and 0.40 mM serine and varied (between 2.00-8.00 mM) glutamine. Fig. 6.4 shows the lactate concentrations and ammonia concentrations produced at each glucose and glutamine concentration used. Table 6.10 shows the lactate to glucose ratios and ammonia to glutamine ratios with the various glucose and glutamine concentrations used. (1) Maximum Vero cell yields were affected by the concentrations of glucose and glutamine in the DMEM in cultures of either bead type and by serine in cultures of Cultispher-G. (2) Maximum cell yields (Table 6.9) were significantly higher (34%, $P < 0.01$) in Cultispher-G cultures compared to Cytodex-1 with respect to all nutrient concentrations used. (3) There were significantly higher maximum cell yields in Cytodex-1 cultures in DMEM at ≥ 17.50 mM glucose. In Cultispher-G cultures there were significantly higher ($P < 0.05$) maximum cell yields at ≥ 25.00 mM glucose. (4) Maximum cell yields in cultures of either bead were proportional to the glutamine concentration. Cell yields were significantly

Table 6.9 - The optimisation of the **glucose, glutamine and serine concentrations** for Vero cultures in 100 mL spinner flasks on **1.00 g/L Cytodex-1 (A)** and **1.70 g/L Cultispher-G (B)**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS enriched with 10.00-32.50 mM glucose, 2.00-8.00 mM glutamine at each glucose concentration and with 0.40-4.00 mM serine at each glutamine concentration. Spinner flasks were stirred at 40 rpm continuously and were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Table 6.9A - Cytodex-1.

Glutamine Concentration (mM)		Maximum Cell Concentration			
		(x 10 ⁶ /mL)			
		Glucose Concentration (mM)			
	Serine Concentration (mM)	10.00	17.50	25.00	32.50
2.00	0.40	0.90 ± 0.07	1.10 ± 0.08	0.97 ± 0.06	1.05 ± 0.07
	2.00	0.93 ± 0.07	1.11 ± 0.08	0.97 ± 0.06	1.08 ± 0.07
	4.00	0.94 ± 0.07	1.14 ± 0.07	1.09 ± 0.07	1.14 ± 0.07
4.00	0.40	0.93 ± 0.06	1.46 ± 0.11	1.46 ± 0.10	1.41 ± 0.07
	2.00	0.93 ± 0.06	1.52 ± 0.11	1.50 ± 0.10	1.49 ± 0.07
	4.00	0.97 ± 0.07	1.55 ± 0.12	1.51 ± 0.10	1.53 ± 0.11
6.00	0.40	0.90 ± 0.05	1.44 ± 0.10	1.51 ± 0.11	1.48 ± 0.09
	2.00	0.94 ± 0.05	1.49 ± 0.10	1.58 ± 0.11	1.49 ± 0.09
	4.00	0.92 ± 0.07	1.56 ± 0.11	1.62 ± 0.11*	1.52 ± 0.10
8.00	0.40	0.83 ± 0.06	1.34 ± 0.08	1.32 ± 0.08	1.22 ± 0.07
	2.00	0.84 ± 0.06	1.37 ± 0.08	1.39 ± 0.08	1.24 ± 0.07
	4.00	0.86 ± 0.05	1.46 ± 0.09	1.48 ± 0.09	1.26 ± 0.07

* - Highest cell yield.

Table 6.9B) - Cultispher-G.

Glutamine Concentration (mM)	Serine Concentration (mM)	Maximum Cell Concentration ($\times 10^6/\text{mL}$)			
		Glucose Concentration (mM)			
		10.00	17.50	25.00	32.50
2.00	0.40	0.89 ± 0.06	1.21 ± 0.08	1.37 ± 0.08	1.37 ± 0.07
	2.00	0.92 ± 0.06	1.25 ± 0.08	1.38 ± 0.08	1.42 ± 0.08
	4.00	0.91 ± 0.06	1.24 ± 0.07	1.39 ± 0.08	1.42 ± 0.09
4.00	0.40	1.23 ± 0.08	1.65 ± 0.11	2.08 ± 0.13	2.00 ± 0.12
	2.00	1.32 ± 0.09	1.75 ± 0.12	2.10 ± 0.13	2.04 ± 0.12
	4.00	1.33 ± 0.10	1.73 ± 0.12	2.11 ± 0.12	2.01 ± 0.12
6.00	0.40	1.21 ± 0.06	1.66 ± 0.11	2.40 ± 0.14	$2.42 \pm 0.13^*$
	2.00	1.27 ± 0.06	1.76 ± 0.11	2.40 ± 0.14	$2.42 \pm 0.13^*$
	4.00	1.28 ± 0.07	1.75 ± 0.11	$2.42 \pm 0.14^*$	$2.42 \pm 0.14^*$
8.00	0.40	1.21 ± 0.07	1.58 ± 0.11	2.01 ± 0.12	2.01 ± 0.12
	2.00	1.29 ± 0.08	1.68 ± 0.12	2.03 ± 0.12	2.05 ± 0.12
	4.00	1.26 ± 0.08	1.69 ± 0.12	2.04 ± 0.11	2.06 ± 0.13

* - Highest cell yield.

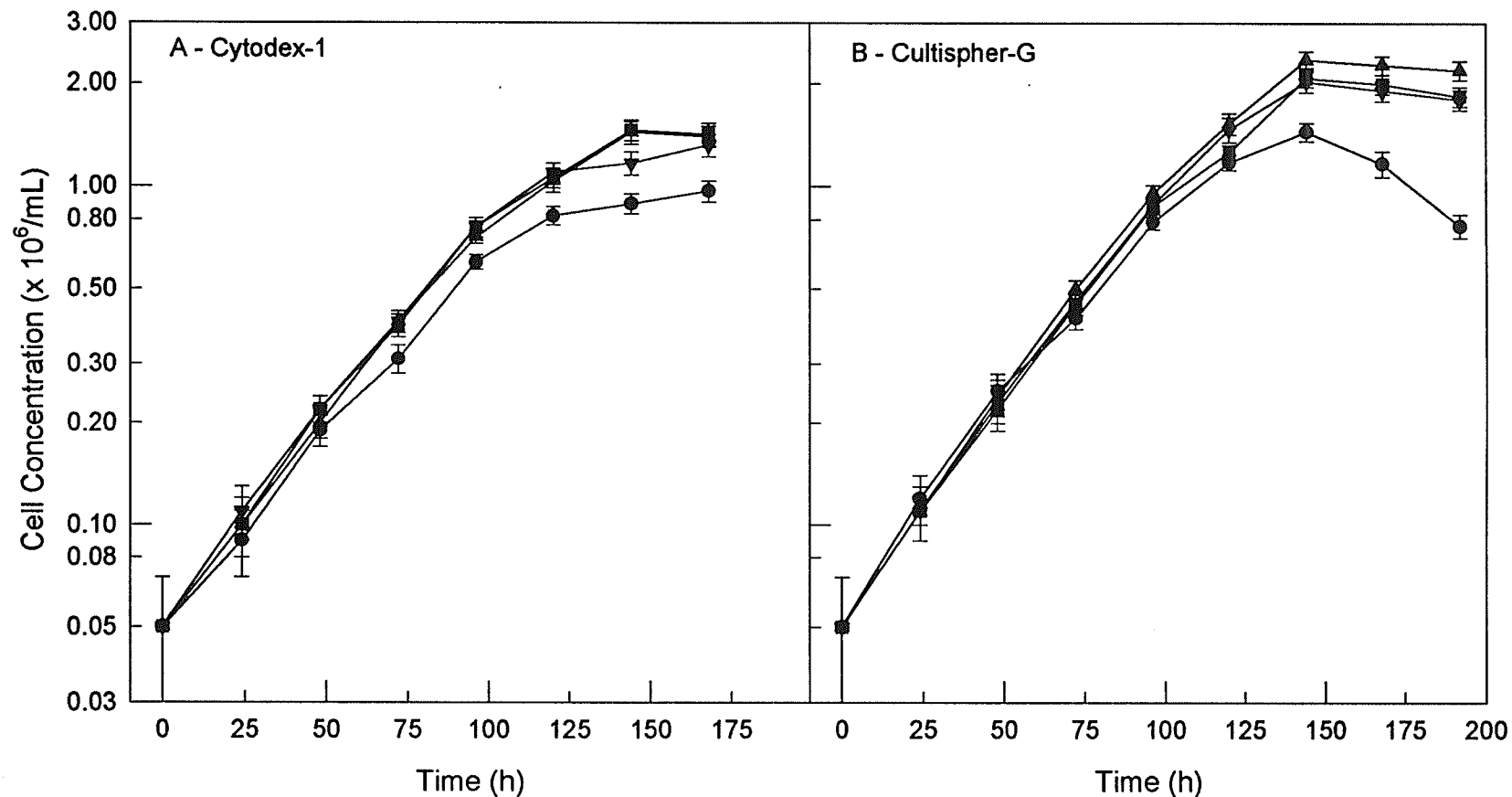


Fig.6.3 - The effect of the glutamine concentration on Vero cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A) and 1.70 g/L Cultispher-G (B). The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS enriched with 25.00 mM glucose, 0.40 mM serine and either $\bullet$ 2.00, $\blacksquare$ 4.00, $\blacktriangle$ 6.00 or $\blacktriangledown$ 8.00 mM glutamine. The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

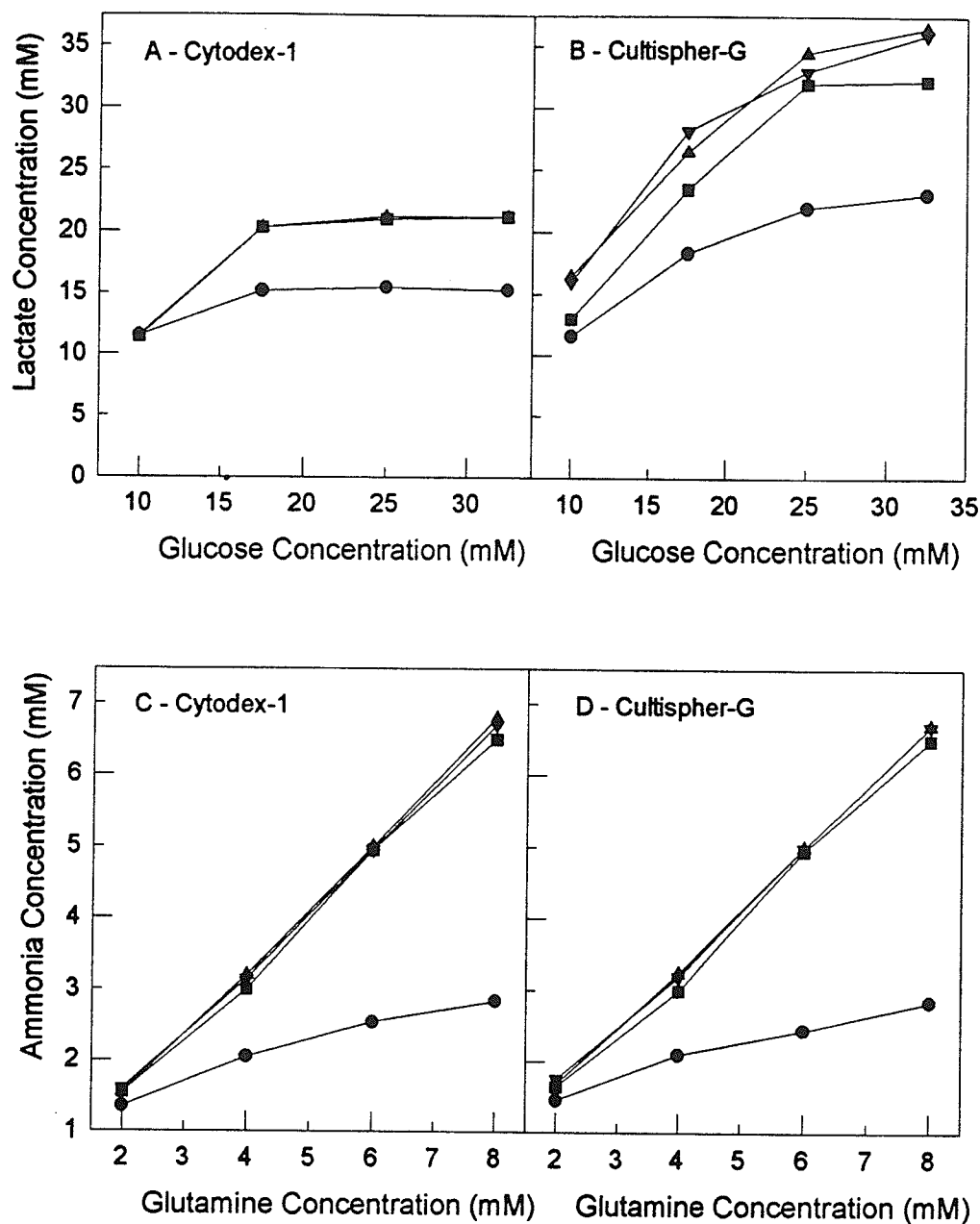


Fig. 6.4 - The effect of the **glucose** and **glutamine** concentration in the medium on the **lactate** and **ammonia** production at maximum Vero cell yields in 100 mL spinner flasks on **1.00 g/L Cytodex-1** (A and C, respectively) and **1.70 g/L Cultispher-G** (B and D, respectively) in DMEM + 5.0% v/v SCS. The DMEM was enriched with up to 32.50 mM glucose and either ● 2.00, ■ 4.00, ▲ 6.00 or ▼ 8.00 mM glutamine (plots A and B) or up to 8.00 mM glutamine and ● 10.00, ■ 17.50, ▲ 25.00 or ▼ 32.50 mM glucose (plots C and D). The cells were inoculated at 0.05×10^6 cells/mL. The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means of cultures with 0.40-4.00 mM serine and are from $n=6$.

Table 6.10 - The effects of the **glucose** and **glutamine concentrations** in the medium on the Vero cell **lactate to glucose ratio** and **ammonia to glutamine ratio** produced in 100 mL spinner flask cultures of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS containing various concentrations of glucose (up to 32.5 mM) and glutamine (up to 8.00 mM). The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means from cultures with 0.40-4.00 mM serine and are from n=6.

Glucose Concentration (mM)	Lactate to Glucose Ratio Produced with the Glutamine Concentration Used			
	Cytodex-1			
	2.00	4.00	6.00	8.00
10.00	1.16	1.15	1.17	1.17
17.50	1.17	1.20	1.19	1.21
25.00	1.19	1.18	1.18	1.18
32.50	1.19	1.18	1.18	1.18
Cultispher-G				
10.00	1.17	1.31	1.67	1.62
17.50	1.07	1.37	1.47	1.64
25.00	1.39	1.36	1.47	1.41
32.50	1.43	1.40	1.48	1.35
	Ammonia to Glutamine Ratio Produced with the Glutamine Concentration Used			
	Cytodex-1			
10.00	0.71	0.72	0.72	0.71
17.50	0.80	0.78	0.86	0.84
25.00	0.80	0.81	0.84	0.86
32.50	0.82	0.80	0.85	0.84
Cultispher-G				
10.00	0.74	0.75	0.74	0.71
17.50	0.83	0.79	0.84	0.83
25.00	0.86	0.82	0.87	0.84
32.50	0.81	0.81	0.87	0.84

higher ($P < 0.05$) in Cytodex-1 cultures at 4.00-6.00 mM glutamine and in Cultispher-G cultures at 6.00 mM glutamine compared to all other concentrations used. (5) There was no significant difference in maximum cell yields in Cytodex-1 cultures with respect to any serine concentration used. However, maximum cell yields in Cultispher-G cultures were significantly higher ($P = 0.05$) at ≥ 2.00 mM serine compared to the other concentrations used. (6) The total lactate production (Fig. 6.A)) in Cytodex-1 cultures was proportional to the initial glucose concentration with respect to all glutamine concentrations, except at 2.00 mM glutamine. The total lactate production (Fig. 6.4B)) in Cultispher-G cultures was proportional to the initial glucose and glutamine concentrations. There were significantly higher (44%, $P < 0.01$) total lactate concentrations produced in Cultispher-G cultures compared to Cytodex-1. There were significantly lower (54%, $P < 0.01$) total lactate concentrations in cultures of either bead at 2.00 mM glutamine with respect to all glucose concentrations used. (7) The ammonia production (Fig. 6.4C)-D)) was proportional to the initial glutamine concentration in cultures of either bead with respect to all the glutamine and glucose concentrations used. (8) The lactate to glucose ratios (Table 6.10) were significantly higher (20%, $P < 0.05$) with respect to all glucose and glutamine concentrations used in Cultispher-G compared to Cytodex-1 cultures. The total lactate concentration and the lactate to glucose ratio were significantly higher (13%, $P = 0.05$) in Cultispher-G cultures at 25.00 and 32.50 mM glucose with 6.00 and 8.00 mM glutamine compared to all other glucose and glutamine concentrations used. (9) There was (Table 6.10) no significant difference in the ratio of ammonia to glutamine (0.81) in cultures of either bead with respect to the glucose and glutamine concentrations used. The ammonia to glutamine ratios were significantly lower (14%, $P < 0.05$) in cultures of either bead at 10.00 mM glucose compared to all other glucose concentrations used with respect to all glutamine concentrations used. (10) There was no significant difference in the cell growth rates ($0.035 \pm 0.003 \text{ h}^{-1}$) in cultures of either bead with respect to the nutrient concentrations used.

Free Cells. After maximum Vero cell yield was reached cells appeared to detach from either

bead. The appearance of free cells was dependent on the glucose concentrations present in the cultures. There were no free cells in cultures of either bead 72 h after maximum cell yield at 32.50 mM glucose with respect to all the glutamine and serine concentrations used. However, the time free cells first appeared after maximum cell yield was proportional to the initial glucose concentration in Cultispher-G cultures. The free cells were seen earliest (24 h) with 10.00 and 17.50 mM glucose. Free cells were also found 24 h after maximum cell yield at 2.00 mM glutamine with respect to all the glucose concentrations used. Free cells were only seen in Cytodex-1 cultures at 10.00 and 17.50 mM glucose (24-48 h after maximum cell yield). Large numbers of free cells (>40%) in cultures of either bead formed immediately after the maximum cell yield at 10.00 mM glucose.

Discussion. The results in this study show Vero cell growth in cultures of either bead was highly dependent on the glucose and glutamine concentration in the DMEM. However, maximum cell yields were dependent more on the glutamine which confirms the results above (6.3.3). The optimal glutamine concentrations also differed between the beads in this study showing there are several bead dependent nutrient differences. Cells consumed the major nutrients (serum, glucose, glutamine and serine) more efficiently in Cultispher-G compared to Cytodex-1 cultures. The differences could also be explained by cellular attachment mechanism differences between the bead types, as described above (6.3.2).

The similar ammonia to glutamine ratios in cultures of either bead suggest the fate of the glutamine was the same no matter what the substrate was. However, the reduced lactate to glucose ratios in Cytodex-1 cultures could implicate glucose in a major biosynthesis required for cell growth, as less was excreted as a waste product.

The free cell dependence on the residual glucose concentration suggests glucose must be involved in maintaining Vero cell attachment and viability. This could be explained by the cells utilising glucose for anabolic substrates, as with HeLa cells (Wice et al., 1974) or for cell adhesion through glycosylation of membrane gangliosides (e.g. integrins). Kleinman et al.

(1981) showed glucose starvation affected sialyl residues of glycoproteins and Akiyama et al. (1990) showed that cell adhesion was lost if the Golgi body, the site of glycosylation reactions (Goochee and Monica, 1990, Voet and Voet, 1990 and Fast et al., 1993), function was affected. Kleinman et al. (1981) showed the loss of sialylation lead to inactive BHK cell integrin receptors and ultimately loss of cell adherence. The cell detachment results in this study could be explained by lack of glycoprotein sialylation. However, glucose starvation could also affect internal energy and nucleotide pools such as adenosine triphosphate (ATP) or UDP-N-Acetyl-Galactosamine or UDP-N-Acetyl-Glucosamine (Ryll et al., 1994). A depletion in the internal pools could significantly affect many metabolic processes involved with cell viability, glycosylation and attachment. Grinnell (1982) showed human fibroblast cell attachment to substrata was prevented by inhibiting electron transport pathways and glycolysis.

The nutrient results in Cytodex-1 cultures confirm cells required another important growth factor which was independent of the major basal medium nutrients. This could implicate a serum factor but a matrix experiment with serum concentrations was not performed in this study.

Refed cultures of Cultispher-G beads were shown to reach higher (an order of magnitude) Vero and CHO cell yields (Reiter et al., 1990) compared to Cytodex-1 beads. The batch results in this study also show Cultispher-G cultures reaching higher yields. However, the previous authors suggested the higher surface area for cell growth in macroporous beads was responsible for this effect whereas, this study suggests cellular nutrient consumption could also be involved.

6.4 Conclusions

The purpose of this study was to determine the major nutrients for Vero cell growth in batch microcarrier cultures and to optimise their concentrations. The results above suggest that:

- **significantly higher cell yields** (30-40%, $P < 0.01$) were produced in Cultispher-G compared to Cytodex-1 cultures, with the same medium and with semi-optimised physical parameters.

- **the preferable basal media** for Vero cell growth were DMEM and IMDM. However, IMDM

was significantly more expensive (210%, $P < 0.01$) compared to DMEM. Maximum cell yields were significantly lower (41%, $P < 0.01$) in DMEM/F12, L-15, M199, Optimem-1 and RPMI 1640 with 5.0% or 2.0% v/v iron enriched, supplemented calf serum (sCS).

- **the preferable serum types** were sCS and foetal bovine serum (FBS) in cultures of either bead. However, FBS was significantly (342%) more expensive, so the sCS was the preferable serum. Maximum cell yields were significantly lower (18%, $P < 0.05$) with calf serum (CS).

- **the optimal serum concentration** was 5.0% v/v in cultures of either bead with all serum types used in this study. Maximum Vero cell yields in cultures of either bead were proportional to the serum concentration supplemented with CS at $< 5.0\%$ v/v.

- **the major nutrients** for batch Vero cell growth were glucose, glutamine and serine.

- **the optimal glucose concentration** was 17.50 mM for Cytodex-1 and 25.00 mM for Cultispher-G cultures. Complete glucose consumption resulted in cell detachment from either microcarrier. Hence, glucose metabolism could be involved in cellular attachment and adherence factor biosynthesis or in regulation of internal nucleotide pool concentrations.

- **the optimal glutamine concentration** was 4.00 mM for Cytodex-1 and 6.00 mM for Cultispher-G cultures.

- **the optimal serine concentration** was 0.40 mM for Cytodex-1 and 4.00 mM for Cultispher-G cultures. The differences in maximum cell yields between basal media could be explained by differences in their initial serine concentrations (0.40, 0.40, 0.25, 0.28 mM, respectively), excepting L-15 or M-199, both of which had significantly higher (478% and 20%, respectively) serine concentrations compared to DMEM.

- **many other amino acids** (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine) were consumed to $< 50\%$ of their original concentration by Vero cell growth.

- **specific nutrient consumption and production rates** were significantly higher (27%, $P < 0.05$) in Cytodex-1 cultures compared to Cultispher-G, except glucose.

Chapter 7. Optimisation of Growth Medium for Vero Cell Microcarrier cultures.

2. Minor Nutrients.

7. Optimisation of Growth Medium for Vero Cell Microcarrier Cultures. 2. Minor Effects.

7.1 Introduction

The major nutrients for the batch growth of African green monkey kidney (Vero) cells in Cytodex-1 and Cultispher-G microcarrier cultures are glucose, glutamine and serine (6.3.3). Normally, these are added to basal medium when purchased in liquid or powdered forms (Gibco, 1992) as all are stable when solid. However, glutamine naturally decomposes in solutions (Lin and Agrawal, 1988). Glutamine decomposition is significantly higher (232-327%) at (i) 10.0% v/v FBS compared to serum-free at pH 7.2 and 35.5°C, (ii) at pH 7.6 compared to pH 7.2 and (iii) at 35.5°C compared to 4°C. Therefore, the storage age of the medium could affect Vero cell growth. No previous reports could be found in the literature defining growth in stored medium.

Serum contains many growth promoting factors such as insulin, transferrin, epidermal and fibroblastic growth factors (EGF and FGF) and fibronectin which could naturally decompose or precipitate out proportional to the storage temperature and time (Barnes et al., 1984). Serum can be purchased either in 500 or 100 mL bottles and could be stored at 4°C for >30 d prior to complete usage. This could also influence the cell growth potential. However, no reports were found defining the effects of storing thawed serum on Vero cell growth.

Others have substituted glucose and glutamine with alternative compounds to reduce the production of lactate and ammonia which are potential growth inhibitors of many murine and human cell lines (Ryan and Cardin, 1966, Butler and Spier, 1984, Glacken et al., 1986, Kimura et al., 1987, Duval et al., 1992 and Rutz and Little, 1995). Glucose has been successfully replaced by fructose, galactose, maltose and mannose for Madin-Darby canine kidney (MDCK), Vero and murine 3T3 cells (Rheinwald and Green, 1974, Barngrover et al., 1985, Nahapetian et al., 1986 and Mendonca et al., 1993). Glutamine has been successfully replaced by glutamate and asparagine for baby hamster kidney (BHK), Chinese hamster ovary (CHO), McCoy and Vero cells (Hassell and Butler, 1990 and Kurano et al., 1990).

Many other parameters could also influence Vero cell growth. Pyruvate from glycolysis can

remove lag phases of human and murine cell lines after subculturing in a similar way to incorporating cell "conditioned" medium in the following subculture (O'Donnell-Tormey et al., 1987). The authors suggested the action of pyruvate was similar to extracellular catalase and could involve the removal of H_2O_2 to prevent membrane damage.

Ceccarini and Eagle (1971) and Ceccarini (1975) showed pH was critical for growth and attachment of KL-2, HeLa and WI-38 cells. Maximum Vero cell yields (5.3.5) in cultures of either bead had an optimal range of pH 6.8-7.1 which was lower compared to DMEM (44.00 mM bicarbonate) (Gibco, 1992) equilibrated with a 10% v/v CO_2 overlay (pH 7.5). $pH \geq 7.5$ resulted in long lag phases (>15 h) and low cell yields (5.3.5). No one has reported if lowering the buffer concentration improves the pH and therefore, cell yields.

The Ca^{2+} content in the medium is important to human fibroblast cell adhesion (Curtis and McMurray, 1986) and growth (Lengheden, 1994). However, the latter author showed the growth response to Ca^{2+} was reduced with decreased membrane charges at high culture pH ($pH > 7.5$). Ng (1995) previously showed Ca^{2+} could be more important than serum proteins for Vero cell adhesion to microcarriers. Thus, the Ca^{2+} concentration could affect cell yields.

Many amino acids present in DMEM were consumed during Vero cell growth (6.3.3) and their concentrations could have affected maximum yields. These were all reported previously to be consumed by Vero cells, except tyrosine (Polastri et al., 1984 and Chotteau and Bastin, 1994) and also agree with the amino acids consumed by HeLa, human (U-12) and rabbit (RM3-56) fibroblast cell growth (Haff and Swim, 1957). However, no one has determined whether the concentrations of these less consumed amino acids require further supplementation to improve Vero cell growth in microcarrier culture.

The daily refed growth of Vero cells in microcarrier spinner flasks previously reached higher cell yields (25%) in Leibovitz L-15 medium diluted (1:1) with phosphate buffered saline solution (PBS) compared to original L-15 (Nahapetian et al., 1986). This could be due to reductions in either concentrations of inhibitory components (6.3.1) or the medium osmolarity to an optimal level for growth (Waymouth, 1970). No one has determined which is more important.

This paper presents an evaluation of the effects of several minor medium nutrients on the batch growth of Vero cells in T25 flasks or spinner flasks of Cytodex-1 or Cultispher-G to assess growth differences between the beads. The parameters investigated were: (a) medium and serum storage time at 4°C, (b) substitution of glucose and glutamine with alternative substrates, (c) reduction of the bicarbonate concentration, (d) addition of pyruvate to fresh and stored medium, (e) increased Ca^{2+} concentrations, (f) supplementation of minor amino acids and (d) dilution of basal medium with nutrient supplemented D-PBS⁻ (with and without Ca^{2+}).

7.2 Materials and Methods

The methods for culturing and counting the cells and analyses of medium nutrients have been described previously (2.1-2.5).

7.2.1 Preadaptation of Cell Stocks

The Vero cells were grown unadapted or preadapted to the media used in the experiments either (a) as described previously (2.1) or (b) to glutamate containing DMEM by the methods that follow.

(i) The method of Hassell and Butler (1990) was to inoculate the cells at 0.20×10^6 cells/mL and grow them in T75 culture flasks in DMEM + 5.0% v/v iron enriched, supplemented calf serum (sCS) (containing 4.0 mM glutamine) (35 mL). Once the cells reached 80% confluency the medium was replaced with DMEM + 5.0% v/v sCS containing glutamate (20.00 mM) with no glutamine. The cells were grown until the yield increased ($\geq 10\%$) and were recovered by the standard trypsinisation procedure (2.2). The cells were inoculated in fresh T75 flasks and passaged twice at each subculture dilution below following growth to confluency at each glutamate concentration used. The recovered cells were diluted (1:2 and 1:3) in DMEM (20.00 mM). One flask was retained at high glutamate and a second was grown in a gradually reducing glutamate concentration. Low glutamate cells were diluted (1:4) at 15.00 and 10.00 mM glutamate and (1:5) at 6.00 mM glutamate prior to use.

(ii) Gradually adapted cells were subcultured (0.50×10^6 cells/mL) in T75 culture flasks every 48 h with various dilutions of DMEM + 5.0% v/v sCS containing either glutamine (4.00 mM) or glutamate (20.00 mM). Every two subcultures the proportion of glutamine containing DMEM was reduced (50%). After two passages in DMEM containing 0.25 mM glutamine the cells were subcultured in DMEM containing only glutamate (20.00 mM). The cells were subcultured twice and one flask was retained at the high glutamate concentration and a second was adapted to low glutamate (6.00 mM) by subculturing the cells twice at 12.00 mM and 6.00 mM glutamate prior to use.

7.3 Results and Discussion

7.3.1 Growth Medium Storage Time

Introduction. Glutamine is the most important nutrient for Vero cell growth in DMEM in cultures of either Cytodex-1 or Cultispher-G. However, glutamine naturally decomposes in stored solutions proportional to the time, pH, temperature and serum concentration. Significant proportions (20%) of the initial glutamine would decompose in DMEM at 5.0% v/v FBS stored at 4°C for 28 d and could result in reduced cell yields in cultures using this media. No previous reports were found in the literature describing cell yields in stored media. Therefore, this study determined the effect of the DMEM storage time at 4°C on Vero cell growth in microcarrier cultures to define the optimal medium batch preparation size and storage conditions.

Experimental. The natural decomposition of glutamine in the medium was determined by adding DMEM + 5.0% v/v iron enriched, supplemented calf serum (sCS) into a 100 mL spinner flask. The DMEM contained 4.00 mM glutamine and either (a) 36.00 mM bicarbonate preadjusted to pH 7.2 or (b) 44.00 mM bicarbonate preadjusted to pH 7.5 with 2.00 M NaOH and 1.00 M HCl. Flasks were stirred continuously at 40 rpm and 37°C. The pH was readjusted daily to the original values.

Stock Vero cells were recovered by the standard trypsinisation protocol (2.2) and counted by the trypan blue method (2.5.1-2). The cells were inoculated in spinner flasks (2.4.3) in DMEM + 5.0% v/v sCS prepared (2.3) and stored at 4°C for various times (up to 28 d) $\pm$ 5.0% v/v sCS.

Results. Fig. 7.1 shows the glutamine decomposition in DMEM + 5.0% v/v sCS at 37°C in spinner flask cultures stirred continuously at 40 rpm at pH 7.2 and 7.6 and Vero cell growth in cultures of either Cytodex-1 or Cultispher-G in DMEM media stored $\pm$ sCS for various times. Table 7.1 shows the cell growth rates and lag phases in cultures of either bead with the various stored media. (1) There were significantly higher (155%, $P < 0.01$) glutamine decomposition rates (Fig. 7.1) at pH 7.5 compared to pH 7.2 with respect to serum containing or serum-free DMEM. (2) Glutamine decomposition rates were significantly higher (74%, $P < 0.01$) in serum containing compared to serum-free DMEM with respect to either pH used. (3) Maximum cell yields (Fig. 7.1B-C)) were significantly higher (29%, $P < 0.01$) in Cultispher-G cultures compared to Cytodex-1 with respect to all the media used. (4) The analyses allowed the media storage to be ranked and showed significantly higher ($P < 0.05$) maximum cell yields in Cytodex-1 cultures in DMEM stored serum-free for up to 28 d or stored with serum for 14 d compared to stored with serum for 28 d. Maximum cell yields in Cutispher-G cultures were significantly higher ($P < 0.05$) in DMEM stored serum-free for 14 d compared to all other storage formulations and times. (5) There (Table 7.1) was no significant difference in the cell growth rates in cultures of either bead with respect to all the stored media used.

(6) Lag phases (Table 7.1) were observed in cultures of either bead and were proportional to the DMEM storage age and serum composition. Lag phases were significantly longer in all stored media types (between 100-800%, $P < 0.01$) and in DMEM stored containing serum compared to serum-free for 28 d (100%, $P < 0.01$).

Discussion. The natural glutamine decomposition rates (Fig. 7.1) from DMEM + 5.0% v/v sCS at 37°C in this study were similar to those of Lin and Agrawal (1988) at similar temperature and

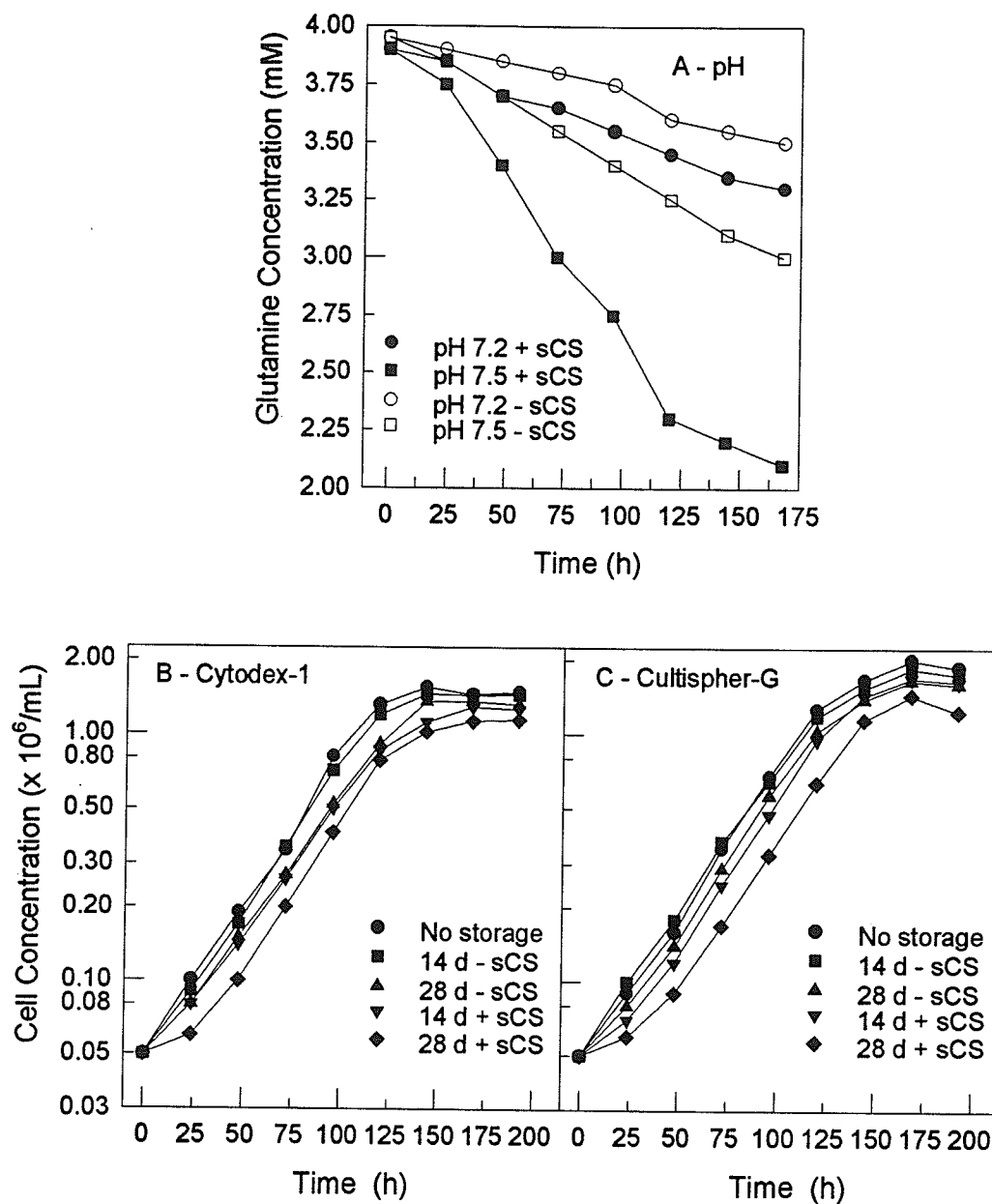


Fig. 7.1 - The effect of either pH at 37°C on natural glutamine decomposition (A) or storage time at 4°C of DMEM $\pm$ 5.0% v/v sCS on Vero cell yields on 1.00 g/L Cytodex-1 (B) or 1.70 g/L Cultispher-G (C) in 100 mL spinner flasks. Flask A had no cells and B and C were inoculated at 0.05×10^6 cells/mL. Flasks were stirred either at 40 rpm continuously (A and B) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (C). Flasks in A were readjusted daily to the initial pH values and flasks in B and C were readjusted to pH 7.1 from 72 h onwards with 2.00 M NaOH or 1.00 M HCl. The points are means of $n=2$.

Table 7.1 - The effect of the **DMEM storage composition** and **time** at **4°C** on the Vero cell **growth rate** and **lag phases** in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS prepared either on the day of use or stored for 14 or 28 d with and without serum. The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

DMEM Storage Protocol	Cell Growth Rate and Lag Phase in the Stored Media Used			
	Cytodex-1		Cultispher-G	
	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)
0 d	0.034 ± 0.003	3.00 ± 1.00	0.037 ± 0.003	3.00 ± 1.00
14 d Without Serum	0.034 ± 0.003	6.00 ± 1.00	0.036 ± 0.003	6.00 ± 1.00
14 d With Serum	0.033 ± 0.003	12.00 ± 1.00	0.035 ± 0.003	15.00 ± 1.00
28 d Without Serum	0.033 ± 0.003	12.00 ± 1.00	0.035 ± 0.003	12.00 ± 1.50
28 d With Serum	0.031 ± 0.003	24.00 ± 2.00	0.034 ± 0.003	24.00 ± 2.00

pH values (pH 7.2 - 0.077 mmol.d⁻¹ and pH 7.5 - 0.196 mmol.d⁻¹). The glutamine decomposition was not determined following storage at 4°C for 28 d in this study but could be expected to have reduced by a similar amount to that of the previous authors (i.e. approximately 13%).

Maximum Vero cell yields were proportionally and significantly lower with the increasing medium storage time, as could be expected from the glutamine decomposition which would have occurred. However, maximum cell yields were similar in DMEM prepared fresh or stored serum-free for up to 28 d. All maximum cell yield reductions could be correlated to the amount of glutamine decomposition during the storage. The results show DMEM could be prepared in litre-sized batches and stored serum-free for 28 d without affecting cell yields.

7.3.2 Serum Storage Time Before Use

Introduction. The previous study (7.3.1) showed complete DMEM could not be prepared and stored supplemented with serum for long periods without significantly reducing cell yields. The iron enriched, supplemented calf serum (sCS) could be stored at 4°C for long time periods prior to use which could result in losses of many serum proteins. In previous studies (6.3.2) Vero cell growth was shown to be highly dependent on the sCS concentration in cultures of either bead. Therefore, this study determined the effects of using serum stored at 4°C for various times on Vero cell growth in microcarrier cultures to define how long thawed sCS could be stored without affecting cell growth.

Experimental. Vero cultures were grown in spinner flasks (7.3.1) in DMEM prepared (2.3) immediately prior to use and supplemented at 5.0% v/v sCS. The sCS was thawed and stored at 4°C for various periods of time (up to 28 d) prior to medium supplementation.

Results. Table 7.2 shows maximum Vero cell yields and growth rates in cultures of Cytodex-1

Table 7.2 - The effect of the **thawed age** of the **sCS** on the maximum Vero cell yield and growth rate in 100 mL spinner flasks on **1.00 g/L Cytodex-1** or **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS. The sCS was thawed and stored at 4°C for either 0, 14 d or 28 d prior to use. The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Serum Storage Time (d)	Maximum Cell Concentration ($\times 10^6$ cells/mL)	
	Cytodex-1	Cultispher-G
0	$1.43 \pm 0.11^*$	$1.99 \pm 0.13^*$
14	$1.43 \pm 0.11^*$	1.90 ± 0.13
28	1.37 ± 0.10	1.93 ± 0.13
	Specific Cell Growth Rate (h^{-1})	
0	0.035 ± 0.003	0.037 ± 0.003
14	0.035 ± 0.003	0.037 ± 0.003
28	0.034 ± 0.003	0.037 ± 0.003

* - Highest cell yield.

and Cultispher-G in DMEM supplemented with the various stored sCS solutions. (1) Maximum Vero cell yields in cultures of either bead were not affected by the serum storage time. (2) There were significantly higher (38%, $P < 0.01$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the media used. (3) There were no significant differences in cell growth rates with respect to any sCS storage time used.

Discussion. The results (Table 7.2) showed the serum storage time (up to 28 d) at 4°C did not affect Vero cell growth in cultures of either bead. This was not expected, as fibronectin and insulin concentrations in the serum could have been highly reduced or completely removed after 28 d of storage. Fibronectin concentrations are already significantly reduced (90%) during serum clotting and processing (Hayman et al., 1985) and further losses could significantly influence the growth stimulation. Hughes et al. (1979) showed fibronectin was required by BHK cells from young animals for adherence and growth but was only required for adherence with Vero cells from older animals. The results above could be explained by this effect. However, maximum cell yields suggest insulin and fibronectin were either not required at high concentrations or were not the major serum proteins for Vero cell growth in microcarrier cultures. Hence, the important growth promoting components in the serum were not sensitive to storage at 4°C for up to 28 d.

Ng (1995) previously reported serum and therefore, fibronectin were not required for Vero cells to attach to either microcarrier type. The cell attachment rate was higher in Cultispher-G cultures when the beads were precoated with serum but previous studies (6.3.2) suggested cell aggregation in serum supplemented DMEM and not fibronectin caused this effect. Collagen or other attachment factors could also have affected such attachment (Doillon et al., 1987).

7.3.3 Substitution of Glucose

Introduction. Glucose metabolism in Vero cultures was shown previously (6.3.3) to produce lactate at high ratios ($>1:1$) per millimole of glucose consumed. Lactate inhibits cell growth and

also reduces the culture pH. Glucose has been replaced by fructose, galactose, maltose and mannose for cell growth to lower the lactate production. No one has previously reported whether Vero cell yields are affected by growth in medium containing alternative sugars to glucose. Therefore, this study determined the optimal sugar for Vero cell growth in microcarrier cultures.

Experimental. Stock Vero cells were preadapted (6 passages) to glucose-free DMEM + 5.0% v/v sCS containing either fructose, galactose, maltose or mannose (25.00 mM) and glutamine (4.00 mM) (2.1). The cells were passaged ($> \times 6$) in each medium with subcultures (1:6 dilutions) every 72 h. The cultures were grown in spinner flasks, as described above (7.3.1).

Results. Fig. 7.2 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G in DMEM + 5.0% v/v sCS containing various sugars. Table 7.3 shows the cell growth rates and lag phases in cultures of either bead with the various sugars used. (1) Vero cell growth in cultures of either bead was affected by the sugar used in the DMEM. (2) Maximum cell yields were significantly higher (19%, $P < 0.05$) in Cultispher-G cultures compared to Cytodex-1 with respect to all the sugars used. (3) There were significantly higher ($P < 0.05$) maximum cell yields in cultures of either bead in DMEM + 5.0% v/v sCS containing glucose or maltose compared to all other sugars used. (4) There (Table 7.3) were significantly higher cell growth rates in cultures of either bead in DMEM containing glucose, fructose or maltose (22%, $P < 0.05$) compared to either galactose or mannose. (5) The lag phases (Table 7.3) in cultures of either bead were significantly shorter ($P < 0.01$) with glucose compared to all other sugars used.

pH. The pH fluctuated during cell growth in cultures of either bead and declined from pH 7.4 to < 6.7 in Cultispher-G cultures and to pH < 7.0 in Cytodex-1 after 72 h in DMEM containing glucose. However, the pH declined from pH 7.4 to 7.1 in cultures of either bead with respect to all the other sugars used. A reduced lactate concentration could explain this effect but was not determined in this study.

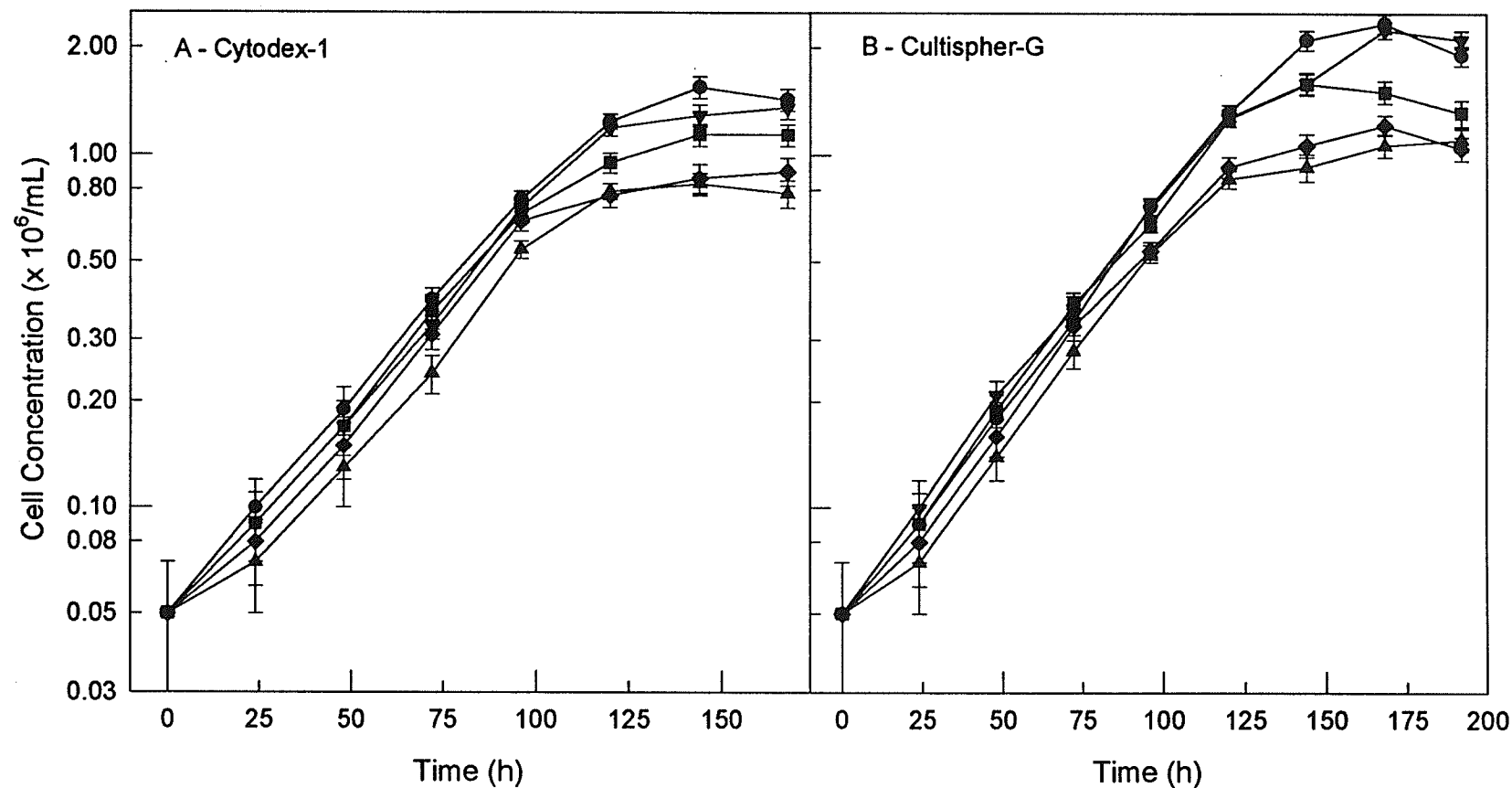


Fig. 7.2 - The effect of alternate carbohydrate sources on Vero cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A) and 1.70 g/L Cultispher-G (B). The cells were preadapted and inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS prepared with 6.00 mM glutamine and either 25.00 mM ● glucose, ■ fructose, ▲ galactose, ▼ maltose or ◆ mannose. The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

Table 7.3 - The effect of the DMEM sugar on the Vero cell growth rate and lag phases in 100 mL spinner flasks on 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS prepared containing 25.00 mM of either fructose, galactose, glucose, maltose or mannose. The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means of $n=2$.

DMEM Sugar	Cell Growth Rate and Lag Phase in DMEM with the Sugar Used			
	Cytodex-1		Cultispher-G	
	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
Fructose	0.031 ± 0.003	6.00 ± 1.00	0.035 ± 0.003	6.00 ± 1.00
Galactose	0.028 ± 0.003	12.00 ± 1.00	0.027 ± 0.003	12.00 ± 1.00
Glucose	0.034 ± 0.003	3.00 ± 1.00	0.033 ± 0.003	3.00 ± 1.00
Maltose	0.032 ± 0.003	6.00 ± 1.00	0.033 ± 0.003	3.00 ± 1.00
Mannose	0.025 ± 0.002	9.00 ± 1.00	0.027 ± 0.003	9.00 ± 1.00

Discussion. Vero cells (Fig. 7.2) could grow in DMEM + 5.0% v/v SCS containing all the sugars used in this study agreeing with previous reports with MDCK, Vero and 3T3 cells (Rheinwald and Green, 1974, Barngrover et al., 1985, Nahapetian et al., 1986 and Mendonca et al., 1993). However, maximum cell yields were significantly higher with glucose and maltose compared to all the other sugars used. Glucose was previously removed from culture medium to reduce the lactate production and therefore, to prevent any possible cell growth inhibition.

Similar cell growth with glucose and maltose could be expected as maltose could be enzymically cleaved into two glucoses. The results suggest Vero cells could cleave maltose. Crespi et al. (1981) previously grew MDCK cells successfully in medium with maltose at a low concentration (5.00 mM) and produced lower lactate concentrations compared to glucose. The Vero cells in this study required less pH readjustment during growth with maltose compared to glucose which supports a reduced lactate concentration, agreeing with the previous authors. However, lactate was not monitored to confirm this.

Vero cells also grew with fructose but to significantly lower maximum yields compared to glucose and maltose. Barngrover et al. (1985) previously showed MDCK cells grew similarly with glucose and fructose at 10.00 mM but maximum cell yields were reduced at 20.00 mM fructose compared to glucose. The Vero cell results above would support this. The authors suggested fructose maintained a better pH and a favourable pyruvate to lactate ratio for cell growth. Imamura et al. (1982) showed MDCK cells produced less lactate from fructose compared to glucose. However, the Vero cells in this study still required pH readjustment during growth with fructose but a lower volume of 2.00 M NaOH was required to maintain optimal pH compared to glucose. Hence, fructose could also be producing lower lactate concentrations.

MDCK cells in galactose and HeLa and L cells in mannose grow to similar cell yields but with significantly slower growth rates compared to glucose. (Eagle et al., 1958 and Barngrover et al. 1985). Maximum Vero cell yields in this study were significantly lower with both galactose and mannose which disagrees with the previous authors. However, cell growth rates were reduced and lag phases increased with galactose and mannose which agrees with the previous authors

results.

Wice et al. (1974) and Barsh and Cunningham (1977) showed glucose metabolism is separated from DNA synthesis and growth in HeLa and human fibroblast cells (i.e. was required for the biosynthesis of anabolic substrates), as it could be completely replaced by cytidine and uridine nucleosides. Growth with alternative sugars could be explained by the cells requiring small amounts of glucose for particular components such as protein glycosylation (Kleinman et al., 1981) or internal nucleotide pool maintenance (Ryll et al., 1994). All the alternative sugars produce glucose via gluconeogenesis or glucose could be generated from glutamine by this pathway (Voet and Voet, 1990).

The extended lag phases in cultures with galactose and mannose could be evidence of differences in the metabolism of these sugars. The glucose or metabolic intermediates of glucose could be supplied more easily from fructose and maltose rather than from galactose or mannose. Gluconeogenesis from galactose and mannose requires 6 and 4 reactions and from fructose and maltose requires 3 and 1, respectively (Voet and Voet, 1990). If any reactions are inhibited or occur at slow rates, then cell growth could be reduced. The cell yields in this study were proportional to the number of gluconeogenesis reactions for each sugar used.

Galactose and mannose could also have complex uptake mechanisms with increased energy usage for membrane transport or are not taken up at all by the cells. The latter could mean that some glutamine has to be diverted into gluconeogenesis which would result in reduced cell growth.

7.3.4 Substitution of L-Glutamine

Introduction. Glutamine is the most important growth nutrient for Vero cells (6.3.3-4). The cell yields could be reduced by glutamine naturally decomposing (7.3.1) or from the ammonia produced which could inhibit cell growth. Vero cells have been cultured with more stable glutamate in the absence of glutamine which also significantly reduced the ammonia produced,

as glutamate has only one amino group compared to two for glutamine. Therefore, this study determined the optimal major amino acid for the growth of Vero cells in microcarrier cultures.

Experimental. Stock Vero cells were preadapted (6 passages) (7.2.1) to DMEM prepared (2.3) with either (a) "original" formulation or (b) "enriched" with serine and pyruvate (to total concentrations of 2.00 and 0.50 mM, respectively). These media were supplemented with 5.0% v/v sCS and either (i) 6.00 mM or (ii) 20.00 mM glutamate or (iii) 4.00 or (iv) 6.00 mM glutamine. The cultures were grown in spinner flasks, as described above (7.3.1).

Results. Table 7.4 shows maximum Vero cell yields and growth rates in cultures of Cytodex-1 and Cultispher-G in DMEM + 5.0% v/v sCS containing either glutamine or glutamate. (1) Maximum Vero cell yields in cultures of either bead were affected by the major amino acid present in the DMEM. (2) Maximum cell yields were significantly higher (24%, $P=0.01$) in cultures of Cultispher-G compared to Cytodex-1 with respect to either glutamine or glutamate at any concentration used. (3) Maximum cell yields in Cytodex-1 cultures were significantly higher (20%, $P<0.05$) at 4.00-6.00 mM glutamine compared to either glutamate concentration used. (4) Maximum cell yields in Cultispher-G cultures were significantly higher (54%, $P<0.01$) at 6.00 mM glutamine compared to all other media used. (5) The cell growth rates were significantly higher (21%, $P<0.05$) in cultures of either bead with glutamine compared to glutamate. (6) The time taken to reach maximum cell yield in cultures of either bead was significantly longer (28%, $P<0.01$) with glutamate compared to glutamine. (7) There were no significant differences in the maximum cell yields and growth rates with either adaptation protocol to glutamate. (8) There was no significant difference in the maximum cell yields with original compared to enriched DMEM.

Discussion. Vero cells could be grown in cultures of either bead with glutamate substitution of glutamine. However, maximum cell yields and growth rates were significantly reduced with

Table 7.4 - The effect of using **glutamine** or **glutamate** as the **primary amino acid** in DMEM on the maximum batch Vero cell yield, time taken to reach maximum yield and growth rate in 100 mL spinner flasks on **1.00 g/L Cytodex-1** or **1.70 g/L Cultispher-G**. The cells were preadapted and inoculated at 0.05×10^6 cells/mL in original or serine and pyruvate enriched DMEM + 5.0% v/v sCS and either glutamine (4.00 or 6.00 mM) or without glutamine and with glutamate (6.00 or 20.00 mM). The spinner flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Primary Amino Acid	Serine Concentration (mM)	Time Taken to Reach Maximum Cell Yield (h)		Maximum Cell Concentration ($\times 10^6$ /mL)		Specific Cell Growth Rate (h^{-1})	
		Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G
4.00 mM	0.40	144	144	1.46 ± 0.09	2.08 ± 0.13	0.032 ± 0.002	0.035 ± 0.002
Glutamine	2.00	144	144	1.51 ± 0.11	2.11 ± 0.13	0.033 ± 0.002	0.035 ± 0.003
6.00 mM	0.40	144	168	1.44 ± 0.10	2.40 ± 0.15	0.032 ± 0.002	0.032 ± 0.003
Glutamine	2.00	144	168	$1.52 \pm 0.11^*$	$2.42 \pm 0.16^*$	0.032 ± 0.003	0.032 ± 0.002
6.00 mM	0.40	192	192	1.14 ± 0.06	1.31 ± 0.09	0.028 ± 0.002	0.027 ± 0.002
Glutamate ¹	2.00	192	192	1.21 ± 0.07	1.48 ± 0.10	0.025 ± 0.002	0.028 ± 0.003
6.00 mM	0.40	192	192	1.19 ± 0.06	1.33 ± 0.08	0.028 ± 0.002	0.027 ± 0.002
Glutamate ²	2.00	192	192	1.27 ± 0.06	1.52 ± 0.11	0.025 ± 0.002	0.028 ± 0.003
20.00 mM	0.40	192	192	1.21 ± 0.08	1.48 ± 0.12	0.026 ± 0.002	0.028 ± 0.003
Glutamate ¹	2.00	192	192	1.25 ± 0.08	1.50 ± 0.13	0.026 ± 0.003	0.029 ± 0.002
20.00 mM	0.40	192	192	1.27 ± 0.08	1.54 ± 0.11	0.026 ± 0.002	0.028 ± 0.003
Glutamate ²	2.00	192	192	1.29 ± 0.08	1.52 ± 0.11	0.026 ± 0.003	0.029 ± 0.002

* Highest cell yield.

1 Cells adapted by the protocol of Hassell and Butler (1991).

2 Cells adapted by the gradual adaptation protocol (7.2.1).

glutamate containing DMEM. Hassell and Butler (1990) previously adapted Vero cells to glutamate containing medium using a protocol which took 30 d and reported similar growth rates and cell yields compared to glutamine. The Vero cells used in this study were adapted by either a similar protocol to the previous authors or by a gradual weaning from glutamine but even after 50 d the cells grew to significantly lower cell yields and with slower growth rates in glutamate containing DMEM. Thus, the results in this study agree with those of Lazo et al. (1981) and Rapaport et al. (1981) who showed ascites tumour and rabbit reticulocytes had poor uptake and growth in glutamate medium. However, they disagree with Hassell and Butler (1990). Thus, glutamate was a poorer growth substrate compared to glutamine.

The reduced cell yield and growth rate with glutamate could indicate it was consumed at a slower rate compared to glutamine. Glutamate is an intermediate in amino acid biosynthesis and TCA cycle energy generation via conversion to α -ketoglutarate (Voet and Voet, 1990) and many pathways could be competing for it. The transport of glutamate across the cell and mitochondrial membranes could involve an energy inefficient uptake mechanism, as it has to enter the mitochondria before it is converted into α -ketoglutarate and ammonia. The ammonia is then transported out of the mitochondria for nucleotide synthesis in the cytoplasm. However, glutamine is deaminated on entry into the cytoplasm and the ammonia can be used directly for nucleotide synthesis (McKeehan, 1982). Hence, slower growth rates with glutamate.

7.3.5 Addition of Pyruvate

Introduction. DMEM medium usually contains sodium pyruvate (Gibco, 1992). However, the DMEM in this study was purchased without pyruvate. Pyruvate could detoxify the medium resulting in improved initial growth rates of the cells following subculture. No reports were found in the literature on pyruvate affecting Vero cell yields. Therefore, this study determined whether pyruvate enrichment of the DMEM affected Vero cell growth in microcarrier cultures.

Experimental. Stock Vero cells were preadapted (>6 passages) (2.1) to DMEM + 5.0% v/v sCS containing sodium pyruvate at various concentrations (up to 3.00 mM) and glutamine (4.00 mM). The cultures were grown in spinner flasks, as described above (7.3.1).

Results. Fig. 7.4 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G in DMEM containing sodium pyruvate. Table 7.5 shows lag phases in cultures of either bead with the various pyruvate concentrations used. (1) Vero cell growth in cultures of either bead was affected by the sodium pyruvate concentration used. (2) Maximum cell yields were significantly higher (32%, $P < 0.01$) in Cultispher-G cultures compared to Cytodex-1 with respect to all pyruvate concentrations used. (3) Maximum cell yields in cultures of either bead were significantly higher ($P < 0.05$) at ≤ 1.00 mM pyruvate compared to 3.00 mM pyruvate. (4) There (Table 7.5) was no significant difference in the lag phase in cultures of either bead in DMEM at up to 0.50 mM pyruvate. However, lag phases were increased proportional to the pyruvate concentration and were significantly longer (280%, $P < 0.01$) at 3.00 mM pyruvate compared to all other concentrations used. (5) There (Table 7.5) was no significant difference in the cell growth rates in cultures of either bead with respect to any concentration of pyruvate used.

Discussion. The maximum Vero cell yield and growth rate in cultures of either bead were not significantly different in DMEM at up to 0.50 mM pyruvate. However, there were significantly longer lag phases and lower maximum cell yields at 3.00 mM pyruvate. This result could explain the previous poor growth in L-15 medium (6.3.1) which contained 5.00 mM pyruvate. However, the pyruvate (up to 0.50 mM) did not affect the initial growth of Vero cells in this study which disagrees with O'Donnell-Tormey et al. (1987) who showed improved growth of several human and murine cells in pyruvate containing (1.00 mM) medium. This could be explained by cell line dependent differences. Pyruvate is secreted from glycolysis and forms an equilibrium with lactate. This equilibrium could explain the majority of the glucose consumption in Vero cell cultures (6.3.3), as lactate is produced at a high ratio per glucose consumed (1.18 and 1.40 in

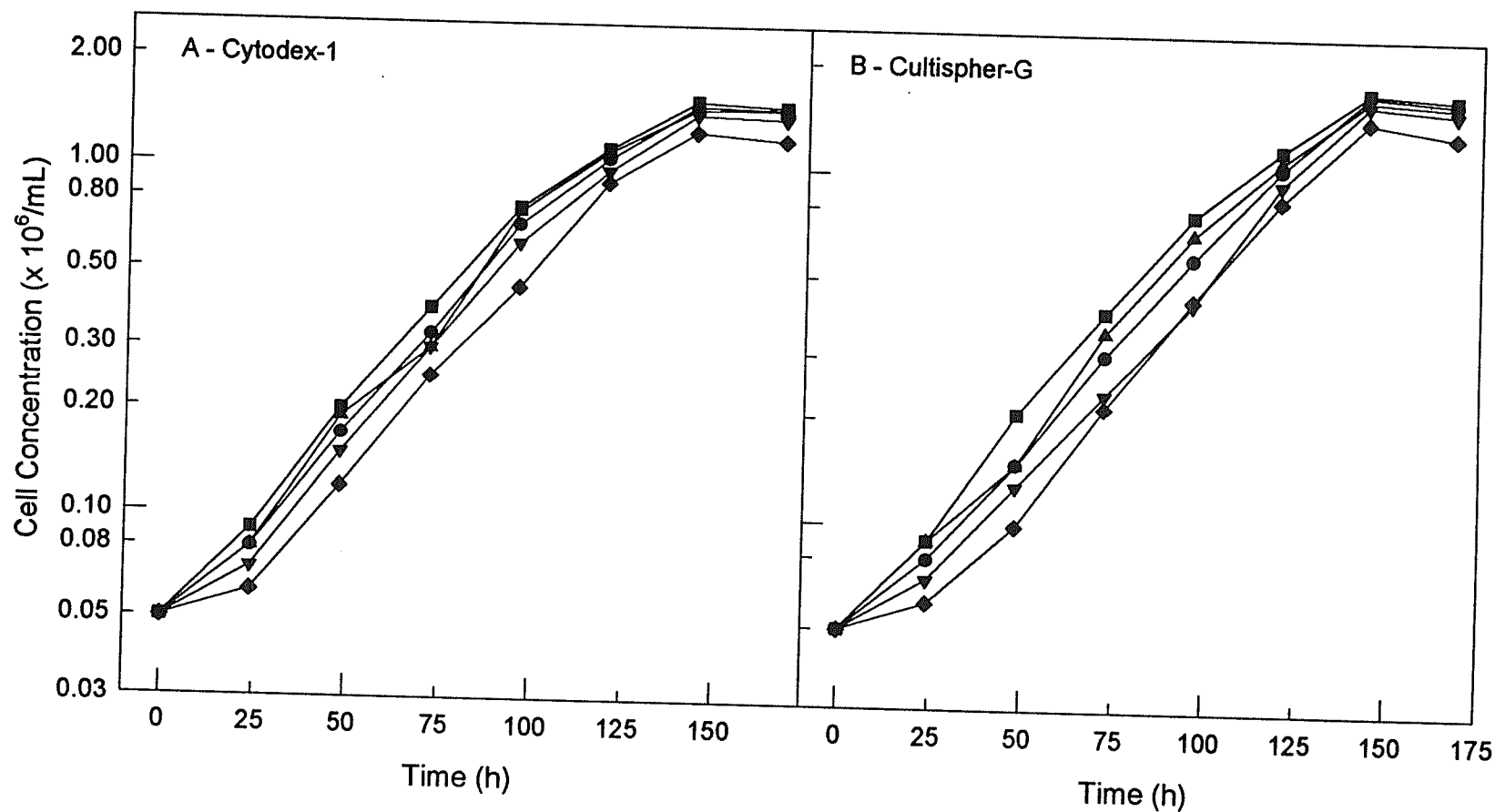


Fig. 7.3 - The effect of the **sodium pyruvate concentration** on Vero cell growth in 100 mL spinner flasks on **1.00 g/L Cytodex-1 (A)** and **1.70 g/L Cultispher-G (B)**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS prepared **●** without sodium pyruvate 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.4 after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

Table 7.5 - The effects of pyruvate in the DMEM on Vero cell lag phases and growth rates in 100 mL spinner flask cultures of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS prepared with up to 3.00 mM pyruvate. Spinner flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.4 after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Pyruvate Concentration (mM)	Specific Cell Growth Rate and Lag Phase in DMEM with the Sugar Used			
	Cytodex-1		Cultispher-G	
	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
0.00	0.034 ± 0.003	6.00 ± 1.00	0.036 ± 0.003	9.00 ± 1.00
0.15	0.034 ± 0.003	9.00 ± 1.00	0.036 ± 0.003	6.00 ± 1.00
0.50	0.034 ± 0.003	9.00 ± 1.00	0.035 ± 0.003	6.00 ± 1.00
1.00	0.033 ± 0.003	12.00 ± 1.00	0.035 ± 0.003	12.00 ± 1.00
3.00	0.032 ± 0.002	21.00 ± 2.00	0.034 ± 0.003	21.00 ± 2.00

Cytodex-1 and Cultispher-G cultures, respectively).

7.3.6 Addition of Pyruvate to Aged Complete Medium

Introduction. Pyruvate had no significant effects (7.3.5) on Vero cell growth at up to 0.50 mM in fresh DMEM. However, pyruvate has improved the initial growth of other cells after subculture. This effect could be explained by free ammonia being sequestered by pyruvate resulting in alanine. Therefore, this study determined the effects of adding sodium pyruvate to DMEM which was stored at 4°C for 28 d prior to use for Vero cell growth in microcarrier cultures.

Experimental. Preadapted stock Vero cells (>6 passages) (2.1) were inoculated in DMEM + 5.0% v/v SCS prepared (2.3) with and without sodium pyruvate (0.50 mM) and stored at 4°C for 28 d. The cultures were grown in spinner flasks, as described above (7.3.1).

