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The Production, Harvest and Adsorptive Recovery of an Infectious  
Herpes Simplex Virus Vaccine

Roderic Simon O'Keeffe

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## Summary

At present there is not a reliable vaccine against herpes virus. Viral protein vaccines as yet have proved unsuccessful to meet the challenge of raising an appropriate immune response. Cantab Pharmaceuticals has produced a virus vaccine that can undergo one round of replication in the recipient in order to produce a more specific immune reaction. This virus is called Disabled Infectious Single Cycle Herpes Simplex Virus (DISC HSV) which has been derived by deleting the essential gH gene from a type 2 herpes virus. This vaccine has been proven to be effective in animal studies.

Existing methods for the purification of viruses rely on laboratory techniques and for vaccine production would be on a far too small a scale. There is therefore a need for new virus purification methods to be developed in order to meet these large scale needs.

An integrated process for the manufacture of a purified recombinant DISC HSV is described. The process involves culture of complementing Vero (CR2) cells, virus infection and manufacture, virus harvesting and subsequent downstream processing. The identification of suitable growth parameters for the complementing cell line and optimal times for both infection and harvest are addressed. Various traditional harvest methods were investigated and found not to be suitable for a scaled up process. A method of harvesting, that exploits the elution of cell associated viruses by the competitive binding of exogenous heparin to virus envelope gC proteins, is described and is shown to yield significantly less contaminated process streams than sonication or osmotic approaches that involve cell rupture (with >10-fold less complementing cell protein). High concentrations of salt (>0.8M NaCl) exhibit the same effect, although the high osmotic strength ruptures cells and increase the contamination of the process stream. This same heparin-gC protein affinity interaction is also shown to provide an efficient adsorptive purification procedure for herpes viruses which avoids the need to pre-treat the harvest material, apart from clarification, prior to chromatography. Subsequent column eluates provide product fractions with a 100-fold increase in virus titre and low levels of complementing cell protein and DNA (0.05 pg protein/pfu and  $1.2 \times 10^{-4}$  pg DNA/pfu respectively).

**Keywords:** Herpes Simplex Virus type 2; DISC HSV; heparin; virus release; affinity adsorption; virus purification.

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## Abbreviations

ADCC	Antibody Dependant Cell mediated Cytotoxicity
BHK	Baby Hamster Kidney cells
BSA	Bovine serum albumin
CMV	Cytomegalovirus
Cpe	Cytopathic effect
CR1	code given to Vero cells expressing HSV 1 glycoprotein H
CR2	code given to Vero cells expressing HSV 2 glycoprotein H
CTL	Cytotoxic T Lymphocytes
CTX	Clinical Trial Exemption
DAB	3,3-Diaminobenzidine tetrahydrochloride
dCTP	deoxycytidine triphosphate
DISC HSV	Disabled Infectious Single Cycle Herpes Simplex Virus (type2)
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
EDTA	Ethylenediaminetetra-acetic acid
EI	Early Intermediate gene expression of viral proteins
ELISA	Enzyme Linked Immuno Sorbant Assay
FBS	Foetal Bovine Serum
FMDV	Foot and Mouth Disease Virus
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
gE	Glycoprotein E
gG	Glycoprotein G
gH	Glycoprotein H
gI	Glycoprotein I
gJ	Glycoprotein J
gK	Glycoprotein K

gL	Glycoprotein L
gM	Glycoprotein M
MBSC	Microbiological Safety Cabinet
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
HSV	Herpes simplex virus
Pfu	Plaque forming units
PIPES	Piperazine-N,N'-bis-[2-ethanesulphonic acid]
RB	Roller bottle
SDS-PAGE	Sodium Dodecylsulphate – Polyacrylamide Gel Electrophoresis
TCID <sub>50</sub>	Tissue Culture Indicated Dose – 50%
TE	Tris, EDTA buffer
TK	Thymidine Kinase
<i>tk</i>	Thymidine Kinase gene
U	unit of activity
UHP	Ultra high pure water
VP	Viral protein
X-gal	5-Bromo-4-chloro-3-β-D-galactosidase

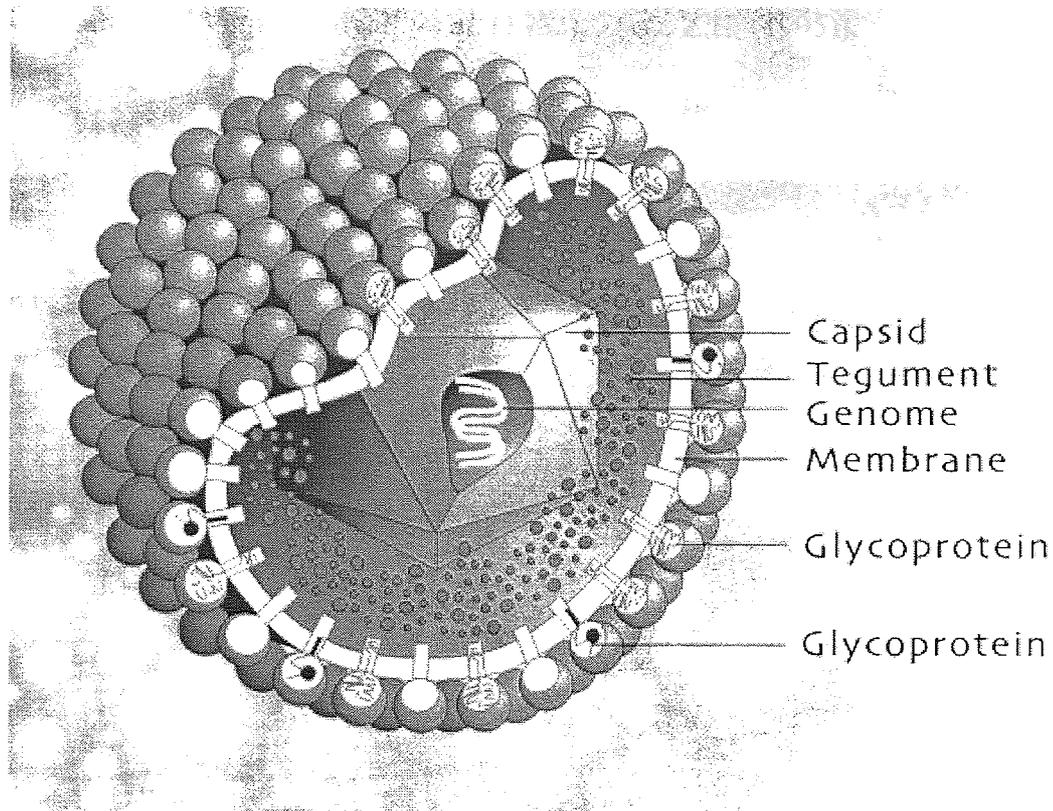
# 1 Introduction

## 1.1 *The Herpes Simplex Virus*

The herpes simplex virus is a member of the Herpesviridae group, which consists of a large number, approximately seventy, different viruses classified into three sub-groups. The alphaherpesviruses include amongst others HSV-1, HSV-2 and the pseudorabies virus, the betaherpesviruses include human and feline cytomegalovirus and the gammaherpesviruses include epstein barr virus and herpesvirus saimiri. This sub classification is based on a number of biological characteristics such as host range, polypeptide composition and virus replication. Although this family consists of a wide range of viruses there are characteristics that are common even between members of different groups and there are close relationships between viruses of the same groups, such as HSV-1, HSV-2 and pseudorabies viruses. Although most of the research has been conducted on the HSV-1 virus it is possible to infer certain characteristics about other alphaherpesviruses.

## 1.2 *The Structure of Herpes Simplex Virus*

To aid the understanding of the complexities of isolating and purifying a virus the following section describes, briefly, the structure of a herpes virus and where in a cell the relative components are found. This will also illustrate the level of present understanding of how the herpes virus invades cells, replicates, forms new virus particles and egresses from the cell. Although there is a great deal known about this process there are certain parts which are less well known as in the exact process of where a new capsid forms a viable virion from its egress from the nucleus to outside the cell. A schematic representation of a *herpesviridae* is shown in Figure 1.1 and which is explained in the following text.



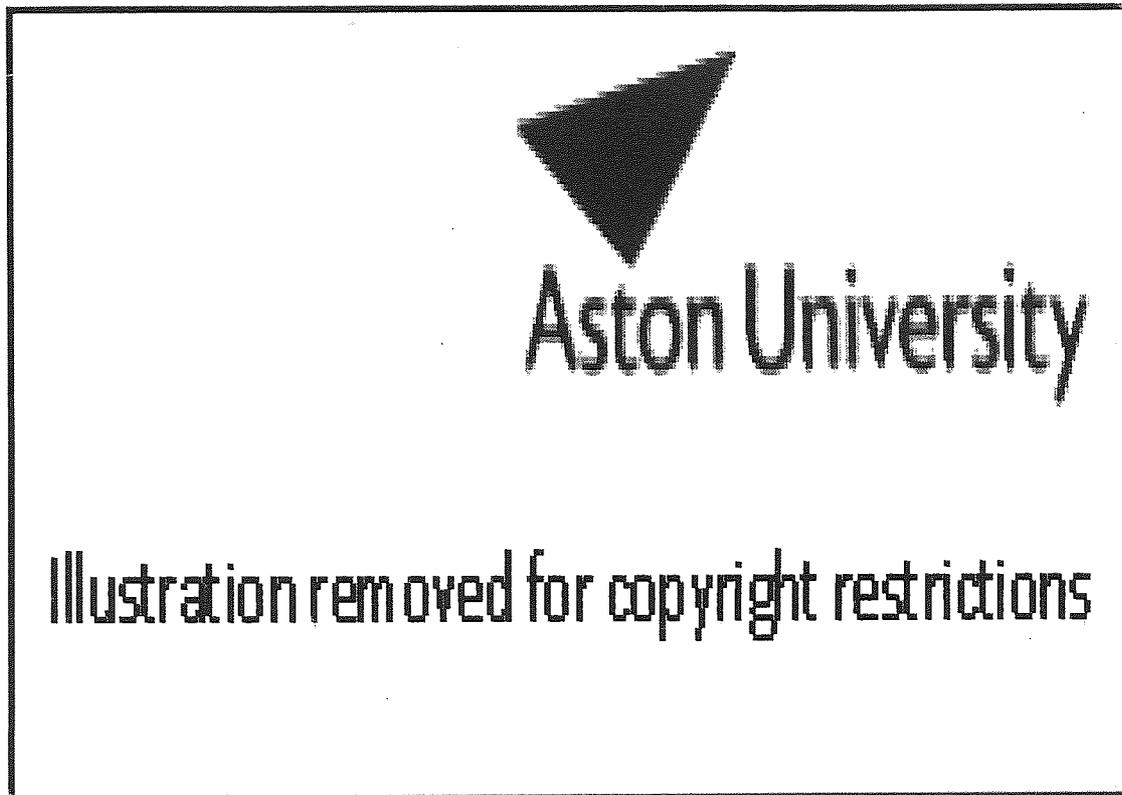
**Figure 1.1** Schematic representation of a *herpesviridae*.

*The capsid encloses the viral genome and is surrounded by an amorphous layer of protein called the tegument. As the virus egresses from a cell it acquires a lipid membrane with embedded viral glycoproteins and has an overall diameter of approximately 180 nm (see text).*

### 1.2.1 The Capsid

The capsid of the HSV-1 virus was elucidated by electron microscopy utilising negative staining and was found to consist of 150 hexons and 12 pentons which correlates with an icosahedral structure. The capsids' major component is the VP5 protein and accounts for 72% of the capsid by weight. It was subsequently found that the pentons and hexons were hexomers and pentomers of the same VP5 protein. The protein VP26 binds specifically around the hexamers, six copies per hexamer and the

proteins VP19c and VP23 form triplexes, which bind between the hexamers and pentomers in different conformational states, see Figure 1.2. The width of the capsid structure is 125 nm (Wildy, P. *et al.* (1963), Zhou, Z.H. (1995)).



**Figure 1.2** Capsid structure of HSV-1.

*The diagram shows the assembly of the VP26 protein and its orientation with the VP5 hexon. Then the assembly of the pentons and hexons to form the virus capsid (Zhou et al. 1995).*

In the process of the assembly of the capsid, in the nucleus of an infected cell, the VP22a protein is utilised as a scaffold protein. This protein is then replaced by the virus DNA that is replicated as a concatamer and therefore needs cleaving into the correct lengths for packaging. There are a number of proteins that are involved in the packaging and cleaving of DNA into the capsid; the VP24 protease is essential to cleave the concatameric DNA as it is packaged and approximately 152 kilobase pairs are packaged into each capsid for HSV-2 (Roizman (1979)). The absence of the scaffold protein VP22a also inhibits packaging. A host of other proteins are involved

in this process (products of the genes UL6, UL15, UL25, UL28, UL32 and UL33) but as yet only UL6, nucleocapsid, and UL25, tegument, have been associated as a constituent of the virion (Deiss *et al.* (1996)).

### 1.2.2 Tegument

Before the nucleocapsid becomes enveloped it acquires tegument protein. These proteins, when observed by electron microscopy, appear as an electron dense layer between the capsid and the phospholipid membrane. Two of these proteins VP16 and VP11 have been associated with assembly of the virus. VP16 is necessary for the capsid to acquire the nuclear membrane. VP11, although not essential, increases the efficiency of envelopment and virion egress. Other tegument proteins are involved in the de-envelopment upon infection, the targeting of the capsid to the nucleus, the delivery of the viral DNA to the nucleus (VP1/2) and the regulation of the expression of viral proteins.

### 1.2.3 Envelope

On leaving the nucleus the nucleocapsid buds into the perinuclear space, the space between the inner and outer nuclear membranes, and in the process gains a membrane composed of the inner nuclear membrane containing virally expressed glycoproteins. This can be clearly seen when viewed under high power using transmission electron microscopy (see Figure 5.2, page 105). It is unclear quite what happens next but the virion egresses through the golgi apparatus and in the process either de-envelops and re-envelops (Stackpole (1969)) or the viral membrane is enriched with membrane of the golgi, since mature virions contain membrane components from the golgi. As the virion progresses through the golgi the glycoproteins become glycosylated either in situ on the virion or by exchange of the phospholipid envelope with that of the golgi (Schwartz *et al.* (1969)).

#### 1.2.4 Glycoproteins

The glycoproteins provide the virion with the mechanism for binding and entry into a susceptible cell and some may provide the virion with a method for targeting the egress through the golgi apparatus and release from the cell. Glycoproteins gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, have had at least some of their structure resolved, but not all have had their function elucidated. gB and gC are involved in the initial binding of the virus to the cell surface which then allows the secondary binding to occur through the gD glycoprotein. The gH glycoprotein then mediates the fusion of the virion with the plasma membrane of the cell. Glycoproteins gI and gE have been associated with cell to cell spread of the virus. Other glycoproteins have been found to be dispensable for the growth of the virus in tissue culture, therefore discerning their function in infecting *in vivo* has been problematic (Stannard *et al.*, 1987).

### 1.3 The Herpes Simplex Virus Disease

The herpes simplex virus is one of the most widespread diseases in humans. It has been reported that up to 80% of adults in the United States are seropositive for HSV type 1 virus and up to 20% harbour the HSV type 2 virus (Corey, 1993). Usually HSV-1 is transmitted in a non-sexual manner while HSV-2 is associated with sexual transmission, although the reverse of this is not uncommon; up to 25% of genital herpes is caused by HSV-1, and leads to indistinguishable infections. Once infection has occurred the virus is transmitted to the peripheral sensory nervous system and after entering an axon travels to the nucleus of the nerve cell and enters a latent state, which lasts for life. In this state the virus effectively eludes the immune system since there are few viral antigens being produced; the only activity noted is the production of RNA of the LAT (latency associated transcripts) region of the virus. Also the nervous system is not well patrolled by the immune system. After an indeterminate amount of time the virus becomes re-activated and travels back to the epithelial cells and causes recurrent disease. This recurrent disease can be asymptomatic and in general is less severe than the original infection. In immunocompetent individuals

infection is usually localised and self-limiting; it is also controlled by the immediate immune response. In an immunocompromised or newborn the infection can be severe leading to conditions such as stomatitis, meningitis and encephalitis. There is therefore a need to produce a vaccine that can both protect against HSV infection, preferably against both types, and to treat the disease.

The following is a description of how the body's immune system fights such infections and provides reasoning why a live virus vaccine should prove more beneficial in the fight against the disease over a more simple protein vaccine.

### 1.3.1 Natural defence against HSV infection

Once a viral pathogen has passed the physical barriers of either the skin or mucosal membranes, then the immune system becomes engaged in attempting its elimination. The immune system uses a complex and diverse range of specific and non-specific mechanisms to attempt removal. The type and strength of response depends on whether the host has previously been challenged by the same infectious agent. Unchallenged host's immediate response is that of the non-specific or innate response. The major components of this innate response are natural killer cells, macrophages, monocytes, neutrophils and cytokines, such as tumour necrosis factor and interferons.

### 1.3.2 The innate response

Once an organism has invaded a host, macrophages begin to phagocytose them. Macrophages then become stimulated to produce factors, which in turn are recognised by neutrophils. These neutrophils are stimulated to move from the blood into the tissue down the concentration gradient set up by these molecules to the site of infection. Neutrophils are also armed by these factors and are then capable of killing invading pathogens by phagocytosis. Once neutrophils have completed their task they die and are removed by macrophages. Natural Killer (NK) cells are able to lyse virally

infected cells without having prior exposure to virus. These cells are therefore important in the immediate immune response to a viral infection. Another part of the innate immune system is antibody dependent cell mediated cytotoxicity (ADCC). Antibodies that recognise cell surface antigens bind in a specific manner and then bind, in a non specific manner, Fc receptor on the surface of Killer cells. Killer cells are a heterologous group of cells including some T cells and some macrophages. For this response to occur, only a small amount of antibody is required; therefore maternally derived antibodies can give protection to unexposed infants. It is thought that a depletion of either the levels of Natural Killer cells or the circulating levels of antibodies specific for HSV are responsible for disseminated disease in neonates.

### 1.3.3 The specific response

Specific responses to infection, although not completely separate from the innate system, are mainly described as the T cell response. T cells, which are derived from bone marrow stem cells, undergo maturation in the thymus and are screened for binding of their T cell receptor (TcR) to any self peptide. If binding is strong then they are eliminated but moderate binding is tolerated. TcR are then able to recognise antigens presented on the surface of an antigen presenting cell when bound to either class I or class II major histocompatibility complex (MHC). When a naive T cell binds to an antigen presenting cell an immune response is only generated when other factors stimulate the T cell. T cells differentiate either into effector cytotoxic T lymphocytes (CTL), which can lyse virally infected cells, or into T helper cells that regulate the immune response by production of cytokines, for example IL-2. The main route for the spread of herpes simplex virus is predominantly from cell to cell. Therefore, CTL would play a greater role than antibodies in the defence against an established infection.

Considering the parts of the immune system described above, the goal of a herpes virus vaccine must be to elicit the CTL response. This will provide a response that is directed to cell presenting antigens since whole viral particles will most likely not be

seen. It also gives the best chance of destroying cells that contain viral genomes in a latent state. Although the genome does not produce much in the way of transcripts, viral proteins may be present in small numbers on the cell surface.

#### **1.4 HSV Vaccines**

The ultimate goal of an HSV vaccine is to establish CTL response to enable the immune system to clear infected cells, although the sensory neurones associated with latency do not express either class I or class II MHC. To achieve this many different vaccine techniques have been employed in trials.

Trials of HSV vaccines date back to the 1920's and 30's and consisted mainly of killed HSV, usually by formalin treatment. Unfortunately data gained from these trials was not compared to a control group and the impression was given that time between recurrent disease was elongated. Other trials in this era used non-specific vaccines such as vaccinia, to test for cross-reactivity with HSV, but were subsequently abandoned. It was then clear that a more sophisticated vaccine had to be developed to produce a better immune response (Stanberry, 1991).

A more refined procedure for vaccine preparation is to extract the major components of the virus that elicit an immune response. These are the envelope glycoproteins and can be isolated by treatment of inactivated virus particles with detergents. Several companies have held trials of either single glycoproteins such as gD, from Lederle (Wayne, NJ), or whole envelope extracts, Merck (West Point, PA) and Porton International (London). Some of these preparations do not work while others are still being analysed. Instead of extracting proteins from viruses genetically engineered proteins can be expressed in bacteria. Chiron (Emeryville, CA) has conducted trials of an engineered gD, but has since found results to be poor (Burke, 1993; Stanberry, 1991)

In all of the above cases the vaccine does not emulate a wild type infection, by infecting cells which then display viral proteins on the cell surface. Therefore the immune response made may not be sufficient to protect an individual against infection or recurrent disease (Inglis, 1995). The new approach of genetically engineering viruses to enable them to infect cells but render them unable to replicate, by deleting essential genes from their DNA, has led to the hope of a new method to raise immunity in patients that is similar to natural infection. One such vaccine is the disabled infectious single cycle herpes simplex virus type 2 (DISC HSV) vaccine produced by Cantab Pharmaceuticals (Boursnell *et al.*, 1997).

## **1.5 The DISC-HSV virus**

### **1.5.1 Introduction**

The aim, therefore, of any vaccine against the herpes virus is to produce a CTL response of the body's immune system. To enable this to happen the vaccine must have the same action as the wild type virus, that is the antigens must be presented on the surface of MHC producing cells. Also since HSV-1 and HSV-2 infections are becoming less distinct from each other, as observed in the clinic, the vaccine should give resistance to challenge to either virus type.

Historically the method of delivering such a virus was to attenuate the virus. This means the virus is selected, by serially passaging in alternate hosts, to produce a vaccine that does not properly infect the recipient. Although this has been achieved for the VZV virus there has been no attenuated HSV virus vaccine. Other drawbacks with this method is that it is not understood what changes happen to the virus while it is being passaged. Therefore it is not known if, after inoculation, the virus has the potential to revert and become virulent.

With the advent of recombinant DNA technology there is an alternative method of producing a live virus vaccine that does not cause pathogenesis. The deletion of one

or more essential genes from the viral genome will lead to a virus that could infect a host but will be unable to replicate. Initial vaccine based on this method deleted the thymidine kinase gene (*tk*<sup>-</sup>). These viruses can then be produced in cells that express thymidine kinase. More recently the HSV virus has been inactivated by deletion of an essential glycoprotein gene. Again these virus can only replicate in a cell line that expresses the corresponding gene that is deleted in the viral genome.

### 1.5.2 Construction of the virus and complementing cell line

It has been shown that virus lacking the gH gene is unable to replicate (Desai, P.J. et al. 1988) so the approach taken was to produce a virus vaccine that lacked this glycoprotein.

The gH gene was initially constructed into the vector pIMCO8 and was inserted in to Vero cells by calcium phosphate mediated transfection. Cells containing a stable insert were selected for by resistance to the antibiotic G418, encoded by a neomycin selectable marker gene on the vector

The construction of the deleted viral genome was accomplished by a two stage recombination strategy. Firstly the gH and half of the upstream TK genes were replaced by the lacZ gene with the CMV EI promoter. This construct could then grow on BHK cells expressing the gH gene and differentiated from non recombinant virus genomes by the addition of X-gal which turn blue by the action of the lacZ gene. The second stage of the recombination process inserts the previously deleted second half of the TK gene and a short linker sequence in place of the lacZ gene and its promoter. When these constructs are grown on gH<sup>+</sup>/tk-BHK cells in the presence of methotrexate only virus with the functional TK gene grow. These can then be colony picked and grown up in the gH<sup>+</sup> Vero cells (CR2 cells).

The sequence of the gH gene inserted into the vero cells starts at the ATG of the gH and ends exactly at the stop codon. The deletion of the gH gene from the viral genome starts three base pairs upstream of the start ATG to halfway between the end

of gH and the start of the UL21 genes which means that there are no overlapping sequences to aid recombination (Bournnell *et al.*, 1997).

## **1.6 Virus purification methods**

There are at present a number of purification strategies used for viruses. The most simple have been used in the laboratory for a number of years and have enabled viruses to be concentrated and purified. This has enabled researchers to study the properties of the viruses without interference that may occur from using crude stocks. The major drawback of any laboratory process when considering a manufacturing process is the ability to scale up. Also one important consideration that is often overlooked at the laboratory scale is the recovery of virus through the process.

This section will introduce a number of purification processes both at laboratory and manufacturing scale for a number of different viruses. The positive and negative aspects of these processes will be discussed and used to explain the strategy employed to develop the purification process for the DISC HSV product.

It is worth noting that during this investigation from 1995-1999, there were no full scale purification processes for the production of virus vectors or vaccines. Recently, however, late 1998, a process for the production of adenovirus vectors was presented (Giroux, D. (1998)) which is included in the discussion below.

### **1.6.1 Sonication**

The primary method of preparation of a virus is largely based on the classical virology methods. Sonication is widely used in the laboratory to generate homogeneous material. This method is quick and, when using a water bath sonicator clean, which enables aseptic preparation of virus for uses such as virus stocks (Killington (1985), Harland).

Infected cells, usually grown in either flat flasks or roller bottles, are scraped into either growth media or buffer and decanted into a suitable closed container. To maintain sterility of the virus a water bath sonicator is used, filled with cold water. The virus is then immersed in the water bath and sonicated for about 1 minute. The size of the container that is used depends on the available size of the water bath, but usually laboratory sized sonicators can only accommodate a 20 to 30 ml container. Although this method produces a homogeneous virus suspension there are obvious drawbacks. There is no attempt in this method to separate contaminants from the virus, which results in the product being heavily loaded with growth media, host cell proteins and DNA. DNA contamination is particularly high since sonication disrupts all cellular membranes resulting in the release of mitochondrial and nuclear DNA. DNA causes many problems making the feed stream viscous and difficult to filter, apart from having to be removed to stringent levels to meet regulatory requirements. The ability of scaling up sonication from the laboratory is also another problem. Sonication is usually performed on samples of around 1 to 20 ml, although there is a process that uses sonication for a Turkey herpes virus vaccine production (Fioentine, D. (1985)), but this only appears to be at around the 100 ml scale.

### 1.6.2 Gradient centrifugation

The use of gradient centrifugation has been used for virus preparation for many years and has become a standard method of purifying viruses (Killington, R.A (1985)). This process relies on the density of virus particles being different to other components of the cell, once a homogeneous suspension has been accomplished, in order for them to be separated. Usually infected cells are harvested and disrupted by methods such as sonication, freeze thaw cycles or Dounce homogenisation followed by removal of large debris by low speed centrifugation. Supernatant is then layered over a 10 – 40% sucrose gradient, poured using a gradient former, and centrifuged at around 20,000 g for 1 hour. The virus will band in the gradient and appear as a fluffy white band which is collected by piercing the centrifuge tube, within this layer, and syringing out

the virus. If necessary a further centrifugation step (20,000 g for 1 hour) can be included to pellet the virus in order to concentrate and remove the sucrose.

The advantages of this process is that it produces virus which is very pure and can be resuspended in any volume to give a high titre if needed. Also this process separates viable virus from certain non viable species such as non enveloped and empty capsids since these sediment at different densities. For these reasons gradient centrifugation is usually used as the 'best method' of purifying virus to which all other methods are compared when evaluating purity of the product.

Obtaining high purity of virus in this process comes at the cost of yield and scalability. The usual recoveries of such a process are around 10 percent, even without the final centrifugation step. The capital outlay for high speed centrifugation equipment is also high making the process unfavourable.

### 1.6.3 Primary purification method use by Cantab Pharmaceuticals

In order to provide material for initial clinical trials a suitable process was needed that could be used quickly with minimal development. The sucrose gradient centrifugation method was therefore employed since there was no need to purify large quantities of virus and the low recoveries of this process did not severely affect the small scale production process. This method also ensured that the virus was pure enough for human use and enabled the virus to be formulated to any desired titre for the trials. The following is a description of the process used using Dounce homogenisation to rupture cells and two centrifugation steps to recover the virus free from sucrose.

Virus was harvested by scraping infected cells into DMEM media and collected into polypropylene tubes and centrifuged at 2000 g for 5 minutes to pellet the cells. Cell pellets were suspended in DMEM and homogenised with a Dounce homogeniser. Cell debris was removed by a further centrifugation and homogenised as before. Supernatants of the homogenates were pooled and stored at -80°C. On thawing the harvested virus was laid over a 10-40% sucrose gradient and centrifuged for 1 hour at 12,000 rpm. The lower band, containing the infectious virus, was removed and

pelleted by further centrifugation in PBS. The viral pellet was then suspended in PBS and sonicated, for 1 minute, to produce a homogeneous viral suspension. Human serum albumin was added, to a final concentration of 5% (w/v), to help stabilise the virus and the bulk purified virus was then stored at -80°C. Once the bulk titre was known the bulk could then be defrosted, diluted, where appropriate, and filled. Filled vials were then stored at -80°C.

#### 1.6.4 Tangential Flow Filtration

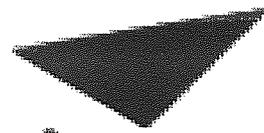
To enable larger clinical trials to be conducted larger quantities of the DISC-HSV vaccine needed to be purified. Tangential flow filtration was used for the purification process instead of sucrose gradient centrifugation due to the cost and difficulties in scaling up high speed centrifugation.

Roller bottle produced virus was harvested by scraping the infected cells into hypotonic saline (10 ml/roller bottle) then pooled in a jacketed glass bottle kept at 4°C. Large cell debris and cell nuclei were removed by tangential filtration through a 0.8 µm membrane. Filtrate from this membrane was then subjected to DNA digestion using the endonuclease Benzonase<sup>TM</sup> for 1 hour. The virus was then diafiltered on a Filtron tangential flow ultrafiltration rig using a 300 kDa membrane with 10 volumes of PBS. This method was the first scaled up virus purification method used at Cantab Pharmaceuticals. This process allowed the quantity of harvest to be used to be increased to any scale that was desired since tangential flow filtration is directly scaleable. The drawback with this process was the purity of the virus obtained was far inferior to that obtained from the sucrose gradient process. Also the recoveries through the process were poor and would not have been viable for manufacturing.

#### 1.6.5 Influenza

There are at present no existing large scale purification methods for herpes viruses, but other virus purification methods can provide useful information in deciding a strategy for DISC HSV purification. Influenza is an RNA virus which is

encapsulated and enveloped. Influenza can be observed in one of two forms, either spherical or filamentous. In the spherical form the overall diameter is between 50 and 120 nm (see Figure 1.3) or 20 nm and 200-300 to 3000 nm long for the filamentous form. The surface of the virus has approximately 500 projections dispersed evenly giving the virus its spiky appearance. The influenza virus had already been studied in a larger scale process (Merton, 1996) and is described below.



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**Figure 1.3 Electron micrograph of Influenza virus.**

*The particles shown are in the special form and are approximately 120 nm in diameter (Hayase et al. 1995).*

Cells were grown to confluency on microcarriers and then infected with influenza virus, A/Shanghai strain, and incubated. Virus was harvested from the cell culture media and cell debris removed by centrifugation, 1500 g for 30 minutes; then virus was pelleted from the supernatant by high speed centrifugation, 15,000 g for 3 hours. The virus was further purified by banding in a 10-50 % gradient of potassium tartrate at 20,000 g for 18 hours. The virus is extracted from the gradient using a hypodermic needle and syringe.

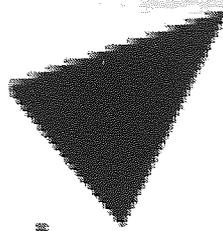
This method would probably produce highly purified virus although information on this and the recoveries obtained is not present in the literature. Although this process has been used to purify virus on a scale larger than flat flasks, it does not seem possible to use this on a larger scale due to the limitation of the high speed centrifugation steps. The best process scale centrifuges will usually go up to only

15,000 g for a disc centrifuge and that is operating in a batch mode (Verrall, M. (1996) Chapter 2). Also the gradient centrifugation step has to be a batch process making production more problematic. One other problem with using the influenza virus as a model for a purification strategy for DISC HSV is that influenza is not cell associated and therefore does not require cells to be harvested. In short this process does not seem to address the problems of scaling up but just uses a laboratory process to its maximum capacity, which would not meet the requirements for full-scale vaccine production.

#### 1.6.6 Foot and Mouth disease virus

Foot and Mouth disease virus (FMDV) is another virus that has been studied for large scale production due to the commercial potential for a vaccine against this disease and is described below (Navarro del Canizo *et al.* (1996)). This virus is also a better model than influenza since it is cell associated, like the herpes virus, and problems of harvesting and removal of high contamination levels need to be overcome. FMDV is a member of the *Picornaviridae* family, (genera *aphthovirus*) (Perez-Bercoff, 1979), which are small 22-30 nm single stranded RNA viruses that are non-enveloped making them very different to herpes viruses, see Figure 1.1.

Infected cells are lysed by a cycle of freezing then thawing followed by removal of large debris by centrifugation, 3,000 g for 10 minutes. Supernatant was applied to a Sephadex G-25 PD-10 gel filtration column to exchange the virus into 0.01 M phosphate, pH 7.0, 0.15 M NaCl buffer. This step enables the virus to be used for chromatographic purification. 3.5 ml of conditioned virus was then loaded onto a 3 ml heparin Ultrogel (IBF Biotechniques) chromatography column and following a wash step, using the conditioning buffer, the virus was eluted in 1.25 M NaCl.