Results. Fig. 7.4 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G with complete DMEM stored for various times with or without pyruvate. Table 7.6 shows the cell growth rates and lag phases in these cultures. Fig. 7.5 shows the glutamine consumption and ammonia production profiles for these cultures. Table 7.7 shows the results of high performance liquid chromatography (HPLC) analysis of the media. (1) Vero cultures of either bead in DMEM + 5.0% v/v SCS were affected by the medium composition after storage (28 d). (2) There were significantly higher (29%, $P < 0.01$) maximum cell yields (Fig. 7.4) in Cultispher-G cultures compared to Cytodex-1 with respect to any medium used. (3) The highest maximum cell yields in cultures of either bead were produced with fresh DMEM. However, maximum cell yields were significantly lower (25%, $P < 0.01$) in DMEM + 5.0% v/v SCS stored (28 d) with or without pyruvate. (4) There (Table 7.6) were lag phases in cultures of either bead. The lag phases were significantly longer (500%, $P < 0.01$) in DMEM + 5.0% v/v SCS stored (28 d) without pyruvate compared to either DMEM + 5.0% v/v SCS prepared fresh or stored with pyruvate. (5) There

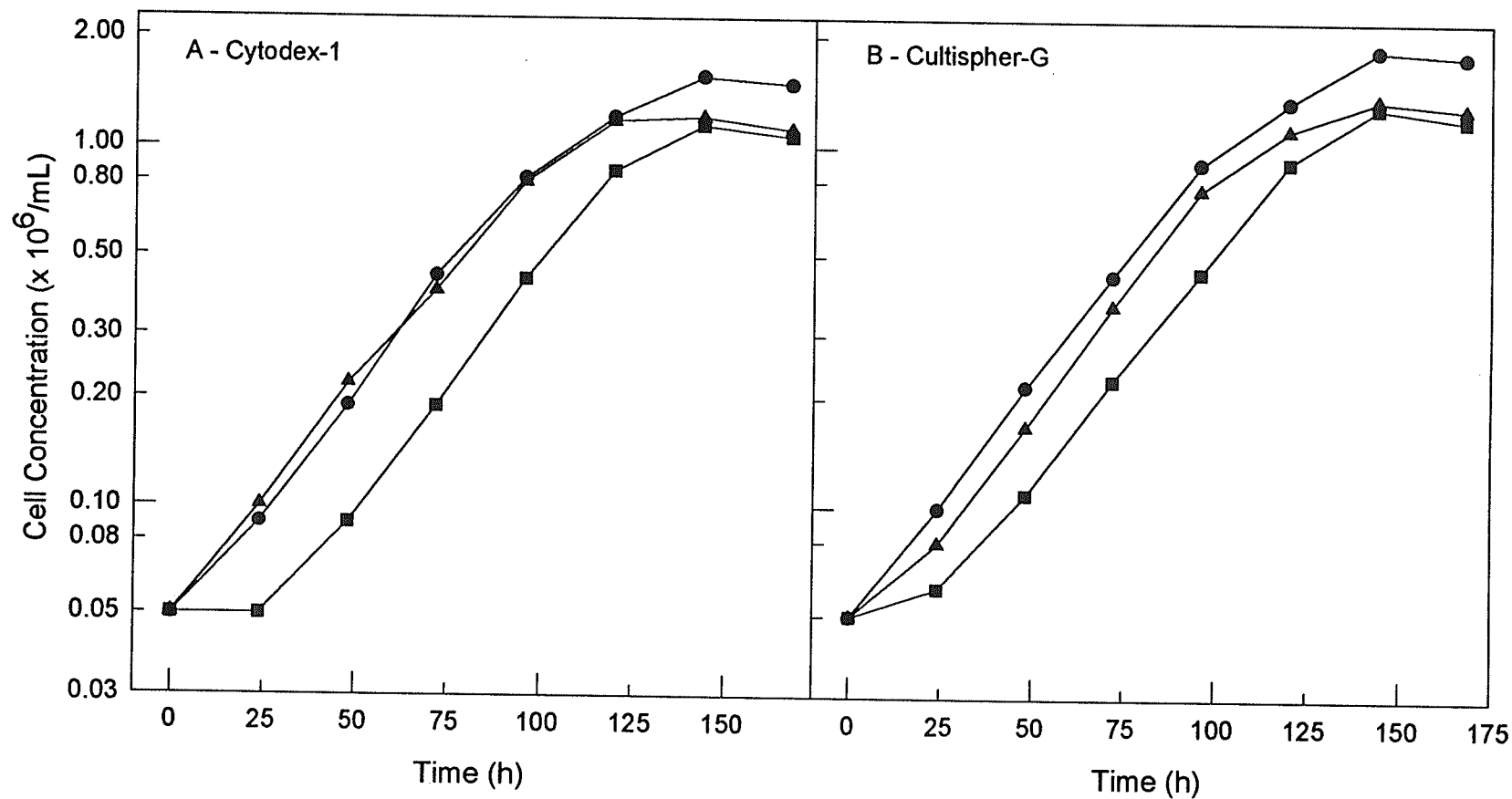


Fig. 7.4 - The effect of sodium pyruvate addition to medium stored at 4°C prior to use on Vero cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A) and 1.70 g/L Cultispher-G (B). The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS. DMEM + 5.0% v/v sCS was prepared ● 0 d without sodium pyruvate, ■ 28 d without pyruvate or ▲ 28 d with 0.5mM pyruvate. Spinner flasks were stirred at 40 rpm either continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means of n=2.

Table 7.6 - The effects of **pyruvate addition** to **stored DMEM** on Vero cell lag phases and growth rates in 100 mL spinner flask cultures of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS. The DMEM + 5.0% v/v SCS was prepared with or without 0.50 mM pyruvate and was stored for 28 d at 4°C. Spinner flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.4 after 72 h in culture with 2.00 M NaOH. The values are means of $n=2$.

Storage Medium	Specific Cell Growth Rate and Lag Phase in DMEM with the Sugar Used			
	Cytodex-1		Cultispher-G	
	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)
DMEM + 5.0% v/v SCS Without Storage	0.034 ± 0.003	3.00 ± 1.00	0.036 ± 0.003	3.00 ± 1.00
DMEM + 5.0% v/v SCS Stored 28 d With Pyruvate	0.030 ± 0.003	4.50 ± 1.00	0.030 ± 0.003	6.00 ± 1.00
DMEM + 5.0% v/v SCS Stored 28 d Without Pyruvate	0.030 ± 0.003	24.00 ± 2.00	0.030 ± 0.003	21.00 ± 2.00

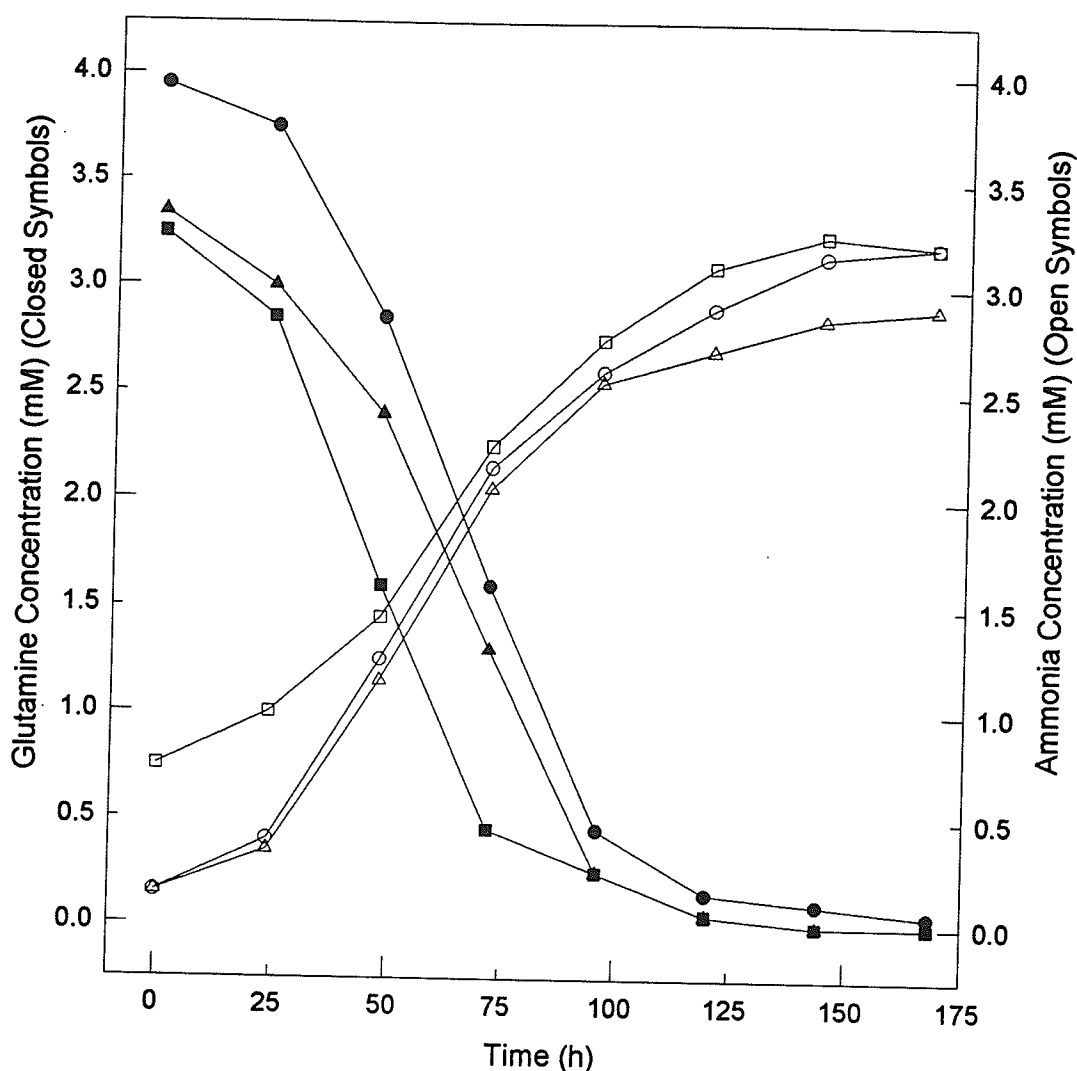


Fig. 7.5 - The effects of pyruvate addition to stored DMEM on the glutamine and ammonia concentrations of 100 mL spinner flask Vero cultures. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS prepared without pyruvate ● 0 d, ■ stored for 28 d or ▲ stored for 28 d with 0.50 mM pyruvate. Spinner flasks were stirred at 40 rpm continuously (1.00 g/L Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (1.70 g/L Cultispher-G). The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means of $n=4$.

Table 7.7 - The effects of **pyruvate addition** to **stored DMEM** on the **consumed** and **produced amino acid concentrations** at maximum Vero cell yield in 100 mL spinner flask cultures of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS prepared without pyruvate immediately prior to use or stored for 28 d or stored for 28 d with 0.50 mM pyruvate. Spinner flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The amino acids were analysed by HPLC. The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means of cultures of Cytodex-1 and Cultispher-G and are from n=4.

Amino Acid	Consumed or Produced Amino Acid Concentration at Maximum Cell Yield (mM)		
	DMEM + 5.0% v/v sCS (Not Stored)	DMEM + 5.0% v/v sCS (Stored 28 d)	DMEM + 5.0% v/v sCS + 0.50 mM Pyruvate (Stored 28 d)
Alanine	+0.75 ± 0.10	+0.85 ± 0.10	+0.70 ± 0.10
Arginine	-0.16 ± 0.05	-0.14 ± 0.04	-0.13 ± 0.03
Asparagine	+0.05 ± 0.01	+0.07 ± 0.02	+0.04 ± 0.01
Cystine	UND	UND	UND
Glutamate	+1.00 ± 0.20	+1.08 ± 0.20	+1.15 ± 0.20
Glutamine	-3.90 ± 0.50	-3.85 ± 0.50	-3.90 ± 0.50
Glycine	+0.15 ± 0.05	+0.17 ± 0.05	+0.17 ± 0.05
Histidine	-0.12 ± 0.03	-0.11 ± 0.03	-0.10 ± 0.03
Isoleucine	-0.25 ± 0.08	-0.22 ± 0.08	-0.23 ± 0.08
Leucine	-0.31 ± 0.08	-0.31 ± 0.08	-0.30 ± 0.08
Lysine	-0.20 ± 0.05	-0.20 ± 0.05	-0.22 ± 0.05
Methionine	-0.06 ± 0.02	-0.05 ± 0.02	-0.08 ± 0.02
Phenylalanine	-0.11 ± 0.02	-0.13 ± 0.03	-0.13 ± 0.02
Serine	-0.32 ± 0.07	-0.30 ± 0.07	-0.33 ± 0.07
Threonine	-0.10 ± 0.02	-0.11 ± 0.02	-0.13 ± 0.02
Tryptophan	-0.03 ± 0.01	-0.05 ± 0.01	-0.05 ± 0.01
Tyrosine	-0.05 ± 0.01	-0.04 ± 0.01	-0.05 ± 0.01
Valine	-0.13 ± 0.04	-0.12 ± 0.04	-0.12 ± 0.04
Total Concentration of Consumed Amino Acids	5.74	5.63	5.77
Total Concentration of Produced Amino Acids	1.95	2.17	2.06

(Table 7.6) were significantly higher (17%, $P < 0.05$) cell growth rates in DMEM + 5.0% v/v SCS prepared fresh compared to medium stored with or without pyruvate.

Nutrient Analysis. Nutrient analysis (Fig. 7.5) showed glutamine had naturally decomposed in DMEM during storage. There was a significantly higher (500%, $P < 0.01$) concentration of ammonia in the DMEM stored without pyruvate compared to fresh DMEM or DMEM stored with pyruvate for 28 d. There were no significant differences in the concentrations of any produced or consumed amino acids (Table 7.7) analysed with respect to any medium used.

Discussion. Maximum cell yields in cultures of either bead were similar following storage of the DMEM for 28 d with or without pyruvate but were significantly lower compared to fresh DMEM. Thus, pyruvate addition did not affect maximum cell yields. There were no significant differences in the lag phases with fresh DMEM or DMEM stored for 28 d containing pyruvate which shows pyruvate did detoxify the DMEM. This agrees with the subculture improvements described by O'Donnell-Tormey et al. (1987).

Nutrient analysis could explain the effects of the pyruvate as glutamine had decomposed during storage. This gave significantly more ammonia in the stored compared to fresh DMEM without pyruvate at inoculation. The reduced glutamine concentration could explain the cell yields in stored DMEM. The similar ammonia concentration at inoculation in fresh and stored DMEM containing pyruvate suggests pyruvate could prevent ammonia inhibition by forming alanine (Voet and Voet, 1990) which could explain the production of alanine in previous cultures (6.3.3). However, nutrient analysis in this study showed no significant difference in the concentrations of alanine between any of the media types used.

7.3.7 Reduction of Sodium Bicarbonate Concentration

Introduction. pH is important to the growth and lag phases of Vero cells (5.3.5) in microcarrier

cultures of either bead type. One approach to generating the optimal pH range would be to reduce the bicarbonate buffer concentration in the DMEM. No one has previously shown the effects of altering the buffer concentrations on cell growth. Therefore, this study determined the effect of reducing the sodium bicarbonate concentration in the DMEM on Vero cell growth in microcarrier cultures.

Experimental. Stock Vero cells were preadapted (>6 passages) (2.1) to DMEM + 5.0% v/v sCS prepared (2.3) with various sodium bicarbonate concentrations (32.00-44.00 mM) and containing glutamine (4.00 mM). The pH of the media was equilibrated prior to inoculation with a 10.0% v/v CO₂ in air overlay. The cultures were grown in spinner flasks, as described above (7.3.1).

Results. Fig. 7.6 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G with the DMEM containing various bicarbonate concentrations. Table 7.8 shows the lag phases and cell growth rates in cultures of either bead with the various bicarbonate concentrations used. (1) There was no significant difference in the maximum cell yields in cultures of either bead with respect to any bicarbonate concentration used. (2) There were significantly higher (34%, $P < 0.01$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the bicarbonate concentrations used. (3) There (Table 7.8) was no significant difference in the lag phases in cultures of either bead in DMEM at 32.00 and 36.00 mM bicarbonate. There were significantly longer (166%, $P < 0.01$) lag phases in cultures of either bead at 44.00 mM bicarbonate. (4) There (Table 7.8) were significantly higher (16%, $P < 0.05$) cell growth rates in cultures of Cultispher-G compared to Cytodex-1 with respect to all bicarbonate concentrations used.

pH. The culture pH during growth differed between the bead types and bicarbonate concentrations used. Cultures of either bead were inoculated at pH 7.1, 7.3 and 7.5 with 32.00, 36.00 and 44.00 mM bicarbonate in the DMEM, respectively. The Cultispher-G cultures had

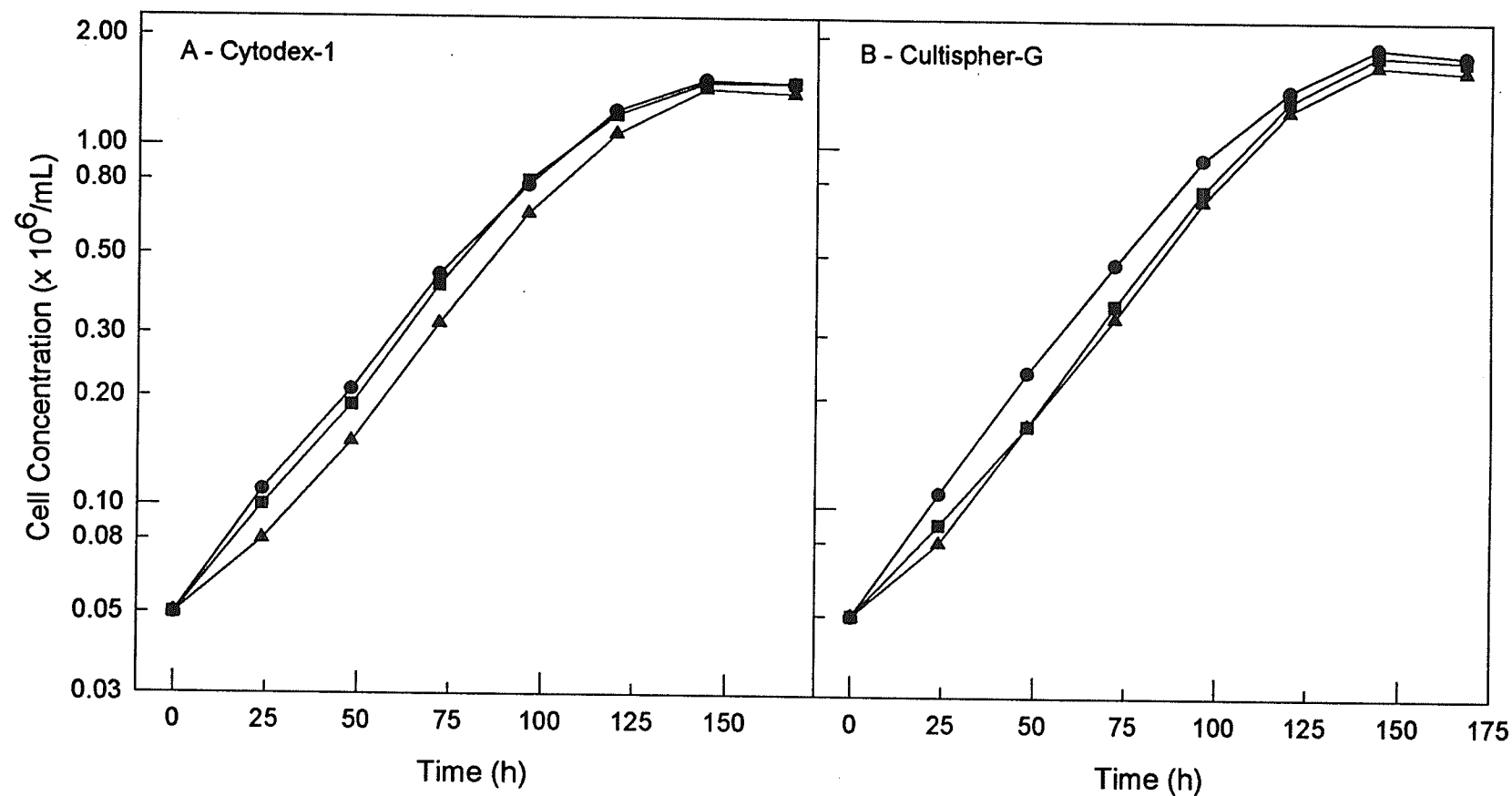


Fig. 7.6 - The effect of the sodium bicarbonate concentration in the DMEM on Vero cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A) and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS prepared with sodium bicarbonate at either ● 32.00, ■ 36.00 or ▲ 44.00 mM. Spinner flasks were stirred at 40 rpm either continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). The flasks were readjusted daily to pH 7.4 after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

Table 7.8 - The effects of the **bicarbonate concentration** in the DMEM on Vero cell lag phases and growth rates in 100 mL spinner flask cultures of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS prepared with 32.00-44.00 mM bicarbonate. Spinner flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.4 after 72 h in culture with 2.00 M NaOH. The values are means of $n=2$.

Bicarbonate Concentration (mM)	Specific Cell Growth Rate and Lag Phase in DMEM Containing Bicarbonate Concentration			
	Cytodex-1		Cultispher-G	
	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
32.00	0.032 ± 0.003	3.00 ± 1.00	0.037 ± 0.003	3.00 ± 1.00
36.00	0.030 ± 0.003	3.00 ± 1.00	0.036 ± 0.003	4.50 ± 1.00
44.00	0.030 ± 0.003	9.00 ± 1.00	0.036 ± 0.003	9.00 ± 2.00

decreased to pH 6.6, 6.7 and 6.8 and the Cytodex-1 cultures to pH 6.7, 6.9 and 7.0, respectively, after 72 h. The cultures at 32.00 mM bicarbonate had decreased to the same values at each daily readjustment time as found at 72 h in culture and required a higher volume (up to x2) of 2.00 M NaOH to maintain the optimal pH range. Cultures of either bead with 36.00 and 44.00 mM bicarbonate were maintained within the optimal pH range from 72 h onwards.

Discussion. No one has previously described the effects of altering the buffer concentrations in the medium on cell growth. Maximum Vero cell yields in cultures of either bead were similar with respect to all the bicarbonate concentrations used. However, the lag phases were significantly shortened proportional to the bicarbonate concentration. The shorter lag phases at low (32.00 mM) bicarbonate concentrations could reflect the more favourable pH during the first few hours in culture. This could be explained by improved cell attachment characteristics and more favourable pH for enzyme functions with the low bicarbonate concentration (Hu et al., 1985). However, pH readjustment of spinner flask cultures was affected by the low bicarbonate concentration. The preferable bicarbonate concentration based on pH control was 36.00 mM.

7.3.8 Addition of Calcium Chloride

Introduction. Cell adhesion could involve Ca^{2+} and cell surface charge interactions. Thus, an increased Ca^{2+} concentration could improve cell growth by enhancing the adhesion process in cultures with an optimal pH for cell growth. Therefore, this study determined the effects of adding extra CaCl_2 to the DMEM on Vero cell growth in microcarrier cultures.

Experimental. Preadapted stock Vero cells (>6 passages) (2.1) were inoculated in DMEM + 5.0% v/v SCS enriched with extra CaCl_2 (up to 5.40 mM). The cultures were grown in spinner flasks, as described above (7.3.1).

Table 7.9 - The effect of enriching the DMEM with extra CaCl_2 on maximum Vero cell yields, growth rates and lag phases in microcarrier culture in 100 mL spinner flasks. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS in 1.00 g/L Cytodex-1 or 1.70 g/L Cultispher-G. The DMEM was prepared with extra CaCl_2 (up to 5.40 mM). The spinner flasks were stirred at 40 rpm either continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). The flasks were readjusted to pH 7.4 after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Concentration of Supplemented CaCl_2 (mM)	Lag Phase		Maximum Cell Concentration		Specific Cell Growth Rate	
	(h)		$(\times 10^6 \text{ cells/mL})$		(h^{-1})	
	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G
0.00	6.00 ± 0.50	6.00 ± 0.50	1.39 ± 0.10	$1.90 \pm 0.13^*$	0.034 ± 0.003	0.037 ± 0.003
1.80	6.00 ± 0.50	6.00 ± 0.50	1.37 ± 0.10	1.81 ± 0.12	0.034 ± 0.003	0.037 ± 0.003
3.60	6.00 ± 0.50	6.00 ± 0.50	$1.41 \pm 0.10^*$	1.83 ± 0.11	0.034 ± 0.003	0.037 ± 0.003
5.40	6.00 ± 0.50	6.00 ± 0.50	1.32 ± 0.09	1.85 ± 0.12	0.034 ± 0.003	0.037 ± 0.003

* Highest cell yield.

Results. Table 7.9 shows the maximum cell yields, cell growth rates and lag phases in cultures of Cytodex-1 and Cultispher-G in DMEM containing various concentrations of CaCl_2 . (1) There was no significant difference in the maximum cell yields in cultures of either bead with respect to the CaCl_2 concentrations used. (2) There were significantly higher (32%, $P < 0.01$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1. (3) There was no significant difference in the cell growth rates or lag phases in cultures of either bead with respect to the CaCl_2 concentrations used.

Cell Recovery. The CaCl_2 did affect cell recoveries from either bead but particularly from Cytodex-1 cultures. Cell detachment from Cytodex-1 beads and the production of a single cell suspension for counting from cultures of either bead were progressively harder the more Ca^{2+} in the DMEM. The cells detached with the standard trypsinisation procedure were in large aggregates and could not be singly suspended from cultures with added CaCl_2 . Increased trypsin (0.50% w/v) and EDTA (3.40 mM) concentrations in the detachment solution were required to generate single cell suspensions from these cultures.

Discussion. Maximum cell yields in cultures of either bead were not affected by Ca^{2+} enrichment (up to 5.40 mM). This agrees with Lengheden (1994) who showed fibroblast cell required Ca^{2+} for attachment and growth but with a pH dependent mechanism where growth was affected at high pH ($\text{pH} > 7.5$). The pH effect has also been described by Hu et al. (1985). Indeed, the extra Ca^{2+} was detrimental in this study, as it caused cell detachment problems especially in Cytodex-1 cultures where high Ca^{2+} concentrations increased cell attachment which agrees with Curtis and McMurray (1986). This was overcome with increased concentrations of both trypsin and EDTA in detachment solutions. The trypsin and EDTA concentrations were shown previously (3.3) not to affect Vero cell recoveries or viabilities from T25 culture flasks.

7.3.9 Addition of Other Amino Acids and Vitamins

Introduction. Vero cells consumed many amino acids in the DMEM (6.3.3) but most were consumed significantly less compared to glutamine and serine. Cell growth could have reduced these amino acids to levels too low for cells to take up. Therefore, this study determined the effects of enriching DMEM with the lesser consumed amino acids either singly or in combinations and with or without extra vitamins on Vero cell growth in microcarrier cultures.

Experimental. Preadapted stock Vero cells (>6 passages) (2.1) were inoculated in DMEM + 5.0% v/v SCS enriched with the amino acids arginine, cysteine, cystine, histidine, methionine, phenylalanine, proline, taurine, threonine, tryptophan, tyrosine and valine (at 2.00 mM total concentration). The cultures were grown in T25 culture flasks and one flask of each medium type was sacrificed daily for cell counts by the trypan blue method (2.5.1-2).

Results. Table 7.10 shows the maximum Vero cell yields in cultures of either bead in DMEM enriched with the various combinations of amino acids. (1) There were no significant differences in maximum cell yields with respect to any amino acid additions. (2) DMEM supplemented with taurine showed a significantly higher maximum cell yield (17%, $P < 0.05$) in 1 of the 3 cultures used for this experiment, the other 2 were not significantly different compared to the control. (3) There were no significant differences in the cell growth rates or lag phases with respect to any of the supplemented amino acids or vitamins used.

Discussion. Vero cell growth was not affected by enriching the DMEM with the less consumed amino acids. Taurine was absolutely required for serum-free growth of human hybridoma cells (Toyoda et al., 1991) although no further reports of taurine in cell cultures were found. Previous results (3.3-6.3) show Vero cells grow well in the absence of taurine. Taurine addition increased maximum cell yields in 1 of 3 cultures, although the precise effect of taurine on cell growth was

Table 7.10 - The effect of **enriching** various amino acids to the **DMEM** on maximum batch Vero cell yields, growth rates and lag phases in T25 culture flasks. The cells were preadapted and inoculated at 0.10×10^6 cells/mL with 10 mL of DMEM + 5.0% v/v sCS supplemented to 2.00 mM total cysteine, cystine, glycine, histidine, homocysteic acid, isoleucine, leucine, lysine, methionine, phenylalanine, proline, taurine, threonine, tryptophan, tyrosine or valine both singly or in combinations and with or without extra vitamins (1X). The values are means of $n=3$.

Amino Acid Supplementation (To 2.00 mM)	Lag Phase (h)	Maximum Cell Concentration ($\times 10^6$ /mL)	Specific Cell Growth Rate (h^{-1})
None	4.50 ± 0.50	1.85 ± 0.13	0.043 ± 0.004
Cysteine	4.50 ± 0.50	1.78 ± 0.12	0.043 ± 0.004
Cystine	4.50 ± 0.50	1.78 ± 0.12	0.043 ± 0.004
Glycine	4.50 ± 0.50	1.84 ± 0.12	0.044 ± 0.004
Histidine	4.50 ± 0.50	1.88 ± 0.13	0.044 ± 0.004
Homocysteic acid	4.50 ± 0.50	1.83 ± 0.12	0.044 ± 0.004
Isoleucine	4.50 ± 0.50	1.77 ± 0.12	0.043 ± 0.004
Leucine	4.50 ± 0.50	1.78 ± 0.12	0.043 ± 0.004
Lysine	4.50 ± 0.50	1.85 ± 0.13	0.043 ± 0.004
Methionine	4.50 ± 0.50	1.85 ± 0.13	0.043 ± 0.004
Phenylalanine	4.50 ± 0.50	1.81 ± 0.13	0.044 ± 0.004
Proline	4.50 ± 0.50	1.80 ± 0.12	0.044 ± 0.004
Taurine	4.50 ± 0.50	$1.92 \pm 0.13^*$	0.044 ± 0.004
Threonine	4.50 ± 0.50	1.87 ± 0.12	0.044 ± 0.004
Tryptophan	4.50 ± 0.50	1.80 ± 0.11	0.044 ± 0.004
Tyrosine	4.50 ± 0.50	1.82 ± 0.13	0.044 ± 0.004
Valine	4.50 ± 0.50	1.82 ± 0.13	0.044 ± 0.004
Cysteine and Cystine	4.50 ± 0.50	1.68 ± 0.11	0.042 ± 0.004
Histidine and Threonine	4.50 ± 0.50	1.77 ± 0.12	0.043 ± 0.004
Cysteine, Cystine and Homocysteic Acid	4.50 ± 0.50	1.71 ± 0.11	0.042 ± 0.004
Isoleucine, Leucine, Lysine, Phenylalanine and Valine	4.50 ± 0.50	1.79 ± 0.12	0.043 ± 0.004
Methionine, tryptophan and tyrosine	4.50 ± 0.50	1.79 ± 0.12	0.043 ± 0.004
All The Above	4.50 ± 0.50	1.68 ± 0.11	0.042 ± 0.004
2X Vitamins	4.50 ± 0.50	1.73 ± 0.12	0.042 ± 0.004
2X Vitamins + All amino Acids Above	4.50 ± 0.50	1.65 ± 0.11	0.042 ± 0.004

* - Highest cell yield.

unclear. Taurine can increase membrane fluidity (Guertin et al., 1993) and Ca^{2+} uptake (Schacter, 1984) of hepatocyte cells, to increase Zn^{2+} uptake of MRC-5 cells (Harraki et al., 1994) and to affect the internal osmolarity of renal cells (Hammerman and Sacktor, 1978 and Huxtable and Sebring, 1986). All could explain the taurine effects in this study.

7.3.10 Dilution of the Basal Medium with D-PBS

Introduction. The dilution of basal medium with PBS could increase Vero cell yields by dilution of inhibitor components in the basal formulation or by medium osmolarity effects. Therefore, this study determined whether the growth of Vero cells in microcarrier cultures was affected by diluting the complete DMEM with nutrient supplemented D-PBS⁻.

Experimental. Preadapted Vero cells (> 6 passages) were cultured in spinner flasks, as described above (7.3.1) in DMEM + 5.0% v/v sCS diluted (up to 1:3) with various formulations of D-PBS⁻. The D-PBS⁻ was supplemented with 25.00 mM glucose, 4.00 mM glutamine, 2.00 mM serine, 36.00 mM bicarbonate, 15.00 mg/L phenol red, 0.90 mM MgCl_2 and ± 1.80 mM CaCl_2 and 5.0% v/v sCS. The medium osmolarity was determined as described previously (2.5.9).

Results. Fig. 7.7 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G in DMEM diluted with supplemented D-PBS⁻ with and without Ca^{2+} . Table 7.11 shows cell growth rates and lag phases in these cultures. (1) Vero cell growth in cultures of either bead were affected by dilution of the DMEM with D-PBS⁻ with and without Ca^{2+} . (2) Maximum cell yields were significantly higher (35%, $P < 0.05$) in Cultispher-G cultures compared to Cytodex-1 with respect to all medium dilutions used. (3) There were significantly higher maximum cell yields (Fig. 7.7A)-B)) in cultures of either bead in DMEM and DMEM diluted 1:1 with supplemented D-PBS⁻ with and without Ca^{2+} compared to all other dilutions used. (4) There were significantly higher (17%, $P < 0.05$) maximum cell yields in Cytodex-1 cultures in DMEM diluted with supplemented

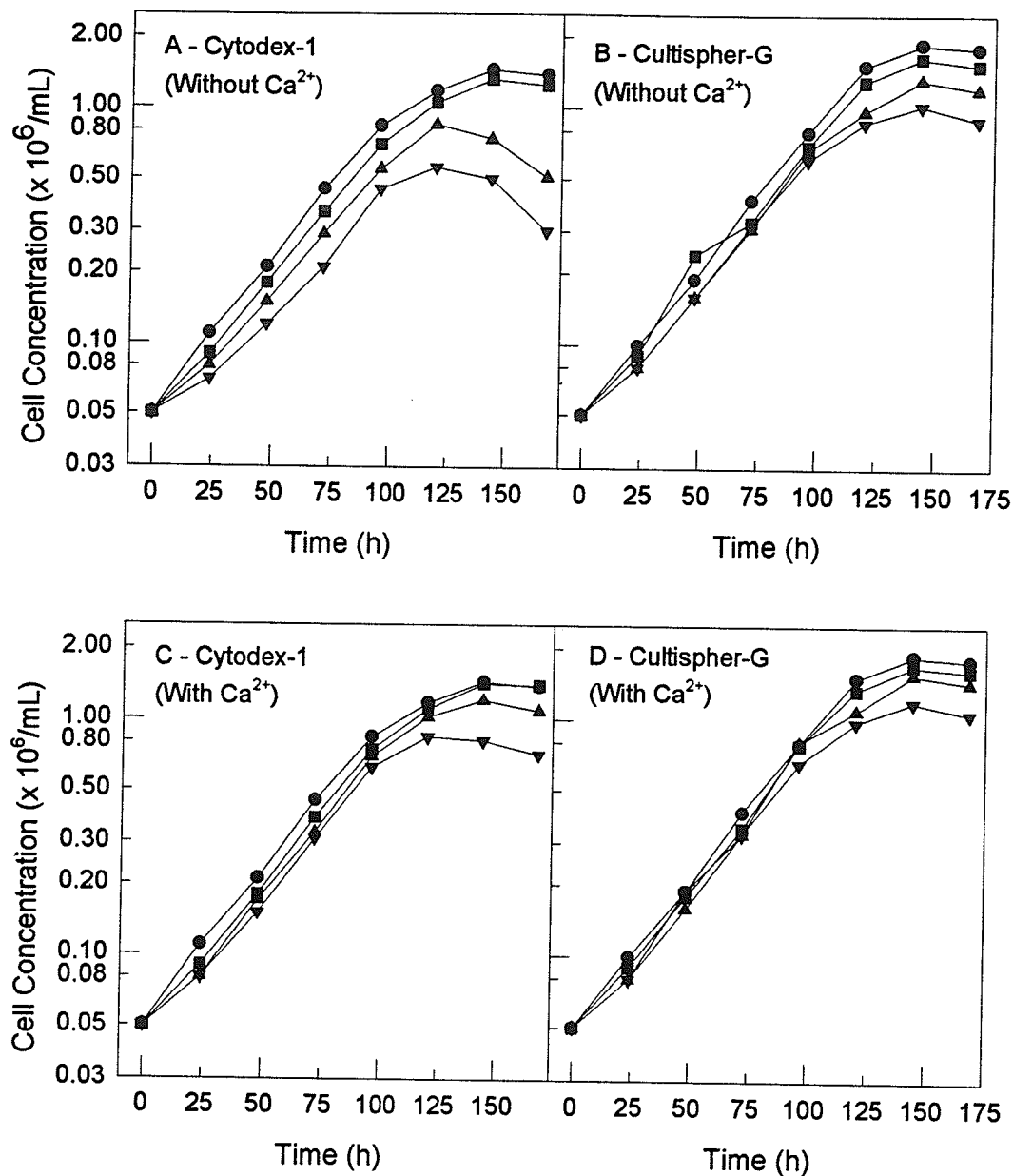


Fig. 7.7 - The effect of diluting DMEM with nutrient supplemented D-PBS⁻, with and without Ca^{2+} , on Vero cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A and C) and 1.70 g/L Cultispher-G (B and D). The cells were inoculated at 0.05×10^6 cells/mL in 5.0% v/v SCS in either ● DMEM or DMEM diluted with D-PBS⁻ ■ 1:1, ▲ 1:2 or ▼ 1:3. Spinner flasks were stirred at 40 rpm either continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.4 after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

Table 7.11 - The effects of **DMEM dilution with D-PBS⁻ solutions** on **medium osmolarity** and **Vero cell lag phases and growth rates** in 100 mL spinner flask cultures of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS diluted to various levels (up to 1:3) with D-PBS⁻ supplemented with 5.0% v/v sCS, glucose, glutamine, serine, MgCl₂ and with or without CaCl₂ (25.00, 4.00, 2.00, 0.90 and 1.80 mM, respectively). Spinner flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.4 after 72 h in culture with 2.00 M NaOH. The values are means of the results from dilution with D-PBS⁻ with and without CaCl₂ and are of n=4.

Medium Dilution	Medium Osmolarity (mOsmol.L ⁻¹)	Specific Cell Growth Rate and Lag Phase in DMEM Containing Bicarbonate Concentration			
		Cytodex-1		Cultispher-G	
		Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)
None	317 ± 10	0.034 ± 0.003	3.00 ± 1.00	0.036 ± 0.003	3.00 ± 1.00
1:1	315 ± 10	0.033 ± 0.003	6.00 ± 1.00	0.035 ± 0.003	4.50 ± 1.00
1:2	315 ± 10	0.034 ± 0.003	9.00 ± 1.00	0.034 ± 0.003	9.00 ± 1.00
1:3	308 ± 10	0.030 ± 0.003	10.50 ± 1.00	0.032 ± 0.003	9.00 ± 1.00

D-PBS⁻ with CaCl₂ (Fig. 7.7C-D)) compared to without CaCl₂. Cultispher-G cultures were unaffected by Ca²⁺ addition. (5) Lag phases (Table 7.11) were increased proportional to the medium dilution ratio. There were significantly longer (325%, $P < 0.01$) lag phases in cultures of either bead in DMEM diluted 1:3 with D-PBS⁻ (with and without Ca²⁺) compared to undiluted DMEM. (6) There (Table 7.11) was no significant difference in the cell growth rates in cultures of either bead with all the media dilutions used. (7) There was no significant difference in the medium osmolarity with respect to any dilution of DMEM + 5.0% v/v sCS used.

Discussion. Vero cell yields in cultures of either bead were unaffected by DMEM diluted with supplemented D-PBS⁻ at 1:1, with or without Ca²⁺ addition. However, the addition of Ca²⁺ was more important to, and produced significantly higher maximum cell yields with dilutions of 1:2 and 1:3 in Cytodex-1 cultures. The Vero cell yield results with 1:1 dilution disagree with the findings of Nahapetian et al. (1986). However, their Vero cells were cultured in both a different growth system (daily refed cultures) and basal media (L-15) compared to this study which could explain this effect.

There were small osmolarity changes in L-15 and none in DMEM diluted with D-PBS⁻ which suggests this was not responsible for the improved cell growth. However, this does indicate that media component dilution resulting in reduced competitive inhibition between components could be responsible. For example, dilution of the pyruvate in L-15 could explain the cell yield effect, as discussed above (7.3.5).

7.4 Conclusions

The purpose of this study was to investigate the effects of minor medium components on Vero cell growth in Cytodex-1 and Cultispher-G cultures and showed that:

- **maximum cell yields** were significantly higher (30%, $P < 0.01$) in Cultispher-G cultures compared to Cytodex-1 with all the nutrients tested.

- **the highest cell yields** were in fresh DMEM and maximum cell yields were significantly lower ($P < 0.05$) in medium stored with (28%) or without serum (20%) for 28 d.
- **there were significantly longer ($P < 0.01$) lag phases** (24 h) in medium stored for 28 d at 4°C prior to use. Pyruvate (0.50 mM) addition removed these but maximum cell yields were unaffected. Pyruvate sequestration of ammonia forming alanine could explain this effect.
- **the serum storage time at 4°C** (up to 28 d) did not affect maximum cell yields in cultures of either bead and showed serum factors normally considered to decompose or cold precipitate were not significantly involved in Vero cell growth.
- **the optimal carbohydrate** for Vero cell growth in cultures of either bead was glucose. Glucose could be substituted by maltose with no effect on maximum cell yields. Cell yields were significantly lower ($P < 0.01$) and lag phases longer with fructose, galactose and mannose.
- **the optimal amino acid** for Vero cell growth in cultures of either bead was glutamine. Cells could grow with glutamine substituted by glutamate but maximum cell yields were significantly lower and lag phases and growth rates were increased with glutamate.
- **the optimal sodium bicarbonate concentration** was 36.00 mM.
- **CaCl₂ enrichment** increased Vero cell attachment to Cytodex-1 and adhesion to other cells. Increased trypsin (0.50% w/v) and EDTA (3.40 mM) concentrations could generate single cell suspensions from high CaCl₂ cultures. Maximum cell yields were unaffected by CaCl₂ addition.
- **supplementing lesser utilised amino acids and vitamins** did not affect maximum Vero cell yields in cultures of either bead.
- **dilution of DMEM with D-PBS⁻** (1:1), with and without Ca²⁺ supplementation, produced similar maximum Vero cell yields in cultures of either bead. However, maximum cell yields were significantly lower (45%, $P < 0.01$) at high (1:3) dilutions. Cell yields were significantly higher in cultures of Cytodex-1 with DMEM diluted with D-PBS⁻ enriched with Ca²⁺.

Chapter 8. The Effects of Inhibitors on Vero Cell Growth in Microcarrier Culture

8. The Effects of Inhibitors on Vero Cell Growth in Microcarrier Cultures

8.1 Introduction

Cell growth can be inhibited by many chemical components and physical culture parameters. The metabolism of amino acids and sugars from the medium results in the production of waste components such as ammonia and lactate which could accumulate to inhibitory concentrations in the cultures. Physical parameters affecting cell growth include medium osmolarity and pH. pH was discussed previously (5.3.5-6).

Vero cells have been infrequently used in growth inhibition research and only Hassell and Butler (1990) and Hassell et al. (1991) have used them to assess ammonia inhibition. However, no reports were found on the effects of other potential growth inhibitors on these cells.

Ammonia inhibits the growth of murine L, baby hamster kidney (BHK), Madin-Darby canine kidney (MDCK), African green monkey kidney (Vero), murine hybridoma and human diploid fibroblast cells (Ryan and Cardin, 1966, Butler and Spier, 1984, Glacken et al., 1986, Reuveny et al., 1986 and Kimura et al., 1987). The inhibitory effect is cell line dependent with some (L cells) sensitive to low (1.00 mM) concentrations (Ryan and Cardin, 1966) and others (MDCK and Vero) to higher concentrations (4.00 mM) of ammonia (Glacken et al., 1986 and Hassell et al., 1991). The natural decomposition of glutamine also produces pyroglutamate. No one has previously determined whether pyroglutamate inhibits or stimulates cell growth.

Anaerobic metabolism of glucose produces lactate which also inhibits murine hybridoma, human diploid and murine 3T3 fibroblast cell growth (Kimura et al., 1987, Duval et al., 1992 and Rutz and Little, 1995). The inhibitory effect is also cell line dependent with some (murine hybridomas and 3T3) inhibited at low (10.00-20.00 mM) and others (murine hybridoma) at higher concentrations (20.00-30.00 mM) of lactate.

The metabolism of certain amino acids (histidine, tryptophan and glycine) produces formate (Rabinovitz and Tabor, 1958) in a mechanism which involves folate, excepting catabolism of tryptophan. Formate is produced by Chinese hamster ovary (CHO) cell growth in a serine/folate

dependent mechanism (Ihrig et al., 1994) and significantly inhibits them at 5.00-10.00 mM.

The medium salt concentration affects the initial culture osmolarity. The optimal osmolarity for growth varies between animal species and cell lines within the species (Waymouth, 1970) (e.g. the optimal for human lymphocyte cell growth was 230-240 mOsmol.L⁻¹ and for most murine cells was 340 mOsmol.L⁻¹). The osmolarity of normal culture medium is the same as human blood (310 mOsmol.L⁻¹). However, high osmolarities (400-520 mOsmol.L⁻¹) result in significantly lower (between 50-60%) hybridoma cell yields but also higher specific monoclonal antibody productivity (Ozturk et al., 1991 and Oyaas et al., 1994). High osmolarities (500-680 mOsmol.L⁻¹) also cause BHK cells to detach from growth surfaces (Wentz and Schugerl, 1992).

pH is important to Vero cell growth in cultures of either bead (5.3-5.6) with an optimal range of pH 6.8-7.1. However, ammonia inhibition of murine hybridomas is pH dependent and could act synergistically with lactate inhibition (Doyle and Butler, 1991).

This paper presents a study of the effects of several chemical and physical inhibitors on Vero cell growth in batch spinner flask cultures of Cytodex-1 and Cultispher-G microcarriers. The chemical inhibitors used were ammonia, lactate and formate and the physical inhibitor was medium osmolarity. The effects of pyroglutamate were also investigated. The cells were grown either unadapted or preadapted to various concentrations of the chemical inhibitors and to various osmolarities. Nutrient analyses were performed to determine any metabolic effects of the inhibitor compounds.

8.2 Materials and Methods

The methods of culturing and counting the cells and analyses of the nutrients have been described previously (2.1-2.5).

8.2.1 Preadaptation of Stock Cells

Stock Vero cells were grown (>6 passages) in DMEM + 5.0% v/v iron enriched, supplemented calf serum (SCS) either (i) unadapted or (ii) preadapted (2.1) to the inhibitor

compounds. DMEM was prepared (2.3) with either (a) "original" formulation or (b) "enriched" with serine and pyruvate (to 2.00 and 0.50 mM, respectively). Cells were preadapted to either DMEM containing various growth inhibitors at each concentration used in the experiments. The preadaptation protocol was to inoculate the cells ($0.10\text{--}0.15 \times 10^6$ cells/mL) in T75 culture flasks (35 mL) in the various media. After 72 h the cells were subcultured to the inoculum cell count.

8.2.2 Formate Assay

The formate assay was developed from Schutte et al. (1976). Sodium formate standards were prepared (0.018–10.000 mM) in 0.05 M potassium phosphate buffer (pH 7.5) containing phenol red (15 mg/L). Samples and standards were diluted (1:1 or 1:5) in phosphate buffer (0.05 M) and were added (100 μ L) to 96 well plates. β -nicotinamide adenine dinucleotide (100 μ L at 50 mM in 0.05 M potassium phosphate buffer) was added to each well. Formate dehydrogenase (50 μ L at 1 unit/mL) in RO water was added and the plates were mixed on a Molecular Devices Thermomax (Molecular Devices Inc., Sunnyvale, CA, USA) microplate reader. The plates were wrapped in cling-film and incubated (37°C) for 1.25 h before reading at 340 nm. Sample concentrations were determined against the standard curve (Appendix 3.2).

8.3 Results and Discussion

8.3.1 Effects of Ammonium Chloride

Introduction. Ammonia inhibits the growth of many mammalian cell lines and is produced in culture from amino acid metabolism. Therefore, this study determined the effects of supplementing ammonium chloride to DMEM on Vero cell growth in microcarrier cultures.

Experimental. Preadapted stock Vero cells were inoculated in original and enriched DMEM + 5.0% v/v SCS supplemented with various concentrations (up to 10.00 mM) of ammonium chloride. The cells were grown in spinner flasks, as described previously (2.4.3).

Results. Fig. 8.1 shows unadapted Vero cell growth in cultures of Cytodex-1 and Cultispher-G in original and enriched DMEM supplemented with various ammonium chloride concentrations. Fig. 8.2 shows the growth of preadapted cells in the same media. Fig. 8.3 shows the effect of the supplemented ammonium chloride concentration on maximum cell yields. Table 8.1 shows the cell growth rates and lag phases in these cultures. Table 8.2 shows the effects of the ammonium chloride concentration on the residual serine and glutamine concentration at maximum cell yield and the specific serine and glutamine consumption rates in cultures of either bead. Table 8.3 shows the IC_{50} concentrations of ammonium chloride in cultures of either bead.

(1) Maximum cell yields in cultures of either bead were affected by the supplemented ammonium chloride concentration in the DMEM, by the DMEM formulation and by preadaptation of the cells. (2) There were significantly higher (31%, $P<0.01$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all supplemented ammonium chloride concentrations and DMEM formulations used. (3) Maximum yields were significantly higher (18%, $P<0.05$) from preadapted compared to unadapted cells in cultures of either bead. (4) Maximum cell yields were significantly higher (18%, $P<0.05$) in enriched compared to original DMEM in cultures of either bead. (5) There were no interactions between the ammonia concentration, bead type or medium type used. (6) There were significantly higher ($P<0.05$) maximum yields in cultures of either bead from unadapted and preadapted cells at ≤ 3.00 mM supplemented ammonium chloride compared to all other concentrations used with respect to either DMEM formulation. (7) Preadapted cells showed similar inhibitory effects, except there was no significant difference in yields at ≤ 5.00 mM supplemented ammonium chloride.

(8) There were no significant differences in cell growth rates (Table 8.1) in cultures of either bead with respect to the supplemented ammonium chloride concentrations used. (9) There were lag phases (Table 8.1) in all cultures proportional to the supplemented ammonium chloride concentrations used and these were significantly longer (450%, $P<0.01$) at 10.00 mM supplemented ammonium chloride compared to the controls. (10) Lag phases were significantly longer (75%, $P<0.01$) from unadapted compared to preadapted cells with respect to all

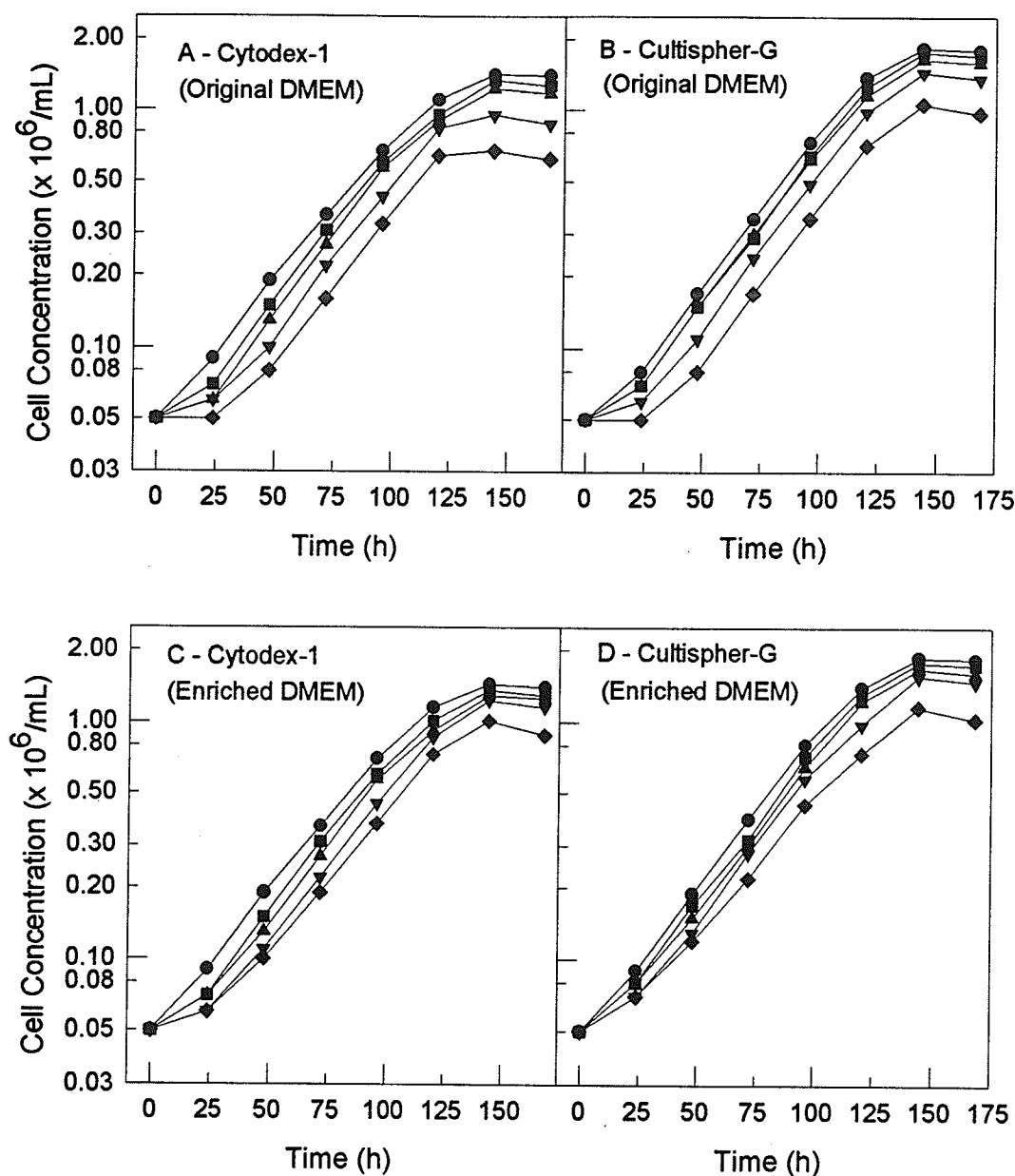


Fig. 8.1 - The effect of the supplemented ammonium chloride concentration in **original** or **enriched DMEM** + 5.0% v/v SCS on **unadapted Vero** cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A and C) and 1.70 g/L Cultispher-G (B and D). The cells were inoculated at 0.05×10^6 cells/mL with either $\bullet$ 0.00, $\blacksquare$ 1.00, $\blacktriangle$ 3.00, $\blacktriangledown$ 5.00 or $\blacklozenge$ 10.00 mM ammonium chloride. The cultures were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

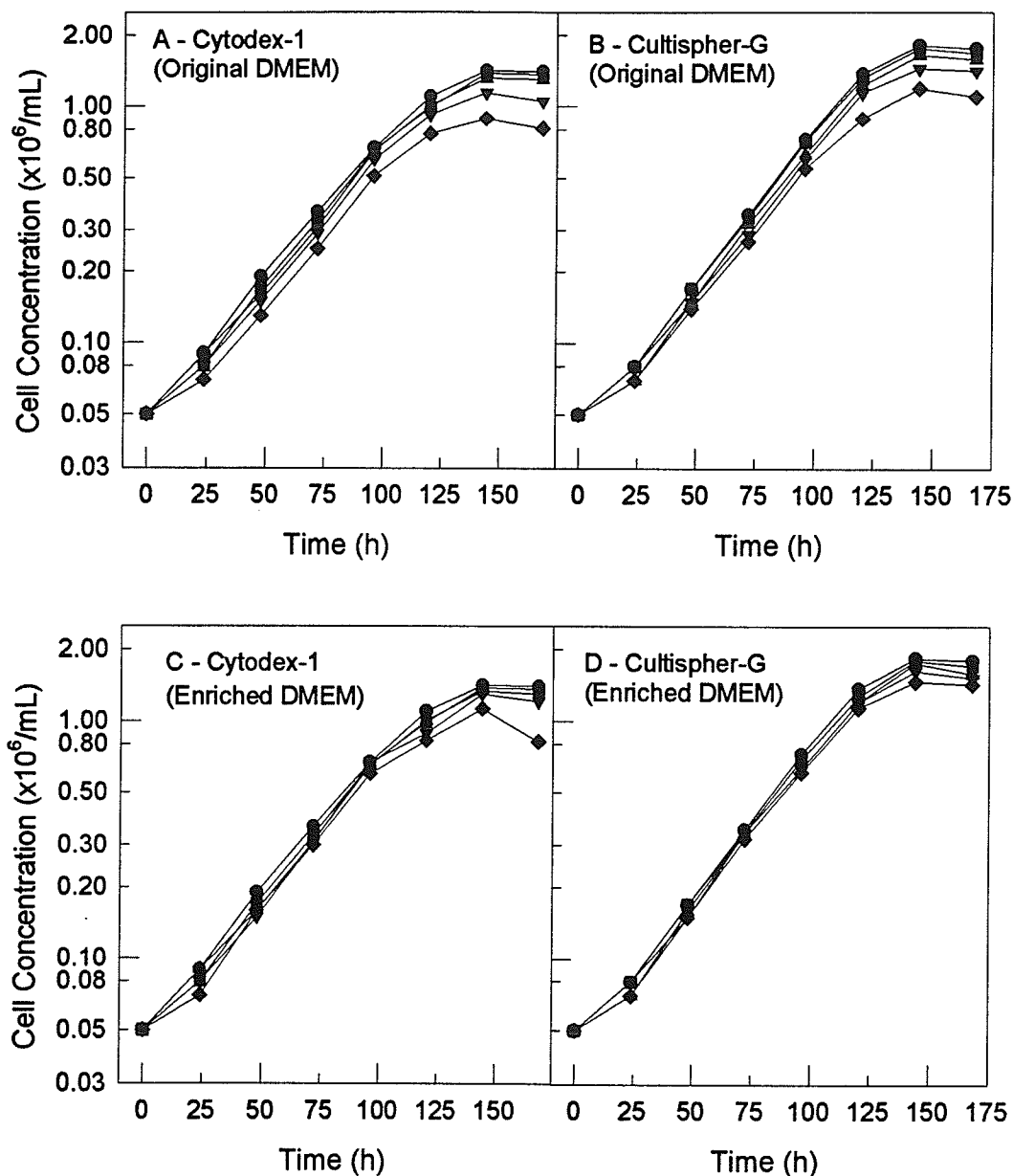


Fig. 8.2 - The effect of the supplemented ammonium chloride concentration in **original** or **enriched DMEM + 5.0% v/v sCS** on **preadapted Vero cell growth** in 100 mL spinner flasks on **1.00 g/L Cytodex-1 (A and C)** and **1.70 g/L Cultispher-G (B and D)**. The cells were inoculated at 0.05×10^6 cells/mL with either $\bullet$ 0.00, $\blacksquare$ 1.00, $\blacktriangle$ 3.00, $\blacktriangledown$ 5.00 or $\blacklozenge$ 10.00 mM ammonium chloride. Cultures were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

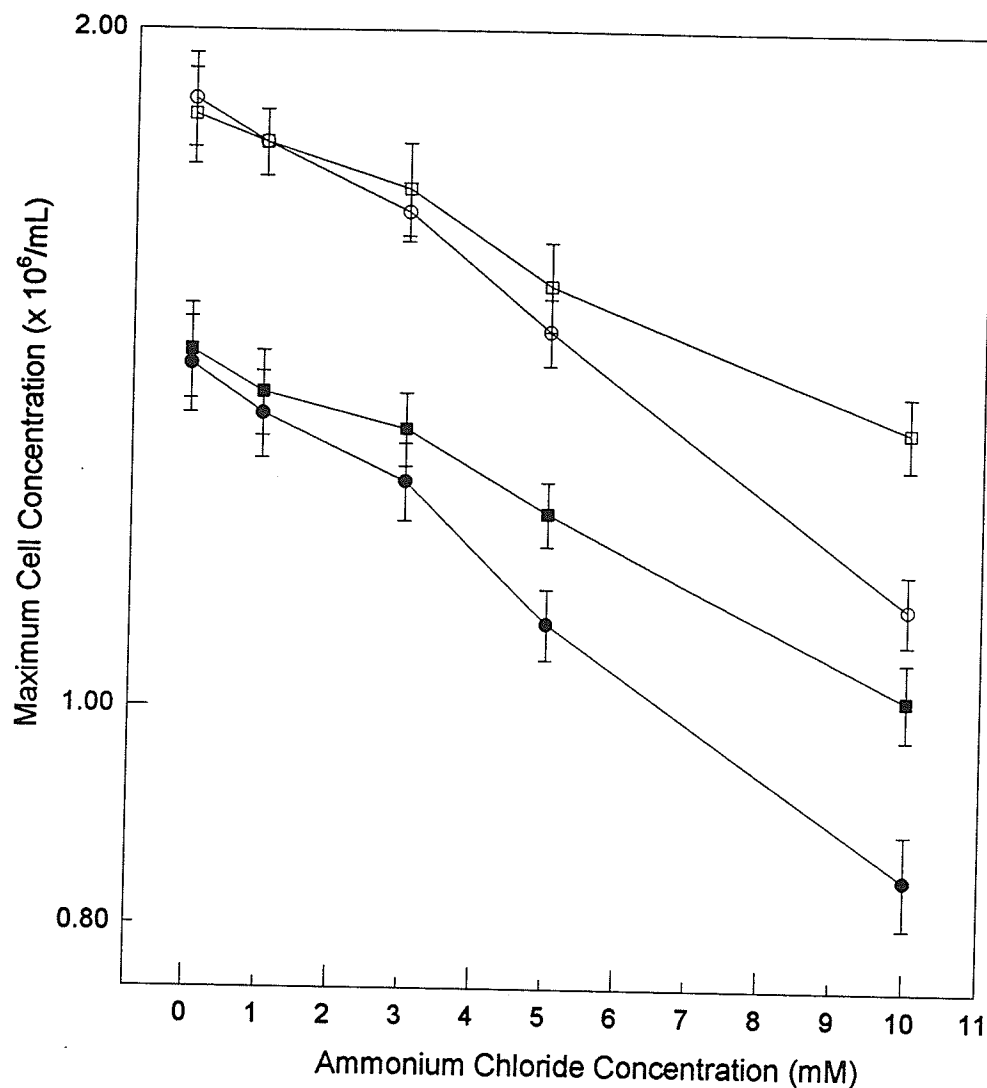


Fig. 8.3 - The effect of the supplemented ammonium chloride concentration on the maximum yields of unadapted and preadapted Vero cells in 100 mL spinner flask cultures of 1.00 g/L Cytodex-1 (Closed symbols) and 1.70 g/L Cultispher-G (Open symbols). The $\bullet$ unadapted or $\blacksquare$ preadapted cells were inoculated at 0.05×10^6 cells/mL with original or enriched DMEM + 5.0% v/v SCS. The cultures were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). Flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means of $n=4$.

Table 8.1 - The effects of the supplemented **ammonium chloride** concentration on **unadapted (A)** and **preadapted (B)** Vero cell **growth rates** and **lag phases** in 100 mL spinner flask cultures of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL with original or enriched DMEM + 5.0% v/v SCS. The cultures were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). Flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means of $n=2$.

Table 8.1A) - Unadapted Cells.

Ammonium Chloride Conc. (mM)	Specific Cell Growth Rate and Lag Phase			
	Cytodex-1		Cultispher-G	
Original DMEM	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
0.00	0.036 ± 0.003	3.00 ± 0.50	0.037 ± 0.003	4.00 ± 0.50
1.00	0.035 ± 0.003	6.00 ± 1.00	0.036 ± 0.003	6.00 ± 1.00
3.00	0.035 ± 0.003	15.00 ± 1.50	0.035 ± 0.003	6.00 ± 1.00
5.00	0.034 ± 0.003	15.00 ± 1.50	0.034 ± 0.003	15.00 ± 1.50
10.00	0.033 ± 0.003	27.00 ± 2.50	0.034 ± 0.003	27.00 ± 2.50
Enriched DMEM				
0.00	0.036 ± 0.003	3.00 ± 0.50	0.037 ± 0.003	3.00 ± 0.50
1.00	0.035 ± 0.003	9.00 ± 1.00	0.036 ± 0.003	6.00 ± 1.00
3.00	0.035 ± 0.003	9.00 ± 1.00	0.035 ± 0.003	6.00 ± 1.00
5.00	0.034 ± 0.003	18.00 ± 2.00	0.035 ± 0.003	15.00 ± 1.50
10.00	0.034 ± 0.003	18.00 ± 2.00	0.034 ± 0.003	15.00 ± 1.50

Table 8.1B) - Preadapted cells.

Ammonium Chloride Conc. (mM)	Specific Cell Growth Rate and Lag Phase			
	Cytodex-1		Cultispher-G	
Original DMEM	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)
0.00	0.036 ± 0.003	3.00 ± 0.50	0.038 ± 0.003	6.00 ± 1.00
1.00	0.036 ± 0.003	3.00 ± 0.50	0.037 ± 0.003	6.00 ± 1.00
3.00	0.036 ± 0.003	6.00 ± 1.00	0.037 ± 0.003	6.00 ± 1.00
5.00	0.035 ± 0.003	6.00 ± 1.00	0.036 ± 0.003	12.00 ± 0.50
10.00	0.034 ± 0.003	12.00 ± 1.00	0.035 ± 0.003	12.00 ± 0.50
Enriched DMEM				
0.00	0.036 ± 0.003	3.00 ± 0.50	0.038 ± 0.003	3.00 ± 0.50
1.00	0.036 ± 0.003	3.00 ± 0.50	0.038 ± 0.003	3.00 ± 0.50
3.00	0.036 ± 0.003	6.00 ± 1.00	0.037 ± 0.003	9.00 ± 1.00
5.00	0.036 ± 0.003	6.00 ± 1.00	0.036 ± 0.003	9.00 ± 1.00
10.00	0.035 ± 0.003	15.00 ± 1.50	0.036 ± 0.003	9.00 ± 1.00

Table 8.2 - The effects of the supplemented **ammonium chloride** concentration on **serine (A)** and **glutamine (B) residual concentrations** at maximum cell yield and **specific consumption rates** from **unadapted** and **preadapted** Vero cells in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS. Flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). Flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means from unadapted and preadapted cultures in each medium and are of n=4.

Table 8.2A) - Serine.

Ammonium Chloride Conc. (mM)	Residual Serine Conc. In DMEM at Maximum Cell Yield			
	(mM)			
	Cytodex-1		Cultispher-G	
	Original	Enriched	Original	Enriched
0.00	0.10	1.70	0.09	1.67
1.00	0.10	1.68	0.08	1.60
3.00	0.07	1.65	0.07	1.60
5.00	0.00	1.55	0.00	1.55
10.00	0.00	1.45	0.00	1.51
Specific Serine Consumption Rate ($\mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$)				
0.00	1.29	1.34	0.99	1.10
1.00	1.32	1.50	1.04	1.34
3.00	1.58	1.65	1.15	1.44
5.00	2.25	2.60	1.51	1.74
10.00	2.65	3.85	1.79	2.23

Table 8.2B) - Glutamine.

Ammonium Chloride Conc. (mM)	Residual Glutamine Conc. In DMEM at Maximum Cell Yield			
	(mM)			
	Cytodex-1		Cultispher-G	
	Original	Enriched	Original	Enriched
0.00	0.25	0.25	0.25	0.25
1.00	0.20	0.15	0.25	0.15
3.00	0.10	0.10	0.15	0.10
5.00	0.00	0.00	0.05	0.10
10.00	0.00	0.00	0.00	0.05
Specific Glutamine Consumption Rate ($\mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$)				
0.00	15.21	15.21	11.77	11.77
1.00	16.09	16.30	12.17	12.50
3.00	18.10	17.28	13.39	13.57
5.00	21.67	19.50	16.04	14.62
10.00	27.88	22.94	20.53	17.73

Table 8.3 - The supplemented ammonium chloride IC_{50} concentration for **unadapted** and **preadapted** Vero cells in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in either original or enriched DMEM + 5.0% v/v SCS supplemented with up to 10.00 mM ammonium chloride. Flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). Flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means from cultures of unadapted and preadapted cells in each medium and are of $n=2$.

Cell and or Medium Type	IC ₅₀ Concentration of Ammonium Chloride (mM)	
	Cytodex-1	Cultispher-G
Unadapted Cells		
Original DMEM	9.50 ± 0.70	11.52 ± 0.82
Enriched DMEM	14.03 ± 1.00	12.41 ± 0.90
Preadapted Cells		
Original DMEM	12.62 ± 0.90	13.15 ± 0.70
Enriched DMEM	15.93 ± 1.13	16.07 ± 1.14

supplemented ammonium chloride concentrations used. (11) Lag phases from unadapted Vero cells were significantly longer (37%, $P < 0.01$) with original compared to enriched DMEM at 10.00 mM ammonium chloride.

IC₅₀. The inhibitory effects of supplemented ammonium chloride were determined as the concentration causing a 50% reduction in cell yields compared to the controls (i.e. the IC₅₀). (1) There was no significant difference in the supplemented ammonium chloride IC₅₀s (Table 8.3) in cultures of either bead. (2) There were significantly higher (15%, $P = 0.05$) IC₅₀'s with preadapted cells compared to unadapted in cultures of either bead. (3) There were significantly higher (25%, $P < 0.01$) IC₅₀s in enriched compared to original DMEM in cultures of either bead. (4) The IC₅₀s determined in this study were significantly higher ($P < 0.01$) compared to the total ammonia concentration (3.10 mM) produced at maximum cell yield in normal batch (with no supplemented ammonia) Vero cultures (6.3.3). This suggests the ammonium chloride concentration reached in batch cultures would not significantly affect maximum cell yields.

Nutrient Analysis. Nutrient analyses showed serine (Table 8.2A)) was completely consumed, prior to maximum cell yield in cultures of either bead in original DMEM supplemented at 5.00 and 10.00 mM ammonium chloride. However, there was a higher residual serine concentration in all cultures in enriched DMEM due to the higher initial serine concentration in the medium. The specific serine consumption rates in cultures of either bead were increased proportional to the supplemented ammonium chloride concentration and were significantly higher (30%, $P < 0.01$) in control cultures of Cytodex-1 compared to Cultispher-G. The consumption rates were significantly higher (119%, $P < 0.01$) in cultures of either bead at 10.00 mM supplemented ammonium chloride compared to the controls in either DMEM. The concentration of glycine increased in all cultures proportional to the serine concentration consumed with a constant glycine to serine ratio (0.27:1).

Glutamine (Table 8.2B)) was almost or completely consumed prior to maximum cell yield in

all cultures. The specific glutamine consumption rates were increased proportional to the supplemented ammonium chloride concentration in either DMEM and were significantly higher (18%, $P < 0.05$) in Cytodex-1 cultures compared to Cultispher-G. The consumption rates were significantly higher in cultures of either bead from unadapted (78%, $P < 0.01$) and preadapted cells (51%, $P < 0.01$) at 10.00 mM supplemented ammonium chloride compared to the controls. Hence, the specific glutamine consumption rates were significantly higher (19%, $P < 0.01$) from unadapted compared to preadapted cells at 10.00 mM supplemented ammonium chloride.

There was no significant difference in the consumption rates of any other nutrients with respect to the supplemented ammonium chloride concentration used.

Discussion. Vero cell yields were affected by the supplemented ammonium chloride concentration used. The cell yields were unaffected at 3.00 mM supplemented ammonium chloride which agrees with the Vero cell results of Hassell et al. (1991). The IC_{50} concentrations were also significantly higher compared to the total ammonia concentration produced during batch cultures of either bead type (3.10 mM from 6.3.3). The IC_{50} s were affected by preadaptation of the cells and by the medium formulation used. The cells could be adapting to the supplemented ammonium chloride concentrations by utilising nutrients such as serine more efficiently, as serine was important to cell growth at high supplemented ammonium chloride concentrations. This could explain the higher IC_{50} s in serine enriched DMEM compared to original DMEM. Pyruvate from glycolysis (Voet and Voet, 1990) or in the medium could be involved and will be discussed below. The adaptation to ammonium chloride could be expected, as Vero cells derive from monkey kidneys; the site of ammonia removal from the body.

Cell Growth Rates. Lag phases were increased proportional to the initial concentrations of supplemented ammonium chloride suggesting it inhibited initial cell growth. However, the effect could be overcome with time, as cell growth rates were similar in all cultures. This could be explained by pyruvate production and will be discussed below.

Nutrient Effects. There was high serine and glutamine consumption in cultures at 5.00-10.00 mM supplemented ammonium chloride. None of the previous reports on ammonia inhibition has discussed any nutrient effects other than for glutamine. Maximum cell yields were highly dependent on the serine concentration. Growth ceased in original DMEM immediately after serine depletion and the higher serine concentration in enriched DMEM could explain the higher cell yields compared to original DMEM. The increased glycine concentration in all cultures could be linked to serine C₁ metabolism through the tetrahydrofolate pathway (6.4.3 and Voet and Voet, 1990). The metabolism of glutamine has been discussed previously (6.4.3 and McKeehan, 1982).

Previous studies (7.3.6) showed pyruvate addition (0.50 mM) to DMEM removed lag phases following storage (28 d at 4°C). O'Donnell-Tormey et al. (1987) observed that pyruvate secreted by the cells could detoxify the medium and the effect was similar to extracellular catalase. Lag phases in all cultures supplemented at 10.00 mM ammonium chloride were reduced in pyruvate enriched DMEM agreeing with the previous authors. However, the effect could involve pyruvate sequestration of ammonia (Voet and Voet, 1990) which should have increased the alanine concentration. No such increase was found. Pyruvate reacts in a 1:1 reaction with ammonia and the pyruvate enrichment (0.50 mM) was significantly less compared to the high supplemented ammonium chloride concentration (10.00 mM). Therefore, cell yield increases in such cultures might not involve pyruvate.

Inhibition Mechanism. Several mechanisms have been proposed for ammonia/ammonium inhibition of cell growth. Ryll et al. (1994) suggested ammonium ions affect intracellular UDP-NAcetyl-galactosamine nucleotide pools in a synergistic effect with glucose. This could affect glycoprotein glycosylation (Voet and Voet, 1990) and cell energy charge ratios.

The species (ammonia/ammonium ion) causing cell inhibition is unclear. Doyle and Butler (1990) showed extracellular ammonia was the more likely inhibitor and could readily diffuse through cell membranes. Once in the cytoplasm it could be protonated to form ammonium ions

resulting in the effects described by Ryll et al. (1994). However, Martinelle and Haggstrom (1993) suggest ammonium ions are transported across cell membranes in competition with potassium ions via the Na^+/K^+ -ATPase or $\text{Na}^+\text{K}^+\text{Cl}^-$ -cotransporter systems. Excess potassium in the medium prevented the inhibition of SP2/0-Ag-14 myeloma cells. This would contradict Doyle and Butler (1990). The protective effect of potassium ions was not tested in this study.

Mitochondria are the suggested site of action for ammonia toxicity (Tagler et al., 1975 and Kovacevic and McGiven, 1983). The ammonia could inhibit glutamate dehydrogenase resulting in decreased formation of α -ketoglutarate, affecting the tricarboxylic acid (TCA) cycle and amino acid biosyntheses. This could result in accumulation of alanine, glutamate and glycine, as shown with MDCK, Vero and hybridoma cells (Butler et al., 1983, Polastri et al., 1984 and Miller et al., 1989). The previous results (6.3.3) agree with this.

8.3.2 Effects of Pyroglutamate

Introduction. The natural decomposition of glutamine produces pyroglutamate and ammonia. The inhibitory or stimulatory effects of pyroglutamate has not been determined for any cells. Therefore, this study determined the effects of adding L-pyroglutamate to the DMEM on Vero cell growth in microcarrier cultures.

Experimental. Preadapted stock Vero cells were inoculated in original and enriched DMEM + 5.0% v/v SCS supplemented with various concentrations (up to 10.00 mM) of pyroglutamate. The cultures were grown in spinner flasks, as described above (8.3.1).

Results. Fig. 8.4 shows unadapted and preadapted Vero cell growth in cultures of Cytodex-1 and Cultispher-G with the various supplemented pyroglutamate concentrations used. Fig. 8.5 shows the effects of the pyroglutamate concentration on maximum yields in these cultures. Table 8.4 shows the cell growth rates and lag phases in cultures of either bead with the various

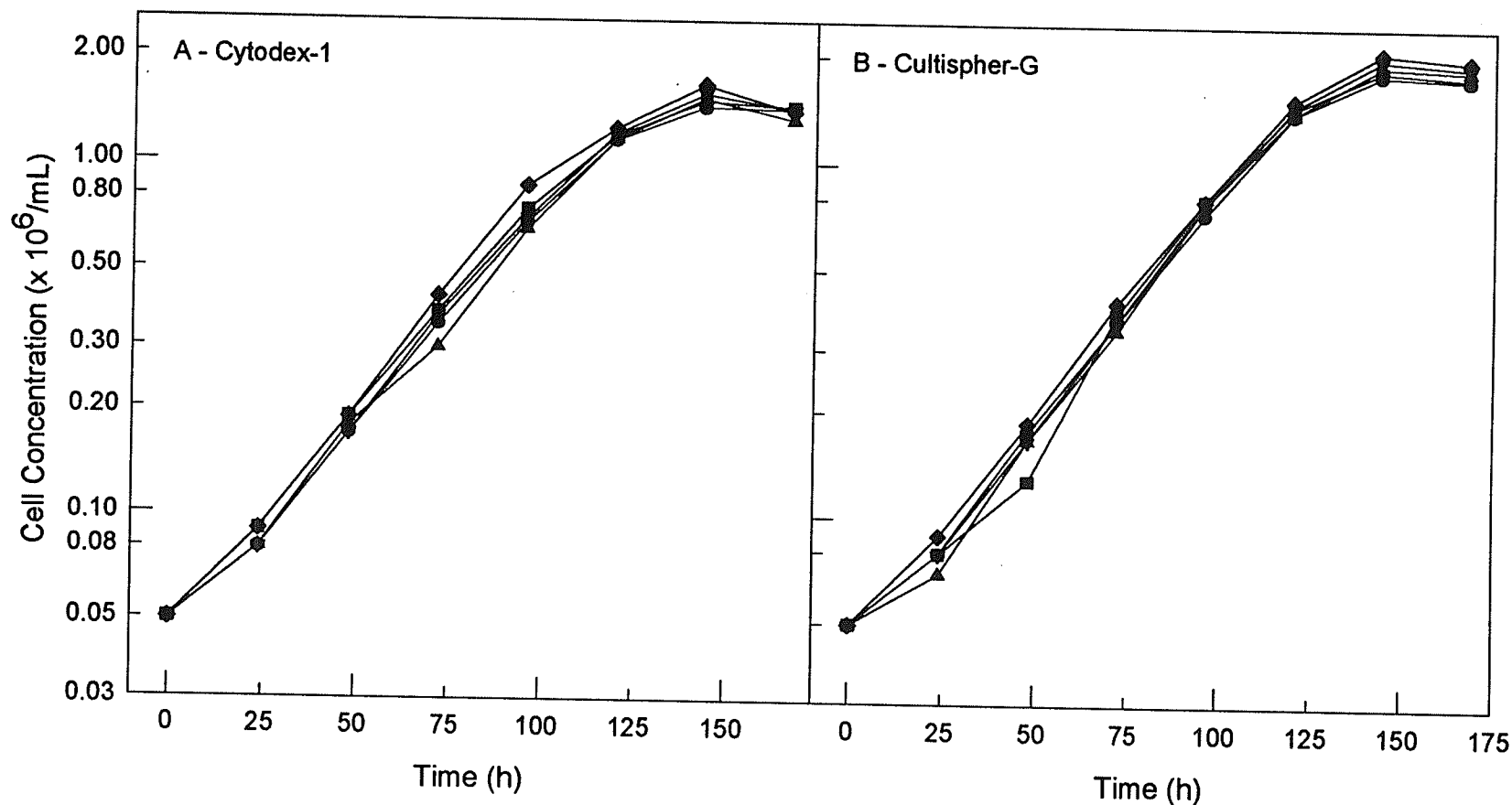


Fig. 8.4 - The effect of the supplemented **L-pyroglutamate** concentration in DMEM + 5.0% v/v sCS on **preadapted** Vero cell growth in 100 mL spinner flasks on **1.00 g/L Cytodex-1 (A)** and **1.70 g/L Cultispher-G (B)**. The cells were inoculated at 0.05×10^6 cells/mL with either $\bullet$ 0.00, $\blacksquare$ 2.50, $\blacktriangle$ 5.00, $\blacktriangledown$ 7.50 or $\blacklozenge$ 10.00 mM L-pyroglutamate. The cultures were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). Flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means from cultures of original and enriched DMEM and are of $n=4$.

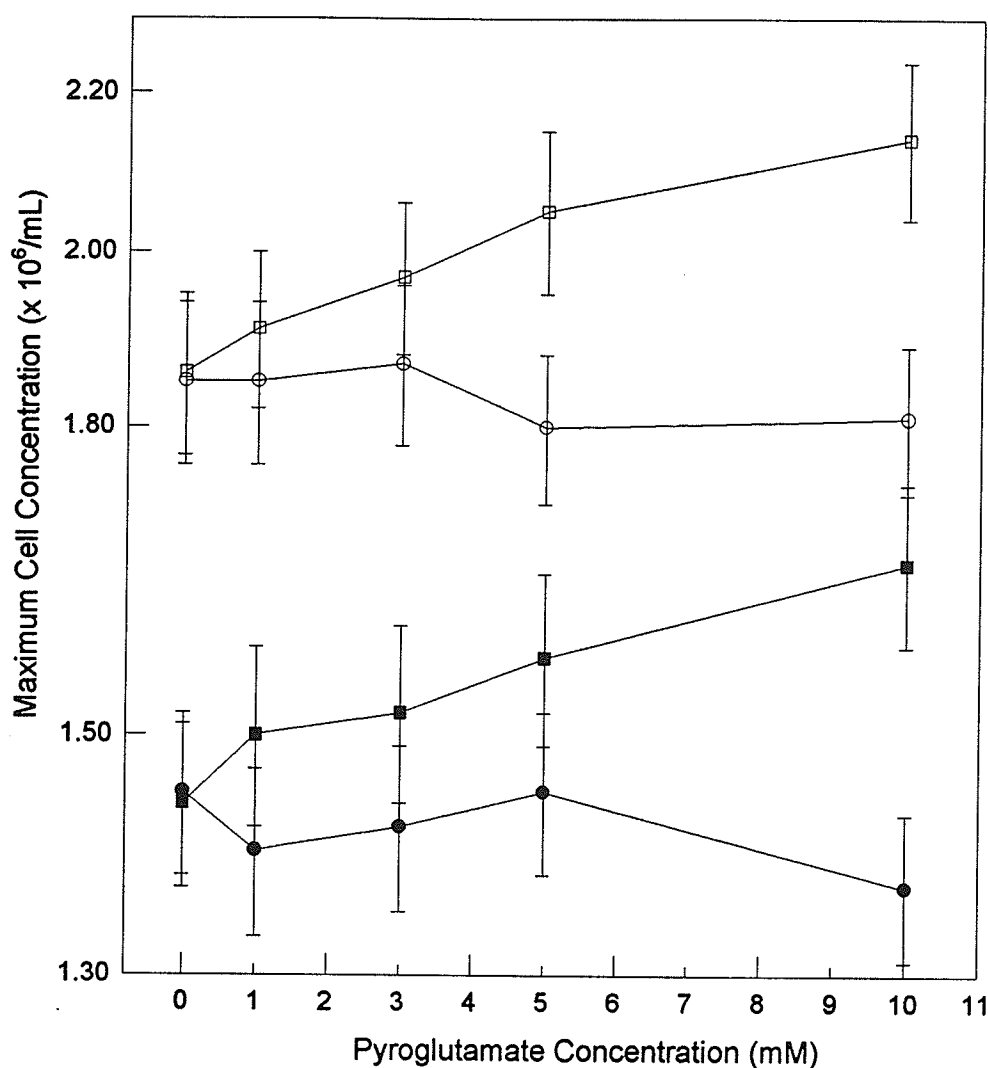


Fig. 8.5 - The effects of the supplemented **pyroglutamate** concentration in the DMEM on **maximum Vero cell yields** in 100 mL spinner flasks on **1.00 g/L Cytodex-1** (Closed symbols) and **1.70 g/L Cultispher-G** (Open symbols). The ● unadapted or ■ preadapted cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). The points are means from cultures of original and enriched DMEM and are of $n=4$.

Table 8.4 - The effects of the supplemented **pyroglutamate** concentration (up to 10.00 mM) in the DMEM on the Vero **cell growth rates** and **lag phases** in 100 mL spinner flasks of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). Flasks were readjusted to pH 7.1 daily after 72 h in culture with 2.00 M NaOH. The values are means from cultures of original and enriched DMEM from unadapted and preadapted cells and are of n=8.

Supplemented Pyroglutamate Concentration (mM)	The Specific Vero Cell Growth Rate and Lag Phase in Cultures of Either Bead Type			
	Cytodex-1		Cultispher-G	
	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
0.00	0.035 ± 0.003	4.00 ± 0.50	0.037 ± 0.003	4.00 ± 0.50
2.50	0.035 ± 0.003	4.00 ± 0.50	0.037 ± 0.003	4.00 ± 0.50
5.00	0.035 ± 0.003	7.00 ± 0.50	0.037 ± 0.003	9.00 ± 1.00
7.50	0.035 ± 0.003	6.00 ± 0.50	0.037 ± 0.003	6.00 ± 0.50
10.00	0.035 ± 0.003	6.00 ± 0.50	0.037 ± 0.003	6.00 ± 0.50

supplemented pyroglutamate concentrations used. (1) Vero cell growth was not affected by the pyroglutamate concentrations used in this study. (2) There were significantly higher (22%, $P < 0.05$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the pyroglutamate concentrations used. (3) ANOVA and paired Student's t-tests showed there were no significant differences in maximum cell yields from unadapted or preadapted cells with respect to any pyroglutamate concentration used. (4) There were no significant differences in maximum cell yields in original DMEM compared to enriched. (5) There (Table 8.4) were no significant differences in the cell growth rates or lag phases in cultures of either bead with respect to the supplemented pyroglutamate concentrations used.

Regression analysis was also performed (Fig. 8.5), as preadapted cells were showing proportional increases with the supplemented pyroglutamate concentrations used. This analysis showed there were no significant differences ($r = 0.425$) in the maximum cell yields from unadapted cells with respect to any pyroglutamate concentrations used. However, maximum cell yields were significantly increasing in a linear manner ($r = 0.967$) from preadapted cells in cultures of either bead.

A complete nutrient analysis was not performed on these cultures.

Discussion. Maximum cell yields in cultures of either bead from unadapted cells were not affected by the pyroglutamate concentrations used. However, maximum cell yields from preadapted cells were increasing proportional to the supplemented pyroglutamate concentration. The highest cell yield (10.00 mM pyroglutamate) from preadapted cells was significantly different from the control by regression analysis. This suggests the cells were either metabolising glutamine more efficiently with pyroglutamate present or were metabolising pyroglutamate once glutamine had been depleted. No one has previously described any effect with pyroglutamate.

8.3.3 Effects of Lactate

Introduction. Lactate inhibits human and murine cell growth. Therefore, this study determined the effects of supplementing lactate in the DMEM on Vero cell growth in microcarrier cultures.

Experimental. Preadapted stock Vero cells were inoculated in original and enriched DMEM + 5.0% v/v sCS supplemented with various concentrations (up to 25.00 mM) of sodium lactate. The cells were grown in spinner flasks, as described above (8.3.1). All media types was adjusted to pH 7.4 with 2.00 M NaOH prior to inoculation of the experiment.

Results. Table 8.5 shows the maximum Vero cell yield in cultures of either Cytodex-1 or Cultispher-G at the various supplemented lactate concentrations used. Fig. 8.6 shows the effects of the supplemented lactate concentration on maximum cell yields. Table 8.6 shows the effects of the supplemented lactate on Vero cell growth rates and lag phases. Table 8.7 shows the supplemented lactate IC_{50} concentrations for Vero cells in cultures of either bead type. (1) Maximum Vero cell yields in cultures of either bead were affected by the supplemented lactate concentration in the medium. (2) Maximum cell yields were significantly higher (22%, $P < 0.05$) in Cultispher-G cultures compared to Cytodex-1 with respect to all the supplemented lactate concentrations used. (3) Maximum cell yields in cultures of either bead were significantly higher ($P < 0.01$) at ≤ 10.00 mM supplemented lactate compared to all other concentrations used with respect to either DMEM formulation used. (4) There were no significant differences in maximum cell yields with original compared to enriched DMEM with respect to any supplemented lactate concentration used. (5) There was no significant difference in the maximum cell yields in cultures of either bead from unadapted and preadapted cells with respect to the supplemented lactate concentrations used. (6) There was no significant difference in the cell growth rates (Table 8.6) or lag phases in cultures of either bead with respect to the supplemented lactate concentrations used. (7) There was no significant difference in the IC_{50} concentration (Table

Table 8.5 - The effect of the supplemented **lactate** concentration in **original** and **enriched DMEM** + 5.0% v/v SCS on maximum yields of **unadapted** and **preadapted** Vero cells in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL with various concentrations (up to 25.00 mM) of lactate. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). Flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Stock Cells	Medium Enrichment	Supplemented Lactate Conc. (mM)	Maximum Cell Concentration ($\times 10^6$ /mL)	
			Cytodex-1	Cultispher-G
Unadapted	None	0.00	$1.47 \pm 0.11^*$	$1.81 \pm 0.13^*$
		5.00	1.44 ± 0.10	1.80 ± 0.12
		10.00	1.29 ± 0.09	1.62 ± 0.12
		17.50	1.17 ± 0.07	1.56 ± 0.11
		25.00	1.05 ± 0.07	1.27 ± 0.08
Preadapted	None	0.00	$1.47 \pm 0.11^*$	$1.81 \pm 0.13^*$
		5.00	1.45 ± 0.11	1.77 ± 0.11
		10.00	1.36 ± 0.09	1.60 ± 0.11
		17.50	1.25 ± 0.08	1.57 ± 0.11
		25.00	1.12 ± 0.07	1.37 ± 0.09
Unadapted	Serine and Pyruvate	0.00	$1.47 \pm 0.11^*$	$1.81 \pm 0.13^*$
		5.00	1.46 ± 0.11	1.78 ± 0.12
		10.00	1.38 ± 0.08	1.64 ± 0.11
		17.50	1.30 ± 0.08	1.57 ± 0.11
		25.00	1.14 ± 0.07	1.34 ± 0.08
Preadapted	Serine and Pyruvate	0.00	$1.47 \pm 0.11^*$	$1.81 \pm 0.13^*$
		5.00	1.47 ± 0.10	1.76 ± 0.12
		10.00	1.42 ± 0.11	1.64 ± 0.11
		17.50	1.33 ± 0.09	1.53 ± 0.10
		25.00	1.22 ± 0.08	1.44 ± 0.09

* Highest cell yield.

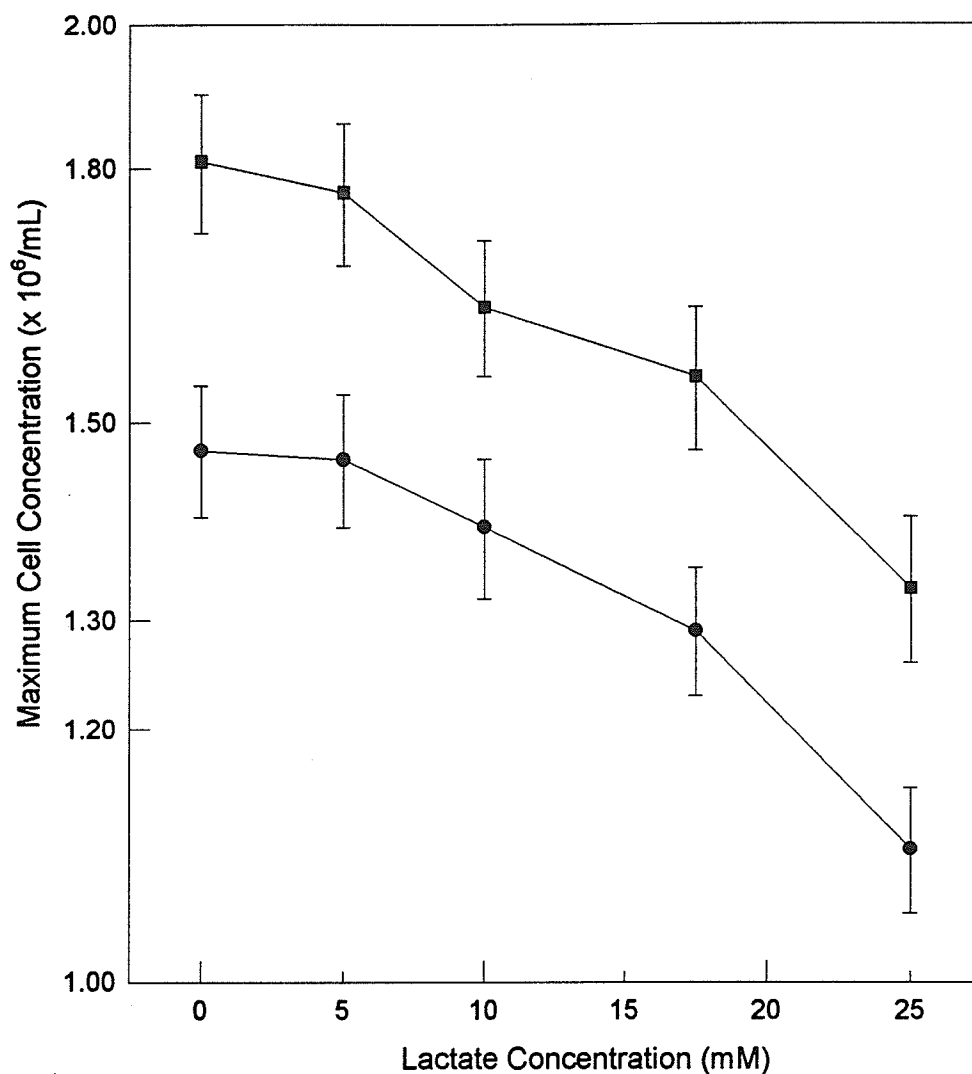


Fig. 8.6 - The effects of the supplemented lactate concentration in the DMEM on maximum Vero cell yields in 100 mL spinner flasks on 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS on either ● Cytodex-1 or ■ Cultispher-G. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). Flasks were readjusted to pH 7.1 daily after 72 h in culture with 2.00 M NaOH. The points are means from cultures of original and enriched DMEM and are of $n=8$.

Table 8.6 - The effects of the supplemented lactate concentration (up to 25.00 mM) in the DMEM on the Vero cell growth rates and lag phases in 100 mL spinner flasks of 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v SCS. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). Flasks were readjusted to pH 7.1 daily after 72 h in culture with 2.00 M NaOH. The values are means from cultures of original and enriched DMEM with unadapted and preadapted cells and are of n=8.

Supplemented Lactate Conc. (mM)	The Specific Vero Cell Growth Rate and Lag Phase in Cultures of Either Bead Type			
	Cytodex-1		Cultispher-G	
	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
0.00	0.035 ± 0.003	6.00 ± 0.50	0.037 ± 0.003	6.00 ± 0.50
5.00	0.035 ± 0.003	6.00 ± 0.50	0.037 ± 0.003	6.00 ± 0.50
10.00	0.034 ± 0.003	6.00 ± 0.50	0.036 ± 0.003	9.00 ± 1.00
17.50	0.034 ± 0.003	9.00 ± 1.00	0.036 ± 0.003	9.00 ± 1.00
25.00	0.034 ± 0.003	6.00 ± 0.50	0.035 ± 0.003	9.00 ± 1.00

Table 8.7 - The supplemented lactate IC_{50} concentration for Vero cells in 100 mL spinner flasks on 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in either original or enriched DMEM + 5.0% v/v SCS containing up to 25.00 mM lactate. Flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). Flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means from cultures of unadapted and preadapted cells in each medium and are of n=2.

Cell and or Medium Type	IC_{50} Concentration of Lactate (mM)	
	Cytodex-1	Cultispher-G
Unadapted Cells		
Original DMEM	35.71 ± 0.25	35.08 ± 0.25
Enriched DMEM	38.09 ± 0.27	37.85 ± 0.27
Preadapted Cells		
Original DMEM	38.78 ± 0.28	37.00 ± 0.26
Enriched DMEM	41.50 ± 0.29	39.78 ± 0.28

8.7) of supplemented lactate for cultures of either bead from unadapted or preadapted cells in either DMEM. The average supplemented lactate IC_{50} concentration (37.97 mM) (from the cultures in this study) was significantly higher ($P < 0.05$) compared to the total lactate concentration produced at maximum cell yield in batch cultures (with no supplemented lactate) of Cytodex-1 (73%) and Cultispher-G (21%) in previous studies (6.3.3). The results suggest there could be significant inhibition (31%, $P < 0.01$) of maximum cell yields in cultures of either bead by lactate at the normal total lactate concentrations produced.

Detailed nutrient analyses were not performed on these cultures.

Culture pH. All cultures were initially at pH 7.4 following equilibration with 10.0% v/v CO_2 overlay. The control cultures had decreased to pH 7.0 in Cytodex-1 and to pH < 6.8 in Cultispher-G after 72 h. These cultures decreased to the same values prior to daily pH readjustment. However, cultures with 25.00 mM supplemented lactate decreased to pH < 6.7 prior to each daily readjustment and could not be maintained within the optimal range (6.8-7.1) for growth by a single daily readjustment using more (two-fold) 2.00 M NaOH. The 25.00 mM lactate cultures decreased to pH < 6.8 following readjustment to pH 7.1 significantly faster (6 h) compared to the controls (18 h).

Discussion. Maximum Vero cell yields in cultures of either bead were lowered proportional to the increase in the supplemented lactate concentration. Thus, lactate was inhibitory to Vero cell growth. However, the IC_{50} s in this study agree with previous values of between 30.00-40.00 mM for human fibroblast (Kimura et al. 1987) and murine hybridoma cells (Glacken et al. 1988). The results with 25.00 mM supplemented lactate suggest there could be significant growth inhibition in cultures reaching this concentration (i.e. Cultispher-G). The cells could not be preadapted to growth at high supplemented lactate concentrations. The culture pH fluctuations could explain this effect, as the pH became very low at high lactate concentrations. This will be discussed below. The maximum cell yields were also not significantly affected by growth in

enriched or original DMEM. This suggests the inhibitory effect of lactate was not to increase the consumption of any of the major nutrients, as was observed with ammonia above (8.3.1).

Several mechanisms have been proposed for lactate inhibition of cell growth. Rutz and Little (1995) showed lactate interfered with 3T3 cell cycle control and exponentially growing cells accumulated in the G₂/M phase at high lactate concentrations. Growth from low cell inoculum concentrations (0.03×10^6 cells/cm²) was inhibited whilst it was stimulated at high inoculum concentrations (0.51×10^6 cells/cm²). There were several problems with their data including significantly lower (>30%) actual cell concentrations at inoculation compared to the expected and differences between inoculum concentrations of cultures in the same experiment. Indeed, their data could be reinterpreted to show lactate had no effect on cell growth.

Duval et al. (1992) showed lactate inhibition of hybridoma cell growth could involve culture pH effects, as inhibition was removed by preadjusting the lactate solution pH with NaOH prior to addition to cultures. However, the inhibitory effect remained if the culture pH was adjusted after the lactate addition. The cultures in this study could agree with this, as the medium was prepared containing lactate before readjustment to pH 7.4 and cell inoculation. However, the culture pH during growth was always significantly lower (pH <6.7) at each sample time in cultures of either bead with 25.00 mM supplemented lactate compared to the controls (pH 7.0 in Cytodex-1 cultures and 6.8 in Cultispher-G). The pH of the high supplemented lactate cultures also decreased faster compared to the controls which caused these cultures to be outside the optimal pH range for growth almost all of the time. Thus, lactate inhibition could be due to culture pH effects, as discussed previously (5.3.6). Vero cell growth was inhibited at 25.00 mM supplemented lactate but significantly less (40%) compared to the hybridoma cells used by the previous authors. A cell line dependent difference could explain this.

Low culture pH could inhibit enzymes such as phosphofructokinase (Duval et al., 1992). This would reduce glycolytic flux, limiting both pyruvate production and membrane glycoprotein glycosylation (Kleinman et al., 1981 and Akiyama et al., 1990) producing reduced cell growth. The supplemented lactate could be interfering with the buffering capacity of the medium, as the

culture pH decreased faster in high supplemented lactate cultures compared to the controls.

Meats et al. (1989) suggested exogenous lactate interfered with membrane transport in hamster HIT-T15 pancreatic cells. The lactate changed the plasma membrane potential and triggered intracellular calcium accumulation. This could have increased nuclear envelope breakdown in the M phase (Patel et al., 1989 and 4.3.7) which could have shortened the G₂/M phase and not lengthened it, as suggested by Rutz and Little (1995). However, if the cells were unable to counteract this accumulation after division, this could explain longer cell cycle times.

Lactate and ammonia could inhibit cells synergistically (Hassell et al., 1991). The authors showed >12.00 mM lactate and 1.00-4.00 mM ammonia lowered maximum McCoy and MDCK cell yields. This effect could explain the similar cell yields in all cultures over the first 72 h in culture in this study. Only after this time was any inhibitory effect seen at high supplemented lactate concentrations. Based on previous results (6.3.3) there could be approximately 2.00 mM ammonia and an extra 12.50 mM lactate produced from cell growth at this time.

8.3.4 Effects of Formate

Introduction. Formate is produced by CHO cells during growth in culture flasks and inhibits their growth. Therefore, this study determined the effects of supplementing sodium formate in the DMEM on Vero cell growth in microcarrier cultures.

Experimental. Preadapted stock Vero cells were grown in original or enriched DMEM + 5.0% v/v SCS supplemented with various concentrations (up to 10.00 mM) of sodium formate. The cultures were grown in spinner flasks, as described above (8.3.1).

Results. Fig. 8.7 show unadapted and Fig. 8.8 preadapted Vero cell growth in cultures of Cytodex-1 and Cultispher-G in original and enriched DMEM with the various formate concentrations used. Fig. 8.9 shows the effects of the supplemented formate concentration on

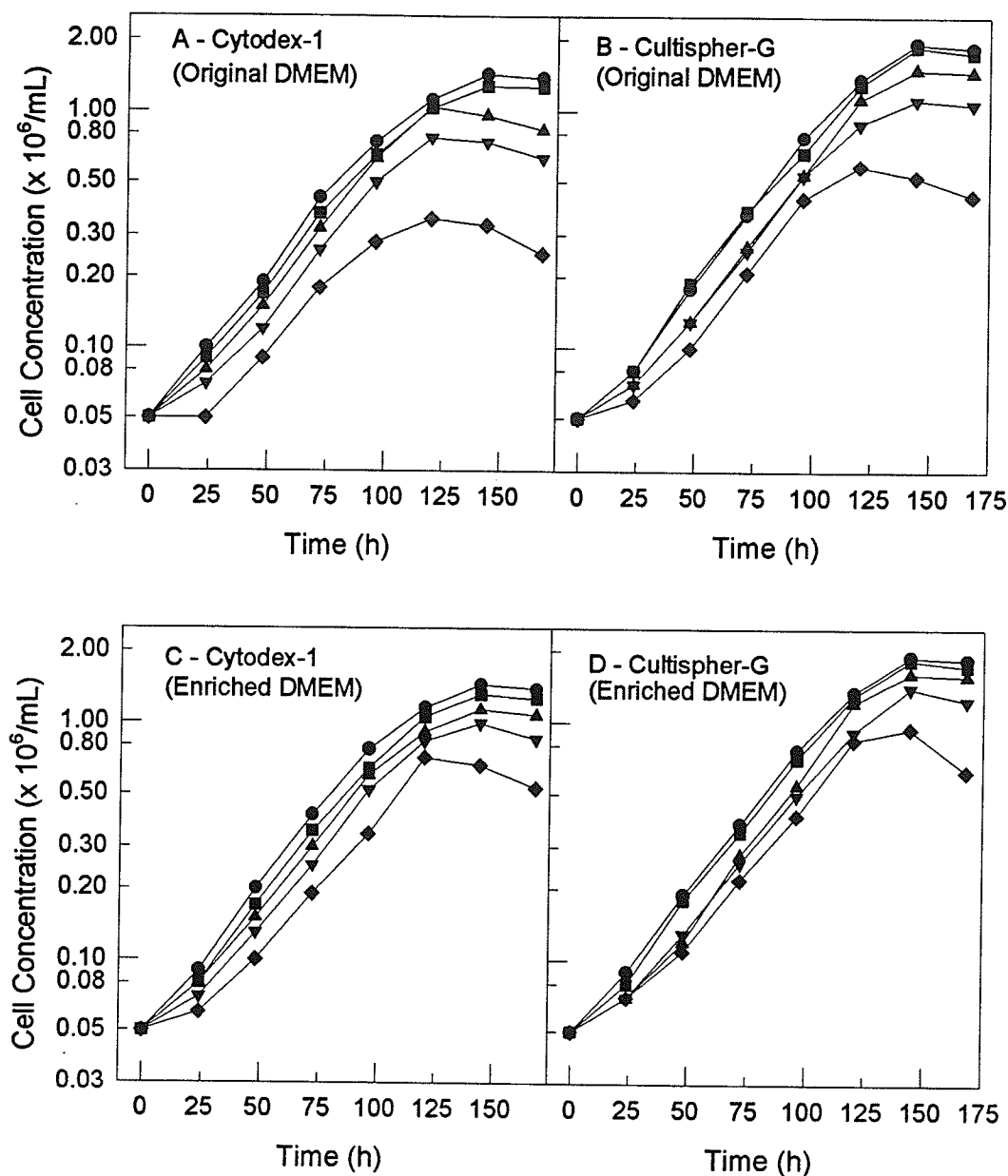


Fig. 8.7 - The effect of the supplemented formate concentration in **original** and **enriched DMEM** + 5.0% v/v SCS on **unadapted** Vero cell growth in 100 mL spinner flasks on **1.00 g/L Cytodex-1** (A and C) and **1.70 g/L Cultispher-G** (B and D). The cells were inoculated at 0.05×10^6 cells/mL with either $\bullet$ 0.00, $\blacksquare$ 2.50, $\blacktriangle$ 5.00, $\blacktriangledown$ 7.50 or $\blacklozenge$ 10.00 mM formate. The cultures were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). Flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

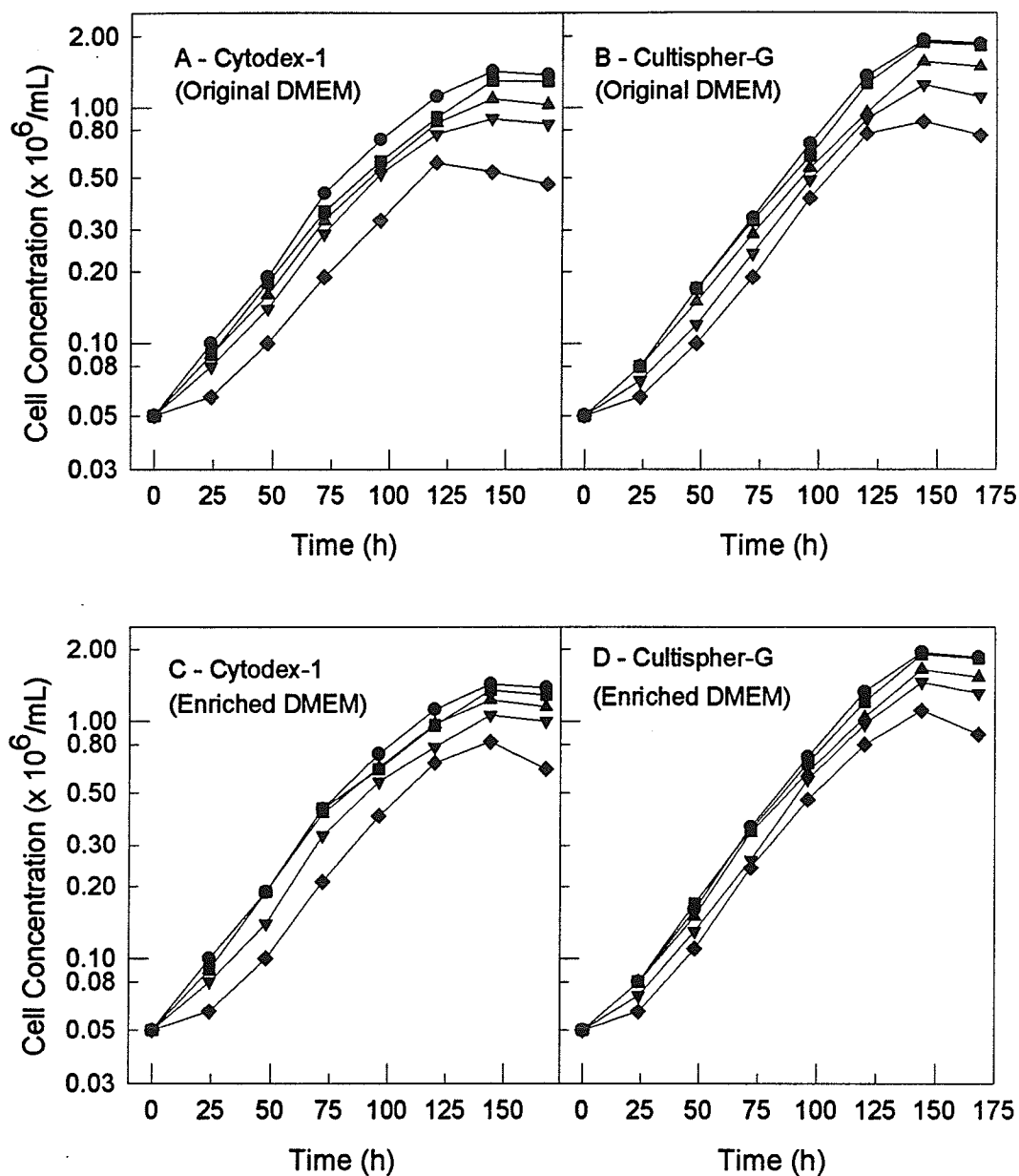


Fig. 8.8 - The effect of the supplemented formate concentration in **original** and **enriched DMEM** + 5.0% v/v SCS on **preadapted** Vero cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A and C) and 1.70 g/L Cultispher-G (B and D). The cells were inoculated at 0.05×10^6 cells/mL with either $\bullet$ 0.00, $\blacksquare$ 2.50, $\blacktriangle$ 5.00, $\blacktriangledown$ 7.50 or $\blacklozenge$ 10.00 mM sodium formate. The cultures were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). Flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means of $n=2$.

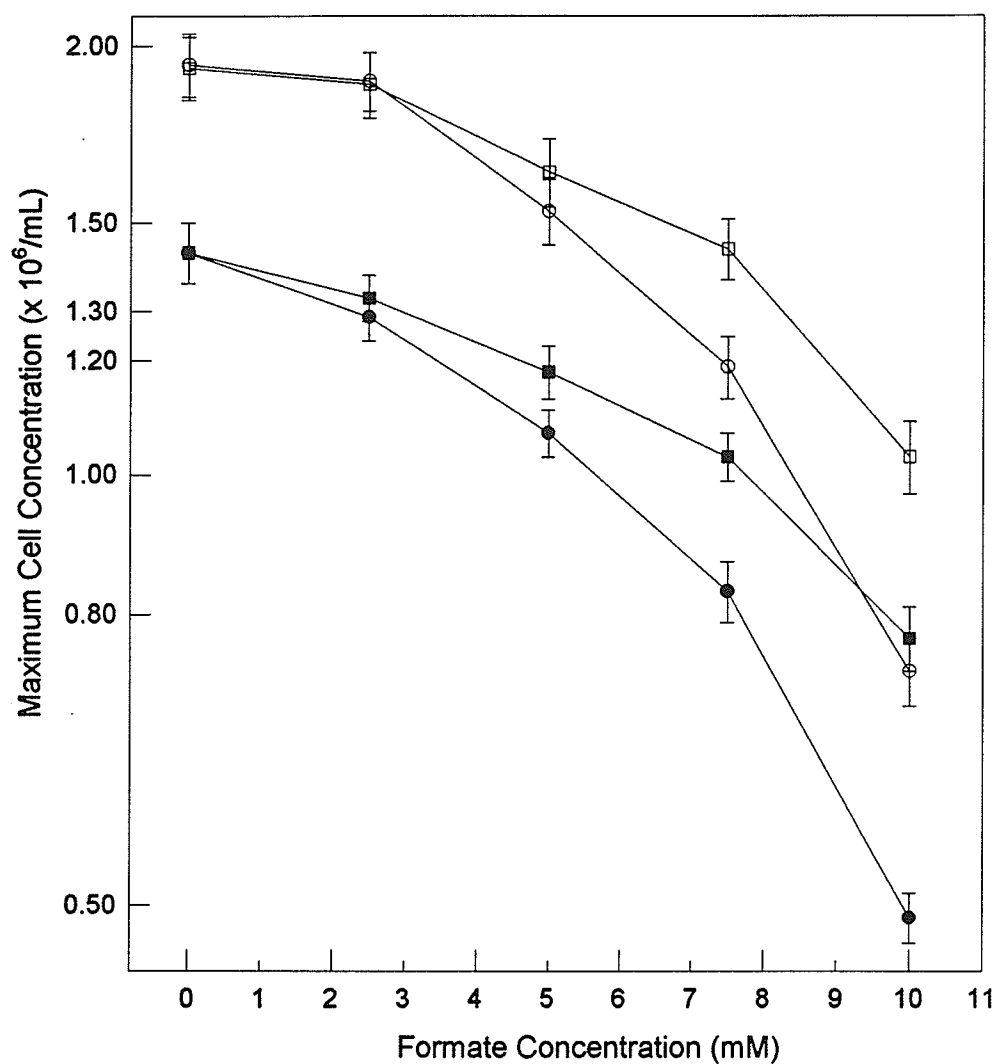


Fig. 8.9 - The effects of the supplemented **formate** concentration on **maximum Vero cell yields** in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in either $\bullet$ original or $\blacksquare$ enriched formulations DMEM + 5.0% v/v sCS. The flasks were stirred at 40 rpm continuously (Cytodex-1 - Closed symbols) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G - Open symbols). The flasks were readjusted to pH 7.1 daily after 72 h in culture with 2.00 M NaOH. The points are means from cultures of unadapted and preadapted cells and are of $n=4$.

the maximum cell yields in these cultures. Table 8.8 shows the Vero cell growth rates and lag phases in cultures of either bead with the supplemented formate concentrations used. Table 8.9 shows the IC_{50} concentrations of supplemented formate on the cells used in these studies. (1) Maximum Vero cell yields in cultures of either bead were affected by the supplemented formate concentration in the DMEM. (2) There were significantly higher (41%, $P<0.05$) cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all supplemented formate concentrations used. (3) Maximum cell yields in original DMEM (Fig. 8.7A)-B) and 8.8A)-B)) were significantly higher ($P<0.01$) at ≤ 2.50 mM supplemented formate and in enriched DMEM (Fig. 8.7C)-D) and 8.8C)-D)) at ≤ 5.00 mM supplemented formate compared to all other concentrations used with either preadapted or unadapted cells. (4) Maximum cell yields were significantly higher (14%, $P=0.05$) in enriched compared to original DMEM at all supplemented formate concentrations used. (5) There were no significant differences in maximum cell yields from preadapted compared to unadapted cells at any supplemented formate concentration. (6) There was no significant difference in the cell growth rates (Table 8.8) in cultures of either bead at any supplemented formate concentration used. (7) The lag phase (Table 8.8) was proportionally longer with the increase in the supplemented formate concentration and was significantly longer ($P<0.01$) in cultures of either bead at 10.00 mM supplemented formate compared to the controls. (8) Lag phases were significantly longer (20%, $P<0.05$) in cultures of either bead from unadapted cells compared to preadapted at 10.00 mM supplemented formate. (9) There were no significant differences in the IC_{50} concentrations (Table 8.9) for supplemented formate in cultures of either bead. (10) IC_{50} concentrations for supplemented formate were significantly higher (30%, $P<0.01$) in cultures of either bead from preadapted compared to unadapted cells. (11) IC_{50} concentrations for supplemented formate were significantly higher (52%, $P<0.01$) in cultures of either bead in enriched DMEM compared to original.

pH. The culture pH fluctuated at all supplemented formate concentrations similar to that with lactate (8.3.3). Cultures were all initially at pH 7.4. Flasks at 7.50-10.00 mM supplemented

Table 8.8 - The effects of the supplemented **formate** concentration in DMEM + 5.0% v/v sCS on the Vero cell **growth rates** and **lag phases** in 100 mL spinner flasks of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in flasks and stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). The flasks were readjusted to pH 7.1 daily after 72 h in culture with 2.00 M NaOH. The values are means from cultures of original and enriched DMEM and from unadapted and preadapted cells and are of n=8.

Supplemented Formate Concentration (mM)	The Specific Vero Cell Growth Rate and Lag Phase in Cultures of Either Bead Type			
	Cytodex-1		Cultispher-G	
	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)	Specific Cell Growth Rate (h^{-1})	Lag Phase (h)
0.00	0.033 ± 0.003	3.00 ± 0.50	0.036 ± 0.003	6.50 ± 0.50
2.50	0.033 ± 0.003	7.50 ± 0.50	0.036 ± 0.003	7.50 ± 0.50
5.00	0.032 ± 0.003	8.25 ± 1.00	0.035 ± 0.003	9.00 ± 1.00
7.50	0.032 ± 0.003	12.00 ± 1.00	0.034 ± 0.003	12.00 ± 1.00
10.00	0.031 ± 0.003	22.00 ± 2.00	0.034 ± 0.003	22.50 ± 2.00

Table 8.9 - The **IC₅₀** concentrations of supplemented **formate** on Vero cell growth in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL with either original or enriched DMEM + 5.0% v/v sCS containing up to 10.00 mM formate. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). The flasks were readjusted to pH 7.1 daily after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Cell and Medium Type	IC ₅₀ Concentration of Formate (mM)	
	Cytodex-1	Cultispher-G
Unadapted Cells		
Original DMEM	4.90 ± 0.35	6.05 ± 0.42
Enriched DMEM	9.86 ± 0.70	9.95 ± 0.70
Preadapted Cells		
Original DMEM	8.11 ± 0.57	9.06 ± 0.63
Enriched DMEM	11.47 ± 0.80	11.39 ± 0.80

formate were at pH <6.7 from 72 h onwards at the point of each daily readjustment. These cultures could not be maintained within the optimal pH range for growth with a single daily readjustment. The pH of such cultures was acidic significantly faster (≤ 6 h) following readjustment compared to the controls (15 h). Increasing the volume of 2.00 M NaOH (two-fold) for pH readjustment did not improve the range in high supplemented formate cultures.

Formate Plate Assay. A plate assay for formate analysis was developed to analyse the concentrations of formate from any culture sample. The assay gave a linear standard curve between 0.05-10.00 mM formate (Appendix 2.2). However, the assay generated erratic formate concentrations with wide variations ($\pm 179\%$ in Cytodex-1 cultures and 120% in Cultispher-G cultures from the mean) in experimental culture samples. The assay was not optimised further.

Discussion. Maximum Vero cell yields were lowered proportional to the supplemented formate concentration used and were significantly lower at ≥ 5.00 mM supplemented formate. This concentration agree with those for CHO cells (6.00 mM) (Ihrig et al., 1994). However, Vero cells in this study reached significantly higher yields ($>30\%$) compared to the previous authors CHO at 10.00 mM supplemented formate. This could be explained by differences in either the growth media or cell line used, as CHO cells were grown in DMEM/F12 with 2.0% v/v FBS compared to DMEM + 5.0% v/v sCS in this study. Therefore, differences in nutrient concentrations could explain the cell yield differences.

Formate was shown to be produced by CHO cell growth (Ihrig et al., 1994). Cell growth was significantly inhibited (60%) at 10.00 mM supplemented concentrations of formate. The IC_{50} concentrations for unadapted cells varied from 4.90 mM in Cytodex-1 cultures up to 6.05 mM in Cultispher-G cultures in original DMEM. The IC_{50} concentration could be significantly increased (52%) by growth in enriched DMEM. However, whether this effect involved increased serine consumptions (refer to 8.3.1) was not determined.

No one has previously proposed a mechanism for formate inhibition of cell growth.

However, the results in this study indicate culture pH could be involved by a similar mechanism to lactate (8.3.3). This effect could also involve interference with the buffering capacity of the medium leading to rapid decreases in the culture pH affecting cell yields.

Formate production occurs from amino acid metabolism and is usually dependent on folate. Formate is produced from the catabolism of either tryptophan (folate independent) or histidine (folate dependent) or the oxidation of glycine (folate dependent) (Rabinovitz and Tabor, 1958). Ihrig et al. (1994) showed the formate production was dependent on folate and directly proportional to the concentration of serine in the medium (0.45 mM formate produced/1.00 mM serine initially in the medium). No formate was produced without folate or serine in the medium. However, these authors did not show any data for serine consumption by the CHO cells to correlate formate production to serine consumption.

There were problems with the formate plate assay used in this study. The assay gave linear standard curves between 0.05-10.00 mM formate but could not generate data from experimental samples. This could be explained by enzyme inhibition by metal ions in the medium from the sCS. Schutte et al. (1976) isolated and characterised formate dehydrogenase and showed activity was inhibited by Cu^{2+} , Hg^{+} and NO_2^{-} . The authors did not catalogue all metals or salts. sCS contains many metals (e.g. Cd, Co, Mg, Mn, Mo, Ni, Sn and V) which could interfere with the enzyme. However, Ihrig et al. (1994) reported using formate dehydrogenase to remove the formate but gave no conditions for this procedure and they used an ion chromatography system to determine the formate concentration in their samples.

8.3.5 Effect of Osmolarity

Introduction. The optimal osmolarities for growth are species and cell line dependent. Osmolarity could be included in with medium optimisation, as the medium salt concentration could affect cell metabolism. However, as osmolarity has been shown previously to inhibit cell growth and productivity it has been included in this Chapter. No one has previously reported the

effects of osmolarity on Vero cell growth. Therefore, this study determined the effects of DMEM osmolarity on Vero cell growth in microcarrier cultures.

Experimental. Preadapted stock Vero cells were inoculated in original and enriched DMEM + 5.0% v/v SCS prepared at various osmolarities (from normal DMEM at 320mOsmol.L^{-1} up to 520 mOsmol.L^{-1}). Increased osmolarities were obtained by enriching the DMEM with various concentrations (up to 116.0 mM) of sodium chloride. The cultures were grown in spinner flasks, as described above (8.3.1).

Results. Table 8.10 shows maximum Vero cell yields and growth rates in cultures of Cytodex-1 and Cultispher-G with various medium osmolarities. Fig. 8.10 shows the effects of the DMEM osmolarity on maximum Vero cell yields. Table 8.11 shows the IO_{50} (inhibitory osmolarity at which there was a 50% reduction in cell yield compared to the control) for these cultures. Table 8.12 shows serine and glutamine consumption in these cultures. (1) Maximum Vero cell yields in cultures of either bead were affected by the medium osmolarity. (2) There were significantly higher (47%, $P<0.01$) cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the osmolarities used. (3) Maximum cell yields in cultures of either bead were significantly higher ($P<0.05$) in original and enriched DMEM at the normal osmolarity (320 mOsmol.L^{-1}) compared to all other osmolarities used. (4) The maximum cell yields were significantly higher (16%, $P<0.05$) in enriched compared to original DMEM with respect to all osmolarities used. (5) Maximum cell yields were significantly higher (15%, $P=0.05$) from preadapted compared to unadapted cells with respect to all osmolarities used. (6) The cell growth rates were significantly higher ($P<0.01$) in the control cultures compared to all other osmolarities used and were proportionally reduced with the osmolarity. (7) Lag phases were significantly shorter (6 h) in the control cultures compared to all other osmolarities used and were reduced proportional to the osmolarity.

Table 8.10 - The effect of osmolarity on maximum yields of unadapted and preadapted Vero cells in 100 mL spinner flasks on 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in original and enriched DMEM + 5.0% v/v sCS supplemented with various concentrations of sodium chloride (29.00-116.00 mM). The cultures were stirred at 40 rpm either continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means from preadapted and unadapted cells and are of n=4.

Growth Medium	Medium Osmolarity (mOsmol.L ⁻¹)	Maximum Cell Concentration ($\times 10^6$ /mL)	
		Cytodex-1	Cultispher-G
Original DMEM + 5.0% v/v sCS	320	1.49 $\pm$ 0.11*	1.85 $\pm$ 0.13*
	370	1.04 $\pm$ 0.07	1.21 $\pm$ 0.08
	420	0.71 $\pm$ 0.06	0.90 $\pm$ 0.06
	470	0.51 $\pm$ 0.04	0.84 $\pm$ 0.06
	520	0.11 $\pm$ 0.02	0.67 $\pm$ 0.05
Enriched DMEM + 5.0% v/v sCS	320	1.47 $\pm$ 0.11*	1.79 $\pm$ 0.12*
	370	1.16 $\pm$ 0.08	1.39 $\pm$ 0.10
	420	0.85 $\pm$ 0.06	1.30 $\pm$ 0.09
	470	0.63 $\pm$ 0.04	1.08 $\pm$ 0.06
	520	0.21 $\pm$ 0.02	0.90 $\pm$ 0.06
		Specific Cell Growth Rate (h ⁻¹)	
Original DMEM + 5.0% v/v sCS	320	0.035 $\pm$ 0.003	0.038 $\pm$ 0.003
	370	0.031 $\pm$ 0.003	0.033 $\pm$ 0.003
	420	0.028 $\pm$ 0.003	0.030 $\pm$ 0.003
	470	0.023 $\pm$ 0.002	0.030 $\pm$ 0.002
	520	ND	0.029 $\pm$ 0.003
Enriched DMEM + 5.0% v/v sCS	320	0.035 $\pm$ 0.003	0.037 $\pm$ 0.003
	370	0.032 $\pm$ 0.003	0.034 $\pm$ 0.003
	420	0.030 $\pm$ 0.003	0.034 $\pm$ 0.003
	470	0.026 $\pm$ 0.002	0.033 $\pm$ 0.002
	520	ND	0.033 $\pm$ 0.002

ND - Value not determined due to poor cell growth.

* - Highest cell yield.

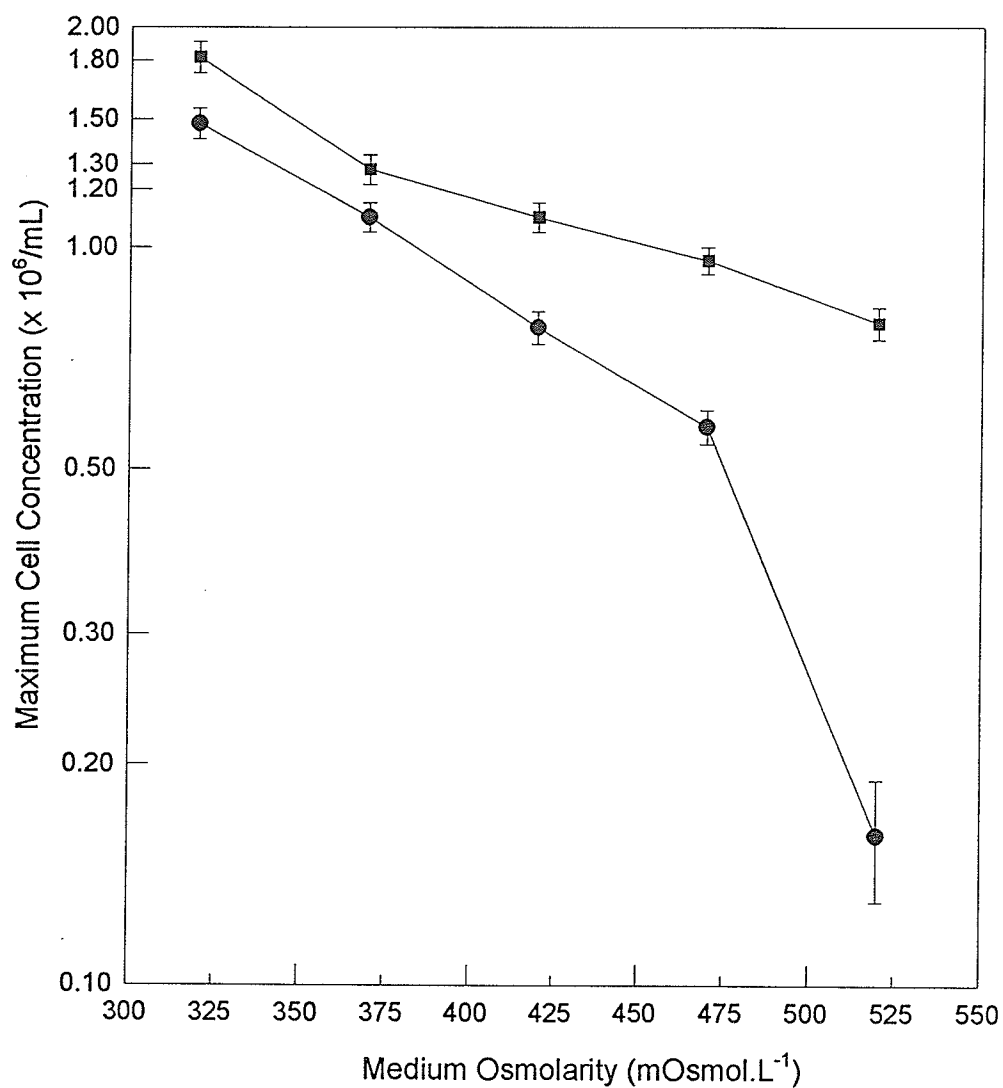


Fig. 8.10 - The effects of osmolarity on the maximum Vero cell yields in 100 mL spinner flasks on 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in either original or supplemented DMEM + 5.0% v/v SCS. The ● Cytodex-1 flasks were stirred at 40 rpm continuously and the ■ Cultispher-G flasks intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture. The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The points are means from unadapted and preadapted cells in each medium and are from $n=4$.

Table 8.11 - The IO_{50} DMEM osmolarity of Vero cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in either original or enriched DMEM + 5.0% v/v SCS prepared at osmolarities from 320-520 mOsmol.L⁻¹. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). The flasks were readjusted to pH 7.1 daily after 72 h in culture with 2.00 M NaOH. The values are means of n=2.

Cell and Medium Type	IO_{50} DMEM Osmolarity (mOsmol.L ⁻¹)	
	Cytodex-1	Cultispher-G
Unadapted Cells		
Original DMEM	415 ± 21	456 ± 23
Enriched DMEM	449 ± 23	521 ± 26

Table 8.12 - The effects of osmolarity on the residual concentration at maximum yield and the specific consumption rates of serine (A) and glutamine (B) of Vero cells in 100 mL spinner flasks of 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in either original or supplemented DMEM + 5.0% v/v SCS. Flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). The flasks were readjusted daily to pH 7.1 after 72 h in culture with 2.00 M NaOH. The values are means from cultures of unadapted and preadapted cells in each medium and are of n=4.

Table 8.12A) - Serine.

Osmolarity (mOsmol.L ⁻¹)	Residual Serine Concentration In DMEM at Maximum Cell Yield (mM)			
	Cytodex-1		Cultispher-G	
	Original	Supplemented	Original	Supplemented
320	0.07	1.70	0.09	1.67
370	0.00	1.55	0.00	1.54
420	0.00	1.47	0.00	1.50
470	0.00	1.40	0.00	1.45
520	ND	ND	0.00	1.40
	Specific Serine Consumption Rate ($\mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$)			
320	1.38	1.27	1.03	1.10
370	2.42	2.45	2.07	2.07
420	3.64	3.99	2.72	2.40
470	4.35	5.17	3.03	3.14
520	ND	ND	3.23	4.29

Table 8.12B) - Glutamine.

Osmolarity (mOsmol.L ⁻¹)	Residual Glutamine Concentration In DMEM at Maximum Cell Yield (mM)			
	Cytodex-1		Cultispher-G	
	Original	Supplemented	Original	Supplemented
320	0.11	0.11	0.15	0.15
370	0.05	0.05	0.10	0.10
420	0.05	0.05	0.05	0.05
470	0.05	0.05	0.00	0.00
520	ND	ND	0.00	0.00
	Specific Glutamine Consumption Rate ($\mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$)			
320	16.04	16.05	12.70	13.14
370	23.00	20.58	20.32	17.55
420	35.55	27.93	27.93	18.62
470	43.44	30.08	30.46	23.29
520	ND	ND	39.60	28.29

ND - Value not determined.

IO₅₀. The inhibitory osmolarity (Table 8.11) (IO₅₀) was not significantly different in cultures of either bead in either DMEM used. There was no significant difference in the IO₅₀ in enriched compared to original DMEM in Cytodex-1 cultures. However, there was a significantly higher (16%, $P < 0.05$) IO₅₀ in Cultispher-G cultures in enriched DMEM. There was no significant difference in the IO₅₀ from preadapted compared to unadapted cells.

Nutrient Analysis. Nutrient analysis showed serine (Table 8.12A)) was completely depleted in original DMEM at $>320 \text{ mOsmol.L}^{-1}$ prior to maximum cell yield but there was a high residual serine concentration in enriched DMEM with all the osmolarities used due to the higher initial concentration. (1) The specific serine consumption rate in controls were significantly higher (24%, $P < 0.01$) in Cytodex-1 cultures compared to Cultispher-G in either DMEM used. (2) The specific serine consumption rates were proportionally and significantly increased in Cytodex-1 (358%, $P < 0.01$) and Cultispher-G (289%, $P < 0.01$) cultures in either DMEM at $470 \text{ mOsmol.L}^{-1}$. (3) There was no significant difference in the specific serine consumption rates in cultures of either bead in either DMEM used. (4) There was no significant difference in the specific serine consumption rates of preadapted compared to unadapted cells in cultures of either bead with respect to any osmolarity used. (5) The glycine concentration increased in the DMEM proportional to the amount of serine consumed with a constant glycine to serine ratio (0.27).

Glutamine (Table 8.12B)) was almost completely consumed prior to maximum cell yield in cultures of either bead at all the osmolarities used. (6) The specific glutamine consumption rates were significantly higher (24%, $P < 0.01$) in control cultures of Cytodex-1 compared to Cultispher-G in either DMEM used. (7) The specific glutamine consumption rates were proportionally and significantly increased with the osmolarity in Cytodex-1 (229%, $P < 0.01$) and Cultispher-G (208%, $P < 0.01$) cultures at $470 \text{ mOsmol.L}^{-1}$. (8) The specific glutamine consumption rates were significantly higher in original DMEM compared to enriched with respect to all osmolarities used.

Discussion. Maximum Vero cell yields were affected by the medium osmolarity above the

normal 320 mOsmol.L⁻¹. These results agree with Ozturk et al. (1991) and Oyaas et al. (1994) who showed murine hybridoma cells reached low maximum cell yields at similar osmolarities (505 mOsmol.L⁻¹). However, cells in Cytodex-1 cultures grew to maximum yields proportional to the osmolarity up to 470 mOsmol.L⁻¹. Vero cells in Cultispher-G cultures grew with similar trends but reached significantly higher yields at all osmolarities compared to Cytodex-1. This difference was most pronounced at 520 mOsmol.L⁻¹ where the cells completed 3.74-4.17 population doublings in Cultispher-G cultures and only 1.14-2.07 in Cytodex-1. Thus, cells were protected from the effects of the osmolarity in Cultispher-G beads. Maximum cell yields were significantly higher in enriched DMEM compared to original at all osmolarities used but there were no significant differences in the IO₅₀ osmolarities in either medium. There was little cell detachment in cultures of either bead at any osmolarity which disagreed with Wentz and Schugert (1992) who reported BHK cell detachment at high osmolarities (500-680 mOsmol.L⁻¹).

The protective effect of Cultispher-G beads could be explained by the cells growing flattened on the outer surface of Cytodex-1 and have a high surface area to volume ratio. This could be beneficial to cellular uptake of nutrients. However, it could be detrimental if it increased inhibitor compound uptake. The cells grow in the inner matrix of the Cultispher-G beads and could form aggregates. Cells in the aggregates could be spherical with a low surface area to volume ratio and this could limit nutrient and inhibitor uptake rates.

Nutrient analysis showed the specific serine consumption rate was significantly increased in original and enriched DMEM at high osmolarities. The serine enrichment could explain the higher cell yields in this medium. Glutamine was also consumed at an increased specific consumption rate, similar to those in serine, proportional to the osmolarity.

8.4 Conclusions

The purpose of this study was to determine the effects of various growth inhibitors on Vero cell yields in cultures of Cytodex-1 and Cultispher-G. The results above show that:

- **maximum Vero cell yields** were significantly higher (32%, $P < 0.05$) in Cultispher-G

cultures compared to Cytodex-1 with all the components and parameters tested in this study.

- **Vero cell growth was inhibited** (Table 8.13) by ammonium chloride, lactate and formate and by DMEM osmolarity. Inhibitor compounds affected cell yields more in Cytodex-1 cultures compared to Cultispher-G which could be due to cell morphology differences on the beads.

- **serine consumption** was affected by the ammonia concentration and DMEM osmolarity. The specific serine consumption rate was proportionally and significantly increased ($P < 0.01$) at high ammonia concentrations and high osmolarities. Thus, Vero cells could require serine for important internal functions related to growth and metabolism when under stress. Maximum cell yields were significantly higher (between 16-31%, $P < 0.05$) in serine enriched DMEM.

- **glutamine was important** to cell growth at high ammonium chloride concentrations and DMEM osmolarities. Specific glutamine consumption rates were proportionally and significantly increased ($P < 0.01$) at high ammonium chloride concentrations and osmolarities.

- **L-pyroglutamate was not inhibitory** to Vero cell growth. Maximum cell yields of preadapted cells were increased at 10.00 mM pyroglutamate and the effect was linear ($r = 0.962$). This suggests that significantly higher cell yields could be reached with a higher (> 10.00 mM) supplemented pyroglutamate concentration. Unadapted cells were unaffected at any pyroglutamate concentration used.

- **preadapted cells** grew to significantly higher (between 15-41%, $P < 0.05$) maximum yields compared to unadapted cells at high concentrations of ammonium chloride, L-pyroglutamate, formate and DMEM osmolarity. Thus, Vero could be adapted to grow to high cell yields in adverse medium conditions.

Table 8.13 - The inhibitor compound **IC₅₀** concentrations and **IO₅₀** osmolarities for **unadapted** and **preadapted** Vero cells in 100 mL spinner flask cultures of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**.

Inhibitory Component	IC ₅₀ Concentration of Compound or IO ₅₀ Osmolarity in Medium Formulation			
	Cytodex-1		Cultispher-G	
	Original	Enriched	Original	Enriched
Unadapted Cells				
Ammonium Chloride	9.50	14.03	11.52	12.41
Sodium Lactate	35.71	38.09	37.08	37.85
Sodium Formate	4.90	9.86	6.05	9.95
Medium Osmolarity	415	449	456	521
Preadapted Cells				
Ammonium Chloride	12.62	15.93	13.15	16.07
Sodium Lactate	38.78	41.50	37.00	39.78
Sodium Formate	8.11	11.47	9.36	11.39
Medium Osmolarity	425	455	470	525

Chapter 9. Serum-Free Growth Medium for Vero Cell Microcarrier Cultures

9. Serum-Free Growth Medium for Vero Cell Microcarrier Cultures

9.1 Introduction

Much of the previous research in mammalian cell culture has used serum supplemented basal media. The serum supplies many important factors for growth such as albumin, fetuin, transferrin, insulin, fibroblast (FGF) and epidermal (EGF) cell growth factors, metal ion cofactors and fibronectin and vitronectin (Barnes et al., 1984, Ruoslahti and Pierschbacher, 1989 and Nie, 1992). Most of the serum protein content (>90%) is in the large molecular weight carrier proteins, albumin and fetuin (Nie, 1992). However, the serum composition is undefined and has been called the "black box" of cell culture (Thilly, 1989). Sera compositions differ between species, age of donor animal and between different processed batches from the same source. Viral and prion contamination of sera also pose serious problems to cell culture (Hodgson, 1991).

The serum problems drove research into defined, serum-free medium formulations. Ham (1963 and 1965) proposed the first serum-free media, Nutrient Mixtures F-10 and F-12, for Chinese hamster (CHBO-C1 and Puck's CHD-3A) cells. However, only recently have serum-free media been developed for murine and human hybridomas, baby hamster kidney (BHK), Madin-Darby canine kidney (MDCK) and Chinese hamster ovary (CHO) cells (Murakami et al., 1982, Bradshaw et al., 1983, Taub, 1984, Toyoda et al., 1991 and Watson et al., 1994).