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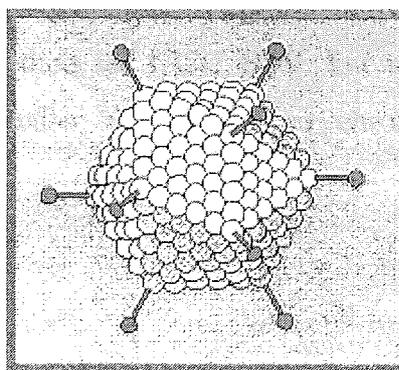
**Figure 1.4 FMDV reconstruction from X-ray crystallography data.**

*Foot and mouth disease virus is a non-enveloped virus which is 22-30 nm in diameter and has been reconstructed using computers from data obtained by X-ray crystallography of crystallised virus particles (Logan, 1993).*

This process looks to be more suitable than using high speed centrifugation since chromatography is scaleable although at only 3 ml the column used here is far from the size that is needed for DSIC HSV production. It is also indicated that the purity of virus eluted from the column is comparable to that obtained from virus prepared by ultra centrifugation on a caesium chloride gradient with the added benefit of obtaining nearly 100 % yield. There are however some drawbacks to this process. The use of freeze thaw cycles at large scale would be time consuming and expensive to operate. The need to centrifuge out debris and condition the virus using a size exclusion column is also expensive with the problem of low yields associated with size exclusion of viruses to overcome, which is not mentioned in the literature. Also the small size of this virus, which is non-enveloped, means recoveries using this process on herpes virus could be vastly different.

### 1.6.7 Process Scale Adenovirus Purification

There are only two processes at present that have attempted to overcome the scale issue; these are processes for a herpes virus type I, discussed in the next section, and for an adenovirus (Giroux (1998)). Adenoviruses are primarily being used as potential gene delivery vectors (Kozarsky and Wilson, 1993) and therefore need to be produced in large quantities and highly purified. In considering the methods used in this process however it must be noted that the adenovirus is greatly different to the herpes virus. The adenovirus virion consists of a non-enveloped icosahedral protein shell, encasing a DNA-protein core complex with a diameter of approximately 45 nm. From the twelve vertices, created by the icosahedral shape, project long fibres (Horne *et al.*, 1959) which give the virus an overall diameter of between 70 and 90 nm, see Figure 1.5. This virus is therefore smaller than the herpes virus and does not have the lipid bilayer membrane associated with herpes.



**Figure 1.5 Representation of an adenovirus.**

*The adenovirus is comprised of hexon and penton subunits, distinguished by having either five or six neighbouring proteins. From each of the vertices projects a fibre protein giving the structure an overall diameter of 70-90 nm.*

The following is a brief description of the large-scale process employed by Canji Inc., San Diego, California (Giroux (1998)). Adenovirus is multiplied in cells grown on microcarriers in a bioreactor. Virus is then released directly from the cells still attached to the microcarriers by a cycle of freeze thaw. Contaminating host cell DNA is then digested using an endonuclease (Benzonase™). Debris is removed by centrifugation, at low force, then filtration. Virus is then column purified using DEAE anion exchange resin followed by a zinc metal chelate affinity column. The resulting eluate is buffer exchanged by either hollow fibre or flat plate systems and finally sterile filtered through a 0.22 µm filter. This process generates an overall yield of 50%.

All parts of this process are scaleable without the technical problems encountered with the processes mentioned above. Also the good yield means that this will probably be an affordable process as well. There are no obvious negative aspects to this process apart from the inclusion of the costly DNA digestion step, which at scale may become one of the most expensive steps in the process and introduces contaminants. It would be useful therefore to release less DNA initially so enabling other steps in the process to remove the remainder of this contaminant. It is also useful to note that this virus can be purified using chromatography, like the FMDV outlined in section 1.6.6, which may suggest a possible method for DISC HSV; but in both cases they are non-enveloped viruses and smaller than HSV, so chromatography may be more problematic.

#### 1.6.8 Process Scale HSV I Purification

A more suitable virus purification method to study is one produced by NeuroVir Inc., Vancouver, Canada, for the purification of a HSV type 1 vector (Johnson, 1998). This method, made public at the end of 1998, was not available when decisions were made on the strategy for the purification of DISC HSV. However, it is useful to make comparisons between this and the other processes outlined above with the eventual final process invented here, which will be discussed further in the general conclusions.

This HSV-1 vector is for infecting brain tumours and has been especially selected not to infect normal brain tissue, which means unmodified cell lines can be used to produce it; in this case Vero cells were used. This therefore closely resembles the production process used for the DISC HSV product (see 1.5).

Vero cells were seeded in roller bottles and incubated at 37°C for 1 day. Cells were then infected with HSV-1 and incubated for three days before harvesting. Infected cells were harvested and 'microfluidised'. Microfluidisation is a process that homogenises the harvest by forcing it through a chamber at high pressure and speed. Following a filtration step to remove debris, the harvest was diafiltered on a hollow fibre membrane into a buffer acceptable for DNA digestion with Benzonase. Harvest is then purified in a size exclusion column. Column eluate is concentrated using hollow fibre ultra-filtration membrane and the product is formulated in PBS/10 % glycerol and then sterile filtered through a 0.22 µm filter. Reported overall recovery for this process was approximately 25 % infectious virus when virus was assayed using the plaque assay.

#### 1.6.9 Aqueous two-phase virus extraction

Partition in aqueous two-phase systems has been widely used for the separation and purification of biological materials and is an established technique. The methodologies involved were extensively developed by Albertsson in the 1950's (Albertsson, 1986). These systems rely on the fact that two solutions of structurally distinct polymers, when over a critical concentration, when mixed separate into two layers, one rich in one polymer and the other layer rich in the second. Particles to be separated are added and mixed then the phases left to settle out. The different physical properties of the polymers create environments where the particles enter into one or the other, or the interface, if conditions are right. Conditions can be altered by using different polymers or altering the salt concentration of the system (Zaslavsky, 1978). If the volume of the phase in which the product of interest is small then a concentration can also be achieved, hence the partitioning of substances from large

cultures can be achieved by addition of polymers directly to the medium. If different polymers are used and their characteristics altered using varying concentrations of salt then selective purification of the particle of interest can be achieved.

In systems containing dextran sulphate, polyethylene glycol and salt viruses tend to partition into the bottom phase, or bottom phase and interface (Philipson, 1969). When added directly to medium up to 100 fold concentrations can be achieved. Viruses that have been separated using this method include Poliovirus (Bengtsson, 1962), Vaccinia virus (Albertsson, 1960), Adenovirus (Philipson, 1960), Influenza virus (Grinrod, 1970) and Newcastle virus (Weslen 1959).

Although a quick and reproducible method with separation taking a short period of time, down to a few seconds in some cases, most methods still rely on centrifugation to separate the polymer from the product. This method though, does have its benefits. The interface tension between the phases will not affect the viruses and the overall mild process will retain high functionality of the product. This method of separation, however, does not seem to be widely used for the separation of viruses at the process scale and is generally used at laboratory scale. Most virus recovery systems have tended to be in favour of chromatography with availability of a variety of matrices meeting the requirements for virus purification.

### ***1.7 Proposed plan for investigation of DISC HSV purification***

The methods of purifying viruses outlined above give a good starting point to the investigation of large scale DISC HSV purification. It is clear, however, that most of the process problems arise from how the virus is harvested, if the virus of interest is cell associated, and the associated levels of contaminants that are released. There, therefore, needs to be detailed effort in the separation of virus from host cells since this will determine how easily the virus can be further purified downstream. For disrupted cell processes a number of steps are required for contaminant removal, especially particulate, before virus can be purified in a process such as chromatography. Also most of the methods discussed above have drawbacks and

these are usually associated with centrifugation and the inability to scale up high speed gradient centrifugation. Any scale process will therefore have to discount this method, as for adenovirus and HSV I, but still produce a product of similar, if not better, purity to that obtained from gradient centrifugation. A common replacement for gradient centrifugation is column chromatography, only the processes that utilise this most are for small non-enveloped viruses such as adenovirus and FMDV.

Productivity is essential for efficient production of any product. Therefore, initial investigations were made on the growth of the host cells, CR2 cells, and the parameters associated with virus and harvest, see Chapter 3. This work was aimed at achieving a consistent production process for virus manufacture, resulting in high titre and consistent levels of impurities to enable accurate assessment of harvest and purification techniques.

The next stage of the investigation was into the method of harvest (Chapters 4 and 5). HSV-2 is cell associated and current harvest methods rely on the harvesting of infected cells then rupturing them to release virus. These methods, as mentioned in the paragraphs above, and others such as glycerol shock were tested for their effectiveness at releasing DISC HSV. Methods to be tested were ones that were able to be used at large scale since small-scale purification was not the objective of this study. Novel methods were also investigated for their potential at large scale.

To test a purification process using all the available methods of harvest would be too time consuming; therefore, only harvest methods found to give high yields with low contaminant burden were used for studies on purification of DISC HSV (Chapter 6). Originally methods such as tangential flow filtration were to be optimised since this was the current best scale up method to use, but this plan was made flexible to allow for novel methods to be investigated. The knowledge that some viruses had been purified by column chromatography (section 1.6.6), even though on small scale and with mainly non-enveloped viruses, gave a useful beginning to identifying a potential method for chromatographic purification of DISC HSV.

## 2 Experimental

### 2.1 Cell Culture

All cell culture was conducted in a laminar flow hood observing good sterile technique to ensure no contamination of the cells and virus used during this study. For the growth of the cells and virus no antibiotic was used and if any microbial contamination was observed the cultures were discarded. Antibiotics were only used in the infectivity assay, since the large number of steps in this assay means a high risk of introducing contamination. The selection of media and supplements was conducted by Cantab Pharmaceuticals prior to the start of the work.

#### 2.1.1 Media

Media used for all cell culture was Dulbecco's Modified Eagles Medium (DMEM) with L-analyl-L-glutamine (0.862 g/l), D-glucose (4.5 g/l), without sodium pyruvate, with pyridoxine (0.004 g/l) (GibcoBRL). To support growth of cells 5% (v/v) foetal bovine serum (FBS) (Pabco Biologicals, NZ) was added to the DMEM. All experiments used serum from the same batch to avoid batch to batch variation. Serum was supplied filter sterilised and gamma irradiated.

#### 2.1.2 Cell lines

CR2 cells were prepared as described by Boursnall *et al.* 1997. Briefly green monkey Kidney (Vero) cells, supplied by the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK) were transfected with an expression vector expressing the HSV2 membrane glycoprotein gH gene enabling the cells to produce gH protein.

The CR1 cell line was constructed in the same manner as the CR2 cell line except the expression vector contained the HSV1 gH gene instead of the HVS2 gH gene.

### 2.1.3 Growth conditions

The Vero cell line used to express the viral glycoprotein gH is an adherent line, therefore requiring a surface to bind to for growth. Two basic methods for growing the cells were used, 75cm<sup>2</sup> flat flasks, vented caps, or 850cm<sup>2</sup> roller bottles (Corning). Flasks were filled with 40ml of culture media for growth of cells and incubated at 37°C in a humidified incubator (Biocenter 2001, Salvis). Incubators were gassed with 5% CO<sub>2</sub> to prevent the culture media from becoming alkaline. Roller bottles were filled with 100 ml of culture media and were gassed with 10% CO<sub>2</sub> for 20 seconds prior to incubation at 37°C in a roller rig incubator at 0.5rpm. All manipulations of cells, media and culture vessels was carried out in a laminar flow microbiological safety cabinet (MBSC) to prevent contamination of the cell cultures.

### 2.1.4 Harvesting and Passaging of cells

Media was decanted from the culture vessel and cells washed briefly with Dulbecco's Phosphate Buffered saline (DPBS) without Magnesium or Calcium, 10 ml for 75cm<sup>2</sup> flasks or 50 ml for roller bottles. DPBS was decanted and replaced with Trypsin-EDTA, 0.5% (w/v) trypsin, 0.2 g/l EDTA in modified Puck's saline (GibcoBRL), 7 ml for flasks or 10 ml for roller bottles and incubated at 37°C. When cells were detached from the plastic, usually less than 10 minutes, viewed under an inverted microscope (Olympus CK2), the trypsin was stopped by addition of approximately 3 volumes media containing serum. Cells were then pipetted into a polypropylene tube for storage at +4°C. Cells were stored for no longer than 6 hours before being re-seeded, although they were greater than 90% viable after 24 hours. Following a viable cells

count the appropriate volume of cells were added to 75cm<sup>2</sup> flasks containing 40 ml serum containing media or roller bottles containing 100 ml and pre-gassed with 10% CO<sub>2</sub>.

#### 2.1.5 Determination of cell number - Trypan Blue

Cells were harvested, as described above and the total volume of cells noted. To ensure an accurate cell count, cells were vortexed to produce a homogeneous cell suspension. One millilitre of cell suspension was diluted with 3 volumes of trypan blue (Sigma). After further vortexing a portion of the cell suspension was pipetted into a disposable plastic counting slide (Kova Glastic). Cells were viewed under an inverted microscope (Olympus CK2) at x200 magnification. Each corner of the counting grid, bordered by a double line and containing a single line grid of 4 by 4 squares, was counted for the total number of cells and the number of cells that appear white, i.e. cells that keep the trypan blue out. The average of the counts from the four corners of the counting grid was used to calculate the concentration of cells. Each corner has a stated volume of 0.1 µl; therefore to obtain a figure for the number of viable cells per ml, the averaged figure of cells, keeping out trypan blue, was multiplied by 10<sup>4</sup> and then multiplied by the dilution factor used when adding the trypan blue, in this case 4. This figure multiplied by the total volume of cells gives the number of viable cells harvested. The calculation is repeated for the total number of cell (white plus blue cells); then viable count divided by the total cell count multiplied by 100 gives the percent viability of the cell population.

#### 2.1.6 Determination of cell growth profile

To discover the best possible inoculation conditions for the CR2 cells a range of seeding densities was chosen for study. Cell growth curves were generated for seeding densities of 10,000, 25,000, 50,000, and 100,000 cell/cm<sup>2</sup> and lasting up to 12 days

post seeding. To ensure more accurate results, each time point on the growth curves was calculated from the average of cell counts from triplicate flasks. The estimated doubling time for these cells was approximately 24 hours; therefore only one time point per day was thought to be sufficient. For each seeding density with twelve time points thirty six 75 cm<sup>2</sup> flasks were filled with 40 ml DMEM containing 5% FBS. The appropriate number of cells was then added to each flask (i.e. for a seeding density of 10,000 cells/cm<sup>2</sup>, 10,000 x 75= 7.5x10<sup>5</sup> cells was added to each flask); then the flasks incubated at 37°C, 5% CO<sub>2</sub> in a humidified incubator. For each time point, at approximately 24 hour intervals, triplicate flasks were removed and the cells harvested as above. For each flask a viable cells count and total cell count was conducted by the trypan blue method, as above; also, cell numbers were estimated by nuclei count using the crystal violet method (Sandford *et al.* (1950)), as above. For viable and total cell counts the values were averaged and errors calculated. Graphs were plotted of the values obtained and from the phase of the graph relating to maximal cell growth the doubling times of the cells was calculated by the gradient of the tangent.

## **2.2 Virus Growth**

### **2.2.1 Virus lines**

The virus used in this study was a gH gene deleted herpes simplex virus type 2 (DISC-HSV2) (Boursnall *et al.* 1997). Stocks of this virus were prepared by infecting CR2 cells and, when ready for harvesting, scraping the infected cells into hypotonic saline. After pooling, the harvested virus was aliquoted into polypropylene cryovials (Nunc) and stored at -80°C. A homogeneous virus suspension was achieved by one cycle of sonication (see 2.4.1) upon complete thawing of the vial.

## 2.3 Growth conditions

### 2.3.1 Determination of virus growth parameters

CR2 cells were seeded, as described above, at the most appropriate seeding density (see results). Fourteen T75 flasks (Corning) for each growth curve were used to enable duplicate flasks to be harvested each day for up to seven days post infection. To determine the maximal yield of virus from CR2 cells, three different multiplicities of infections (MOI) were carried out. MOI's of 0.1, 0.01 and 0.001 pfu/cell were investigated at three different time points on the cell growth curve. The time points were chosen to span the recognised growth states of the cell line (i.e. lag phase, log phase and stationary phase). The quantity of virus for each infection was calculated by multiplying the MOI by the number of cells/flask interpolated from the cell growth curve (see results). This figure is the quantity of virus needed per flask at that time point. Virus stock, of known titre, was thawed, sonicated then diluted with media to the required pfu/flask/ml. Appropriate flasks were then removed from the incubator and 1ml of the diluted virus was added to each flask and incubated at 37°C. Each infection was carried out using virus that had been thawed from -80°C and kept on ice prior to infection. Every day post infection duplicate flasks were removed and the cell monolayers were scraped into the media using sterile scrapers. The media was then decanted into a 50ml polypropylene tube (Corning) and cells pelleted by centrifugation at 675g for 8 minutes at 4°C. The pellets were suspended in 2 ml PBS then sonicated (see 2.4.1) prior to being frozen at -80°C. On thawing the samples were re-sonicated prior to assaying for virus by TCID<sub>50</sub>. Each set of samples was assayed at the same time to reduce variances in the assay. Samples of supernatant media were also assayed for infectious virus to assess the proportion of virus that was cell associated. Infectious virus titres generated from the duplicate flasks were averaged before plotting.

### 2.3.2 Standard virus production in roller bottles

Roller bottles were filled with 100 ml media, as described above, then gassed for 20 seconds with 10% CO<sub>2</sub>. Each roller bottle was seeded with  $2 \times 10^7$  cells, as described in the passaging method, then incubated at 37°C in a roller rig incubator rotating at 0.5 rpm. 5 days post passage media was decanted and cell monolayers rinsed three times with 50 ml DPBS. 100 ml of DMEM was then poured into each roller bottle and incubated at 34°C. Cells from one roller bottle were harvested and cells counted to calculate the quantity of virus required for each roller bottle. Cells were infected with a multiplicity of infection (MOI) of 0.01; therefore cell number (per RB)  $\times$  0.01  $\times$  number of RB's = total amount of virus required. A suitable amount of virus stock was thawed from -80°C in a 37°C water bath and sonicated. The total amount of virus required divided by the stock virus titre (pfu/ml) gives the quantity of stock virus needed (ml). The desired quantity of stock virus was then pipetted into x ml of DMEM, where x is the number of roller bottles to be infected. After gentle mixing 1 ml of virus was pipetted, using an eppendorf repeating pipette, into each roller bottle and incubated at 34°C. When cell monolayers were observed to have greater than 90% cpe, virus was ready for harvest; this was usually 66-69 hours post infection.

## **2.4 Virus Harvest**

### 2.4.1 Sonication

Virus samples to be sonicated were sealed with Nescofilm in a polypropylene tube, for example Nunc cryovial, to prevent the escape of hazardous aerosols. Samples that were frozen were thawed completely, by placing in a 37°C water bath or in a 37°C incubator, before sonication. Tubes were clamped in place in a 200 ml cup horn water

bath sonicator (Sonicator XL2020, Misonix Inc.) filled with cold water completely immersing the sample. Virus was then sonicated for one minute at full power, approximately 550 watts at 20 kHz. On completion of sonication the water bath was filled with fresh water for the next sample to prevent heat build-up which could cause viral inactivation. Sonicated virus was held on ice prior to use.

#### 2.4.2 Estimation of maximum virus yield

For every experiment conducted on the release of virus a positive control was needed to ascertain the maximal yield obtainable from that particular harvest. At least duplicate flasks, or roller bottles, were harvested by scraping the cells into the media and decanting into either 50 ml or 125 ml polypropylene centrifuge tubes (Corning). After centrifugation, 1000 g for 10 minutes at 4°C, the supernatant was decanted and cell pellet re-suspended in a small volume of DPBS. Cells were sonicated prior to assaying for virus by TCID<sub>50</sub> or stored by freezing at -80°C. If samples were frozen prior to assaying then, on thawing, the samples were re-sonicated.

#### 2.4.3 Hypotonic Saline

Media was decanted from infected cells and flasks left to stand on end for about half a minute to allow the remaining media to be pipetted from the bottom. Hypotonic saline (10 mM sodium chloride, 32 mM Disodium hydrogen phosphate anhydrous, 7 mM Sodium dihydrogen phosphate.2H<sub>2</sub>O, pH7.5), 10 ml for roller bottles and 6 ml for flasks, was added and incubated for 10 minutes at 37°C. Cell monolayers were then scraped using sterile cell scrapers (Costar) and harvest pipetted from the flask. Flasks were then rinsed with a further volume of hypotonic saline to ensure good recovery. The wash and the harvest were pooled, total volume noted and kept on ice. To determine the effect of hypotonic saline on the separation of the virus from the cells

the harvest was centrifuged at 3000 rpm for 10 minutes at 4°C and supernatant assayed for virus by TCID<sub>50</sub>. Comparing this result to a sonicated harvest (see 2.4.1) gives the relative amount of dissociation.

#### 2.4.4 Hypotonic saline pH range

The desired pH range for this experiment was 4-8. To enable sufficient buffering capacity over this range two different buffers were required for each end of the scale. pH 4, 5 and 6 were prepared in citrate-phosphate buffer and pH 7 and 8 were prepared using phosphate buffer. All buffers were prepared from concentrated stock solutions of base and acid, see table (Methods in Enzymology 1990). The harvesting of the virus was performed as in the hypotonic saline harvest method (2.4.3).

pH	0.1M citric acid ml	0.2M dibasic sodium phosphate ml
4	30.7	19.3
5	24.3	25.7
6	17.9	32.1

**Table 2.1 Citrate-phosphate buffered hypotonic saline formulation for acidic pH.**

*Following mixing, make up to 100 ml and add 0.058 g sodium chloride.*

pH	0.2M monobasic sodium phosphate ml	0.2M dibasic sodium phosphate ml
7	39.0	61.0
8	5.3	94.7

**Table 2.2 Phosphate buffered hypotonic saline formulation for alkaline pH.**

*Following mixing, make up to 100 ml and add 0.058 g sodium chloride.*

#### 2.4.5 Glycerol Shock

Virus was harvested by scraping infected cells into the culture media and decanting into a 50 ml polypropylene centrifuge tube. Infected cells were pelleted at 1000 g for 10 minutes at room temperature. Media supernatant was decanted and discarded and the cell pellet re-suspended in 4 ml of DPBS. 660 µl of pre-warmed, 37°C, 90% (v/v) glycerol was added to the cell suspension and tubes incubated at 37°C for 5 minutes. The addition of 660 µl of 90% glycerol and incubation was repeated twice giving a final concentration of 30% glycerol in the cell suspension. After the final incubation the cell suspension was chilled on ice for 5 minutes. Cells were pelleted by centrifugation at 1200 g for 10 minutes at 4°C. Supernatant was pipetted off and sampled for virus infectivity assay. Cell pellet was loosened by gentle vortexing, for a few seconds, whilst adding 3 ml of hypotonic saline. After incubating on ice for 5 minutes cell debris was pelleted by centrifugation at 700 g for 10 minutes at 4°C. Supernatant was collected and held on ice; cell debris pellet was washed with a further 3 ml hypotonic saline and centrifuged as before. Wash supernatant was pooled with the first supernatant to produce final harvest, which was assayed for virus. To determine the quantity of virus lost, cell pellets were resuspended in DPBS sonicated then assayed for virus. All experiments were conducted in triplicate and individually assayed for virus by TCID<sub>50</sub>. To enable a comparison to be made with previous processes, triplicate flasks were harvested and assayed by the sonication and hypotonic saline methods as described in 2.4.1 and 2.4.3. Results were calculated as the average of the triplicate samples and errors calculated.

#### 2.4.6 Freeze Thaw

Virus was harvested by scraping infected cells into the culture media and decanting into a 50 ml polypropylene centrifuge tube and held on ice. Infected cells were

centrifuged at 1000 g for 10 minutes at 4°C. Media supernatant was discarded and the cell pellets re-suspended in 4 ml of pre-chilled DPBS, then vortexed, to re-suspend the cell pellets. Cell suspensions were immersed in liquid nitrogen until equilibrium was reached then thawed at 20°C in a water bath. This process was repeated up to three times for duplicate samples. After cell suspensions had thawed they were held on ice prior to assaying for virus. To enable a comparison to be made with previous processes duplicate flasks were harvested and assayed by the sonication method as described in 2.4.1. Results were calculated as the average of the duplicate samples and errors calculated.

#### 2.4.7 Heparin

Monosodium heparin sodium salt was supplied sterile (CP Pharmaceuticals, Wrexham) in 5 and 20 ml vials ready for intravenous injection. Heparin source was the 7000 Dalton molecular weight fraction from porcine intestinal mucosa, formulated in water and pH adjusted with hydrochloric acid.

#### 2.4.8 Heparin concentration range

Concentration range experiments were conducted in 75cm<sup>2</sup> flasks. Monosodium heparin (CP Pharmaceuticals, Wrexham, UK) was supplied at a concentration of 1000 IU/ml. 175 IU  $\equiv$  1 mg/ml (CP pharmaceuticals) therefore 1 ml contains 5.7 mg heparin. Heparin concentrations of 0, 2, 5, 10, 20, 50, 100, 200  $\mu$ g/ml were formulated in hypotonic saline. Media from flasks was discarded and replaced with 6 ml of the above heparin concentrations, then incubated at 37°C for 15 minutes. Cells were scraped into the buffer, pipetted in polypropylene centrifuge tubes and held on ice. Samples were centrifuged at 2000 g for 10 minutes at 4°C and supernatants were carefully sampled. Cell pellets were re-suspended in 1 ml DPBS and sonicated.

Supernatant samples and sonicated cell pellets were assayed for infectivity. Each concentration was carried out in multiples of four. Four flasks were also harvested by the sonication method (2.4.1) to give an indication of maximal yield.

#### 2.4.9 Sodium chloride concentration range

Sodium chloride concentrations of 140, 250, 500, 750, 1000, 1500, 2000 mM were formulated in DPBS and filter sterilised with 0.22  $\mu\text{m}$  hollow fibre filters (Mediacap-5, Microgon). No attempt was made to adjust any pH variation observed due to the addition of salt. The experimental method and virus sampling was conducted as for the heparin concentration range experiment (2.4.8). Supernatant samples and sonicated cell pellets were assayed for infectivity. Each concentration was carried out in multiples of four. Four flasks were also harvested by the sonication method (2.4.1) to give an indication of maximal yield

#### 2.4.10 Heparin release time course

Media was discarded from roller bottles ready for harvest, and replaced with 35 ml of DPBS containing 50  $\mu\text{g/ml}$  heparin, then incubated at 34°C in a roller rig incubator. At 30 minute intervals 0.5 ml samples were taken and stored at 4°C. Samples were centrifuged at 10000  $g$  in a microfuge (Sorvall 12V) for 1 minute and supernatant assayed for infectivity by TCID<sub>50</sub>. The experiment was conducted in triplicate and a control harvested by the sonication method included (2.4.1).

#### 2.4.11 Sodium chloride release time course

The method for this experiment was conducted in a similar manner as for the heparin time course. Sodium chloride was formulated in dPBS to a concentration of 1 molar and filter sterilised using a 0.2 µm hollow fibre filter (Microgon).

#### 2.4.12 Acid wash

Media from roller bottles, ready for harvest, was discarded. To four roller bottles 10 ml of dPBS containing 50 µg/ml heparin was added and incubated at 34°C. Three roller bottles were briefly rinsed (less than 30 seconds), by rolling the bottles in the laminar flow hood, twice with 50 ml of dPBS and after decanting replaced with 20 ml dPBS containing 50 µg/ml heparin and roller bottles incubated at 34°C. Three roller bottles were briefly rinsed with 50 ml of pH 3 glycine buffer, adjusted using citric acid, then rinsed with 50 ml dPBS to return the cells to neutral pH. 20 ml of dPBS containing 50 µg/ml heparin was added to each roller bottle and incubated at 34°C. All roller bottles were sampled, 0.7 ml, at one hour intervals and centrifuged at 10,000 g in a microfuge before supernatants were assayed for infectivity.

#### 2.4.13 Standard heparin harvest

Roller bottles were passaged and infected as in 2.3.2. Infected cells were observed using an inverted microscope (Olympus CK2) and only harvested when cells displayed above 90% cytopathic effect (CPE), usually between 65 and 69 hours post infection. Media was discarded and replaced with 10 ml dPBS containing 50 µg/ml heparin. Roller bottles were incubated at 34°C for 3 hours in a roller rig at 0.5 rpm. On removing roller bottles from the incubator they were left for 1-2 minutes to allow liquid to drain to the bottom, then harvest buffer was collected by pipetting into sterile polypropylene centrifuge tubes. Harvest material was then centrifuged at 1800 g for

10 minutes at 4°C. Supernatant was decanted into a sterile plastic bottle (Nalgene) and stored at 4°C.

#### 2.4.14 Standard salt harvest

Roller bottles were passaged and infected as in 2.3.2. Infected cells were observed using an inverted microscope (Olympus CK2) and only harvested when cells displayed above 90% cytopathic effect (CPE), usually between 65 and 69 hours post infection. Media was discarded and replaced with 10 ml dPBS containing 800mM sodium chloride. Roller bottles were incubated at 34°C for 4 hours in a roller rig at 0.5 rpm. On removing roller bottles from the incubator they were left for 1-2 minutes to allow liquid to drain to the bottom, then harvest was collected by pipetting into sterile polypropylene centrifuge tubes. Harvest material was then centrifuged at 1800 g for 10 minutes at 4°C. Supernatant was decanted into a sterile plastic bottle (Nalgene) and stored at 4°C.

### **2.5 Virus harvest clarification**

#### 2.5.1 Low speed centrifugation

Centrifugation was employed as a simple method to remove cell debris from virus harvests. Harvest material was pipetted into polypropylene centrifuge tubes and centrifuged at 1800 g in a refrigerated bench top centrifuge (Sorvall 6000B) for 10 minutes at 4°C. Supernatant was usually decanted from the centrifuge tube into a sterile plastic bottle (Nalgene). If the cell debris pellet was not solid, supernatant was pipetted off. Supernatant was then stored at 4°C for up to 1 month.

## 2.5.2 Filtration

To enable the virus harvest to be used for chromatography a filtration step was required. For small volumes of salt harvested virus, 50-200 ml, harvest was drawn into a 60 ml syringe and passed through a single 5 µm pore size filter (Gelman Acrodisc 50A) into a sterile plastic bottle. If larger harvest volumes required filtering multiple filters were used. For heparin harvested virus larger volumes, up to 500ml, were passed through a single filter. Volumes of greater than 400 ml were pumped through the filters using a peristaltic pump. All filtration was carried out aseptically in a laminar flow safety cabinet.

## 2.6 Virus purification

### 2.6.1 Chromatography matrices

Various chromatographic matrices were tested for their ability to bind the DISC HSV. The following is a summary of these matrices with information on bead and pore size, ligand and ligand density.

*Heparin sepharose* - (Pharmacia) a cross linked agarose gel (6%) of between 45 and 145 µm in diameter with an exclusion limit of 6,000 kDa. Heparin (porcine intestinal mucosa, 5 kDa) is attached by cyanogen bromide activation.

*Heparin sepharose 6 fast flow* - (Pharmacia) a cross linked agarose gel (6%) of between 45 and 145 µm in diameter with an exclusion limit of 6,000 kDa. Heparin (porcine intestinal mucosa, 5 kDa) is attached by an undisclosed method. This is the process scale version of the heparin HP media, but has about half the ligand density.

*Streamline heparin* - (Pharmacia) solid dense media designed for expanded bed capture of heparin affinity molecules. No information is present on how the heparin is bound to this matrix.

*Heparin -HP* - (Pharmacia) a highly cross linked agarose bead (6%) of between 24 and 44  $\mu\text{m}$  in diameter with an exclusion limit of 4,000 kDa. Heparin (porcine intestinal mucosa, 5 kDa) is attached by N-hydroxysuccinimide activation with a final density of 10 mg heparin per ml gel. Matrix is supplied in pre-packed heparin-HiTrap columns.

*Cellufine sulphate* - a spherical cellulose matrix with a particle size distribution in the range 45 to 105 $\mu\text{m}$  and an exclusion limit of 3 kDa. The matrix is sulphated at the C<sub>6</sub> position to a final sulphate concentration of 8  $\mu\text{M}$  /ml of bead.

*Cellufine heparin* - prepared from the same cellulose matrix as cellufine sulphate with porcine intestinal mucosa heparin of high molecular weight, 130 - 200 kDa, attached to the cellulose through a hydrophilic hydroxysuccinate spacer molecule (-O-CH<sub>2</sub>-CH(OH)-CH<sub>2</sub>-CO-NH-Heparin).

*Bioprocessing matrices* - (Bioprocessing Ltd., UK) The following matrices were obtained as test samples so limited information was available about their physical parameters and the source and type of ligand used.

*BPL-8054* - a sulphated ligand bound to sephacryl base matrix

*BPL-8074* - a sulphated ligand bound to sephacryl matrix with 'small' pore size but of higher ligand density than either BPL 8054 or 8080

*BPL-8080* - a sulphated ligand bound to sephacryl matrix with a 400 nm pore size with a similar ligand density as BPL 8054

*Cellthru heparin* - (Sterogene Bioseparations Inc., USA) a 4% cross linked agarose bead of between 300 and 500  $\mu\text{m}$  diameter with an exclusion limit of 20 MDa. The source and attachment method of the heparin is unknown. The size of heparin used is in the range 5-30 kDa.

*Sulphated-Hiflow* - (Sterogene Bioseparations Inc., USA) a hardened fibrous cellulose matrix with no measurable size distribution. The matrix is sulphated to a concentration of 8 µm/ml of matrix.

### 2.6.2 Batch adsorption chromatography

1 ml chromatography matrix was pipetted into a sterile 50 ml polypropylene centrifuge tube and washed twice with 30 ml DPBS. DPBS was removed by centrifuging the matrix for 30 seconds at 800 g in a bench top centrifuge (Sorvall 6000B). Salt harvested virus (1 molar NaCl in 10mM citrate buffer) was clarified by centrifugation and diluted, 7.25 fold, to isotonic level with 10mM citrate buffer. 25 ml of the virus harvest was added to each of the tubes containing chromatography matrix and to a blank tube as a control. All tubes were laid on a shaker table and gently shaken. After 15, 30 then 30 minute intervals all tubes were centrifuged at 800 g for 30 seconds and a 0.5 ml sample removed. Chromatography matrices were re-suspended before tubes were replaced on the shaker table. Supernatant samples were kept at 4°C, 2-4 hours, before being assayed for infectivity.

### 2.6.3 Packed bed chromatography

A typical column adsorption experiment was conducted as follows. With the exception of heparin-HP, columns (Pharmacia XK16) containing 5 - 15 ml of the appropriate gel were prepared by equilibration with 5 column volumes of PBS (10 mM phosphate, 138 mM NaCl, pH 7.0) at a flow rate of 0.7 ml/min (Pharmacia P-1 pump). Clarified supernatant was 5µ filtered (Gelman 50A), then passed through the columns at a flow rate of 0.7 ml/min. Typically, 100 ml of supernatant containing around  $5 \times 10^7$  pfu/ml were loaded to the columns. The discharges from the columns

were monitored for UV absorption ( $A_{280}$ ) (Parmacia UV-1) and fractions were collected for analysis.

## **2.7 Analytical**

### **2.7.1 Infectivity Assay - TCID<sub>50</sub>**

Infectious virus titres were calculated using the Tissue Culture Indicated Dose–50 (TCID<sub>50</sub>) method (Reed and Munch 1938). This method estimates the infectivity of a sample by calculating the dilution required to produce cytopathic effect (cpe) in 50% of replicate wells. This figure is then converted into plaque forming units (pfu) using a mathematically derived conversion factor based on comparison studies between the TCID<sub>50</sub> method and the plaque assay method.

CR1 cells were harvested, as above, and diluted to  $4.5 \times 10^5$  cell/ml using media containing 50 IU/ml penicillin and 50  $\mu$ g/ml streptomycin. Using an eppendorf repeating pipette with a multi-channel adapter, 100  $\mu$ l of cells were pipetted into each well of a sterile 96 well microtitre plate. Plates were incubated in a humidified incubator at 37°C, 5% CO<sub>2</sub> until cell had adhered and flattened, approximately four hours, or overnight. Samples for assay were prepared as directed in the above methods and serially diluted 1:10 from  $10^{-1}$  to  $10^{-8}$  by pipetting 0.25 ml of sample into 2.25 ml of media containing penicillin/streptomycin and mixing with a new pipette tip. This process was repeated eight times. The  $10^{-1}$  dilution was discarded and each of the  $10^{-2}$  to  $10^{-8}$  dilution were pipetted into 24 wells of columns B-H of two CR1 cell containing microtitre plates (50  $\mu$ l into each well), pipetting the  $10^{-8}$  dilution first with an eppendorf repeating pipette. Column A was left as a blank. If the sample to be assayed was suspected to be of high titre, the sample was diluted to  $10^{-10}$  and the dilutions  $10^{-4}$  to  $10^{-10}$  were plated out instead. Plates were incubated in a humidified incubator at 37°C and 5% CO<sub>2</sub> for three days. Plates were read under an inverted microscope (Olympus CK2) using x400 magnification. A well was scored as positive when cpe was observed, the rounding up of cells due to viral infection. If infection

was advanced clear plaques could be seen as well. To calculate the TCID<sub>50</sub> value the number of positives were added for each column, across both plates. Starting from the 10<sup>-8</sup> column, going to the 10<sup>-2</sup>, positive numbers were cumulatively added, then starting from the 10<sup>-2</sup> column, going to the 10<sup>-8</sup>, the negative scores were cumulatively added. Using these cumulative figures the percent positive for each dilution can be calculated e.g. positive score divided by the positive score plus the negative score multiplied by 100. Then the following calculation is used to calculate the TCID<sub>50</sub> value.

$$\text{Proportional Distance (PD)} = \frac{(\% \text{ rate above } 50\%) - 50\%}{(\% \text{ rate above } 50\% - \% \text{ rate below } 50\%)}$$

$$\log \text{TCID}_{50} = \log \text{dilution next above } 50\% + \text{PD}$$

$$\text{TCID}_{50} / 0.05 = \text{TCID}_{50} / \text{ml}$$

The TCID<sub>50</sub> value was then converted into pfu/ml by a conversion factor. This factor was calculated from the observed difference in results gained from concurrent TCID<sub>50</sub> and plaque assay titres.

$$\text{TCID}_{50} / \text{ml} \times 0.69 = \text{pfu/ml}$$

### 2.7.2 Effect of heparin on the infectivity assay

Standard sample aliquots were assayed for infectivity, as above. The similar aliquots were then serially diluted 10<sup>-1</sup> to 10<sup>-8</sup> in media containing 50 IU penicillin, 50 µg/ml streptomycin and 50 µg/ml heparin and plated out onto CR1 cells in 96 well plates as above. Standard sample aliquots were also diluted in media containing heparin and then plated out onto 96 well plates with CR1 cells seeded with media containing 50 µg/ml heparin. To complete the possible permutations samples were serially

diluted in media and plated out onto CR1 cells seeded in media containing 50 µg/ml heparin.

In a separate experiment virus samples were made up to 0, 50, 100, 200, 400 µg/ml heparin by addition of concentrated heparin (5.7 mg/ml) and all samples were serially diluted and plated out as in the standard method. Resulting titres were then compared for the effect of heparin.

### 2.7.3 Total protein

The total protein assay was conducted using the Sigma Protein Assay Kit based on the Lowrey method (Lowrey OH et al 1951). A standard sample of bovine serum albumin (fraction V) was serially diluted in the range 5-160 µg/ml, including a blank, and made up to a final volume of 1 ml. Samples and positive control were diluted to within the standard concentration range to a final volume of 1 ml. To remove interference from certain buffers and chemicals, such as EDTA, sucrose, citrate, the protein is precipitated by adjusting the samples and standards to 150 µg/ml deoxycholate and then, after a ten minute incubation at room temperature, adjusted to 6% (w/v) trichloroacetic acid. Protein was then centrifuged at 10,000g (Sorvall 12V microfuge) for ten minutes. Supernatant was then decanted and blotted from the tubes. Protein was dissolved in 1 ml Lowreys reagent and transferred to appropriate tubes. Remaining protein and Lowreys reagent was rinsed from the tubes with 1 ml water and transferred to their respective tubes. After a 20 minute incubation at room temperature, 0.5 ml Folin and Ciocalteu's Phenol reagent was added to each tube and incubated at room temperature for 30 minutes. Solutions were then transferred to triplicate wells of a 96 well microtitre plate (Nunc Maxisorp) and their absorbance read at 540 nm on a microtitre plate reader (Titretek Multiscan 340). Averages were taken for each solution and a calibration curve plotted after the blank reading had been taken into account. A best fit curve was plotted using Microsoft Excel version 7.0 and values for the unknown parameters calculated.

#### 2.7.4 Host cell DNA

Complementing cell DNA was assayed by slot blot hybridisation. Triplicate DNA samples were taken in order to gain the average DNA content of each sample. Protein in the samples was extracted by addition of three volumes of sodium iodide sarcosinate extraction buffer (6 M sodium iodide, 13 mM EDTA, 0.5 % N-lauryl sarcosine, 26 mM Tris.HCl pH8.0 and 32 µg/ml glycogen), and incubated at 60°C for 15 minutes (M. Ishizawa *et al.* 1991). DNA was precipitated from the aqueous phase by addition of an equal volume of isopropyl alcohol. Following centrifugation at 13000 g for 5 minutes (Sorvall 12V microfuge), supernatants were removed by aspiration and the DNA pellets were washed with 40 % (v/v) isopropyl alcohol. After a further centrifugation at 13000 g for 5 minutes the isopropyl alcohol was aspirated off and pellets allowed to air dry for 5 minutes. DNA pellets were then dissolved in TE buffer (10 mM EDTA, 100 mM Tris-HCl) and incubated at 65°C for 30 minutes to inactivate nucleases. CR2 DNA standard was prepared by phenol-chloroform extraction, to remove protein, and DNA was ethanol precipitated with 70 % (v/v) ethanol. After a final wash with 70% ethanol DNA was re-suspended in TE buffer. Calibration of the standard was by fluorimetry (Hoefler, TKO 100) using Hoechst 3325 dye (Pharmacia Biotech) against an external standard of calf thymus DNA (Boehringer Mannheim). The standard was then serially diluted 1:2, to the range 250-0.5 ng/ml, then standards, samples and a positive control were transferred to 0.5 ml geneAmp tubes and adjusted to 100 mM sodium hydroxide. DNA was then denatured at 100°C for 5 minutes (PerkinElmer Thermocycler) and sodium hydroxide neutralised by addition of ammonium acetate to a final concentration of 1.5 M. Samples and standard were then applied to a nylon membrane (Nytran 0.45, Schleicher and Schuell), prepared in tri-sodium citrate buffer (90 mM, 900 mM NaCl), using a Minifold II slot blot (Schleicher and Schuell) by application of a vacuum. DNA was cross-linked to the membrane using ultraviolet radiation (BioRad).

To prepare a radio-labelled probe, 25 ng of CR2 standard was incubated with 10  $\mu$ l 9-mer random oligonucleotide primers (Prime It II kit) and incubated at 100°C for 5 minutes. After addition of 10  $\mu$ l 5X dCTP primer buffer (Prime It II kit), 5  $\mu$ l <sup>32</sup>P dCTP (Amersham) and 1 ml Klenow fragment DNA polymerase (Prime It II kit) was added and incubated at 37°C for 10-15 minutes. To remove DNA polymerase, unincorporated radio-nucleotide and oligonucleotides, the reaction mixture was centrifuged at 2000g for 10 minutes through a size exclusion chromatography column. Radio-labelled CR2 DNA probe from the column eluate was incubated with the membrane in hybridisation buffer (40 % formamide, 0.5 g/l Ficoll 400, 0.5 g/l polyvinylpyrrolidone, 0.5 g/l BSA Fraction V in 750 mM NaCl, 75 mM tri-sodium citrate) at 42°C in a Techne HB-2D hybridiser for 15 hours. Following incubation the membrane was washed twice with primary wash buffer (300mM NaCl, 20mM sodium di-hydrogen orthophosphate with 0.74 g/l EDTA and 0.1% SDS) for 15 minutes at 42°C and then twice with secondary wash buffer (15mM NaCl, 1.3mM Sodium di-hydrogen orthophosphate with 0.037 g/l EDTA and 0.1 % SDS) for 30 minutes at 60°C (Yoneyama *et al.*, 1989). Following exposure to Kodak Biomax film for 6 hours the film was developed (X-OGRAPH Compact X2) and scanned using a densitometer (Molecular Dynamics). Peak areas for the standards were plotted and a best fit curve calculated using Multicalc (LKB Wallac). From this the triplicate unknowns were read and averaged.

### 2.7.5 SDS-Page protein gels

SDS-PAGE gels were run using pre-cast 4-12% acrylamide gels (NuPAGE, Novex). Gels were rinsed with distilled water then placed in the buffer chamber (X cell II mini cell, Novex). Samples and high molecular weight standard SDS6H (Sigma) were diluted 1:1 with reducing buffer (10 mM Tris, 10% (v/v) 2-mercaptoethanol) and boiled for 20 minutes in a boiling water bath. 10  $\mu$ l of SDS6H marker was loaded into the end wells of the gels and 20  $\mu$ l of each sample was loaded into the wells. If gels were to be used for western blotting against HSV and CR2 proteins then duplicate

gels were run in the same tank. The gels are then run at 200 V for approximately 45 minutes or until the dye front had run off the bottom of the gel. Using a knife the gels were removed from the plastic plate and soaked in coomassie stain for 20 minutes whilst shaking on a shaker table (Rotatest). Once the gels were stained they were placed in destain (40% (v/v) methanol, 7% (v/v) acetic acid) for approximately 2 hours until the background was clear.

### 2.7.6 Western blots

SDS-PAGE protein gels were produced as above. Gels were removed from the plates and placed in transfer buffer (10 % (v/v) methanol) and left to equilibrate for 5 minutes. Blotting apparatus (Novex) was immersed in transfer buffer. The gel and nitrocellulose membrane (Schleicher and Schuell) were sandwiched together on top of four layers of blotting paper and sponge making sure no air was trapped underneath. The membrane was then covered with a further four pieces of blotting paper. This process was repeated for the next gel. Finally sponge was placed on top to enable the second electrode to hold the gels firmly in place. The transfer apparatus was then placed in the gel running tank and completely immersed in transfer buffer. Protein was then transferred at 200 V for 1 hour. On disassembly the gels were discarded and the membrane was placed in Ponceau stain to visualise protein bands. Molecular weight markers were then cut off and dried on a paper towel. The membranes were then incubated with PBS, 2% (w/v) Bovine albumin (fraction V, Sigma) on a shaker table for 1 hour. Membranes were washed 5 times with PBS-Tween then replaced with 100 ml of PBS-Tween with 2% rabbit sera and with 100 µl of either sheep anti-CR2 or sheep anti-HSV antibodies. After 1 hour blots were washed as before and incubated for another hour with 100 ml of PBS-Tween with 2% rabbit sera and 100 µl of rabbit anti-sheep HRP (Dako). Following a final wash, as before, the membranes were incubated with DAB (Sigma). When the desired strength was achieved, the excess DAB was washed off with UHP water. Blots were then left to air dry on paper towel.

### 2.7.7 Electron Microscopy

Electron microscopy was conducted by Dr. Jeremy Scepper at the Multi Imaging Centre, University of Cambridge. CR2 cells were passaged at  $2.5 \times 10^4$  cells/cm<sup>2</sup> into T75 flasks and incubated at 37°C for 5 days. CR2 monolayers were then infected with DISC-HSV at an MOI of 0.1 and incubated at 37°C. When 90-100% cpe was observed, 3 days post infection, the cells were detached from the plastic into the culture media by vigorous agitation and collected into centrifuge tubes. Infected cells were pelleted by centrifugation at 400 g for 10 minutes at 4°C. Alternatively infected monolayers were harvested with heparin, 50 µg/ml in PBS for 1 hour, and cells collected and pelleted as before. Cell pellets were then suspended in 1 ml of ice cold 3% gluteraldehyde fixative, kept on ice and sent for electron microscopy. Following a fixing period of 1 hour, samples were rinsed in PIPES buffer and resuspended in 1% osmium ferricyanide for 30 minutes. Following a further rinse in buffer, samples were dehydrated through an ascending series of ethanol solutions (50% ethanol 2x10 minutes, then others 3x10 minutes) to propylene oxide (2x10 minutes) then embedded in araldite.

50 µm sections of samples were mounted on copper grids and stained with uranyl acetate and lead citrate and viewed in a Phillips CM100 electron microscope at 80 kV with an objective of 10 µm in diameter.

### 2.7.8 Error Calculation

Data reported in tables and graphs have indicated errors that are associated with the variation and the reproducibility of the assays. The following describes how errors for assay results were calculated.

### 2.7.8.1 Infectious Virus Assay (TCID<sub>50</sub>)

For the virus assay, TCID<sub>50</sub>, errors given are ±37 %. This has previously been demonstrated at Cantab Pharmaceuticals (data not given) to be the average error of the TCID<sub>50</sub> assay when one assay is conducted. This figure is given to data that is either the result of one or two assay determinants. If more than two assays were conducted for a particular data point then a standard error was calculated see Figure 2.1, and presented as a percentage of the average of the assay results.

$$STDError = \frac{STDEV}{\sqrt{N}}$$

#### Figure 2.1 Calculation of Standard Error

*STDEV is the standard deviation; N is the number of assay results*

When expressing the error of a value that is a quotient for example percent recovery from a chromatography column, the error is the combination of both the individual errors and is calculated as in Figure 2.2.

$$\text{For } \frac{A}{B} \quad \text{Error} = \frac{\text{error}B}{A} + \frac{B \times \text{error}A}{(A^2)}$$

#### Figure 2.2 Calculation of error resulting from a quotient

*Where errorA and errorB are calculated as in Figure 2.1*

### 2.7.8.2 Protein Assay

Samples assayed for protein were routinely only assayed once. Therefore, errors stated for protein results were based on the error of the assay. This error was calculated from the variance observed in control samples assayed many times on many occasions. Typical errors were found to be ± 8 %.

For results of specific activity where a combination of virus and protein errors were generated the equation in Figure 2.2 was used to generate overall errors.

### 2.7.8.3 Host Cell DNA

Although the host cell protein assay used triplicate measurements to give a titre the result was generated from one sample. Therefore, a standard error could not be generated from this data. Again as in the protein assay, a general error was calculated from the results of a control sample that was assayed many times on many occasions. A typical error of  $\pm 9\%$  was found and was used for all host cell DNA data generated. Results indicating a ratio between virus titre and levels of host cell DNA used the equation in Figure 2.2 to calculate the final error.

### 3 Growth of Complementing Cells and DISC-HSV

#### 3.1 Introduction

Disabled Infectious Single Cycle HSV (DISC HSV) requires a cell line containing the deleted gH gene to allow continual infection and multiplication of the virus (see 1.5.2). The gH containing cell line has been designated the CR2 cell line and is derived from the Vero cell line (Bournnell *et al.* 1997). Vero cells are adherent and therefore require a solid surface to adhere to in order for them to grow. If no surface is available or has been completely covered by cells the cells exhibit contact inhibition and cease dividing. Also animal cells often require the accumulation of chemicals they produce for maximum doubling times to be reached, so too few cells will result in very slow growth rates. In order to calculate efficient passaging regimes for the CR2 cells, growth curves were studied using different initial seeding densities.

Growth profiles of the complementing (CR2) cell line were conducted in 75 cm<sup>2</sup> flasks. The use of flasks enabled the study of many different seeding densities in a relatively short period of time. Also flasks provide a consistent mechanism for cell growth enabling the generation of consistent data. The major drawback of using flasks is providing a method for a scaled process. The surface area required for a manufacturing campaign would be far too large for flasks to be adequate. Therefore, although useful cell growth data can be generated, it cannot be directly scaled up to manufacturing scale. Alternative methods of producing cells at manufacturing scale must be employed, such as roller bottle cultures and bioreactors. These systems are not stationary, as in the flask system, and require optimisation of the volume of culture media to surface area, control of dissolved gases and pH. These parameters will lead to a change in the growth characteristics of the cells requiring the evaluation of the growth profiles.

The study of cell growth set out to define a seeding density that could be used for routine passaging of cells, enabling the consistent and quick growth of cells for infection with the DISC-HSV virus. The seeding density that was chosen would also have a growth profile that had distinguishable phases. This would enable the study of virus growth in the three stages of cell growth, lag, log and stationary phase, which occur for this adherent cell line. If any difference in virus productivity was seen in infecting at these three stages, it would influence the final design of the passage and infection regime.

### 3.1.1 Objectives

The main objective of the growth of the CR2 complementing cell line and the DISC HSV was to identify a passage and infection regime that would produce reproducible harvest titres with similar contaminating profiles for DNA and protein. Although the identification of optimal conditions was sought, it was recognised that using a small-scale system such as here (75 cm<sup>2</sup> flasks), would not be applicable for a manufacturing process. The value of this optimisation was to enable repeated experiments to be conducted on harvesting the virus with as little variation as possible between experiments.

## **3.2 Results and Discussion**

### 3.2.1 Cell growth profiles

To define a suitable passage regime for the complementing cell line (CR2 cells) various initial seeding densities were tested. The ideal seeding density would probably need to be a compromise. A low seeding density is preferred since this would require fewer cells to be produced to seed the final reactor vessel. For instance if it were only possible to seed at half the final cell density, then each passage would only double the cell number. If on the other hand the seeding density is too low, then

a long lag phase may result in the cell growth being sparse or taking too long to reach confluency.

Four initial seeding densities of  $1 \times 10^4$ ,  $2.5 \times 10^4$ ,  $5 \times 10^4$  and  $1 \times 10^5$  cells/cm<sup>2</sup> were used to seed 75 cm<sup>2</sup> flasks and the growth of the cells was monitored by harvesting duplicate flasks every day post seeding and cell counts made (see Figure 3.2). These seeding densities were chosen to span the possible range of seeding densities likely to be incorporated in the final process. It was thought any higher seeding density would not be viable as explained above and the lowest seeding density tested would only be used if the flasks became confluent in a reasonably short period of time.

Each of the growth curves displays a lag phase and an exponential growth phase. The final cell number per flask is approximately the same for each of the seeding densities. A plot of the normalised growth rates of the cells seeded at the three lower seeding densities is shown in Figure 3.3. The graphs are normalised by dividing the values by initial cell seeding densities. The result suggests very little difference in the behaviour of the cells when seeded at these levels. Indeed a best-fit curve was drawn to generate an equation for the exponential growth of the cells. This equation, Figure 3.1, can therefore be used to determine the cell density at a given seeding density over time.

$$\text{CellDensity} = \text{SeedingDensity} \times 0.8618e^{0.6602x}$$

**Figure 3.1 General Calculation of Cell density.**

*Cell density in cm<sup>2</sup> can be calculated by the equation generated from the graph in Figure 3.3.*

The seeding density therefore has no detrimental effect on the quantity of the cells produced, but the lower the seeding density the longer the lag phase and hence greater time required to reach maximum cell density. Cells seeded at  $1 \times 10^4$  cells/cm<sup>2</sup> became confluent but took at least seven days to do so. If this passage regime was to be scaled up, then the overall process of cell growth and virus infection would take at least 10 days and so was thought to be too long.

Five or six days after the cells reached stationary phase the cell number began to drop. This drop in cell number correlates with the observed drop in the viability of the cells

(Figure 3.4) which was determined by comparing total cell counts to viable cell counts (see experimental). Using an inverted microscope the cells were observed to be very dense and becoming detached from the flasks. Also the cells form multiple layers in clusters. Once this happens the cells tend not to survive since nutrients cannot easily reach the covered cells.

Using the samples generated from the cell growth curves, cell number estimations were conducted by counting nuclei (see experimental). To count nuclei crystal violet is used to stain the nuclear proteins and these are counted using a counting grid as in the cell counts. The difference in the two methods of counting cells is demonstrated in Figure 3.5. The higher three seeding densities  $2.5 \times 10^4$ ,  $5 \times 10^4$  and  $1 \times 10^5$  cells/cm<sup>2</sup> gave almost the same cell number throughout the growth curve by both methods meaning either method can be used. The disparity of the two methods for the low seeding density samples ( $1 \times 10^4$  cells/cm<sup>2</sup>) cannot readily be explained. The results suggest that there were up to twice as many cells as nuclei. This is of course meaningless, but since the cell numbers are low at the beginning of this growth curve the low numbers of cells to count may give rise to large sampling errors. It is also interesting to note that the ratios did not drop below 1, so the population of cells that have undergone mitosis but not divided (i.e. one cell having two nuclei) at the time of sampling must be very low even in the log growth phase.

A measure of the rate at which the cells are growing and dividing is by calculating the doubling time. The maximum rate at which the cells divide occurs after the lag phase and before the stationary phase and this is the exponential growth phase. For all of these growth curves the lag phase was from initial seeding to day 1 and the stationary phase began, for the higher seeding densities at about 4 days (Figure 3.2). The data for days 1-4 were therefore used to estimate the population doubling times for each of the growth curves. These values were plotted on a log scale and a best-fit line was drawn through the points. The gradient of the best-fit lines can then be used to calculate the doubling times. (Figure 3.6). This data shows that the lower the seeding density the faster the cells multiply although the difference in doubling time is minimal and may not be significant. Indeed when a general doubling time estimation is made as in Figure 3.1, there does not appear to be a large variation in the cells numbers. This general rule may be used in future to generate optimal seeding

densities for cell growth. the main drawback with this equation is the stage at which cell reach confluency and stop growing. Here this has not been addressed and so only initial growth is modelled.

The consumption of an energy source or build up of inhibitory by products are other factors which may effect the growth rate of the cells. The main source of energy provided in the culture media for the cells is glucose, which is supplied at an initial concentration of 4.5 g/L. The main metabolic pathway used by the cells to utilise glucose is the glycolysis pathway. This pathway is inefficient at converting glucose into energy, but if a cell has a plentiful supply of glucose it will only use this method to generate energy. In only using this pathway inhibitory waste products are produced namely lactate. If lactate reaches too high a concentration then it will inhibit growth; therefore the levels of both glucose and lactate were monitored for the  $1 \times 10^4$  cells/cm<sup>2</sup> seeding density (Figure 3.7). It can be seen that the highest rate of lactate production and glucose consumption are during the exponential phase and that even after 12 days of culture glucose is still present in the culture media. The presence of glucose does not suggest that the cells were prevented from growing due to lack of an energy source. The level of 1.5 mg/ml lactate is regarded as low but does not mean it has no effect on the rate of cell growth. Here the reason the cessation of growth is lack of available surface area.

It is clear that in deciding on a seeding density a number of factors have to be balanced. If the seeding density is too high then the quantity of cells that need to be harvested for the passage becomes excessively large. But seeding at lower cell densities means a longer time before any pre-determined cell density is reached for, say, infection with virus or passaging of cells. Also at low seeding densities the cells actually decline in number before growth starts; this occurs because adherent cells require contact with other cells for optimal growth. If the cells are seeded at too low a density then they do not make contact with other cells which results in an extended lag phase.

The lower seeding density therefore takes too long to reach maximal cell density, 9 days, and the decline in cell number at the lag phase means the reproducibility in a scaled system may well be affected. For these reasons this profile was ruled out. The highest seeding density tested proved that cells could reach saturation quickly but at a

cost of needing large amounts of cells for the passage. Such a low ratio passage is acceptable on a lab scale but at manufacturing scale would require too large a quantity of cells for each passage and hence a costly passage train to reach the final production vessel. For the above reasons the seeding density of  $2.5 \times 10^4$  was chosen. This gave a good rate of cell growth with a minimal lag phase.

### 3.2.2 Virus Growth

To determine the optimum parameter for the growth of DISC-HSV a suitable CR2 cell growth profile was chosen to represent cells in all states of their growth i.e. lag, log and stationary phases. This correlates with days 1, 3 and 5 post passage of the  $2.5 \times 10^4$  cells/cm<sup>2</sup> seeding density. At each of these time points a range of multiplicity of infections (MOI) were tested 0.1, 0.01 and 0.001. This range, spanning three logs, is broad but due to the inaccuracies of the virus assay (TCID<sub>50</sub>) a narrower range may give more confusing results. For each infection time point at each MOI a series of flasks were set up and duplicate flasks were harvested each day for 8 days post infection. Flasks were harvested by scraping infected cells into the culture media and centrifuging at 1000 g for 10 minutes. Cell pellets were suspended in PBS and subjected to 1 minute of sonication prior to assaying for virus by TCID<sub>50</sub>. Figure 3.8, 3.7, 3.8 show the results of the virus growth profiles. It can be seen that when cells were infected at MOI of 0.001 virus production was slow in appearing and did not reach the same level as the other infections. Even after 8 days the quantity of virus produced was not as great as infecting at MOI of 0.1 or 0.01. The best MOI, in this study, was 0.1, which gave greater than  $1.0 \times 10^8$  pfu/75cm<sup>2</sup> flask when infecting cells 3 or 6 days post passage.

Titres of the culture media (data not shown) at the point of harvest showed the maximum quantity of virus present was at least a log lower than the values for the cell associated virus. Culture media was therefore not regarded as a necessary constituent of the virus harvest.

Although the primary aim is to establish a method for the routine production of as high a yield of virus as possible, without reducing the reproducibility, the product

should also be separated from contaminants as much as possible. To help achieve this, points through the process can be identified which may allow the lowering of the contaminant burden for the downstream processing. The time at which the cells are infected provides a possible step for reducing levels of contaminants from the growth of the cells, such as foetal bovine serum. The optimal time for infection in the cell growth curve has been identified as 3 days post seeding, which is after the cells have become confluent and growth and division has all but stopped. Therefore, cells at this point do not need the growth factors provided by the FBS and so its removal at this point would reduce the levels of bovine protein in the eventual harvest. To test whether the removal of FBS at this point had an effect on the viral growth, cell monolayers were washed three times with PBS then incubated with DMEM without FBS. Controls flasks that had media containing FBS were used and both sets of flasks infected with DISC-HSV at an MOI of 0.1 and incubated at 34°C. The results of virus production 3 and 4 days post infection are shown in Figure 3.11. It can be seen that there is little or no affect on virus production when this wash step is included in the process and so it could be used as an initial step in removing bovine proteins.

### **3.3 Conclusions**

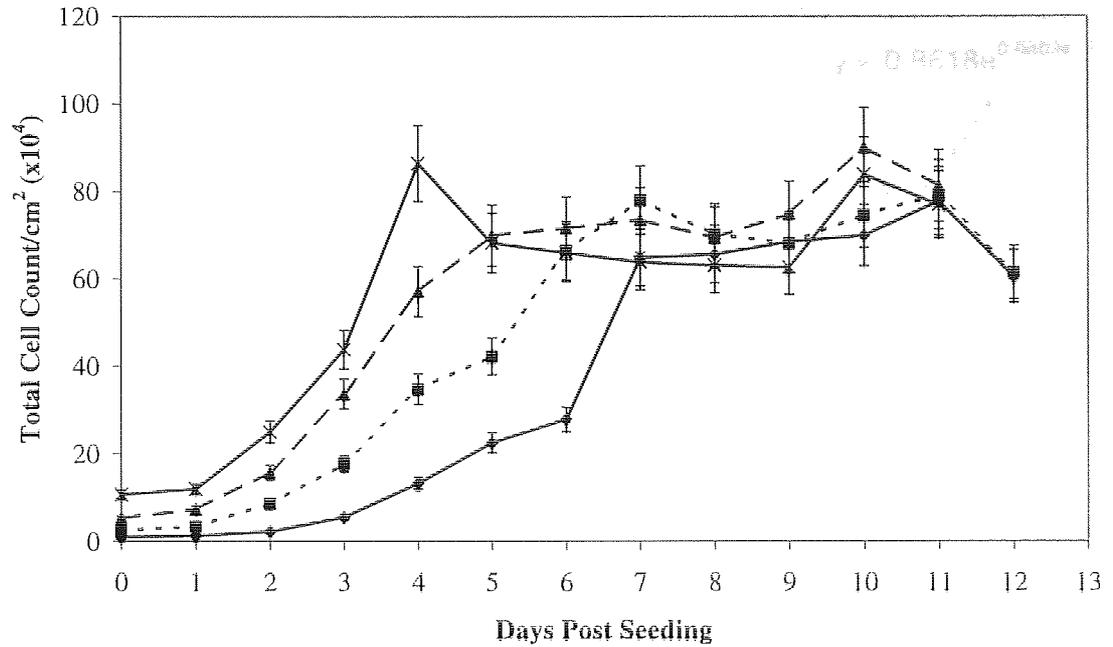
The choice of cell seeding density is a compromise between the time required to reach the required state for infection and the low efficiency of passaging cells too often. The decision to use a seeding density of  $2.5 \times 10^4$  cells/cm<sup>2</sup> gives a growth profile with the distinguishable stages of lag, log and stationary phase. This enabled the study of virus growth from cells infected at each of these stages. Also this seeding density, taking only 6 days to reach confluency, allows the final process to be completed in a time scale suitable for manufacturing.

The virus growth profiles demonstrate the variability in virus production. Too low an MOI takes too long to produce a significant quantity of virus, too high means to large an input to make a viable scale process. An MOI of 0.01 pfu/cell is probably not the optimum, although somewhere near it, but allows the preparation of small virus

aliquots for the infection of cells produced in large scale. The MOI of 0.1 pfu/cell infecting at three days post seeding was chosen to produce virus on a small scale in flasks because of the reproducible high titres generated.