Hybridomas grow in suspension, as they require no proteinaceous growth or attachment factors due to their myeloma parentage (Cherington et al., 1979). This makes them easy to grow in serum-free media composed of basal medium (e.g. DMEM:F12 (1:1)) supplemented with insulin, transferrin, selenium, ethanolamine and trace elements (Murakami et al., 1982) and in protein-free media (Schneider and Veithen, 1990). However, serum-free media for BHK and MDCK cells (i.e. adherent) require more complex supplements such as fibronectin (Bradshaw et al., 1983), prostaglandin E₁, hydrocortisone and triiodothyronine (T₃) (Taub, 1984).

Few reports were found using serum-free media for culturing African green monkey kidney (Vero) cells and none were in microcarrier culture. However, Vero cells can be cultured as

aggregates using medium supplemented with a bovine serum ultrafiltrate of an undefined nature (Litwin, 1992) and also in culture flasks with serum-free (Merten et al., 1995) and defined protein-free media (Yasumara et al., 1978 and Cinatl et al., 1993). The medium of Cinatl et al. (1993) contained 94 components and cell growth could have been influenced by the modified substrata (a polyvinyl-formal surface) used. However, as MDCK and BHK cells grow in defined, serum-free media then Vero cells should also grow in such media. Serum-free media for adherent cells are also being developed by commercial suppliers (e.g. Gibco BRL®, Grand Island, NY, U.S.A.). However, there are no commercial serum-free formulations currently for adherent cells. The American Type Culture Collection (ATCC) has adapted Vero to a serum-free medium using defined TCM™ (Celox® Corp., Hopkins, MN, U.S.A) serum replacement supplement in the basal medium but they do not define the preferable basal medium to use.

There are several approaches to defining serum-free medium formulations. The first step in is to decide the components to test from the many described in the literature including biotin, cell growth factors, choline chloride, copper, cyclic AMP, cytidine fibronectin, hormones, hydrocortisone, hypoxanthine, insulin, iron, phospholipids, prostaglandin E₁, T₃, trace metals, thymidine, transferrin, uridine, vitamin B₁₂ and zinc (Clarke and Smith, 1973, Werrlein and Glinos, 1974, Mierzewski et al., 1976, Zielke et al., 1976, Hatten et al., 1977, Messmer and Young, 1977, Ham and McKeehan, 1979, Hughes et al., 1979, Young et al., 1979, Valentich et al., 1979, Bottenstein and Sato, 1980, Giguere, 1982, Hoshi et al., 1982, Cherington, 1984, Ham, 1984, Taub, 1984, Taub et al., 1984, Cinatl et al., 1992, Murakami, 1992, Sarantos et al., 1993 and Tucker et al., 1993). Once these are known two approaches exist for defining the required components:- (1) Prepare a complete mixture of all the components and determine the important ones by selective removal. (2) Prepare a minimal mixture of components and add combinations of the others at varied concentrations in a factorial design experiment. The results from factorial experiments can also suggest optimal concentrations of the required components.

This paper presents the development of a completely defined, hormonally supplemented serum-free medium for the growth of Vero cells in static and spinner flask cultures of Cytodex-1

and Cultispher-G. The original formulation of the Vero cell serum-free medium (VSFM) was a combination of several previous reports but was based on the medium of Taub (1984). The important components were determined by method 1 above. The performance of VSFM was compared to 3 serum-free media from commercial suppliers in spinner flask cultures.

9.2 Materials and Methods

The methods of culturing and counting the cells and the nutrient analyses have been described previously (2.1-2.5).

9.2.1 Preparation of Serum-Free Medium

9.2.1.1 Vero Cell Serum-Free Medium

Vero cell serum-free medium (VSFM) was DMEM containing supplements from reports on fibroblast cell growth (Hatten et al., 1977, Cherington et al., 1979, Rockwell et al., 1980, Crespi et al., 1981, Bettger et al., 1981, Bradshaw et al., 1983, Ham, 1984, Taub, 1984, Schneider and Veithin, 1990, Schmid et al., 1992, Bashir et al., 1992 and Watson et al., 1994). The DMEM contained glucose (25.00 mM), glutamine (4.00 or 6.00 mM) and bicarbonate (36.00 or 44.00 mM) and was "enriched" with serine and pyruvate (2.00 and 0.50 mM, respectively). All components were prepared in stock concentrate solutions (100X) stored at -20°C . The final formulation of VSFM is a trade secret awaiting patent application review at the time this document is being printed.

9.2.1.2 Commercial Serum-Free Media

Commercial serum-free media were obtained as TCM concentrate (Celox Corp., MN, U.S.A.) (10 mL) which replaced serum in enriched DMEM (500 mL) and Gibco BRL (Grand Island, NY, U.S.A.) generously supplied two developmental stage complete Vero Maintenance Medium Formulations II and III. All were proprietary formulations.

9.2.2 Preadaptation of Stock Cells

Stock Vero cells were detached and recovered by the standard trypsinisation procedure (2.2) and counted by the trypan blue method (2.5.1-2). The cells were inoculated (0.25×10^6 cells/mL) in T75 culture flasks (35 mL) and adapted to VSFM either (a) directly or (b) gradually from DMEM + 5.0% v/v iron enriched, supplemented calf serum (sCS). Directly adapted cells were subcultured every 72 h as above (5 passages). Gradually adapted cells were inoculated (0.25×10^6 cells/mL) in a mixture (5:1) of DMEM + 5.0% v/v sCS:VSFM. The cells were subcultured every 48 h as above and twice at each DMEM + 5.0% v/v sCS:VSFM dilution (4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:5 and 1:10).

9.3 Results and Discussion

9.3.1 Testing Vero Cell Serum-Free Medium Components

Introduction. MDCK and BHK cells grow in serum-free media which suggests Vero cells should also grow in such media. A Vero cell serum-free medium (VSFM) was formulated based on previous reports with other transformed fibroblast cell lines. However, it was unknown whether all the supplemented components were required. Therefore, this study determined the effects of the different components added to VSFM on Vero cell growth in T25 culture flasks to define whether (1) All the components in the medium were required. (2) Further optimisation of the component concentrations was required. (3) Additional components were required.

Experimental. Preadapted stock Vero cells were inoculated in T25 culture flasks in VSFM prepared with and without various combinations of components (insulin and transferrin or biotin, vitamin B₁₂ and/or choline chloride or glutathione and β -mercaptoethanol or T₃, prostaglandin E₁ and/or hydrocortisone or metal ions or trace elements or putrescine and vitamins or FGF, fetuin and/or fibronectin). The cell attachment time (the time taken for >90% of the cell inoculum to attach to the flask) was determined (2.5.5) and one culture flask of each medium was

sacrificed daily for cell counts by the trypan blue method (2.5.1-2).

Results. Table 9.1 shows Vero cell growth in T25 culture flasks using VSFM prepared without various combinations of components. Table 9.2 shows the costs of VSFM compared to DMEM + 5.0% v/v sCS. Table 9.3 shows the lag phases, cell growth rates and attachment times in the media used. Fig. 9.1 shows the effects of multiple passaging on cell growth in VSFM or DMEM + 5.0% v/v sCS. (1) Maximum cell yields in VSFM were dependent on certain combinations of components. (2) There were significantly higher (37%, $P<0.01$) cell yields in DMEM + 5.0% v/v sCS compared to any VSFM formulations used. (3) Maximum cell yields in VSFM were not affected by the removal of (i) putrescine and extra vitamins or (ii) glutathione and β -mercaptoethanol. (4) Maximum cell yields were significantly higher (183%, $P<0.01$) in original VSFM compared to VSFM with all other removed components tested. A lack of fibronectin caused poor attachment and the cells completed 1.5 population doublings over 7 d. The poor attachment promoted cell aggregate formation (25 cells) and these could be easily shaken off the flask surface. Fibronectin was the only component in VSFM which ensured strong attachment to the flasks. Cell yields in VSFM without (a) insulin and transferrin, (b) T_3 , prostaglandin E_1 and hydrocortisone, (c) iron, zinc and copper, (d) trace elements or (e) fibronectin, FGF and fetuin were not significantly different from each other. (5) The VSFM (Table 9.2) was significantly more expensive (forty nine-fold, $P<0.01$) compared to DMEM + 5.0% v/v sCS. The VSFM cost was mostly (94%) from fibronectin and FGF. (6) The cell growth rate (Table 9.3) was significantly higher (25%, $P<0.01$) in DMEM + 5.0% v/v sCS compared to any VSFM formulation. (7) The lag phase (Table 9.3) was significantly longer ($P<0.01$) in VSFM compared to DMEM + 5.0% v/v sCS. (8) The attachment time (Table 9.3) was significantly longer ($P<0.01$) in VSFM compared to DMEM + 5.0% v/v sCS and the cells did not completely flatten onto the flask surface in VSFM cultures. The cells flattened within 6.0 h of attaching to the surface in DMEM + 5.0% v/v sCS. (9) Cells subcultured in VSFM (Fig. 9.1) gradually ceased to grow over multiple passages (35 passages) in T25 culture flasks.

Table 9.1 - The effects of removing various combinations of components from the original VSFM on the growth of preadapted Vero cells in T25 culture flasks. The cells were inoculated at 0.10×10^6 cells/mL in 10 mL of each medium and grown with a 10% v/v CO₂ in air overlay. One culture flask was sacrificed daily for cell counts by the trypan blue method. The values are means from cultures of cells either preadapted directly or gradually (>6 passages) from DMEM + 5.0% v/v SCS into the VSFM formulation and are of n=2.

Removed Components	Maximum Cell Concentration ($\times 10^6$ /mL)
DMEM + 5.0% v/v SCS	$1.79 \pm 0.12^*$
None (36.00 mM Bicarbonate) ¹	1.13 ± 0.07
None (36.00 mM Bicarbonate) ²	1.10 ± 0.07
None (44.00 mM Bicarbonate) ¹	0.94 ± 0.07
None (44.00 mM Bicarbonate) ²	0.89 ± 0.06
Insulin and Transferrin	0.28 ± 0.03
Biotin, Vitamin B ₁₂ and Choline Chloride	0.45 ± 0.03
Biotin and Vitamin B ₁₂	0.55 ± 0.04
Glutathione and β -Mercaptoethanol	0.98 ± 0.05
Triiodothyronine, Prostaglandin E ₁ and Hydrocortisone	0.32 ± 0.03
Triiodothyronine and Prostaglandin E ₁	0.52 ± 0.04
MnSO ₄ , Na ₂ SeO ₃ , Na ₂ SiO ₃ .5H ₂ O, (NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O, NH ₄ VO ₃ , NiCl ₂ .6H ₂ O and SnCl ₂ .2H ₂ O	0.30 ± 0.03
FeSO ₄ .7H ₂ O, ZnSO ₄ .7H ₂ O and CuSO ₄ .5H ₂ O	0.25 ± 0.07
Putrescine and 1X D-Vitamins	1.09 ± 0.07
Fibronectin, FGF and Fetuin	0.32 ± 0.03
Fibronectin	0.29 ± 0.03

* - Highest cell yield.

1 - Directly adapted cells.

2 - Gradually adapted cells.

Table 9.2 - The component costs of DMEM + 5.0% v/v sCS compared to the original VSFM medium. Prices sourced from Gibco BRL (Grand Island, NY, U.S.A) Price List (1995) and from Sigma Chemical Co. (St. Louis, MO, U.S.A.) Catalogue (1995). The prices are based on purchasing the largest amount available of each component in powdered form. The prices of the serum-free additives have been omitted, as the medium has a proprietary formulation at the current time.

Medium Component	Component Cost in Medium Formulation (\$ Cdn./L medium)	
DMEM	2.56	2.56
sCS	7.92	
Glucose	0.03	0.03
Glutamine	0.21	0.21
Serine	0.12	0.12
Pyruvate	0.04	0.04
NaHCO ₃	0.09	0.09
Phenol Red	0.02	0.02
Other Components Required for VSFM		527.41
Total Cost/L	10.99	538.40

Table 9.3 - The effect of the medium formulation on specific Vero cell growth rate, lag phase and attachment times in T25 culture flasks. The cells were inoculated at 0.10×10^6 cells/mL in 10 mL of VSFM and DMEM + 5.0% v/v sCS and grown with a 10% v/v CO₂ in air overlay. One culture flask was sacrificed daily for cell counts by the trypan blue method. The values are means from cultures of cells preadapted either directly or gradually into VSFM and are of n=4.

Effect	Time Taken for Particular Effect	
	DMEM + 5.0% v/v sCS	VSFM
Cell Attachment Time	1.50 ± 0.50 h	6.00 ± 1.00 h
Cell Flattening Time	6.00 ± 1.00 h	NF
Lag Phase	6.00 ± 0.50 h	24.00 ± 2.00 h
Specific Cell Growth Rate	0.036 ± 0.003 h ⁻¹	0.027 ± 0.003 h ⁻¹

NF - Never became flattened on the flask surface.

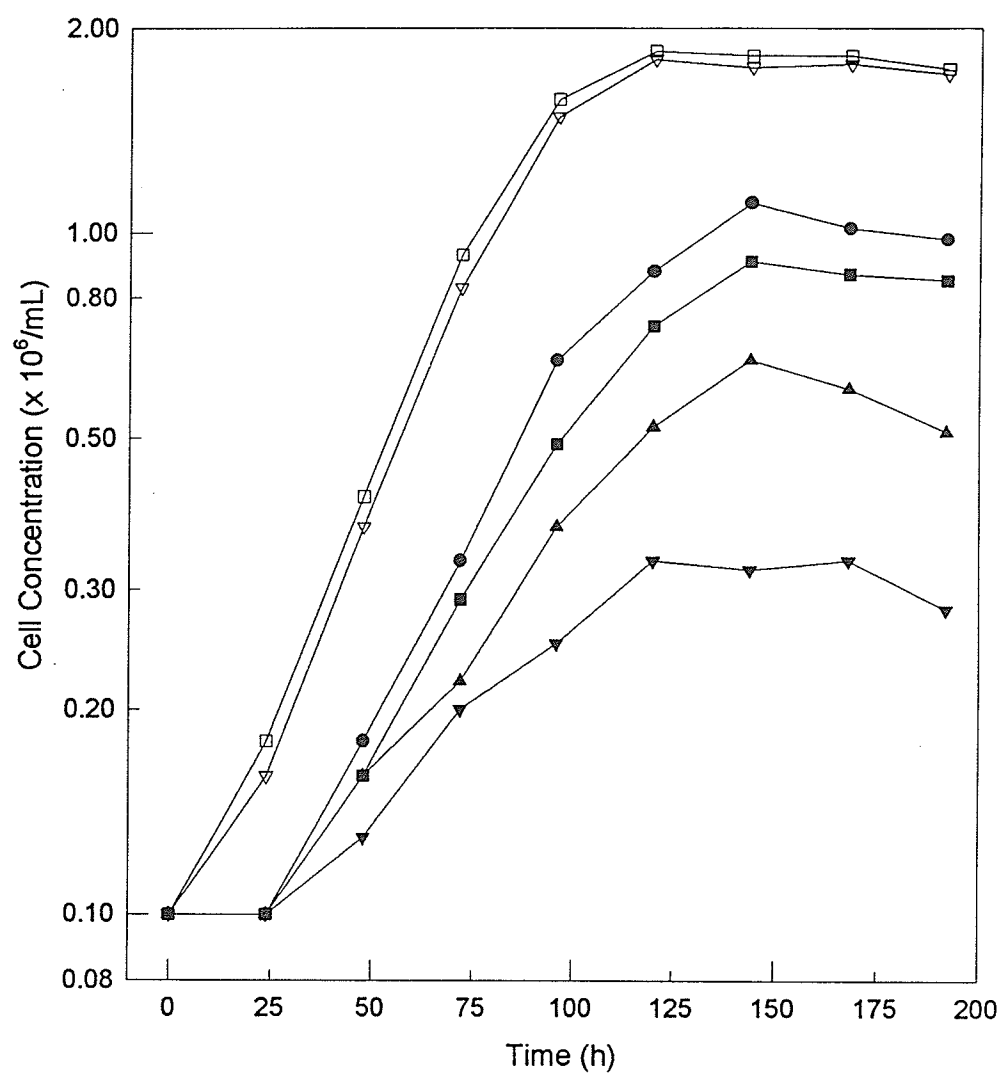


Fig. 9.1 - The effect of multiple passages on Vero cell growth in DMEM + 5.0% v/v sCS and VSFM in T25 culture flasks. The cells were passaged for either ● 6, ■ 20, ▲ 30 or ▼ 35 passages in each medium. The cells were inoculated at 0.10×10^6 cells/mL in 10 mL of either VSFM (Closed symbols) or DMEM + 5.0% v/v sCS (Open symbols). The flasks were incubated with a 10.0% v/v CO_2 overlay. The points are means of $n=2$.

pH. The culture pH was affected by the bicarbonate buffer concentration in the VSFM. VSFM prepared with the original DMEM (44.00 mM bicarbonate) equilibrated to pH 7.4 with a 10% v/v CO₂ overlay and remained at this pH throughout the cultures. VSFM at 36.00 mM bicarbonate equilibrated to pH 7.2 and remained at this throughout the cultures. Maximum cell yields were significantly higher (17%, $P < 0.05$) in VSFM containing 36.00 mM bicarbonate compared to 44.00 mM.

Discussion. Vero cells could grow in a serum-free media similar to that for BHK and MDCK cells (Bradshaw et al., 1983 and Taub, 1984). Putrescine and extra vitamins (twice) were the only components from original VSFM which did not affect Vero cell yields. These components were then omitted from the medium. Glutathione and β -mercaptoethanol also had no effect on maximum cell yields but were retained in VSFM, as both could be important to spinner flask cultures, as found for hybridoma and human diploid lung fibroblast (MRC-5) cells (Tharakan et al., 1986 and Forestall et al., 1992). Glutathione protects cell membranes from free-radical damage and β -mercaptoethanol is a strong reducing agent to maintain the glutathione and other components in an active state (Meister and Anderson, 1983 and Voet and Voet, 1990). All other components were absolutely required for cell growth in this study. The maximum cell yields in T25 culture flasks in VSFM were significantly higher (314%) compared to the previous yields in protein-free medium reported by Cinatl et al. (1993).

The cells could be passaged directly into VSFM with no loss of maximum cell yield compared to gradually adapted cells. However, the directly adapted cells required subcultures of 1:3 every 72 h for the first 5-10 passages to prevent loss of the cultures. Overall, the most reproducible results were obtained with gradual adaptation from DMEM + 5.0% v/v SCS. The VSFM supported Vero cell growth in T25 culture flasks over multiple passages (>10) but not indefinitely (Fig. 9.1). Cells in VSFM showed progressively slower cell growth rates and growth eventually ceased (>35 passages). This suggests important nutrients required for cell growth were absent from the VSFM. However, this could also be explained by the cells differentiating

(Bayreuther et al., 1991). Any of the factors described below could be responsible for this effect and would normally have been present in complete DMEM from the serum.

Fibronectin. The cells rapidly lost their adherence in the absence of fibronectin growing poorly and forming cell aggregates. Fibronectin was used previously in serum-free cultures of BHK and bovine corneal endothelial (BCE-1) cells (Hughes et al., 1979 and Werrlein and Glinos, 1982). The effects of fibronectin were discussed previously (6.3.2 and 7.3.2). Insulin, transferrin, hydrocortisone, prostaglandin E₁, T₃, biotin, vitamin B₁₂, choline chloride, iron, copper, zinc and the trace metals were also very important and the cells grew poorly in medium lacking any of these components. However, cell viability was high in all the latter media.

Metabolic Effects of Components. The essential components in VSFM have different effects on cell growth. Hughes et al. (1979) showed BHK cells (i.e. young animals) required fibronectin to attach and grow on surfaces but Vero cells (i.e. adult animals) only require it for attachment. Grinnell and Phan (1983) showed fibronectin is the most important adhesion factor at low serum concentrations (0.1% v/v) which could explain the results above in fibronectin-free VSFM. However, at >2.0% v/v serum other attachment factors were more important. No explanation was given for this effect. Cherington et al. (1979) found primary Chinese hamster cells required transferrin, insulin and EGF for cell growth but the EGF requirement was lost following chemical transformation. Transferrin was required for all the cell cycle and insulin for progression from G₁ into S phase (refer to 4.3.6). The insulin could be regulating glucose metabolism (Voet and Voet, 1990). Many trace elements were required for Vero and BCE-1 cell growth in serum and protein-free medium (Werrlein and Glinos, 1974 and Cinatl et al., 1992). The trace elements could be important as metalloenzyme cofactors for many reactions such as Mn for isocitrate dehydrogenase in TCA cycle or for protein glycosylation, as discussed previously (6.3.2). Hoshi et al. (1982) showed hydrocortisone was required for fibroblast cell spreading and Taub (1984) showed it acted synergistically with prostaglandin E₁ in stimulating MDCK cell growth and

flattening. Messmer and Young (1977) and Hatten et al. (1977) showed 3T3 cells required biotin for coenzyme structures such as acetyl CoA carboxylase for fatty acid biosynthesis (Voet and Voet, 1990). Mierzewski et al. (1976) showed human diploid fibroblast cells required vitamin B₁₂ and insulin for growth stimulation. The vitamin B₁₂ could be involved in two processes: (a) the synthesis of methionine from homocysteine which involves methylcobalamin, and (b) the isomerisation of methyl-malonyl-CoA to succinyl-CoA which involves deoxyadenosylcobalamin (Murakami, 1992). Young et al. (1979) showed SV40 transformed 3T3 cells (and therefore, possibly Vero) required iron, transferrin and biotin for growth. The iron is transported to the cells by transferrin and could be important in the cytochrome oxidative phosphorylation cascade mechanism or for enzyme activity such as aconitase in TCA cycle (Voet and Voet, 1990). The effects of cell growth factors were reviewed by Cherington (1984). Fetuin is a large molecular weight carrier protein for growth factors (Fisher and Lam, 1974 and Nie, 1992). However, it was unclear what other components of the many which have growth effects in other cells could be required in VSFM.

Lag Phases. There were significantly longer lag phases in VSFM compared to DMEM + 5.0% v/v SCS. Crespi et al., (1981) showed similar lag phases (24 h) with MDCK cells in serum-free medium. This could be caused by many things such as a slow biosynthesis of one or more components required for cell attachment and/or growth which were normally found in serum. These could include tenascin, collagen and laminin (Grinnell and Phan, 1983 and Akimoto et al., 1992) which all require several hours to be biosynthesised. The absence of vitronectin could affect cell spreading on the substrata increasing the time taken for the cells to reorganise their actin fibrils and begin further cell divisions (Sasger, 1975 and Knox and Griffiths, 1980). Fibronectin was shown to successfully replace vitronectin for BHK cell growth (Underwood and Bennett, 1989) but Vero cells could require both. High concentrations (100 μ M) of hydrocortisone could replace fibronectin and vitronectin for fibroblast cell growth (Hoshi et al., 1982) but this was not determined in this study.

Many other serum factors could enhance Vero cell growth rates. These factors include hormones, growth factors, phospholipids and nucleotide precursors. Dexamethasone regulates the glutamine synthetase activity of murine L cells (Feng et al., 1990). Growth factors such as EGF and PDGF stimulate human diploid fibroblast cell growth (Mierzewski et al., 1976, Bettger et al., 1981 and Ham, 1984) probably by activating protein kinases (Cherington, 1984). Phospholipids such as linoleic, oleic, nervonic and arachidonic acids etc. were required by 3T3 and MDCK cells (Hatten et al., 1977, Rockwell et al., 1980 and Bashir et al., 1992) for membrane biosynthesis. Nucleotide precursors such as hypoxanthine and thymidine etc. were required by BHK and 3T3 cells (Clarke and Smith, 1973) for nucleotide biosynthesis. However, none of these components were tested in VSFM in this study.

Medium Cost. VSFM was expensive (Table 9.2) to prepare and was not optimised owing to the high cost. The fibronectin and FGF requirements cost in excess of Cdn.\$500/L. The commercial use of Vero cells for vaccine production would not permit the use of such an expensive medium, especially as virus purification contains steps to remove the risks from serum components. However, should the use of Vero cells be for a high value secreted product then the rationale and importance of VSFM could justify further development, although, further optimisation could require many experiments.

9.3.2 Growth in Microcarrier Cultures

Introduction. The previous study (9.3.1) showed Vero cells could be grown in defined serum-free media in T25 culture flasks. However, for commercial success the cells would require to be grown in microcarrier bioreactors. Few adherent cell lines have been cultured in serum-free medium in microcarrier culture and no one has previously reported serum-free growth of Vero cells in microcarrier cultures. There are also few commercial serum-free media for the growth of adherent cells. Therefore, this study determined the effects of growing Vero cells in microcarrier

spinner flask cultures using the VSFM and three commercial formulations.

Experimental. Preadapted stock Vero cells were inoculated in VSFM, Gibco Vero Maintenance Medium II and III and enriched DMEM supplemented with Celox TCM supplement (9.2.2). The cells were grown in 100 mL spinner flasks, as described previously (2.4.3).

Results. Fig. 9.2 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G using the various serum-free media. Table 9.4 shows cell attachment times, lag phases and cell growth rates in these cultures. Table 9.5 shows glucose consumption and lactate production in these cultures. (1) Vero cells could grow in cultures of either bead in all the serum-free medium formulations (VSFM, Gibco II and III and TCM) used. (2) Maximum cell yields were significantly higher (22%, $P < 0.05$) in Cultispher-G cultures compared to Cytodex-1 with respect to all serum-free medium formulations used. (3) Maximum cell yields in cultures of either bead were significantly higher (83%, $P < 0.01$) in DMEM + 5.0% v/v sCS compared to all serum-free media used. (4) In serum-free media maximum cell yields in cultures of either bead were significantly higher (44%, $P < 0.01$) in VSFM compared to the other serum-free media used. Maximum cell yields in Cultispher-G cultures were not significantly different in VSFM and TCM. (5) The cell growth rate (Table 9.4) in cultures of either bead was significantly higher (40%, $P < 0.01$) in DMEM + 5.0% v/v sCS compared to all serum-free media used. (6) Lag phases (Table 9.4) were significantly longer (246%, $P < 0.01$) in all serum-free media compared to DMEM + 5.0% v/v sCS. (7) The cell attachment times were significantly faster (40%, $P < 0.01$) in DMEM + 5.0% v/v sCS compared to all serum-free media used. The cells were strongly attached to the beads in DMEM + 5.0% v/v sCS and there were few (<5% of the total count) free cells. However, cells were weakly attached to the beads in all serum-free media and were not flattened. The weak attachment caused cells to detach from the beads and there were significant proportions of freely suspended cells (up to 0.28×10^6 cells/mL) throughout cultures in Gibco II and III and TCM serum-free media. There were significantly fewer (64%, $P < 0.01$) free cells in cultures of VSFM.

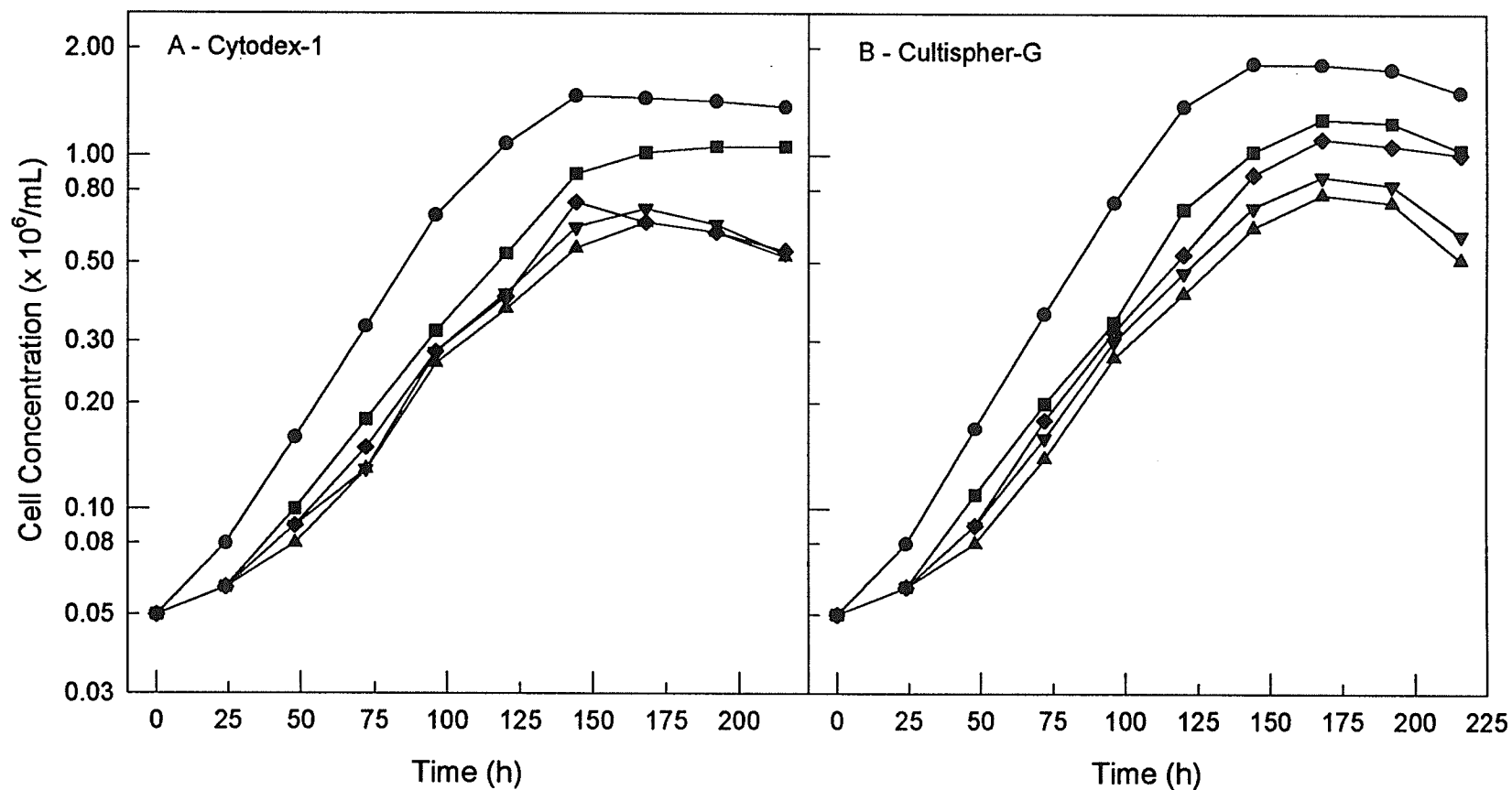


Fig. 9.2 - The effect of various serum-free medium formulations on preadapted Vero cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A) and 1.70 g/L Cultispher-G (B). The cells were inoculated at 0.05×10^6 cells/mL in either ● DMEM + 5.0% v/v sCS, ■ VSFM, Gibco serum-free Vero maintenance media ▲ II, or ▼ III or ◆ Celox TCM supplemented, enriched DMEM. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted daily to pH 7.1 after 72 h in culture with 1.00 M HCl or 2.00 M NaOH. The points are means of $n=2$.

Table 9.4 - The effect of the **serum-free medium formulation** and DMEM + 5.0% v/v sCS on **cell growth rate, lag phase and attachment times** in 100 mL spinner flask cultures of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were preadapted either directly or gradually (>6 passages) from DMEM + 5.0% v/v sCS into VSFM, Gibco Maintenance Medium II and III and TCM supplemented DMEM formulations. The cells were inoculated at 0.05×10^6 cells/mL. The flasks were readjusted to pH 7.1 daily after 72 h in culture with 1.00 M HCl or 2.00 M NaOH. The values are means of n=2.

Cultures Effect	Time Taken for Particular Effect				
	DMEM + 5.0% v/v sCS	VSFM	Gibco II	Gibco III	TCM
Cytodex-1					
Cell Attachment Time	1.00 ± 0.25 h	2.50 ± 1.00 h	2.50 ± 1.00 h	2.50 ± 1.00 h	2.00 ± 1.00 h
Cell Flattening Time	5.00 ± 1.00 h	12.00 ± 1.00 h [#]	12.00 ± 1.00 h [#]	12.00 ± 1.00 h [#]	12.00 ± 1.00 h [#]
Lag Phase	4.00 ± 1.00 h	21.00 ± 2.00 h	24.00 ± 2.00 h	24.00 ± 2.00 h	21.00 ± 2.00 h
Specific Cell Growth Rate	0.036 ± 0.003 h ⁻¹	0.028 ± 0.003 h ⁻¹	0.024 ± 0.002 h ⁻¹	0.024 ± 0.002 h ⁻¹	0.025 ± 0.002 h ⁻¹
Cultispher-G					
Cell Attachment Time	9.00 ± 3.00 h	15.00 ± 1.00 h	15.00 ± 1.00 h	18.00 ± 2.00 h	15.00 ± 1.00 h
Cell Flattening Time	UNK	UNK	UNK	UNK	UNK
Lag Phase	9.00 ± 2.00 h	21.00 ± 2.00 h	24.00 ± 2.00 h	24.00 ± 2.00 h	21.00 ± 2.00 h
Specific Cell Growth Rate	0.035 ± 0.003 h ⁻¹	0.027 ± 0.003 h ⁻¹	0.024 ± 0.002 h ⁻¹	0.025 ± 0.002 h ⁻¹	0.026 ± 0.002 h ⁻¹

UNK - Unknown as impossible to visualise.

- Cells never fully flattened but this was the time at which they became flattest.

Table 9.5 - The effects of the **serum-free medium formulation** on Vero cell **residual concentration** and **consumption rates** of **glucose (A)** and **lactate (B)** in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in DMEM + 5.0% v/v sCS, VSFM, Gibco Maintenance Medium II and III and TCM supplemented, enriched DMEM. The flasks were readjusted to pH 7.1 daily after 72 h in culture with 1.00 M HCl or 2.00 M NaOH. The values are means of n=2.

Table 9.5A) - Glucose.

Nutrient Effect	Residual Glucose Concentrations at Maximum Cell Yield and Specific Consumption and Production Rates in the Various Media Formulations				
	DMEM+5.0%v/vsCS	VSFM	Gibco II	Gibco III	TCM
Cytodex-1					
Residual Conc.	7.25 mM	12.80 mM	9.00 mM	9.20 mM	13.60 mM
Sp. Consump. Rate	$67.00 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$86.15 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$94.44 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$83.00 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$86.67 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$
Cultispher-G					
Residual Conc.	2.25 mM	8.70 mM	8.60 mM	8.80 mM	10.40 mM
Sp. Consump. Rate	$72.50 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$85.00 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$80.91 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$72.50 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$85.00 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$

Table 9.5B) - Lactate.

Nutrient Effect	Residual Lactate Concentrations at Maximum Cell Yield and Specific Consumption and Production Rates in the Various Media Formulations				
	DMEM+5.0%v/vsCS	VSFM	Gibco II	Gibco III	TCM
Cytodex-1					
Residual Conc.	22.25 mM	12.60 mM	10.70 mM	10.90 mM	13.00 mM
Sp. Consump. Rate	$89.00 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$96.92 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$118.89 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$109.00 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$108.33 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$
Cultispher-G					
Residual Conc.	31.55 mM	20.90 mM	13.65 mM	13.35 mM	15.20 mM
Sp. Consump. Rate	$105.17 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$116.11 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$124.09 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$111.25 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$	$95.00 \mu\text{mol} \cdot 10^6 \cdot \text{d}^{-1}$

pH. The culture pH varied between the media types used during growth. The cultures of DMEM + 5.0% v/v sCS had decreased after 72 h from pH 7.4 to pH 7.0 and 6.7 in Cytodex-1 and Cultispher-G, respectively. Following readjustment to pH 7.1 the daily pH was not significantly different from that after 72 h in cultures of either bead. However, all cultures of serum-free media were still at pH 7.4 after 24 h. The cultures were then readjusted to pH 7.1-2 with 1.00 M HCl daily up to 72 h. After 72 h in culture none of the serum-free cultures required further pH readjustment, except the Cultispher-G cultures in VSFM which were readjusted with 2.00 M NaOH after 96 h.

Nutrient Analysis. (1) There was a significantly higher (83%, $P<0.01$) glucose concentration (Table 9.5A)) consumed in cultures of either bead in DMEM + 5.0% v/v sCS compared to all serum-free media used. (2) There was a significantly higher residual glucose concentration at maximum cell yield in cultures of Cytodex-1 (63%, $P<0.01$) and Cultispher-G (404%, $P<0.01$) in all serum-free media compared to DMEM + 5.0% v/v sCS. (3) There were significantly higher (21%, $P<0.05$) specific glucose consumption rates in cultures of either bead in all serum-free media used compared to DMEM + 5.0% v/v sCS. (4) There was a significantly higher (95%, $P<0.01$) lactate concentration (Table 9.5B)) produced in cultures of either bead in DMEM + 5.0% v/v sCS compared to all serum-free media used. No other nutrients were analysed from these cultures. (5) There were no significant differences in the specific lactate production rates in cultures of either bead with respect to any media used.

Discussion. The only reports of serum-free microcarrier culture were for rBHK cells in cultures of Cytodex-3 (Schmid et al., 1992) and human foreskin fibroblast (FS-4) and MRC-5 cells on SuperbeadsTM (Crespi et al., 1981). However, Vero cells could also grow in 100 mL spinner flask cultures of either Cytodex-1 or Cultispher-G in all the serum-free media used in this study.

Maximum cell yields were significantly higher in VSFM compared to all other media used. The lower cell yields in Gibco media could be expected, as these were developed for Vero cell

maintenance and not for growth. The assumed intention of these formulations is to grow the cells in serum containing medium prior to infection with viruses or production of a secreted product. After infection or reaching a high cell yield the serum medium could be replaced with serum-free medium for cell maintenance in the production phase. The rationale behind these media could be to prevent the purification problems associated with serum. The use of serum-free media for virus production would have little effect on virus recoveries, as the purification early steps in vaccine production are not prone to serum induced losses. Cell maintenance in these media could benefit purification recoveries of secreted proteins produced by Vero cells. However, Vero cells currently have no commercial significance for such products (Thilly, 1989), although there have been reports of these cells being used for producing measles virus fusion protein F and human immunodeficiency virus coat proteins (Alkhatib et al., 1990 and Rovinski et al., 1992).

Cell attachment to Cytodex-1 beads differed between the media and there was little flattening on the bead surface in either of the Gibco media or TCM medium. This suggests these formulations contained little or no fibronectin or hydrocortisone to promote cell attachment and flattening (Hoshi et al., 1982). Cells were flattened more in VSFM compared to the other serum-free media but nowhere near so well as in DMEM + 5.0% v/v SCS. This could be explained by vitronectin deficiency (Knox and Griffiths, 1980).

All serum-free cultures of Cytodex-1 required acidification for the first 72 h to maintain the optimal pH for growth compared to alkalination in DMEM + 5.0% v/v SCS from 72 h onwards. This could explain the early lag phases in serum-free cultures where they were at pH 7.4 after 24 h. This pH reduced maximum cell yields and increased lag phases in previous cultures of either bead (5.3.5). Only Cultispher-G cultures in VSFM required NaOH readjustment after 96 h to raise the pH from <pH 6.8 back to the optimal range. The pH effect could be explained by the lactate production in these cultures. Both glucose consumption and lactate production were significantly reduced in all serum-free media compared to DMEM + 5.0% v/v SCS (6.3.3). This suggests cells growing in serum-free medium were metabolising the nutrients differently

compared to those with serum. The serum-free media could lack regulatory proteins normally present in serum containing medium (Feng et al., 1990 and Hundal et al., 1991) and this could have major implications to previous studies on the effects of serum factors using serum-free medium. If the cellular metabolism were regulated by a hormone not present in the serum-free medium used then the observed effects could be exaggerated or misleading. For example, the results of serum-free studies with growth factors and lipids could be significantly affected if fetuin and BSA (responsible for transport of these factors to the cells) were absent from the medium (Fisher and Lam, 1974 and Nie, 1992).

9.3.3 Precoated Microcarriers

Introduction. Vero cells required fibronectin to attach to substrata and flatten for growth (9.3.1). However, fibronectin is cold insoluble globulin and has a high affinity for adsorption to surfaces, in the absence of other serum proteins, which could cause losses during medium storage. Fibronectin precoated beads could prevent this problem. Therefore, this study determined the effects of precoating the microcarriers with DMEM + 5.0% v/v sCS or fibronectin on Vero cell growth in microcarrier cultures. Fibronectin was also removed from the VSFM for cell growth.

Experimental. Vero cultures were grown in the serum-free media in spinner flasks, as described above (9.3.2). The microcarriers were either (a) precoated or (b) were uncoated. Precoating was with (i) DMEM + 5.0% v/v sCS or (ii) DMEM + 5.0 mg/L bovine fibronectin. Cytodex-1 (0.50 g) and Cultispher-G (0.80 g) microcarriers were aseptically transferred into siliconised, sterile 100 mL glass bottles. The beads were suspended in the precoating solutions (50 mL) at 20°C and swirled for 5 min every 15 min for 6 h. The beads were settled and retained in these solutions for 12 h. The supernatant (45 mL) was removed and the beads were washed with D-PBS⁻ (x2 at 100 mL). The beads were settled and the supernatant (95 mL) removed. The beads were resuspended in DMEM (10 mL) and aseptically transferred (0.10 g Cytodex-1 and

0.17 g Cultispher-G) into 100 mL spinner flasks. The VSFM was prepared without fibronectin.

Results. Table 9.6 shows maximum Vero cell yields in cultures of serum or fibronectin precoated and uncoated Cytodex-1 and Cultispher-G in the various serum-free media used. Fig. 9.3 shows cell growth in these cultures. Table 9.7 shows cell attachment times, lag phases and growth rates in cultures of either bead with the various precoatings used. (1) Maximum cell yields in cultures of either bead in the various serum-free media (VSFM, Gibco II and III and TCM) were affected by bead precoating. (2) There were significantly higher (15%, $P=0.05$) cell yields (Table 9.6) in Cultispher-G cultures compared to Cytodex-1 with respect to all serum-free medium formulations used. (3) Maximum cell yields were not significantly different in cultures of either bead with precoated compared to uncoated beads in the commercial serum-free media used. Maximum cell yields were significantly higher (159%, $P<0.01$) in cultures of precoated Cultispher-G and Cytodex-1 in VSFM (without fibronectin). However, maximum cell yields (Fig. 9.3) would have been significantly higher (37%, $P<0.01$) in cultures of either bead, with or without precoating, in DMEM + 5.0% v/v SCS compared to all serum-free media used. (4) The maximum cell yields in cultures of either precoated bead were significantly higher (50%, $P<0.01$) in VSFM compared to the other serum-free media. (5) There was no significant difference (Table 9.7.1-2) in the cell growth rates in cultures of either precoated beads with respect to all the serum-free media used. Cell growth rates were not significantly different with uncoated compared to precoated beads of either type in commercial serum-free media. However, cell growth rates in VSFM were significantly higher (29%, $P<0.01$) in cultures of precoated beads of either type. (6) Cell attachment times were not significantly different in any cultures of Cytodex-1. However, cell attachment times were significantly faster (15%, $P=0.05$) in Cultispher-G beads precoated with SCS compared to fibronectin precoated or uncoated beads. (7) Lag phases (Table 9.7) were significantly shorter (15%, $P=0.05$) in cultures of either bead precoated with SCS compared to fibronectin coated or uncoated beads.

The cells were poorly attached in all the cultures with uncoated beads of either type and

Table 9.6 - The effect of **precoating Cytodex-1 (A) and Cultispher-G (B)** beads with either DMEM containing sCS or bovine fibronectin on the **maximum Vero cell yields** in serum-free medium. Preadapted cells were inoculated at 0.05×10^6 cells/mL on 1.00 g/L Cytodex-1 or 1.70 g/L Cultispher-G in 100 mL spinner flasks. The microcarriers were precoated (18 h) at 20°C. The cells were grown in VSFM, Gibco Vero cell maintenance media II or III or in Celox TCM supplemented, enriched DMEM. The flasks were readjusted daily after 72 h in culture to pH 7.1 with 1.00 M HCl or 2.00 M NaOH. The values are means of n=2.

Table 9.6A) - Cytodex-1.

Serum-Free Medium Formulation	Maximum Cell Concentration ($\times 10^6$ /mL)		
	Precoated		Uncoated
	DMEM + 5.0% v/v sCS	5.0 mg/L Fibronectin	
VSFM – Fibronectin	$1.17 \pm 0.07^*$	1.16 ± 0.07	0.31 ± 0.03
VSFM + Fibronectin	ND	ND	1.06 ± 0.07
Gibco II	0.81 ± 0.06	0.78 ± 0.05	0.68 ± 0.04
Gibco III	0.76 ± 0.06	0.76 ± 0.05	0.75 ± 0.05
TCM-DMEM + Serine and Pyruvate	0.88 ± 0.07	0.85 ± 0.06	0.77 ± 0.06

Table 9.6B) - Cultispher-G.

Serum-Free Medium	Maximum Cell Concentration ($\times 10^6$ /mL)		
	Precoated		Uncoated
	DMEM + 5.0% v/v sCS	5.0 mg/L Fibronectin	
VSFM – Fibronectin	$1.47 \pm 0.10^*$	1.29 ± 0.08	0.66 ± 0.04
VSFM + Fibronectin	ND	ND	1.27 ± 0.08
Gibco II	0.90 ± 0.07	0.80 ± 0.05	0.76 ± 0.05
Gibco III	0.96 ± 0.06	0.87 ± 0.05	0.83 ± 0.06
TCM-DMEM + Serine and Pyruvate	0.94 ± 0.07	0.89 ± 0.06	0.77 ± 0.06

* - Highest cell yield.

ND - Value not determined.

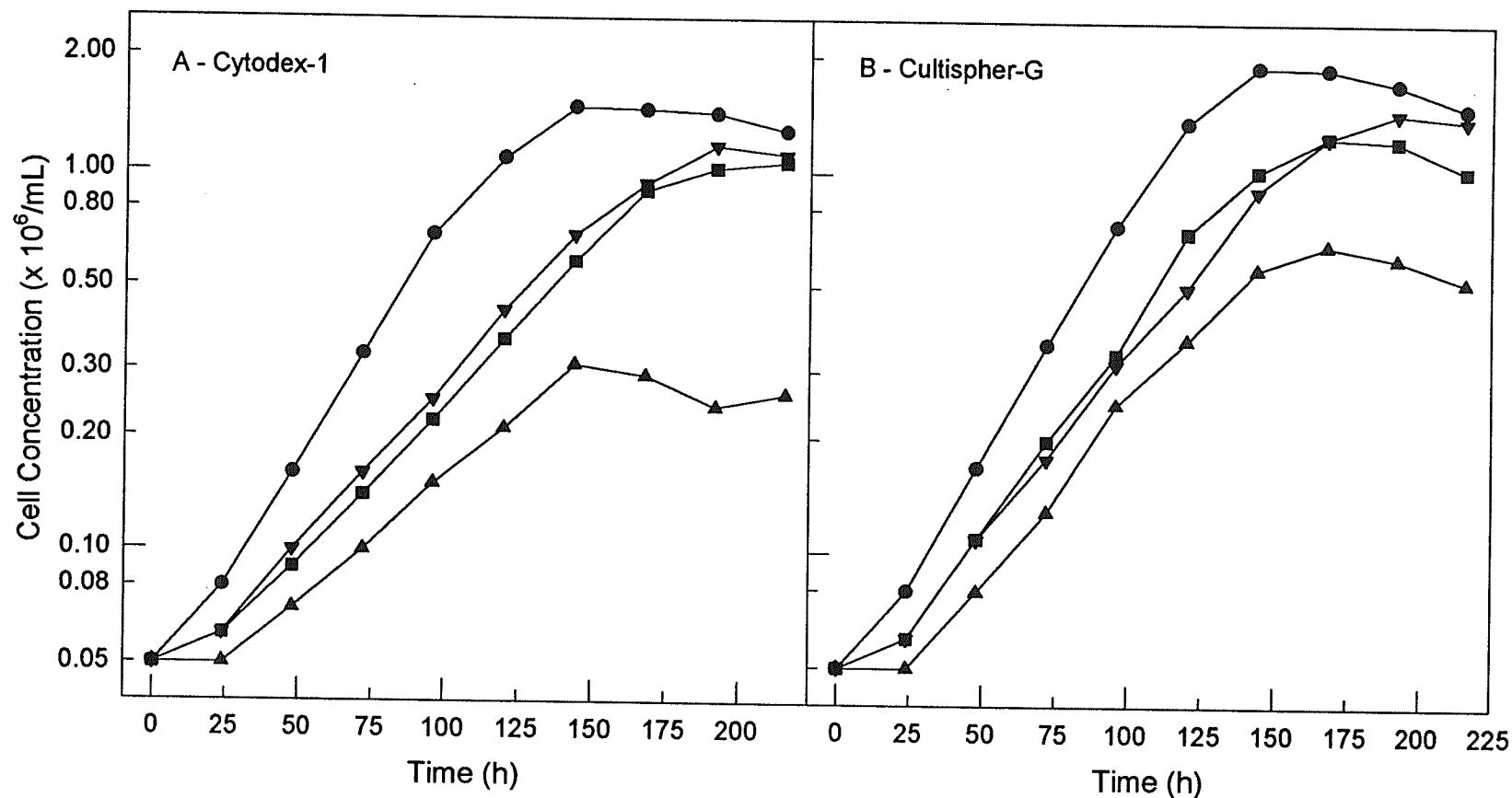


Fig. 9.3 - The effect of bead precoating and medium formulation on Vero cell growth in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (A) and 1.70 g/L Cultispher-G (B). The cells were inoculated at 0.05×10^6 cells/mL in either ● DMEM + 5.0% v/v sCS on uncoated beads, ■ VSFM on uncoated beads, ▲ VSFM (- Fibronectin) on uncoated beads or ▼ VSFM (- Fibronectin) on DMEM + 5.0% v/v sCS precoated beads. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h in culture (Cultispher-G). The flasks were readjusted to pH 7.1 daily after 72 h in culture with 1.00 M HCl or 2.00 M NaOH. The points are means of $n=2$.

Table 9.7 - The effects of the **serum-free medium formulation** on specific Vero cell growth rate, lag phase and attachment times in 100 mL spinner flasks on **1.00 g/L Cytodex-1 (A)** and **1.70 g/L Cultispher-G (B)**. The cells were inoculated at 0.05×10^6 cells/mL and grown in VSFM (without fibronectin), Gibco II and III and TCM serum-free media on beads which were either precoated with DMEM + 5.0% v/v sCS or DMEM + 5.0 mg/L fibronectin for 18 h at 20°C or were uncoated. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously and ramped to 60 rpm after 72 h (Cultispher-G). The flasks were readjusted to pH 7.1 daily after 72 h in culture with 1.00 M HCl or 2.00 M NaOH. The values are means of n=2.

Table 9.7A) - Cytodex-1.

Precoating	Time Taken for Particular Effect			
	VSFM	Gibco II	Gibco III	TCM
DMEM + 5.0% v/v sCS				
Cell Attachment Time	1.00 ± 0.50 h	1.00 ± 0.50 h	1.00 ± 0.50 h	1.00 ± 0.50 h
Lag Phase	18.00 ± 2.00 h	21.00 ± 2.00 h	21.00 ± 2.00 h	18.00 ± 2.00 h
Specific Cell Growth Rate	0.027 ± 0.003 h ⁻¹	0.025 ± 0.002 h ⁻¹	0.025 ± 0.002 h ⁻¹	0.026 ± 0.002 h ⁻¹
5.0 mg/L Fibronectin				
Cell Attachment Time	2.50 ± 1.00 h	2.50 ± 1.00 h	2.50 ± 1.00 h	2.50 ± 1.00 h
Lag Phase	24.00 ± 2.00 h	24.00 ± 2.00 h	24.00 ± 2.00 h	24.00 ± 2.00 h
Specific Cell Growth Rate	0.027 ± 0.003 h ⁻¹	0.025 ± 0.002 h ⁻¹	0.025 ± 0.002 h ⁻¹	0.026 ± 0.002 h ⁻¹
Uncoated				
Cell Attachment Time	2.50 ± 1.00 h	2.50 ± 1.00 h	2.50 ± 1.00 h	2.50 ± 1.00 h
Lag Phase	36.00 ± 4.00 h	30.00 ± 3.00 h	30.00 ± 3.00 h	30.00 ± 3.00 h
Specific Cell Growth Rate	0.020 ± 0.002 h ⁻¹	0.024 ± 0.002 h ⁻¹	0.024 ± 0.002 h ⁻¹	0.027 ± 0.003 h ⁻¹

Table 9.7B) - Cultispher-G.

Precoating	Time Taken for Particular Effect			
	VSFM	Gibco II	Gibco III	TCM
DMEM + 5.0% v/v sCS				
Cell Attachment Time	9.00 ± 1.00 h	9.00 ± 1.00 h	9.00 ± 1.00 h	9.00 ± 1.00 h
Lag Phase	24.00 ± 3.00 h	24.00 ± 3.00 h	24.00 ± 3.00 h	24.00 ± 3.00 h
Specific Cell Growth Rate	0.026 ± 0.003 h ⁻¹	0.024 ± 0.002 h ⁻¹	0.024 ± 0.002 h ⁻¹	0.026 ± 0.002 h ⁻¹
5.0 mg/L Fibronectin				
Cell Attachment Time	9.00 ± 1.00 h	9.00 ± 1.00 h	9.00 ± 1.00 h	9.00 ± 1.00 h
Lag Phase	28.00 ± 3.00 h	28.00 ± 3.00 h	28.00 ± 3.00 h	28.00 ± 3.00 h
Specific Cell Growth Rate	0.027 ± 0.003 h ⁻¹	0.024 ± 0.002 h ⁻¹	0.024 ± 0.002 h ⁻¹	0.027 ± 0.002 h ⁻¹
Uncoated				
Cell Attachment Time	18.00 ± 2.00 h	18.00 ± 2.00 h	18.00 ± 2.00 h	18.00 ± 2.00 h
Lag Phase	30.00 ± 3.00 h	30.00 ± 3.00 h	30.00 ± 3.00 h	30.00 ± 3.00 h
Specific Cell Growth Rate	0.022 ± 0.002 h ⁻¹	0.023 ± 0.002 h ⁻¹	0.023 ± 0.002 h ⁻¹	0.023 ± 0.002 h ⁻¹

especially in VSFM (without fibronectin). The majority of the cells (>60%) were in aggregates (>30 cells) in cultures of either uncoated bead in VSFM.

Discussion. Maximum Vero cell yields were similar in cultures of either bead with serum or fibronectin precoating in all serum-free media used. This confirms the cells could attach and grown on fibronectin precoated beads and agrees with the results of Bottenstein and Sato (1980) with neuroblastoma cells on fibronectin precoated Cytodex-1. The maximum cell yields with precoated and uncoated beads of either type were similar in all commercial serum-free media suggesting bead precoating had no effect on cell growth. This could be explained by these media being deficient in nutrients, hormones or growth factors required for regulation, as discussed above (9.3.1).

The maximum cell yields in VSFM were significantly higher in cultures of precoated compared to uncoated beads. The cells in uncoated Cytodex-1 cultures were mainly present as aggregates, not attached to the beads, which confirms that attachment factors were required in this medium. Increasing the hydrocortisone concentration could have improved cell attachment and flattening to Cytodex-1 (9.3.1) but was not investigated in this study.

Almost all of the cells were attached to the uncoated Cultispher-G beads in cultures of VSFM without fibronectin. This agrees with previous attachment results (6.3.2) and shows that Vero cells attach either by a passive entrapment of aggregated cells or by an active protein mediated mechanism. However, Ruoslahti et al. (1982) stated that fibroblast cells required fibronectin to attach to collagen surfaces. Vero cells clearly did not but could be secreting another adhesion protein (e.g. collagen, laminin etc.) on their surfaces to mediate the attachment.

The bead precoating could affect the process costs. Precoating with serum containing medium would be a relatively cheap process but would not reduce the risks of using serum. Precoating the beads with fibronectin solutions would not reduce the process costs as the purified fibronectin would still need to be purchased. Therefore, precoating did not benefit the serum-free growth of Vero cells.

9.4 Conclusions

The purpose of this study was to define and develop a serum-free medium for the growth of Vero cells in spinner flask cultures of Cytodex-1 and Cultispher-G. The results suggest that:

- **Vero cells can be grown in serum-free medium** in T25 flask or spinner flask cultures of solid Cytodex-1 and macroporous Cultispher-G. However, maximum cell yields were significantly higher (22%, $P < 0.05$), lag phases were shorter (233%, $P < 0.01$) and cell growth rates were higher (23%, $P < 0.05$) in DMEM + 5.0% v/v sCS compared to all serum-free media used.

- **the Vero cell serum-free medium (VSFM)** produced significantly higher (22-44%, $P < 0.05$) cell yields but was more expensive (>Cdn.\$550/L) to prepare compared to commercially available TCM supplemented medium. The VSFM also requires further optimisation to enhance cell growth and replace the expensive components (e.g. fibronectin, FGF and fetuin).

- **the concentration of glucose consumed** by cells was significantly reduced in all serum-free media compared to DMEM + 5.0% v/v sCS. However, the specific rate of glucose consumption was significantly higher in serum-free media compared to that in serum. This resulted in significantly lower lactate production which caused less acidification in cultures of either bead with respect to all the serum-free media used.

- **serum or fibronectin precoating of the microcarriers** allowed removal of the fibronectin from the VSFM with no loss of maximum cell yields. Serum precoating significantly lowered the process and medium cost but fibronectin precoating did not affect the overall process cost.

Chapter 10. Optimisation of Medium Refeeding for Vero Cell Microcarrier Cultures

10. Optimisation of Medium Refeeding for Vero Cell Microcarrier Cultures

10.1 Introduction

Standard T25-T150 culture flasks, with 10 and 50 mL of medium, respectively, provide a surface area of between 2.5-3.0 cm²/mL of medium. To increase their surface area requires more flasks causing handling, labour and sterility problems (Reuveny, 1990). However, microcarriers such as solid Cytodex-1TM have a surface area of 4,400 cm²/g dry weight (Pharmacia, 1981) and at a concentration of 1.00 g/L provide 4.4 cm²/mL of culture. Hence, microcarriers significantly increase surface areas for cell growth. The microcarrier concentration can also be increased to provide more surface area per volume of medium.

Few, if any, process parameters have been optimised for either batch or perfusion growth of adherent cells in microcarrier cultures. These cultures can produce significantly higher cell yields compared to static flasks by using fed-batch or perfusion processes, (i.e. continuous or periodic medium exchange). Many researchers used these processes to increase Chinese hamster ovary (CHO), murine hybridoma and African green monkey kidney (Vero) cell yields (Clark and Hirtenstein, 1981, Nahapetian et al., 1986, Vournakis and Runstadler, 1988, Smiley et al., 1989, Reiter et al., 1990, Forestall et al., 1992 and Ohlson et al., 1994). However, the microcarrier types and concentrations used previously vary greatly from 2.00 g/L Cytodex-1 (Smiley et al., 1989) up to 20.00 g/L SuperbeadsTM (Nahapetian et al., 1986). The refeeding methods and medium volume changes have also varied from one medium exchange every few days in spinner flasks to continuous perfusion in a fluidised bed bioreactor at 30.00 v/v/d (Clark and Hirtenstein, 1981 and Reiter et al., 1990). Clark and Hirtenstein (1981) showed 1.00 g/L Cytodex-1 is sub-optimal for batch and refed growth of Vero and human diploid lung fibroblast (MRC-5) cells in spinner flasks, so they used 3.00-5.00 g/L Cytodex-1 in DMEM + 10.0% v/v foetal bovine serum (FBS). The cells were refed with one 50% v/v (i.e. equivalent to 0.50 v/v/d perfusion rate) medium exchange every 3-4 d. Their maximum cell yields increased from c.1.00 x 10⁶ cells/mL (batch) up to 5.00 x 10⁶ cells/mL (refed). Nahapetian et al. (1986) also grew

Vero cells but used 20.00 g/L Superbeads in spinner flasks in L-15 (5.60 mM fructose and without galactose) + 5.0% v/v FBS and refed at 8.00 v/v/d. They reported maximum cell yields $>30.00 \times 10^6$ cells/mL. Reiter et al., (1990) grew Vero cells in 5.00 g/L Cultispher-G cultures in DMEM (5.60 mM glucose) + 5.0% v/v FBS at a perfusion rate of 10.00-30.00 v/v/d. These authors reported maximum cell yields of 35.00×10^6 cells/mL of bed volume in a 1.2 L bioreactor. However, none of these authors proved their bead concentrations to be optimal for cell growth in the systems used. It must be remembered that the cells were counted by the crystal violet nuclei staining method in many of the studies above which could mean the results are overestimated by up to 70% due to inaccuracies with this method (refer to 4.3.3). These errors make it difficult to compare results between authors.

No one has previously determined the important parameters for high cell yield cultivation of any adherent cells. High cell yield cultivation on microcarriers requires three steps:- (1) Identify and optimise the important physical and medium parameters for growth (refer to 5.3-8.3). (2) Define the maximum cell yields in refed cultures using fixed but low microcarrier concentrations and determine the maximum cell/bead loadings and limiting culture parameters. This was the purpose of this Chapter. (3) Scale the system up to higher microcarrier concentrations and determine if the maximum cell/bead loadings can be reached or if other parameters become limiting. This will be discussed in Chapter 11.3.

This study presents the optimisation of high yield culture of Vero cells at low concentrations of solid Cytodex-1 (1.00 g/L) and macroporous Cultispher-G (1.70 g/L) microcarriers in spinner flasks and Celligen™ bioreactors. Optimal batch parameter values were used in the initial refeeding strategies. The daily medium exchange of spinner flasks and perfusion of bioreactor cultures was optimised and the maximum cell/bead loadings on the two beads defined.

10.2 Materials and Methods

The methods for culturing and counting the cells and nutrient analyses have been described previously (2.1-2.5).

10.2.1 Preadaptation of Stock Cells

Stock Vero cells were preadapted (>x5 passages) to original DMEM (Medium I), serine and pyruvate enriched (2.00 mM and 0.50 mM, respectively) DMEM (Medium II) and enriched DMEM supplemented with taurine (2.00 mM) (Medium III) . All media were supplemented with 5.0% v/v iron enriched, supplemented calf serum (sCS). The cells were detached and recovered (2.2) and counted by the trypan blue method (2.5.1-2).

10.3 Results and Discussion

10.3.1 Formulation of Refeeding Medium

Introduction. Few, if any, process parameters have been optimised for daily refed or perfusion growth of adherent cell lines in microcarrier cultures. Therefore, this study determined the effects of the refeeding medium formulation on Vero cell growth in microcarrier cultures.

Experimental. Preadapted stock Vero cells were inoculated at 0.05×10^6 cells/mL in original DMEM (Medium I), serine and pyruvate enriched (2.00 mM and 0.50 mM, respectively) DMEM (Medium II) and enriched DMEM supplemented with taurine (2.00 mM) (Medium III) and grown in 100 mL spinner flasks on 1.00 g/L Cytodex-1 or 1.70 g/L Cultispher-G (2.4.3). Culture refeeding at one daily 50% v/v medium exchange was initiated after 72 h (2.4.4).

Results. Fig. 10.1 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G in Media I, II and III. Table 10.1 shows the maximum cell yields and maximum cell/bead loadings produced in the cultures. Table 10.2 shows the cell growth rates and lag phases in these cultures. Fig. 10.2 shows glucose consumption and lactate production, glutamine consumption and ammonia production and serine concentration in these cultures. Table 10.3 shows lactate/glucose, ammonia/glutamine production ratios and serine and taurine concentrations at maximum cell yields in these cultures. (1) Maximum cell yields in daily refed cultures of either bead were

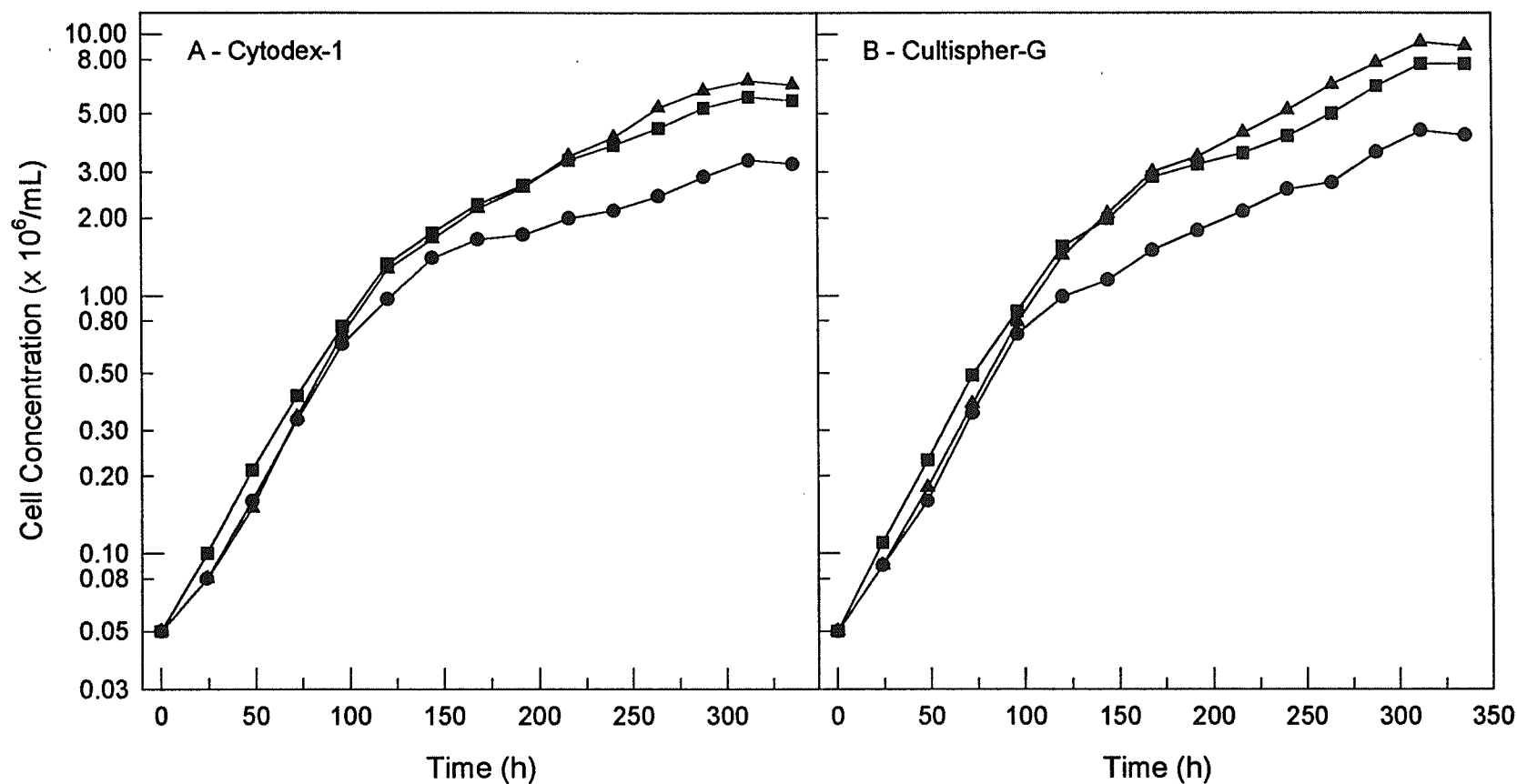


Fig. 10.1 - The effect of the daily refeeding DMEM formulation on the growth of preadapted Vero cells in 100 mL spinner flasks on **1.00 g/L Cytodex-1 (A)** and **1.70 g/L Cultispher-G (B)**. The cells were inoculated at 0.05×10^6 cells/mL in either ● Medium I, ■ Medium II or ▲ Medium III all containing 5.0% v/v SCS. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and ramped to 60 rpm after 72 h in culture. The flasks were refed one daily 50% v/v medium exchange and readjusted to pH 7.1 with 1.00 M HCl or 2.00 mM NaOH after 72 h in culture. The points are means of $n=2$.

Table 10.1 - The effect of the daily refeeding DMEM formulation on maximum yields and cell/bead loadings of Vero cells in 100 mL spinner flasks on 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in either Medium I, II or III all containing 5.0% v/v SCS. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and ramped to 60 rpm after 72 h in culture. The flasks were refed one daily 50% v/v medium exchange and readjusted to pH 7.1 with 1.00 M HCl or 2.00 mM NaOH after 72 h in culture. The values are means of n=2.

Medium Formulation	Maximum Cell Yield and Cell/Bead Loading in the Medium Formulation Used			
	Cytodex-1		Cultispher-G	
	Maximum Cell Yield ($\times 10^6$ cells/mL)	Maximum Cell/Bead Loading (Cells/Bead)	Maximum Cell Yield ($\times 10^6$ cells/mL)	Maximum Cell/Bead Loading (Cells/Bead)
Medium I	3.32 ± 0.24	738	4.31 ± 0.31	2,817
Medium II	5.78 ± 0.41	1,284	7.71 ± 0.55	5,032
Medium III	$6.65 \pm 0.48^*$	1,478	$9.33 \pm 0.66^*$	6,098

* - Highest cell yield.

Table 10.2 - The effect of the daily refeeding DMEM formulation on growth rates and lag phases of Vero cells in 100 mL spinner flasks on 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The cells were inoculated at 0.05×10^6 cells/mL in either Medium I, II or III all containing 5.0% v/v SCS. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and ramped to 60 rpm after 72 h in culture. The flasks were refed one daily 50% v/v medium exchange and readjusted to pH 7.1 with 1.00 M HCl or 2.00 mM NaOH after 72 h in culture. The values are means of n=2.