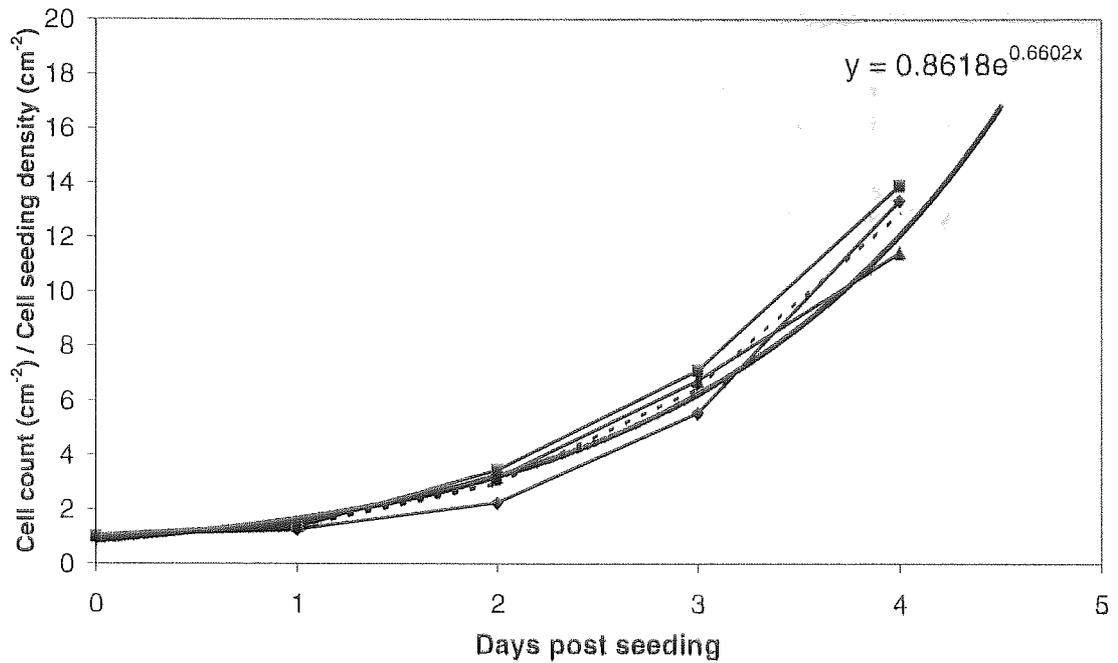
The step of washing the cell monolayers prior to infection to remove FBS and replaced with only DMEM was also included to provide an initial step to remove some of the contaminants from the downstream process. Virus growth was not affected by the removal of the serum as greater than 90 % of BSA was removed. This method was employed in the production of virus for the investigation of harvesting DISC HSV.

At this point the virus used to infect the cells for the study was produced by addition of hypotonic saline to the infected cells and harvesting by mechanical scraping then decanting into a container, see Figure 3.12. This process was used throughout the course of this work to produce working seed DISC HSV virus.



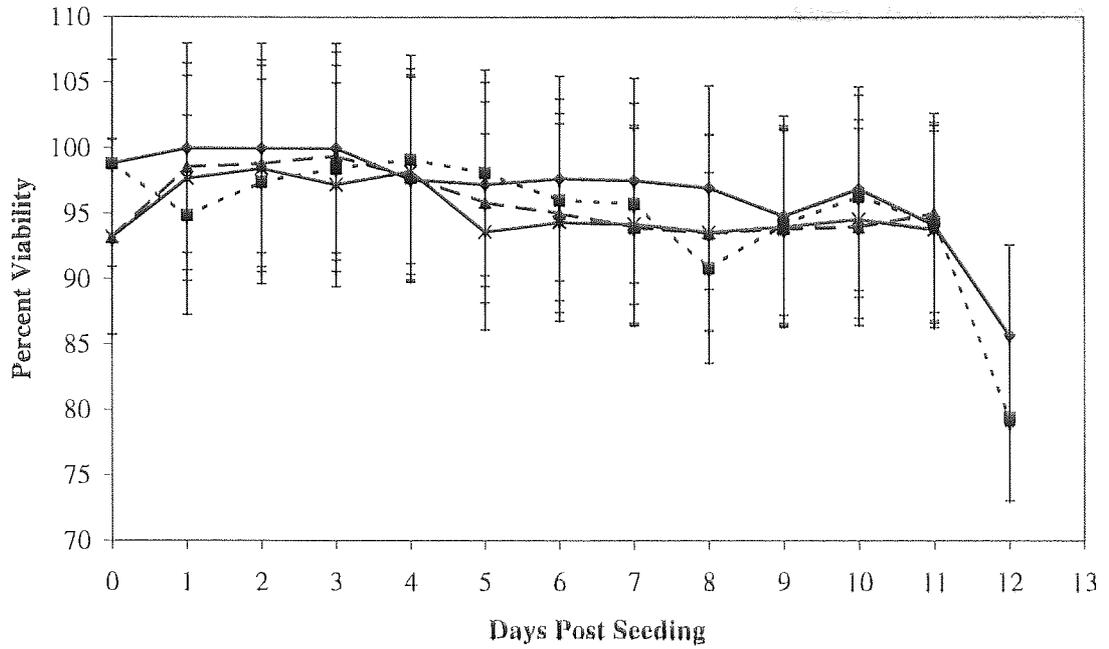
**Figure 3.2 Optimisation of Seeding Density**

CR2 cells were grown in T75 flasks with 40 ml DMEM containing 5% FBS and incubated at 37°C with 5% CO<sub>2</sub>. For each time point cells from duplicate flasks were harvested by decanting off the media, washing the cell monolayers with 10 ml DPBS without Magnesium and Calcium and incubating with 7 ml of Trypsin. Following incubation for 10 minutes at 37°C the Trypsin was inhibited by addition of 10 ml of DMEM containing 5% FBS and cells were collected in 50 ml centrifuge tubes. Total cells were counted using a haemocytometer and the average cell count per cm<sup>2</sup> of tissue culture flask plotted. Four initial seeding densities were tested 10,000 cells/cm<sup>2</sup> (◆), 25,000 cells/cm<sup>2</sup> (■), 50,000 cells/cm<sup>2</sup> (▲) and 100,000 cells/cm<sup>2</sup> (×).



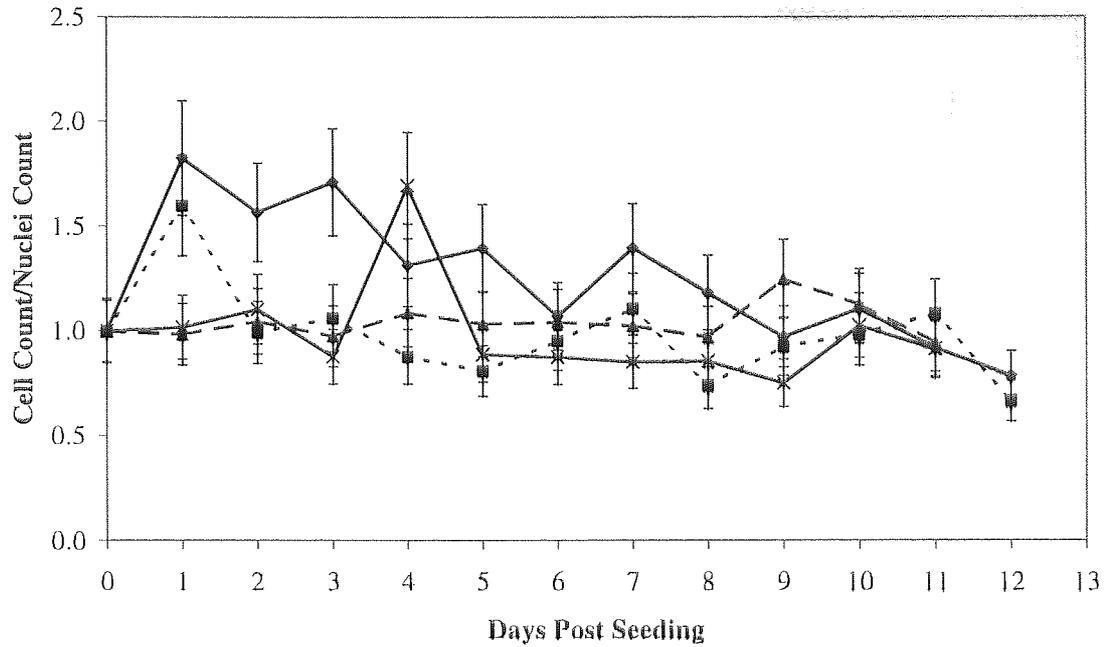
**Figure 3.3** Average population doubling times.

Cells in exponential phase of growth, up to day 4, at three different seeding densities are plotted by dividing the cell count/cm<sup>2</sup> by the initial seeding in cells/cm<sup>2</sup>. 10,000 cells/cm<sup>2</sup> (◆), 25,000 cells/cm<sup>2</sup> (■), 50,000 cells/cm<sup>2</sup> (▲) and the average (dashed line). A best fit exponential curve (thick line) was fitted using Microsoft® Excel and equation displayed. The equation represent the average growth rate of cells seeded at varying densities.



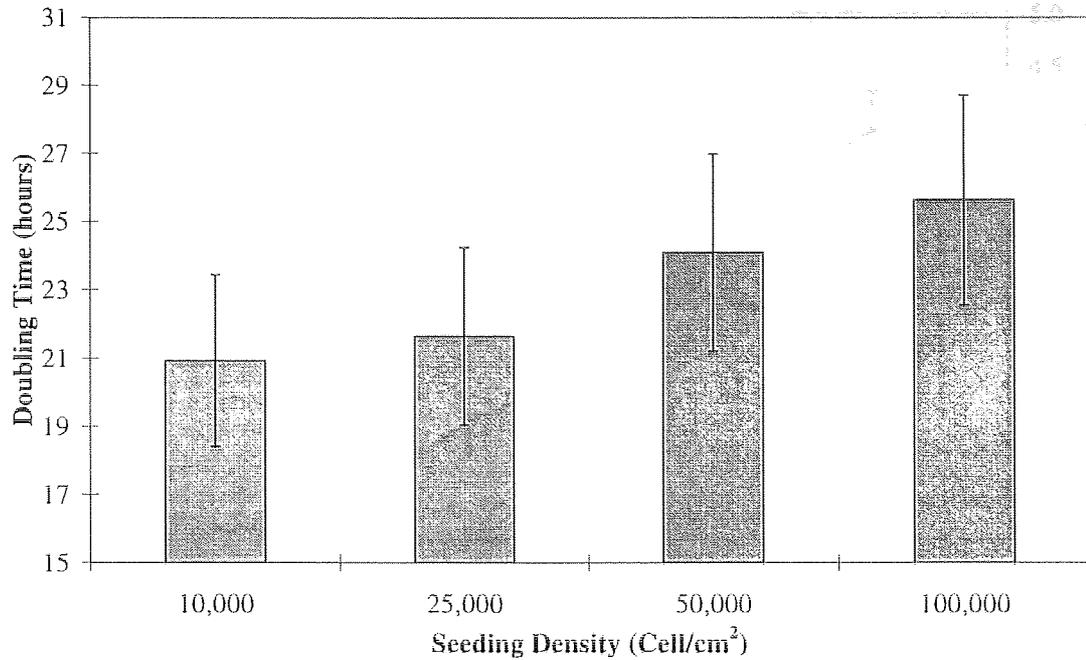
**Figure 3.4 Cell Viability**

Cells harvested and counted for growth profiles (see figure1) were also counted for viable cells by the Trypan Blue method. The percentage of viable cells in any one sample was calculated by dividing the viable cell count by the total cell count multiplied by 100. Cell viabilities for seeding densities of 10,000 cells/cm<sup>2</sup> (◆), 25,000 cells/cm<sup>2</sup> (■), 50,000 cells/cm<sup>2</sup> (▲) and 100,000 cells/cm<sup>2</sup> (×) are plotted.



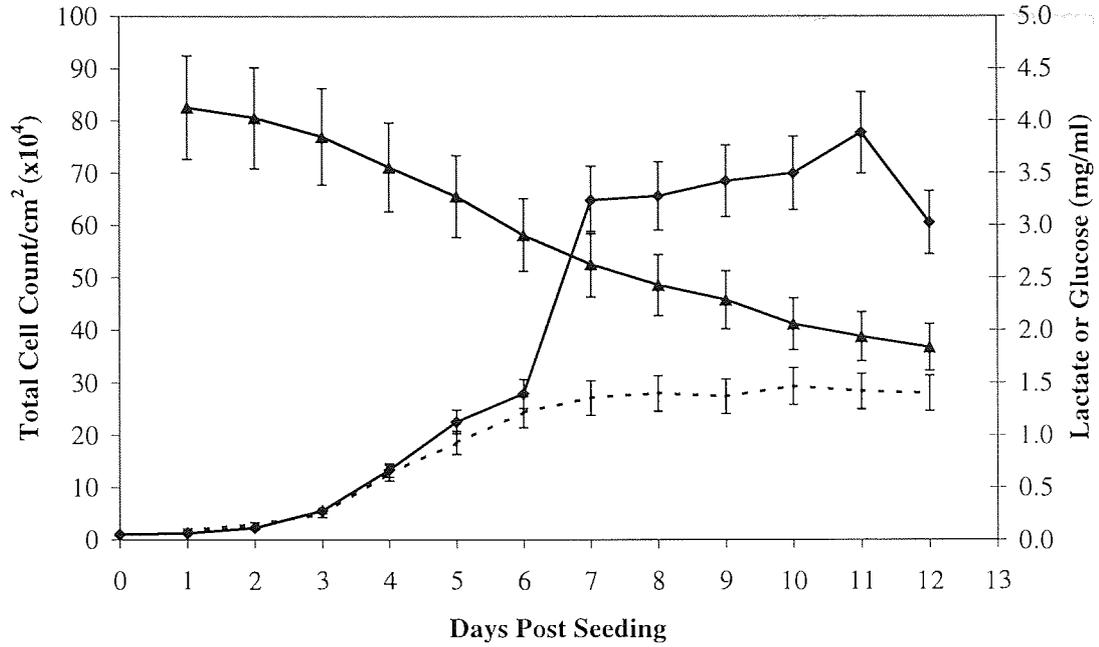
**Figure 3.5** Ratio between whole cell and nuclei count methods of estimating cell population

Ratio of total cell count to nuclei count are plotted for cell growth curves of seeding densities 10,000 cells/cm<sup>2</sup> (◆), 25,000 cells/cm<sup>2</sup> (■), 50,000 cells/cm<sup>2</sup> (▲) and 100,000 cells/cm<sup>2</sup> (×). Total cell count was carried out by harvesting cells by the trypsin method and counting whole cells using a haemocytometer. Nuclei counts were carried out by incubating 1 ml of harvested cells with 1 ml of Crystal Violet in citric acid for 2 hours at 37°C and counting nuclei using a haemocytometer. A ratio of 1 means the different methods of cell enumeration gave identical quantification of cell number.



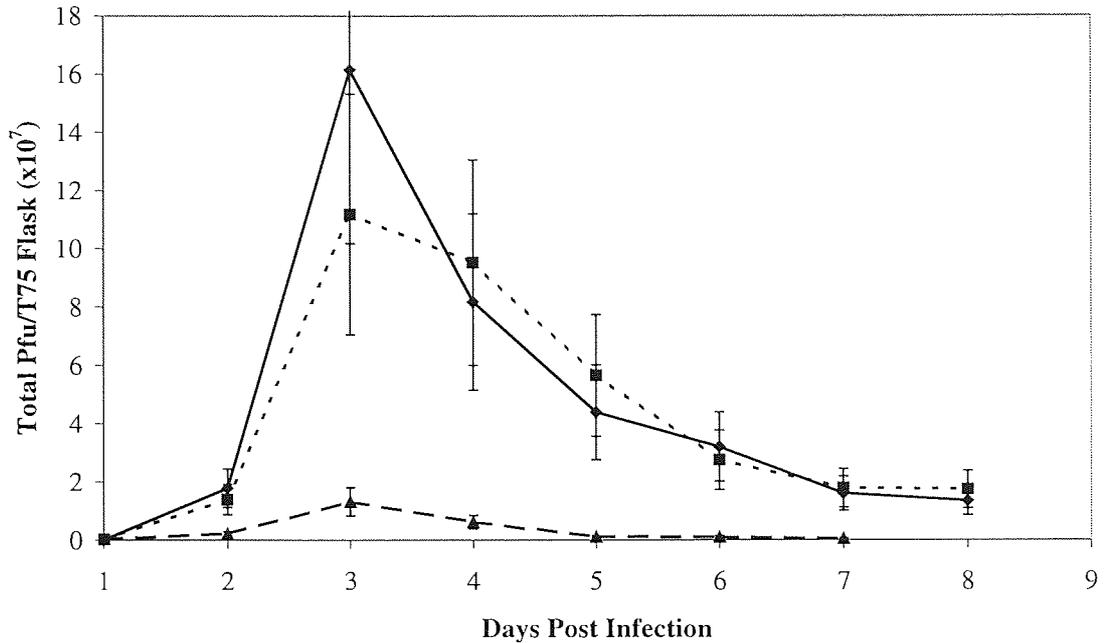
**Figure 3.6 Estimation of population doubling times**

Maximum cell doubling times were calculated by plotting the exponential growth phase (from day 1 post seeding to day 4, see figure 1) of the four initial seeding densities (10,000, 25,000, 50,000 and 100,000 cells/cm<sup>2</sup>) on a log scale and finding the gradient of a best fit line. The gradient can then be used to calculate the doubling time of cell growth.



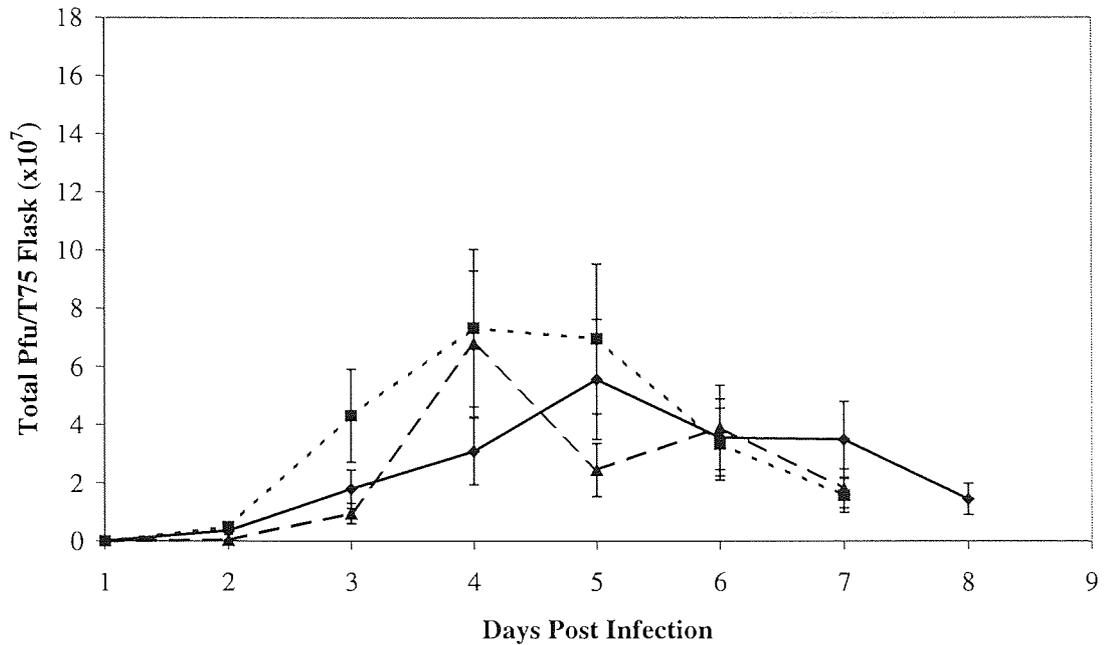
**Figure 3.7** Glucose consumption and lactate production during cell growth

Samples of growth media were taken for the 10,000 cells/cm<sup>2</sup> cell growth curve (◆) and assayed for glucose and lactate on a YSI2000 analyser. Values for glucose (▲) and lactate (■) are plotted as mg/ml of media. It can be clearly seen that as the cells are multiplying glucose is consumed and lactate begins to build up in the media. Once the cells reach confluency the rate of lactate production declines as well as the rate of glucose consumption.



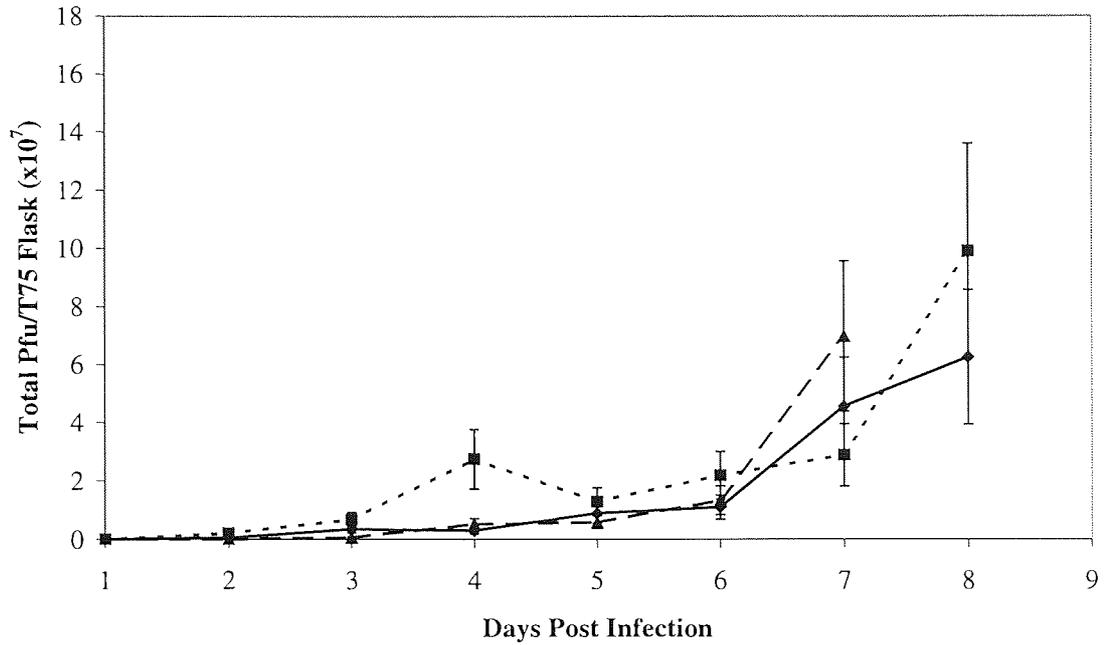
**Figure 3.8 Virus production in cells infected with an MOI of 0.1**

CR2 cells seeded at 25,000 cells/cm<sup>2</sup> in T75 flasks and grown in DMEM containing 5% FBS at 37°C. 1 day (▲), 3 days (◆) and six days (■) post seeding; the cells were infected with DISC-HSV virus at a multiplicity of infection of 0.1. Cell number was estimated from the seeding density experiment growth profile (see figure 1) and the quantity of virus added to each flask was one tenth of the total cell number per flask. For each time point duplicate flasks were harvested, by scraping the cells into the culture media and centrifuging at 2,000 g for 5 minutes; cell pellets were suspended in hypotonic saline and sonicated for 1 minute prior to assaying for virus by TCID<sub>50</sub>.



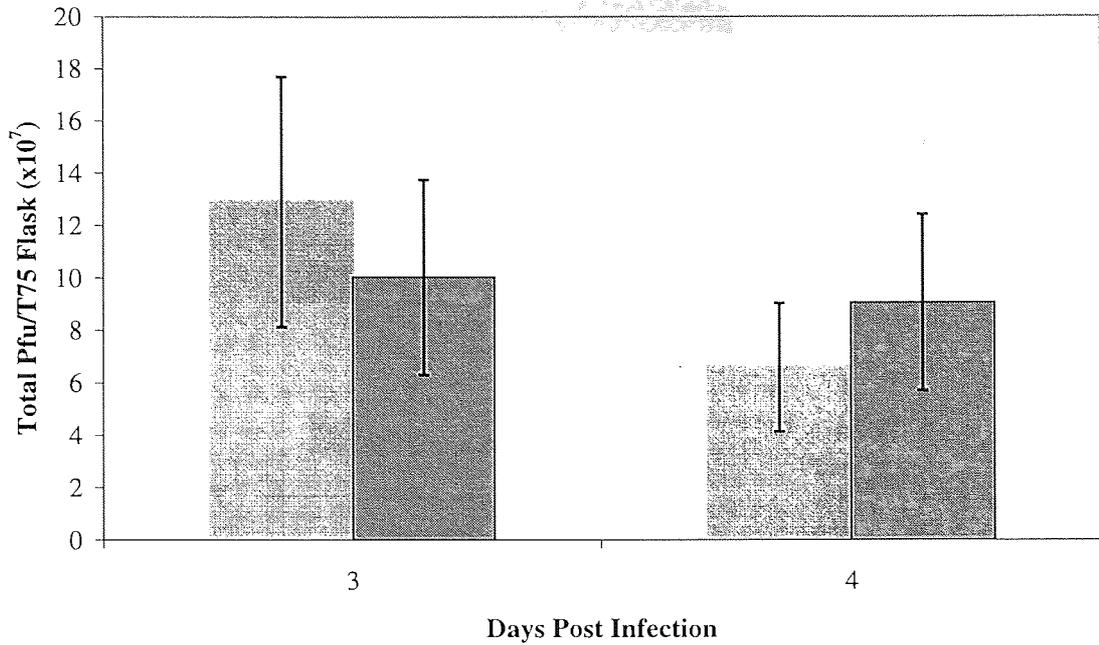
**Figure 3.9 Virus production in cells infected with an MOI of 0.01**

CR2 cells seeded at 25,000 cells/cm<sup>2</sup> in T75 flasks and grown in DMEM containing 5% FBS at 37°C. 1 day (▲), 3 days (◆) and six days (■) post seeding; the cells were infected with DISC-HSV virus at a multiplicity of infection of 0.01. Cell number was estimated from the seeding density experiment growth profile (see figure 1) and the quantity of virus added to each flask was one hundredth of the total cell number per flask. For each time point duplicate flasks were harvested, by scraping the cells into the culture media and centrifuging at 2,000 g for 5 minutes; cell pellets were suspended in hypotonic saline and sonicated for 1 minute prior to assaying for virus by TCID<sub>50</sub>.



**Figure 3.10** Virus production in cells infected with an MOI of 0.001

CR2 cells seeded at 25,000 cells/cm<sup>2</sup> in T75 flasks and grown in DMEM containing 5% FBS at 37°C. 1 day (▲), 3 days (◆) and six days (■) post seeding; the cells were infected with DISC-HSV virus at a multiplicity of infection of 0.01. Cell number was estimated from the seeding density experiment growth profile (see figure 1) and the quantity of virus added to each flask was one thousandth of the total cell number per flask. For each time point duplicate flasks were harvested, by scraping the cells into the culture media and centrifuging at 2,000 *g* for 5 minutes; cell pellets were suspended in hypotonic saline and sonicated for 1 minute prior to assaying for virus by TCID<sub>50</sub>.



**Figure 3.11 Effect of withdrawing FBS at infection on virus production.**

CR2 cells were seeded at 25,000 cells/cm<sup>2</sup> in T75 flasks and incubated with 40 ml DMEM containing 5% FBS at 37°C. Three days post seeding media was discarded and cell monolayers were washed with 3x15 ml of DPBS and replaced with 40 ml of DMEM without FBS (dark grey bars). Control flasks were flasks that were infected directly into the growth media and not media changed (light grey bars). Cells were then infected with DISC-HSV at an MOI of 0.1 and incubated at 37°C. At three and four days post infection triplicate flasks were harvested by scraping cells into media and centrifuging for 5 minutes at 2,000 g. Cell pellets were suspended in hypotonic saline and sonicated for 1 minute prior to assaying for virus by TCID<sub>50</sub>. The results show that the difference between washed and control samples is minimal and within the variation of the TCID<sub>50</sub> assay.

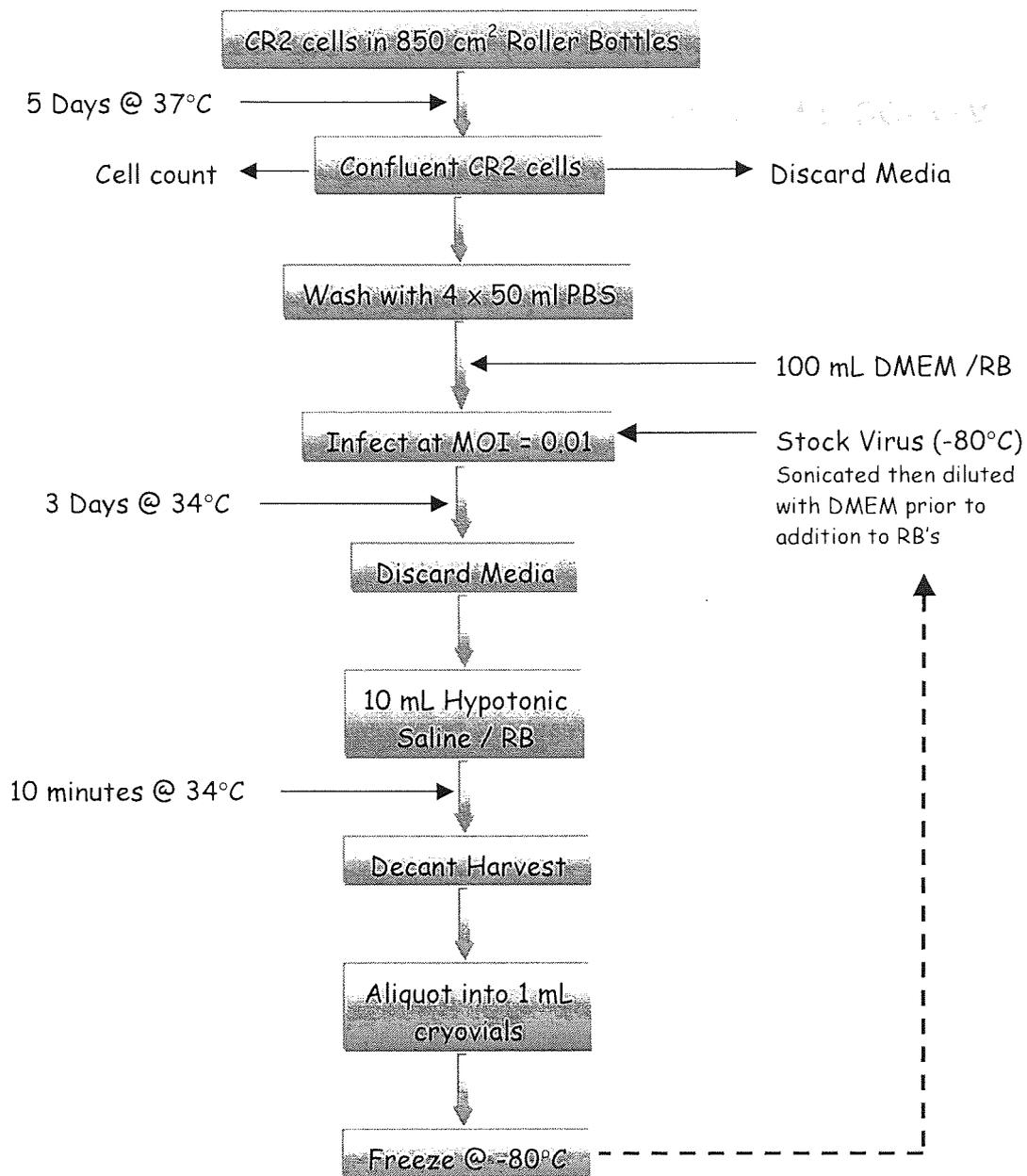


Figure 3.12 Flow diagram of routine infection and harvesting of DISC HSV for production of working seed virus stocks.

## 4 Lytic Methods of Harvesting Cell Associated DISC-HSV

### 4.1 Introduction

Once the optimal time for harvesting the virus had been decided, see previous chapter, the next objective was to successfully recover virus from the cell culture. The harvest method used must be scalable, efficient and able to produce material that is suitable for further downstream purification. A successful harvest method must also be one that is consistently repeatable and with satisfactory yield and purity.

The harvesting of non-cell associated viruses, such as HSV-1 and Pseudorabies, is a relatively simple procedure and therefore has not been widely reviewed in the literature. As an infection proceeds in cultured cells, infectious virus accumulates in the culture media. Harvesting of the virus can therefore be accomplished by collecting the media and discarding the cells once the maximum titre has been reached, usually at a predetermined time following the initial infection of the cells. In some cases, such as in the production of polio vaccine (I.R. Paul Van Hemert, *et al.* (1977)), any cellular debris in the harvest can be removed simply by letting it settle out over two or three weeks. The poliovirus being small does not settle out and can therefore be decanted from the cellular debris. It is clear in this process that there is a holding time to allow for the settling of the cellular debris but if no holding time is required then cell debris can be removed by other methods, such as centrifugation. Another method to remove cell debris is filtration, but factors such as pore size, membrane composition and flow rate must also be considered, so making the process more complex although ultimately more scalable.

When it comes to the harvesting of cell associated viruses a wide range of techniques have been previously employed to separate the virus from the host cells. All methods rely on cell breakage to release intracellular virus particles.

#### 4.1.1 Mechanical Disruption

Disrupting cells using mechanical force has been achieved using homogenisation, bionebulisation and sonication. Spear and Roizman (1972) described homogenisation of cells infected with herpes simplex virus type 2 to yield intracellular virus. This method uses a Dounce homogeniser to rupture the cell membranes and release the virus. A Dounce homogeniser is a thick walled tube in which the sample to be homogenised is placed. A rotating plunger with a diameter a fraction less than the internal diameter of the tube is then inserted into the tube. The liquid flows past the plunger, which creates a high shear force between itself and the tube wall. This shear force breaks open the cells. Following homogenisation the virus is purified by high speed centrifugation over a dextran gradient. Although this process produces very pure virus there are some major drawbacks. High speed centrifugation has low yields and takes a long time to process small volumes, so it is not ideal for a downstream process; therefore the harvest must be subjected to alternative purification methods. The product of the homogenisation has high levels of host cell DNA, due the breakage of cell nuclei, which produces a viscous harvest. This harvest is therefore not suitable for purification processes such as filtration or chromatography since the feed will most likely foul the membrane or chromatography matrix. Another operating problem is maintaining an aseptic process since the Dounce homogeniser is not a closed system and is therefore open to contamination. Although this system does separate the virus from the cells it also produces virus that cannot easily be separated from the host cell contaminating DNA and protein. Therefore this method was discounted as an alternative for the scale up of a manufacturing process.