Medium Formula	Primary and Secondary Stage Specific Cell Growth Rates and Lag Phases in the Medium Formulation Used				
	Cytodex-1			Cultispher-G	
		Specific Cell Growth Rates (h^{-1})	Lag Phase (h)	Specific Cell Growth Rates (h^{-1})	Lag Phase (h)
Medium I	High	0.033 ± 0.003	3.00 ± 0.50	0.034 ± 0.003	0.00
	Low	0.009 ± 0.002		0.011 ± 0.002	
Medium II	High	0.036 ± 0.003	4.50 ± 0.50	0.035 ± 0.003	3.00 ± 0.50
	Low	0.010 ± 0.002		0.010 ± 0.002	
Medium III	High	0.035 ± 0.003	4.50 ± 0.50	0.035 ± 0.003	3.00 ± 0.50
	Low	0.012 ± 0.002		0.011 ± 0.002	

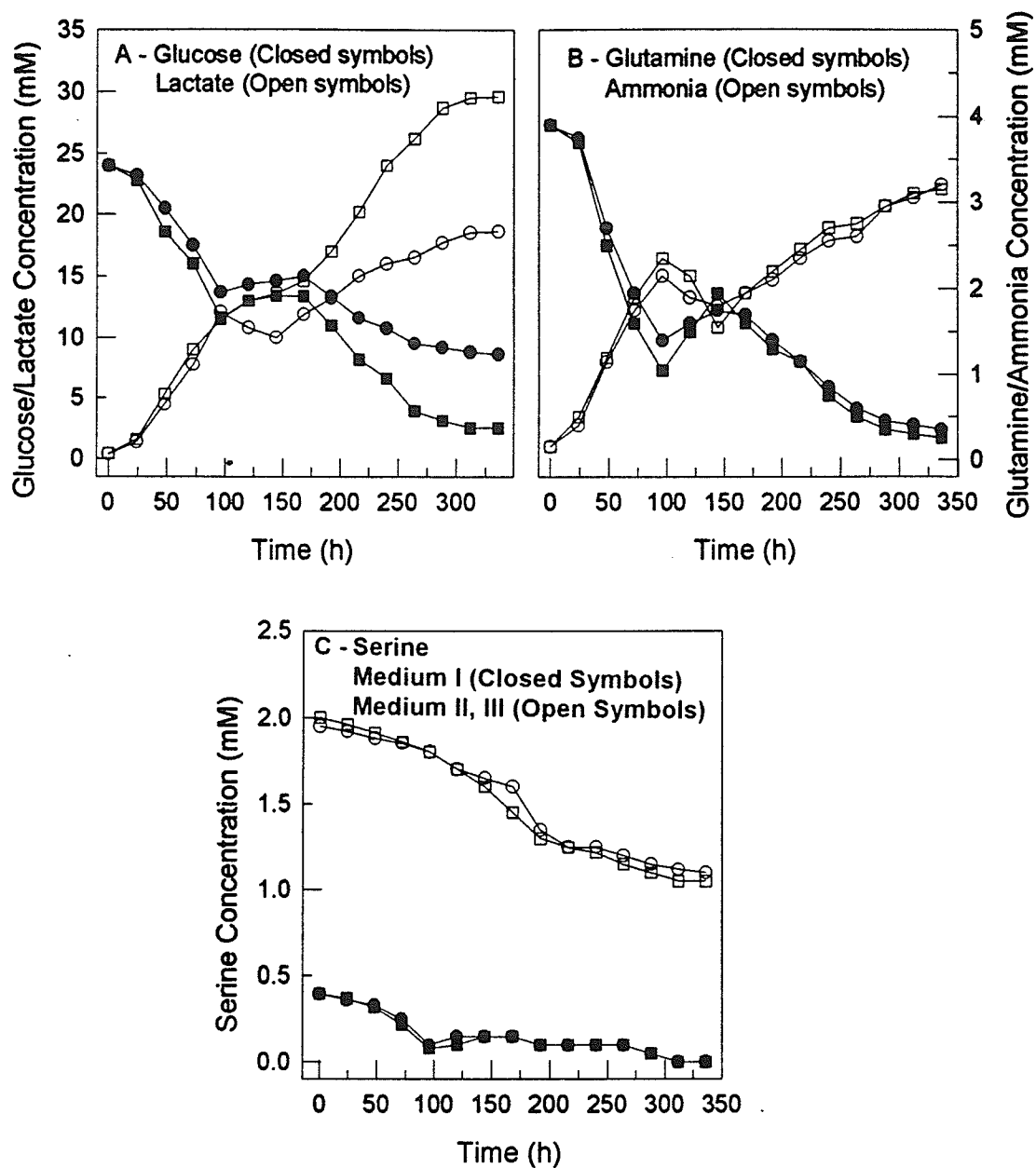


Fig. 10.2 - The effect of the daily refeeding DMEM formulation on the glucose/lactate (A), glutamine/ammonia (B) and serine (C) concentrations prior to each refeed time in 100 mL spinner flask Vero cultures on ● 1.00 g/L CytoDEX-1 and ■ 1.70 g/L Cultispher-G. The flasks were refeed with one 50% v/v medium exchange daily after 72 h in culture. The values are means from cultures of Media I, II and III and are of n=6.

Table 10.3 - The effect of the daily refeeding DMEM formulation on lactate/glucose, ammonia/glutamine, glutamate to glutamine, alanine to glutamine and glycine to serine production ratios and taurine concentration at maximum Vero cell yield in 100 mL spinner flasks on 1.00 g/L Cytodex-1 and 1.70 g/L Cultispher-G. The values were determined in cultures grown in Medium I, II or III. The flasks were refed daily with one 50% v/v medium exchange after 72 h in culture. The values are means of n=2.

Nutrient Effect	Residual Concentration (mM) or Production Ratio in the Medium at Maximum Cell Yield					
	Medium I		Medium II		Medium III	
	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G
Glucose Concentration	11.00 ± 1.10	3.55 ± 0.40	7.90 ± 0.80	2.00 ± 0.20	7.50 ± 0.75	1.95 ± 0.20
Lactate Concentration	14.85 ± 1.50	27.70 ± 3.00	19.20 ± 2.00	30.90 ± 3.00	19.50 ± 2.00	31.25 ± 0.20
Lactate to Glucose Ratio	1.15	1.36	1.20	1.41	1.19	1.42
Glutamine Concentration	0.30 ± 0.05	0.25 ± 0.03	0.35 ± 0.04	0.20 ± 0.05	0.25 ± 0.05	0.25 ± 0.05
Ammonia Concentration	3.05 ± 0.30	3.25 ± 0.03	3.15 ± 0.30	3.25 ± 0.30	3.15 ± 0.30	3.15 ± 0.30
Ammonia to Glutamine Ratio	0.84	0.88	0.88	0.87	0.85	0.85
Glutamate Concentration	0.95 ± 0.10	0.95 ± 0.10	1.15 ± 0.10	1.25 ± 0.10	1.10 ± 0.10	1.15 ± 0.10
Glutamate to Glutamine Ratio	0.26	0.26	0.32	0.33	0.30	0.31
Alanine Concentration	0.45 ± 0.05	0.50 ± 0.05	0.55 ± 0.06	0.55 ± 0.05	0.50 ± 0.05	0.55 ± 0.05
Alanine to Glutamine Ratio	0.13	0.14	0.15	0.15	0.14	0.15
Serine Concentration	0.00	0.00	1.15 ± 0.80	1.15 ± 0.10	1.05 ± 0.10	1.05 ± 0.10
Glycine Concentration	0.095 ± 0.01	0.092 ± 0.01	0.220 ± 0.05	0.230 ± 0.05	0.265 ± 0.05	0.268 ± 0.05
Glycine to Serine Ratio	0.24	0.24	0.26	0.27	0.28	0.28
Taurine Concentration	NP	NP	NP	NP	1.51 ± 0.15	1.43 ± 0.15

NP - None present.

dependent on the medium formulation. (2) There were significantly higher (36%, $P < 0.01$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the refeeding medium formulations used. (3) There were significantly higher (51%, $P < 0.01$) maximum cell yields in cultures of either bead in Medium III compared to the other media used. (4) There were significantly higher (298%, $P < 0.01$) maximum cell/bead loadings (Table 10.1) in Cultispher-G compared to Cytodex-1 with respect to all medium formulations used. (5) The cell growth rates (Table 10.2) showed biphasic patterns in cultures of either bead in all cultures. There was a significantly higher initial growth rate (up to 120 h in culture), to a cell yield of between $1.50\text{--}1.80 \times 10^6$ cells/mL, compared to the rate from this point up to maximum cell yields. (6) There was no significant difference in the lag phases (Table 10.2) in all cultures.

Nutrient Analysis. (1) The general trends in the glucose and lactate concentrations (Fig. 10.2A)) in cultures of either bead were to decrease and increase, respectively, as the cell yield increased. However, there was a short period (between 72-144 h in culture) where their concentrations at each refeed time plateaued in cultures of either bead. This coincided with the start of culture refeeding (72 h). The general trend then continued up to maximum cell yields. (2) There was a significantly higher (300%, $P < 0.01$) residual glucose concentration at maximum cell yield in Cytodex-1 cultures compared to Cultispher-G with respect to all medium formulations used. (3) There was a significantly higher (60%, $P < 0.01$) lactate concentration at maximum cell yield in Cultispher-G cultures compared to Cytodex-1 with respect to all medium formulations used. Thus, there were constant ratios of lactate/glucose in cultures of either bead. However, the lactate/glucose ratio was significantly higher (20%, $P < 0.05$) in Cultispher-G (1.40) cultures compared to Cytodex-1 (1.17). (4) The general trend in the glutamine and ammonia concentrations (Fig. 10.2B)) in cultures of either bead were to decrease and increase, respectively, as the cell yields increased. There was a short period (96-120 h) where their concentrations increased and decreased, respectively, prior to each daily refeed time. However, there was a low residual glutamine concentration at maximum cell yield in cultures of either

bead. (5) There was no significant difference in the ammonia concentration or ammonia/glutamine ratios (Table 10.3) in cultures of either bead at maximum cell yield. (6) There was (Fig. 10.2C) and Table 10.3) no significant difference in the residual serine concentration at maximum cell yield in cultures of either bead with respect to the medium formulations used. There was a low residual serine concentration in Medium I prior to each daily refeed time after 96 h in culture. However, there was a high residual serine concentration at maximum cell yield in cultures of Medium II and III due to the serine enrichment (2.00 mM). (7) There was a significant increase (between 23-67%, $P < 0.01$) in the glycine concentration which was proportional to the serine concentration used in cultures of either bead. This gave a constant ratio of glycine/serine in all media formulations used. (8) There was no significant difference in the taurine concentration (Table 10.3) consumed (0.53 mM) in cultures of either bead in the taurine supplemented (2.00 mM) Medium III. (9) No other detectable nutrients were at limiting concentrations at maximum cell yields in any of these cultures. (10) There were high concentrations of glutamate and alanine (Table 10.3) at maximum cell yield in all cultures. There were significantly higher (23%, $P < 0.05$) glutamate/glutamine production ratios in cultures of enriched Media II and III compared to Medium I. However, there were no significant differences in the alanine/glutamine production ratios in any cultures.

Cell Multi-Layers and Bead Aggregates. Vero cells grew as multi-layers (>2 cells deep) on Cytodex-1 beads (by microscopic observation) in all cultures. The beads were aggregated (2-10 beads) in all cultures similar to those described previously (5.3-8.3). At maximum cell yield the void spaces between Cytodex-1 aggregates were completely filled with cells. However, free cells detached from the beads at between $4.00\text{--}4.50 \times 10^6$ cells/mL (i.e. before the maximum cell yields) in Media II and III. The free cells formed spherical aggregates of a similar diameter (150-180 μm) to the beads and left significant numbers (>30%) of empty beads at maximum cell yield. There were few free cells (<5%) in any Cultispher-G cultures at any time.

pH. The pH of Cultispher-G cultures decreased from pH 7.2 at inoculation to pH <6.7 prior to the first refeed time. This could be caused by lactate production from glucose, as discussed previously (5.3.3). After 144 h in culture the pH was maintained within the optimal range (pH 6.8-7.1) for growth at each refeed time and little pH readjustment was necessary. The pH in Cytodex-1 cultures was at >pH 7.0 prior to all refeed times and required acidification with 1.00 M HCl after refeeding to lower the pH to the optimal range.

Discussion. There were significant differences in maximum cell yields in cultures of either bead between the various refeeding media used. Nutrient analyses suggested serine (Fig. 10.2C)) was the most important nutrient in refeed Vero cultures, as it was completely consumed prior to each daily refeed time in Medium I. However, there was a high residual serine concentration in Media II and III due to the serine enrichment. This result confirms the importance of serine to Vero cell growth, and the metabolism was discussed previously (6.4.3). Hence, the improved cell yields in the enriched DMEM could be explained by the extra serine. Pyruvate could also be involved through sequestration of ammonia, as discussed previously (7.3.5-6).

There was also a residual glutamine concentration at each refeed time and the concentration was low in all cultures at maximum cell yield. Thus, maximum cell yields could also be dependent on the glutamine concentration. The metabolism of glutamine was discussed previously (6.3.3). Ammonia accumulated gradually and gave constant ammonia/glutamine ratios 0.86 at maximum cell yield which not significantly different from those (0.81) in batch cultures (6.3.3). Glutamate and alanine were produced to high concentrations in all cultures and reached similar concentrations to those in batch cultures (6.3.3).

The lactate/glucose ratios (1.18 and 1.40, for Cytodex-1 and Cultispher-G, respectively) were also similar to values (1.17 and 1.39, respectively) produced in batch cultures (6.3.3).

Therefore, the metabolic profiles of Vero cells were not significantly different in daily refeed cultures compared to batch.

Taurine addition to enriched refeeding medium significantly increased maximum cell yields

compared to taurine free medium. There were significantly higher maximum cell yields in 3 of 5 cultures in this study suggesting the preadaptation process could be critical to the effect. Increased maximum cell yields were also shown previously in some batch cultures (7.3.9). The mechanism of the taurine increased yields was unclear but was discussed previously (7.3.9).

Cell Growth Rate. The cell growth rates were biphasic and were not significantly different in all refed cultures. This effect has been shown previously with Vero cells in refed cultures using 20.00 g/L Superbeads (Nahapetian et al., 1986). These authors observed the onset of the effect at a significantly higher cell concentration ($1.00\text{--}1.50 \times 10^7$ cells/mL) compared to this study ($1.50\text{--}1.80 \times 10^6$ cells/mL) which could be explained by the higher inoculum concentration used (0.70×10^6 cells/mL) compared to this study (0.05×10^6 cells/mL).

The reduction in cell growth rates could arise from reduced nutrient supply in high cell yield cultures. Nahapetian et al. (1986) and Werrlein and Glinos (1974) showed medium nutrient and oxygen supply limitations in Vero and murine L-WRL10A cultures with cell multi-layers. The culture dissolved oxygen tension (DOT) declines rapidly in spinner flasks at high cell concentrations and could not be controlled. Nahapetian et al. (1986) attempted to partially control the DOT by gassing the headspaces when the DOT was <10%. Their results indicate the strategy was unsuccessful, as the DOT fluctuated with a range of 0-45% DOT. However, the point the DOT first fell to 0% coincided with the beginning of their low growth rate phase which could explain the low growth rate phase in this study. This would agree with inhibition of human fibroblast cell growth at <2% DOT (Graff and McCarty, 1957), as discussed previously (5.3.6).

Free Cells. Vero cells were strongly attached/entrapped in Cultispher-G beads and there were few free cells at any time in culture. However, cells grew as multi-layers in Cytodex-1 cultures and filled the void spaces in bead aggregates. This caused detachment at cell yields of $4.00\text{--}4.50 \times 10^6$ cells/mL which could be related to stirrer speed shear effects or to nutrient diffusion limitations resulting in adhesion problems in lower cell layers, as discussed previously (6.3.3-4)

(Werrlein and Glinos, 1974, Kleinman et al., 1981, Grinnell, 1982 and Nahapetian et al., 1986).

No one has previously reported this effect in refed microcarrier cultures.

10.3.2 Concentration of Glutamine in the Refeeding Medium

Introduction. The optimal glutamine concentration for batch Vero cell growth was 4.00 mM in Cytodex-1 and 6.00 mM in Cultispher-G cultures. The study above (10.3.1) showed serine and pyruvate enriched DMEM with 4.00 mM glutamine reached significantly higher (93%, $P < 0.01$) cell yields compared to original DMEM in cultures of either bead. However, the glutamine concentration was not optimal in Cultispher-G cultures which could have reduced the maximum cell yields. No previous reports in the literature have discussed the effects of altered nutrient concentrations on refed cell growth. Therefore, this study determined the effects of the glutamine concentration in the refeeding medium on Vero cell growth in microcarrier cultures.

Experimental. Preadapted stock Vero cells inoculated in enriched DMEM (Medium II from 2.1) + 5.0% v/v SCS enriched with either 4.00 or 6.00 mM glutamine. The cultures were grown in daily refed spinner flasks, as described above.

Results. Fig. 10.3 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G in refeeding medium containing 4.00 or 6.00 mM glutamine. Table 10.4 shows lag phases and cell growth rates in these cultures. Table 10.5 shows the nutrient analyses results for these cultures. (1) Maximum Vero cell yields in daily refed cultures of either bead were not affected by the glutamine concentration in the medium. (2) There were significantly higher (45%, $P < 0.01$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1. (3) There was no significant difference in the biphasic cell growth rates (Table 10.4) in all cultures. (4) There was a significantly higher (44%, $P < 0.01$) consumed concentration of glutamine in cultures of either bead from an initial 6.00 mM glutamine compared to 4.00 mM. However, there was no

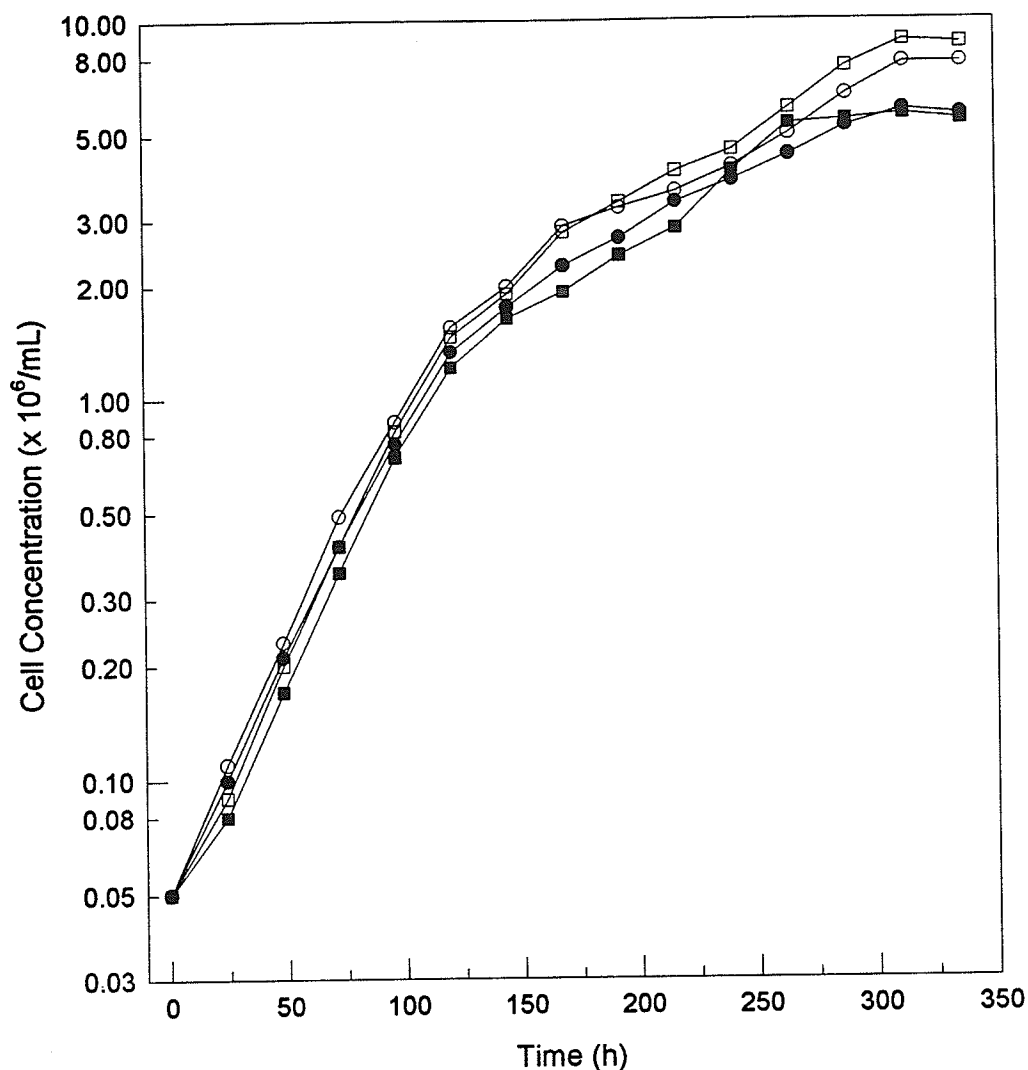


Fig. 10.3 - The effect of the daily refeeding DMEM glutamine concentration on the growth of preadapted Vero cells in 100 mL spinner flasks on 1.00 g/L Cytodex-1 (Closed symbols) or 1.70 g/L Cultispher-G (Open symbols). The cells were inoculated at 0.05×10^6 cells/mL in Medium II + 5.0% v/v sCS enriched with either ● 4.00 mM or ■ 6.00 mM glutamine. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and ramped to 60 rpm after 72 h in culture. After 72 h in culture the flasks were refed daily with one 50% v/v medium exchange and the pH was readjusted daily to pH 7.1 with 1.00 M HCl or 2.00 M NaOH. The points are means of $n=2$.

Table 10.4 - The effect of the daily refeeding DMEM glutamine concentration on **growth rates** and **lag phases** of Vero cells in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in either Medium II + 5.0% v/v sCS enriched with either 4.00 or 6.00 mM glutamine. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and ramped to 60 rpm after 72 h in culture. The flasks were refed one daily 50% v/v medium exchange and readjusted to pH 7.1 with 1.00 M HCl or 2.00 mM NaOH after 72 h in culture. The values are means of $n=2$.

Medium Glutamine Conc.	Primary and Secondary Stage Specific Cell Growth Rates and Lag Phases in the Medium Formulation Used				
	Cytodex-1			Cultispher-G	
		Specific Cell Growth Rates (h^{-1})	Lag Phase (h)	Specific Cell Growth Rates (h^{-1})	Lag Phase (h)
4.00 mM	High	0.035 ± 0.003	3.00 ± 0.50	0.037 ± 0.003	3.00 ± 0.50
	Low	0.011 ± 0.002		0.012 ± 0.002	
6.00 mM	High	0.035 ± 0.003	3.00 ± 0.50	0.037 ± 0.003	3.00 ± 0.50
	Low	0.010 ± 0.002		0.013 ± 0.002	

Table 10.5 - The effect of the daily refeeding DMEM glutamine concentration on **lactate to glucose**, **ammonia to glutamine**, **glutamate to glutamine**, **alanine to glutamine** and **glycine to serine** production ratios at maximum Vero cell yield in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The values were determined in cultures grown in Medium II + 5.0% v/v sCS containing 4.00 or 6.00 mM glutamine. The flasks were refed daily with one 50% v/v medium exchange after 72 h in culture. The values are means of $n=2$.

Nutrient Effect	Residual Concentration or Production Ratio in the Medium at Maximum Cell Yield			
	4.00 mM Glutamine		6.00 mM Glutamine	
	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G
Glucose Concentration	7.75 ± 0.80	2.15 ± 0.20	7.50 ± 0.75	2.05 ± 0.20
Lactate Concentration	19.40 ± 2.00	31.50 ± 0.20	19.70 ± 2.00	31.15 ± 0.20
Lactate to Glucose Ratio	1.20	1.45	1.20	1.43
Glutamine Concentration	0.25 ± 0.05	0.25 ± 0.05	0.35 ± 0.05	0.35 ± 0.05
Ammonia Concentration	3.10 ± 0.30	3.15 ± 0.30	4.70 ± 0.50	4.80 ± 0.50
Ammonia to Glutamine Ratio	0.84	0.85	0.84	0.85
Glutamate Concentration	1.05 ± 0.10	1.10 ± 0.10	1.60 ± 0.15	1.60 ± 0.15
Glutamate to Glutamine Ratio	0.28	0.30	0.29	0.28
Alanine Concentration	0.50 ± 0.05	0.50 ± 0.05	0.84 ± 0.10	0.85 ± 0.05
Alanine to Glutamine Ratio	0.14	0.14	0.15	0.15
Serine Concentration	1.05 ± 0.10	1.05 ± 0.10	1.05 ± 0.10	1.05 ± 0.10
Glycine Concentration	0.260 ± 0.05	0.255 ± 0.05	0.260 ± 0.05	0.260 ± 0.05
Glycine to Serine Ratio	0.27	0.27	0.27	0.27

significant difference in the residual glutamine concentration (0.30-0.40 mM) at maximum cell yield in all cultures. (5) There was a significantly higher (52%, $P < 0.01$) ammonia concentration at maximum cell yield in cultures of either bead with an initial 6.00 mM glutamine compared to 4.00 mM. However, there was no significant difference in the ammonia/glutamine ratios in cultures of either bead. (6) There were significantly higher concentrations of glutamate (49%, $P < 0.01$) and alanine (69%, $P < 0.01$) produced in cultures of either bead at 6.00 compared to 4.00 mM glutamine. (7) There was no significant difference in the concentration of any other consumed or produced components in cultures of either bead with respect to the glutamine concentrations used.

The cells detached in Cytodex-1 cultures at a cell yield of 4.00×10^6 cells/mL and formed aggregates.

Discussion. Maximum cell yields in cultures of either bead were not affected by the glutamine concentration in the refeeding DMEM medium. No one has previously shown whether cell yields could be improved using altered nutrient concentrations in the medium. Although cell yields were not affected there were significant differences in the glutamine concentrations consumed and the ammonia, alanine and glutamate concentrations produced at maximum cell yields. The cells consumed all of the glutamine from either initial concentration used and generated constant ratios of ammonia, alanine or glutamate/glutamine in cultures of either bead. This could be expected as glutamine is one of the major growth nutrients for Vero cells (6.3.3 and 10.3.1). Results from inhibitor studies (8.3.1) could explain this effect, as the ammonia concentration produced (4.75 mM) from 6.00 mM glutamine could be causing growth inhibition.

10.3.3 Time of Initiating Daily Refeeding

Introduction. Refed Vero cultures of either bead have significantly reduced concentrations of the major growth nutrients, glutamine and serine (10.3.1). Daily refeeding increases maximum

cell yields probably by maintaining more favourable nutrient concentrations during the growth phase or reduces growth inhibitor concentrations. The day of refeed initiation is important as medium nutrients could be wasted if refeeding is initiated too early or conversely, nutrient limitations could be introduced prior to refeeding if it is started too late. No one has previously determined whether the day of refeed initiation affects cell growth. Therefore, this study determined the latest culture time to initiate refeeding without affecting maximum Vero cell yields or the time taken to reach it in microcarrier cultures.

Experimental. The Vero cultures were grown in daily refed spinner flasks in Medium II (10.3.1). Refeeding was by one daily 50% v/v medium exchange initiated after various culture times (between 48-120 h).

Results. Fig. 10.4 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G with refeeding initiated after various times in culture. Table 10.6 shows the cell growth rates and lag phases in these cultures. Table 10.7 shows nutrient concentrations at the point of refeed initiation and Table 10.8 shows nutrient concentrations at maximum cell yield for these cultures.

(1) Maximum Vero cell yields in cultures of either bead were not affected by the day of initiating the refeeding. (2) Maximum cell yields were significantly higher (37%, $P < 0.01$) in Cultispher-G cultures compared to Cytodex-1 with respect to all refeed initiation times. (3) There were significantly longer ($P < 0.05$) times to maximum cell yield with refeed initiation times of 96 or 120 h (336 h) compared to 48 or 72 h (288 h) in cultures of either bead. (4) There were no significant differences in lag phases (Table 10.6) in cultures of either bead following inoculation. There were significantly longer ($P < 0.01$) lag phases immediately after refeed initiation in all cultures with refeed initiation after 96 and 120 h compared to 48 and 72 h. (5) There was no significant difference in the biphasic cell growth rates (Table 10.6) in all cultures.

Nutrient Analysis. Nutrient analyses (Table 10.7) could explain the lag phases in the cultures

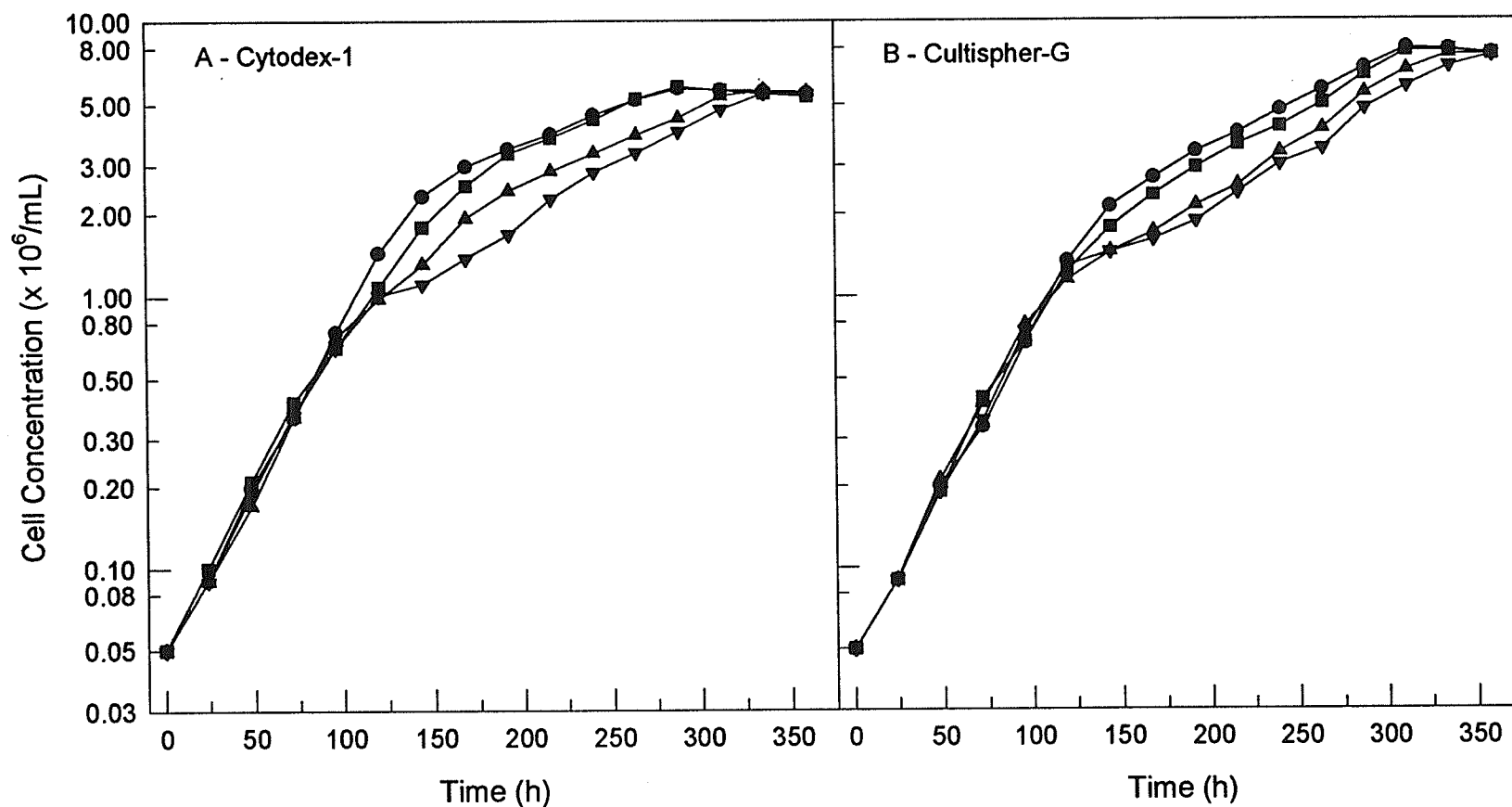


Fig. 10.4 - The effect of the daily refeed initiation time on Vero cell growth in 100 mL spinner flask cultures on 1.00 g/L Cytodex-1 (A) and 1.70 g/L Cultispher-G (B). The cells were inoculated at 0.05×10^6 cells/mL in Medium II + 5.0% v/v SCS. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and were ramped to 60 rpm after 72 h. Refeeding at one daily 50% v/v medium exchange was initiated after either ● 48, ■ 72, ▲ 96 or ▼ 120 h in culture and the pH was readjusted daily to pH 7.1 after 72 h in culture with 1.00 M HCl or 2.00 M NaOH. The points are means of $n=2$.

Table 10.6 - The effect of the **daily refeed initiation time** on **growth rates** and **lag phases** of Vero cells in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in Medium II + 5.0% v/v SCS. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and ramped to 60 rpm after 72 h in culture. Refeeding at one daily 50% v/v medium exchange was initiated at various times between 48-120 h in culture. Flasks were readjusted to pH 7.1 with 1.00 M HCl or 2.00 mM NaOH after 72 h in culture. The values are means of n=2.

Refeed Initiation Time (h)	Primary and Secondary Stage Specific Cell Growth Rates and Lag Phases				
	Cytodex-1			Cultispher-G	
		Specific Cell Growth Rates (h^{-1})	Lag Phase (h)	Specific Cell Growth Rates (h^{-1})	Lag Phase (h)
48	High	0.037 ± 0.003	3.00 ± 0.50	0.038 ± 0.003	0.00
	Low	0.009 ± 0.002		0.011 ± 0.002	
72	High	0.036 ± 0.003	3.00 ± 0.50	0.036 ± 0.003	4.50 ± 0.50
	Low	0.012 ± 0.002		0.013 ± 0.002	
96	High	0.036 ± 0.003	4.50 ± 0.50	0.038 ± 0.003	4.50 ± 0.50
	Low	0.012 ± 0.002		0.013 ± 0.002	
120	High	0.036 ± 0.003	4.50 ± 0.50	0.038 ± 0.003	4.50 ± 0.50
	Low	0.011 ± 0.002		0.011 ± 0.002	

Table 10.7 - The effect of the **daily refeed initiation time** on the major **consumed and produced component concentrations** at the **point of refeed initiation** from Vero cell growth in 100 mL spinner flask cultures on **1.00 g/L Cytodex-1 (A)** and **1.70 g/L Cultispher-G (B)**. The cells were inoculated at 0.05×10^6 cells/mL in Medium II + 5.0% v/v sCS. Refeeding at one daily 50% v/v medium exchange was initiated at various times between 48-120 h in culture. Culture supernatants were analysed for glucose, lactate, glutamine, ammonia and serine prior to the refeed time. The values are means of n=2.

Table 10.7A) - Cytodex-1.

Refeed Initiation Time (h)	Concentration of Major Component Prior to Refeeding				
	(mM)				
	Glucose	Lactate	Glutamine	Ammonia	Serine
48	18.50 ± 1.90	4.90 ± 0.50	2.70 ± 0.20	1.30 ± 0.15	1.88 ± 0.20
72	16.20 ± 1.60	11.00 ± 1.10	1.50 ± 0.15	2.30 ± 0.30	1.85 ± 0.20
96	9.80 ± 1.00	18.10 ± 1.80	0.70 ± 0.10	2.70 ± 0.30	1.78 ± 0.20
120	7.20 ± 0.70	21.55 ± 2.20	0.25 ± 0.05	2.95 ± 0.30	1.75 ± 0.20

Table 10.7B) - Cultispher-G.

Refeed Initiation Time (h)	Concentration of Major Component Prior to Refeeding				
	(mM)				
	Glucose	Lactate	Glutamine	Ammonia	Serine
48	17.85 ± 1.80	5.60 ± 0.60	2.60 ± 0.20	1.30 ± 0.15	1.86 ± 0.20
72	15.20 ± 1.50	14.00 ± 1.40	1.60 ± 0.15	2.20 ± 0.30	1.81 ± 0.20
96	8.60 ± 0.90	22.80 ± 2.30	0.65 ± 0.10	2.60 ± 0.30	1.76 ± 0.20
120	5.70 ± 0.60	26.60 ± 2.70	0.20 ± 0.05	3.05 ± 0.30	1.70 ± 0.20

Table 10.8 - The effect of the **daily refeed initiation time** on the major **consumed** and **produced component concentrations** at **maximum Vero cell yield** in 100 mL spinner flask cultures on **1.00 g/L Cytodex-1 (A)** and **1.70 g/L Cultispher-G (B)**. The cells were inoculated at 0.05×10^6 cells/mL in Medium II + 5.0% v/v sCS. Refeeding at one daily 50% v/v medium exchange was initiated at various times between 48-120 h in culture. Culture supernatants were analysed for glucose, lactate, glutamine, ammonia and serine prior to the refeed. The values are means of n=2.

Table 10.8A) - Cytodex-1.

Refeed Initiation Time (h)	Concentration of Major Component Prior to Refeeding				
	(mM)				
	Glucose	Lactate	Glutamine	Ammonia	Serine
48	7.65 ± 0.80	19.40 ± 2.00	0.40 ± 0.10	3.20 ± 0.30	1.15 ± 0.10
72	7.30 ± 0.70	19.25 ± 2.00	0.30 ± 0.10	3.30 ± 0.30	1.15 ± 0.10
96	7.40 ± 0.70	19.50 ± 2.00	0.35 ± 0.10	3.15 ± 0.30	1.10 ± 0.10
120	7.70 ± 0.80	19.35 ± 2.00	0.35 ± 0.10	3.25 ± 0.30	1.15 ± 0.10

Table 10.8B) - Cultispher-G.

Refeed Initiation Time (h)	Concentration of Major Component Prior to Refeeding				
	(mM)				
	Glucose	Lactate	Glutamine	Ammonia	Serine
48	3.65 ± 0.50	31.40 ± 3.10	0.30 ± 0.10	3.15 ± 0.30	1.15 ± 0.10
72	3.30 ± 0.50	31.25 ± 3.10	0.35 ± 0.10	3.20 ± 0.30	1.15 ± 0.10
96	3.40 ± 0.50	31.50 ± 3.10	0.50 ± 0.10	3.15 ± 0.30	1.10 ± 0.10
120	3.50 ± 0.50	31.35 ± 3.10	0.30 ± 0.10	3.30 ± 0.30	1.15 ± 0.10

with refeed initiation after 96 or 120 h, as the glucose and glutamine concentrations were proportionally reduced with the increasing refeed initiation time. These components were at low concentrations in cultures of either bead type with refeed initiation times of 120 h. Lactate and ammonia had also accumulated to high concentrations in these cultures at this time. There was an excess of serine in all the cultures at all refeed initiation times used. No other analysed nutrients were at limiting concentrations at the refeed initiation times used.

There were few differences (Table 10.8) between the concentrations of the components at maximum cell yield with respect to any refeed initiation time used. There was a significantly lower glucose concentration (46%, $P < 0.01$) and higher lactate concentration (62%, $P < 0.01$) in Cultispher-G cultures compared to Cytodex-1. However, there were no significant differences between the concentrations of any other nutrients.

Bead and Cell Aggregates. The beads became aggregated in all cultures and cells detached from Cytodex-1. The aggregate sizes and onset of cell detachment occurred after similar culture times and to similar levels, as were described above (10.3.1).

Discussion. The day of refeed initiation affected Vero cell growth in cultures of either bead but did not affect maximum cell yields. No one has previously discussed whether the day of refeed initiation affected cell growth. The day of refeed initiation proportionally increased the time taken to reach the maximum cell yield. The lag phases immediately after initiating refeeding after 96 and 120 h could explain the longer time to maximum cell yield.

Nutrient analyses explained the lag phases in the 96 and 120 h cultures. Cultures refed from 96 and 120 h had low glucose and glutamine concentrations at the point of refeeding initiation. Ammonia and lactate were at high concentrations at this time. These, either alone or in synergy, could cause the lag phases, although, the low glutamine and glucose are the most likely. No other detectable nutrients were limiting at the refeed initiation times in any cultures.

At maximum cell yield both glutamine and glucose were at low concentrations in all cultures.

This suggests that the refeed rate used (one daily 50% v/v medium exchange) was sub-optimal and was not supplying sufficient nutrients for cell growth at this time. However, as serine was in excess in all cultures this could not be responsible for the cessation of growth.

10.3.4 Volume of Refeed Medium

Introduction. To generate the highest Vero cell yields the glucose, glutamine and serine concentrations must be maintained as high as possible. These concentrations could be affected by the refeed rates and strategy used. Therefore, this study determined the effects of various refeed rates on Vero cell yields and cell/bead loadings in microcarrier cultures.

Experimental. The Vero cultures were grown in Medium II + 5.0% v/v sCS in refed spinner flasks, as described above (10.3.1). Culture refeeding was initiated after 72 h with one daily medium exchange of various volumes (between 13-90% v/v).

Results. Table 10.9 shows maximum Vero cell yields and cell/bead loadings in cultures of Cytodex-1 and Cultispher-G using the various refeeding rates. Table 10.10 shows the nutrient analyses for the major consumed and produced components in these cultures at maximum cell yield. (1) Maximum Vero cell yields and cell/bead loadings in cultures of either bead were affected by the medium exchange rate used. (2) There were significantly higher (44%, $P < 0.01$) maximum cell yields (Table 10.9) in Cultispher-G cultures compared to Cytodex-1 with respect to all medium exchange rates used. (3) Maximum cell yields and cell/bead loadings in Cytodex-1 cultures were significantly higher ($P < 0.01$) at medium exchange rates of $\geq 50\%$ v/v compared to all other rates used. Maximum yields and loadings in Cultispher-G cultures were significantly higher ($P < 0.01$) at $\geq 75\%$ v/v compared to all other rates used. (4) Maximum cell/bead loadings were significantly higher (322%, $P < 0.01$) in Cultispher-G cultures compared to Cytodex-1 at all the medium exchange rates used.

Table 10.9 - The effect of the **daily refeeding medium volume** on maximum Vero cell yields in 100 mL spinner flask cultures of **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in Medium II + 5.0% v/v sCS. Refeeding was initiated after 72 h in culture at various daily medium exchange rates (between 13-90% v/v). Flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and were ramped to 60 rpm after 72 h. The flasks were readjusted daily to pH 7.1 after 72 h in culture with 1.00 M HCl or 2.00 M NaOH. The values are means of $n=2$.

Refeed Medium Exchange Rate (% v/v)	Maximum Cell Concentration		Maximum Cell/Bead Loading	
	(x 10^6 /mL)		(Cells/Bead)	
	Cytodex-1	Cultispher-G	Cytodex-1	Cultispher-G
13	2.21 ± 0.15	2.67 ± 0.17	491	1,745
25	2.98 ± 0.25	3.60 ± 0.30	662	2,353
38	4.01 ± 0.35	5.35 ± 0.40	891	3,497
50	5.58 ± 0.45	7.57 ± 0.65	1,240	4,948
75	5.51 ± 0.45	8.72 ± 0.75	1,224	5,699
90	5.67 ± 0.45	9.35 ± 0.85	1,260	6,111

Table 10.10 - The effect of the **medium exchange volume** on the major **consumed** and **produced component concentrations** at maximum Vero cell yield in 100 mL spinner flask cultures on **1.00 g/L Cytodex-1 (A)** and **1.70 g/L Cultispher-G (B)**. The cells were inoculated at 0.05×10^6 cells/mL in Medium II + 5.0% v/v sCS. Refeeding was initiated after 72 h in culture at one daily medium exchange of between 13-90% v/v. Culture supernatants were analysed for glucose, lactate, glutamine, ammonia and serine. The values are means of n=2.

Table 10.10A) - Cytodex-1.

Medium Exchange Rate (% v/v)	Concentration of Major Component at Maximum Cell Yield				
	(mM)				
	Glucose	Lactate	Glutamine	Ammonia	Serine
13	3.70 ± 0.40	21.40 ± 2.40	0.00 ± 0.01	3.20 ± 0.35	1.10 ± 0.10
25	4.10 ± 0.40	21.25 ± 2.30	0.00 ± 0.01	3.20 ± 0.30	1.10 ± 0.10
38	5.30 ± 0.60	20.10 ± 2.00	0.15 ± 0.02	3.20 ± 0.30	1.15 ± 0.10
50	7.75 ± 0.80	19.25 ± 2.00	0.30 ± 0.10	3.10 ± 0.30	1.10 ± 0.10
75	11.85 ± 1.20	15.90 ± 1.60	0.72 ± 0.10	2.90 ± 0.30	1.25 ± 0.10
90	14.30 ± 1.40	11.35 ± 1.20	1.25 ± 0.15	2.60 ± 0.25	1.35 ± 0.10

Table 10.10B) - Cultispher-G.

Medium Exchange Rate (% v/v)	Concentration of Major Component at Maximum Cell Yield				
	(mM)				
	Glucose	Lactate	Glutamine	Ammonia	Serine
13	2.15 ± 0.20	31.40 ± 3.10	0.30 ± 0.10	3.20 ± 0.30	1.10 ± 0.10
25	2.30 ± 0.20	31.25 ± 3.10	0.30 ± 0.10	3.15 ± 0.30	1.10 ± 0.10
38	3.65 ± 0.40	31.40 ± 3.10	0.35 ± 0.10	3.15 ± 0.30	1.15 ± 0.10
50	4.30 ± 0.50	31.25 ± 3.10	0.30 ± 0.10	3.25 ± 0.30	1.10 ± 0.10
75	4.40 ± 0.50	30.50 ± 3.10	0.40 ± 0.10	3.10 ± 0.30	1.15 ± 0.10
90	4.50 ± 0.50	29.40 ± 3.00	0.35 ± 0.10	3.20 ± 0.30	1.15 ± 0.10

Nutrient Analysis. Nutrient analyses (Table 10.10A)) showed there was an excess of glutamine and glucose at maximum cell yield in Cytodex-1 cultures with a medium exchange of 90% v/v. However, the residual concentrations of glucose and glutamine at maximum cell yield were proportionally lower with refeed rates at 75-25% v/v. There was no residual glutamine at medium exchange rates of 13-25% v/v. The ammonia and lactate concentrations were not significantly different at 13-50% v/v but were significantly lower at 90 (ammonia) and 75-90 (lactate) % v/v medium exchange rates. There was no significant difference in the residual serine concentration with respect to any medium exchange rate used.

Nutrient analysis in Cultispher-G cultures (Table 10.10B)) showed no significant differences in the residual concentrations of any component with respect to the medium exchange rates used.

Bead Aggregates. Bead aggregates were present in Cytodex-1 cultures at all medium exchange rates, as described above (10.3.1). The cells began to detach from Cytodex-1 beads at times which were proportionally longer with the increasing medium exchange rate used. Significant proportions (16-28%, $P < 0.05$) of free cells were found at maximum cell yield with medium exchange rates of 13-25% v/v. There were few free cells at 50-90% v/v medium exchange rates at any time up to maximum cell yield in Cultispher-G cultures. However, significant numbers of cells ($>15\%$, $P = 0.05$) were detached within 48 h of reaching the maximum cell yield at 13-50% v/v medium exchange rates.

Discussion. The refeed rates in the previous literature have varied even with the same cell line. For example Forestall et al. (1992) grew Vero cells in DMEM/F12 + 10.0% v/v FBS in cultures of 5.00 g/L Cytodex-1 and refed with a single 50% v/v medium exchange when the glucose concentration reached 11.11 mM. Glutamine was shown to be at 0.90-2.00 mM throughout their cultures. The cells grew from 0.20 up to 4.00×10^6 cells/mL in approximately 10 d. However, Lee et al. (1992) grew Vero cells on 0.50 g/L porous polystyrene beads and

refed the cells with a 100% v/v medium exchange when the glucose was at 5.55 mM. These authors estimated their cell count from the glucose consumption as the cells could not be easily recovered from the beads and their cell counts are highly speculative. Nahapetian et al. (1986) grew Vero cells with L-15 + 5.0% v/v FBS, diluted (1:1) with phosphate buffered saline (PBS), in cultures of 20.00 g/L Superbeads and refed at 8.00 v/v/d with no rationale. The cells grew to 30.00×10^6 cells/mL. None of these authors showed any optimisation of these conditions.

The results in this study show maximum Vero cell yields in cultures of either bead were proportional to the medium exchange rate used. Maximum cell yields were not increased in Cytodex-1 cultures at medium exchange rates above 50% v/v whereas maximum yields were increased at all medium exchange rates in Cultispher-G cultures.

The Cytodex-1 results could be explained by two effects:- (1) Limiting bead surface area for cell growth at 1.00 g/L Cytodex-1 with one >50% v/v medium exchange. This effect agrees with the refed culture results of Clark and Hirtenstein (1981) who suggested 1.00 g/L Cytodex-1 was limiting to growth and therefore, they used 3.00-5.00 g/L Cytodex-1. (2) There was nutrient limitation in Cytodex-1 cultures at the medium exchange rates used. No nutrients which could be detected were found to be at limiting concentrations with a 90% v/v medium exchange rate. However, oxygen (which was not monitored) could explain this effect, as described above (10.3.1). The bead concentration appears to be responsible, as cells were detaching prior to maximum cell yield. Cell multi-layering could increase the nutrient limitation effect by preventing diffusion to lower cell layers (Nahapetian et al., 1986). A lack of glucose, as found at 13-25% v/v medium exchange could lead to reduced cell adhesion (Kleinman et al., 1981) or viability (Grinnell, 1982), as was discussed previously (6.3.3).

The Cultispher-G results support the limiting surface area argument, as maximum cell yields were significantly higher at all refeeding rates in these cultures. Macroporous Cultispher-G beads have highly increased surface areas for growth (Leighton et al., 1967) which could explain their higher yields. Hence, oxygen could not have been limiting to cultures of either bead stirred at 60 rpm. Oxygen limitation could have effects on maximum cell yields in Cultispher-G cultures

which could explain the non-linear increase in maximum cell yields at 50-90% v/v medium exchange rates. It is likely the DOT was low throughout most of these cultures, as shown by Nahapetian et al. (1986), except immediately after refeeding when the DOT could be $\geq 100\%$ due to temperature and supersaturation effects. Thus, it could be speculated that Vero cell yields $> 9.35 \times 10^6$ cells/mL could be achieved in Cultispher-G cultures with no nutrient limitations.

The results suggest that a ramped refeeding strategy could be a preferable method of growing Vero cells to high yields and will be discussed below (11.3.4)

Nutrient analyses confirm that refeeding strategies must be optimised to maintain non-limiting concentrations of essential nutrients. Glutamine was the most important nutrient to cell growth, as discussed previously (6.3.4). Glucose was also important in cultures of either bead. However, the results in this study contradict previous strategies of controlling culture refeeding rates based on glucose concentrations (Forestall et al., 1992 and Lee et al., 1992).

The cell/bead loadings show there were differences between the bead types. Maximum cell/bead loadings were significantly higher in Cultispher-G cultures compared to Cytodex-1. Others have shown that macroporous beads produce higher cell/bead loadings (Leighton et al., 1967 and Reiter et al., 1990). However, Reiter et al. (1990) claimed Cultispher-G produced an order of magnitude more cells compared to solid beads. The cell/bead results in this study were not an order of magnitude higher in Cultispher-G compared to Cytodex-1 cultures but this could be explained by the unoptimised conditions (i.e. spinner flasks) used.

10.3.5 Spinner Flask Volume

Introduction. Processes require to be scaled-up to generate more cells and products. However, the spinner flask volume affects maximum cell yields in cultures of either bead proportional to the stirrer speed used (5.3.13). Therefore, this study determined the effects of spinner flask volume on daily refeed growth of Vero cells in microcarrier cultures to define the optimal spinner flask volume for process development work.

Experimental. Preadapted stock Vero cells were inoculated in Medium II + 5.0% v/v sCS and grown in daily refed (one 50% v/v medium exchange from 72 h onwards) cultures in various spinner flask working volumes (100-500 mL working volume), as described above (10.3.1).

Results. Table 10.11 shows maximum Vero cell yields in refed cultures of Cytodex-1 and Cultispher-G in the various spinner flask working volumes used and stirring at different speeds. (1) Maximum cell yields in cultures of either bead were affected by the spinner flask working volumes and stirrer speeds used. (2) There were significantly higher (28%, $P < 0.01$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all spinner flask volumes and speeds used. (3) There were significantly higher ($P < 0.01$) maximum cell yields in cultures of either bead in 100 mL working volume flasks compared to the others used in this study with either stirring speed. (4) The maximum cell yields in cultures of either bead were significantly higher (18%, $P < 0.05$) with stirring at 40 rpm ramped to 60 rpm after 72 h compared to 40 rpm continuously.

Discussion. Most of the previously reported refeeding work has used 100 mL spinner flasks of various designs for Vero and CHO cells (Nahapetian et al., 1986 and Smiley et al., 1992) or bioreactors (Martin et al., 1987 and Reiter et al., 1990). Few reports have discussed whether differences in the engineering design of culture flasks affects cell yields. Clark and Hirtenstein (1981) discussed using 5,000 mL working volume flasks with Vero cells refeeding with one 50% v/v medium exchange after 3-4 d. The authors saw no differences in maximum cell yields with 3.00-5.00 g/L Cytodex-1 (5.00×10^6 cells/mL) in any flask volumes used.

The results in this study show that maximum Vero cell yields in cultures of either bead were significantly and proportionally lower as the spinner flask volume increased. Ramped stirrer speeds (40-60 rpm) significantly increased the maximum cell yields in the 250-500 mL flasks compared to continuous stirring at 40 rpm. These results reflect the differences in the spinner flask design ratios described previously (5.3.13). All of these differences could affect the oxygen

Table 10.11 - The effect of the **spinner flask working volume** on maximum Vero cell yields in daily refeed cultures of **1.00 g/L Cytodex-1** or **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL with enriched DMEM + 5.0% v/v sCS in 100, 250 and 500 mL spinner flasks. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) or were ramped to 60 rpm after 72 h in culture. Refeeding was initiated after 72 h in culture at one daily 50% v/v medium exchange. The pH was readjusted daily to pH 7.1 after 72 h in culture with 1.00 M HCl or 2.00 M NaOH. The values are means of $n=2$.

Spinner Flask Working Volume (mL)	Maximum Cell Concentration			
	(x 10^6 /mL)			
	Cytodex-1 (40 rpm)	Cytodex-1 (40-60 rpm)	Cultispher-G (40 rpm)	Cultispher-G (40-60 rpm)
100	5.65 ± 0.45	5.52 ± 0.40	7.66 ± 0.60	7.71 ± 0.55
250	3.75 ± 0.25	4.63 ± 0.35	4.37 ± 0.35	5.98 ± 0.45
500	2.65 ± 0.16	3.85 ± 0.30	3.22 ± 0.25	4.27 ± 0.30

entrainment into the system explaining the cell yields. Thus, the results in this study contradict those of Clark and Hirtenstein (1981). Several reasons could explain this:- (1) The previous authors used different spinner flasks to the ones in this study. (2) There were differences in the refeeding strategies and media used. Clark and Hirtenstein (1981) used original DMEM + 10.0% v/v FBS with one 50% v/v medium exchange every 3-4 d compared to the serine and pyruvate enriched DMEM + 5.0% v/v sCS in this study refed 50% v/v every day after 72 h in culture. (3) The previous authors used the crystal violet nuclei stain method of cell counting compared to the trypan blue method in this study which could have given similar cell counts ($2.50-3.00 \times 10^6$ cells/mL by the trypan blue method in 100-500 mL spinner flasks) if adjusted for count differences between the methods (refer to 4.3).

10.3.6 Serum Concentration of the Refeeding Medium

Introduction. Low serum concentrations (<5.0% v/v) reduced cell yields proportionally in cultures of Cytodex-1 (6.3.2) suggesting serum nutrients were important to cell growth in such cultures. The concentrations of these components could be depleted more quickly from medium supplemented at low (1.0% v/v) compared to high (>5.0% v/v) serum concentrations. However, daily refeeding with complete medium could prevent these limitations and allow lower serum concentrations to be used in the refeeding medium. No reports were found describing the optimal serum concentration for culture refeeding. Therefore, this study determined the optimal refeeding medium serum concentration for Vero cells in microcarrier cultures.

Experimental. Preadapted Vero cells were inoculated in Medium II supplemented with iron enriched, supplemented calf serum (sCS) at either (a) 1.0-5.0% v/v or (b) trace element "enriched" 1.0% v/v sCS (1.0 mg/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.0025 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). The cultures were grown in daily refed spinner flasks (one 50% v/v medium exchange from 72 h onwards), as described above (10.3.1).

Results. Table 10.12 shows maximum Vero cell yields in cultures of Cytodex-1 and Cultispher-G with the various serum concentrations in the medium. (1) Maximum Vero cell yields in cultures of either bead were affected by the serum concentration in the refeeding medium. (2) There were significantly higher (55%, $P<0.01$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all the serum concentrations used. (3) Maximum cell yields in cultures of either bead were significantly higher ($P<0.05$) at 5.0 % v/v serum compared to all other concentrations used. (4) There was no significant difference in the maximum cell yield in Cytodex-1 cultures refed with trace metal enriched 1.0% v/v sCS compared to 1.0% v/v sCS. However, there was a significantly higher (20%, $P<0.05$) maximum cell yield in Cultispher-G cultures refed with the enriched 1.0% v/v sCS.

A complete nutrient analysis was not performed on these cultures.

Free Cells. Cells were detached in Cytodex-1 cultures prior to maximum cell yield and proportional to the serum concentration used in the medium. Many cells (35%) were detached at 1.0% v/v sCS. The free cells aggregated in large clumps (50-100 cells). However, few free cells (<5% of the total cell count) were found in any of the Cultispher-G cultures.

Discussion. Previous research into high cell yield culture with CHO, Vero and monkey kidney (CV-1) cells has used FBS supplemented media (Nahapetian et al., 1986, Smiley et al., 1989, Reiter et al., 1990, Adema et al., 1990, Forestall et al., 1992 and Ohlson et al., 1994). However, the serum concentration in these reports varied between 2.0-10.0% v/v.

The results in this study show maximum Vero cell yields in refed Cytodex-1 cultures were proportional to the serum concentration at <5.0% v/v sCS. Thus, there was no difference in the effects of reduced serum concentrations in refed compared to batch cultures (6.3.2). However, Refed Cultispher-G cultures also reached proportionally reduced cell yields at <5.0% v/v sCS which differed from batch results (6.3.2) where the cell yield was only significantly reduced at 1.0% v/v sCS. Therefore, serum factors were important to cell yields in cultures of either bead.

Table 10.12 - The effect of the **serum concentration** and **trace metal enrichment** in the refeeding medium on maximum Vero cell yields in 100 mL spinner flasks on **1.00 g/L Cytodex-1** or **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in Medium II + 5.0% v/v sCS. Flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and were ramped to 60 rpm after 72 h. Refeeding was initiated after 72 h at one daily 50% v/v medium exchange. The pH was readjusted daily to pH 7.1 after 72 h in culture with 1.00 M HCl or 2.00 M NaOH. The values are means of n=2.

Refeeding Medium	Maximum Cell Concentration ($\times 10^6$ /mL)	
	Cytodex-1	Cultispher-G
DMEM + 5.0% v/v sCS	$5.63 \pm 0.40^*$	$7.71 \pm 0.55^*$
DMEM + 2.5% v/v sCS	4.73 ± 0.35	6.18 ± 0.50
DMEM + 1.0% v/v sCS	3.25 ± 0.25	5.72 ± 0.45
DMEM + 1.0% v/v sCS + Metals	3.47 ± 0.30	6.85 ± 0.50

* - Highest cell yield.

However, the cells were more affected by low serum concentrations in refed Cytodex-1 cultures.

Previously (6.3.2) it was speculated that metal ions could be responsible for the growth differences between the serum concentrations. However, there was no significant difference in the maximum cell yields in Cytodex-1 cultures refed with 1.0% v/v sCS with or without metal enrichment. The cells did grow to significantly higher maximum yields in Cultispher-G cultures with enriched 1.0% v/v sCS and reached a yield which was not significantly different from that at 5.0% v/v sCS.

Several reasons could explain this. Cells growing in Cultispher-G cultures could have a requirement for high concentrations of either iron, zinc or copper for a biosynthetic process for growth. Iron could be required for the cytochrome oxidative phosphorylation mechanism for energy generation (Voet and Voet, 1990) or for maintenance of cell adhesion (Grinnell, 1982), zinc could be required as an enzyme cofactor for DNA synthesis (Epstein, 1982) and copper as an enzyme cofactor (Prescott et al., 1990).

10.3.7 Perfusion Growth in the Celligen Bioreactor

Introduction. The optimal method of growing cells is in a fully controlled bioreactor which can be scaled-up linearly to large volumes (>1,000 L) for production purposes. This could give increased cell yields through improved physical environments. A major limitation to high cell yield spinner flask growth is that medium replacement once a day could cause cyclical changes in nutrient and inhibitor concentrations affecting cell growth. Perfusion fermentations prevent this effect. Therefore, this study determined the effects of transferring the daily refeeding methods, defined in spinner flasks (10.3.1-6), into the controlled Celligen bioreactor on Vero cell growth in microcarrier perfusion fermentations to define maximum cell/bead ratios.

Experimental. Preadapted Vero cells were inoculated into the 1,300 mL Celligen bioreactor in Medium II + 5.0% v/v sCS, as described above (10.3.1). After 72 h in culture perfusion was

initiated at various rates (0.50-1.50 v/v/d). Other physical parameters were controlled at set-points described previously (2.4.5). The pH was controlled by CO₂ sparging to lower and by pumping a solution of 1.00 M Na₂CO₃ to raise the pH and the DOT was controlled by sparging compressed air blended with pure oxygen to raise or sparging pure N₂ to lower the DOT.

Results. Fig. 10.5 shows Vero cell growth in Cytodex-1 and Cultispher-G cultures with various perfusion rates in the Celligen bioreactor. Table 10.13 shows maximum cell yields and cell/bead loadings in these cultures. Table 10.14 shows the cell growth rates and lag phases in these cultures. Table 10.15 shows the major nutrient concentrations at maximum cell yield in these cultures. (1) Maximum Vero cell yields were unaffected in Cytodex-1 cultures but were affected in Cultispher-G cultures by the perfusion rates used. (2) There were significantly higher (91%, $P<0.01$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all perfusion rates used. (3) There was no significant difference in the maximum cell yield in Cytodex-1 cultures at any perfusion rate used. (4) There were significantly higher ($P<0.01$) maximum cell yields in Cultispher-G cultures at ≥ 1.00 v/v/d compared to 0.50 v/v/d. (5) There was no significant difference (Table 10.13) in the maximum cell/bead loadings in Cytodex-1 cultures at all perfusion rates used. Maximum cell/bead loadings in Cultispher-G cultures were significantly higher (57%, $P<0.01$) at ≥ 1.00 v/v/d compared to 0.50 v/v/d. (6) Therefore, there were significantly higher (540%, $P<0.01$) maximum cell/bead loadings in cultures of Cultispher-G compared to Cytodex-1. (7) The maximum cell/bead loadings gave optimal perfusion rates of 0.50-0.60 v/v/d/g beads with respect to either bead type. (8) There was no significant difference (Table 10.14) in the biphasic cell growth rates in cultures of either bead with respect to any perfusion rate used. The cell growth rate was high and linear up to between 168 and 192 h in culture and was significantly higher (227%, $P<0.01$) compared to the low linear growth rate up to maximum cell yield. The onset of the lower growth rate phase occurred at a cell yield of $4.50\text{--}5.00 \times 10^6$ cells/mL in cultures of either bead. (9) The total culture time to reach maximum cell yields in cultures of either bead was significantly longer (19%, $P<0.05$) in 100 mL spinner flasks

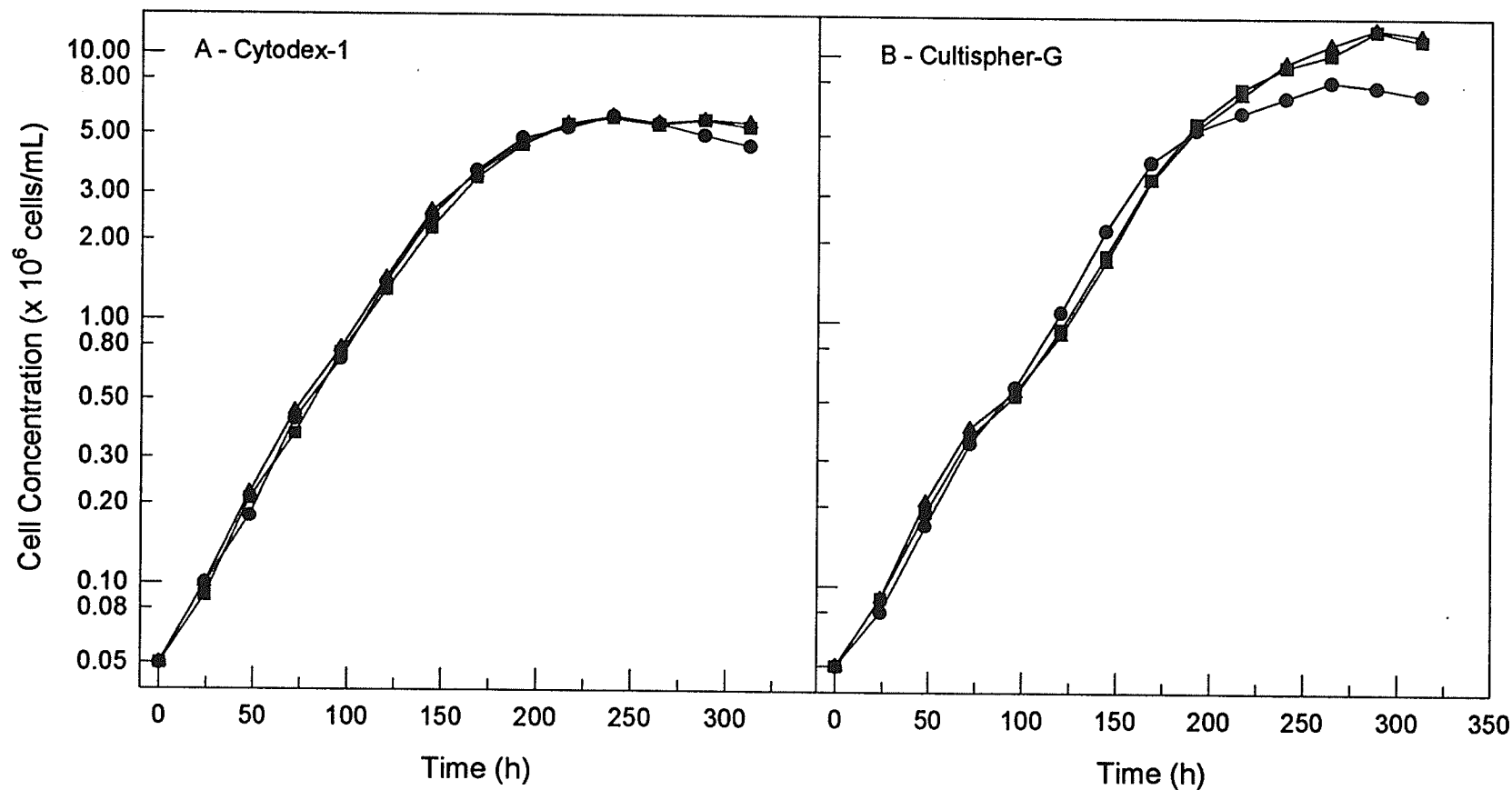


Fig. 10.5 - The effect of the **perfusion rate** on Vero cell growth in the Celligen bioreactor on **1.00 g/L Cytodex-1 (A)** and **1.70 g/L Cultispher-G (B)**. The cells were inoculated at 0.05×10^6 cells/mL in 1,300 mL of Medium II + 5.0% v/v sCS. Perfusion was initiated after 72 h in culture at either ● 0.50 v/v/d, ■ 1.00 v/v/d or ▲ 1.50 v/v/d. Physical parameters were controlled at pH 7.1 (Cytodex-1) and 6.8 (Cultispher-G), DOT at 50%, temperature at 37°C, stirring at 40 rpm continuously and ramped to 60 rpm after 72 h and 0.50 L/min gas flow rate. The points are means of n=2.

Table 10.13 - The effects of the **perfusion rate** on the **maximum Vero cell yields** and **cell/bead loadings** in 1,300 mL Celligen bioreactor on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in Medium II + 5.0% v/v sCS. The bioreactor was perfused from 72 h in culture at 0.50, 1.00 or 1.50 v/v/d. The values are means of n=2.

Perfusion Rate (v/v/d)	Maximum Cell Yields and Cell/Bead Ratios with the Perfusion Rate Used			
	Cytodex-1		Cultispher-G	
	Maximum Yield ($\times 10^6$ cells/mL)	Maximum Cell/Bead Ratio (Cells/Bead)	Maximum Yield ($\times 10^6$ cells/mL)	Maximum Cell/Bead Ratio (Cells/Bead)
0.50	$5.88 \pm 0.42^*$	1,307	8.11 ± 0.57	5,301
1.00	5.78 ± 0.41	1,284	12.60 ± 0.89	8,235
1.50	5.87 ± 0.42	1,304	$12.80 \pm 0.91^*$	8,366

* - Highest cell yield.

Table 10.14 - The effects of the **perfusion rate** on the Vero **cell growth rates** and **lag phases** in 1,300 mL Celligen bioreactor on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL in enriched DMEM + 5.0% v/v sCS. The bioreactor was perfused from 72 h in culture at 0.50, 1.00 or 1.50 v/v/d. The values are means of n=2.

Perfusion Rate (v/v/d)	Specific Cell Growth Rate and Lag phase with the Perfusion Rate Used				
		Cytodex-1		Cultispher-G	
		Specific Cell Growth Rates (h^{-1})	Lag Phase (h)	Specific Cell Growth Rates (h^{-1})	Lag Phase (h)
0.50	High	0.036 ± 0.003	3.00 ± 0.50	0.037 ± 0.003	6.00 ± 1.00
	Low	0.010 ± 0.001		0.009 ± 0.001	
1.00	High	0.035 ± 0.003	4.50 ± 0.50	0.036 ± 0.003	6.00 ± 1.00
	Low	0.010 ± 0.001		0.013 ± 0.001	
1.50	High	0.036 ± 0.003	4.50 ± 0.50	0.036 ± 0.003	6.00 ± 1.00
	Low	0.010 ± 0.001		0.013 ± 0.001	

Table 10.15 - The effects of the **perfusion rate** on the major **consumed** and **produced component concentrations** at maximum Vero cell yield in 1,300 mL Celligen bioreactor on **1.00 g/L Cytodex-1 (A)** and **1.70 g/L Cultispher-G (B)**. The cells were inoculated at 0.05×10^6 cells/mL in Medium II + 5.0% v/v sCS. The bioreactor was perfused from 72 h in culture at 0.50, 1.00 or 1.50 v/v/d. Culture supernatants were analysed for glucose, lactate, glutamine, ammonia and serine. The values are means of n=2.