Bionebulisation (Degouys *et al.* (1997)) relies on the shear force generated when the cell harvest is forced, under pressure, through a nozzle and impinged on to a surface. The desired result from this method is to rupture the cell membranes whilst keeping the cell nuclei intact. This can be achieved by choosing parameters such as the pressure the harvest is forced through the nozzle and distance to the impinging surface

that does not break the nuclei but ruptures the cell membrane. This method therefore is an improvement on the Dounce homogeniser in its ability to optimise the force used; also being a continuous process, it enables the processing of large quantities of harvest.

Nitrogen cavitation (Blough *et al.* (1977)) has also been used to break cell membranes to yield cell associated rabies virus. This method was thought impractical for a variety of reasons; mainly it is a batch process that requires cumbersome and expensive equipment such as a nitrogen cavitation bomb and a high pressure nitrogen source. For these reasons the investigation of this method was not carried out, as it cannot be considered for process scale.

Mechanical shearing of cells can also be accomplished by the action of ice crystals on the cell membranes. Repeated cycles of freeze thawing allows ice crystals to form inside the cell and, when they grow bigger, the cell membrane ruptures and the cell contents are released. This method of release has been used in the production of other viruses, most notably adenovirus (Shabram *et al.* (1997), Huyghe *et al.* (1995)). Other benefits of using freeze thaw is that the process can be conducted in a closed system, so maintaining an aseptic process, and the equipment required is simple to operate and readily scalable.

#### 4.1.2 Osmotic Potential Disruption

The level of cell breakage produced by mechanical disruption can, in some cases, be adjusted to only break open the cell membranes and leave the nuclei intact to aid the removal of DNA. The drawback of mechanical disruption is the need for equipment that can be used for a pharmaceutical grade product. Also the equipment has to be validated and pass stringent sterility tests. Therefore, if cell breakage could be accomplished without the need for such equipment, this would be advantageous for the process.

The most basic method of rupturing cells using osmosis is to replace the culture media with a weak saline solution, such that after a short incubation the majority of the cells

will be lysed. To attempt to improve the efficiency of cell lysis, Sarmiento et al. (1992) pre-incubated cells with a hypertonic solution, glycerol. This resulted in cells becoming crenated due to the osmotic loss of water from the cells. Glycerol was then replaced with hypotonic saline, which induced cell lysis at a higher efficiency due to the increased osmotic potential between the cells and the hypotonic saline.

To provide a comprehensive study of all the methods of rupturing cells would be impractical due to the large variety of techniques available; also some of the techniques can be discounted for not producing suitable material for a downstream process. Although sonication is not suitable for a downstream process it is a very practical method for releasing the total virus content of harvested cells and so was used to gauge the effectiveness of other virus release methods.

## **4.2 Results and Discussion**

### 4.2.1 Sonication

To investigate the ability of a procedure to release virus it is helpful to have an in-built standard to enable correlation between experiments. The standard must therefore be able to release the maximal amount of virus obtainable to give an indication of the recoveries of other harvest methods investigated. Sonication is a common method employed to harvest cell associated viruses (S.M. Brown, A.R. MacLean (1998), D. Fiorentine *et al.* (1985)) because of its ability to completely disrupt cells whilst having no apparent affect on the virus. Sonication was therefore chosen as the standard, in these experiments, to give a value for the maximum obtainable virus yield for the harvest. An arbitrary length of sonication of one minute was used for a 'standard sonication'. Figure 4.1 demonstrates that one minute is long enough to obtain the maximum yield since extra sonication did not increase the recovery and any variation observed was within the variation of the TCID<sub>50</sub> assay. Also the stability of the virus

during longer sonication demonstrates the ability of the virus to be unaffected by the treatment. It does not however follow that this method gives an absolute value for the infectious virus present in a sample but it is quick, repeatable and since sonication completely disrupts cells will give a close approximation to a total infectious virus count.

Sonication might be considered for a scaled up process since it releases a large amount of the virus. Unfortunately there are a number of problems associated with this method. Sonication completely ruptures cells releasing a large amount of cellular DNA and protein and results in a product that is not suitable for further downstream purification. Also there are manufacturing problems with sonication. At the lab scale, sonication is conducted in batches in a sonicated water bath. This enables samples to be sealed preventing the escape of aerosols. At manufacturing scale the process would have to be continuous to cope with the volumes that require sonication so containment becomes a problem. For these reasons sonication was not considered as a possible method for large-scale virus harvest.

#### 4.2.2 Freeze Thaw

A more subtle method for rupturing cells is using successive cycles of freezing and thawing. Figure 4.2 shows virus released by up to three cycles of freeze thaw following the removal of cell debris by low speed centrifugation. After 1 cycle virus yield is low at less than 10% of sonication and the purity of the released virus, at about 1 pg protein/pfu protein is low. Unfortunately further cycles did not increase the yield but reduced it, probably due to inactivation of the virus. This inactivation lead to a rise in the specific purity of the harvest to about the 5 pg protein/pfu level. The reasons why further freeze thaw cycles inactivated the virus are unclear but the formation of ice crystals within the virions may cause the rupturing of their lipid membranes. Other methods that rely on cell rupturing by freeze thaw are for non enveloped viruses such as adenovirus (Huyghe, B.G., *et al.* (1995)) which have no reported losses of infectivity on freeze thawing. The lack of recovery and the apparent

drop in titre after three cycles could therefore be because of ice crystals rupturing the membrane of the virus.

Although the specific purity of the released virus was acceptable, the recoveries of this procedure were thought to be too low for practical use. Therefore this method was rejected for the scale up of the process.

#### 4.2.3 Osmotic Rupture of Cells

There are a number of practical methods to achieve the rupturing of cells using a change in the extracellular osmotic potential. The simplest of methods is to change the culture media with a buffer containing a hypotonic concentration of salt. A range of salt concentrations, 1-10 mM NaCl in phosphate buffer (10mM), was tested for their ability to release infectious virus from infected cells (Figure 4.3). To distinguish released virus from virus remaining cell associated, the harvests were centrifuged to remove cell debris and the supernatants assayed; sonicated harvests were also performed to give a value for the maximum obtainable virus. The quantity of virus released by hypotonic saline rupture of cells reached approximately 20 percent of that released by sonication. The variation in the titres across the range of salt concentrations was probably due to variation in the assay since there is no trend to the data.

To try and improve the cell lysis, and therefore the release of virus, glycerol shock treatment of the cells was tried (Sarmiento and Batterson (1992)). The method pre-treats the cells with a high concentration of glycerol to osmotically remove water. Following a short centrifugation the cells are resuspended in hypotonic saline to rupture the membranes. The hypotonic saline was then centrifuged to remove debris and samples of all the steps were taken and assayed for virus. Table 1 shows the glycerol shock results including a sonicated control and a hypotonic saline control. The hypotonic saline control falls below 10% which was observed in the previous experiment. When comparing the glycerol shock hypotonic saline supernatant there is not a significant improvement in recovery. Although Sarmiento and Batterson reported comparable recoveries to other mechanical methods for virus harvest, in this

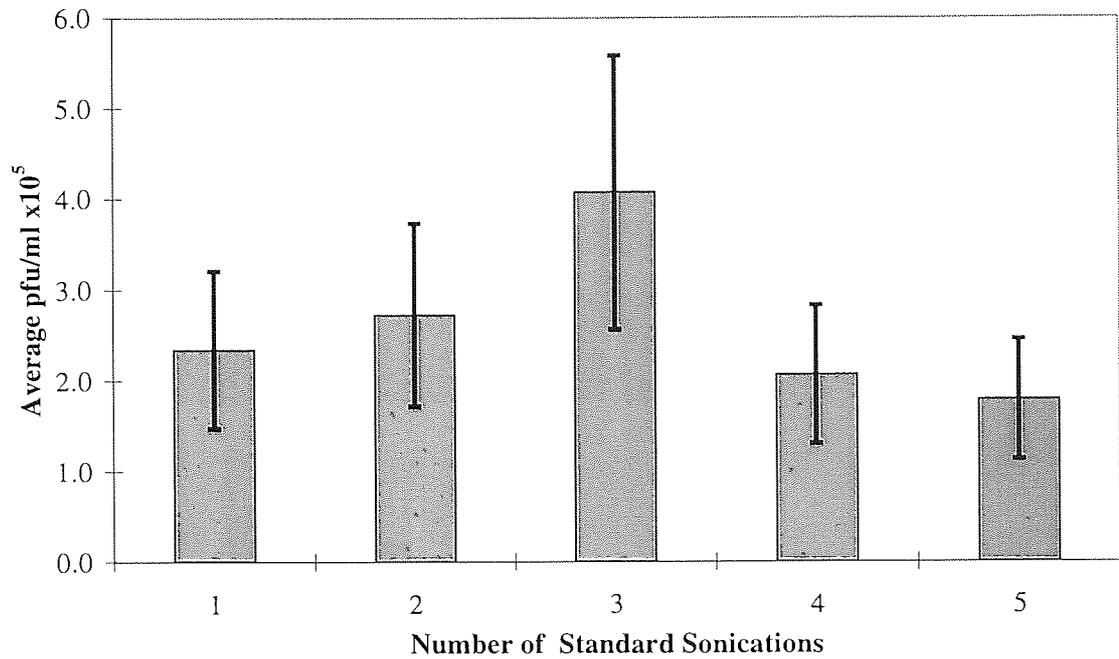
case the recoveries are low. One possible explanation for this is the difference in host cells and virus type used. It is well known that different herpesviruses have different affinities for different cell lines. Therefore if DISC-HSV has a stronger affinity then the glycerol shock method may not be as efficient. It can also be seen that the infectious virus is spread amongst the glycerol supernatant, cell pellets and the hypotonic saline supernatants. Therefore cell rupture and virus release is not confined to a particular step. The hypotonic saline supernatant after sonication does not alter in titre giving the indication that it is not aggregated, but again the recovery in this step is unfortunately too low to make it a viable process.

### **4.3 Conclusions**

The work in this chapter set out to investigate current methods of harvesting cell associated virus from tissue culture. All the methods used a positive control of virus released by sonication to measure the success of any particular experiment. Although sonication is seen to release by far the most virus, it also completely disrupts cells. This leads to a product that cannot readily be used in a downstream process.

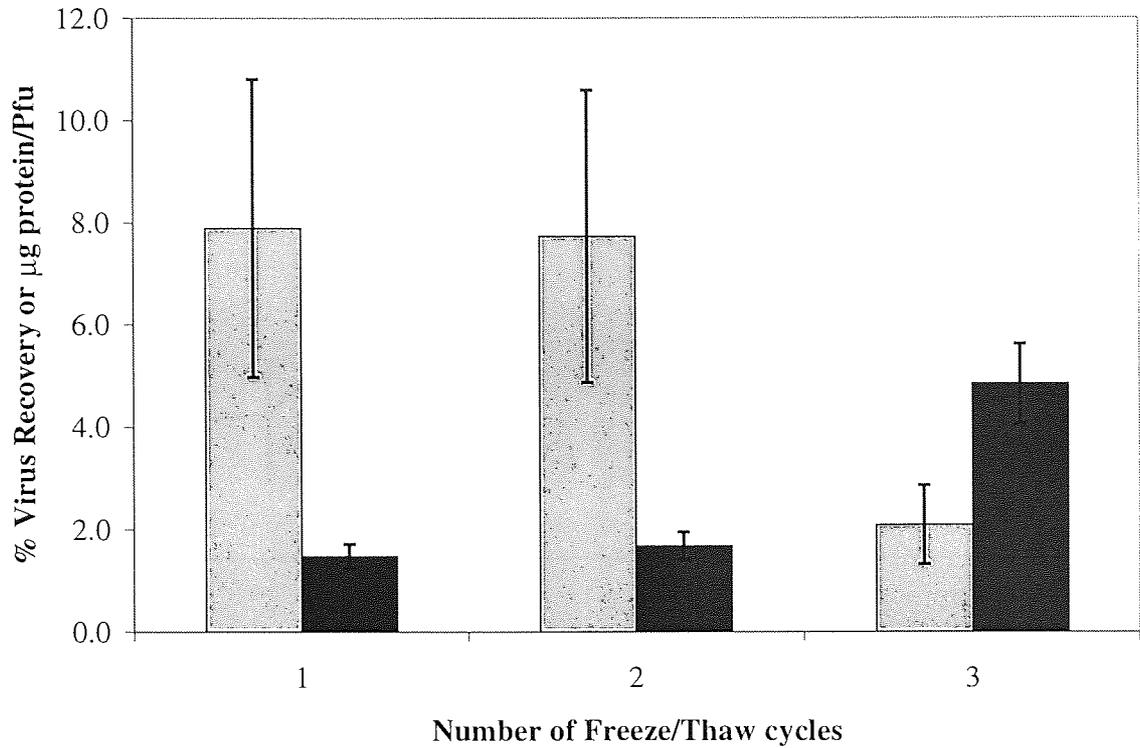
In each method investigated, the yield of virus was low and was thought not to provide enough material for a production process. Even the improved method of glycerol shock to rupture cell membranes did not yield any more virus than other methods, such as freeze thaw. The purity of the harvested material was therefore not significant since the lack of recovery meant that these methods were not appropriate and that an alternative strategy was needed.

There are other methods of disrupting cells that have not been investigated for their potential to release DISC HSV. These include homogenisation using Dounce homogenisation, microfluidisation or bionebulisation. These have been shown to be productive in different systems, such as freeze thaw for adenovirus (Giroux, 1998), but due to complexities of scale-up were not chosen for inclusion in this study.



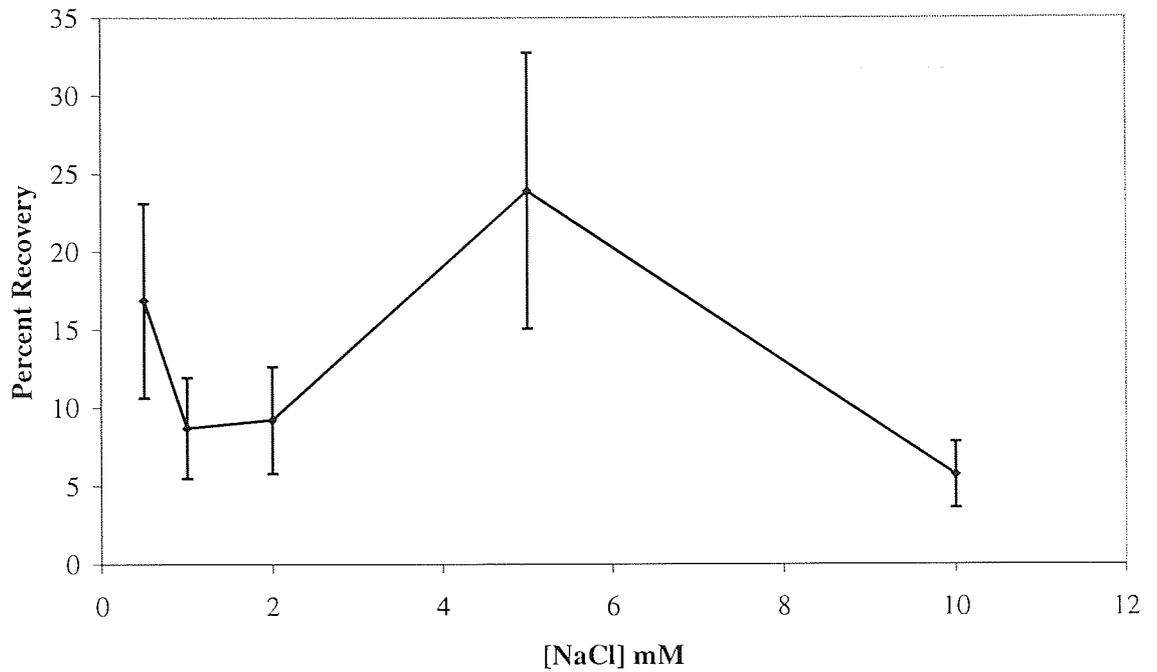
**Figure 4.1 Virus release by sonication**

DISC-HSV virus was harvested with hypotonic saline, 10ml per roller bottle; then infected cells scraped and collected in a polypropylene tube and kept on ice, 1.5 ml aliquots were sonicated in a cup horn sonicator 1 to 5 times by the standard method (see Experimental). Following sonication the aliquots were assayed in triplicate for virus by TCID<sub>50</sub>.



**Figure 4.2 The freeze thaw release of virus**

DISC-HSV infected CR2 cells were scraped into culture media and collected in polypropylene tubes. Tubes were then immersed in liquid nitrogen until frozen then thawed in a 37°C water bath. Upon the final thaw cycle the samples were centrifuged, 2,000g for 10 minutes, to remove cell debris and the supernatants assayed for virus by TCID<sub>50</sub> (light grey bars). Supernatant samples were also assayed for protein (see Experimental) (dark grey bars) to give an indication of the level of contamination released during cell rupture. Error bars indicate variance of triplicate samples. The greater the number of cycles increases protein release whilst significantly reducing infectious virus.



**Figure 4.3 Hypotonic saline osmolality range**

Infected cells were harvested by incubating with varying concentrations of hypotonic saline, 10 ml/T75 flask for 10 minutes, and collected by scraping then pipetting into centrifuge tubes. Cell debris was removed by centrifugation, 2,000 g for 10 minutes, and supernatants assayed for virus by TCID<sub>50</sub>. Data is plotted as a percentage of the yield obtained from a flask harvested by hypotonic saline and then sonicated. No significant increase in released infectious virus is observed since the errors for the individual points are large and distinction between the results cannot be made.

Sample	Average pfu/T75 Flask	%age of Sonication
Sonicated Cells	1.00x10 <sup>8</sup>	100.00
Hypotonic Control:		
Hypotonic Supernatant	2.60x10 <sup>6</sup>	2.60
Hypotonic Cell Pellet	2.78x10 <sup>5</sup>	0.28
Glycerol Shock:		
Glycerol Supernatant	2.75x10 <sup>6</sup>	2.75
Hypotonic Supernatant	1.19x10 <sup>6</sup>	1.19
Sonicated Supernatant	1.29x10 <sup>6</sup>	1.29
Cell Pellet	7.02x10 <sup>6</sup>	7.01

**Table 4.1 Glycerol shock treatment of cells to release virus**

DISC-HSV infected CR2 cells were harvested by scraping into the culture media, decanting into centrifuge tubes and centrifuging at 2,000 g for 10 minutes. Culture media was discarded and cell pellets suspended in 4 ml DPBS and kept at 37°C. 660µl of 90% glycerol was added three times with 5 minute incubations at 37°C between each addition. Cells were then centrifuged at 1,200 g for 10 minutes and the glycerol supernatant assayed for virus. Cell pellets were suspended in 3 ml hypotonic saline and incubated on ice for 5 minutes. Samples were then centrifuged for 10 minutes at 2,000 g and supernatant collected, cells were incubated with a further 3 ml hypotonic saline and centrifuged as before, and supernatants were pooled and assayed for virus. The final cell pellets were suspended in hypotonic saline, sonicated then assayed for virus. Replicate flasks were harvested, sonicated then assayed for virus to indicate the maximum obtainable yield. A 'hypotonic saline harvest' control of virus harvested by scraping cells into hypotonic saline, centrifuged at 2,000 g for 10 minutes, then assaying the supernatant and cell debris pellet for virus was conducted to test the effect of the glycerol shock release.

## **5 Non-Lytic Methods of Harvesting Cell Associated DISC- HSV**

### ***5.1 Introduction***

As described in the previous chapter, harvesting the virus is crucial to the ability of a downstream process to be operable. Not only does the harvest method have to give high yields, it must be versatile enough to be used with different culture methods. For instance, if the harvest method requires the physical removal of cell from the support, the scraping of cells from flasks or roller bottles can be accomplished using cell scrapers. When other methods of cell culture are used, such as bioreactors, the harvesting of cells needs to be more automated. Microcarriers, agarose beads of approximately 100  $\mu\text{m}$  in diameter, are used to provide a surface for adherent cells to attach too and grow. Microcarriers can therefore be used to keep adherent cell lines in a suspension of growth media by continual stirring; therefore scale-up can be achieved by using larger vessels. The major drawback of using this system is that the harvesting of the infected cells becomes more complicated since they are no longer attached to a flat plastic surface and scraping cells off their support is impossible. If cells are harvested with microcarriers the bulk of the agarose makes any downstream process impractical. The investigation of alternative methods of virus release was therefore necessary to overcome the need for the harvesting of the cells.

#### **5.1.1 An alternative strategy**

To aid the investigation of alternative methods an ideal solution was sought. If the eventual method of harvest was to be carried out on cells grown in bioreactors, then the method should be scalable and hands free to ensure the product would be sterile, easily processed and cost effective. An ideal harvest method would be to chemically

separate the infectious virus particles from the host cells. If possible the host cells should be left intact and therefore give the extra bonus of having a harvest with less contaminating host cell protein and DNA, which would aid the downstream processing. Therefore it was decided to investigate compounds and chemicals for this effect. To aid in the search for a potential candidate, the background of why HSV-2 virus particles are cell associated was researched. From this, possible methods of virus release were investigated.

### 5.1.2 Objectives

The objectives therefore were to investigate non-lytic methods of harvesting DISC HSV, which would provide harvest material with high recovery of virus and low contaminant burden, which could lead to an efficient downstream purification process.

## **5.2 Results and Discussion**

### 5.2.1 Potential Mechanism for Chemical Release

The goal of any harvest is to recover all, or near all the available product in a form that can be readily processed in a scaled up downstream process. Also if the harvest is as free as possible from culture contaminants, such as host cell protein and DNA, or from exogenous contaminants, i.e. by keeping a closed system so not allowing contaminants to enter, then the further purification of the product can be simplified.

For these reasons a different approach to the harvesting of DISC HSV was investigated. All the harvest methods that have been reported in the literature and assessed in the previous chapter have the same function – to break open the cells to effect the release of the cell associated virus. The point at which viruses become infectious during the process of their transport out of the host cell is not clear. For this reason electron micrographs of DISC HSV infected CR2 cells were taken (see

Experimental) to attempt to demonstrate where the viable virus was residing in the cells, see Figure 5.1. One of the major problems with this approach is that only 10 infectious particles are harvested per cell when using sonication. When looking at E.M.'s of infected cells only a small portion of any cell is visualised at any one time, since when using transmission electron microscopy only a slice through a cell is viewed; so drawing conclusions as to where the infectious virus resides is problematic. It can be safe to say, though, that the large quantities of viral capsids present in the cell nucleus, appearing as if crystallised (Figure 5.1), cannot be infectious due to their lack of lipid membrane. It is only after the virus has egressed into the peri-nuclear space and acquired a lipid bilayer membrane that the discussion whether they are viable becomes contentious (see Figure 5.2). It is clear from the electron microscopy that membrane bound virus is apparent throughout the cell and on the outside, in close proximity to the cell membrane. Glycosylation of proteins occurs in the Golgi apparatus of the cell and since there is a high degree of glycosylation on the viral glycoproteins these viral proteins are processed here. So for virus to become infective it is assumed they must egress from the nucleus to at least as far as the Golgi to acquire glycosylated membrane proteins. If rupturing the cell membrane is essential for the release of infective virus, then it is not clear why only a small proportion of the total virus is released using methods such as hypotonic saline or freeze thaw which break open cells. One hypothesis is that if the virus is associated with cellular membranes at the point of harvest, then sonication will yield an individual virus suspension with small fragments of cell membrane still attached. Hypotonic saline will yield only large fragments of cell membrane which may have more than one virus particle bound to it. This will have the effect of aggregating the virus or virus will be cleared by centrifugation, if the fragments are sufficiently large. If this hypothesis is true then discovering a mechanism to dissociate the virus from the membrane fragments should increase the recoveries when using hypotonic saline to harvest the virus.

There have been a large number of studies conducted to elucidate the function of the viral surface glycoproteins and these have mainly been focused on the attachment and entry of the herpes virus into cells. It has become clear that the initial attachment ligand for the herpes viruses (for example HSV-1, HSV-2 and Pseudorabies) is the

ubiquitous cell membrane proteoglycan, heparan sulphate. It has further been shown that this initial binding of the virus to heparan sulphate is reversible and the virus can be eluted from the surface of the cells up to 20 minutes, at 4°C, after initial contact using heparin, a soluble form of heparan sulphate. After this, binding of virus becomes insensitive to heparin because of further viral glycoprotein interactions with cell surface molecules resulting in eventual fusion with the cells.

The heparan sulphate proteoglycan is present on the cell surface and is comprised of alternating units of L-iduronic acid and D-glucosamine and is found in almost all animal cell membranes and in extracellular matrix. The soluble analogue of heparan sulphate is heparin that has a diverse range of functions most notably that of an anticoagulant. Heparin is a very heterologous molecule with large variation in length of the sugar chains and also large variation in the sulphate substitution, which affects the overall charge of the molecule. These variations can have an effect on the ability of heparin to elute virus from the surface of cells as described by Trybala *et al.* (1996). This also indicates that the effect of the heparin molecule is not just a charge interaction but an affinity interaction since the removal of certain sulphate groups disproportionately affects the efficacy of the heparin.

The predominance of information relating to heparan sulphate and its role in virus binding leads to the investigation of soluble heparin for its potential to dissociate the virus from cell debris in a hypotonic saline harvest.

### 5.2.2 The effect of heparin on harvesting virus

Figure 5.3 demonstrates the effect of heparin concentration on the release of virus when added to hypotonic saline. The experiment was repeated and both sets of data are reproduced to show the consistency of the effect. As with previous experiments the quantity of virus released by hypotonic saline alone (0 µg/ml heparin) is less than 10 % of a sonicated harvest. The quantity of virus released increases in a dose dependant manner up to the 50 µg/ml heparin level. Above 50 µg/ml heparin the level of released virus appears to decline. Reasons for this decline are not understood but

one hypothesis is that the extra heparin in the harvest is itself aggregating the virus and leads to the apparent fall in virus titre, but this was not further investigated. Although the released virus only achieved a maximum of about 20% of sonication there was a significant effect attributable to the addition of the heparin and so further studies were conducted to try and maximise the effect of heparin on virus release.

Table 5.1 shows the release of virus by the glycerol shock method and also the effect of including heparin in the experiment. In all samples the effect of the heparin was to increase the yield of virus by a significant level, but again the recovery compared to a sonicated harvest was low.

In view of the above results it was not certain that the apparent effect of the heparin was due to the release of the virus or an effect on the TCID<sub>50</sub> assay. Since the assay relies on the observation of plaques following the dispensing of the virus onto cell monolayers there were obvious analogies to experiments conducted in elucidating virus-cell interactions (Trybala *et al.* (1996), Lycke E. *et al.* (1991), Herold B.C *et al.* (1991)). For this reason, experiments were conducted to determine if heparin in a sample of virus altered the titre of that sample. Figure 5.4 demonstrates that the effect of heparin added to identical aliquots of hypotonic saline harvested virus does not have an affect on the assay, even when added at up to a final concentration of 400 µg/ml. An explanation why the heparin had no affect is that in a TCID<sub>50</sub> assay the samples are serially diluted and each sample aliquotted into 24 replicate wells of a 96 well plate. Infection is then observed and the dilution at which 50% of the wells are infected is calculated. In this experiment the titre of the samples is in the 10<sup>6</sup> range and so the dilutions where 50% positives are observed were at the 10<sup>-6</sup> to 10<sup>-7</sup> dilutions and therefore the heparin concentration would have been in the range 0.04-0.4 µg/ml. This concentration is too low to affect the binding of virus to the cells in view of the observed results that infection is only hindered when using 50 µg/ml heparin in the tissue culture media (Mettenleiter *et al.* 1996, Ramos-Kuri *et al.* 1996, Trybala *et al.* 1996 and Tal-Singer *et al.* 1995).

To further investigate the effect of heparin on virus binding to cells, heparin was added to the sample, dilution series or microtitre plate in a TCID<sub>50</sub> assay at a concentration of 50 µg/ml. Table 5.2 shows the results of this experiment. When heparin was present throughout the dilution series or added to the cells in the

microtitre plate at 50 µg/ml then the observed titre of the sample was reduced by 90%. It is clear that the presence of heparin at this concentration hinders the ability of DISC-HSV to infect cells *in vitro*. Therefore the heparin present in virus harvested in heparin does not seem to have a detrimental affect on the observed titre, only when the heparin is present at a higher concentration throughout the assay plate is any affect observed.

The interaction between heparin and viral glycoprotein has a large ionic component; therefore it was hypothesised that changing the pH of the harvest buffer would alter the net charge on the glycoprotein and encourage this interaction to yield a greater recovery of virus. Hypotonic saline containing 50 µg/ml heparin across a pH range of 4 to 8 was subsequently used to harvest virus. The results (Figure 5.5) suggest that the optimal pH for the buffer was around pH 7. At the alkaline pH 8 the virus recovery is lower but it is difficult to distinguish between alkaline inactivation of the virus and loss of interaction between the heparin and the glycoprotein. At the acidic pH's of 4 and 5 the observed recovery is very poor. It is known that the HSV virus is not stable at pH 4, indeed buffers at pH 4, or lower, are used to inactivate the HSV virus (Mettenleiter, 1987). The low recovery at pH 5 is also probably due to acidic inactivation. Information on the pH stability of DISC-HSV gained in this experiment means that any further processing of the virus would have to be achieved between pH 6 and 8 at the most extreme, since virus was substantially inactivated outside this range. Since all previous work to this experiment was conducted using phosphate buffer within the pH range 7.2-7.5 there was no need to repeat the work.

In an attempt to increase the recovery of virus, the length of time infected cells were exposed to heparin was investigated. Infected roller bottles displaying greater than 90% cpe were incubated with dPBS containing 50 µg/ml heparin. At 15 to 30 minute time intervals, for up to 24 hours, samples were taken and assayed for infectious virus (Figure 5.6). Surprisingly the quantity of virus released reached the level obtained by sonication after around three hours exposure, which also remained stable for at least 24 hours, the length of the experiment. This observation explains why only a small rise in virus released, when using heparin, was seen in previous experiments (see Figure 5.3), since the maximum length of exposure before was 30 minutes and here 30

minutes is approximately 20% of the final yield. The use of PBS as the harvest buffer also means that the cell membranes are not ruptured during the harvest process. It is therefore not clear where the viable virus is, in relation to the cell, at the point of harvest.

Since heparin has such a profound effect upon release of the virus, the virus must in some way be associated with cellular heparan sulphate. It seems logical therefore that, when virus egresses from a cell, it could then bind to the heparan sulphate on the surface of that cell or a neighbouring cell. In an attempt to answer this hypothesis roller bottles were acid wash treated before being harvested (see 2.4.12). The result, Table 5.3, shows a two log reduction in virus harvested from the roller bottles washed with pH 3 buffer. If the virus originated from within the cells then they would have been, at least partially, protected from the acid wash. But if the virus was already bound to the outside of the cell then the acid wash would, as it has appeared to do, inactivate the vast majority of the virus. This experiment, though, is by no means conclusive. The use of pH 3 washing has mainly been used in experiments to determine viral entry into cells (Brown 1998), by inactivating viral particles that have not entered the cell, and so in these cases the cell membranes are in much better condition. Infected cells at the time of harvest, when infected with DISC HSV appear spherical and not epithelial like when not infected. Therefore the integrity of the cell membranes may not be intact, and certainly different to cells that have not, or just recently been infected. This change in the state of the cell membranes could possibly allow the entry of the pH 3 glycine buffer and so inactivate the internal virus. If this were true, then the results of the above experiment would be misleading. An explanation and proof of where the virus is at the time of harvest cannot therefore be made.

Another hypothesis is that the presence of heparin in the harvest buffer prevents virus particles from binding to the cell membrane as soon as they have egressed into the extracellular space. Any virus that has egressed prior to exposure to heparin buffer reattaches and is rendered inactive. Figure 5.7 shows the effect of harvesting the virus at different time points post infection in an attempt to investigate such a mechanism. Four roller bottle cultures displaying 100% cpe (69 hours post infection) and two

roller bottle cultures displaying <70% cpe (65 hours post infection) were harvested with dPBS containing 50 µg/ml heparin for up to 5.5 hours. The viral harvests from the cultures with 100% cpe increased rapidly up to about 3 hours and thereafter remained fairly constant. By contrast, the viral harvests from the cultures with 70% cpe increased minimally up to 3 hours after harvesting and only marginally thereafter. These results suggest that the long length of time taken to harvest the virus is not resulting from the cells producing more virus. The earlier harvested cells would have released more virus over time and instead of a plateau the titre would have increased to equal the 69 hour post infection roller bottles.

Attempting to shed light on how the heparin is working in the above experiments proved to be difficult. No one experiment was without its flaws and it was impossible to say exactly where infectious virus was at the time of harvest. The length of time taken to reach maximal harvest titres is not typical of a solely ionic interaction. Other virus glycoproteins have been identified as binding to other cell surface receptors, such as gD binding to the Manose-6-phosphate receptor of cells (Brunetti *et al.*, 1994). Secondary binding such as these may explain the slow rate of harvesting infectious virus.