Table 10.15A) - Cytodex-1.

Perfusion Rate (v/v/d)	Concentration of Major Component at Maximum Cell Yield				
	(mM)				
	Glucose	Lactate	Glutamine	Ammonia	Serine
0.50	7.25 ± 0.70	21.30 ± 2.30	0.25 ± 0.04	3.15 ± 0.30	1.15 ± 0.10
1.00	12.85 ± 1.30	13.20 ± 1.30	1.15 ± 0.15	2.45 ± 0.30	1.40 ± 0.15
1.50	16.60 ± 1.70	8.85 ± 0.90	1.45 ± 0.15	2.10 ± 0.20	1.65 ± 0.15

Table 10.15B) - Cultispher-G.

Perfusion Rate (v/v/d)	Concentration of Major Component at Maximum Cell Yield				
	(mM)				
	Glucose	Lactate	Glutamine	Ammonia	Serine
0.50	2.35 ± 0.25	35.30 ± 3.50	0.30 ± 0.10	3.20 ± 0.30	1.10 ± 0.10
1.00	8.85 ± 0.90	24.50 ± 2.50	0.75 ± 0.10	2.50 ± 0.25	1.30 ± 0.10
1.50	12.55 ± 1.30	18.50 ± 1.90	1.35 ± 0.15	1.30 ± 0.15	1.45 ± 0.10

at one daily 50% v/v medium exchange compared to the Celligen bioreactor at 0.50 v/v/d. However, there was no significant difference in the maximum cell yields produced in these cultures with respect to either bead type. There was a significantly higher maximum cell yield (36%, $P < 0.01$) in bioreactor cultures of Cultispher-G at 1.00 v/v/d compared to spinner flasks with one daily 90% v/v medium exchange.

Nutrient Analysis. Nutrient analysis results (Table 10.15) could explain the differences in maximum cell yields between the perfusion rates used. In cultures of either bead the residual concentrations at maximum cell yield of all the nutrients were proportionally increased with the perfusion rate used. However, residual concentrations of all the nutrients were significantly higher ($P < 0.01$) in Cytodex-1 cultures compared to Cultispher-G at all perfusion rates used. Glutamate (1.25 mM) and alanine (0.65 mM) were produced to high concentrations at maximum cell yield in cultures of either bead at 0.50 v/v/d perfusion rate. There were significantly lower ($P < 0.01$) concentrations of glutamate (0.70 and 0.45 mM) and alanine (0.45 and 0.25 mM) produced at maximum cell yield at perfusion rates of 1.00 and 1.50 v/v/d, respectively.

Cell Detachment. The cells began to detach from Cytodex-1 beads prior to reaching the maximum cell yield at all the perfusion rates used. The free cells detached from the beads after reaching a cell yield of $4.00\text{--}4.50 \times 10^6$ cells/mL. The cells formed cell aggregates of a similar size to the beads. Free cells were also found in Cultispher-G cultures 24 h after reaching the maximum cell yield at a perfusion rate of 0.50 v/v/d. However, there were few free cells in Cultispher-G cultures perfused at 1.00–1.50 v/v/d.

Discussion. Others have previously used perfusion bioreactor microcarrier cultures for CHO and Vero cell growth (Reiter et al., 1990, Avgerinos et al., 1990 and Ohlson et al., 1994). Reiter et al. (1990) and Ohlson et al. (1994) grew CHO cells with Cultispher-G. However, the perfusion rates were very different with the former authors ramping from 10.00–30.00 v/v/d and the latter

authors from 0.50-6.00 v/v/d. These perfusion rates appear high and would not be cost effective, unless the product had a high commercial value. The results suggest the media were not completely optimised and were limiting the supply of an important nutrient for high cell yields.

The results in this study show maximum Vero cell yields could not be increased in 1.00 g/L Cytodex-1 cultures with perfusion rates >0.50 v/v/d and in 1.70 g/L Cultispher-G cultures at >1.00 v/v/d. This agrees with the previous spinner flask results (10.3.1-6). Previously (10.3.4) it was speculated that Cultispher-G cultures could reach yields $>9.50 \times 10^6$ cells/mL. The bioreactor results confirm this showing that spinner flasks should only be used for preliminary refeeding studies to identify important nutrients and to suggest initial refeed strategies. Accurate assessments for system scale-up must be performed in controlled bioreactors.

The nutrient concentration results confirm these findings. The nutrients were in excess at perfusion rates > 0.50 v/v/d in 1.00 g/L Cytodex-1 cultures. However, in 1.70 g/L Cultispher-G cultures the nutrients were only in excess with 1.50 v/v/d.

The maximum cell/bead loadings were 6.44 times higher in Cultispher-G cultures compared to Cytodex-1. The maximum loadings were 1,300 cells/bead for Cytodex-1 and 8,300 cells/bead for Cultispher-G cultures. The cell/bead number of Cultispher-G compares favourably with previous values for Verax[®] collagen beads (8,000-12,000 cells/bead - Vournakis and Runstadler, 1989). These loadings reflect both the differences in the bead number/g dry weight and the surface area for growth. Cytodex-1 contain $4.3\text{-}4.5 \times 10^6$ beads/g and Cultispher-G contain $0.9\text{-}1.0 \times 10^6$ beads/g dry weight. The maximum cell/bead loading difference between the beads was not an order of magnitude but would agree with results showing macroporous beads produce higher cell/bead loadings (Leighton et al., 1967 and Reiter et al., 1990). However, if we compare the cell/bead loadings at the point where cell detachment began in Cytodex-1 cultures, then the difference would be >9.00 times.

The results can be used to identify the optimal perfusion rate/g beads used in the system. The optimal perfusion rate for maximum cell/bead loadings was at 0.50 v/v/d/g of either beads. Therefore, Vero cell growth would be maximal in cultures of either bead with the same perfusion

rate/g beads used. The maximum cell/bead loading defines the maximum number of cells grown on each bead before either nutrient diffusion limitations prevent further growth or the cells become sloughed off the beads. The value sets the target loading for scale-up cultures using higher microcarrier concentrations.

There were similar biphasic growth rates in cultures of either bead type in bioreactors and spinner flasks. However, the cells grew with the linear, high rate throughout most of the bioreactor cultures reaching significantly higher cell yields ($4.00\text{--}4.50 \times 10^6$ cells/mL) compared to spinner flasks ($<2.00 \times 10^6$ cells/mL) before reducing. This difference could be explained by oxygen limitations in spinner flasks, as discussed above (10.3.4) whereas, there were probably no oxygen limitations in bioreactor cultures. Glutamine could be responsible for the onset of the low growth rate phase in bioreactor cultures of either bead, as the glutamine concentration at each sampling time was <1.00 mM in cultures at 0.50-1.00 v/v/d. However, there were no significant differences in the maximum cell yields in cultures of either bead at 0.50 v/v/d in the bioreactor or with one daily 50% v/v medium exchange in spinner flasks. There were significantly higher maximum cell yields in Cultispher-G cultures in the bioreactor at 1.00 v/v/d compared to spinner flasks at one daily 90% v/v medium exchange. This would agree with the oxygen limitation described above.

10.4 Conclusions

The purpose of this study was to define the refeeding medium, refeeding rates and maximum cell/bead loadings for Vero cell cultures of Cytodex-1 and Cultispher-G beads. The results above show that:

- **maximum cell yields** were significantly higher (% , $P<0.01$) in Cultispher-G cultures compared to Cytodex-1 with respect to all the studies described above (10.3.1-7).
- **the serine concentration** governed the maximum Vero cell yields in refed or perfusion cultures. Hence, serine must be enriched to a total of 2.00 mM for refed cell growth.

- **the optimal glutamine concentration** in the refeeding medium was 4.00 mM for either bead.

- **the surface area for cell growth** was limiting to refed growth at 1.00 g/L Cytodex-1. Cell aggregates appeared in the medium after cell yields of approximately 4.00×10^6 cells/mL in spinner flasks and after 5.00×10^6 cells/mL in Celligen bioreactor. Nutrient limitations, particularly oxygen or serum nutrients, are suggested as the cause of this effect.

- **maximum cell/bead loadings** were significantly higher (540%, $P < 0.01$) in Cultispher-G beads compared to Cytodex-1 with respect to the perfusion rates used in this study. Cultures of Cultispher-G could grow 6.40-9.00 times the cell/bead loading of Cytodex-1.

- **the cell growth rates** in spinner flasks and Celligen bioreactor cultures of either bead were biphasic with an initial linear high growth rate phase which was significantly higher compared to the following low growth rate up to maximum cell yield. Spinner flask cultures grew at the high rate up to cell yields between 1.50 - 2.00×10^6 cells/mL. However, Celligen bioreactor cultures had the high growth rate up to a significantly higher cell yield before lowering ($>4.00 \times 10^6$ cells/mL). This could be caused by improved nutrient availability in the bioreactor. Oxygen diffusion is suggested to control the onset of the low growth rate phase in spinner flask cultures.

- **the 250 and 500 mL working volume Bellco spinner flasks** produced significantly lower maximum cell yields compared to 100 mL. Hence, process development of refed cultures was impossible with linear increases of process parameters. The non-linear flask scale-up was responsible for this.

- **the optimal refeeding or perfusion rates** were at 0.50-0.60 v/v/d (or one daily 50-60% v/v medium change) per g of either bead type.

Chapter 11. High Density Cultivation of Vero Cells in Perfusion Microcarrier Cultures

11. High Density Cultivation of Vero Cells in Perfusion Microcarrier Cultures

11.1 Introduction

Previous literature on culture refeeding to increase maximum cell yields has always involved high concentrations of microcarriers to provide sufficient surface area for growth. Cytodex-1 and Cultispher-G microcarrier concentrations of 3.00-5.00 g/L are most common with African green monkey kidney (Vero), Chinese hamster ovary (CHO), baby hamster kidney (BHK), Madin-Darby canine kidney (MDCK) and monkey kidney (CV-1) cells (Clark and Hirtenstein, 1981, Martin et al., 1987, Cherry and Papoutsakis, 1988, Smiley et al., 1989, Adema et al., 1990, Reiter et al., 1990, Forestall et al., 1992, Schmid et al., 1992 and Ohlson et al., 1994).

CHO and MDCK cells have been grown in Celligen bioreactors on either 6.00 g/L Cytodex-3 or 4.00 g/L Cultispher-S (Martin et al., 1987 and Ohlson et al., 1994) and CHO and Vero have been grown in a fluidised bed (Reiter et al., 1990). MDCK cells reached a high yield (23.00×10^6 cells/mL) at a perfusion rate of 1.00 v/v/d (Martin et al., 1987). The CHO cell yields varied between the reports. Reiter et al. (1990) used ramped perfusion rates from 10.00-30.00 v/v/d over a 30 d culture and obtained maximum CHO cell yields of 35.00×10^6 cells/mL of bed volume in a 1,200 mL fluidised bed with 10.00 g/L Cultispher-G beads. Ohlson et al. (1994) also used ramped perfusion rates of 0.50-6.00 v/v/d and achieved maximum CHO cell yields of 4.00×10^6 cells/mL with 4.00 g/L beads. Indeed, there are several reports with similar maximum CHO cell yields (3.00 - 4.00×10^6 cells/mL) in refed cultures in either DMEM/F12 or DMEM at 2.0-10.0% v/v foetal bovine serum (FBS) (Smiley et al., 1989, Forestall et al., 1992 and Ohlson et al., 1994). However, these authors used different beads (5.00 g/L Cytodex-1 or 4.00 g/L Cultispher-G) and refeed rates (0.50-6.00 v/v/d) which suggests the parameters were incompletely optimised in these studies. Vero cells have also been grown in a Celligen bioreactor at a perfusion rate of 0.50 v/v/d on 3.00 g/L Cytodex-3 (New Brunswick Scientific® (NBS) Report No.: CC114, 1991). The cells grew to a low maximum yield (2.00×10^6 cells/mL) after 6 d but could be maintained for a further 3 d at this value. Vero cells have grown to high

yields (30.00×10^6 cells/mL) in refed/perfusion systems in L-15 medium (4.00 mM glutamine) at 5.0% v/v FBS (Nahapetian et al., 1986). The authors used high bead concentrations (20.00 g/L Superbeads™) and refeed rates (8.00 v/v/d) without proving this requirement and they also found that diluting the medium with PBS (1:1) gave higher cell yields compared to undiluted basal medium. The differences in maximum cell yields could be explained by the systems, perfusion rates, bead types or media used in these studies.

Glutamine is the major growth nutrient for Ehrlich ascites, HeLa, human fibroblast and Vero cells (Rabinowitz et al., 1956, Griffiths, 1972, Reitzer et al., 1979 and 6.3.3). However, previous reports on refed cultures used DMEM (25.00 mM glucose and 4.00 mM glutamine), DMEM/F12 (17.50 mM glucose and 2.50 mM glutamine), L-15 (0 glucose, 10.00 mM fructose or galactose and 2.00 mM glutamine) and M199 (5.55 mM glucose and 0.70 mM glutamine) at different glucose and glutamine concentrations (Nahapetian et al., 1986, Smiley et al., 1989, Reiter et al., 1990, New Brunswick Scientific® Report No.: CC114, 1991 and Forestall et al., 1992). Hence, the glutamine concentrations could explain the differences in cell growth and refeed rates required.

Vero cells are used to commercially produce human and animal virus vaccines (Beale, 1992 and Mendonca et al., 1993) in bioreactors of 1,000 L using microcarrier technology (Montagnon et al., 1981). These cells have no commercial significance for producing secreted products (Thilly, 1989). However, increasing the cell yields through culture refeeding could lead to increased virus yields and also make the cells attractive for secreted protein production.

No one has previously determined the limiting culture factors or the maximum cell/bead loadings for any cells prior to using increased bead concentrations. Previous studies (10.3.7) in Celligen bioreactor cultures of 1.00 g/L solid Cytodex-1 and 1.70 g/L macroporous Cultispher-G produced finite maximum Vero cell/bead loadings. The Cultispher-G cultures produced 6.40-9.00 times more cells per bead. The study also showed these loadings were obtained with the same medium exchange/perfusion rate (0.50-0.60 v/v/d/g beads) in cultures of either bead. As the maximum cell/bead loadings have been identified the system can be scaled-up. This paper

attempts to confirm the optimal refeeding values by growing Celligen bioreactor cultures using increased microcarrier concentrations. The system limitations to further increasing cell yields are also defined.

11.2 Materials and Methods

The methods involved in culturing and counting the cells and analyses of nutrients have been described previously (2.1-2.5).

11.2.1 Stock Cell Maintenance

Stock Vero cells were grown in T150 culture flasks and adapted to DMEM enriched with serine and pyruvate (2.00 mM and 0.50 mM, respectively) + 5.0% v/v iron enriched, supplemented calf serum (sCS). The cells were detached and recovered by the standard trypsinisation protocol (2.2) and counted by the trypan blue method (2.5.1-2).

11.3 Results and Discussion

11.3.1 Microcarrier Concentration in Refed Spinner Flasks

Introduction. The microcarrier concentration used in culture refeeding has varied from 3.00-20.00 g/L with many different bead types and cell lines. However, no one has proved their microcarrier concentration as optimal for cell growth. Bead to bead collisions cause cell damage in Cytodex-3 cultures and increased microcarrier concentrations could result in significantly reduced cell yields due to collisions. Therefore, this study determined the effects of using various microcarrier concentrations and medium exchange rates on Vero cell growth in spinner flask microcarrier cultures.

Experimental. Preadapted stock Vero cells were inoculated in 100 mL spinner flasks at various concentrations of Cytodex-1 (1.00-5.00 g/L) and Cultispher-G (1.70-5.10 g/L) in enriched DMEM

+ 5.0% v/v iron enriched, supplemented calf serum (sCS), as described above (11.2.1). The cultures were refed daily from 48 h onwards at either (i) 1 x 50% v/v (ii) 2 x 50% v/v or (iii) 2 x 75% v/v medium exchanges.

Results. Table 11.1 shows maximum Vero cell yields in cultures with various concentrations of Cytodex-1 and Cultispher-G refed at various medium exchange rates. Table 11.2 shows cell growth rates and lag phases in these cultures. (1) Maximum Vero cell yields in cultures of Cytodex-1 were affected by both the microcarrier concentration and the medium exchange rates used. However, cultures of Cultispher-G were only affected by the medium exchange rates. (2) There were significantly higher (28%, $P < 0.01$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all microcarrier concentrations and medium exchange rates used. (3) There were significantly higher (29%, $P < 0.01$) maximum cell yields at ≥ 2.00 g/L Cytodex-1 compared to 1.00 g/L with respect to all medium exchange rates used. Maximum cell yields in Cultispher-G cultures were not significantly different at any microcarrier concentration used. (4) Maximum cell yields in cultures of either bead were significantly higher (39%, $P < 0.01$) at 2 x 75% and 2 x 50% v/v medium exchanges compared to 1 x 50% v/v with respect to all microcarrier concentrations, except 1.00 g/L Cytodex-1. (5) There were biphasic cell growth rates (Table 11.2) which were not significantly different in cultures of either bead. The high growth rate extended up to cell counts of 2.00×10^6 cells/mL and was significantly higher (27%, $P < 0.01$) compared to the low growth rate phase from this cell count up to maximum yield. (6) There was no significant difference in the lag phases (Table 11.2) in any cultures.

A complete nutrient analysis was not performed on these cultures.

Discussion. Maximum Vero cell yields in Cytodex-1 cultures were dependent on the bead concentration at high medium exchange rates. However, the increased bead concentrations did not give linear increases in maximum cell yields in spinner flask cultures. This suggests there was still a major nutrient limitation in these cultures. The maximum cell yields in Cultispher-G

Table 11.1 - The effect of **microcarrier concentration** and **medium exchange rate** on **maximum Vero cell yield** in 100 mL spinner flasks on **Cytodex-1** and **Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL on Cytodex-1 (1.00-5.00 g/L) and Cultispher-G (1.70-8.50 g/L) in enriched DMEM + 5.0% v/v sCS. Spinner flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and were ramped to 60 rpm after 72 h in culture. Refeeding with complete medium was initiated daily after 72 h in culture at either 1 x 50%, 2 x 50% or 2 x 75% v/v medium exchanges. The pH was readjusted daily to pH 7.1 after 72 h in culture with 1.00 M HCl or 2.00 M NaOH. The values are means of n=2.

Microcarrier Concentration (g/L)	Maximum Cell Yield at the Medium Exchange Rate Used		
	(x 10^6 /mL)		
	1 x 50% v/v	2 x 50% v/v	2 x 75% v/v
Cytodex-1			
1.00	5.75 $\pm$ 0.45*	5.58 $\pm$ 0.45	5.67 $\pm$ 0.45
2.00	5.62 $\pm$ 0.45	7.72 $\pm$ 0.50	7.64 $\pm$ 0.55
3.00	5.48 $\pm$ 0.45	7.86 $\pm$ 0.50*	8.58 $\pm$ 0.55
5.00	5.73 $\pm$ 0.45	7.73 $\pm$ 0.55	8.93 $\pm$ 0.60*
Cultispher-G			
1.70	7.65 $\pm$ 0.55*	9.55 $\pm$ 0.75*	9.45 $\pm$ 0.70
3.40	7.44 $\pm$ 0.55	9.54 $\pm$ 0.75	9.41 $\pm$ 0.65
5.10	7.50 $\pm$ 0.55	9.40 $\pm$ 0.70	9.35 $\pm$ 0.75
8.50	7.42 $\pm$ 0.50	9.42 $\pm$ 0.75	9.62 $\pm$ 0.70*

* Highest cell yield.

Table 11.2 - The effect of the microcarrier concentration and medium exchange rate on Vero cell growth rates and lag phases in 100 mL spinner flasks on **1.00 g/L Cytodex-1** and **1.70 g/L Cultispher-G**. The cells were inoculated at 0.05×10^6 cells/mL on Cytodex-1 (1.00-5.00 g/L) and Cultispher-G (1.70-8.50 g/L) in enriched DMEM + 5.0% v/v SCS. Spinner flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and were ramped to 60 rpm after 72 h in culture. Refeeding with complete medium was initiated daily after 72 h in culture at either 1 x 50%, 2 x 50% or 2 x 75% v/v medium exchanges and the pH was readjusted daily to pH 7.1 with 1.00 M HCl or 2.00 M NaOH. The values are means of $n=2$.

Microcarrier Concentration (g/L)	Specific Cell Growth Rate and Lag Phase at the Perfusion Rate Used						
		1 x 50% v/v		2 x 50% v/v		2 x 75% v/v	
Cytodex-1		Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)
1.00	High	0.035 ± 0.003	4.50 ± 1.00	0.036 ± 0.003	3.00 ± 0.50	0.036 ± 0.003	3.00 ± 0.50
	Low	0.011 ± 0.001		0.010 ± 0.001		0.012 ± 0.001	
2.00	High	0.035 ± 0.003	6.00 ± 1.00	0.036 ± 0.003	4.50 ± 1.00	0.037 ± 0.003	3.00 ± 0.50
	Low	0.011 ± 0.001		0.011 ± 0.001		0.012 ± 0.001	
3.00	High	0.035 ± 0.003	6.00 ± 1.00	0.036 ± 0.003	4.50 ± 1.00	0.036 ± 0.003	4.50 ± 1.00
	Low	0.011 ± 0.001		0.010 ± 0.001		0.010 ± 0.001	
5.00	High	0.035 ± 0.003	4.50 ± 1.00	0.036 ± 0.003	6.00 ± 1.00	0.036 ± 0.003	4.50 ± 1.00
	Low	0.011 ± 0.001		0.011 ± 0.001		0.011 ± 0.001	
Cultispher-G							
1.70	High	0.037 ± 0.003	3.00 ± 0.50	0.037 ± 0.003	4.50 ± 1.00	0.037 ± 0.003	4.50 ± 1.00
	Low	0.012 ± 0.001		0.013 ± 0.001		0.012 ± 0.001	
3.40	High	0.036 ± 0.003	4.50 ± 1.00	0.037 ± 0.003	4.50 ± 1.00	0.037 ± 0.003	3.00 ± 0.50
	Low	0.012 ± 0.001		0.012 ± 0.001		0.012 ± 0.001	
5.10	High	0.037 ± 0.003	4.50 ± 1.00	0.037 ± 0.003	6.00 ± 1.00	0.037 ± 0.003	4.50 ± 1.00
	Low	0.011 ± 0.001		0.012 ± 0.001		0.012 ± 0.001	
8.50	High	0.036 ± 0.003	6.00 ± 1.00	0.037 ± 0.003	6.00 ± 1.00	0.037 ± 0.003	6.00 ± 1.00
	Low	0.010 ± 0.001		0.012 ± 0.001		0.012 ± 0.001	

cultures were not bead dependent but were significantly higher compared to Cytodex-1. The results confirm that spinner flasks are poor environments for developing scale-up processes.

Oxygen supply could be the limiting nutrient in refed spinner flask cultures, as was discussed previously (10.3.1). However, serum components could also be involved in lower cell yields in Cytodex-1 cultures, as discussed previously (6.3.2 and 10.3.6). The oxygen limitation could help to explain the similar CHO cell yields ($3.00\text{--}4.00 \times 10^6$ cells/mL) in refed 100 mL spinner flask cultures of 5.00 g/L Cytodex-1 obtained by previous authors (Smiley et al., 1989 and Forestall et al., 1992). However, it does not explain why CHO cells grown in a controlled Celligen bioreactor culture of 4.00 g/L Cultispher-S and perfused at 6.00 v/v/d (Ohlson et al., 1994) only reached the same cell yield. It is hard to believe that a perfusion rate of 6.00 v/v/d could not supply all the vital medium nutrients for cell growth. Another difference could be the bead type used, as the latter authors grew CHO cells in Cultispher-S beads which have a smaller pore size compared to Cultispher-G. This could produce a low void volume in Cultispher-S for growth.

The Vero cell growth rates showed biphasic patterns, as seen and discussed previously (10.3.1). Nahapetian et al., (1986) showed similar growth rate phases with refed Vero cultures in 100 mL spinner flasks.

11.3.2 Perfusion Bioreactor Cultures

Introduction. The previous study (11.3.1) confirmed spinner flasks as a poor growth environment for developing refeeding strategies. Thus, it was impossible to clearly determine the effects of higher microcarrier concentrations and refeeding rates on Vero cell growth. Therefore, this study determined the effects of using various microcarrier concentrations and perfusion rates on Vero cell growth in Celligen bioreactor microcarrier cultures.

Experimental. Preadapted stock Vero cells were inoculated in enriched DMEM + 5.0% v/v sCS, described above (11.2.1) on Cytodex-1 beads (1.00-5.00 g/L) and Cultispher-G beads (1.70-5.10

g/L) in 1,300 mL Celligen bioreactor. Culture parameters were controlled at the set points described previously (2.4.5).

Results. Fig. 11.1 and 11.2 show Vero cell growth in perfusion Celligen bioreactor cultures of various concentrations of Cytodex-1 and Cultispher-G with various perfusion rates. Table 11.3 shows the cell growth rates and lag phases in these cultures. Table 11.4 shows the cell concentrations at which the growth rates changed from high to low values. Table 11.5 shows the maximum cell/bead loadings and the estimated number of cell layers at maximum cell yield in these cultures. (1) Maximum Vero cell yields in cultures of either bead were affected by the microcarrier concentration and perfusion rates used. (2) There were significantly higher (36%, $P<0.01$) maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to all microcarrier concentrations and perfusion rates used. (3) There were no significant differences (Fig. 11.1A) and 11.2A)) in maximum cell yields in cultures at any bead concentration with perfusion at 0.50 v/v/d. However, there were significantly higher ($P<0.01$) maximum cell yields in cultures of Cytodex-1 at ≥ 3.00 g/L and in Cultispher-G cultures at ≥ 3.40 g/L at perfusion rates of 1.00 and 1.50 v/v/d compared to all other bead concentrations used. (4) There were significantly higher maximum cell yields in cultures of either bead at 1.50 v/v/d compared to all other perfusion rates with respect to all the microcarrier concentrations used. (5) There was no significant difference in the lag phase times (Table 11.3) with respect to either bead type. (6) There were biphasic cell growth rates (Table 11.3) in all cultures. The high growth rate phase was extended to different cell yields (Table 11.4) in cultures of either bead and was significantly higher (182%, $P<0.01$) compared to the low rate. The cell yield at which the rate reduced (Table 11.4) was proportional to the microcarrier concentration and perfusion rate used. The high cell growth rate phase was extended to significantly higher ($P<0.05$) cell yields in Cultispher-G cultures compared to Cytodex-1. (7) The maximum cell/bead loadings (Table 11.5) were significantly higher (545%, $P<0.01$) in Cultispher-G cultures compared to Cytodex-1 at all perfusion rates used. However, the maximum cell/bead loadings were significantly lower at 5.00

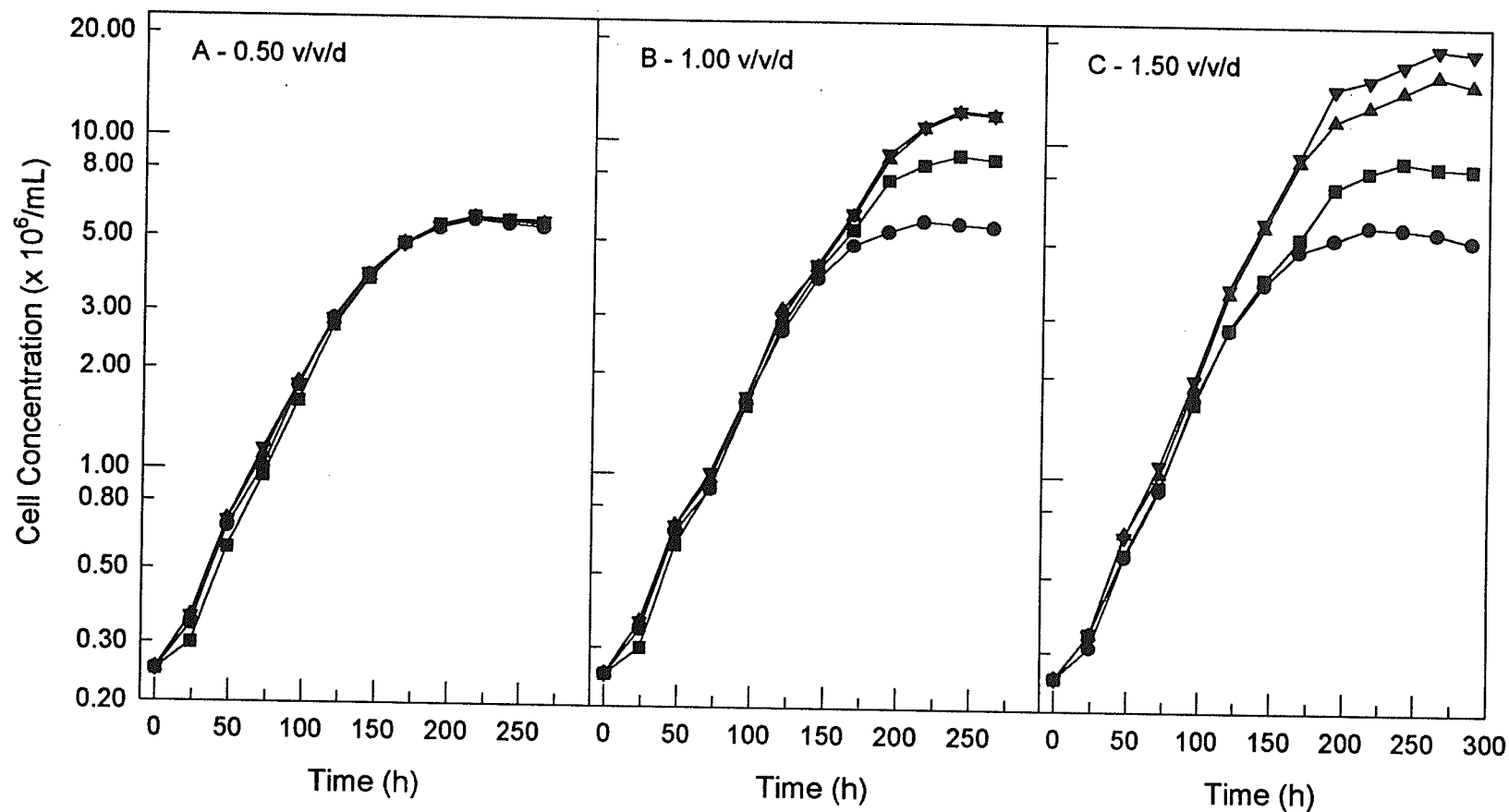


Fig. 11.1 - The effect of the **Cytodex-1 microcarrier concentration** on Vero cell growth in the Celligen bioreactor at perfusion rates of **0.50 (A), 1.00 (B) and 1.50 (C) v/v/d**. The cells were inoculated at 0.25×10^6 cells/mL in enriched DMEM + 5.0% v/v sCS. Cytodex-1 microcarriers were added at either ● 1.00, ■ 2.00, ▲ 3.00 or ▼ 5.00 g/L. Controls were set to pH 7.1, stirring at 40 rpm ramped to 60 rpm after 48 h in culture, 37°C, 50% DOT and 0.50 L/min gas flow rate. Perfusion was initiated after 48h in culture. The points are means of n=2.

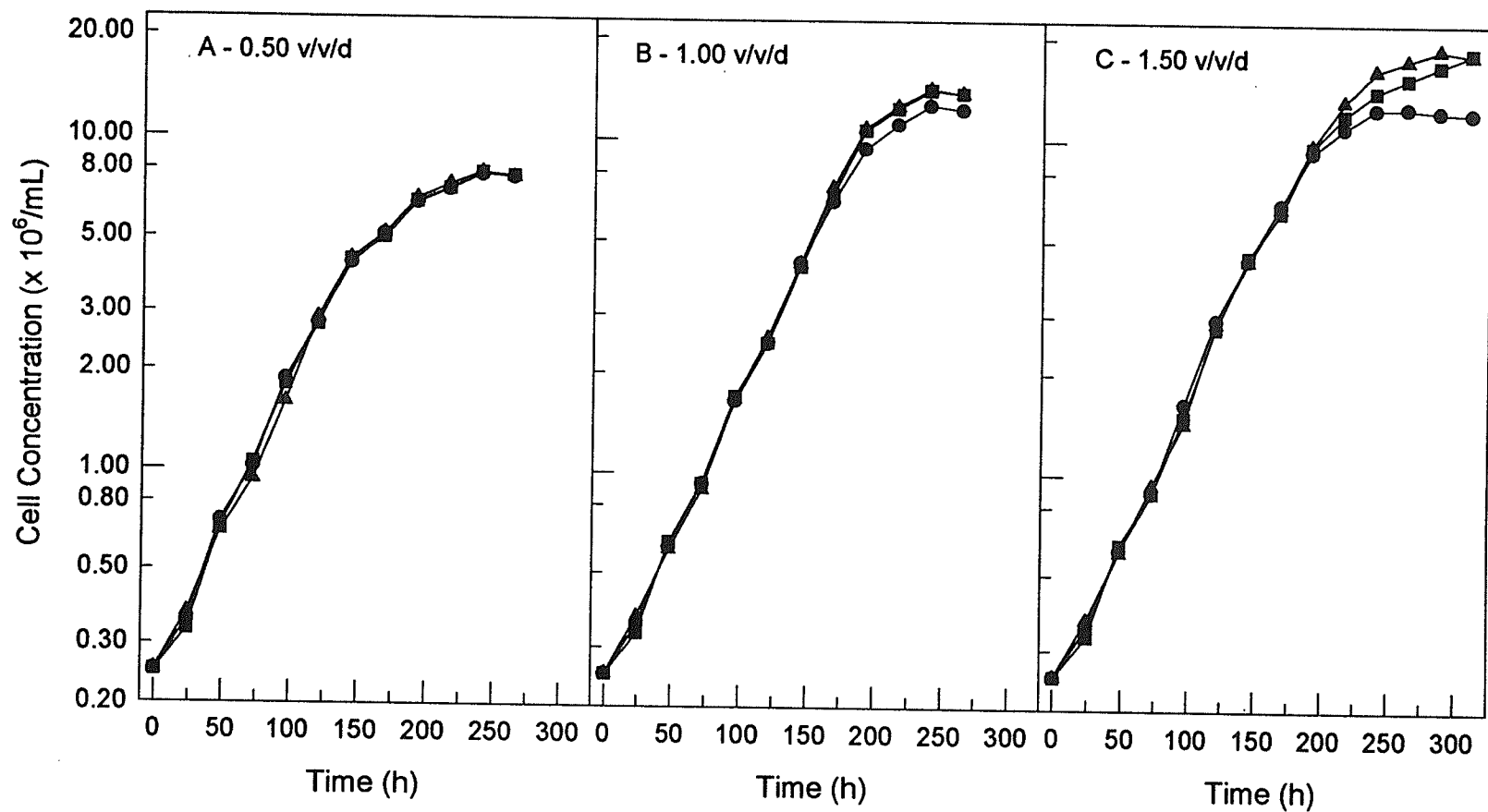


Fig. 11.2 - The effect of the **Cultispher-G** microcarrier concentration on Vero cell growth in the Celligen bioreactor at perfusion rates of **0.50 (A)**, **1.00 (B)** and **1.50 (C) v/v/d**. The cells were inoculated at 0.25×10^6 cells/mL in enriched DMEM + 5.0% v/v sCS. Cultispher-G microcarriers were added at either ● 1.70, ■ 3.40 or ▲ 5.10 g/L. Controls were set to pH 6.8, stirring at 40 rpm after 48 h and 80 rpm after 120 h in culture, 37°C, 50% DOT and 0.50 L/min gas flow rate. Perfusion was initiated after 48 h in culture. The points are means of $n=2$.

Table 11.3 - The effect of the microcarrier concentration and perfusion rate on the Vero cell growth rate and lag phase in 1,300 mL perfusion Celligen bioreactor cultures of **Cytodex-1** and **Cultispher-G**. The cells were inoculated at 0.25×10^6 cells/mL in enriched DMEM + 5.0% v/v SCS. Cytodex-1 were added at 1.00-5.00 g/L and Cultispher-G at 1.70-5.10 g/L. Controls were set to pH 7.1 (Cytodex-1) or 6.8 (Cultispher-G), stirring at 40 rpm ramped to 60 rpm after 48 h (either microcarrier) and to 80 rpm after 120 h (Cultispher-G) in culture, 37°C, 50% DOT and 0.50 L/min gas flow rate. Perfusion was initiated at 0.50-1.50 v/v/d after 48 h in culture. The values are means of n=2.

Microcarrier Concentration (g/L)	Specific Cell Growth Rate and Lag Phase at the Perfusion Rate Used						
Cytodex-1		0.50 v/v/d		1.00 v/v/d		1.50 v/v/d	
		Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)	Specific Cell Growth Rate (h ⁻¹)	Lag Phase (h)
1.00	High	0.032 ± 0.003	12.00 ± 1.00	0.031 ± 0.003	12.00 ± 1.00	0.032 ± 0.003	12.00 ± 1.00
	Low	0.011 ± 0.001		0.010 ± 0.001		0.011 ± 0.001	
2.00	High	0.032 ± 0.003	15.00 ± 1.50	0.031 ± 0.003	12.00 ± 1.00	0.032 ± 0.003	15.00 ± 1.00
	Low	0.011 ± 0.001		0.011 ± 0.001		0.010 ± 0.001	
3.00	High	0.031 ± 0.003	12.00 ± 1.00	0.031 ± 0.003	12.00 ± 1.00	0.031 ± 0.003	15.00 ± 1.50
	Low	0.011 ± 0.001		0.010 ± 0.001		0.010 ± 0.001	
5.00	High	0.032 ± 0.003	12.00 ± 1.00	0.031 ± 0.003	12.00 ± 1.00	0.032 ± 0.003	15.00 ± 1.50
	Low	0.011 ± 0.001		0.011 ± 0.001		0.011 ± 0.001	
Cultispher-G							
1.70	High	0.030 ± 0.003	9.00 ± 1.00	0.031 ± 0.003	9.00 ± 1.00	0.030 ± 0.003	9.00 ± 1.00
	Low	0.012 ± 0.001		0.012 ± 0.001		0.011 ± 0.001	
3.40	High	0.030 ± 0.003	9.00 ± 1.00	0.032 ± 0.003	9.00 ± 1.00	0.029 ± 0.003	9.00 ± 1.00
	Low	0.012 ± 0.001		0.011 ± 0.001		0.012 ± 0.001	
5.10	High	0.031 ± 0.003	9.00 ± 1.00	0.031 ± 0.003	12.00 ± 1.00	0.029 ± 0.003	12.00 ± 1.00
	Low	0.011 ± 0.001		0.012 ± 0.001		0.012 ± 0.001	

Table 11.4 - The effect of the **microcarrier concentration** and **perfusion rate** on the Vero cell count at which the **specific cell growth rate** was significantly reduced from a **high to low linear rate** in 1,300 mL perfusion Celligen bioreactor cultures of **Cytodex-1** and **Cultispher-G**. The cells were inoculated at 0.25×10^6 cells/mL in enriched DMEM + 5.0% v/v sCS. Cytodex-1 were added at 1.00-5.00 g/L and Cultispher-G at 1.70-5.10 g/L. Controls were set to pH 7.1 (Cytodex-1) or 6.8 (Cultispher-G), stirring at 40 rpm after 48 h (either microcarrier) and to 80 rpm after 120 h (Cultispher-G) in culture, 37°C, 50% DOT and 0.50 L/min gas flow rate. Perfusion was initiated at 0.50-1.50 v/v/d after 48 h in culture. The values are means of n=2.

Microcarrier Concentration (g/L)	Cell Count at which the Specific Cell Growth Rate was Significantly Reduced from High to Low Rate in the Perfusion Culture ($\times 10^6$ cells/mL)		
	0.50 v/v/d	1.00 v/v/d	1.50 v/v/d
Cytodex-1			
1.00	4.00 ± 0.50	4.00 ± 0.50	4.00 ± 0.50
2.00	4.00 ± 0.50	7.50 ± 0.80	7.50 ± 0.80
3.00	4.00 ± 0.50	8.50 ± 0.80	12.50 ± 1.00
5.00	4.00 ± 0.50	8.50 ± 0.80	15.00 ± 1.50
Cultispher-G			
1.70	4.70 ± 0.50	9.00 ± 0.75	9.00 ± 0.75
3.40	4.75 ± 0.50	10.20 ± 1.00	14.50 ± 1.50
5.10	4.75 ± 0.50	10.20 ± 1.00	16.50 ± 1.50

Table 11.5 - The effect of the **microcarrier concentration** and **perfusion rate** on the maximum Vero **cell/bead loadings** and the estimated **number of cell layers** at maximum yield in Celligen bioreactor cultures of **Cytodex-1** and **Cultispher-G**. The cells were inoculated at 0.25×10^6 cells/mL in enriched DMEM + 5.0% v/v sCS. Cytodex-1 were added at 1.00-5.00 g/L and Cultispher-G at 1.70-5.10 g/L. Controls were set to pH 7.1 (Cytodex-1) or 6.8 (Cultispher-G), stirring at 40 rpm ramped to 60 rpm after 48 h (either microcarrier) and to 80 rpm after 120 h (Cultispher-G) in culture, 37°C, 50% DOT and 0.50 L/min gas flow rate. Perfusion was initiated at 0.50-1.50 v/v/d after 48 h in culture. The number of cell layers was estimated from the maximum cells/bead compared to the number of cells in a monolayer on each bead. The values are means of n=2.

Microcarrier Concentration (g/L)	Maximum Cell/Bead Loading at the Perfusion Rate used (Cells/Bead)					
	0.50 v/v/d		1.00 v/v/d		1.50 v/v/d	
Cytodex-1	Maximum Cell/Bead Loading	Number of Cell Layers	Maximum Cell/Bead Loading	Number of Cell Layers	Maximum Cell/Bead Loading	Number of Cell Layers
1.00	1,260	3.93	1,291	4.02	1,282	3.99
2.00	644	2.01	1,016	3.17	1,003	3.13
3.00	427	1.33	910	2.83	1,200	3.74
5.00	256	0.80	552	1.72	867	2.70
Cultispher-G						
1.70	5,065	8.79	8,327	14.46	8,281	14.34
3.40	2,565	4.45	4,608	8.00	6,095	10.58
5.10	1,717	2.98	3,094	5.37	4,150	7.21

g/L Cytodex-1 and 3.40-5.10 g/L Cultispher-G compared to the other microcarrier concentrations used. This suggests these cultures were not perfused at their optimal rates. The maximum cell/bead loadings suggested there were up to 4.02 cell layers on 1.00 g/L Cytodex-1 beads at maximum cell yield. The Cultispher-G beads produced maximum cell/bead loadings which were up to 14.46 times the number of cells which could be produced by a monolayer on the outer bead surface. However, the number of cell layers were reduced proportional to the microcarrier concentration and there were significantly fewer layers (56% at 5.00 g/L Cytodex-1, $P < 0.01$) at > 1.00 g/L Cytodex-1.

Discussion. Maximum Vero cell yields in perfusion Celligen bioreactor cultures could be significantly increased by using high microcarrier concentrations. At high perfusion rates maximum cell yields were dependent on the Cytodex-1 concentration and 5.00 g/L Cytodex-1 produced the highest cell yield. The maximum cell yields were similar to those achieved with MDCK cells (23.00×10^6 cells/mL) on 6.00 g/L Cytodex-3 at 1.00 v/v/d (Martin et al., 1987). However, the cell yields were significantly higher (188%, $P < 0.01$) in this report on 3.00 g/L Cytodex-1 at 0.50 v/v/d compared to those in a previous New Brunswick Scientific technical report with Vero cells on 3.00 g/L Cytodex-3 (NBS, 1991). There was no significant difference in the maximum cell yields produced in cultures of 3.40-5.10 g/L Cultispher-G suggesting the perfusion rates were sub-optimal at these concentrations. Thus, Vero cells grew differently in perfusion bioreactor cultures from CHO cells in previous reports (Ohlson et al., 1994) which could be a cell-line dependent effect but more likely reflects the optimised process parameters used in this study. The results also confirm that Vero cell yields could be linearly increased with the bead concentration if the optimal perfusion rate per gram of beads was used.

The maximum cell yields were dependent on the perfusion rate used and were significantly increased at 1.50 v/v/d compared to 0.50 v/v/d with 5.00 g/L Cytodex-1 and 3.40-5.10 g/L Cultispher-G. The maximum cell/bead loadings confirmed the optimal perfusion rate/g of either beads to be 0.50-0.60 v/v/d/g, as suggested previously (10.3.7). This suggests the yields in the

high bead concentration cultures were below their maximal, as the perfusion rates were not at their optimal levels. Yields could be expected to reach between $30.00\text{--}40.00 \times 10^6$ cells/mL in optimal cultures of 5.00 g/L Cytodex-1 and 5.10 g/L Cultispher-G.

11.3.3 Effects of the Glutamine Concentration in the Perfusion Medium

Introduction. Vero cell yields in Cultispher-G cultures could be significantly increased with perfusion medium containing 6.00 mM glutamine compared to 4.00 mM. Cells in cultures of Cytodex-1 were unaffected by the glutamine concentration. No one has previously shown differences in the perfused growth of any cell line due to using different medium glutamine concentrations. Therefore, this study determined the effects of varying the glutamine concentration in the perfusion medium on Vero cell growth in Celligen bioreactor microcarrier cultures.

Experimental. Preadapted stock Vero cells were inoculated in enriched DMEM + 5.0% v/v SCS containing either 4.00 or 6.00 mM glutamine. The cultures were grown in the Celligen bioreactor on 5.00 g/L Cytodex-1 or 5.10 g/L Cultispher-G with perfusion initiated after 48 h at 1.50 v/v/d.

Results. Fig. 11.3 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G perfused with medium containing various concentrations of glutamine. Table 11.6 shows the maximum cell yields and growth rates in these cultures. (1) There were no significant differences in the maximum cell yields in Cytodex-1 cultures with respect to either glutamine concentration used. However, maximum cell yields (Table 11.6) in Cultispher-G cultures were significantly higher (15%, $P=0.05$) at 6.00 mM glutamine compared to 4.00 mM in the perfusion medium. (2) There were biphasic cell growth rates (Table 11.6) which were not significantly different in all cultures.

Discussion. No one has previously described the effects of different glutamine concentrations

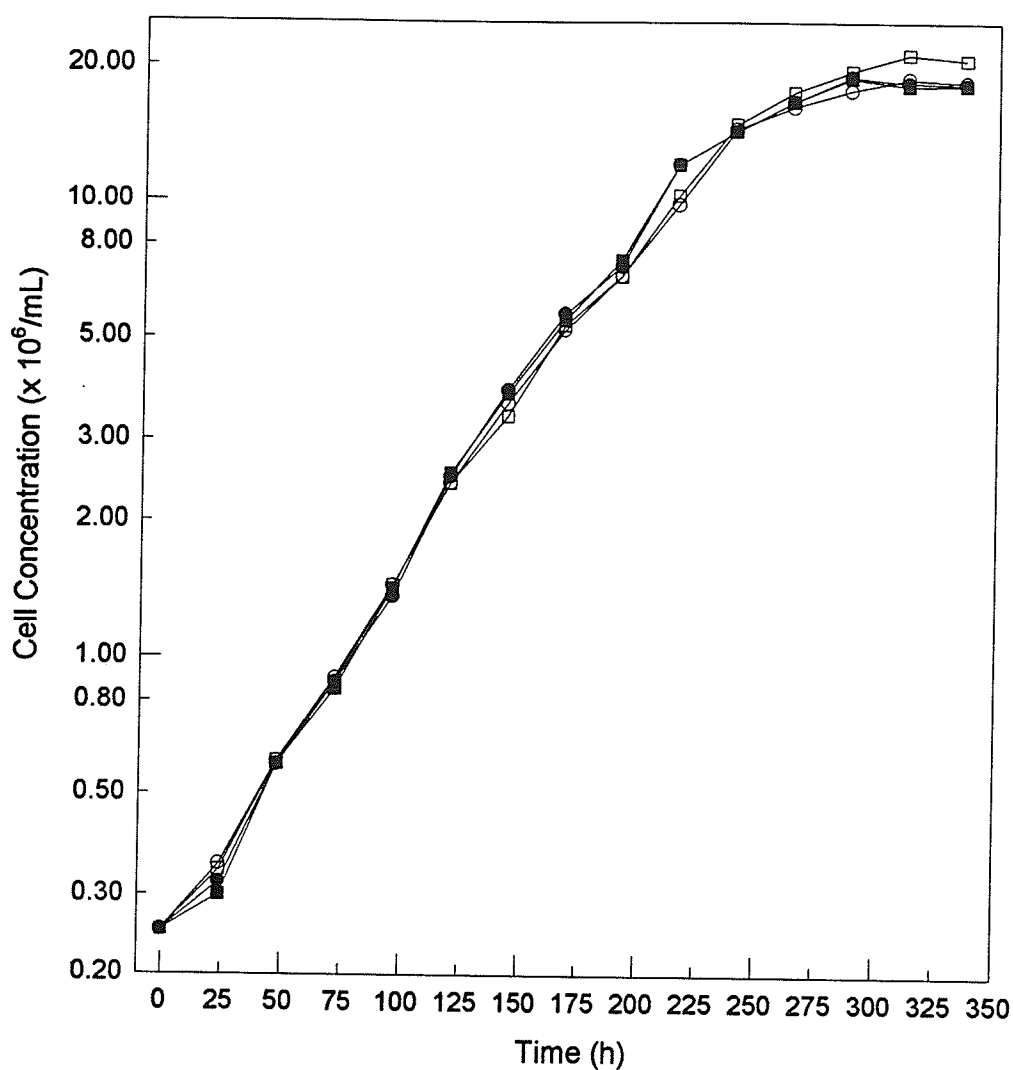


Fig. 11.3 - The effect of the glutamine concentration on the maximum Vero cell growth in perfused Celligen bioreactor on 5.00 g/L Cytodex-1 or 5.10 g/L Cultispher-G. The cells were inoculated at 0.25×10^6 cells/mL with enriched DMEM + 5.0% v/v sCS and were attached stirring continuously at 40 rpm (Cytodex-1) or intermittently for 24 h in a 500 mL spinner flask (Cultispher-G) prior to inoculation. All cultures were manually ramped to 60 rpm after 48 h. Glutamine was used at either ● 4.00 mM or ■ 6.00 mM with Cytodex-1 (Closed symbols) and Cultispher-G (Open symbols). The points are means of $n=2$.

Table 11.6 - The effect of the **glutamine concentration** on the **maximum Vero cell yield** and **growth rate** in perfused Celligen bioreactor on **5.00 g/L Cytodex-1** or **5.10 g/L Cultispher-G**. The cells were inoculated at 0.25×10^6 cells/mL with enriched DMEM + 5.0% v/v sCS and were attached stirring continuously at 40 rpm (Cytodex-1) or intermittently for 24 h in a 500 mL spinner flask (Cultispher-G) prior to inoculation. All cultures were manually ramped to 60 rpm after 48 h. Glutamine was used at either 4.00 mM or 6.00 mM with Cytodex-1 and Cultispher-G. The values are means of n=2.

Parameter		Maximum Cell Yield, Specific Growth Rate and Lag Phase of the Culture at the Glutamine Concentration Used			
		Cytodex-1		Cultispher-G	
		4.00 mM	6.00 mM	4.00 mM	6.00 mM
Maximum cell yield ($\times 10^6$ cells/mL)		18.45 $\pm$ 1.35	18.20 $\pm$ 1.35	18.80 $\pm$ 1.45	21.25 $\pm$ 1.51
Specific Cell growth rate (h ⁻¹)	High	0.031 $\pm$ 0.003	0.030 $\pm$ 0.003	0.030 $\pm$ 0.003	0.030 $\pm$ 0.003
	Low	0.010 $\pm$ 0.001	0.011 $\pm$ 0.001	0.010 $\pm$ 0.001	0.011 $\pm$ 0.001
Lag phase (h)		12.00 $\pm$ 1.00	15.00 $\pm$ 1.50	9.00 $\pm$ 1.00	9.00 $\pm$ 1.00

in the perfusion medium. The medium glutamine concentration did not affect maximum Vero cell yields in Cytodex-1 cultures which agrees with previous results (10.3.2). However, Cultispher-G cultures grew to significantly higher maximum cell yields at 6.00 mM glutamine compared to 4.00 mM. This agreed with batch results (6.3.4) but disagreed with refed spinner flask cultures (10.3.2). The controlled conditions of the bioreactor could explain this difference, as oxygen could have limited the cell yield in spinner flasks but not in the Celligen.

11.3.4 Ramped Refeeding Strategies

Introduction. Previous results (10.3.7 and 11.3.2) showed that maintaining high perfusion rates could significantly increase maximum Vero cell yields at 5.00 g/L Cytodex-1 and 5.40 g/L Cultispher-G compared to 1.00 g/L and 1.70 g/L, respectively. However, the results also suggested perfusion did not have to be initiated at the high rate but could be gradually increased (ramped) every few days without reducing final cell yields. This strategy could maintain favourable nutrient levels and reduce the total medium throughput during perfusion to reduce process costs. Therefore, this study determined the effects of ramping the perfusion rate every 48 h on Vero cell growth in Celligen bioreactor microcarrier cultures.

Experimental. Preadapted Vero cells were inoculated in enriched DMEM + 5.0% v/v SCS and grown in Celligen bioreactor cultures on 5.00 g/L Cytodex-1 and 5.10 g/L Cultispher-G, as described above (11.3.2). After 48 h the cultures were perfused at either (a) 1.50 v/v/d continuously or (b) at 0.25 v/v/d and ramped to 0.50 after 120 h, 1.00 after 168 h and 2.00 v/v/d after 216 h in culture.

Results. Fig. 11.4 shows Vero cell growth in cultures of Cytodex-1 and Cultispher-G using ramped perfusion rates. Table 11.7 shows the maximum cell yields and growth rates in these cultures. (1) Maximum cell yields in perfusion cultures of either bead were affected by the

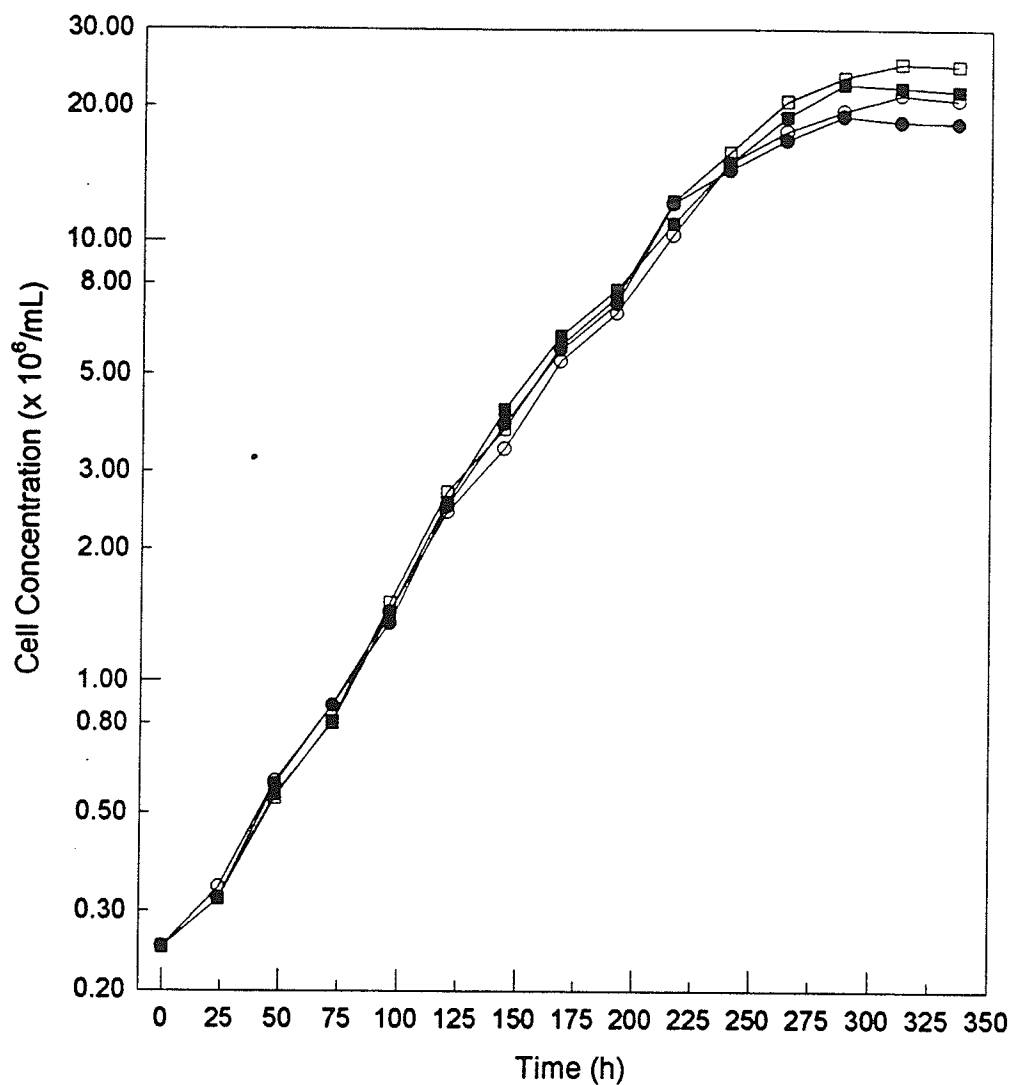


Fig. 11.4 - The effect of using a **ramped perfusion rate** on Vero cell growth in Celligen bioreactor on **5.00 g/L Cytodex-1** and **5.10 g/L Cultispher-G**. The cells were inoculated at 0.25×10^6 cells/mL in enriched DMEM + 5.0% v/v sCS. Perfusion was initiated after 48 h in culture at either ● a fixed rate of 1.50 v/v/d or ■ at 0.25 v/v/d, increased to 0.50 after 120 h, 1.00 after 192 h and 2.00 v/v/d after 240 h. Controls were set to pH 7.1 (Cytodex-1 - Closed symbols) or 6.8 (Cultispher-G - Open symbols), stirring at 40 rpm ramped to 60 rpm after 48 h and 80 rpm after 120 h in culture, 37°C, 50% DOT and 0.50 L/min gas flow rate. The points are means of n=2.

Table 11.7 - The effect of the **perfusion strategy** on the **maximum Vero cell yield** and **specific growth rate** in Celligen bioreactors on **5.00 g/L Cytodex-1** or **5.10 g/L Cultispher-G**. The cells were inoculated at 0.25×10^6 cells/mL in enriched DMEM + 5.0% v/v SCS containing 4.00 mM (Cytodex-1) or 6.00 mM (Cultispher-G) glutamine. The cells were attached stirring continuously at 40 rpm (Cytodex-1) or intermittently for 24 h in a 500 mL spinner flask (Cultispher-G) prior to inoculation. All cultures were manually ramped to 60 rpm after 48 h. Perfusion was initiated after 48 h in culture at either ● a fixed rate of 1.50 v/v/d or ■ at 0.25 v/v/d, increased to 0.50 after 120 h, 1.00 after 192 h and 2.00 v/v/d after 240 h. The values are means of n=2.

Parameter		Maximum Cell Yield, Specific Growth Rate and Lag Phase of the Culture at the Glutamine Concentration Used			
		Cytodex-1		Cultispher-G	
		1.50 v/v/d	0.25-2.00 v/v/d	1.50 v/v/d	0.25-2.00 v/v/d
Maximum cell yield ($\times 10^6$ cells/mL)		19.00 $\pm$ 1.35	22.45 $\pm$ 1.59	21.25 $\pm$ 1.51	25.05 $\pm$ 1.78
Specific Cell growth rate (h^{-1})	High	0.029 $\pm$ 0.003	0.029 $\pm$ 0.003	0.029 $\pm$ 0.003	0.029 $\pm$ 0.003
	Low	0.010 $\pm$ 0.001	0.011 $\pm$ 0.001	0.010 $\pm$ 0.001	0.011 $\pm$ 0.001
Lag phase (h)		15.00 $\pm$ 1.50	15.00 $\pm$ 1.50	15.00 $\pm$ 1.50	12.00 $\pm$ 1.00

ramped perfusion rate used. (2) There were no significant differences in the maximum cell yields in Cultispher-G cultures compared to Cytodex-1 with respect to the perfusion rates used. (3) There were significantly higher (18%, $P < 0.05$) maximum cell yields (Table 11.7) in cultures of either bead in ramped perfusion rate cultures compared to continuous perfusion rates. (4) There were biphasic cell growth rates (Table 11.7) in cultures of either bead with high linear rates up to 15.00×10^6 cells/mL and then significantly reduced up to maximum cell yield. (5) The total medium volume used to reach maximum cell yield was significantly higher (56%, $P < 0.01$) with the continuous perfusion rate (22.75 L) compared to the ramped refeeding strategy (14.63 L).

A complete nutrient analysis was not performed on these cultures.

Discussion. Maximum Vero cell yields were significantly higher in cultures of either bead with ramped perfusion rates compared to fixed. This shows that a ramped refeeding strategy could produce high maximum cell yields. Overall the total complete medium volume used to reach the maximum cell yield was significantly lower with the ramped perfusion strategy. Thus, the cell yield/mL of medium used was 1.64×10^6 cells/mL with ramped perfusion and 1.39×10^6 cells/mL with a continuous perfusion rate of 1.50 v/v/d. This difference could also be explained by the differences between the final perfusion rates used (i.e. 2.00 v/v/d in ramped compared to 1.50 v/v/d in continuous). Therefore, using a ramped refeeding strategy significantly reduced the perfusion culture operating costs. There were significantly higher cell yields in Cultispher-G cultures compared to Cytodex-1 which could be explained by the higher glutamine concentration in Cultispher-G perfusion medium, as discussed above (11.3.3). The results agree with the ramped perfusion strategies used by Reiter et al. (1990) and Ohlson et al. (1994). However, the total volumes of medium used by Reiter et al. (1990) and Ohlson et al. (1994) were significantly higher (1,343%, $P < 0.01$ and 107%, $P < 0.01$, respectively) compared to those in this study, even with the continuous perfusion rate. Reiter et al. (1990) reported high Vero cell yields of 35.00×10^6 cells/mL of bed volume (i.e. a cell yield of 11.67×10^6 /mL of reactor volume). Therefore, the cell yields in this study were also significantly higher (114%, $P < 0.01$) compared to the

previous authors. This could be explained by differences between the media types or bead types used between the studies, as Reiter et al. (1990) used DMEM with low glucose (5.60 mM) + 5.0% v/v FBS and Cultispher-GLD (titanium weighted beads). The low glucose concentration in particular could explain the higher perfusion rates required for their cell yields.

11.4 Conclusions

The purpose of this study was to define the scale-up strategy for growing Vero cells in cultures of Cytodex-1 and Cultispher-G. The results above show that:

- **spinner flasks are ineffective** environments for the development refeeding strategies. Maximum cell yields were significantly higher (approximately 100%, $P < 0.01$) in perfused Celligen bioreactors compared to spinner flasks with the same daily medium volume change. However, spinner flasks can be used for preliminary refeeding studies at low bead concentrations to identify nutrient limitations and optimal refeed rates per g beads.
- **high maximum Vero cell yields** in cultures of either bead were reached in the Celligen bioreactor at the optimal control set-points.
- **linear scale-up** was achieved with enriched DMEM at the optimal refeed rates of 0.50-0.60 v/v/d/g beads. Cell yields $> 20.00 \times 10^6$ cells/mL were generated from 5.00 g/L Cytodex-1 and 5.10 g/L Cultispher-G inoculated at 0.25×10^6 cells/mL. These yields show cultures could be transferred from 1-100-10,000 L volume using the same inoculum concentration.
- **ramped refeeding** produced significantly higher cell yields compared to fixed refeeding rates. Continuous perfusion used significantly higher (56%, $P < 0.01$) total medium volumes compared to the ramped strategy and did not reach the same maximum cell yield.
- **glutamine** in the refeeding DMEM was optimal at 4.00 mM for Cytodex-1 and 6.00 mM for Cultispher-G cultures.

Chapter 12. Virus Productivity of Vero Cells in Microcarrier Cultures

12. Virus Productivity of Vero Cells in Microcarrier Cultures

12.1 Introduction

Adherent cells such as baby hamster kidney (BHK), Madin-Darby bovine or canine kidney (MDBK or MDCK), human diploid lung fibroblast (MRC-5) and African green monkey kidney (Vero) cells are grown as hosts for producing virus vaccines (Beale, 1992). Vaccines have a major therapeutic role in human and agricultural healthcare, as it is estimated that measles causes 1.5 million deaths per annum (Makela et al., 1989) and animal virus infections cause annual agricultural losses >\$100 Bn. U.S. (Shevitz et al., 1990).

Early vaccine production used simple technologies such as tissue culture flasks and roller bottles (Shevitz et al., 1990), scaling-up for single vaccine batches by increasing their numbers to several thousand (Nardelli and Panina, 1976 and Mirchamsy et al., 1977). The more complex technology of microcarrier culture allows batches to be produced in large single-unit bioreactors (van Wezel, 1967). However, Varani et al. (1988) reported differences in turkey herpes virus production from chick fibroblast cells with cultures grown on glass Biosil™ producing significantly higher titres compared to polydextran Cytodex-1™ or collagen Ventregel™ beads. The effect could be virus dependent, as the authors found no differences in herpes simplex (HSV) or bovine rhinotracheitis (BRV) virus production in MRC-5 or MDBK cells on the different bead types.

BHK cells were the first commercially successful hosts cultured in microcarrier bioreactors for foot and mouth disease virus (FMDV) vaccine (Spier, 1983). BHK culture technology has been successfully transferred to MDCK, MRC-5 and Vero cells for the production of many other virus types.

Vero are a Simian virus 40 (SV40) transformed cell line capable of being cultured indefinitely (Yasumara and Kawakita, 1963). Vero have WHO acceptance for vaccine production, as they do not shed contaminating sequences of SV40 during growth (WHO, 1987^a and Horaud, 1992). Vero are normally grown to monolayers before virus is added to ensure even infection (House et al., 1972, Nardelli and Panina, 1976, Mirchamsy et al., 1977, Reuveny et al., 1981, Scattergood

et al., 1983) and they have been used in research on polio (Montagnon et al., 1981), bovine vesicular stomatitis (VSV) (Nikolai and Hu, 1992), herpes simplex (HSV) (Griffiths et al., 1981, Griffiths and Thornton, 1982 and Marique et al., 1994) and rabies (Mendonca et al., 1993) viruses. Vero are also cultured for commercial rabies and polio vaccines (El-Karamany, 1987, Shevitz et al., 1990 and Beale, 1992) to replace monkeys (Montagnon et al., 1981).

Reoviruses are a less commonly researched virus and have been reviewed by Schiff and Fields (1990). They are a dsRNA virus which replicate in the cytoplasm and infect vertebrates including humans. They cause diarrheas or respiratory diseases with symptoms of rhinitis, tonsillitis, laryngitis and bronchitis, although infection is not life threatening. The viruses penetrate the cells quickly via phagocytic vesicles which could be a receptor mediated endocytosis and they migrate to the centre of the cells fusing to lysosomes (80% of virus particles found in lysosomes 1 h after infection) (Silverstein and Dales, 1968). The viruses uncoat within 2-3 h of infection and inhibit host cell macromolecular synthesis, blocking DNA and protein synthesis by viral hemagglutinin $\sigma 1$ and $\sigma 3$ (Sharpe and Fields, 1981 and 1982). Reovirus can infect Vero cells and cause lytic or persistent infections in Chinese hamster ovary (CHO) and murine L-cells (Taber et al., 1976 and Davis et al., 1990). There is no current vaccine against reovirus infection and there are no statistics on the number of people contracting illnesses resulting from such infections.

Several bioreactor technologies now exist for virus production including fluidised beds for HSV production from MRC-5 cells (Thornton et al., 1984) and stirred tanks for FMDV vaccine from BHK cells (Whiteside and Spier, 1981). Most bioreactors are operated in batch mode (Mered et al., 1980, Reuveney et al., 1981 and Scattergood et al., 1983) and have been scaled-up to 1,000 L for poliovirus vaccine (Montagnon et al., 1984). However, Griffiths and Thornton (1982) used a semi-continuous perfusion reactor for MRC-5 production of HSV and reported increased cell yields (five-fold) and virus titres (one and a half-fold), although, the virus yield/cell was significantly reduced in this system.

Previous studies (5.3-11.3) defined the process parameters for high yield cultivation of Vero

cells in microcarrier cultures of Cytodex-1 and Cultispher-G. The purpose of high Vero cell yields would be increased virus titres. Therefore, this paper presents a study of Vero microcarrier culture infections with type-1 (Lang) and type-3a (Dearing) reoviruses (identified by differences in protein mobility on polyacrylamide gels - Ramig et al., 1977). Infections were performed in batch static and spinner flask cultures and daily refed spinner flasks to assess the infectivity of highly increased cell/bead loadings.

12.2 Materials and Methods

The methods of culturing and counting the cells have been described previously (2.1-2.5).

12.2.1 Preadaptation of Stock Cells

Stock Vero cells were preadapted (>5 passages) to either (i) "original" DMEM or (ii) serine and pyruvate "enriched" DMEM (2.00 mM and 0.50 mM, respectively) both supplemented with 5.0% v/v iron enriched, supplemented calf serum (sCS). The cells were detached and recovered by the standard trypsinisation protocol (2.2) and counted by the trypan blue method (2.5.1-2).

12.2.2 Preparation of Stock Virus

Host Cells. Stock murine L-929 cells (American Type Culture Collection - CCL 0001) were grown in spinner flasks (500 mL) using Joklik's Modified Eagle's Medium (JMEM) containing 5.55 mM glucose, 2.00 mM glutamine and 100 units/mL of penicillin G, streptomycin sulphate and amphotericin B + 2.5% v/v foetal bovine serum (FBS) + 2.5% v/v neonate bovine serum (VSP) (Biocell® Labs., Los Angeles, CA, U.S.A.) supplement ("complete JMEM"). The spinner flasks were stirred continuously at 100 rpm. Stock cells were diluted to $0.40\text{--}0.45 \times 10^6$ cells/mL in complete JMEM and seeded in 6-well plates (2.5 mL/well) grown overnight at 37°C with 5.0% v/v CO₂ in air overlay.

Preparation of Master Virus Stock. Stock type-1 and 3a reovirus were kindly supplied by Dr.K.M.Coombs, Dept. of Med. Micro., Uni. of Manitoba. The viruses were serially diluted (10^{-1} - 10^{-8}) in gel-saline solution. The L-929 6-well plate supernatants from above were removed and the serially diluted stock reovirus was added (100 μ L of 10^{-1} and 10^{-4} - 10^{-8} dilutions). The plates were rocked and rolled (x5) for 1 h at 20°C and overlaid (3 mL) with 2X Medium 199 (M199) + 5.0% v/v FBS + 5.0% v/v VSP + 100 units/mL of penicillin-G, streptomycin sulphate and amphotericin-B diluted (1:1) with select agar at 37°C. The overlay was allowed to set and the plates incubated at 37°C. After 72 h the wells were refed with overlay (2 mL). After a further 48 h the wells were refed with overlay (2 mL) containing neutral red dye (20 mL/L of 2.0% w/v stock). After a further 24 h the plaques were read using a fluorescent light box and 10-20 plaques from wells containing <50 plaques were picked with a Pasteur pipette and placed into gel/saline solution (1 mL) for 18 h virus extractions.

Preparation of Working Virus Stock. Stock L-929 cells were inoculated (0.40×10^6 cells/mL) in T25 culture flasks in complete JMEM (10 mL) and grown for 18 h. The supernatant was removed and the cells were infected with gel/saline extracted virus solution (0.2-0.5 mL), rocking and rolling as above for 1 h. The cells were fed with complete JMEM (4 mL) and incubated for 168 h prior to virus harvesting by freeze/thawing flasks between 37°C and -80°C (x3). This was passage 1 virus stock and was stored at 4°C.

Passage 1 (P1) virus was expanded in L-929 cells grown (18 h) in T75 flasks in complete JMEM (35 mL). The cells were infected with P1 virus (0.5 mL) rocking and rolling (1 h) as above and refed with complete JMEM (12 mL) and cultured for 168 h at 37°C. Virus was recovered by freeze/thawing as above and the virus was plaque assayed as described in 12.2.3. This was passage 2 (P2) virus stock and was stored at 4°C.

12.2.3 Reovirus Plaque Assay

Stock L-929 cultures were diluted in 6-well plates and grown for 18 h, as described above

(12.2.2).

Experimental samples were freeze/thawed (x3) as above (12.2.2) and serially diluted (10^{-1} - 10^{-9}) in gel-saline solution. The diluted virus samples were added (100 μ L of 10^{-1} and 10^{-5} - 10^{-9} dilutions) to L-929 cells in 6 well plates which were rocked and rolled (1 h), overlaid, refed and wells containing 50-100 plaques were counted, as described above (12.2.2). The total titre was calculated by accounting for the dilution used.

12.2.4 Preparation of Gel/Saline Solution

Gel/saline solution was prepared by adding NaCl (8.00 g/L), CaCl_2 (0.03 g/L), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.17 g/L), H_3BO_3 (1.20 g/L), $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (0.05 g/L) and type A gelatin (3.00 g/L) to 1 L of distilled water. The solution was dissolved by heating to 60°C stirring continuously at 100 rpm. Once dissolved the solution was aliquotted into 100 mL glass bottles and autoclaved at 121°C for 20 min.

12.3 Results and Discussion

12.3.1 Static Culture Flask Batch Growth Infections

Introduction. Vero cells are cultured commercially for the production of human rabies and polio vaccines and can be infected with a wide range of virus types. The previous studies (3.3-11.3) optimising growth only used Vero cell yields to determine the success of the approaches used. Therefore, this study determined the effects of type-1 and 3a reovirus infection of monolayered Vero cells grown in polystyrene T25 culture flasks.

Experimental. Preadapted stock Vero cells were grown in T25 culture flasks (10 mL) for 96 h. The cultures were infected at a multiplicity of infection (MOI) of 5.0 virions/cell with P1 type-1 and type-3a reoviruses, as described above (12.2.2) and refed with original or enriched DMEM + 5.0% v/v SCS (10 mL). The flasks were incubated for 240 h and one flask of each virus type was

sacrificed for cell and supernatant samples 72, 144 and 240 h post-infection. All samples were frozen at -20°C prior to virus plaque assay, as described above (12.2.3).

Results. Fig. 12.1 shows the T25 culture flask growth of Vero cells in enriched DMEM pre- and post-infection with type-1 and 3a reoviruses and the virus titres in the supernatant, cells and cumulative totals. (1) Vero cells could be infected with type-1 and 3a reoviruses. (2) There were no significant differences (Fig. 12.1A)-B)) in the cell counts at any time following infection with either reovirus type. (3) Following infection the cells grew and reached a significantly higher (95%, $P<0.01$) cell yield 72 h post-infection compared to that at the point of infection with either reovirus used. (4) There were no detached cells in the cultures at maximum cell yield (72 h post-infection). However, significant proportions of detached cells were in the supernatants 144 (43%) and 240 (82%) h post-infection in all cultures. (5) The cell viability was low 144 (60%) and 240 (23%) h post infection in all cultures. (6) There were significantly higher (40%, $P<0.01$) maximum cumulative type-3a reovirus titres compared to type-1 in T25 culture flasks. (7) The maximum cumulative reovirus titre was obtained significantly earlier (40%, $P<0.01$) from type-1 compared to type-3a. (8) There was no significant difference in the time (72 h post-infection) the maximum reovirus titres in the cells were obtained in all cultures. (9) There were no significant differences in the cell counts or maximum virus titres in original compared to enriched DMEM with respect to either reovirus type used. (10) Maximum Vero cell yields were significantly higher (90%, $P<0.01$) following reovirus infection at $1.00\text{--}1.20 \times 10^6$ cells/mL compared to those of L-929 cells (Schiff and Fields, 1990). (11) Maximum virus titres were obtained after significantly longer (233%, $P<0.01$) culture times compared to those for L-929 cells with either reovirus used. (12) The maximum cumulative reovirus titres were significantly higher (200%, $P<0.01$) from Vero cells compared to L-929 cells with either reovirus used and could be related to the increased maximum Vero cell yields.

Discussion. Reovirus were chosen to test the susceptibility of Vero cells to infection in T25

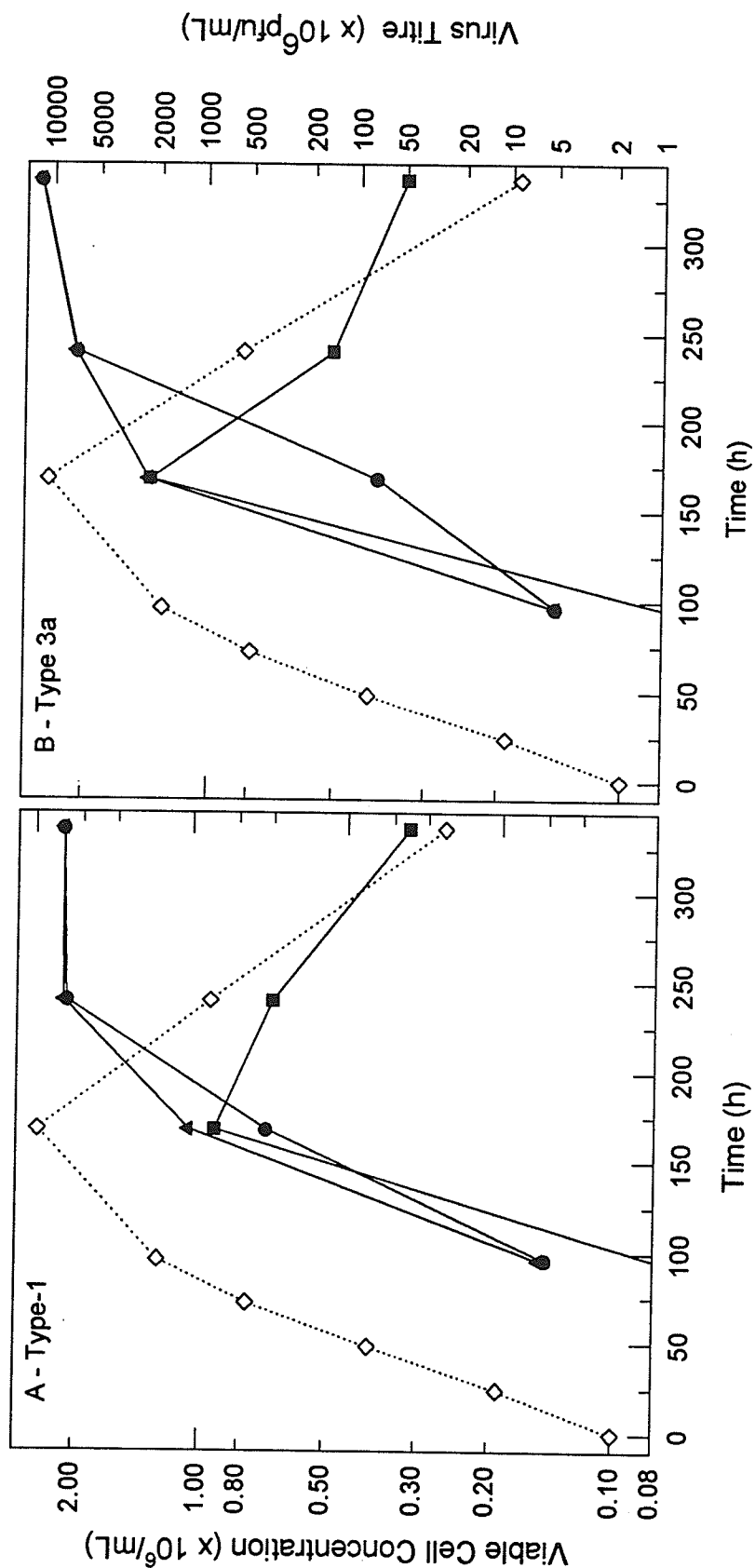


Fig. 12.1 - The effect of type-1 (A) and 3a (B) reovirus infection on Vero cell growth and virus titres in T25 culture flasks. The cells were inoculated at 0.10×10^6 cells/mL in 10 mL of enriched DMEM + 5.0% v/v sCS. The cells were grown for 96 h and infected at 5.0 virions/cell of either reovirus type. Reovirus titres were obtained for ● supernatant, ■ cells and ▲ cumulative total. Cell counts ◇ were obtained by the trypan blue method. The points are means of $n=2$.

culture flasks, as they have been less commonly used previously. Reovirus physiology and replication were discussed above (12.1). Virus replication is also pH dependent with an optimal range of pH 6.8-7.5 (Fields and Eagle, 1973) and these viruses infect a broad range of cells. Most of the previous literature on reoviruses has used murine L-929 cells as the hosts for replication and infections from high MOI (1.0-10.0 virions/cell) reach maximum titres 72 h post-infection (Schiff and Fields, 1990).

Vero cells could be infected with both type-1 and 3a reoviruses, although, there were differences in growth compared to L-929 cells following infection. Vero cells continued to grow for 72 h post-infection prior to cell yields and viability reducing up to 240 h post-infection. However, L-929 cell growth is inhibited within 6 h of reovirus incorporation due to blockage of DNA synthesis (Gomatos and Tamm, 1963, Ensminger and Tamm, 1969 and 1970 and Shaw and Cox, 1973). This effect could be cell line dependent involving differences in virus incorporation.

There was a significantly lower maximum type-1 reovirus titre compared to type-3a and the type-1 titre was reached earlier in culture. However, whilst maximum titres of both reoviruses were significantly higher from Vero cells compared to L-929 cells from similar MOIs the titres were in the same general ranges (10^9 - 10^{10}) (Schiff and Fields, 1990). The higher titres could result from Vero growth following infection, as titre differences were similar to cell yield differences.

12.3.2 Batch Spinner Flask Infections

Introduction. Most virus infection research has used spinner flask or bioreactor cultures with cells grown on microcarriers. However, there could be differences in virus productivity between substrata used for cell growth. The previous study (12.3.1) showed Vero cells could produce high titres of type-1 and 3a reoviruses in T25 culture flasks. Therefore, this study determined the effects of infecting Vero cells grown in spinner flasks on microcarriers.

Experimental. Preadapted stock Vero cells were grown in 100 mL spinner flasks on Cytodex-1 or Cultispher-G in enriched DMEM + 5.0% v/v SCS (12.2.1). The cultures were grown to approximately 1.00×10^6 cells/mL and infected with P1 type-1 and 3a reovirus. The flasks were either (a) "control" infected by swirling 1 min every 12 min for 1 h at 20°C, replacing the medium and stirring at 60 rpm or (b) "stirred" infection by immediately replacing the medium and continuously stirring at 60 rpm at 37°C.

Results. Table 12.1 shows Vero cell yields and reovirus titres in cultures of solid Cytodex-1 and macroporous Cultispher-G with swirled infections. (1) Vero cell infections were affected by the microcarrier type used and by the mode of stirring during infection. (2) The cell grew in cultures of either bead for 72 h following infection with either reovirus and infection stirring method used. There was no significant difference in the maximum cell yields in all cultures. (3) The microcarrier cell count was significantly reduced in all cultures 240 h post-infection with control (93%, $P < 0.01$) and continuously stirred (60%, $P < 0.01$) infections. The cell viability by the trypan blue method was low in control (12%) and continuously stirred (49%) cultures of either bead 240 h post-infection. (4) There were significantly higher titres of reovirus type-1 (83%, $P < 0.01$) and type-3a (555%, $P < 0.01$) in Cytodex-1 cultures compared to Cultispher-G in control cultures. The Cytodex-1 titres were not significantly different from those in T25 culture flasks (12.3.1). (5) There were significantly higher (138%, $P < 0.01$) type-3a reovirus titres compared to type-1 in Cytodex-1 control cultures. However, there were significantly higher (28%, $P < 0.01$) type-1 titres compared to type-3a in Cultispher-G control cultures. (6) Maximum type-1 reovirus titres in the controls were obtained significantly later (67%, $P < 0.01$) in Cultispher-G cultures compared to Cytodex-1. The time taken to reach maximum type-3a reovirus titre was similar in cultures of either bead. (7) There were significantly higher (five hundred and forty-fold, $P < 0.01$) maximum titres of either reovirus in control cultures compared to continuously stirred.

Discussion. Others have used spinner flask or bioreactor cultures of microcarriers in virus

Table 12.1 - The effects of **type-1** and **type-3a reovirus infection** of Vero cells in 100 mL spinner flasks on **1.00 g/L Cytodex-1 (A and C)** and **1.70 g/L Cultispher-G (B and D)** with **intermittent swirling for infection**. The cells were inoculated at 0.10×10^6 cells/mL in enriched DMEM + 5.0% v/v sCS and were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and ramped to 60 rpm after 72 h in culture. The cells were grown to 1.00×10^6 cells/mL, the medium was removed (90%) and the cultures infected at an MOI of 5.0 virions/cell. The flasks were swirled at 40 rpm for 1 min every 12 min for 1 h. Fresh medium (90%) was added and the flasks stirred at 60 rpm. The flasks were readjusted to pH 7.1 daily after infection with 2.00 M NaOH. The cell viability was determined by the trypan blue method. The values are means of n=2.

Table 12.1A) - Cytodex-1 infected with type-1 reovirus.

Time (h)	Cell Concentration ($\times 10^6$ /mL)	Cell Viability (%)	Virus Titre		
			(x 10^9 pfu/mL)		
			Supernatant	Cells	Cumulative
0	0.10 ± 0.02	97			
48	0.32 ± 0.02	95			
96	1.08 ± 0.08	95	0.005 ± 0.001	0.00	0.005 ± 0.001
168	$1.54 \pm 0.12^*$	93	0.600 ± 0.050	0.350 ± 0.035	0.950 ± 0.100
240	0.57 ± 0.05	55	5.800 ± 0.500	0.100 ± 0.010	5.900 ± 0.600
336	0.06 ± 0.01	18	5.900 ± 0.600	0.050 ± 0.005	$5.950 \pm 0.600^\#$

Table 12.1B) - Cultispher-G infected with type-1 reovirus.

Time (h)	Cell Concentration ($\times 10^6$ /mL)	Cell Viability (%)	Virus Titre		
			(x 10^9 pfu/mL)		
			Supernatant	Cells	Cumulative
0	0.10 ± 0.02	100			
48	0.26 ± 0.02	97			
96	0.97 ± 0.06	95	0.005 ± 0.001	0.00	0.005 ± 0.001
168	$1.51 \pm 0.11^*$	95	0.050 ± 0.005	1.350 ± 0.130	1.400 ± 0.140
240	0.51 ± 0.05	57	1.800 ± 0.180	0.160 ± 0.016	1.960 ± 0.200
336	0.09 ± 0.01	20	3.100 ± 0.300	0.150 ± 0.015	$3.250 \pm 0.300^\#$

* - Highest cell yield.

- Maximum virus titre.

Table 12.1C) - Cytodex-1 infected with type-3a reovirus.

Time (h)	Cell Concentration ($\times 10^6/\text{mL}$)	Cell Viability (%)	Virus Titre		
			(x 10^9 pfu/mL)		
			Supernatant	Cells	Cumulative
0	0.10 ± 0.02	95			
48	0.30 ± 0.02	100			
96	1.14 ± 0.06	96	0.005 ± 0.001	0.00	0.005 ± 0.001
168	$1.79 \pm 0.13^*$	93	0.030 ± 0.003	1.200 ± 0.120	1.230 ± 0.120
240	0.71 ± 0.04	59	7.000 ± 0.700	0.200 ± 0.020	7.200 ± 0.700
336	0.10 ± 0.01	21	14.100 ± 1.400	0.050 ± 0.005	$14.150 \pm 1.400^\#$

Table 12.1D) - Cultispher-G infected with type-3a reovirus.

Time (h)	Cell Concentration ($\times 10^6/\text{mL}$)	Cell Viability (%)	Virus Titre		
			(x 10^9 pfu/mL)		
			Supernatant	Cells	Cumulative
0	0.10 ± 0.02	97			
48	0.28 ± 0.02	97			
96	1.02 ± 0.07	93	0.005 ± 0.001	0.00	0.005 ± 0.001
168	$1.78 \pm 0.13^*$	94	0.080 ± 0.008	1.350 ± 0.130	1.430 ± 0.140
240	0.80 ± 0.04	53	0.200 ± 0.020	1.900 ± 0.190	2.100 ± 0.200
336	0.25 ± 0.01	15	2.300 ± 0.230	0.250 ± 0.025	$2.550 \pm 0.250^\#$

* - Highest cell yield.

Maximum virus titre.

research (van Hemert et al., 1969, Mered et al., 1981, Montagnon et al., 1981 and 1984, Reuveny et al., 1981, Scattergood et al., 1983, Varani et al., 1988, Nikolai and Hu, 1992, Marique et al., 1994 and Mendonca et al., 1993). HSV, rabies and VSV can all infect monolayers of Vero cells in cultures of either Cytodex-1 (Griffiths et al., 1981 and Mendonca et al., 1993) or at 2,500 cells/bead in Cultispher-G (Nikolai and Hu, 1992) beads. However, none of these authors directly compared the virus infection from the two bead types. Varani et al. (1988) showed the substrata could affect turkey herpes virus production from chick fibroblast cells with significantly higher titres from glass (Biosil) compared to polydextran (Cytodex-1) compared to collagen (gelatin - Ventregel) beads. The authors found no differences between substrata for the production of either HSV or bovine rhinotracheitis (BRV) viruses in MRC-5 or MDBK cells.

Vero cells could be infected with both types-1 and 3a reoviruses in cultures of either bead with intermittent swirling following infection. Maximum titres in these cultures were similar to those of previous authors with L-929 cells in suspension cultures and T25 culture flasks (Schiff and Fields, 1990 and 12.3.1). The titres were significantly higher in Cytodex-1 cultures compared to Cultispher-G with either reovirus suggesting the substrata could have affected titres. This could show substrata dependent differences in titres agreeing with Varani et al. (1988).

The low titres in Cultispher-G cultures could be explained by infection problems in cell masses in the inner bead matrix. Cells in masses could be inaccessible to virus particles during the infection stage especially with intermittent swirling where diffusion could be limiting and only cells on the outer periphery would be infected leading to low maximum titres. However, cells in Cytodex-1 cultures grow on the surface of the beads and could come into contact with virus particles more easily and thus, more cells infect leading to the higher titres.

The higher titres in Cytodex-1 cultures compared to Cultispher-G could also be explained by pH effects, as Cytodex-1 cultures were always at pH >6.7. However, Cultispher-G cultures were at pH <6.7 every day from 48 h post-infection onwards which could be due to lactate production

(5.3-6.3). The low pH in Cultispher-G cultures was below the optimal (pH 6.8-7.5) for type-3a reovirus (and possibly type-1 as well) reported in cultures of L-929 cells (Fields and Eagle, 1973).

Low titres could also be explained by the culture time, as Fields and Eagle (1973) showed total infection times of 30-72 h for L-929 cells, whereas, Vero cells were 240 h. This could result in the concentrations of growth nutrients becoming limiting due to cell growth prior to the viruses beginning to replicate. The effect could be exaggerated if the cells were not evenly infected (i.e. although the MOI was 5.0 virions/cell it could be the cells only took up 10-20% of the total virus present). The results from the continuously stirred cultures agree with this, as titres were very low in these cultures compared to intermittent swirled suggesting the virus infected the cells unevenly in the continuously stirred cultures. All previous reovirus research has used intermittent swirled infections (Schiff and Fields, 1990).

Nutrient effects could also explain low titres, although previous reports have contradictory data. van Hemert et al. (1969) suggested DOT had little or no effect on rubella virus production from BHK cells in microcarrier cultures, as virus accounts for <5% of the cell dry weight. However, others have shown increases in nutrient consumption following infection such as Marique et al. (1994) who suggested oxygen consumption could be correlated to the titre of cytolytic HSV. Pasternak et al. (1988) showed VSV infection increased hexose (e.g. glucose) transport in BHK cells. These effects could be virus and/or cell line dependent. Oxygen could explain low titres in spinner flasks cultures with Vero cells even in batch mode (Jenssen et al., 1974). No other nutrient limitations to virus titres have been discussed in the literature.

12.3.3 Daily Refed Spinner Flask Infections.

Introduction. Virus infection is almost exclusively used in batch mode with low cell/bead loadings which could result in low virus titres. Daily culture refeeding could significantly increase cell yields and possibly virus titres. Therefore, this study determined the effects of infecting Vero cells grown to various cell/bead loadings in daily refed spinner flasks with type-1 reovirus.

Experimental. Preadapted stock Vero cells were grown in 100 mL spinner flasks of Cytodex-1 and Cultispher-G in enriched DMEM + 5.0% v/v sCS. The cultures were grown to various cell/bead loadings in daily refed Cytodex-1 (150-1,200) and Cultispher-G (750-8,000) cultures prior to infection with the control method (12.2.2) with P2 type-1 reovirus at either (a) "high" MOI of 5.0 virions/cell or (b) "low" MOI of 0.5 virions/cell 48 h prior to reaching loadings of 250, 900 and 1,200 cells/bead in Cytodex-1 and 750, 6,000-8,000 cells/bead in Cultispher-G cultures.

Results. Fig 12.2 shows the maximum virus titres produced in cultures of Cytodex-1 and Cultispher-G with the cell concentration at infection from high and low MOI infections of type-1 reovirus. Fig. 12.3 shows virus yields/cell at the cell/bead loadings in these cultures. (1) Type-1 reovirus titres were affected by the cell concentration at infection (i.e. the cell/bead loading) and by the MOI used. (2) There were significantly higher (70%, $P<0.01$) reovirus titres (Fig. 12.2) in Cytodex-1 cultures compared to Cultispher-G with respect to all cell concentrations and MOI's used. (3) There were significantly higher ($P<0.01$) titres in Cytodex-1 cultures (Fig. 12.3) from high MOI at loadings of 400 cells/bead and in Cultispher-G cultures at 4,000 cells/bead compared to all other loadings used. (4) Maximum reovirus yields/cell in Cytodex-1 cultures from high MOI were significantly higher ($P<0.01$) at loadings up to 400 cells/bead and in Cultispher-G cultures at up to 1,000 cells/bead compared to all other loadings. (5) There was no significant difference in maximum reovirus titres in Cytodex-1 cultures at any cell/bead loadings used from low MOI. There was a significantly higher (18%, $P<0.05$) reovirus titre in Cultispher-G cultures at loadings of 750 and 6,000 cells/bead compared to 8,000 cells/bead from a low MOI. (6) There was no significant difference in the maximum reovirus yields/cell in Cytodex-1 cultures at any cell/bead loading from low MOI. There was a significantly higher maximum reovirus yield/cell (41%, $P<0.01$) in Cultispher-G cultures at 750 and 6,000 compared to 8,000 cells/bead from low MOI. (7) Maximum reovirus titres and yields/cell were significantly higher (458%, $P<0.01$) from low MOI compared to high MOI at 900 and 1,200 cells/bead in Cytodex-1 cultures and 6,000 and 8,000 cells/bead in Cultispher-G cultures. (8) The highest reovirus yields/cell

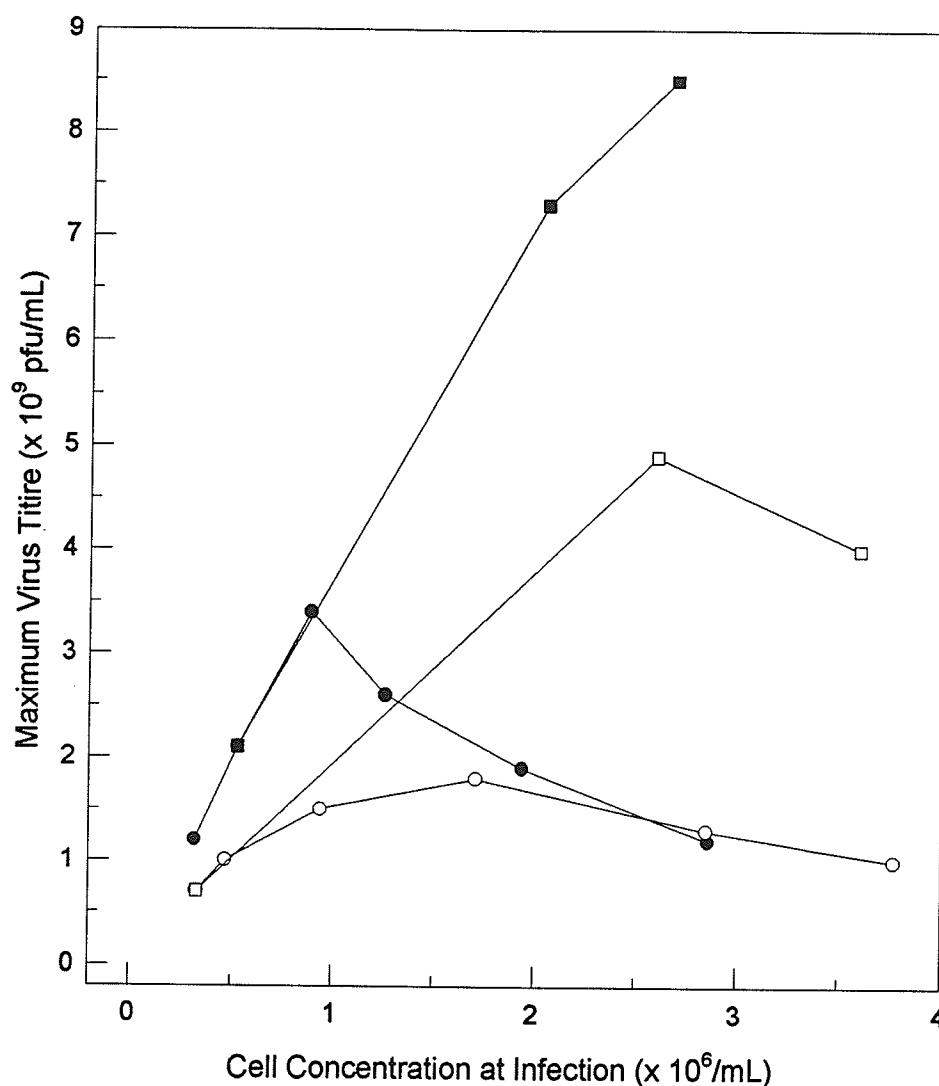


Fig. 12.2 - The effects of the Vero cell concentration at infection on maximum type-1 reovirus titres in 100 mL spinner flasks of 0.50 g/L Cytodex-1 (Closed symbols) and Cultispher-G (Open symbols). The cells were inoculated at 0.20×10^6 cells/mL in enriched DMEM + 5.0% v/v SCS and infected at various cell concentrations with either ● 5.0 at the cell concentration or ■ 0.50 virions/cell 48 h prior to reaching the cell concentration. The flasks were stirred at 40 rpm continuously (Cytodex-1) or intermittently for 24 h then continuously (Cultispher-G) and were ramped to 60 rpm after 72 h. The cultures were refed from 48 h onwards with one daily medium exchange (90% v/v) until the infection cell concentration was reached. The points are means of $n=2$.

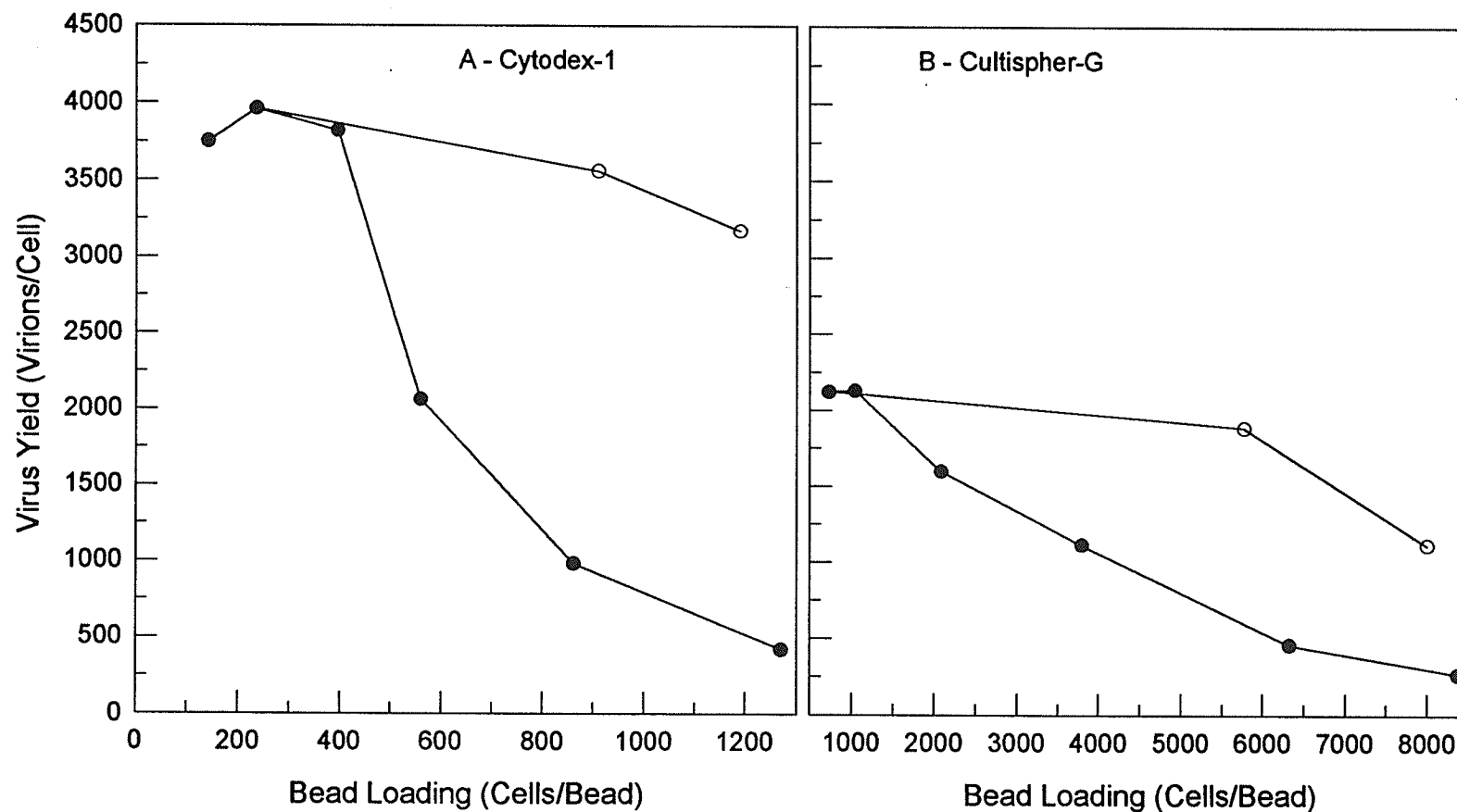


Fig. 12.3 - The effect of type-1 reovirus infection on the virus yield/Vero cell produced in 100 mL spinner flask cultures of 0.50 g/L **CytoDex-1** and **Cultispher-G** infected at either 5.0 (Closed symbols) or 0.50 virions/cell (Open symbols) at various cell/bead loadings. The cells were inoculated at 0.20×10^6 cells/mL in enriched DMEM + 5.0% v/v SCS and refed daily with one 90% v/v medium exchange after 48 h in culture. The cells were infected loadings of 150, 250, 400, 600, 900 or 1,200 cells/bead. The pH was readjusted to pH 7.1 after 48 h in culture with 2.00 M NaOH. The points are means of $n=2$.

were not significantly different from either MOI in cultures of either bead.

Discussion. Griffiths and Thornton (1982) used a semi-continuous perfusion system which increased cell yields (up to x5) and virus titres (x1.5) compared to batch but gave significantly decreased (67%) virus yields/cell. Previous studies (10.3 and 11.3) showed Vero cell/bead loadings could be significantly increased ($>x3$) in cultures of either bead by daily refeeding and if all these cells could be infected with viruses the virus titres could be increased significantly.

Maximum reovirus titres and yields/cell were reduced proportional to the increase in cell/bead loading when infected in cultures of either bead with high MOI. This suggests the infection was unsuccessful once several cell layers had formed on Cytodex-1 beads and the void spaces of Cultispher-G became filled with cells. The results agree with those of Griffiths and Thornton (1982). This could be expected as infection required almost static conditions in this study which could influence diffusion to lower or inner cell layers. Previous results (12.3.2) showed it was impossible to infect Vero cells in cultures of either bead with continuous stirring to improve diffusion. Infections with higher infectivity viruses such as polio, rabies and HSV is possible with continuous stirring (Montagnon et al., 1981, Varani et al., 1988 and Mendonca et al., 1993) and could lead to improved virus titres in cultures with high cell/bead loadings. This was not determined in this study.

Cultures of either bead infected with low MOI prior to reaching maximum cell/bead loading had increased virus titres. This could be due to virus transfer between mother and daughter cells during cell divisions allowing more cells to be infected prior to the production stage. However, the yields/cell were significantly lower at the highest cell/bead loadings showing the process was not completely optimised. Nutrient limitations could explain this and further experiments using a fed-batch approach following infection could lead to significantly increased virus titres. This was not performed in this study. No one has previously reported using a fed-batch approach following infection. Culture pH and DOT control could also improve the productivity of high cell/bead loaded cultures but this was also not determined in this study.

The methods used in this study show Vero cells could produce high reovirus titres with culture refeeding and reduced MOI. This process could have a large impact on vaccine manufacture and shows these cells could be used for commercial production of a reovirus vaccine should interest in one arise.

12.4 Conclusions

The purpose of this study was to determine virus infection strategies and productivity from Vero cells in microcarrier cultures. The results above show that:

- **Vero cells could be infected** with type-1 and 3a reoviruses in both static T25 culture flasks and 100 mL spinner flask cultures of Cytodex-1 and Cultispher-G microcarriers.
- **maximum type-1 reovirus titres** were reached earlier (144 h) in culture compared to type-3a.
- **maximum type-3a reovirus titres** were significantly higher (43%) compared to type-1.
- **reovirus titres were significantly higher** in Cytodex-1 cultures and T25 culture flasks compared to Cultispher-G cultures with respect to either reovirus type. This could be caused by a substrata dependent or bead structure difference affecting the virus infection phase. Diffusion problems during the semi-static infection phase could be responsible for the Cultispher-G results.
- **increased cell/bead loadings** in cultures of either bead infected at high MOI (5.0 virions/cell) produced proportionally decreased maximum virus yields/cell. This could result from poor virus diffusion to lower cell layers on or in the beads and hence, poor infection.
- **high cell/bead loadings** in cultures of Cytodex-1 (900-1,200 cells/bead) and Cultispher-G (6,000-8,000 cells/bead) produced significantly higher titres (458%, $P < 0.01$) when infected at low (0.5 virions/cell) compared to high (5.0 virions/cell) MOI. The semi-static infection phase could be involved with this effect through diffusion problems to lower cell layers.
- **Vero cells were poorly infected** with both type-1 and 3a reovirus in continuously stirred cultures.

Chapter 13. General Discussion

13. General Discussion

At the outset of the research in this Thesis I outlined several aims for the work. I hope that the previous Chapters have answered all of them. The aim of this Chapter is to draw attention to the major achievements from these experiments and to describe how they could impact on the future uses for all adherent cell lines. The work in this Thesis left some questions unanswered and these will be posed in each section below.

13.1 Most Significant Results From the Thesis

My results of Vero cell yields in Cultispher-G cultures are the most important result from this Thesis. The cell yields were consistently higher in these beads and there were differences in some parameter optima (e.g. pH, stirrer speed, optimal glucose, serine and glutamine concentrations) when compared to Cytodex-1. Cells grown in Cultispher-G cultures also consumed the medium nutrients more efficiently compared to Cytodex-1. The maximum cell/bead loadings of Cultispher-G beads were approaching an order of magnitude higher compared to Cytodex-1. This has major implications on the bead concentrations required to generate a given cell yield. However, there were some disadvantages of using Cultispher-G beads which included a rapid decrease in culture pH due to rapid utilisation of glucose leading to the production of lactate. This had the knock on effect of increasing Cultispher-G bead aggregation at the acidic pHs. Cultures of either bead suffered from problems of bead loss in both spinner flasks and Celligen bioreactor. Indeed, the bead numbers stuck on the vessel surfaces and filter screens increased with the culture time. Nothing could be done in these studies to prevent this effect, although, the use of weighted beads such as Cultispher-GLD or Verax could improve this by allowing higher stirring speeds to be used to prevent bead aggregation. Even taking these problems into account I would still recommend growing Vero cells in Cultispher-G beads, as the benefits in cell/bead loadings outweigh the problems.

Vero cells are commercially used in virus vaccine production but are not used for any secreted proteins, although they have been used recently for the production of virus antigen

proteins. My results indicate Vero cells are easy to culture and can grow to significantly higher cell yields compared to suspension cells in the Celligen bioreactor system used with significantly lower total media volumes used. Whilst the cells require beads for growth, which increases process costs, the advantages to perfusion culture of using such supports outweigh their costs. New Brunswick Scientific® would seem to share this view as they now market a Celligen Plus bioreactor using fibrous Biomat™ discs of polystyrene for suspension cells in order to grow higher cell densities. The ease of growing Vero cells and their regulatory acceptance for human therapeutics make them ideal host cell for genetically engineering secreted product genes into. I hope that in the near future others share my views, as this is a much under utilised cell line. It is my view that the methods presented in this Thesis could be applied to the culture of any adherent cell line to high densities. Many such adherent cell lines are used for virus vaccine production including BHK21, MDCK, MRC-5 and WI-38 cells. Perfusion microcarrier culture of adherent cell lines was easy in this Thesis due to the knowledge which was established through medium optimisation. The finding that serine was absolutely required in order to generate maximum cell yields with efficient medium utilisation is also one of the most important results observed. This has never been previously reported and could have major implications to the future of Vero cells as host cell lines. Taurine was not required for high cell densities but could allow further improvement in cultures. This again has never been previously reported for this cell line.

One of my final comments, for which I am sure the reader is glad, is that as of December (1995) Hy-Clone® are no longer commercially marketing the Cultispher range of beads supposedly due to a lack of interest in their use. My condolences to Hy-Clone. This is especially interesting, as Sigma® Chemical Co. (St. Louis, MO, U.S.A.) has announced that they will now be supplying the Cultispher-G beads from the manufacturers (Percell® Biolytica, Lund, Sweden). It should also be mentioned that the methods of producing similar beads have been widely reported in the literature which allows interested parties to manufacture their own gelatin beads.

13.2 Cell Counting

One of my results with the most impact is that binucleated Vero cells could be found throughout cultures and especially in the stationary phases. These binucleates caused significantly higher counts by the crystal violet nuclei stain method compared to the whole cell trypan blue method. A further problem with this was the cyclic and hypervariable nature of the differences. I strongly advise that the crystal violet technique is used with extreme care and only with a prior knowledge of the size of difference which occurs with the chosen cells and growth systems compared to the trypan blue method. This finding draws into question the use of this technique over the last 30 years. I will make the comment that ones results are only as good as the techniques used in obtaining them which agrees with previous comments (Baserga, 1989). Whilst industry is required to validate every minute aspect involved in the production of compounds often academic research fails in this respect which leads to unrepeatable experiments and questionable accuracy. This must stop if academia is to gain the respect of it's industrial peers!

My study also showed that fluorescence activated cell sorting (FACS) analysis was prone to errors from cell losses during sample preparation and from the presence of binucleated cells. The data suggested hybridoma cells were not susceptible whilst adherent cells were. I suggest great care is taken in interpreting FACS analyses and especially Map 2 to ensure there were no significant cell losses during analysis. Questions:

1. Do all adherent cells show similar cell count differences by crystal violet and trypan blue methods (i.e. do they binucleate)?
2. Do other adherent cells have problems with FACS analysis?

These could be tested by growing cells such as MDCK, BHK21 or MRC-5 in similar systems.

13.3 Serum-Free Medium

There has only been one previously reported serum-free medium for the growth of Vero cells

in microcarrier cultures. However, their medium showed poor growth characteristics with long lag phases (>48 h). The chemically and hormonally defined serum-free medium (VSFM) was another major achievement. However, cells could not be passaged indefinitely in VSFM, although, it could be used as a maintenance medium for the production phase of a secreted product. Thus, the expensive components, fibroblastic cell growth factor and fibronectin, could be removed making VSFM highly cost effective whilst retaining the product recovery advantages of serum-free formulations. Questions:

1. What components were missing to allow indefinite culturing?
2. Could Vero cells grown in serum-free media be infected with and produce viruses or other secreted proteins?
3. Could the medium be used to culture other similar adherent cells?

Components such as lipids, EGF, dexamethasone and interleukins could be important to this.

13.4 Cell Yields in Perfusion Culture

My Vero cell yields in perfusion cultures are also very important to the manufacture of secreted products and viruses. The cells reached maximum yields which could be linearly scaled-up by increasing the perfusion rate and bead concentration accordingly but only when DMEM was enriched with serine. No one has previously described the growth of Vero cells being dependent on serine. Growth was also highly dependent on glucose, glutamine and serum factors. The perfusion growths with ramped refeed rates also showed that significantly higher cell yields could be produced from lower total medium volumes compared to previous authors.

Virus infection of cultures at high cell/bead loadings was impossible with normal multiplicities of infection (MOI). However, this problem could be overcome by further optimising the system using a lower MOI at an earlier culture time to allow cell division to pass virus to most of the cells. Questions:

1. What is the absolute maximum Vero cell yield which can be achieved in a Celligen bioreactor?
2. Can the methods developed in the Celligen be transferred to alternate technologies such as fluidised bed bioreactors with similar results?
3. What do the cells use serine for?
4. What is the cells efficiency of glucose and glutamine metabolism?
5. Could the perfusion process be improved by using less ammoniagenic alternatives to glutamine (i.e. glutamine dipeptides)?

13.5 Taurine

My improved yields in media supplemented with taurine was another unusual finding. Taurine has been reported to be required by human cells and is supplemented into feline feedstuffs. The taurine was not essential for cell growth but did significantly increase growth in some batch and refed cultures. Therefore, I would recommend taurine be added to growth medium for all cells even when they can grow in it's absence. Questions:

1. How does taurine affect cell growth?
2. Are other cell lines affected by taurine supplementation of the medium?

13.6 Growth Inhibitors

I found that many cell growth inhibitors affected nutrient consumption rates and in particular serine consumption. Indeed, the effects of several inhibitors were reduced in serine enriched DMEM. This effect could involve membrane damage and repair, as serine could be metabolised for membrane biosynthesis. Elucidation of serine metabolism could lead to better understanding of this effect. The cell growth and inhibitory concentration (IC₅₀) differences between the bead types were also important and suggest the cells could have different morphologies growing in Cultispher-G beads compared to Cytodex-1. The morphological difference could allow increased product secretions if the cells were producing a valuable protein. Questions:

1. Does serine metabolism affect cell membrane biosynthesis?
2. Does the cell morphology differ between the beads?
3. Does the cell morphology affect protein synthesis and secretion?
4. Can other adherent cells be protected from growth inhibitors by medium enrichment?

13.7 Cell Attachment

I found that cell attachment differed between the bead types. Attachment to Cytodex-1 beads was not serum dependent, whereas, it was serum dependent in Cultispher-G cultures. However, cell flattening and growth were serum dependent in cultures of either bead. Attachment to Cultispher-G beads could involve entrapment of cell aggregates and not just strict protein mediated attachment or could involve both mechanisms. Questions:

1. Which serum factors affect cell attachment to Cultispher-G beads?
2. Does aggregate formation precede cell attachment to Cultispher-G beads?

Literature Cited

- Abercrombie, M. and Ambrose, E.J., The surface properties of cancer cells: A review. *Cancer Res.* 22, 525-548. (1962).
- Adema, E., Shnek, D., Cahn, F. and Sinskey, A.J., Use of porous microcarriers in agitated cultures. *BioPharmaceuticals*. 3 (7), 20-24. (1990).
- Akimoto, S.K., Obinata, A., Hiroyoshi, E. and Hirano, H., Immunohistochemical study of basement membrane reconstruction by an epidermis-dermis recombination experiment using chick skin: Induction of tenascin. *J. Histo. and Cyto.* 40 (8), 1129-1137. (1992).
- Akiyama, S.K., Nagata, K. and Yamada, K.M., Cell surface receptors for extracellular matrix components. *Biochem. et Biophys. Acta.* 1031, 91-110. (1990).
- Alkhatib, G., Richardson, C. and Shen, S.H., Intracellular processing, glycosylation and cell-surface expression of the measles virus fusion protein (F) encoded by a recombinant adenovirus. *Virology*. 175 (1), 262-270. (1990).
- Altankov, G., Brodvarova, I. and Rashkov, I., Synthesis of protein coated gelatin microspheres and their use as microcarriers for cell culture. 1. Derivatisation with native collagen. *Biomaterials Science. Polymer Ed.* 2 (2), 81-89. (1991).
- Amadori, M., Berneri, C. and Archetti, I.L., Immunogenicity of foot and mouth disease virus grown in BHK-21 suspension cells: Correlation with cell ploidy alterations and abnormal expression of the $\alpha 5\beta 1$ integrin. *Vaccine*. 12, 159-166. (1993).
- Amos, D.B., Bashir, H., Boyle, W., MacQueen, M. and Tiilikainen, A., A simple micro cytotoxicity assay. *Transplantation*. 7 (3), 220-223. (1969).
- Arathoon, W.R. and Birch, J.R., Large scale cell culture in biotechnology. *Science*. 232, 1390-1395. (1986).
- Avgerinos, G.C., Drapeau, D., Socolow, J.S., Mao, J., Hsiao, K. and Broeze, R.J., Spin filter perfusion system for high concentration cell culture: Production of recombinant utPA in CHO cells. *Biotechnology*. 8, 54-58. (1990).
- Barnes, D.W., Sirbashu, D.A. and Sato, G.H. (Eds.), *Methods for the preparation of media, supplements and substrata for serum-free culture of a) endocrine, b) epithelial and fibroblast, c) neuronal and lymphoid cells*. Plenum Press. New York, NY, USA. (1984).
- Barngrover, D., Thomas, J. and Thilly, W.G., High cell yield mammalian cell growth in Leibovitz bicarbonate free medium: Effect of fructose and galactose on culture biochemistry. *J. Cell Science*. 78, 173-189. (1985).
- Barngrover, D., Substrata for anchorage dependent cells. In: *Mammalian Cell Technology*. Thilly, W.G. (Ed.). Butterworths. Stoneham, MA, U.S.A.. 131-150. (1989).
- Barsh, G.S. and Cunningham, D.D., Nutrient uptake and control of animal cell proliferation. *J. of Supramolecular Structure*. 7, 61-77. (1977).
- Bashir, N., Kuhen, K. and Taub, M., Phospholipids regulate growth and function of MDCK cells in hormonally defined serum-free medium. *In Vitro*. 28A, 663-668. (1992).

Bayreuther,K., Francz,P.I., Gogol,J., Hapke,C., Maier,M. and Meinrath,H-G., Differentiation of primary and secondary fibroblasts in cell culture systems. *Mutation Research.* 256, 233-242. (1991).

Beale,A.J., The production of viruses for human vaccines from animal cells in culture. In: *Animal Cell Biotechnol.* Spier,R.E. and Griffiths,J.B. (Eds.). Academic Press. London, U.K.. 5, 189-200. (1992).

Berman,P.W. and Laskey,L.A., Engineering glycoproteins for use as pharmaceuticals. *TIBTECH* 3, 51-53. (1985).

Bettger,W.J., Boyce,S.T., Walthall,B.J. and Ham,R.G., Rapid clonal growth and serial passage of human diploid fibroblasts in lipid enriched synthetic medium supplemented with EGF, insulin and dexamethasone. *PNAS.* 78, 5588-5592. (1981).

Bibila,T. and Flickinger,M.C., A structured model for MAb synthesis in exponentially growing and stationary phase hybridoma cells. *Biotechnol. and Bioeng.* 37, 210-226. (1991).

Bliem,R. and Katinger,H., Scale-up engineering in animal cell technology. Part I and II. *TIBTECH.* 6, 190-230. (1988).

Billig,D., Clark,J.M., Ewell,A.J., Carter,C.M. and Gebb,C., The separation of harvested cells from microcarriers: A comparison of methods. *Develop. Biol. Standards.* 55, 67-75. (1984).

Birch,J.R., Thompson,P.W., Boraston,R., Oliver,S. and Lambert,K., The large scale production of monoclonal antibodies in airlift fermenters. In: *Plant and Animal Cells: Process Possibilities.* Webb,C. and Mavituna,F. (Eds.). Ellis-Horwood Ltd.. Manchester, U.K.. 162-171. (1987).

Bober,F.J., Birk,D.E. and Raska,K., Expression of varying portions of the adenovirus 12 early region 1 in transformed cells affects tumorigenicity and interactions with extracellular matrix components. *Lab. Investigation.* 56 (1), 37-43. (1987).

Bodeker,B.G.D., Berg,G.J., Hewlett,G. and Schlumberger,H.D., Procedures to test for the serum-free cultivation of adherent cell lines on microcarriers. *J. Tissue Culture Methods.* 8 (4), 161-165. (1984).

Borys,M.C. and Papoutsakis,E.T., Formation of bridges and large cellular clumps in CHO cell microcarrier cultures: Effects of agitation, DMSO and calf serum. *Cytotechnology.* 8, 237-248. (1992).

Bottenstein,J.E. and Sato,G.W., Fibronectin and poly-lysine requirement for proliferation of neuroblastoma cells in defined medium. *Exp. Cell Res.* 129, 361-366. (1980).

Bradshaw,G.L., Sato,G.H., McClure,D.B. and Dubes,G.R., The growth requirements of BHK-21 cells in serum-free medium. *J. Cellular. Phys.* 114, 215-221. (1983).

Broad,D., Boraston,R. and Rhodes,M., Production of recombinant proteins in serum-free media. *Cytotechnology.* 5, 47-55. (1991).

Burger,M., Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. *Nature.* 227, 170-171. (1970).

Butler,M., The characteristics and growth of cultured cells. In: *Mammalian Cell Biotechnol.: A practical approach.* Butler,M. (Ed.). IRL Press. Oxford, U.K.. 1-25. (1991).

- Butler,M. and Dawson,M. (Eds.), *Cell Culture - Labfax*. BIOS-Blackwell. Oxford, U.K.. (1992).
- Butler,M., Hassell,T. and Rowley,A., The use of microcarriers in animal cell cultures. In: *Plant and Animal Cells: Process Possibilities*. Webb,C. and Mavituna,F. (Eds.). Ellis-Horwood Ltd.. Manchester, U.K.. 64-74. (1987).
- Butler,M., Imamura,T., Thomas,J. and Thilly,W.G., High yields from microcarrier cultures by medium perfusion. *J. Cell Science*. 61, 351-363. (1983).
- Butler,M. and Spier,R.E., The effects of glutamine utilisation and ammonia production on the growth of BHK cells in microcarrier cultures. *J. Biotechnol.* 1, 187-196. (1984).
- Butler,M. and Thilly,W.G., MDCK microcarrier culture: Seeding cell concentration effects and amino acid utilisation. *In Vitro*. 18 (3), 213-219. (1982).
- Carr,K., BSE: The questions that need answers. *Nature*. 380, 273-274. (1996).
- Carrell,A., Artificial activation of the growth in vitro of connective tissue. *J. Exp. Med.* 17, 14-17. (1913).
- Cassiman,J.J., Brugmans,M. and van den Berghe,H., Growth and surface properties of disperse dissociated Hu fibroblasts. *Cell Biol. Int. Rep.* 5 (2), 125-132. (1981).
- Ceccarini,C., Effect of pH on plating efficiency, serum requirement and incorporation of radioactive precursors into human cells. *In Vitro*. 11, 78-86. (1975).
- Ceccarini,C. and Eagle,H., pH as a determinant of cellular growth and contact inhibition. *PNAS*. 68 (1), 229-235. (1971).
- Chattopadhyay,D., Rathman,J.F. and Chalmers,J.J. The protective effect of specific medium additives with respect to bubble rupture. *Biotechnol. and Bioeng.* 45, 473-480. (1995).
- Cherington,P.V., Smith,B.L. and Pardee,A., Loss of EGF requirement and malignant transformation. *PNAS*. 76, 3937-3941. (1979).
- Cherington,P.V., Regulation of fibroblast growth by multiple growth factors in serum free media. In: *Mammalian Cell Culture - The use of serum-free hormone supplemented media*. Mather,J.P. (Ed.). Academic Press. New York, NY, U.S.A.. 17-52. (1984).
- Cherry,R.S. and Papoutsakis,E.T., Physical mechanisms of cell damage in microcarrier cell culture bioreactors. *Biotechnol. and Bioeng.* 32, 1001-1014. (1988).
- Cherry,R.S. and Papoutsakis,E.T., Growth and death rates of bovine embryonic kidney cells in turbulent microcarrier bioreactors. *Bioprocess Eng.* 4, 81-89. (1989).
- Chotteau,V. and Bastin,G., A general 2-step procedure for the kinetic modelling of animal cell cultures. In: *Proceedings of 12 ESACT Meeting*. Spier,R.E. and Griffiths,J.B. (Eds.). Butterworths-Heinemann. London, U.K.. 545-548. (1994).
- Christie,A. and Butler,M., GLutamine-based dipeptides are utilised in mammalian cell culture by extracellular hydrolysis catalysed by a specific peptidase. *J. of Biotechnol.* 37, 277-290. (1994).
- Cinatl,J.Jr., Cinatl,J., Rabeneau,H. and Doerr,H.W., Polyvinyl formal surface promotes continuous growth of Vero cells in protein-free medium. *Biologicals*. 20, 59-65. (1992).

- Cinatl,J.Jr., Cinatl,J., Rabenau,H. and Doerr,H.W., Protein free cell culture on polyvinyl formal surface. *In Vitro*. 28A, 147-148. (1992).
- Cinatl,J.Jr., Cinatl,J., Rabeneau,H. and Doerr,H.W., Polyvinyl formal surface promotes continuous growth of Vero cells in protein-free medium. *Biologicals*. 20, 59-65. (1992).
- Cinatl,J.Jr., Cinatl,J., Rabenau,H., Rapp,J., Kornbauer,B. and Doerr,H.W., Protein-free culture of Vero cells: A substrate for replication of human pathogenic viruses. *Cell Biol. Int.* 17 (9), 885-895. (1994).
- Clark,J.M., Hirtenstein,M.D. and Gebb,C., Critical parameters in the microcarrier culture of animal cells. *Develop. Biol. Standards.* 46, 117-124. (1980).
- Clark,J.M. and Hirtenstein,M.D., Optimising culture conditions for the production of animal cells in microcarrier culture. *Annals of New York Academy of Sciences*. C28, 33-45. (1981).
- Clarke,J.D. and Smith,C., The response of normal and polyoma virus transformed BHK21 cells to exogenous purines. *J. Cell. Phys.* 81, 125-132. (1973).
- Cocomartin,J.M., Oberink,J.W., Brunink,F., van der Velden de Groot,T.A.M. and Beuvery,E.C., Instability of a hybridoma cell line in a continuous perfusion culture system. *Hybridoma*. 11 (5), 653-665. (1992).
- Couchman,J.R., Hook,M., Rees,D. and Timpl,J., Adhesion, growth and matrix production by fibroblasts on laminin substrates. *J. Cell Biol.* 96, 177-183. (1983).
- Coulter,W.H., US Patent. No.: 2, 656, 508. (1953).
- Crespi,C.L., Imamura,T., Leong,P-M., Fleischaker,R.J., Brunengraber,H. and Thilly,W.G., Microcarrier culture: Applications in biological production and control. *Biotechnol. and Bioeng.* 23, 2673-2689. (1981).
- Croughan,M.S., Hamel,J-F.P. and Wang,D.I.C., Hydrodynamic effects on animal cells in microcarrier culture. *Biotechnol. and Bioeng.* 29, 130-141. (1987).
- Croughan,M.S., Hamel,J-F.P. and Wang,D.I.C., Effects of microcarrier concentration in animal cell culture. *Biotechnol. and Bioeng.* 32, 975-982. (1988).
- Croughan,M.S. and Wang,D.I.C., Reversible removal and hydrodynamic phenomena in CHO microcarrier culture. *Biotechnol. and Bioeng.* 36, 316-319. (1990).
- Cunningham,J.M. and Hodgson,H.J.F., Liver assist devices. *Int. J. Artificial Organs*. 15, 162-169. (1992).
- Curtis,A.S.G. and McMurray,H.M.J., Conditions for fibroblast adhesion without fibronectin. *J. Cell Science*. 86, 25-34. (1986).
- Dahl,D.C., Tsao,T and Rabkin,R., Ammonium chloride increases kidney cell protein content. *Mineral Electrolyte Metab.* 18, 104-107. (1992).
- Danilov,Y.N. and Juliano,R.L., (Arg-Gly-Asp)_n-albumin conjugates as a model substratum for integrin mediated cell adhesion. *Exp. Cell Res.* 182, 186-196. (1989).

- Darzynkiewicz,Z., Sharples,T., Staiano-Coico,L. and Melamed,M.R., Subcompartments of the G1 phase of the cell cycle detected by flow cytometry. *PNAS*. 77 (11), 6696-6699. (1980).
- Davis,B.D., Dulbecco,R., Eisen,H.N. and Ginsberg,H.S. (Eds.), *Microbiology*. 4 Ed. Lippincott. Philadelphia, PA, U.S.A.. (1990).
- Dean,R.C., Karkare,S.B., Phillips,P.G., Ray,N.G. and Runstadler,P.W.Jr, Continuous cell culture with fluidised sponge beads. In: *Large Scale Animal Cell Culture Technology*. Lydersen,B.K. (Ed.). Plenum Press. New York, NY, U.S.A.. 145-167. (1987).
- Doillon,C.J., Silver,F.H. and Berg,R.A., Fibroblast growth on porous collagen sponge containing hyaluronic acid and fibronectin. *Biomaterials*. 8 (5), 195-200. (1987).
- Dorr,R.T., Clinical properties of yeast derived versus *Escherichia coli* derived granulocyte macrophage colony stimulating factor. *Clin. Ther.* 15 (1), 19-29. (1993).
- Doyle,C. and Butler,M., The effect of pH on the toxicity of ammonia to murine hybridoma. *J. Biotechnol.* 15, 91-100. (1990).
- Dulbecco,R. and Freeman,G., Plaque production by the polyoma virus. *Virology*. 8, 396-397. (1959).
- Duval,D., Demangel,C., Geahel,I., Blondeau,K. and Marcadet,A., Comparison of various methods for monitoring hybridoma cell proliferation. *J. Immunol. Methods*. 134, 177-185. (1990).
- Duval,D., Demangel,C., Mirosec,J. and Geahel,I., Role of metabolic waste products in the control of cell proliferation and antibody production by mouse hybridoma cells. *Hybridoma*. 11 (3), 311-322. (1992).
- Eagle,H., Nutrition needs of mammalian cells in tissue culture. *Science*. 122, 501-504. (1955).
- Eagle,H., Barban,S., Levy,M. and Schultze,H.O., The utilisation of carbohydrates by human cell cultures. *J. Biol. Chem.* 233, 551-558. (1958).
- Egrie,J., The cloning and production of recombinant human erythropoietin. *Pharmacotherapy*. 10, 3S-8S. (1990).
- El-Karamany,R.M., Production in Vero cells of an inactivated rabies vaccine from strain FRV/K for animal and human use. *Acta Virologica*. 31, 321-328. (1987).
- Engelhardt,D.L. and Mao,J.L., A serum factor requirement for the passage of Vero cells through G2. *J. Cell. Physiol.* 90, 307-320. (1977).
- Ensminger,W.D. and Tamm,I., The step in cellular DNA synthesis blocked by reovirus infection. *Virology*. 39, 935-938. (1969).
- Ensminger,W.D. and Tamm,I., Inhibition of synchronised cellular DNA synthesis during Newcastle disease virus, mengovirus or reovirus infection. *J. Virology*. 5, 672-676. (1970).
- Epstein,J., SV40 transformed human cells fail to grow in Zn^{2+} concentration which permit normal human fibroblasts to proliferate. *J. Cell Physiol.* 110, 17-22. (1982).
- Fast,D.G., Jamieson,J. and McCaffrey,G., The role of the carbohydrate chains of Gal- β -1,4-GlucNAc- α 26 sialyltransferases for enzyme activity. *Biochim. et Biophys. Acta.* C1-C26. (1993).

- Feng,B., Hilt,D.C. and Max,S.R., Transcriptional regulation of glutamine synthetase gene expression by dexamethasone in L6 muscle cells. *J. Biol. Chem.* 265 (30), 18702-18706. (1990).
- Fields,B.N. and Eagle,H., The pH dependence of Reovirus synthesis. *Virology*. 52, 581-583. (1973).
- Finter,N.B., Allen,G., Ball,G.D., Fantes,K.H., Johnston,M.D. and Lockyer,M.J., Production of broad spectrum interferons from sendai virus induced Namalwa cells. *Lab. Technol.* March/April. 157-159. (1984).
- Fisher,D.A. and Lam,R.W., Thyroid hormone binding by bovine and ovine fetuin. *Endocrinology*. 94, 49-54. (1974).
- Foran,D.J., Cahn,F. and Eikenberry,E.F., Assessment of cell proliferation on porous microcarriers by means of image analysis. *Anal. and Quantitative Cytology and Histochem.* 13 (3), 215-222. (1991).
- Forestall,S.P., Kalogerakis,N. and Behie,L.A., Low serum medium development for human diploid fibroblast microcarrier cultures. *Appl. Microbiol. Biotechnol.* 38, 165-172. (1992).
- Forestall,S.P., Kalogerakis,N., Behie,L.A. and Gerson,D.P., Development of the optimal inoculation conditions for microcarrier cultures. *Biotechnol. and Bioeng.* 39, 305-313. (1992).
- Fujiwara,K. and Pollard,T.D., Fluorescent antibody localisation of myosin in the cytoplasm, cleavage furrow and mitotic spindle of human cells. *J. Cell Biol.* 71, 848-875. (1976).
- Gebb,C. Lundgren,B., Clark,J.M. and Lindskog,U., Harvesting and subculturing cells growing on denatured collagen coated microcarriers. *Develop. Biol. Standards.* 55, 57-65. (1984).
- Gibco BRL, *Catalogue and Reference Guide*. Gibco, Grand Island, NY, U.S.A.. (1992).
- Gibco BRL, *Price List*. Gibco, Grand Island, NY, U.S.A.. (1995/6).
- Giguere,L., Cheng,J. and Gospodorawicz,D., Factors involved in the control of proliferation of bovine corneal endothelial cells in serum-free medium. *J. Cell Phys.* 110 (1), 72-80. (1982).
- Gille,J.P., Pasman,P., van Berkel,C.G.M. and Joenje,H., Effect of antioxidants on hyperoxia induced chromosomal breakage in CHO cells: Protection by carnosine. *Mutagenesis*. 6 (4), 313-318. (1991).
- Gillies,R.J., Didier,N. and Denton,M., Determination of cell number in monolayer culture. *Anal. Biochem.* 159, 109-113. (1986).
- Glacken,M.W., Fleischaker,R.J. and Sinskey,A.J., Reduction of waste product excretion via nutrient control: Possible strategies for maximising product and cell yields in cultures of mammalian cells. *Biotechnol. and Bioeng.* 28, 1376-1389. (1986).
- Glacken,M.W., Adema,E. and Sinskey,A.J., Mathematical descriptions of hybridoma culture kinetics: Initial metabolic rates. *Biotechnol. and Bioeng.* 32, 491-506. (1988).
- Goetghebeur,S. and Hu,W-S., Cultivation of anchorage dependent animal cells in microsphere-induced aggregate cultures. *Appl. Microbiol. Technol.* 34, 735-741. (1991).

- Gomatos,P.J. and Tamm,I., Macromolecular synthesis in reovirus infected L cells. *Biochim. et Biophys. Acta.* 72, 651-653. (1963).
- Goochee,C.F. and Monica,T., Environmental effects on protein glycosylation. *Biotechnology.* 8 (5), 421-427. (1990).
- Graff,S. and McCarty,K.S., Sustained cell culture. *Exp. Cell Res.* 13, 348-357. (1957).
- Grant,M.M., Koo,H.C. and Rosenfeld,W., Oxygen affects human endothelial cell proliferation by inactivation of FGF's. *Amer. J. Physiol.* 263, L370-375. (1992).
- Green,L.M., Reade,J.L. and Ware,C.F., Rapid colourimetric assay for cell viability: Application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J. Immunol. Methods.* 70, 257-268. (1984).
- Griffiths,J.B., The effect of cell population cell concentration on nutrient uptake and cell metabolism: A comparative study of human diploid and heteroploid cell lines. *J. Cell Science.* 10, 515-524. (1972).
- Griffiths,J.B., Closing the culture gap. *Biotechnology.* 10 (1), 31-32. (1992).
- Griffiths,J.B., Thornton,B. and McEntee,I.D., The development and use of microcarrier and microsphere culture techniques for the production of herpes simplex viruses. *Develop. Biol. Standards.* 50, 103-108. (1981).
- Griffiths,J.B. and Thornton,B., Use of microcarrier culture for the production of herpes simplex virus (type 2) in MRC-5 cells. *J. Chem. Technol. and Biotechnol.* 32, 324-329. (1982).
- Grinnell,F., Cell-collagen interactions: An overview. *Methods in Enzymology.* 82, 499-503. (1982).
- Grinnell,F. and Phan,T.V., Deposition of fibronectin on material surfaces exposed to plasma: Quantitative and biological studies. *J. Cell Physiol.* 116, 289-293. (1983).
- Grossmann,M., Szudlinski,M.W., Tropea,J.E., Bishop,L.A., Thotakura,N.R., Schofield,P.R. and Weintraub,B.D., Expression of human thyrotropin in cell lines with different glycosylation patterns combined with mutagenesis of specific glycosylation sites. *J. Biol. Chem.* 270 (49), 29378-29385. (1995).
- Gu,M.B., Todd,P. and Kompala,D.S., Foreign gene expression (β -galactosidase) during the cell cycle phases in recombinant CHO cells. *Biotechnol. and Bioeng.* 42, 1113-1123. (1993).
- Guertin,F., Roy,C.C., Lepage,G., Yousef,I. and Tuchweber,B., Liver membrane composition after short term parenteral nutrition with and without taurine in guinea-pigs: The effect of taurine. *Proc. Soc. Exp. Biol. Med.* 203 (4), 418-423. (1993).
- Haff,R.F. and Swim,H.E., The amino acid requirements of rabbit fibroblasts strain RM 3-56. *J. Gen. Phys.* 41, 91-99. (1957).
- Hagachi,M., Oh-eda,M., Kuboniwa,H., Tmonoh,K., Shimonaka,Y. and Ochi,N., Role of sugar chains in the expression of the biological activity of human erythropoietin. *J. Biol. Chem.* 267 (11), 7703-7709. (1992).
- Ham,R.G., An improved nutrient solution for diploid Chinese hamster and human cell lines. *Exp. Cell Res.* 29, 515-526. (1963).