We wanted to establish whether other polyanionic or polycationic polymers could enhance HSV recovery, either by binding the gC molecule (as heparin), or by competitively binding the cell surface receptor by mimicking the positive charge distribution of the gC glycoprotein. Dextran sulphate was tested as an alternative polyanionic ligand because its viral binding properties have already been established (Nakashima *et al.*, 1989); poly-L-lysine was tested as a polycationic ligand because it is a polypeptide chain that has been shown to inhibit viral infection of cells (Zsak *et al.*, 1990). In the case of dextran sulphate, a clear enhancement of viral recovery was again noted (see Figure 5.8), though the maximum virus release occurred at a higher concentration and with a lower yield than for heparin. These trends are in line with those for viral infection (Ramos-Kuri *et al.*, 1996). For poly-L-lysine only a small increase in viral recovery was discerned over untreated culture supernatant (see Figure 5.9). Using a polycationic molecule to disrupt the binding should not necessarily work. Dextran sulphate is a direct analogue of heparin whilst poly-L-lysine may not

truly represent the binding region of the viral glycoprotein and so may not confer sufficient affinity for the cell surface heparan sulphate to release the virus from the cells.

### 5.2.3 The effect of high salt concentration on virus release

The addition of chemicals to aid the harvesting of virus has the disadvantage that the downstream process will ultimately have to remove them. To overcome this problem, the use of sodium chloride was tested for its ability to release the virus; since the affinity between virus and cell is based on ionic interactions, sodium chloride may be able to dissociate the virus from heparan sulphate. Figure 5.10 shows the effect of increasing salt concentration of a buffer on the release of DISC-HSV. At somewhere between 500 and 800mM NaCl, or around 400mM Na<sub>2</sub>SO<sub>4</sub> the salt has a marked affect although the percent yield is not high. It is clear that the ionic strength of the salt solution is important. Ionic strength ( $\mu$ ) is calculated by the Debye-Huckel equation:

$$\mu = \frac{1}{2} \sum m z^2$$

where  $m$  is the molality in moles of ions per litre and  $z$  is the valence of the ion.

The ionic strength of 0.8 M NaCl is 0.8 and the ionic strength of 0.4 M Na<sub>2</sub>SO<sub>4</sub> is 1.2, which being similar further suggests that a certain level of ionic strength is required to release the virus. The exposure time for this experiment was again only 30 minutes so a longer time course experiment was carried out to maximise the yield. Figure 5.11 shows the effect of time on virus yield when using 1M NaCl in dPBS to harvest the virus. The pattern of virus release is very similar to that observed for heparin release (Figure 5.6), although to reach maximum titre took at least 4 hours. High concentrations of salt can therefore be used as an alternative method for harvesting DISC HSV. The immediate disadvantage of using a salt solution to harvest the virus over the heparin method is the increased release of protein contamination. For the

heparin method the protein rises to about 60 µg/ml after 5 hours whilst with the salt method the protein rises to over 120 µg/ml in the same time frame. This increase in protein could lead to a more complicated downstream process. Again the perplexing question of why it takes so long for maximum virus release to be reached is raised. One major difference between the heparin and salt harvests is that the salt harvest, at 1 molar NaCl, completely breaks open the cells, hence the increased protein contamination. If it takes so long to harvest the virus because the virus is egressing from the cell and the heparin is preventing attachment to the cell membrane then the salt harvest should not take so long since the majority of cells are lysed. The interaction of the virus with other cell surface molecules may again play an important part in the length of time taken to release the virus. But if such an interaction is taking place, then it cannot be an ionic interaction. This is because the ionic strength would overcome this other interaction; also the salt harvest method still requires a long period of time to have an effect, although slightly shorter than the heparin method.

#### 5.2.4 Comparison of new and old harvest methods

The benefits of the two new approaches to harvesting DISC HSV, either heparin (and its analogues) or high salt solutions can be seen in Table 5.4. In both cases the quantity of virus recovered in the harvest matches that obtained by sonication. Physical methods of releasing virus did not reach that of sonication and therefore gave poor specific impurity profiles in terms of contaminating protein and DNA. The release of contaminants for the high salt method, when compared to the previously used disruption methods, is low even though the cells lyse; this is mainly due to the high recovery of virus. Far more DNA is released by the salt method, since whereas the mild disruption methods used did not rupture cell nuclei, salt is less discriminating. By far the most 'clean' method of harvesting DISC HSV was using heparin. Being isotonic not even the cell membranes were ruptured; hence low levels of protein and DNA were released into the harvest buffer, whilst high yields were maintained.

### **5.3 Conclusions**

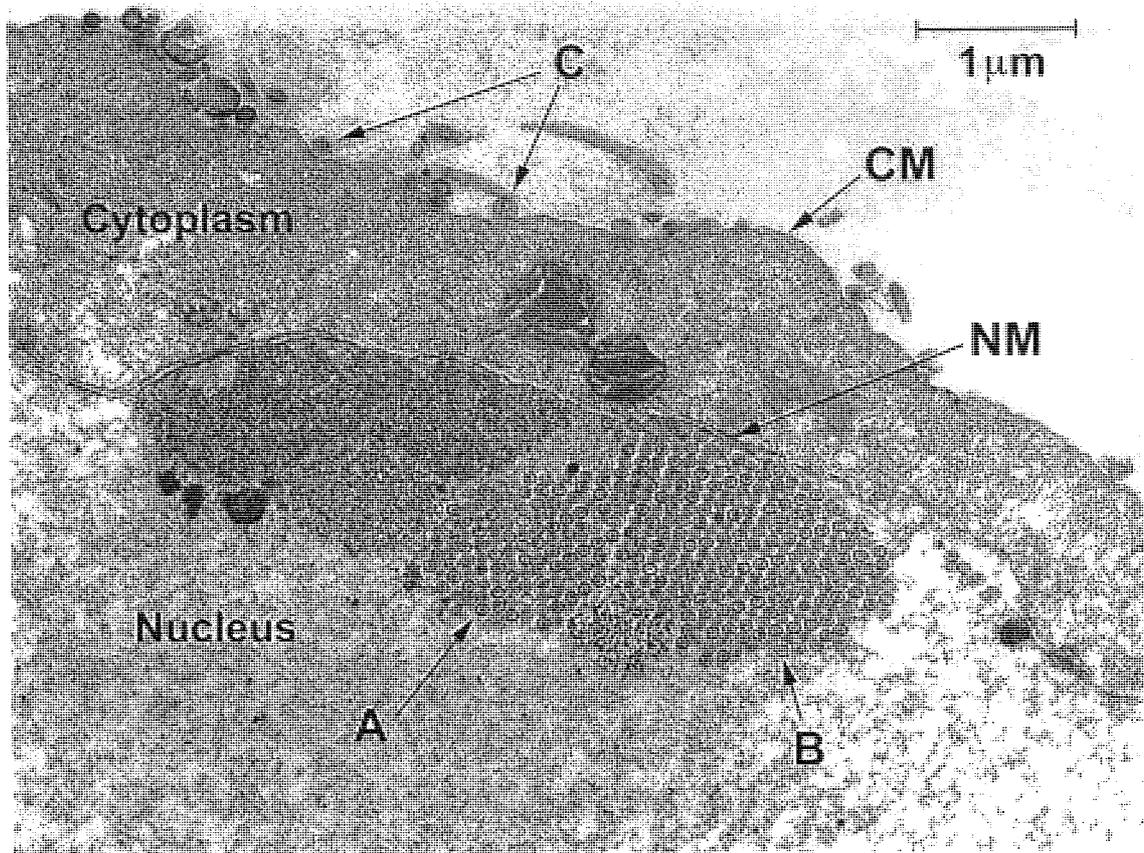
The objective of primary processing is to deliver a process stream that contains a high infectious viral titre characterised by the complete clearance of cell particulates and minimal contamination with lipids, nucleic acids and protein that might interfere with subsequent purification.

Physical methods that rupture cells to release virus proved ineffective for simultaneously achieving high virus yields while keeping the potential for harvest contamination from host cells to a minimum. Sonication gave high yields and the lowest host cell contamination/pfu of all the physical procedures that were studied. Other physical methods of cell rupture gave inadequate virus yields and unacceptable protein release, which led to the investigation of alternative release methods. The addition of heparin to the harvest buffer increases the yield of infectious virus in the culture supernatant, and appears particularly effective when conducted on cells displaying a cytopathic effect (% CPE). To minimise the rupture of cells, cell culture media can be replaced with isotonic phosphate buffer (PBS) containing the heparin, thus removing any ruptured cells before the harvest and reducing the occurrence of further cell rupture. This strategy therefore overcomes the contamination problem due to host cell protein and DNA.

In the case of the high salt harvest method, the increased cell lysis encountered still did not release as much contamination as other physical rupture methods. One advantage gained by using the salt release method over the heparin method is that it does not require the addition of different chemicals to the harvest buffer over the pre-existing method of virus harvest employed by Cantab Pharmaceuticals in their production campaign (i.e. only an increase in salt concentration from 0.01 M to 1.0 M). Therefore, there only needs to be a slight alteration to the CTX application to enable this method to be included in the manufacturing process for production of clinical trial material. The addition of heparin to the process includes not only an extra chemical but one that is of animal origin. Therefore any product produced using

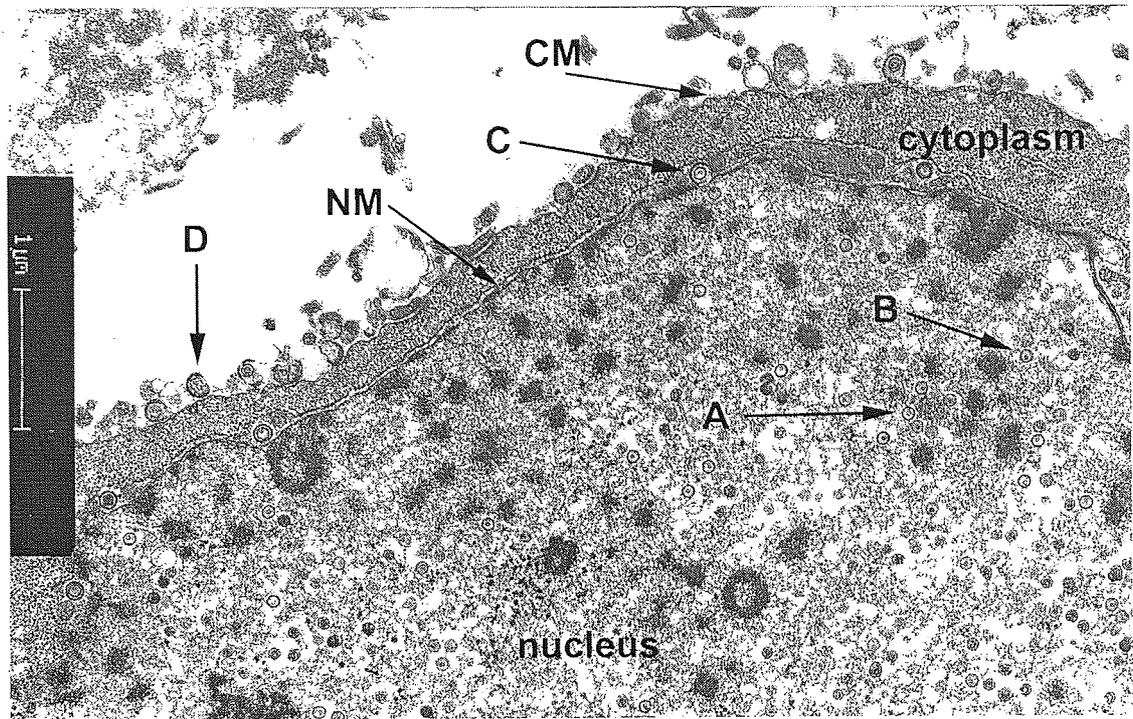
heparin would need more extensive testing to prove removal of the heparin and any possible extraneous agent that may have contaminated the heparin source. However, the problem of addition of animal products can be overcome by the use of dextran sulphate, which is a synthetic analogue of heparin produced from plants. The use of this over a salt harvest would mean far lower levels of contaminants in the process stream, see Table 5.4, especially in terms of DNA release.

There are therefore two possible methods of harvesting the DISC HSV virus summarised in Figure 5.12 and Figure 6.21, both of which have benefits over the other. To fully assess which method is best suited for a final process, both the heparin and salt methods were investigated for their suitability in purification.



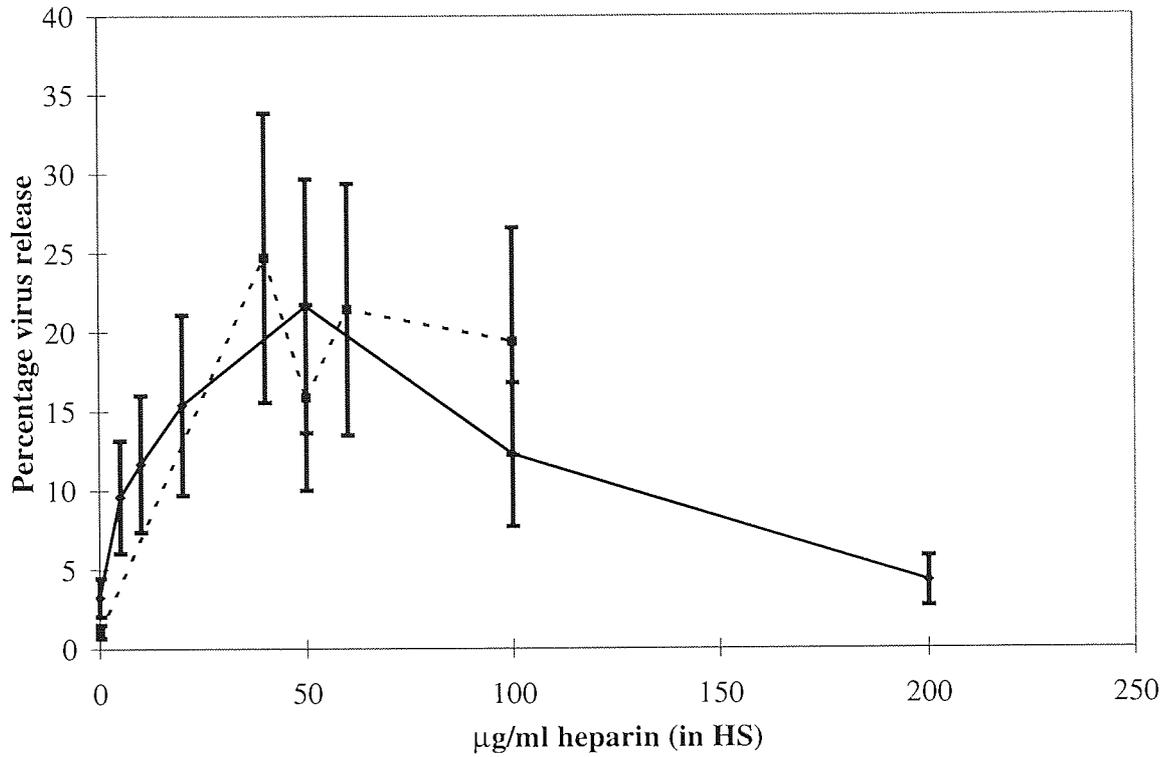
**Figure 5.1** Electron micrograph of DISC HSV infected CR2 cells.

Cells infected with DISC HSV were harvested (three days post infection) and prepared for microscopy (see Experimental). Viral capsids can be seen to form crystallised structures in the nucleus some containing DNA (A) and some empty (B). It is not known whether this crystallisation is an artefact of the preparation of the cells or naturally occurring. Once capsids are formed they egress through the nuclear membrane (NM) and eventually leave the cell at the cellular membrane (CM) as viable virus particles with lipid membranes(C).



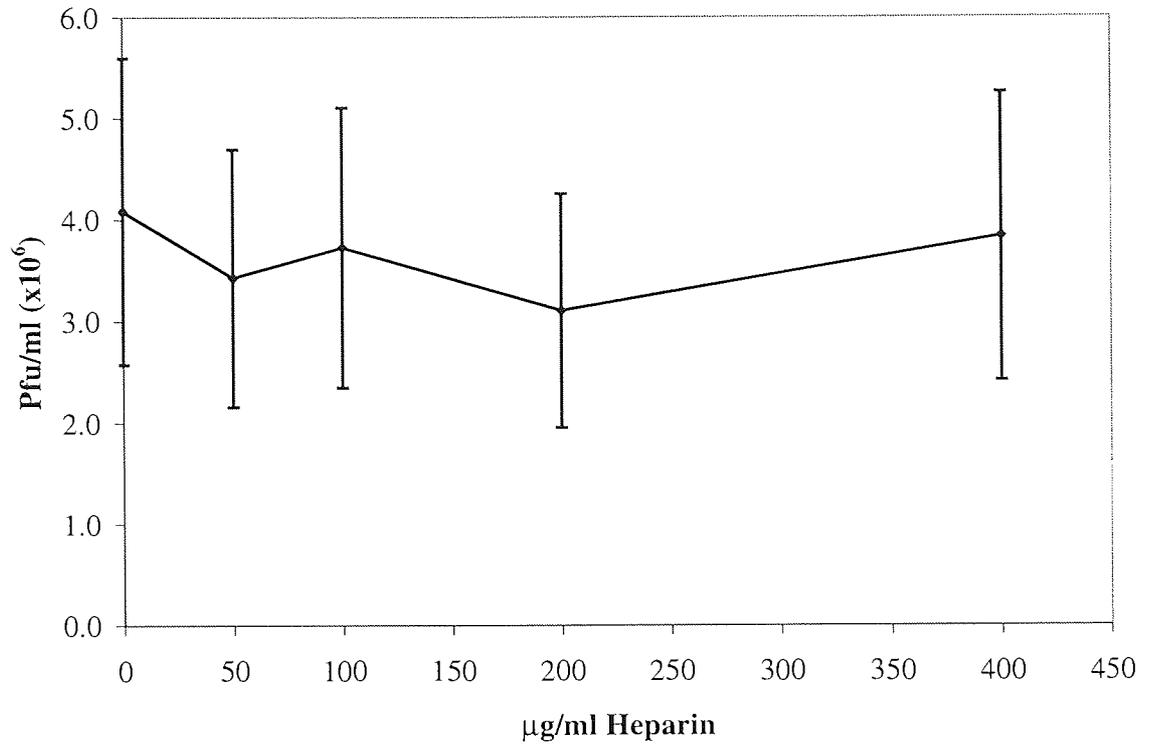
**Figure 5.2** Electron micrograph of DISC HSV infected CR2 cells.

Cells infected with DISC HSV were harvested (three days post infection) and prepared for microscopy (see Experimental). Viral capsids are formed in the nucleus and packed with DNA, which appears as electron dense centres to the capsid (B). Some capsids do not get packaged with DNA (A) and appear with clear centres. Once capsids are formed they egress through the nuclear membrane (NM), gaining a lipid membrane, and enter the peri nuclear space (C). From here the virus particles are transported to the cellular membrane (CM) and leave the cell (D). At some point in this process (from the peri nuclear space to when they leave the cell) they become viable and, for HSV 2, remain associated with the cell.



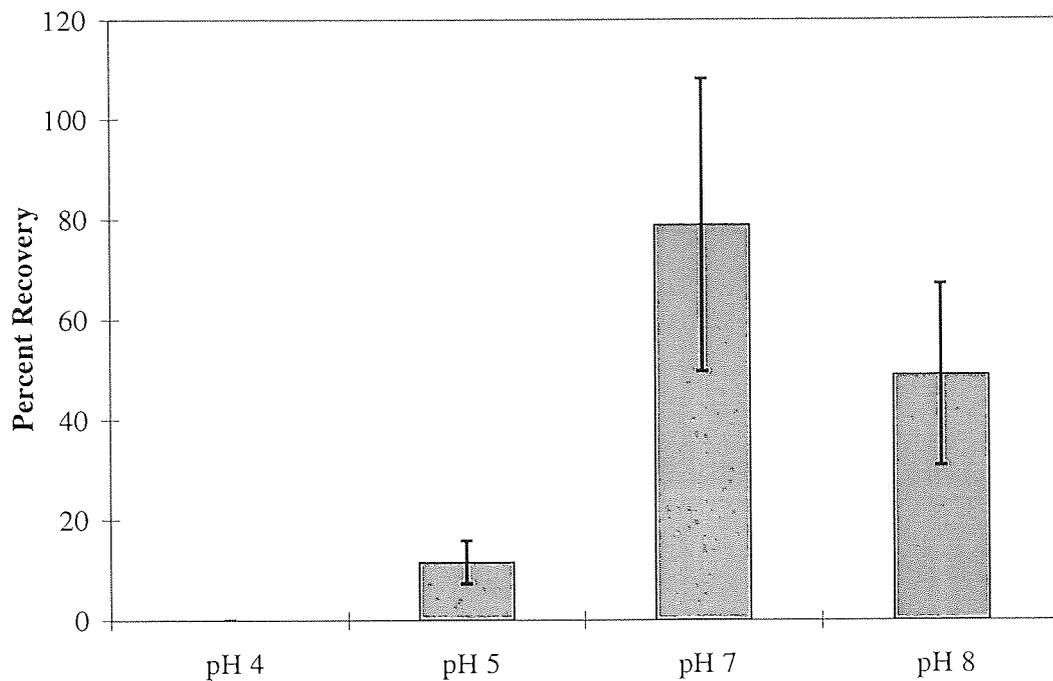
**Figure 5.3 The effect of heparin on virus release**

Media from infected cells was discarded and replaced with 10 ml/T75 flask hypotonic saline with varying concentrations of low molecular weight porcine heparin, 0-100 µg/ml (dashed line) and 0-200 µg/ml (solid line), and incubated for 25 minutes at 37°C. Harvests were decanted into centrifuge tubes and centrifuged for 10 minutes at 2,000 g to remove cell debris. Supernatants were assayed for virus by TCID<sub>50</sub> and figures are displayed as a percentage of virus harvested by the sonication method.



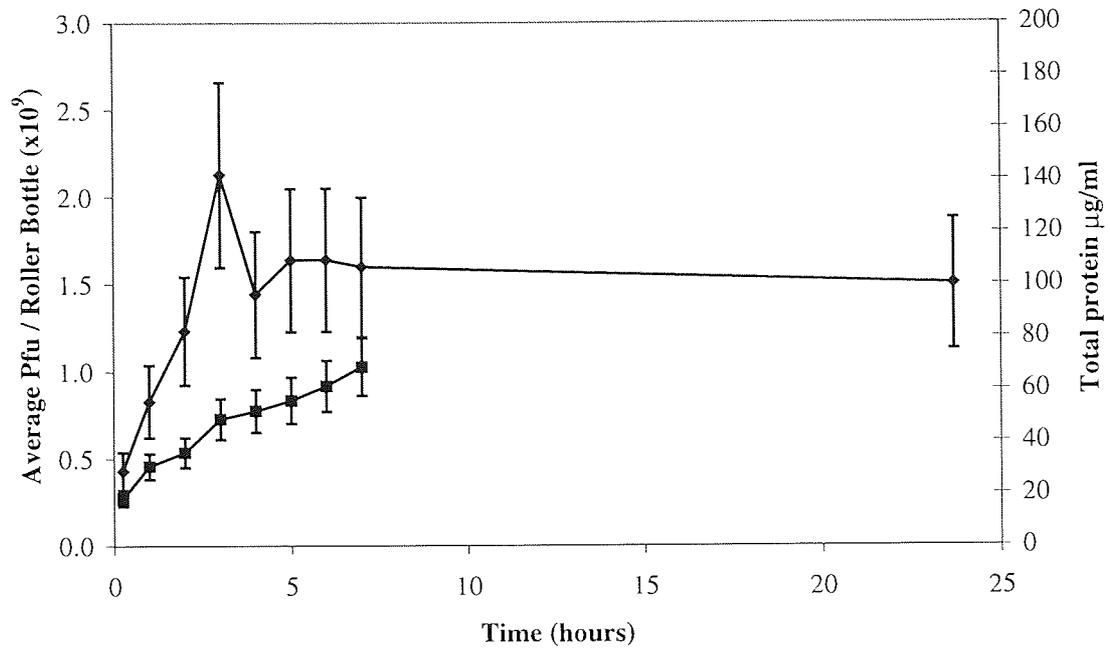
**Figure 5.4** The effect of initial heparin concentrations on the TCID<sub>50</sub> assay.

Hypotonic saline harvested DISC-HSV was sonicated and heparin added to aliquots. A final concentration range of 0-400 µg/ml was achieved using heparin at 5.4 mg/ml and hypotonic saline, to maintain equal dilution across the aliquots. Samples were then assayed for virus by TCID<sub>50</sub>.



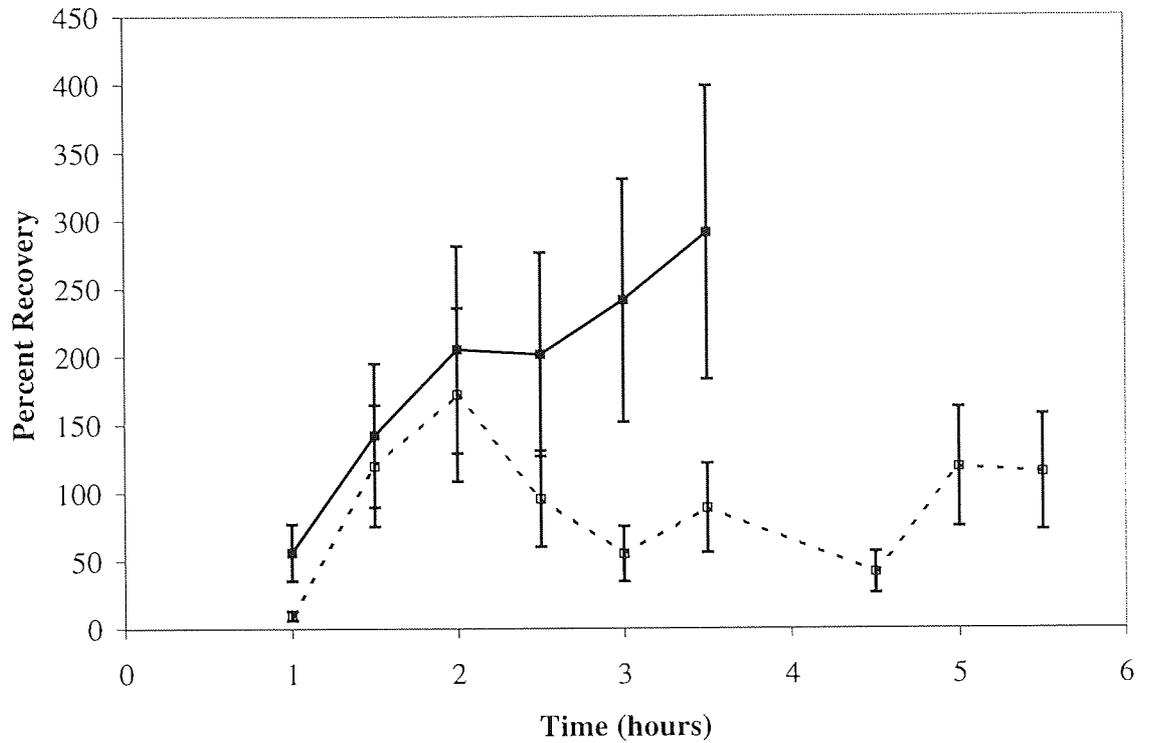
**Figure 5.5 Optimising pH for heparin harvest**

Media from infected cells was replaced with hypotonic saline containing 50  $\mu\text{g/ml}$  heparin of varying pH. Following incubation at 37°C for 30 minutes harvests were decanted to the flasks and centrifuged at 2,000  $g$  for 10 minutes. Supernatants were assayed for virus by  $\text{TCID}_{50}$  and values are displayed as a percentage of virus harvested by the sonication method



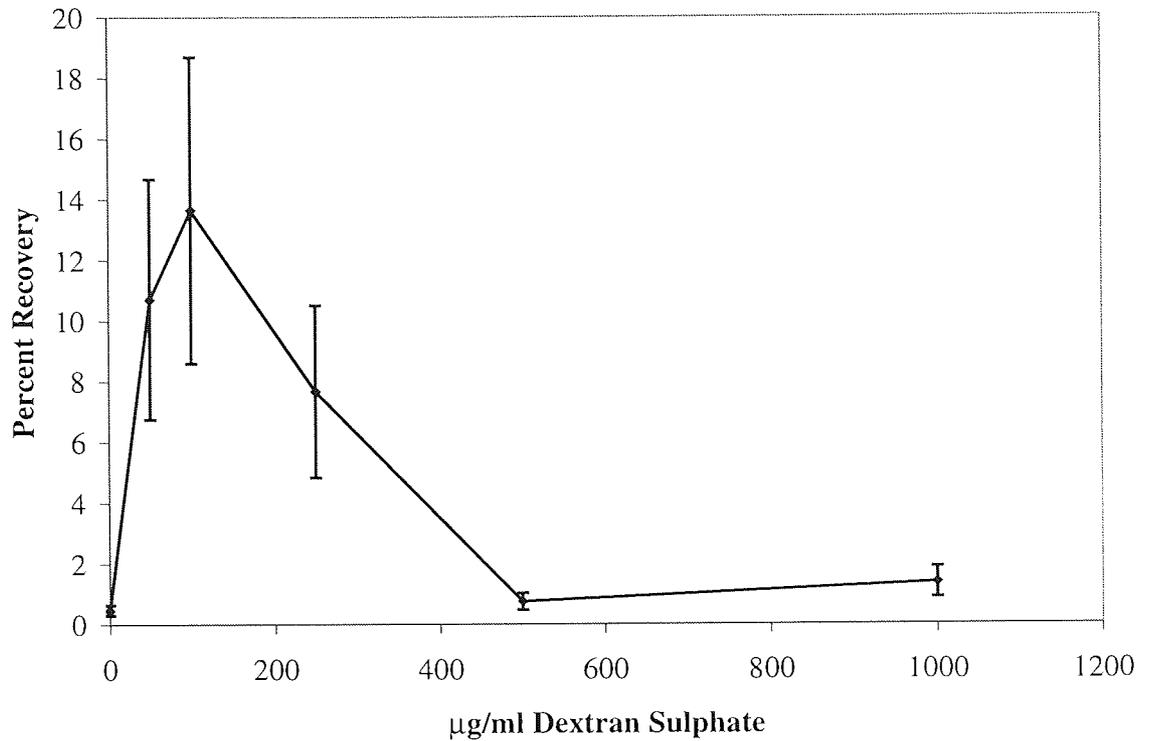
**Figure 5.6 Optimising exposure time for heparin release of virus**

Media from triplicate roller bottles, containing CR2 cells three days post infection, was decanted and replaced with 35 ml of PBS containing 50 µg/ml heparin and incubated at 37°C in a roller rig. At, initially, 15 minutes then every 60 minutes a 0.5 ml sample was taken from each roller bottle (a final sample was taken at approximately 24 hours). Samples were centrifuged at 10,000 g for 1 minute to remove cellular debris and stored at -80°C. Supernatants were thawed at room temperature, then assayed for infectious virus by TCID<sub>50</sub> (◆) and total protein by the Lowry method (■).



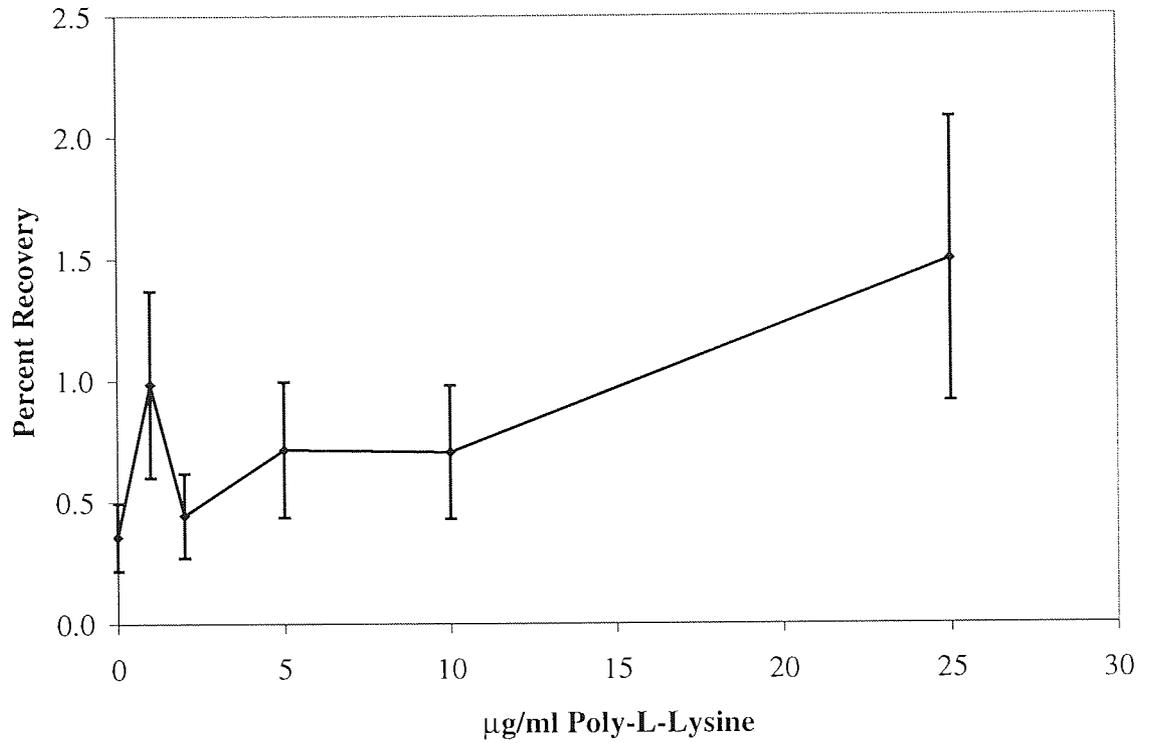
**Figure 5.7 Optimising the harvest time for heparin release of DISC-HSV**

Three of six identically infected roller bottles were harvested at 65 hours post infection (dashed line) and the remaining three harvested 69 hours post infection (solid line). At harvest, media was discarded and replaced with 35 ml PBS containing 50  $\mu\text{g/ml}$  heparin and at each time point a 0.5 ml sample was taken and centrifuged at 10,000 g for 1 minute. Supernatants were stored at  $-80^{\circ}\text{C}$  prior to assaying for virus by  $\text{TCID}_{50}$ .



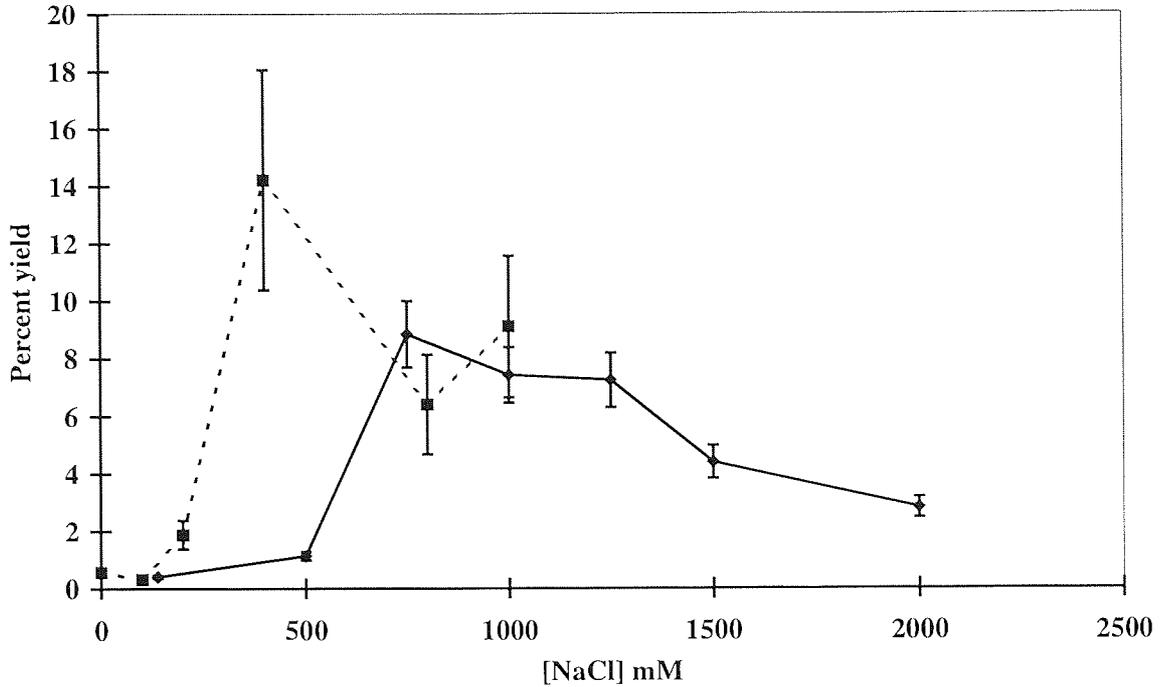
**Figure 5.8 Release of DISC-HSV using the heparin analogue Dextran sulphate**

Media from infected cells was discarded and replaced with 10 ml/T75 flask hypotonic saline with varying concentrations, 0-1000 µg/ml, of low molecular weight (5,000 MW) dextran sulphate and incubated for 25 minutes at 37°C. Harvests were decanted into centrifuge tubes and centrifuged for 10 minutes at 2,000 g to remove cell debris. Supernatants were assayed for virus by TCID<sub>50</sub> and figures are displayed as a percentage of virus harvested by the sonication method.

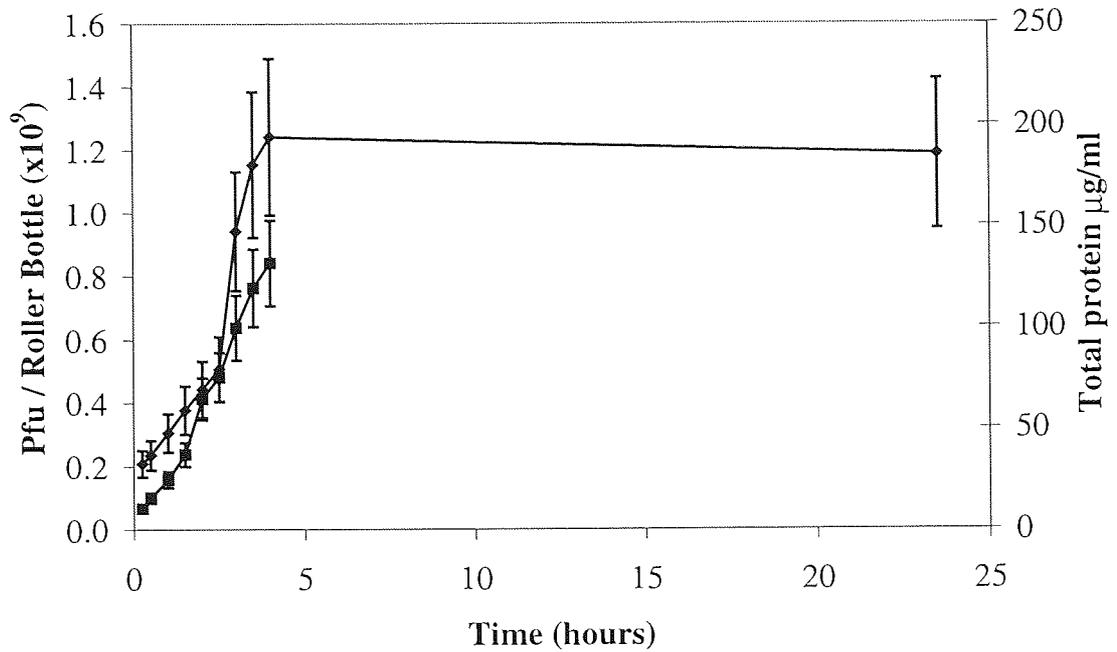


**Figure 5.9 Virus release using poly-L-lysine**

Media from infected cells was discarded and replaced with 10 ml/T75 flask hypotonic saline with varying concentrations, 0-25 µg/ml of poly-L-lysine and incubated for 25 minutes at 37°C. Harvests were decanted into centrifuge tubes and centrifuged for 10 minutes at 2,000 g to remove cell debris. Supernatants were assayed for virus by TCID<sub>50</sub> and figures are displayed as a percentage of virus harvested by the sonication method.



**Figure 5.10 Release of DISC-HSV using sodium chloride and sodium sulphate**  
 Media from infected cells was discarded and replaced with 10 ml/T75 flask phosphate buffer with varying concentrations, 0-2000 mM, of sodium chloride (solid line), or 0-1000 mM sodium sulphate (dashed line) and incubated for 25 minutes at 37°C. Harvests were decanted into centrifuge tubes and centrifuged for 10 minutes at 2,000 *g* to remove cell debris. Supernatants were assayed for virus by TCID<sub>50</sub> and figures are displayed as a percentage of virus harvested by the sonication method.



**Figure 5.11 Optimising exposure time for salt release of virus**

Media from triplicate roller bottles, containing CR2 cells three days post infection, was decanted and replaced with 35 ml of phosphate buffer containing 1 molar sodium chloride and incubated at 37°C in a roller rig. At, initially, 15 minutes then every 30 minutes a 0.5 ml sample was taken from each roller bottle (a final sample was taken at approximately 24 hours). Samples were centrifuged at 10,000 *g* for 1 minute to remove cellular debris and stored at -80°C. Supernatants were thawed at room temperature then assayed for infectious virus by TCID<sub>50</sub> (◆) and total protein by the Lowry method (■).

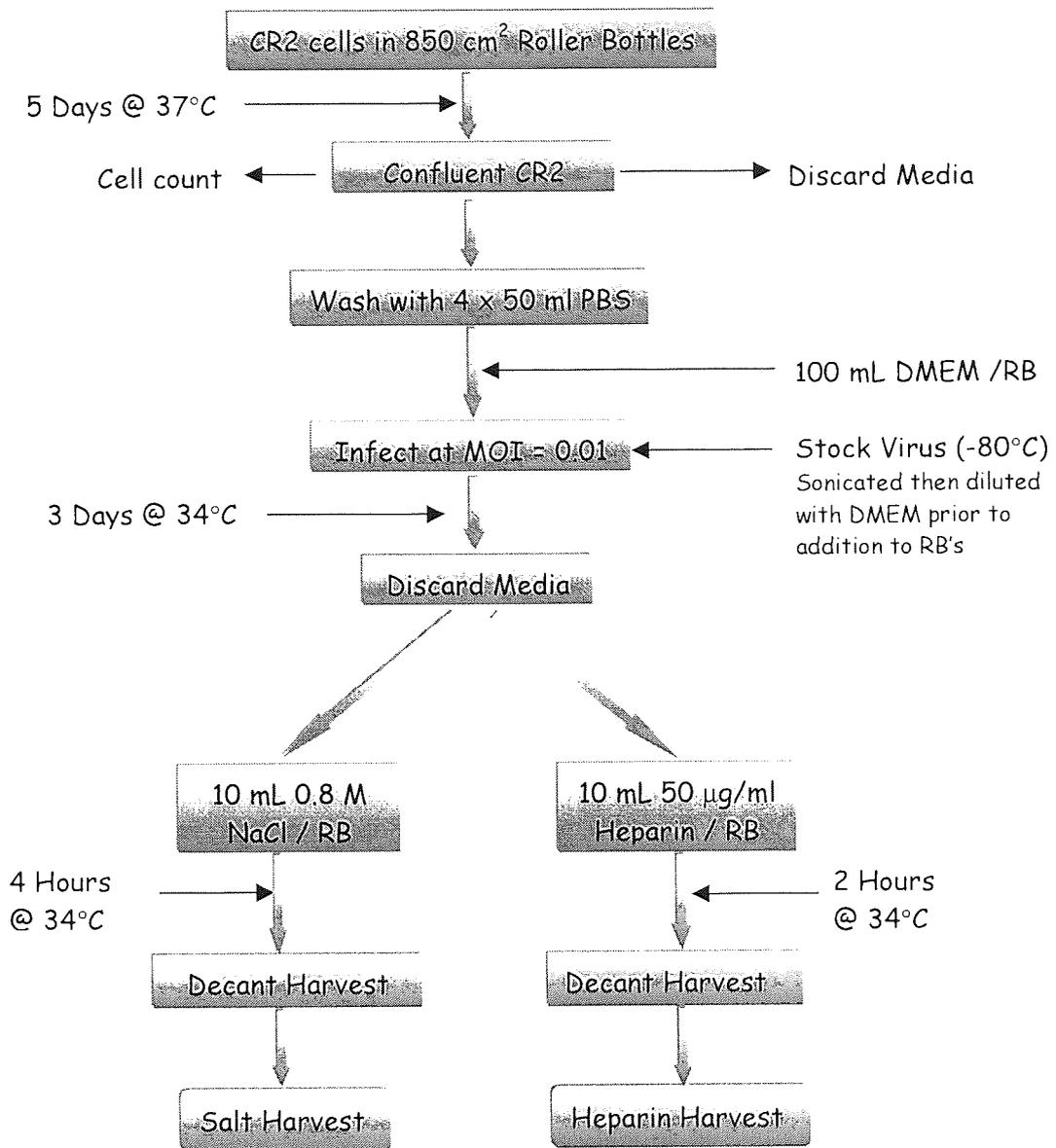


Figure 5.12 Flow diagram of virus growth and harvesting methods salt or heparin.

Sample	Average pfu/T75 Flask	%age of Sonication	With Heparin %age of Sonication
Sonicated Cells	1.00E+08	100.00	
Hypotonic Control:			
Hypotonic Supernatant	2.60E+06	2.60	19.11
Hypotonic Cell Pellet	2.78E+05	0.28	26.63
Glycerol Shock:			
Glycerol Supernatant	2.75E+06	2.75	
Hypotonic Supernatant	1.19E+06	1.19	10.50
Sonicated Supernatant	1.29E+06	1.29	8.28
Cell Pellet	7.02E+06	7.01	4.62

**Table 5.1 Glycerol shock treatment of cells to release virus**

Infected cells were collected by scraping into culture media, centrifuging at 2,000 g for 10 minutes, then discarding the supernatant. Cell pellets were resuspended in 4 ml PBS and kept at 37°C. 660µl of 90% glycerol was added three times with 5 minute incubations at 37°C between each addition. Cells were then centrifuged at 1,200 g for 10 minutes and the glycerol supernatant assayed for virus. Cell pellets were resuspended in 3 ml hypotonic saline and incubated on ice for 5 minutes. Samples were then centrifuged for 10 minutes at 2,000 g and supernatant collected; cells were incubated with a further 3 ml hypotonic saline and centrifuged as before; supernatants were pooled and assayed for infectious virus using the TCID<sub>50</sub> assay. The final cell pellets were resuspended in hypotonic saline, sonicated then assayed for virus. Replicate flasks were harvested, sonicated then assayed for virus to indicate the maximum obtainable yield. A 'hypotonic saline harvest' control of virus harvested by scraping cells into hypotonic saline, centrifuged at 2,000 g for 10 minutes, then assaying the supernatant and cell debris pellet for virus was conducted to test the effect of the glycerol shock release.

Heparin added to			Average Titre (pfu/ml)
Sample	Dilution Series	Microtitre Plate	
-	-	-	$2.13 \pm 0.1 \times 10^7$
+	+	+	$2.39 \pm 0.1 \times 10^6$
-	+	-	$2.34 \pm 0.2 \times 10^6$
+	-	+	$3.05 \pm 0.3 \times 10^6$

**Table 5.2** Effect of heparin in the TCID<sub>50</sub> estimation of infectious virus

CR2 cells infected with DISC-HSV and displaying 95-100% cpe were harvested with 10 ml/roller bottle of hypotonic saline, cells scraped and collected. Following sonication for 1 minute triplicate aliquots were assayed to give a control. A further three aliquots were assayed in the presence of heparin, at 50 µg/ml, in the sample dilution series and in the growth media for the CR1 cells. Three aliquots were assayed with heparin present, at 50 µg/ml, in only the dilution series, and a further three aliquots were assayed with heparin present, at 50 µg/ml, in only the growth media for the CR1 cells. Titres given are the averages of the triplicate samples.

	Total pfu/Roller Bottle		
	pH3	PBS Wash Control	Positive Control
pH3 glycine wash	<10		
1 <sup>st</sup> Wash (PBS)		1.89±0.9x10 <sup>6</sup>	
2 <sup>nd</sup> Wash (PBS)	2.12±1.8x10 <sup>5</sup>	6.82±5.0x10 <sup>6</sup>	
Heparin Release (mins)			
30	9.56±1.6x10 <sup>5</sup>	1.07±0.0x10 <sup>8</sup>	
60	1.41±0.1x10 <sup>6</sup>	1.80±0.1x10 <sup>8</sup>	
120	4.49±0.7x10 <sup>6</sup>	7.25±0.5x10 <sup>8</sup>	
240	6.06±0.3x10 <sup>6</sup>	4.80±0.3x10 <sup>8</sup>	5.9±2.1x10 <sup>8</sup>

**Table 5.3 Virus recovery following acid treatment of infected cells.**

Media from three infected roller bottle cultures displaying >90% cpe was discarded and the cells rinsed with 20 ml glycine buffer (pH3) for approximately 5 seconds. Cells were returned to neutral pH with a second rinse with DPBS and then harvested with 20 ml DPBS containing 50 µg/ml heparin at 37°C in a roller rig, with samples taken at 30 to 60 minute intervals to be assayed for virus by TCID<sub>50</sub>. Three similarly infected roller bottle cultures were rinsed twice with DPBS and then harvested in the same manner to act as a control. The positive control was a further three roller bottle cultures that were harvested with 10 ml/roller bottle DPBS containing 50 mg/ml heparin for 4 hours at 37°C in a roller rig. All samples were centrifuged at 2,000 g prior to assaying for virus by TCID<sub>50</sub>. Figures given are averages of triplicate roller bottles with standard errors.

Cell Treatment	Typical total virus release (/10 <sup>6</sup> pfu)	% of virus released by sonication	pg Protein /pfu	ng DNA /10 <sup>7</sup> pfu
Sonication	100	-	164	200
Freeze/Thaw	1.3	7.9	1479	-
Hypotonic Saline	2.6	2.6	350	14
Glycerol Shock	1.2	1.2	645	-
Bionebulised	60	67	297	90
Heparin	100	100	4	10
High Salt	100	100	10	100
Dextran Sulphate	100	100	7	30

**Table 5.4 Comparison between old and new methods of harvesting DISC-HSV**

Infected roller bottles were harvested by varying methods as described in the experimental chapter (2.4). Bionebulisation was carried out by Cantab Pharmaceuticals as part of the manufacture of clinical trial material. All figures are approximated since large batch to batch variation occurs due to differing harvest titres. In most cases a sonicated control was included in an attempt to normalise the data. For these reasons no error data is given.

## 6 Purification of DISC-HSV

### 6.1 Introduction

Purification of DISC-HSV must be to the extent that the product meets satisfactory contamination levels for a vaccine. The objective of the work done in this chapter was to purify the virus to within these limits whilst maintaining an acceptable recovery of infectious virus.

Over the course of this work the limits on the levels of the various contaminants have changed. The initial clinical trial exemption (CTX) only required the virus to be purified to a moderate level and the concentration of virus in the product was set at a level required for the purposes of the trial. The maximum level of total protein was set at 100 µg/dose, host cell DNA was set at 500 pg/dose and bovine serum albumin was set at 500 ng/ml, with a dose for this trial being  $1 \times 10^6$  pfu.

It is not possible to generalise the allowable levels of contaminants for products to be used for human use since each product is assessed individually, but there are some general guidelines for how pure the product should be.

#### 6.1.1 Host Cell DNA

Host cell DNA is a potential problem since the cells that are used in producing the DISC-HSV virus are immortal. The process of making cells immortal is the result of a change in the expression and structure of cellular proteins. The major concern is the possibility that the ability of these cells to continually divide is transferred to a recipient of the vaccine which could result in the growth of cancerous cells. Experiments to demonstrate the potential of host cell DNA to transform cells *in vivo* (Levinson *et al.*, Palladino *et al.* 1987) showed that subcutaneous injection of up to

100 µg of transformed cell line DNA from HeLa, CHO and T-24 cells, into anti-thymocyte serum treated new born rats did not produce any tumours. Whereas when similarly treated rats were injected with  $10^6$  HeLa cell 55 out of 66 rats developed tumours and 10 out of 10 rats developed tumours when injected with  $10^6$  CHO cells. Further experiments showed that injecting known transforming genes, Ha-ras-DNA and Ki-ras-DNA, at 10 µg per rat did not induce tumours either. To deliver the same amount of activated oncogene using cellular DNA would require injecting 1 g of DNA. In similar experiments Mufson and Gesner injected Chinese Hamsters with 50 µg DNA from the transformed Chinese Hamster Ovary cell line CHO-AJ19 and found no tumour growth. Levinson has however demonstrated that  $10^7$  pg of intravenously injected, highly tumorigenic, Ki-ras DNA does induce tumour formation. With this in mind the level of allowable host cell DNA has been set at the low level of 500 pg/dose. Recently however the World Health Organisation (WHO) has revised the levels of contaminating host cell DNA permitted in products derived from continuous cell lines to 10 ng/dose. This reassessment is based on the fact that the majority of host cell DNA is non-oncogenic and has now become regarded as a 'cellular contaminant rather than a significant risk factor' (WHO (1997)).

As explained in the general introduction, to enable the DISC-HSV virus to grow in tissue culture the deleted gH gene has been inserted into the culture cell line. If gH gene DNA is present in the purified virus there is the possibility that the DISC-HSV virus could recombine with the gH gene DNA in the host cells and revert to a wild type virus with the potential to cause disease. The likelihood of a recombination event has been minimised by the deletion of any overlapping DNA sequences between the gH deletion site in the DISC-HSV virus and the insertion of the gH gene into the Vero cell line (see introduction). This does not however negate the possibility that it may happen since all the genetic information could be present in the product. Recombination can be tested for by infected cells that do not contain the gH gene and looking for the continual growth of virus. In all tests carried out to date, even with crude virus harvests, no recombination events have been detected. Unfortunately a standard assay for the presence of gH DNA was not available for the work carried out here but it is important to consider its existence in the final product.

### 6.1.2 Contaminating Protein

The potential problems associated with the presence of host cell protein in the product are much less than those associated with contaminating host cell DNA. The major implication for the presence of host cell protein is the potential immune response generated when administered. This problem was thought a low risk so the level of 100 µg/dose host cell protein was set for the CTX batches produced.

There are other sources of protein contamination, namely products used in the production of the virus. Foetal bovine serum (FBS) is used to enable the host cells to grow and is carried over into the harvested virus. Bovine protein is regarded as a more hazardous contaminant primarily because of the risk of contracting CJD from bovine products contaminated with BSE. To eliminate the risk of using FBS contaminated with BSE the serum used is supplied from countries where there is no record of BSE infection, which at the present time is only New Zealand. It is quite difficult to quantify the total range of proteins present in FBS so bovine albumin, which comprises the majority of the total protein, is used to set the impurity level. Even with the safeguards against using BSE contaminated FBS, the hazardous potential of FBS means the limit set is 500 ng/ml.

Although there are not any limits set for the final specifications of this product, the aim of the following work was to reduce the overall protein levels to as low a level as possible. The purification methods used could then be compared and a decision made as to which method is the best to use for a manufacturing process.

The methods for the detection of both host cell protein and bovine albumin are ELISA methods. Due to the number of assays being carried out on the samples only total protein was assayed instead of the two ELISA assays.

### 6.1.3 Current purification methods

To enable the DISC-HSV vaccine to be used in clinical trial, the virus was needed in a pure form to be administered into patients. Initial clinical trials also did not require a large quantity of product to be purified so a laboratory scale method was used. This

method relies on high speed centrifugation of the virus on a sucrose gradient (see 1.6.2). This separates the virus from other cellular contaminants by banding at a different density in the sucrose gradient. Although this method produces fairly pure virus (see results), it cannot readily be scaled up for manufacturing because of the small capacity of the centrifugation rotor tubes and the length of time the centrifugation takes. Another major drawback of this method is the low yields associated with the recovered virus. For these reasons alternative, more practical methods were used to produce the larger scale clinical trial batches. Tangential Flow Filtration (TFF) was used to aid the purification of the crude harvested virus by diafiltration (see 1.6.4). Although this method did not get the virus as pure as the sucrose gradient material, larger quantities of virus could be purified and the level of contaminants were below that set by the FDA.

#### 6.1.4 Objectives of the purification study

Although there have been many methods used to purify viruses we did not set out to repeat these methods and compare the results due to the length of time this would take. Instead only methods that were considered scaleable were investigated. The use of tangential flow filtration was not investigated here because the current work conducted at Cantab Pharmaceuticals was concentrating on this aspect of the purification, although results of this work were used for comparison.

The primary objective was to find a method of purifying the virus that would be acceptable to the regulatory authorities for a vaccine product, whilst maintaining high recoveries that would be necessary for the process to become viable at manufacturing scale.

The work conducted on the release of the virus from the host cells gave an indication that there was a specific ligand on the surface of the virus that could be utilised in the purification of the virus. This interaction was to be investigated for potential affinity purification.

If it was possible to purify the virus by this route, then a secondary objective was aimed at identifying the virus ligand for affinity purification.

Virus used in this study was produced and harvested by the two methods as described in Figure 5.12.

## **6.2 Results and Discussion**

The reversible binding of Herpes viruses types 1 and 2, and Pseudorabies virus to cell surface heparan sulphate glycosamino-glycans during the early stages of cell infection occurs through basic amino acid residues on the two virus envelope glycoproteins gC (Mettenleiter *et al.*, (1990)) or gB (Herold *et al.*, (1994), Williams and Straus (1997)). Cell-binding and hence infection are impeded by incubation with soluble heparin and the chemical modification of the sulphate group composition and location on the polymer molecule modifies the affinity of this antagonist for virus envelope proteins (Trybala *et al.*, 1996). Other polyanionic cell-binding antagonists have been reported such as dextran sulphate (Nakashima *et al.*, 1989; Ramos-Kuri *et al.*, 1996), biphenyl disulphonic acid urea co-polymers (Taylor *et al.*, 1995) and protamine sulphate (Ramos-Kuri *et al.*, 1996). HSV-1 envelope glycoproteins gB and gC have been shown to bind heparin-Sepharose affinity gels from physiological saline solution (Herold *et al.*, 1991). In another study (Tal-Singer *et al.*, 1995) HSV-1 gC was absorbed onto a heparin functionalised bead and eluted with either 2 mg/ml heparin in phosphate buffered saline, or a linear salt gradient (0.15 to 1.5 M NaCl). We therefore reasoned that these polysulphonated cell-binding antagonists might be attractive affinity purification ligands for infectious HSV-2 since their specificity may permit the efficient isolation of virus from complementing cell protein and nucleic acid contaminants.

### 6.2.1 Batch adsorption chromatography

Initial batch adsorption experiments screened the binding of HSV-2 to the Cellufine-sulphate, Cellufine-heparin, heparin-Sepharose and heparin-HP matrices. Salt released supernatant was diluted to a final salt concentration of 140 mM and 25 mls of this suspension (containing  $2.8 \times 10^7$  pfu) were shaken with 1 ml of the respective gels at room temperature. The Cellufine-sulphate matrix provided almost complete adsorption of virus ( $>2 \log_{10}$  removal) within 180 minutes of contact (Figure 6.1). By contrast, the supernatant virus titre for the Cellufine-heparin and heparin-Sepharose samples were indistinguishable from the negative control (a shaken sample of virus supernatant) after 210 minutes of contact. Heparin-HP gel adsorbed virus in a similar fashion to Cellufine-sulphate (Figure 6.2), though only marginally more than a single  $\log_{10}$  removal was observed within 180 minutes. Expanded bed matrices were also studied for their ability to bind DISC-HSV in batch adsorption (Figure 6.3). Unfortunately neither of the two matrices tested, Streamline heparin and Procep heparin, bound virus when compared to the negative control. A further study to examine the influence of Cellufine-sulphate gel loading upon the kinetics and extent of adsorption (Figure 6.4) indicates that the extent of virus adsorption scales with the gel loading, and hence the external gel surface area, with a capacity exceeding  $1.5 \pm 0.55 \times 10^9$  pfu/ml gel.

The variation in adsorption characteristics of the different heparin matrices is noticeable. It has been reported (Trybala *et al.*, 1996) that the attachment of Pseudorabies virus to cell monolayers is influenced to varying extents by the addition of exogenous porcine intestine mucosal heparan sulphate preparations with different degrees of sulfation, as well as by heparin oligomers of different chain lengths. The steric accessibility, distribution and density of sulphate groups are thus inferred to influence gC binding and might contribute to the low degree of virus attachment to Cellufine-heparin gel which contains a high molecular weight heparin fraction (130 - 200 kDa) bound to the matrix by a hydroxysuccinate spacer. By contrast, heparin-Sepharose and heparin-HP are both made from a low molecular weight heparin fraction. The inability of heparin-Sepharose to adsorb virus conflicts with its earlier

use for gC protein adsorption (Herold *et al.*, 1991) but may arise from the lower density of bound heparin for this matrix (3 mg heparin/ml heparin-Sepharose compared with 10 mg heparin/ml heparin-HP). Other factors such as steric influences due to the lack of a spacer moiety for the heparin coupling chemistry, and the lower accessible specific surface area of heparin-Sepharose matrix relative to heparin-HP (due to the larger mean particle size) may also influence binding.

## 6.2.2 Gradient elution profiles

### 6.2.2.1 Cellufine-Sulphate Matrix

Cellufine-sulphate was evaluated as a packed bed adsorbent. Salt released virus was diluted to 0.14 M NaCl and 360 mls of 5 $\mu$  filtered virus suspension were passed through a 15 ml column. The column was washed with 5 column volumes of PBS and eluted with a linear salt gradient (0.14 M to 2 M NaCl, phosphate buffered over 10 column volumes) (Figure 6.5). Sequential column volume fractions were collected and assayed for infectious virus, total protein and CR2-DNA as described in Chapter 2.

Figure 6.6 shows that the major part of the infectious virus elutes in fractions 7 and 8 and is clearly separated from the protein which is at its maximum in fraction 2 and the CR2-DNA in fraction 5.

Samples of all fractions were subjected to western blot analysis against the complementing cell CR2 protein and the HSV-2 proteins. Equal volume samples were loaded onto duplicate gels so that a direct comparison could be made. From the anti-CR2 blot (Figure 6.7) it can be seen that only a trace of complementing cell protein co-elutes with the virus in fraction 8. The anti-HSV-2 blot (Figure 6.8) shows large quantities of virus protein in the fractions preceding the infectious virus. This may originate from harvested non-infectious particles which might not exhibit the same binding characteristics to the matrix (see, for example, Huyghe *et al.*, 1994) who used the differential binding of Ad-5 infectious virus and non-infectious versions to ion-exchange matrices to infer P/I ratios).

#### *6.2.2.2 Heparin-HP Matrix*

The only other matrix tested that exhibited binding of DISC HSV in the batch adsorption experiments was the heparin-HP medium. To enable a comparison with Cellufine-sulphate, salt released virus was diluted to 0.14 M NaCl and loaded onto a pre-packed 5 ml heparin-HP column at a flow-rate of 1.3 ml/min. Approximately 300 ml of this suspension was fed to the column which was then washed with PBS and eluted with a gradient of 0.14 to 2M NaCl in phosphate buffer over 10 column volumes. Eluate from the column was continuously monitored for UV absorbance ( $A_{280}$ ), see Figure 6.9, and sequential 5 ml fractions were collected for analysis as before (Figure 6.10). The patterns of protein and DNA elution are similar to those of the gradient eluted Cellufine-sulphate matrix though the viable virus is now eluted at a lower salt concentration from the heparin-HP matrix (fraction 5 rather than fraction 8) and coincides with the peak DNA elution. In this case, the virus fraction is less well resolved from the peak contaminant fractions.

Western blots of the heparin-HP eluted fractions were conducted, as for the Cellufine-sulphate fractions, to examine if there was correspondingly greater amounts of both complementing cell and virus protein contamination due to the earlier elution of the viable virus. Photographs of the blots are shown in Figure 6.11 and Figure 6.12, which are similar to those for the Cellufine-sulphate fractions; the early total protein elution profile corresponds to that for CR2 protein while there are large quantities of HSV-2 protein in the fractions directly preceding the viable virus peak. Although the viable virus is eluted earlier, the anti-CR2 blot (Figure 6.12) suggests that there is little contaminating CR2 protein in this fraction.

#### *6.2.2.3 Treatment of Undiluted Harvest Material*

The above experiments demonstrate that DISC-HSV virus binds to certain polyanionic chromatography matrices and can be eluted with a salt gradient in a substantially purer form. However, we envisage practical problems with operating this process at scale due to the need to dilute the harvest material to 0.14 M NaCl prior to loading, which increases the overall process volumes and time for purification. Direct loading of clarified salt or heparin released virus was therefore tested.

A 5 ml Cellufine-sulphate column was loaded with salt released virus (phosphate buffered in 0.8 M NaCl). Following a wash step (0.7 M NaCl, 10 mM phosphate) the

column was eluted with a 0.7 - 2 M salt gradient (Figure 6.13). The UV absorbance of the column eluate was continuously monitored and sequential 5 ml elution fractions were collected for analysis as before (Figure 6.14). A higher proportion of non-absorbed protein was present in the column flow-through during loading (data not shown), corresponding to a much lower total protein concentration in the fractions preceding the virus and a somewhat lower specific protein contamination of the peak virus fraction (0.6 pg protein/pfu (Figure 6.14) compared with 2.4 pg protein/pfu (Figure 6.6)).

Heparin-HP matrix was similarly loaded with undiluted salt released virus and gradient eluted. Again the virus is able to bind in the presence of high salt (Figure 6.15) whereas the major part of the contaminating protein passes from the column in the flow-through. As with the diluted harvests, the virus is eluted from the heparin-HP matrix earlier in the gradient than from the Cellufine-sulphate matrix, but is now resolved from the CR2-DNA peak (Figure 6.16). This may suggest that co-purification of DNA in these experiments is not attributable to binding to virus.

High salt concentrations, such as those used for salt release of virus, disrupt CR2 cells and release cellular protein (see Table 6.2 and Table 6.4). The heparin release method, being isotonic, produces lower process stream contamination and therefore provides a cleaner starting material for chromatography. We therefore investigated the binding of virus to a heparin adsorbent directly from a heparin released virus suspension. Heparin released harvest was clarified and loaded directly onto a heparin-HP column. After washing with PBS the virus was eluted with a salt gradient (0.14 - 2 M NaCl) over 10 column volumes and collected in 10 sequential fractions (Figure 6.17) that were assayed for total protein and complementing cell DNA (Figure 6.18). Contrary to expectation the assay data show that the presence of heparin in the feed material at 50 µg/ml does not inhibit the binding of the virus to the heparin matrix. Little protein is eluted in the early eluate fractions as compared to the loading of diluted salt harvest material (Figure 6.9) and the protein level in all fractions are the lowest observed in this work. Unfortunately, peak DNA corresponds with the peak virus fraction.