- Ham, R.G., Clonal growth of mammalian cells in a chemically defined synthetic medium. *PNAS*. 53, 288-293. (1965).
- Ham, R.G., Growth of human fibroblast cultures in serum-free media. *Cell Culture Methods for Molecular and Cell Biology*. 3, 249-264. (1984).
- Ham, R.G. and McKeehan, W.L., Media and growth requirements. *Methods in Enzymology*. 58, 44-93. (1979).
- Hammerman, M and Sacktor, B., Transport of β -alanine in renal brush border membrane vesicles. *Biochim. et Biophys. Acta.* 509, 338-347. (1978).
- Handa, A., Emery, A.N. and Spier, R.E., On the evaluation of gas-liquid interfacial effects on hybridoma viability in bubble-column reactors. *Develop. Biol. Standards.* 66, 241-245. (1987).
- Hansen, H.A. and Emborg, C., Extra- and intra-cellular amino acid concentrations in continuous Chinese hamster ovary cell culture. *Appl. Microbiol. Biotechnol.* 41, 560-564. (1994).
- Harraki, B., Guiraud, P., Rochat, M-H., Faure, H., Richard, M-J., Fussellier, M. and Favier, A., Effect of taurine, L-glutamate, L-histidine, addition in amino acid glucose solution on the cellular bioavailability of zinc. *Bio Metals*. 7, 237-243. (1994).
- Hassell, J.A., PhD Thesis. Uni. of Chicago. (1975).
- Hassell, T. and Butler, M., Adaptation to non-ammoniogenic medium and selective substrate feeding lead to enhanced yields in animal cell cultures. *J. Cell Science*. 96, 501-508. (1990).
- Hassell, T., Gleave, S. and Butler, M., Growth inhibition in animal cell culture: The effect of lactate and ammonia. *Appl. Biochem. and Biotechnol.* 30, 29-41. (1991).
- Hatten, M.E., Horowitz, A.F. and Burger, M.M., The influence of membrane lipids on the proliferation of transformed and untransformed cell lines. *Exp. Cell Res.* 107, 31-34. (1977).
- Hawboldt, K.A., Linardos, T.I., Kalogerakis, N. and Behie, L.A., Cell cycle dynamics of microcarrier cultures. *J. Biotechnol.* 34, 133-147. (1994).
- Hayman, E.G., Pierschbacher, M.D., Suzuki, S. and Ruoslahti, E., Vitronectin: A major cell attachment promoting protein in FBS. *Exp. Cell Res.* 160, 245-258. (1985).
- Hayter, P.M., Curling, E.M.A., Baines, A.J., Jenkins, N., Salmon, I., Strange, P.G., CHO cell growth and interferon production kinetics in stirred batch culture. *Appl. Microbiol. and Biotechnol.* 34, 559-564. (1991).
- van Hemert, P., Kilburn, D.G. and van Wezel, A.L., Homogeneous cultivation of animal cells for the production of virus and virus products. *Biotechnol. and Bioeng.* 11, 875-885. (1969).
- Hodgson, J., Cell culture oversold. *Biotechnology*. 9 (9), 810-811. (1991).
- Holley, R.W. and Kiernan, J.A., "Contact inhibition" of cell division in 3T3 cells. *PNAS*. 60, 300-304. (1968).
- Horaud, F., Absence of viral sequences in the WHO Vero cell bank: A collaborative study. *Develop. Biol. Standards.* 76, 43-46. (1992).

- Hoshi,H., Kan,M., Minamoto,Y. and Yamane,I., Hydrocortisone potentiates cell proliferation and promotes cell spreading on tissue culture substrates of human diploid fibroblast cells in a serum-free hormone supplemented medium. *Biomed. Res.* 3, 546-552. (1982).
- House,W., Shearer,M. and Maroudas,G., Method for bulk culture of animal cells on plastic film. *Exp. Cell Res.* 71, 293-297. (1972).
- Hovgaard,D., Mortensen,B.T., Schifter,S. and Nissen,N.I., Comparative pharmacokinetics of single dose administration of mammalian and bacterially derived recombinant human granulocyte macrophage colony stimulating factor. *Eur. J. Haematol.* 50, 32-36. (1993).
- Hu,W-S., Giard,D.J. and Wang,D.I.C., Serial propagation of mammalian cells on microcarriers. *Biotechnol. and Bioeng.* 27, 1466-1476. (1985).
- Hu,W-S., Meier,J. and Wang,D.I.C., A mechanistic analysis of the inoculum requirements for the cultivation of mammalian cell on microcarriers. *Biotechnol. and Bioeng.* 27, 585-595. (1985).
- Hu,W-S. and Peshwa,M.V., Animal cell bioreactors - Recent advances and challenges to scale-up. *Can. J. Chem. Eng.* 69, 409-420. (1991).
- Hu,W-S. and Wang,D.I.C., Selection of microcarrier diameter for the cultivation of mammalian cells on microcarriers. *Biotechnol. and Bioeng.* 30, 548-557. (1987).
- Hughes,R.C., Pena,S.D.J., Clark,J. and Dourmashkin,R.R., Molecular requirements for the adhesion and spreading of hamster fibroblasts. *Exp. Cell Res.* 121, 307-314. (1979).
- Hussein,A.M., Ross,M., Vredenbergh,J., Meisenberg,B., Hars,V., Gilbert,C., Petros,W.P., Coniglio,D., Kurtzberg,J., Rubin,P. and Peters,W.P., Effects of granulocyte macrophage colony stimulating factor produced in chinese hamster ovary cells (regramostim), *Escherichia coli* (molgramostim) and yeast (sargramostim) on primary peripheral blood progenitor cells for use with autologous bone marrow after high dose chemotherapy. *Eur. J. Haematol.* 54, 281-287. (1995).
- Huxtable,R.J. and Sebring,L.A., Towards a unifying theory for the action of taurine. *Trends in Pharmaceutical Science.* 7, 481-485. (1986).
- Hy-Clone[®], The use of Cultispher-S microcarriers. *Application Report*. Salt Lake City, UT, U.S.A.. (1993).
- Hynes,R.O., Integrins: A family of cell surface receptors. *Cell.* 48, 549-554. (1987).
- Ihrig, T.J., Maulawizada,M.A., Thomas,B.D. and Jacobson,F.S., Formate production by CHO cells: Biosynthetic mechanism and potential cytotoxicity. *Paper presented at 5 American Cell Engineering Symposium*. San Diego, CA, U.S.A.. May. (1994).
- Imamura,T., Crespi,C.L., Thilly,W.G. and Brunengraber,H., Fructose as a carbohydrate source yields stable pH and redox potential in microcarrier culture. *Anal. Biochem.* 124, 353-358. (1982).
- Ito,Y., Imanishi,Y. and Suido,M., Attachment and proliferation of fibroblasts cells on polyetherurethane urea derivatives. *Biomaterials.* 8 (11), 464-472. (1987).
- Jager,V., Lehmann,J. and Friedl,P., Serum-free growth medium for the cultivation of a wide spectrum of mammalian cells in stirred bioreactors. *Cytotechnology.* 1, 319-329. (1988).

Jenssen, M.D., Wallach, D.F.H. and Liss, P.S., Comparative growth characteristics of Vero cells on gas permeable and conventional supports. *Exp. Cell Res.* 84, 271-281. (1974).

Johnson, I.S., Human insulin from recombinant DNA technology. *Science*. 219, 632-637. (1983).

Kaufman, R.J., Wasley, L.C., Spiliotes, A.J., Gossels, S.D., Latt, S.A., Larsen, G.R. and Kay, R.M., Coamplification and coexpression of human tissue type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. *Mol. and Cell. Biol.* 5, 1750-1759. (1985).

Kennedy, M.J., The evolution of the word 'Biotechnology'. *TIBTECH*. 9 (9), (1991).

Kersting, U., Schwab, A., Treidtel, M., Pfaller, W., Gstraunthaler, G., Steigner, W. and Oberleithner, H., Differentiation of Madin-Darby canine kidney cells depends on cell culture conditions. *Cell Physiol. Biochem.* 3, 42-55. (1993).

Kessler, N., Bertrand, S. and Aymard, M., Stability of a murine hybridoma is dependent on the clonal line and culture media. *In Vitro*. 29A, 203-207. (1993).

Kilberg, M.S., Stevens, B.R. and Novak, D.A., Recent advances in mammalian amino acid transport. *Ann. Rev. Nutrition*. 13, 137-165. (1993).

Kim, B-S., Choi, Y.H., Choi, C.Y. and Kim, B.G., Mammalian cell cultivation on serum coated microcarriers. *Biotechnol. Techniques*. 6 (4), 347-352. (1992).

Kimura, T., Iijima, S. and Kobayashi, T., Effects of lactate and ammonia on the oxygen uptake rate of human cells. *J. Ferment. Technol.* 65 (3), 341-344. (1987).

Kiremitci, M., Ozilgen, M. and Piskin, E., Attachment and growth kinetics of anchorage dependent BHK cells on microcarriers. *Enzyme Microbiol. Technol.* 11, 830-836. (1989).

Kleinman, H.K., Klebe, R.J. and Martin, G.R., Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* 88, 474-485. (1981).

Knox, P. and Griffiths, J.C., The distribution of cell spreading activities in sera: A quantitative approach. *J. Cell Science*. 46, 97-112. (1980).

Kohler, G. and Milstein, C., Continuous cultures of fused cells secreting antibodies of predefined specificity. *Nature*. 256, 495-498. (1975).

Kovacevic, Z. and McGiven, J.D., Mitochondrial metabolism of glutamine and glutamate and its physiological significance. *Physiol. Rev.* 63, 547-605. (1983).

Kovar, J. and Franek, F., Iron compounds at high concentrations enable hybridoma growth in a protein-free medium. *Biotechnol. Letters*. 9, 259-267. (1987).

Krebs, H.A., Body size and tissue respiration. *Biochim. et Biophys. Acta.* 4, 249-264. (1950).

Kriat, M., Fantani, J. and Vion-Dury, J., Energetic metabolism of glucose, mannose and galactose in glucose starved rat insulinoma cells anchored on microcarrier beads. A ³¹P phosphorous NMR study. *Biochimie*. 74 (9/10), 949-957. (1992).

- Kurano,N., Leist,c., Messi,F., Kurano,S. and Feichter,A., Growth behaviour of CHO in a compact loop bioreactor. 2 Effects of medium components and waste products. *J. Biotechnol.* 15,113-128. (1990).
- Lakhotia,S. and Papoutsakis,E.T., Agitation induced cell injury in microcarriers: Protective effect of viscosity is agitation intensity dependent: Experiments and modelling. *Biotechnol. and Bioeng.* 39, 95-107. (1992).
- du Laney,T.V., Cherry,R.S., Coppinger,J.A. and Truskey,G.A., Altered distribution of mitochondria and actin fibers in 3T3 cells cultured on microcarriers. *Biotechnol. Progress.* 8, 572-575. (1992).
- Lang,A.B., Scuerch,U. and Cryz,S.J.Jr., Optimisation of growth and secretion of human MAb by hybridomas cultured in serum-free medium. *Hybridoma*.10 (3), 401-409. (1991).
- Langer,R. and Vasanti,J.P., Tissue engineering. *Science.* 260, 920-926. (1993).
- Lanks,K.W. and Kasambalides,E.J., Factors that regulate proliferation of normal and transformed cells in culture. *Pathol. Annu.* 10, 35-50. (1980).
- Laskey,R.A., Fairman,M.P. and Blow,J.J., S phase of the cell cycle. *Science.* 246, 609-614. (1989).
- Lazo,P.A., Amino acids and glucose utilisation by different metabolic pathways in ascites tumour cells. *European. J. Biochem.* 117, 19-25. (1981).
- Lee,D.W., Grace,J.R., Chow,B.K.C., MacGillivray,R.T.A. and Kilburn,D.G., High yield cultivation of BHK cells on sintered alumina ceramic foam support. *Cytotechnology.* 5, 233-241. (1991).
- Lee,D.W., Piret,J.M., Gregory,D., Haddow,D.J. and Kilburn,D.G., Polystyrene macroporous bead support for mammalian cell culture. *Biochemical Engineering VII. Ann. New York Acad. Science.* 665, 137-145. (1992)
- Leibovitz,A., The growth and maintenance of tissue cell cultures in free gas exchange with the atmosphere. *Amer. J. Hygeine.* 78, 173-180. (1963).
- Leighton,J., Justh,G., Esper,M. and Kronenthal,R.L., Collagen-coated sponge: Three dimensional matrix for tissue culture of Walker tumor 256. *Science.* 155, 1259-1261. (1967).
- Lengheden,A., Influence of pH and calcium on growth and attachment of human fibroblasts in vitro. *Scandanavian J. Dental Res.* 102, 130-136. (1994).
- Lewin,B. (Ed.), *Genes IV*. Cell Press. Cambridge, MA, U.S.A.. (1990).
- Lin,A.A., Nguyen,T. and Miller,W.M., A rapid method for counting cell nuclei using a particle sizer/Coulter counter. *Biotechnol. Techniques.* 5 (2), 153-156. (1991).
- Lin,A. and Agrawal,P., Glutamine decomposition in DMEM: Effect of pH and serum concentration. *Biotechnol. Letters.* 10 (10), 695-698. (1988).
- Linardos,T.I., Kalogerakis,N.,Behie,L. and Montagne,L., The effects of specific growth rate and death rate on MAB production in hybridoma chemostat cultures. *Can. J. Chem. Eng.* 69, 429-438. (1991).

Lindner,E., Arvidsson,A.-C., Wergeland,I. and Billig,D., Subpassaging cells on microcarriers: The importance for scaling up to production. *Develop. Biol. Standards.* 66, 299-305. (1987).

Lindskog,U, Lundgren,B., Billig,D. and Lindner,E., Alternatives for harvesting cells grown on microcarriers: Effect on subsequent attachment and growth. *Develop. Biol. Standards.* 66, 307-313. (1987).

Litwin,J., The growth of Vero cells as suspended aggregates in serum-free medium. In: *Proceedings of 11 ESACT Meeting*. Spier,R.E., Griffiths,J.B. and MacDonald,C. (Eds.). Butterworths-Heinemann. Oxford, U.K.. 414-418. (1992).

Lubiniecki,A.S.; Arathoon,W.R.; Polastri,G.; Thomas,J.; Wiebe,M.; Garnick,R.; Jones,A.; van Reis,R.; Builder,S. Selected strategies for manufacture and control of recombinant tPA prepared from cell culture. In: *Advances in Animal Cell Biology and Technology for Bioprocesses*. Spier,R.E., Griffiths,J.B., Stephanie,J. and Crooy,P.J. (Eds.). Butterworths. Oxford, U.K.. 442-451. (1989).

Lubiniecki,A.S. (Ed.), *Large scale mammalian cell technology*. Dekker. New York, NY, U.S.A.. (1990).

Lubiniecki,A.S. and Lupker,J.H., Purified protein products of rDNA technology expressed in animal cell culture. *Biologicals.* 22, 161-169. (1994).

Makela,J.M., Marusyk,G.R., Norrby,E., Tyrrel,J.L.D. and Salmi,A.A., Antibodies to measles virus surface polypeptides in an immunised student population before and after booster vaccination. *Vaccine.* 7, 541-545. (1989).

Maller,J.L., Gautier,J., Langan,T.A., Lohka,M.J., Shenoy,S., Shalloway,D. and Nurse,P., Maturation promoting factor and the regulation of the cell cycle. *J. Cell Science. Supplement.* 12, 53-63. (1989).

Marique,T.H., Malarme,D., Stagier,P. and Werenne,J., On-line monitoring of growth and viral infection of Vero cells in collagen microspheres in a fluidised-bed reactor. In: *Proceedings of 12 ESACT Meeting*. Spier,R.E., Griffiths,J.B. and Berthold,W.G. (Eds.). Butterworths-Heinemann. Oxford, U.K.. 369-372. (1994).

Martin,N., Brennan,A, Denome,L and Shevitz,J., High production in mammalian cell culture. *Biotechnology.* 5, 838-840. (1987).

Martinelle,K. and Haggstrom,L., Mechanisms of ammonia and ammonium ion toxicity in animal cells: Transport across cell membranes. *J. Biotechnol.* 30, 339-350. (1993).

McKeehan,W.L., Glycolysis, glutaminolysis and cell proliferation. *Cell Biol. Int. Reports.* 6 (7), 3417-3421. (1982).

McKeehan,K.A. and McKeehan,W.L., Serum factors modify the cellular requirement for Ca^{2+} , K^{+} , Mg^{2+} , inorganic phosphate and 2-oxocarboxylic acids for multiplication of normal fibroblasts. *PNAS.* 77, 3417-3421. (1980).

Meats,J.E., Tuersley,M.D., Best,L., Lynch,A.M. and Tomlinson,S., Lactate alters plasma membrane potential, increases the concentration of cytosolic Ca^{2+} and stimulates the secretion of insulin by the hamster β -cell line HIT-T15. *J. Mol. Endocrinol.* 3, 121-128. (1989).

Meister,A. and Anderson,E., Glutathione. *Ann. Rev. Biochem.* 52, 711-760. (1983).

- Mendonca,R.Z., Ioshimoto,L.M., Mendonca,R.M.Z., de-Franco,M., Valentini,E.J.G., Becak,W., Raw,I. and Pareira,C.A., Preparation of human rabies vaccine in Vero cell culture using a microcarrier system. *Brazilian J. Med. Biol. Res.* 26, 1305-1317. (1993).
- Mercille,S., Johnson,M., Lemieux,R. and Massie,B., Filtration-based perfusion of hybridoma cultures in protein-free medium: Reduction of membrane fouling by medium supplementation with DNase I. *Biotechnol. and Bioeng.* 43, 833-846. (1993).
- Mered,B., Albrecht,P. and Hopps,H.E., Cell growth optimisation in microcarrier culture. *In Vitro.* 16, 859-865. (1980).
- Mered,B., Albrecht,P., Hopps,H.E., Petricciani,J.C. and Salk,J., Monkey kidney cell growth optimisation and poliovirus propagation in microcarrier culture. *Develop. Biol. Standards.* 47, 41-46. (1980).
- Mered,B., Albrecht,P., Hopps,H.E., Petricciani,J.C. and Salk,J., Propagation of poliovirus in microcarrier cultures of 3 monkey cell lines. *J. Biol. Standard.* 9, 137-145. (1981).
- Merten,O.W., Kierulff,J.V. and Perrin,P., Evaluation of the new serum-free medium (MDSS2) for the production of different biologicals: Use of various cell lines. *Cytotechnology.* 14 (1), 47-53. (1994).
- Messmer,T.O. and Young,D.V., The effects of biotin and fatty acids on SV40 transformed 3T3 cell growth in the presence of normal calf serum. *J. Cell. Phys.* 90, 265-270. (1977).
- Mierzejewski,K. and Rozengurt,E., Stimulation of DNA synthesis and cell division in a chemically defined medium: Effect of insulin, EGF and Vitamin B12 on resting cultures of 3T6 cells. *Biochem. Biophys. Res. Comm.* 73, 271-278. (1976).
- Miller,W.M., Wilke,C.R. and Blanch,H.W., The transient responses of hybridoma cells to nutrient additions in continuous culture. II. Glutamine pulse and step changes. *Biotechnol. and Bioeng.* 33, 487-489. (1989).
- Mirchamsy,H., Shafiyi,A., Bahrami,S., Kamali,M. and Nazari,P., Use of human diploid cell MRC-5 for production of measles and rubella virus vaccines. *Develop. Biol. Standards.* 37, 297-305. (1977).
- Montagnon,B.J., Fanget,B. and Nicolas,A.J., The large scale cultivation of Vero cells in microcarrier culture for virus vaccine production: Preliminary results for killed poliovirus vaccine. *Develop. Biol. Standards.* 47, 55-64. (1981).
- Montagnon,B.; Vincent-Falquet,J.C.; Fagnet,B., Thousand litre scale microcarrier culture of Vero cells for killed polio virus vaccine:- Promising results. *Develop. Biol. Standards.* 55, 37-38. (1984).
- Mookerjee, S., Cole,D.E.C. and Chow,A., Glycoprotein biosynthesis: Stimulation from UDP[¹⁴C] galactose into microsomal proteins by cytidine-5-diphospho choline. *FEBS Letters.* 23 (2), 257-261. (1972).
- Moore, G.E., Gerner,R.E. and Franklin,H.A., Culture of normal human leukocytes. *J. Amer. Med. Assoc.* 199 (8), 519-524. (1967).
- Morandi,M., Bandinelli,L. and Valeri,A., Growth of MRC-5 diploid cells on three types of microcarriers. *Experientia.* 38, 668-670. (1982).

- Morgan, P.L., Morton, L. and Parker, F.N., Nutrition of animal cells in tissue culture. I. Clinical Studies in a synthetic medium. *Proc. Soc. Exp. Biol. Med.* 73 (1), 1-8. (1950).
- Morgan, S.J. and Darling, D.C. (Eds.), *Animal Cell Culture*. BIOS Scientific Publishers. Oxford, U.K.. (1993).
- Mossin, L., Blankson, H., Huitfeldt, H. and Seglen, P.O., Ploidy dependent growth and binucleation in cultured rat hepatocytes. *Exp. Cell Res.* 214 (2), 551-560. (1994).
- Mukhopadhyay, A., Mukhopadhyay, S. and Talwar, G.P., Influence of serum proteins on the kinetics of attachment of Vero cells to Cytodex microcarriers. *J. Chem. Technol. Biotechnol.* 56, 369-374. (1993).
- Murakami, H., Masui, H. and Sato, G.H., Suspension culture of hybridoma cells in serum-free medium: Soybean phospholipids are the essential components. In: *Growth of Cells in Hormonally Defined Media*. Sato, G.H., Pardee, A.B. and Sirbachu, D.A. (Eds.). Plenum Press. New York, NY, U.S.A.. 711-715. (1982).
- Murakami, H., Serum-free media used for cultivation of hybridomas. In: *Monoclonal Antibodies: Production and Application*. Murakami, H. (Ed.). Sci. Soc. Proc.. Tokyo, Japan. 107-141. (1991).
- Nahapetian, A.T., Thomas, J.N. and Thilly, W.G., Optimisation of environment for high cell concentration Vero cell culture: Effect of dissolved oxygen and nutrient supply on cell growth and changes in metabolites. *J. Cell Science*. 81, 65-103. (1986).
- Nardelli, L. and Panina, G.F., 10-years experience with a 28,800 roller bottle plant for FMD vaccine production. *Develop. Biol. Standards.* 37, 133-137. (1976).
- New Brunswick Scientific®, Report No.: CC114. *Application Reports in Mammalian Cell Culture*. Compiled by Vosper, N. (1991).
- Ng, Y-C., M.Sc. Thesis. Uni. of Manitoba. (1995).
- Nie, Z., Fetuin: It's enigmatic property of growth promotion. *Amer. J. Physiol.* 263, C551-C562. (1992).
- Nikolai, T.J. and Hu, W-S., Cultivation of mammalian cells in macroporous microcarriers. *Enzyme and Microb. Technol.* 14, 203-208. (1992).
- Nilsson, K., Buzsaky, F. and Mosbach, F., Growth of anchorage dependent cells on macroporous microcarriers. *Biotechnology*. 4 (10), 989-990. (1986).
- O'Donnell-Tormey, J., Nathan, C.F., Lanks, K., DeBoer, C.J. and De La Harpe, J., Secretion of pyruvate - An antioxidant defense of mammalian cells. *J. Exp. Med.* 165, 500-514. (1987).
- Ogata, M., Wakita, K-I., Kimura, K., Marumoto, Y., Oh-i, K. and Shimizu, S., High level expression of recombinant human soluble thrombomodulin in serum-free medium by CHO-K1 cells. *Appl. Microbiol. Technol.* 38, 520-525. (1993).
- Ogawa, T., Kamihara, M., Terashima, S., Yasuda, K., Iijima, S. and Kobayashi, T., High cell density cultivation of anchorage dependent cells using a novel macroporous cellulosic support. *J. Ferment. and Bioeng.* 74 (1), 27-31. (1992).

- Ohlson, S., Branscomb, J. and Nilsson, K., Bead to bead transfer of CHO cells using macroporous microcarriers. *Cytotechnology*. 7, 67-72. (1994).
- Omasa, T., Higashiyama, K.-I., Shioya, S. and Suga, K.-I., Effects of lactate concentration on hybridoma culture in lactate-controlled fed-batch operation. *Biotechnol. and Bioeng.* 39, 556-564. (1992).
- Ong, C.P., Portner, R., Markl, H., Yamazaki, Y., Yasuda, K. and Matsumura, M., High density cultivation of hybridoma in charged porous carriers. *J. Biotechnol.* 34, 259-268. (1994).
- Orly, J. and Sato, G.W., Fibronectin mediates cytokinesis and growth of rat follicular cells in serum-free medium. *Cell*. 17 (2), 295-301. (1979).
- van Oss, C.J., Chorny, C.K., Absolom, D.R. and Flanagan, T.D., Detachment of cultured cells from microcarrier particles and other surfaces by repulsive van der Waals forces. *Biotechniques*. 5 194-197. (1983).
- Oyaas, K., Elligren, T.E., Dyrset, N. and Levine, D.W., Utilisation of osmoprotective compounds by hybridoma cells exposed to hyperosmotic stress. *Biotechnol. and Bioeng.* 43, 77-89. (1994).
- Page, M.J. and Sydenham, M.A., High level expression of humanised monoclonal antibody Campath 1H in chinese hamster ovary cells. *Biotechnology*. 9 (1), 64-68. (1991).
- Pardee, A., Cherington, P.V. and Medrano, E.E., On deciding which factors regulate cell growth. In: *Control of Cellular Division and Development. Part A*. Cunningham, D., Goldswasser, E., Watson, J. and Fox, C.F. (Eds.). Cell Press. Cambridge, MA, U.S.A.. 495-502. (1980).
- Parekh, R.B. and Patel, R.P., Comparing the glycosylation patterns of recombinant glycoproteins. *TIBTECH*. 10, (8), 276-280. (1992).
- Pasternak, C.A., Whittaker-Dowling, P.A. and Widnell, C.C., Stress induced increase of hexose transport as a novel index of cytopathic effect in virus infected cells: Role of the L protein in the action of vesicular stomatitis virus. *Virology*. 166, 379-386. (1988).
- Patel, R., Twigg, J., Crossley, I., Golsteyn, R., Whitaker, M., Calcium-induced chromatin condensation and cyclin phosphorylation during chromatin condensation cycles in ammonia-activated sea urchin eggs. *J. Cell Science. Supplement*. 12, 129-144. (1989).
- Paul, J. (Ed.), *Cell and Tissue Culture*. 5 ed. Churchill Livingstone. Edinburgh, U.K.. 1-8. (1975).
- Pearlstein, E., Substrate activation of cell adhesion factor as a prerequisite for cell attachment. *International J. Cancer*. 22, 32-35. (1978).
- Pennica, D., Holmes, W.E., Kohr, W.E., Harkins, R.N., Vedar, G.A., Ward, C.A., Bennett, W.F., Yelverton, E., Seeburg, P.H., Heyneker, H.L., Goddel, D.V. and Collen, D., Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*. *Nature*. 301, 214-221. (1983).
- Pharmacia®, *Microcarrier Culture Handbook*. Almqvist and Wicksell Tryckeri. Uppsala, Sweden. (1981).
- Polastri, G.D., Friesen, H.J. and Maurer, R., Amino acid utilisation by Vero cells in microcarrier culture. *Develop. Biol. Standards.* 55, 53-56. (1984).

- Pons,M-N, Wagner,A., Vivier,H. and Marc,A., Application of quantitative image analysis to a mammalian cell line grown on microcarriers. *Biotechnol. and Bioeng.* 40 (1), 187-193. (1992).
- Prescott,L.M., Harley,J.P. and Klein,D.A. (Eds.), *Microbiology*. Wm.C.Brown. Dubuque, IA, U.S.A.. (1990).
- Prokop,A. and Rosenberg,M.Z., Bioreactors for mammalian cells. *Adv. Biochem. Eng.* 39, 30-71. (1989).
- Prusiner,S.B., Molecular biology of prion diseases. *Science*. 252, 1515-1522. (1991).
- Qualitz,J., Kwong,S.C.W., Trinh,L.B., Basic considerations of scale-up and optimisation in mammalian cell culture. *Amer. Biotechnol. Lab.* 11 (6), 98-100. (1993).
- Rabinovitz,M., Olson,M.E. and Greenberg,D.M., Role of glutamine in protein synthesis by th Ehrlich ascites cells. *J. Biol. Chem.* 222, 879-893. (1956).
- Rabinovitz,J.C. and Tabor,H., The urinary excretion of formic acid and formiminoglutamic acid in folic acid deficiency. *J. Biol. Chem.* 233, 252-258. (1958).
- Radlett,P.J., Pay,T.W.F. and Garland,A.J.M., The use of BHK suspension cells for the commercial production of FMD vaccine over a 20 year period. *Develop. Biol. Standards.* 60, 163-170. (1985).
- Ramig,R.F., Cross,R.K. and Fields,B.N., Genome RNAs and polypeptides of reovirus serotypes 1, 2 and 3. *J. Virol.* 22, 726-733. (1977).
- Rapaport,S, Rost,J. and Schultze,M., Glutamine and glutamate as respiratory substrates of rabbit reticulocytes. *European J. Biochem.* 23, 166-170. (1981).
- Reiter,M., Hohenwarter,O., Gaida,T., Zach,N., Schmatz,C., Bluml,G., Weigang,F., Nilsson,K. and Katinger,H., The use of macroporous gelatin microcarriers for the cultivation of mammalian cells in fluidised bed reactors. *Cytotechnology*. 3, 271-277. (1990).
- Reiter,M., Bluml,G., Gaida,T., Zach,N., Unterluggauer,O. and Dobloff-Dier,M., Modular integrated fluidised bed bioreactor technology. *Biotechnology*. 9 (11), 1100-1102. (1991).
- Reitzer,L.J., Wice,B.M. and Kennell,D., Evidence that glutamine not sugar is the major energy source for cultured HeLa cells. *J. Biol. Chem.* 254, 2669-2676. (1979).
- Reuveny,S., Silberstein, L., Shahar,A., Freeman,E. and Mizrahi,A., Cell and virus propagation on cylindrical cellulose based microcarriers. *Develop. Biol. Standards.* 50, 115-121. (1981).
- Reuveny,S., Velez,D., Miller,L. and MacMillan,J.D., Factors affecting cell growth and monoclonal antibody production in stirred reactors. *J. Immunol. Methods*. 86, 53-59. (1986).
- Rheinwald,J.G. and Green,H., Growth of cultured mammalian cells on secondary glucose sources. *Cell*. 2, 287-293. (1974).
- Rhodes,M. and Birch,J.R., Recombinant antibodies from CHO cells. In: *Advances in Animal Cell Biology and Technology for Bioprocesses*. Spier,R.E., Griffiths,J.B., Stephanie,J. and Crooy,P.J. (Eds.). 472-474. Butterworths. Oxford, U.K.. (1989).

- Rockwell,G.A., Sato,G.H. and McClure,D.B., The growth requirements of SV40 virus transformed Baln/c3T3 cells in serum-free monolayer culture. *J. Cell. Phys.* 103, 323-331. (1980).
- Roder,B., Zuhlke,A., Widdecke,H. and Klein,J., Synthesis and application of new microcarriers for animal cell culture.2. Applications of polystyrene microcarriers. *J. Biomaterials Science. Polymer Ed.* 5 (1/2), 79-88. (1993).
- Rovinski,B., Haynes,J.R., Cao,S.X., James,O., Sia,C., Zolla-Pazner,S., Matthews,T.J. and Klein,M.H., Expression and characterisation of genetically engineered human immunodeficiency virus-like particles containing modified envelope glycoproteins: Implications for development of a cross-protective AIDS vaccine. *J. Virology.* 66 (7), 4003-4012. (1992).
- Royce,P.N., A need to refocus research in the operation of fermenters? *Biotechnology. 10, Forum.* (1992).
- Ruoslahti, E., Proteoglycans as modulators of growth factor activities. *Cell.* 64, 867-869. (1991).
- Ruoslahti,E., Hayman,E.G., Pierschbacher,M.D. and Engvall,E., Fibronectin: Purification, immunochemical properties and biological activities. *Methods in Enzymology.* 82, 803-822 (1982).
- Ruoslahti,E. and Pierschbacher,M.D., New perspectives in cell adhesion: RGD and integrins. *Science.* 238, 491-497. (1987).
- Rutz,H.P. and Little,J.B., Exogenous lactate interferes with cell cycle control in BALB/3T3 mouse fibroblasts. *Int. J. Radiation Oncology.* 31 (3), 525-528. (1995).
- Ryan,W.L. and Cardin,C., Amino acids and ammonia of fetal calf serum during storage. *Proc. Soc. Exp. Biol. Med.* 123, 27-30. (1966).
- Ryll,T., Valley,U. and Wagner,R., Biochemistry of growth inhibition by ammonium ions in mammalian cells. *Biotechnol.and Bioeng.* 44, 184-193. (1994).
- Sanford,K.K., Earle,W.R., Evans,V.J., Waltz,H.K. and Shannon,J.E., The measurement of proliferation in tissue cultures by enumeration of cell nuclei. *J. Natl. Cancer Inst.* 11, 773-795. (1951).
- Sarantos,P., Howard,D. and Souba,W.W., Dexamethasone regulates glutamine synthetase expression in rat lung. *Metabolism - Clinical and Experimental.* 42, 795-800. (1993).
- Sasger,J.W., Changing patterns of actin localisation during cell division. *PNAS.* 72, 1913-1916. (1975).
- Scattergood,E.M., Schlabach,A.J., McAleer, W.J. and Hillman,M.R., Scale-up of chick cell growth on microcarriers in fermenters for vaccine production. *Biochemical Engineering III. Annals New York Academy Sciences.* 413, 332-337. (1983).
- Schacter,D., Fluidity and function of hepatocyte plasma membranes. *Hepatology.* 4, 140-151. (1984).
- Scher,C.D., Pledger,W.J., Martin,P., Antoniades,H. and Stiles,C.D., Transforming viruses directly reduce the cellular growth requirements for a PDGF. *J. Cell Phys.* 97, 371-380. (1979).

- Schiff, L.A. and Fields, B.N., Reoviruses and their replication. In: *Virology*. Fields, B.N., Knipe, D.M. et al. (Eds.). 1275-1306. Raven Press Ltd., New York, NY, U.S.A.. 1275-1306. (1990).
- Schmid, G., Zilg, H. and Johanssen, R., Repeated batch cultivation of rBHK cells on Cytodex-3 microcarriers: Antithrombin III, amino acid and fatty acid metabolic quotients. *Appl. Microbiol. Biotechnol.* 38, 328-333. (1992).
- Schneider, Y.-J. and Veithen, X., Hybridoma cell growth and monoclonal antibody secretion upon continuous cultivation in bioreactor with a serum and protein-free nutritive medium. *Biotechnol. Letters*. 12 (7), 481-486. (1990).
- Schrimpf, G. and Friedl, P., Growth of human vascular endothelial cells on various types of microcarriers. *Cytotechnology*. 13, 203-211. (1993).
- Schutte, H., Flossdorf, J., Sahm, H. and Kula, M.-R., Purification and properties of formaldehyde dehydrogenase and formate dehydrogenase from *Candida boidinii*. *Eur. J. Biochem.* 62, 151-160. (1976).
- Sefton, B.M. and Rubin, H., Release from density dependent growth inhibition by proteolytic enzymes. *Nature*. 227, 843-845. (1970).
- Seglen, P.O., Preparation of rat liver cells. III. Enzymatic requirements for tissue dispersion. *Exp. Cell Res.* 82, 391-398. (1973).
- Shaffer, M.C., Foley, T.P. and Barnes, D.W., Quantitation of spreading factor in human biological fluids. *J. Lab. Clin. Med.* 103, 783-791. (1984).
- Shahar, A., Reuveny, S., David, Y., Budu, C. and Shainberg, A., Cerebral neurons, skeletal myoblasts and cardiac muscle cells cultured on macroporous beads. *Biotechnol. and Bioeng.* 43, 826-831. (1994).
- Shapiro, H.M. (Ed.), *Practical flow cytometry*. Academic Press. London, U.K.. (1988).
- Sharpe, A.H. and Fields, B.N., Reovirus inhibition of cellular DNA synthesis: Role of the S1 gene. *J. Virol.* 38, 389-392. (1981).
- Sharpe, A.H. and Fields, B.N., Reovirus inhibition of cellular RNA and protein synthesis: Role of the S4 gene. *Virology*. 122, 381-391. (1982).
- Shaw, J.E. and Cox, D.C., Early inhibition of cellular DNA synthesis by high multiplicities of infections and UV-irradiated reovirus. *J. Virology*. 12, 704-710. (1973).
- Shevitz, J., LaPorte, T.L. and Stinnett, T.E., Production of viral vaccines in stirred bioreactors. In: *Advances in biotechnological processes. Viral Vaccines*. Mizrahi, A. (Ed.). Wiley-Liss Publishers. New York, NY, U.S.A.. 14, 1-35. (1990).
- Silverstein, S.C. and Dales, S., The penetration of reovirus RNA and initiation of its genetic function in L-strain fibroblasts. *J. Cell Biol.* 36, 197-230. (1968).
- Smiley, A.L., Hu, W.-S. and Wang, D.I.C., Production of human immune interferon by rec mammalian cells cultivated on microcarriers. *Biotechnol. and Bioeng.* 33, 1182-1190. (1989).

Sokal, R.R. and Rohlf, F.J. (Eds.), *Introduction to Biostatistics*. 2 Ed.. W.H. Freeman Publishers. New York, NY, U.S.A.. (1987).

Spier, R.E., Production of veterinary vaccines. In: *Advances in Biotechnological Processes. Viral Vaccines*. Mizrahi, A. and van Wezel, A. (Eds.). Alan R. Liss, New York, NY, U.S.A.. 2, 33-39. (1983).

Spier, R.E., Chairmans note. In: *Proceedings of 11 ESACT Meeting*. Spier, R.E., Griffiths, J.B. and MacDonald, C. (Eds.). Butterworths-Heinemann. London, U.K.. (1992).

Spier, R.E. and Griffiths, J.B. (Eds.), *Animal Cell Biotechnology*, Vols. 1-5. Academic Press. London, U.K.. (1985-92).

Sytkowski, A.J., Feldman, L. and Zurbuch, D.J., Biological activity and structural stability of N-deglycosylated recombinant human erythropoietin. *Biochem. and Biophys. Res. Comm.* 176 (2), 698-704. (1991).

Tagler, J.M., Akerboom, T.P.M., Hoek, J.B., Meifer, A.J., Vaartjes, W., Ernster, L. and Williamson, J.R., Ammonia and energy metabolism in isolated mitochondria and intact liver cells. In: *Normal and Pathological Development of Energy Metabolism*. Hommes, F.A. and van der Berg, C.J. (Eds.). Academic Press, London, U.K.. (1975).

Tao, T.Y., Ji, G-Y. and Hu, W-S., Serial propagation of mammalian cells on gelatin coated microcarriers. *Biotechnol. and Bioeng.* 32, 1037-1052. (1988).

Taub, M., Kidney cell cultures in hormonally defined serum-free medium. In: *Mammalian Cell Culture - The use of serum-free hormone supplemented media*. Mather, J.P. (Ed.). Plenum Press. New York, NY, U.S.A.. 129-150. (1984).

Taub, M., Devis, P.E. and Hiller-Grobel, S., PGE1 independent MDCK cells have elevated intracellular cAMP levels, but retain the growth stimulating effects of glucagon and EGF in serum-free medium. *J. Cell. Phys.* 120, 19-29. (1984).

Temin, H., Study on carcinogenesis by avian sarcoma viruses. VI. Differential multiplication of infected and converted cells in response to insulin. *J. Cell. Phys.* 69, 377-384. (1967).

Tharakan, J.P., Lucas, A. and Chau, P.C., Hybridoma growth and antibody secretion in serum supplemented and low protein serum-free media. *J. Immunol. Meth.* 94, 225-235. (1986).

Thilly, W.G., The rationale for and elements of mammalian cell technology. In: *Mammalian Cell Technology*. Thilly, W.G. (Ed.). Butterworths. Stoneham, MA, U.S.A.. 1-8. (1989).

Thornton, B., Griffiths, J.B. and Walkland, D., Herpes simplex vaccine using cell membrane associated antigen in an animal model. *Develop. Biol. Standards.* 50, 201-205. (1984).

Tolman, K.G., Peterson, P., Gray, P. and Hammar, S.P., Hepatotoxicity of salicylates in monolayer cell cultures. *Gastroenterology*. 74, 205-208. (1978).

Toyoda, K. and Inouye, K., Development of a new protein and hormone-free medium for hybridoma cultivation. *Agricultural Biol. Chem.* 55 (6), 1631-1633. (1991).

Tsao, E.I., Bohn, M.A., Numsawan, V., Omstead, D.R. and Munster, M., Effects of heat shock on the production of human EPO from recombinant CHO cells. *Biotechnol. and Bioeng.* 40, 1190-1196. (1992).

- Tsuda,E., Kawanashi,G., Ueda,M., Masuda,S. and Sasaki,R., The role of carbohydrate in recombinant human erythropoietin. *Eur. J. Biochem.* 188, 405-411. (1990).
- Tucker, R.P., Hammerback,J.A., Jenrath,D.A., Mackie,E.J. and Xu,Y., Tenascin expression in the mouse in situ localisation and induction in vitro by bFGF. *J. Cell Science.* 104, 69-76. (1993).
- Underwood,P.A. and Bennett,F.A., A comparison of the biological activities of the cell-adhesive proteins vitronectin and fibronectin. *J. Cell Science.* 93, 641-649. (1989).
- Valdivieson-Garcia,A., Clarke,R.C. and Rahn,K., Neutral red assay for measurement of quantitative Vero cell cytotoxicity. *Appl. Environ. Microbiol.* 59 (6), 1981-1986. (1993).
- Valentich,J.D., Tchao,R. and Leighton,J., Hemicyst formation is stimulated by cAMP in dog kidney cell line - MDCK. *J. Cell. Phys.* 100, 291-304. (1979).
- van der Valk,P., Gille,J.J.P., Oostra,A.B., Roubos,E.W., Sminia,T. and Joenje,H., Characterisation of an oxygen tolerant cell line derived from Chinese hamster ovary cells. *Cell Tissue Res.* 239, 314-322. (1985).
- Varani,J., Dame,M. and Beals,T.F., Growth of 3 established cell lines on glass microcarriers. *Biotechnol. and Bioeng.* 25, 1359-1372. (1983).
- Varani,J., Bendelow,M.J. and Hillegas,W.J., The effect of substrate on the production of infectious virus by cells in culture. *J. Biol. Standard.* 16, 333-338. (1988)
- Vincent,N., Martin,G. and Bavarel,G., Glycine, a new regulator of glutamine metabolism in isolated rat-liver cells. *Biochim. et Biophys. Acta.* 1175, 13-20. (1992).
- Voet,D. and Voet,J.G. (Eds.), *Biochemistry*. John Wiley and Sons. New York, NY, U.S.A.. (1990).
- Vournakis,J.N. and Runstadler,P.W. Jr., Optimisation of the microenvironment for mammalian cell culture in flexible collagen microspheres in a fluidised bed bioreactor. In: *Mammalian Cell Technology*. Thilly,W.G. (Ed.). Butterwoths. Stoneham, MA, U.S.A.. 305-326. (1988).
- Vournakis,J.N. and Runstadler,P.W.Jr., Microenvironment: The key to improved cell culture products. *Biotechnology.* 7 (2), 143-145. (1989).
- Wada,M.L.F. and Vidal,B.C., Growth and differentiation of Vero cells cultivated in 3-dimentional type-1 collagen. *Cytobios.* 67, 101-107. (1991).
- Wang,S. and Haslam,S.Z., Serum-free primary culture of normal mouse mammary epithelial and stromal cells. *In Vitro.* 30A, 859-866. (1994).
- Wasley,L., Timony,G., Murtha,P., Stoudemire,J., Dorner,A.J., Caro,J., Krieger,M. and Kaufman,R.J., The importance of N and O linked oligosaccharides for the biosynthesis and in vitro and in vivo biologic activities of erythropoietin. *Blood.* 77 (12), 2624-2632. (1991).
- Watson,E., Shah,B., Leiderman,L., Hsu,Y-R., Karkare,S., Lu,H.S. and Lim,F-K., Comparison of N-linked oligosaccharides of recombinant human tissue kallikrein produced by CHO cell in microcarrier beads and in serum-free suspension culture. *Biotechnol. Prog.* 10, 39-44. (1994).
- Waymouth,C., Osmolality of mammalian blood and of media for culture of mammalian cells. *In Vitro.* 6, 109-127. (1970).

Weller,A., Beck,S. and Ekblom,P., Amino acid sequence of mouse tenascin and differential expression of two tenascin isoforms during embryogenesis. *J. Cell Biol.* 112 (2), 355-362. (1991).

Weinstein,R., Hoover,G.A., Majure,J., van der Spek,j., Stemerman,M.D. and Maciag,T., Growth of human FS4 in serum-free defined medium without PDGF. *J. Cell Physiol.* 110, 23-28. (1982).

Wentz,D. and Schugerl,K., Influence of lactate, ammonia and osmotic stress on adherent and suspension BHK cells. *Enzyme Microb. Technol.* 14, 68-75. (1992).

Werrlein,R.J. and Glinos,A.D., Oxygen microenvironment and respiratory oscillation in cultured mammalian cells. *Nature*. 251, 317-319. (1974).

van Wezel,A.L., Growth of cell strains and primary cells on microcarriers in homogeneous culture. *Nature*. 216, 64-69. (1967).

van Wezel,A.L., Monolayer growth systems: Homogeneous unit processes. In: *Animal Cell Biotechnology*. Spier,R.E. and Griffiths,J.B. (Eds.). 1, 266-283. Academic Press. London, U.K.. (1985).

Wharton,W., Loef,E., Pledger,W.J. and O'Keefe,E.J., Modulation of the EGF receptor by PDGF and cholera toxin: Effects on mitogenesis. *PNAS*. 79, 5567-5571. (1982).

Wheatley,D.N., Binucleation in mammalian liver. *Exp. Cell Res.* 74, 455-465. (1972).

White,L.A. and Ades,E.W., Growth of Vero E6 cells on microcarriers in a cell bioreactor. *J. Clin. Micro.* 28 (2), 283-286. (1990).

Whiteside,J.P. and Spier,R.E., The scale-up from 0.1 to 100 L of a unit process based on 3 mm diameter glass spheres for the production of four strains of FMDV in BHK monolayer cells. *Biotechnol. and Bioeng.* 23, 551-555. (1981).

Wice,B.M., Reitzer,L.J. and Kennell,D., The continuous growth of vertebrate cells in the absence of sugar. *J. Biol. Chem.* 256, 7812-7819. (1974).

World Health Organisation - Acceptability of cell substrates for production of biologicals. WHO Technical Report Series. 747, 5-24. (1987).

World Health Organisation - Requirements for continuous cell lines used for biological substances. WHO Technical Report Series. 745, 99-115. (1987).

World Health Organisation - Requirements for rabies vaccine (inactivated) for human use produced in continuous cell lines. WHO Technical Report Series. 760, 167-189. (1987).

World Health Organisation - Transfer of technology for rabies vaccine production: New strains available for Vero and BHK. *Bulletin of WHO*. 65, 941-945. (1987).

Xie,L. and Wang,D.I.C., Stoichiometric analysis of animal cell growth and its application to medium design. *Biotechnol. and Bioeng.* 43, 1164-1174. (1994).

Xie,L. and Wang,D.I.C., Fed-batch cultivation of animal cells using different medium design concepts and feeding strategies. *Biotechnol. and Bioeng.* 43, 1175-1189. (1994).

Yamada,K.M., Yamada,S.S. and Pastan, I., Cell surface protein partially restores morphology, adhesiveness and contact inhibition of movement to transformed fibroblasts. PNAS. 73, 1217-1221. (1976).

Yamazi,H., Fukuda,H., Nojima,Y and Webb,C., Immobilisation of anchorage independent animal cells using reticulated polyvinyl formal resin biomass support particles. Appl. Microbiol. Biotechnol.. 30, 609-613. (1989).

Yasumara,S. and Kawakita,H., Immortalisation of an African green monkey kidney cell using simian virus 40. Nippon Rinsho. 21, 1209-1212. (1963).

Yasumara,Y., Nissua,A. and Yamamoto,K., Phenotypic requirements for gln of kidney cells and for glu and arg of liver cells in culture. In: *Nutritional requirements of cultured cells*. Katsuta,H.(Ed.). Sci. Soc. Proc.. Tokyo, Japan. 223-255. (1978).

Young,D.V., Cox,F.W.III, Chipman,S. and Hartman,S.C., The growth retardation of SV transformed 3T3 cells by transferrin and it's dependence on biotin. Exp. Cell. Res.. 118,410-414. (1979).

Young,M.W. and Dean,R.C.Jr., Optimisation of mammalian cell bioreactors. Biotechnology. 5 (9), 835-837. (1987).

Appendix

Appendix 1. Abbreviations Used in the Thesis

ADP	- Adenine Dinucleotide Phosphate.
ANOVA	- Two way Analysis of Variance with replication.
Balb/c3T3	- Murine B-Lymphocyte Cell - Clone 3T3.
BCE-1	- Bovine Corneal Endothelial Cell - Clone 1.
BHK	- Baby Hamster Kidney Cells.
BRV	- Bovine Rhinotracheitis Virus.
BSA	- Bovine serum albumin (Fraction V).
BSE	- Bovine spongiform encephalopathy.
CC9C10	- Murine Anti-Insulin Producing Hybridoma Cells.
CHO	- Chinese Hamster Ovary Cells.
CS	- Calf Serum (Normal unsupplemented).
sCS	- Iron Enriched, Supplemented Calf Serum.
CV-1	- Primary Monkey Kidney Cells - Clone CV-1.
DMEM	- Dulbecco's Modified Eagle's Medium.
DMEM/F12	- Dulbecco's Modified Eagle's Medium:Ham's F12 Nutrient Mixture (1:1).
DOT	- Dissolved Oxygen Tension.
D-PBS	- Dulbecco's Phosphate Buffered Saline.
D-PBS ⁻	- Dulbecco's Phosphate Buffered Saline (Ca ²⁺ and Mg ²⁺ Free).
EDTA	- Ethylenediaminetetraacetic Acid.
FACS	- Fluorescence Activated Cell Sorting.
FBS	- Foetal Bovine Serum.
FMDV	- Foot and Mouth Disease Virus.
FS-4	- Human Foreskin Fibroblast Cell Line - Clone FS-4.
GMEM	- Glasgow's Modified Eagle's Medium.
HPLC	- High Pressure Liquid Chromatography.

HSV	- Herpes Simplex Virus.
IMDM	- Iscove's Modified Eagle's Medium.
JMEM	- Joklik's Modified Eagle's Medium.
k_{La}	- Oxygen transfer coefficient.
L-15	- Leibovitz's L-15 Medium.
M199	- Medium 199.
MDBK	- Madin-Darby Bovine Kidney.
MDCK	- Madin-Darby Canine Kidney Cells.
MEM	- Minimal Essential Medium.
MOI	- Multiplicity of Infection.
MRC-5	- Human Diploid Lung Fibroblast Cells - Medical Research Council Clone 5.
NAD	- β -Nicotinamide Adenine Dinucleotide.
NBCS	- New Born Calf Serum.
OPA	- Orthophthalaldehyde.
PID	- Proportional, Integral and Derivative control algorithm's.
RO	- Reverse Osmosis.
RPMI 1640	- Rosewell Park Memorial Institute Medium 1640.
TCA	- Trichloroacetic Acid.
TCM	- Tissue Culture Medium Supplement.
Vero	- African Green Monkey Kidney Cells - CCL81
VSFM	- Vero cell Serum-Free Medium.
VSP	- Bovine Serum Ultrafiltrate Serum Substitute.
VSV	- Bovine Vesicular Stomatitis Virus.
v/v	- volume/volume
v/v/d	- volumes added per volume of vessel per day
v/v/d/g	- volume/volume/day/gram of beads

Appendix 2 - Standard Curves

A2.1 Ammonium Chloride

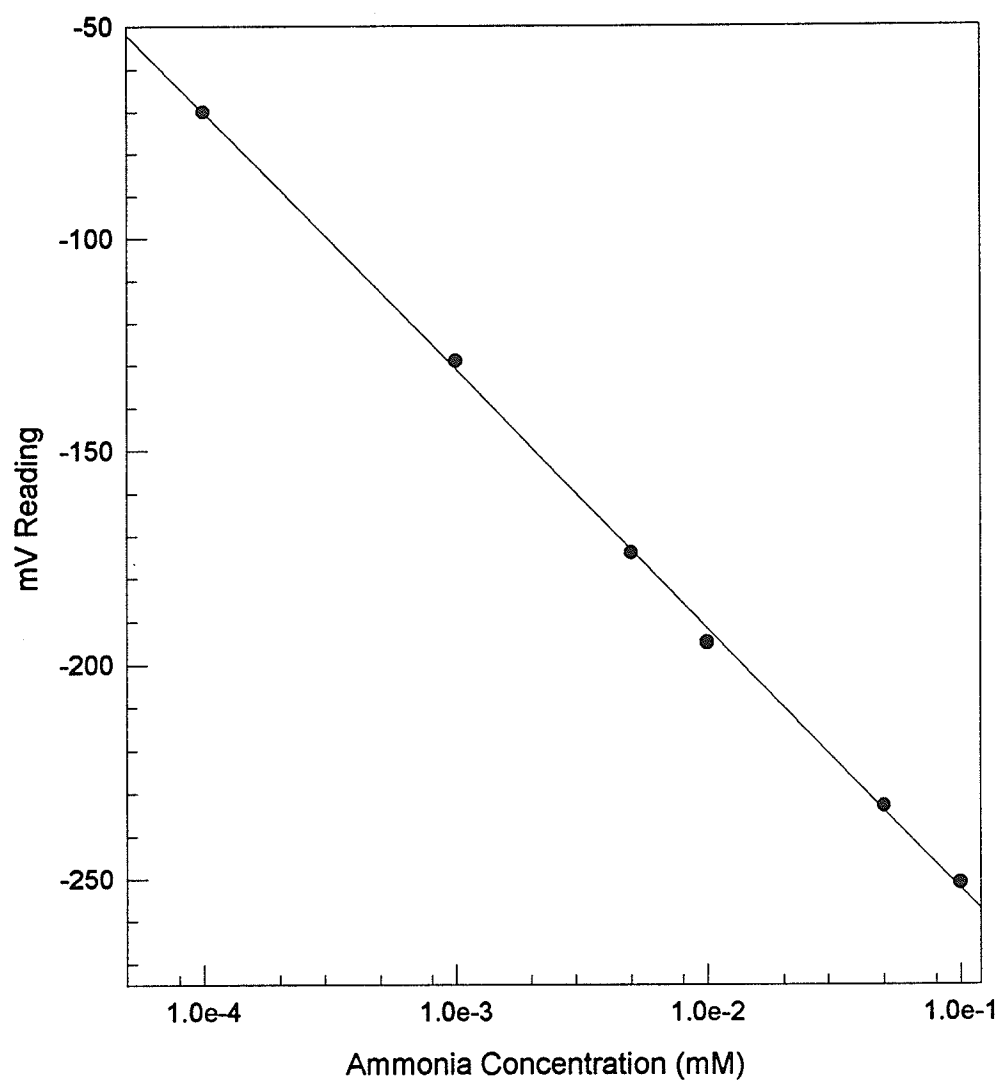


Fig. A2.1 - The ammonium chloride standard curve. Solutions of ammonium chloride were prepared at 0.10 mM and serially diluted in distilled water to 0.0001 mM. Samples (0.90 mL) of each solution were placed under an Orion 9512 ammonia electrode and alkalinized (100 μ L of 10.00 M NaOH). The mV reading was allowed to stabilise before recording.

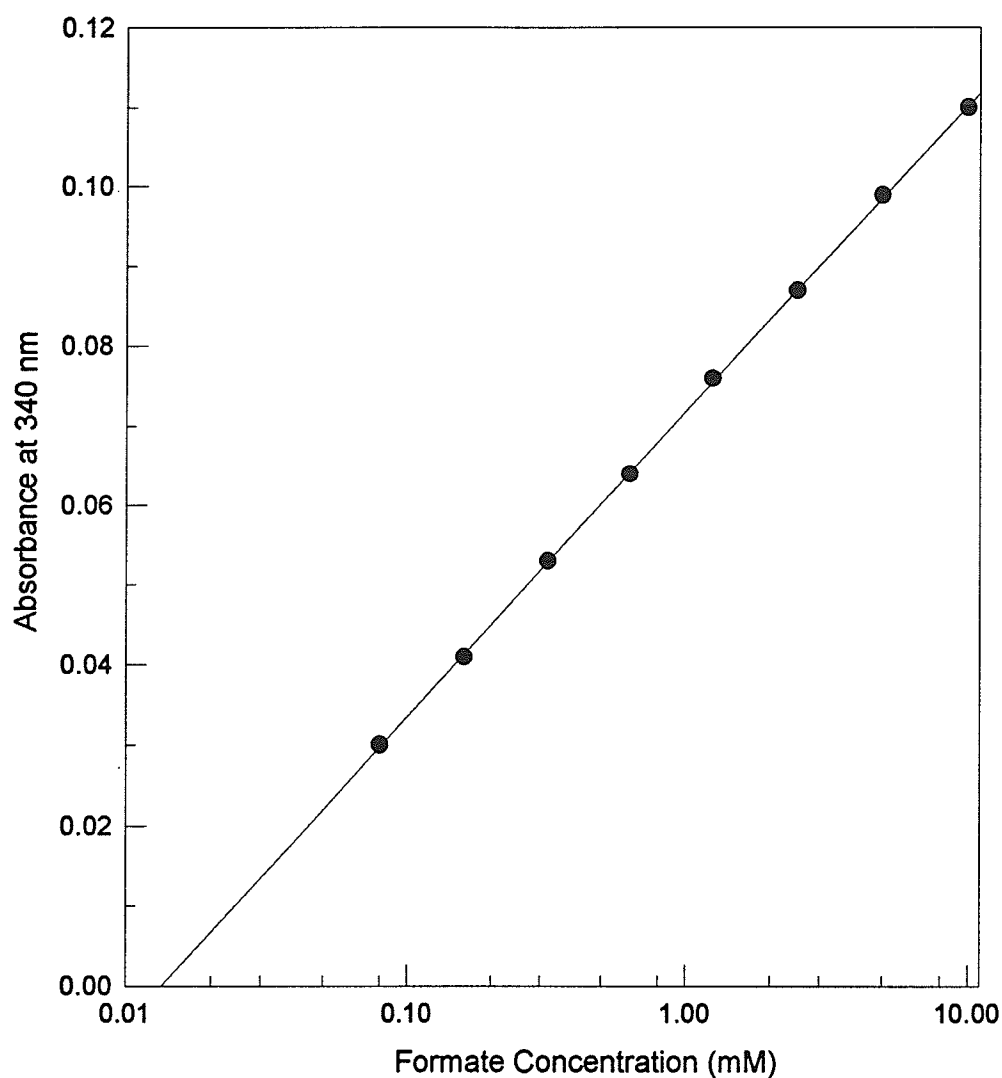
A2.2 - Sodium Formate

Fig. A2.2 - The sodium formate standard curve. Solutions of sodium formate were prepared at 10.00 mM and serially diluted indistilled water to 0.019 mM. Samples (100 μ L) were assayed using formate dehydrogenase monitoring the concentration of NADH at 340 nm.

Appendix 3. Sample Calculations

A3.1 Poisson Cell to Bead Distribution Profile

The Poisson cell to bead distribution profile was determined using Equation 2.1 described previously (2.5.5).

$$P(n) = ((e^{-\lambda} \times \lambda^n) / n!)$$

where P = the probability of observing a number of beads with a given cell to bead number.

λ = the cell to bead ratio in the inoculum.

n = the observed cell to bead ratio.

The data used for the sample calculation is taken from Chapter 5.3.1 - Speed and Mode of Stirring for Cell Attachment.

$\lambda = 10$ cells/bead.

Therefore, at:

n	P
0	0.000045
1	0.00045
2	0.0023
3	0.0076
4	0.0189
5	0.0378
6	0.0631
7	0.0901
8	0.1126
9	0.1251
10	0.1251
11	0.1137
12	0.0948
13	0.0729
14	0.0521
15	0.0347
16	0.0217
17	0.0128
18	0.0071
19	0.0037
20	0.0019

A3.2 Lag Phase

The lag phase was estimated using Equation 2.2 described previously (2.5.6).

$$L_p = (t_T - t_e)$$

where L_p = the lag phase time.

t_T = the total time of the cell growth.

t_e = the time that would have elapsed for the total number of cell generations at the maximum cell generation time in the exponential phase.

The data used for the sample calculation is taken from Chapter 3.2 - Attachment Time and Cell Growth of EDTA Detached Cells.

Inoculum cell concentration = 0.20×10^6 cells/mL.

Cell concentration after 24 h = 0.34×10^6 cells/mL.

Maximum cell concentration = 1.41×10^6 cells/mL.

t_T = 72 h.

$$\begin{aligned} \text{Total number of cell generations} &= ((\ln (1.41 \times 10^6) - \ln (0.20 \times 10^6)) / \ln (2)) \\ &= ((14.16 - 12.21) / 0.69) = (1.95 / 0.69) \\ &= 2.83 \end{aligned}$$

$$\begin{aligned} \text{Number of gens. at } \mu_{MAX} &= ((\ln (1.41 \times 10^6) - \ln (0.34 \times 10^6)) / \ln (2)) \\ &= ((14.16 - 12.74) / 0.69) = (1.42 / 0.69) \\ &= 2.06 \end{aligned}$$

$$\begin{aligned} \text{Maximum cell generation time} &= ((72 - 24) / 2.06) &= (48 / 2.06) \\ &= 23.30 \text{ h} \end{aligned}$$

$$\begin{aligned} t_e &= (2.83 \times 23.30) \\ &= 65.94 \text{ h} \end{aligned}$$

$$\begin{aligned} \text{Therefore, } L_p &= (72 - 65.94) \\ &= 6.06 \text{ h} \end{aligned}$$

A3.3 Sample Calculation of Standard Error of the Mean

The data used for the sample calculation below was taken from Chapter 4.3.4 - Origin of the Inoculum Vero Cells. The Standard Deviation (SD) was calculated using Microsoft Excel v. 5.0a.

Cell Counts from Culture 1 = 0.64×10^6 and 0.67×10^6 cells/mL

$$\bar{x}_{\text{Mean1}} = 0.65 \times 10^6 \text{ cells/mL}$$

Cell Counts from Culture 2 = 0.76×10^6 and 0.80×10^6 cells/mL

$$\bar{x}_{\text{Mean2}} = 0.78 \times 10^6 \text{ cells/mL}$$

SD = 0.0919

Standard error of the mean = $(\text{SD}/\sqrt{n})$ = $(0.0919/\sqrt{2})$
 = $(0.0919/1.414)$
 = **0.064**

Therefore, this culture sample time cell count was:

$$= 0.72 \pm 0.06 \times 10^6 \text{ cells/mL}$$

A3.4 Sample Calculation of Paired Student's t-test

The data used for the sample calculation was taken from Chapter 5.3.3 - Maximum Cell Concentrations Following Cell Attachment. The probabilities for differences between cell concentrations were determined using Sigmaplot for Windows v.1.02.

Cell concentrations	1/2 volume	= 1.48×10^6 and 1.38×10^6 cells/mL
	30 rpm (Int.)	= 1.55×10^6 and 1.39×10^6 cells/mL
	40 rpm (Int.)	= 1.40×10^6 and 1.50×10^6 cells/mL
	60 rpm (Int.)	= 1.37×10^6 and 1.23×10^6 cells/mL
	100 rpm (Int.)	= 1.17×10^6 and 1.05×10^6 cells/mL
1/2 vol.:30 rpm	P	= >0.10 Not significantly different.
30 rpm:40 rpm	P	= >0.10 Not significantly different.
40 rpm:60 rpm	P	= <0.05 Significantly different.
40 rpm:100 rpm	P	= <0.05 Significantly different.

A 3.5 Sample Calculation of Two Way Analysis of Variance with Replication

The method was that described by Sokol and Rohlf (1987).

The data used for the sample calculation below was taken from Chapter 6.3.1 - Basal Medium Formulation. Each culture was replicated at least twice ($n=2$).

Factor B: Basal Medium Formulation (b=7)	Factor A: Serum Concentration (a=2)		Σ Row	Σ^2 Row
	5.0% v/v	2.0% v/v		
DMEM	2.03	1.52	3.55	6.43
	1.75	1.32	3.07	4.80
DMEM/F12	1.30	1.21	2.51	3.16
	1.12	1.07	2.19	2.40
IMDM	1.97	1.57	3.54	6.35
	1.75	1.35	3.10	4.88
L-15	0.39	0.25	0.64	0.21
	0.31	0.19	0.50	0.13
M199	0.95	0.71	1.66	1.41
	0.83	0.63	1.46	1.09
Optimem-1	1.55	1.52	3.07	4.71
	1.33	1.30	2.63	3.46
RPMI1640	1.17	0.88	1.85	1.83
	1.01	0.58	1.59	1.35
Σ Column	17.46	13.90	31.36	
Σ^2 Column	25.47	16.75		42.21

From the table:

1. Grand Total:-

$$= \sum_a \sum_b \sum_n Y = (2.03 + 1.75 + \dots + 0.58) = 31.36.$$

2. Sum of squared observations:-

$$= \sum_a \sum_b \sum_n Y^2 = (2.03^2 + 1.75^2 + \dots + 0.58^2) = 42.21.$$

3. Sum of squared replicate totals divided by number of replicates:-

$$= \sum_a \sum_b (\sum_n Y)^2 / n = (3.78^2 + 2.42^2 + \dots + 1.26^2) / 2 = 42.04.$$

4. Sum of squared column totals divided by sample size of column:-

$$= \sum_a (\sum_b \sum_n Y)^2 / bn = (17.46^2 + 13.9^2) = 35.58.$$

5. Sum of squared row totals divided by sample size of row:-

$$= \sum_b (\sum_a \sum_n Y)^2 / an = (3.55^2 + 3.07^2 + \dots + 1.59^2) = 41.34.$$

6. Grand total squared divided by total sample size = correction term (CT):-

$$= (\sum_a \sum_b \sum_n Y)^2 / abn = (1.)^2 / 28 = 35.12.$$

7. Sum of Squares_{total}:-

$$= \sum_a \sum_b \sum_n Y^2 - CT = 2. - 6. = 7.09.$$

8. Sum of squares_{subgroup}:-

$$= \sum_a \sum_b (\sum_n Y)^2 / n - CT = 3. - 6. = 6.92.$$

9. Sum of squares_A (SS Columns):-

$$= \sum_a (\sum_b \sum_n Y)^2 / bn - CT = 4. - 6. = 0.46.$$

10. Sum of squares_B (SS Rows):-

$$= \sum_b (\sum_a \sum_n Y)^2 / an - CT = 5. - 6. = 6.22.$$

11. Sum of squares_{A x B} (Interaction SS):-

$$= SS_{\text{subgroup}} - SS_A - SS_B = 8. - 9. - 10. = 0.24.$$

12. Sum of squares_{within} (Within subgroups) = Error SS:-

$$= SS_{\text{total}} - SS_{\text{subgroup}} = 7. - 8. = 0.17.$$

Source of Variation	Degrees of Freedom (df)	Sum of Squares (SS)	Mean Square (MS)	Expected MS
A (Columns)	a - 1	9.	9./(a - 1)	$(\sigma^2 + (n \times b / (a - 1)) \times \sum \alpha^2)$
B (Rows)	b - 1	10.	10./(b - 1)	$(\sigma^2 + (n \times a / (b - 1)) \times \sum \beta^2)$
A x B (Interaction)	(a - 1) x (b - 1)	11.	11./(a - 1) x (b - 1)	$(\sigma^2 + (n / ((a - 1) \times (b - 1))) \sum \alpha\beta^2)$
Within Subgroups	a x b x (n - 1)	12.	12./a x b x (n - 1)	σ^2
Total	a x b x n - 1	7.		

Source of Variation	Degrees of Freedom (df)	Sum of Squares (SS)	Mean Square (MS)	Expected MS
A (Columns)	1	0.46	0.46	46.00 ^S
B (Rows)	6	6.22	1.04	104.00 ^S
A x B (Interaction)	6	0.24	0.04	4.00 ^{NS}
Within Subgroups	14	0.17	0.01	
Total	27	7.09		

From $F_{CRIT.}$ tables with the df above the values would be as follows:

df of 1 v. 14 P = 0.05 F = 4.61 P = 0.01 F = 9.11

df of 6 v. 14 P = 0.05 F = 2.86 P = 0.01 F = 4.65

Therefore, as the calculated F was higher compared to the $F_{CRIT.}$ for sources of variation between columns and rows there was a significant difference between these groups. However, there was no interaction between the groups as the calculated F was below the $F_{CRIT.}$.

A 3.6 Sample Calculation of Number of Cells at Monolayer on the Beads

Average Cytodex-1 diameter = 180 μm Radius = 90 μm

Average Cultispher-G diameter = 240 μm Radius = 120 μm

Average Vero cell diameter = c. 20 μm Radius = 10 μm

Surface Area (SA) of sphere = $4\pi r^2$

SA Cytodex-1 = $(4\pi (90 \times 90))$ = $(12.57 \times 8,100)$
= 101,817.00 μm^2

SA Cultispher-G = $(4\pi (120 \times 120))$ = $(12.57 \times 14,400)$
= 181,008.00 μm^2

Assuming Vero cells are flat discs when attached on the bead surfaces, then:

SA Vero cell = πr^2
= $(3.142 \times (10 \times 10))$ = (3.142×100)
= 314.20 μm^2

Number of cells which would totally cover bead surface assuming no gaps between cells:

Cytodex-1 = (SA Cytodex-1/SA Vero)
= $(101,817.00/314.20)$
= **324.05 cells**

Cultispher-G = $(181,008.00/314.20)$
= **576.09 cells**