### 6.2.3 Step elution profiles

The gradient elution of DISC-HSV2 from the chromatography matrices is a complex procedure to implement in manufacturing. Step elution is a more convenient procedure and was therefore evaluated. Virus was loaded onto Cellufine-sulphate or heparin-HP columns as above, a stringent wash step was conducted (using 0.7 M NaCl in 10 mM phosphate buffer) and virus was eluted using 1.5 M NaCl in 10 mM phosphate buffer.

To determine which harvest method and matrix would be most appropriate for the final process, Cellufine-sulphate and heparin-HP columns were loaded with diluted and undiluted salt released harvests. Table 6.1 and Table 6.3 summarise the pfu loaded/ml gel, the amount of virus absorbed and the proportions of infectious virus recovered. The results show that both matrices have a capacity of greater than  $10^9$  pfu/ml gel, and in the case of heparin-HP it was more than  $5 \times 10^9$  pfu/ml gel. In both cases the 0.7 M NaCl wash removed low, but acceptable levels of virus for a production process. In these studies the recoveries of virus was generally better for the heparin-HP matrix.

The data on purity of the eluted virus are summarised in Table 6.2 and Table 6.4. These results suggest that the heparin-HP medium had generally better purification characteristics than Cellufine-sulphate. For example, eluates from 0.8 M NaCl loaded virus had good recovery in both cases but four fold less protein was observed from the heparin-HP matrix. The levels of DNA observed in the eluate from the heparin-HP matrix were also low, especially for virus loaded in the presence of heparin. At 1.2 ng DNA/ $10^7$  pfu this is within the present limits accepted by the FDA. This should therefore overcome the need for a DNA digestion step, often included in virus purification processes ( Huyghe *et al.*, (1995)).

### 6.2.4 Comparison of Affinity purified DISC-HSV with previous methods

As described in the introduction, the previous method used to purify the DISC-HSV virus was either sucrose gradient centrifugation or tangential cross flow filtration.

These methods although good enough for production of material used in clinical trial were not suitable for full scale production. Sucrose gradient purification could not be used at the scale required for production and tangential flow filtration does not purify the virus to sufficient levels. The use of affinity chromatography has the advantage of being scalable.

Table 6.5 provides a brief summary of purity and virus recoveries obtained from the different processes discussed. Sucrose gradient and tangential cross flow data were obtained from Cantab Pharmaceuticals clinical trial production runs. Sucrose gradient preparation although producing virus with a low burden of protein had very poor recoveries of typically less than 10 percent. The other major drawback of this method is the need to scale up high speed centrifugation, which requires the commissioning of large industrial centrifuges which is time consuming and expensive. This method is also lengthy, requiring centrifugation over many hours, and is a batch process and therefore not flexible in the quantity of material to be processed.

The originally proposed scale up method of disruption, cross flow filtration followed by diafiltration also has its drawbacks. Although the recoveries were markedly better than the sucrose gradient preparations, at between 1 and 35 percent, they were not consistent and still low for a manufacturing process. For this method the removal of host cell DNA was accomplished with a DNA digestion enzyme, Benzonase™, to below the then upper level of 1 ng/dose. Where this method did not accomplish the manufacturing requirements was in removing host cell protein, which at about 230 µg/10<sup>7</sup> pfu, was too high a level for the final product. When these methods are compared to the proposed method of heparin release followed by heparin chromatography the advantages gained can be clearly seen. Recoveries of around 75 percent and protein levels down to 0.5 µg/10<sup>7</sup> pfu mean a purer product with a recovery level which would require an upstream production almost a tenth of the size of previously proposed systems to generate the same number of final doses. Another benefit of this method is the low level of contaminating host cell DNA, at 1.2 ng/10<sup>7</sup> pfu without the aid of a DNA digestion enzyme. The harvesting of the virus, not being lytic, does not release as much DNA; also DNA does not bind the heparin column since it is essentially a cation exchange column and therefore the DNA is washed away. Even if Benzonase™ use is to be included in the process, the heparin

chromatography should still be able to remove remaining traces of DNA to aid the polishing of the final product.

#### 6.2.5 Identification of the virus ligand responsible for binding in the heparin chromatography

For both the HSV1 and the HSV2 viruses, the two glycoproteins, gB and gC, have been observed to bind to cell surface heparan sulphate (Herold, 1994; Williams, 1997; Griffiths, 1998). No other herpes glycoprotein has been shown to have this affinity for heparan sulphate. It would therefore be reasonable to assume that one, or possibly both, of these molecules provide the strong affinity observed between the virus and the heparin chromatography matrix. In an attempt to provide evidence of this, chromatography was used as an analytical tool to isolate the protein.

The isolation of virus particle glycoproteins has been studied previously (Abidi, 1984). These methods rely on dissolving the viral membrane with detergents. There are many detergents that can be used but some can be discarded because of possible interference with the chromatography. Tween, for example, absorbs at 280 nm; therefore if this was used the chromatography of the proteins would be masked. Also ionic detergents cannot be used as these may change the loading and elution conditions of the proteins. The detergent octylglycoside was therefore chosen to disperse the virus membranes, and since it does not absorb at 280 nm it will not interfere with the chromatography of the samples as described below.

The approach taken to achieve this was to first to purify the DISC HSV on a heparin-HP column to obtain a pure starting material with as little interference from non specific proteins. Also since the virus was purified on this matrix, there must be ligand present on their surface that binds to heparin. Second, the purified virus was digested with a non-ionic detergent, 1% (v/v) octylglycoside, to dissolve the viral envelope whilst leaving envelope glycoproteins in their natural state (Hjelmeland, 1984; VanAken, 1986; Compton, 1993). Finally the digested virus preparation was passed through a new heparin-HP column using the same load, wash and elution

conditions as before. The eluted protein was then subjected to SDS-PAGE electrophoresis and then Western blotted using anti-HSV antibodies.

Figure 6.19 shows a coomassie stained SDS-PAGE gel of the isolated protein using octylglycoside as the detergent at 1 % final concentration. It can clearly be seen that the final column eluate contains a band at approximately 20 kDa which has been concentrated and purified from other viral and cellular components, as seen in the centrifugation pellet. Eluted virus from the initial column purification step can only just be seen due to the low protein concentration of the individual bands.

To further qualify the origin of this single band the same samples were tested by Western blot (see experimental) using sheep anti-HSV. The results (Figure 6.20) show two bands, one 20 kDa band seen in the coomassie stained gel and one at a higher molecular weight, 66 kDa, which did not appear on the coomassie stained gel. The explanation for this is probably that the western blot technique is a more sensitive assay than coomassie staining; therefore the extra band was present in the coomassie stained gel, but at too low a concentration to be visualised using this technique.

If this result is true then two proteins could be involved in the binding of the virus to the heparin-HP chromatography media. There are two glycoproteins that are possible candidates to bind to heparin, gB and gC. Glycoprotein C is the most probable protein to affect binding but reports (Herold *et al.* 1994) suggest that in its absence gB can still bind the virus to cell surface heparan sulphate, albeit at a lower efficiency. Both of these proteins therefore may have been purified in the process above. The hypothetical weights of these two proteins based on their DNA sequence is 100.2 kDa for gB and 51.7 kDa for gC. The higher molecular weight band observed on the western blot could possibly be gC. An explanation for the lower band is more elusive since at about 20 kDa it is too small to be any of the glycoproteins, but could be a degraded protein originally of a higher molecular weight. If this were the case then the rest of the protein did not show in the Western blot or it was part of the higher molecular weight band, which would mean that only one protein causes the virus to bind to the heparin-HP column.

An attempt was made to produce enough material for amino acid sequencing, but low concentrations of protein prevented successful results. Discovering the glycoprotein responsible for the binding can be of importance in selecting better chromatographic

matrices and working out how the virus binds to the column. This information could then be used to design better columns and improve recoveries and purities.

### **6.3 Conclusions**

The objective of purification is to deliver a final product with a high infectious virus titre characterised by complete clearance of cell particulates and minimal contamination with nucleic acids and proteins. In addition, we sought high titre eluate fractions to permit dilution and the addition of excipients for the manufacture of a formulated product.

We had previously observed that the heparin release procedure leads to a particularly clean feed stream due to the avoidance of cell lysis (Chapter 5, O'Keeffe *et al.*, 1998). This work showed that the heparin concentration required for virus release from cells does not impede the subsequent adsorption of virus onto the heparin-HP matrix. This unanticipated result may arise from competitive binding by the higher concentration of heparin at the matrix surface (the heparin-HP gel contains 10 mg heparin per ml gel compared with 50 µg heparin per ml in the harvest buffer). The observation is useful in permitting the direct processing of the heparin released material without prior conditioning.

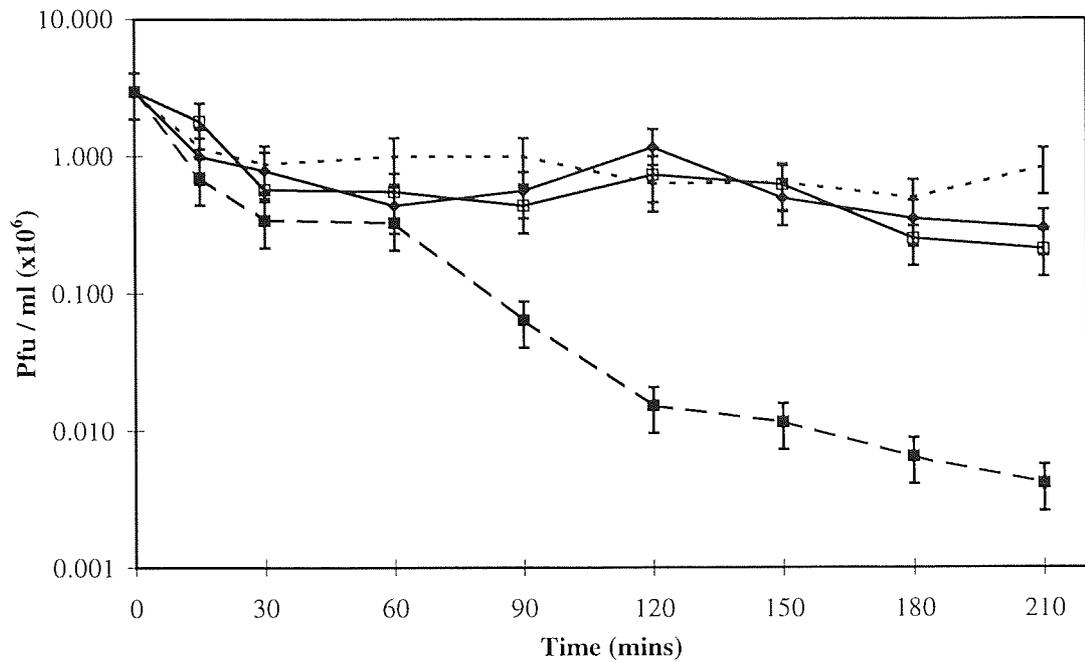
Regarding loading, the lower molality of viral suspensions as compared with typical protein solutions makes the need for high specific surface area adsorbents redundant. Indeed the relative impenetrability of HSV2 into the majority of pores in conventional gel matrices (diameter 30 - 400 nm) restricts virus adsorption to the outer surfaces of adsorbents. Simple packing estimates of the peak capacity for spherical, 120 nm diameter viruses (as HSV-2) on the external surface of heparin-HP adsorbent particles (diameter 34 µm) suggests that more than  $10^{13}$  pfu/ml could theoretically be accommodated as a monolayer. For the heparin-HP column with simple step elution (1.5 M NaCl) of heparin released virus, a total of  $5 \times 10^9$  pfu were recovered in the eluate, corresponding to a very low degree of virus breakthrough during loading and a substantially higher peak capacity.

Despite the satisfactory binding capacity of poly-sulphonated ligands, we were initially concerned that such macroporous matrices may be unduly affected by non-specific binding of contaminant proteins. Though the impenetrability of HSV2 reduces the specific surface area for virus adsorption, the effective surface area for non-specific adsorption of proteins is undiminished. This concern proved unfounded for the heparin-HP matrix (Table 6.4) since the eluted virus suspension contained only 0.05 pg protein/pfu and  $1.2 \times 10^{-4}$  pg DNA/pfu (corresponding to 1.2 ng DNA/dose for a typical vaccine dose of  $10^7$  pfu). The combination of a heparin harvest with heparin-HP chromatography therefore provides a substantially pure viral suspension that satisfies regulatory requirements on levels of contaminants with a single purification step and without the need for DNA digestion.

In comparing the purity of the virus purified by affinity chromatography to virus purified by either sucrose gradient or tangential flow filtration (Table 6.5), it can be clearly seen that this method produces higher purity product. Also the virus yield using this method is far greater than other methods, which reduces the cost of producing large batches of virus upstream to accommodate for low recoveries downstream.

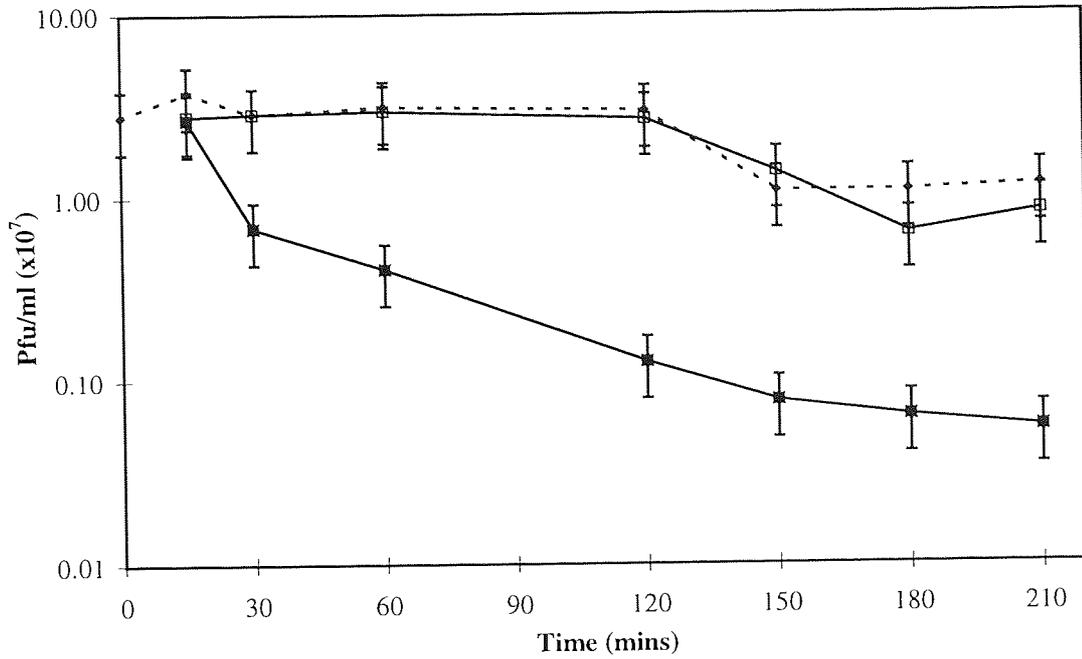
If the use of harvesting agents and chromatography matrices, which were originated from animals, becomes a problem then the alternative strategy of salt harvest followed by Cellufine-sulphate could be used in direct replacement, see Figure 6.21. Although this method does not have as high a purity as heparin harvest followed by heparin chromatography.

Finally, the method of purifying the proteins responsible for binding the virus to the chromatography media was successful in that it gave a clear picture as to two possible proteins that may be acting. The definitive answer was elusive but this went a long way to solving the problem of how to isolate the protein or proteins responsible.



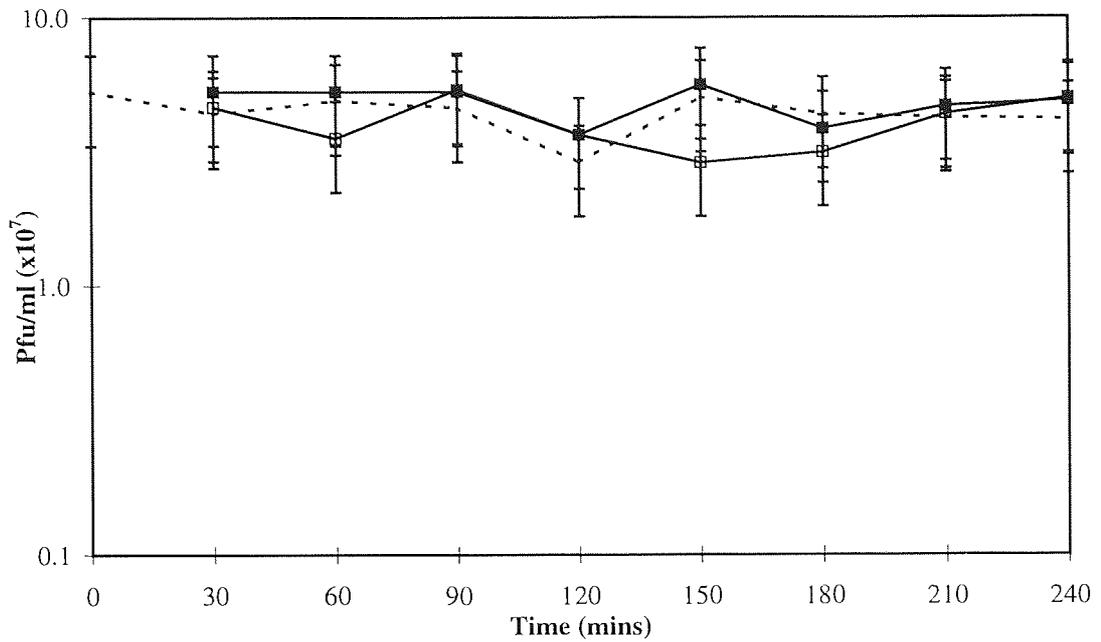
**Figure 6.1 Batch absorption of DISC-HSV to chromatography matrices.**

0.8M NaCl harvested virus was diluted with hypotonic saline to 0.14M NaCl and 25 ml was shaken, at room temperature, with 1 ml of each matrix. At each time point, 0.5 ml samples were taken after the matrices were pelleted by centrifugation, 400 rpm for 1 minute, then assayed for infectivity by TCID<sub>50</sub>. Negative control (short dashed line), Cellufine-sulphate (■), heparin-Sepharose (□) and Cellufine-heparin (◆).



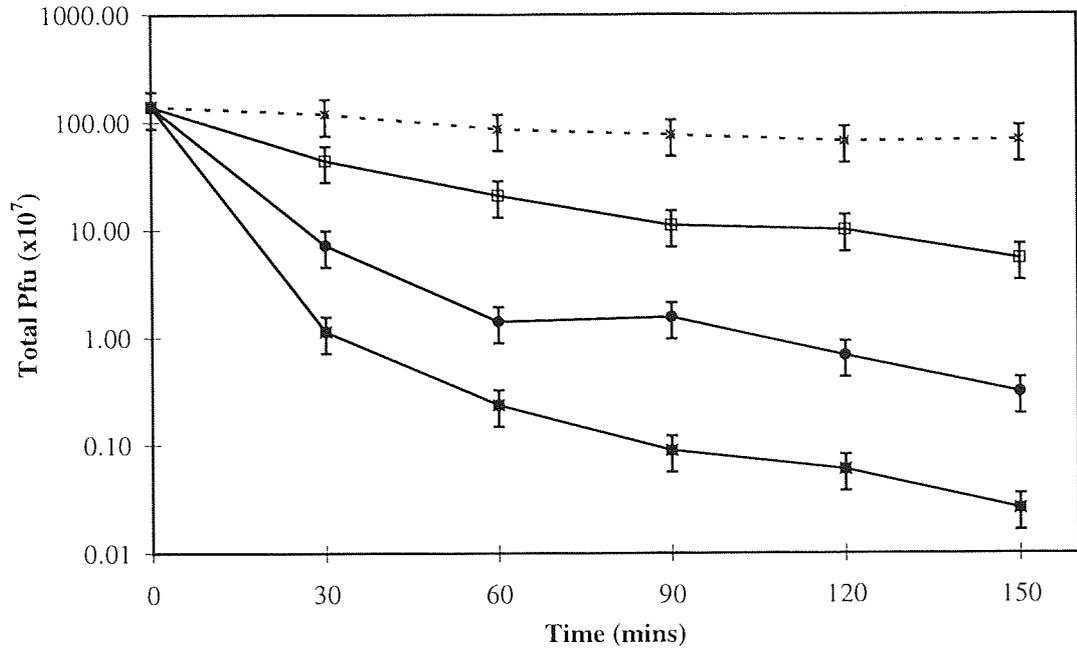
**Figure 6.2 Batch absorption of DISC-HSV to chromatography matrices**

0.8M NaCl harvested virus was diluted with hypotonic saline to 0.14M NaCl and 25 ml was shaken, at room temperature, with 1 ml of each matrix. At each time point, 0.5 ml samples were taken after the matrices were pelleted by centrifugation, 400 rpm for 1 minute, then assayed for infectivity by TCID<sub>50</sub>. Negative control (short dashed line), heparin-HP (■) and Cellufine-heparin (□).



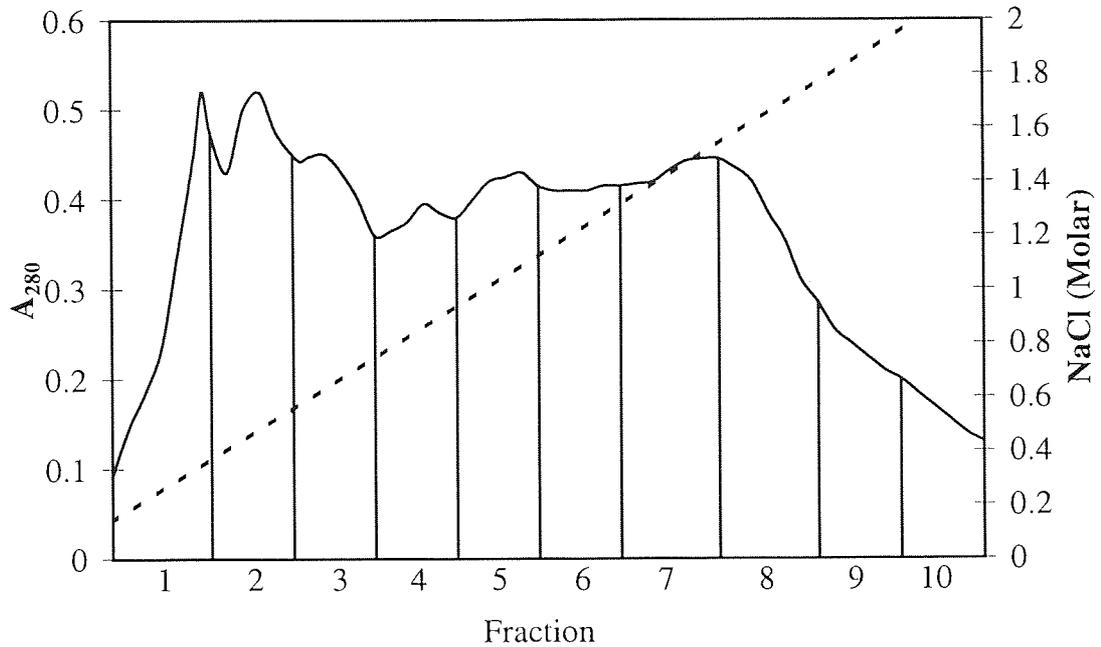
**Figure 6.3 Batch Adsorption of DISC-HSV to Expanded bed matrices**

0.8M NaCl harvested virus was diluted with hypotonic saline to 0.14M NaCl and 25 ml was shaken, at room temperature, with 1 ml of each matrix. At each time point, 0.5 ml samples were taken after the matrices were pelleted by centrifugation, 400 rpm for 1 minute, then assayed for infectivity by TCID<sub>50</sub>. Negative control (short dashed line), Procep Heparin (□), Streamline heparin (■).



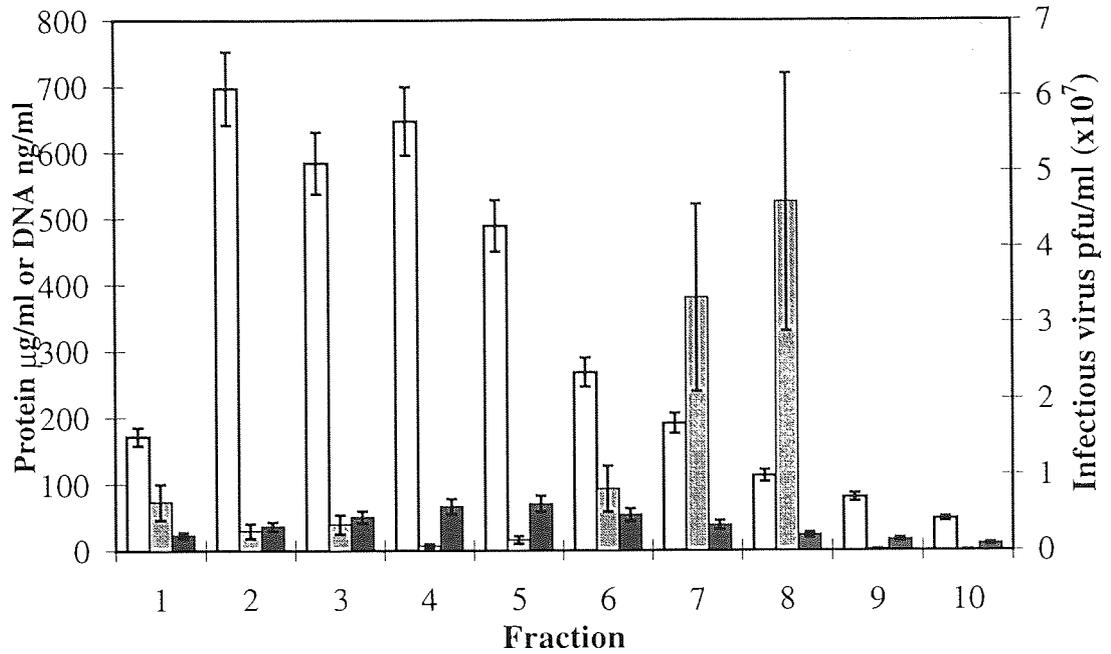
**Figure 6.4 Binding Kinetics of DISC-HSV to Cellufine sulphate**

0.8M NaCl harvested virus was diluted with hypotonic saline to 0.14M NaCl and 25 ml was shaken, at room temperature, with 1 ml (dashed line), 2 ml (□), 3 ml (●) and 4 ml (■) of cellufine sulphate matrix to investigate the kinetics of binding. At each time point, 0.5 ml samples were taken after the matrices were pelleted by centrifugation, 400 rpm for 1 minute, then assayed for infectivity by TCID<sub>50</sub>.



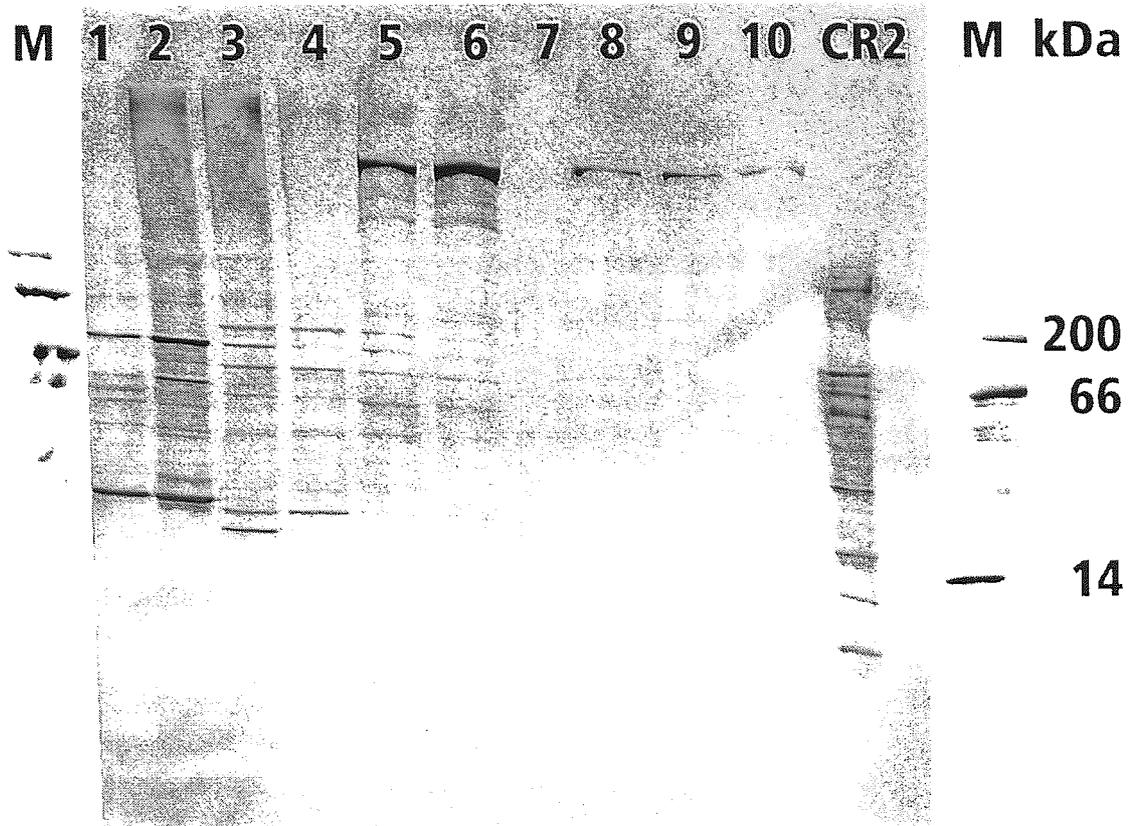
**Figure 6.5 Gradient elution of DISC-HSV from Cellufine-sulphate**

A 15 ml Cellufine-sulphate column was loaded with 360 ml of salt released virus diluted to 0.14 M NaCl with 10 mM phosphate, at 0.7 ml/min. Following washing with PBS the column was gradient eluted (0.14 M to 2 M NaCl, 10 mM phosphate). The  $A_{280}$  of the column eluate is drawn (solid line) with solid vertical lines indicating the collection of equal volume fractions 1 to 10. Also, the conductivity of the column eluate is drawn (dashed line) to indicate the salt molality of each fraction.



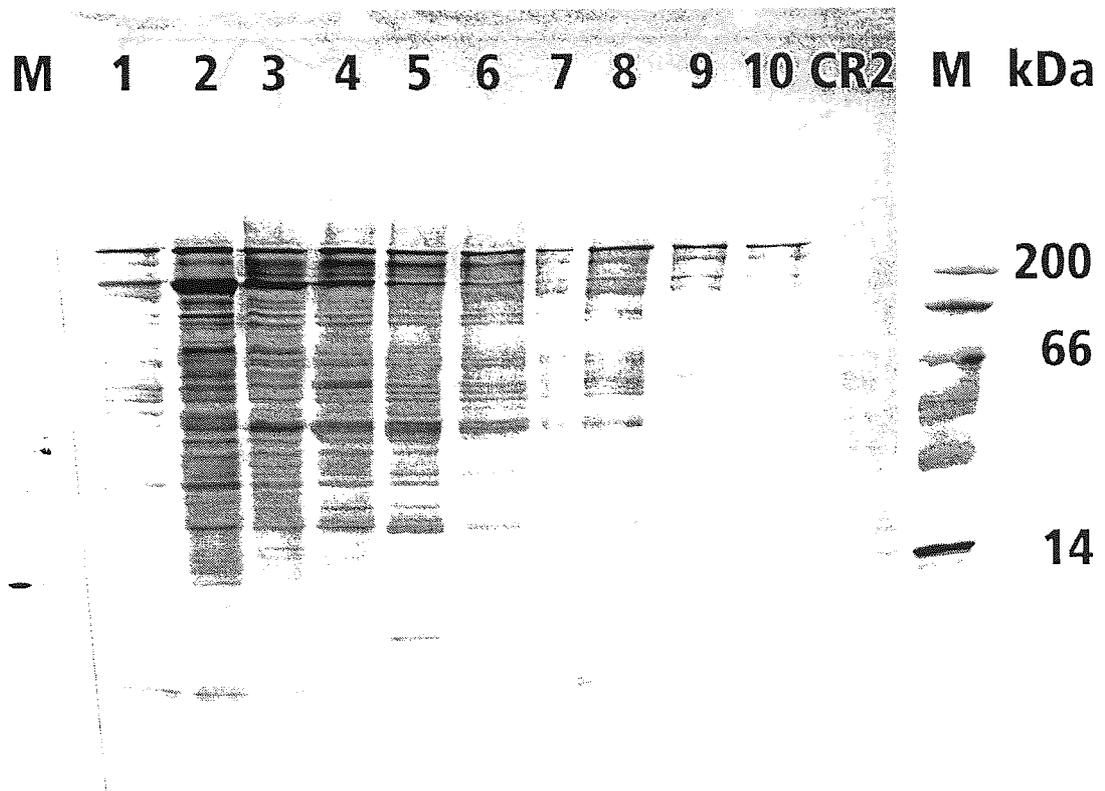
**Figure 6.6** Virus, protein and DNA assays for DISC-HSV gradient eluted from Cellufine sulphate.

A 15 ml Cellufine-sulphate column was loaded with 360 ml of salt released virus diluted to 0.14 M NaCl with 10 mM phosphate, at 0.7 ml/min. Following washing with PBS the column was gradient eluted (0.14 M to 2 M NaCl, 10 mM phosphate) see Figure 6.5. Each fraction was assayed for infectious virus (light grey bars), total protein (clear bars) and CR2-DNA (dark grey bars).



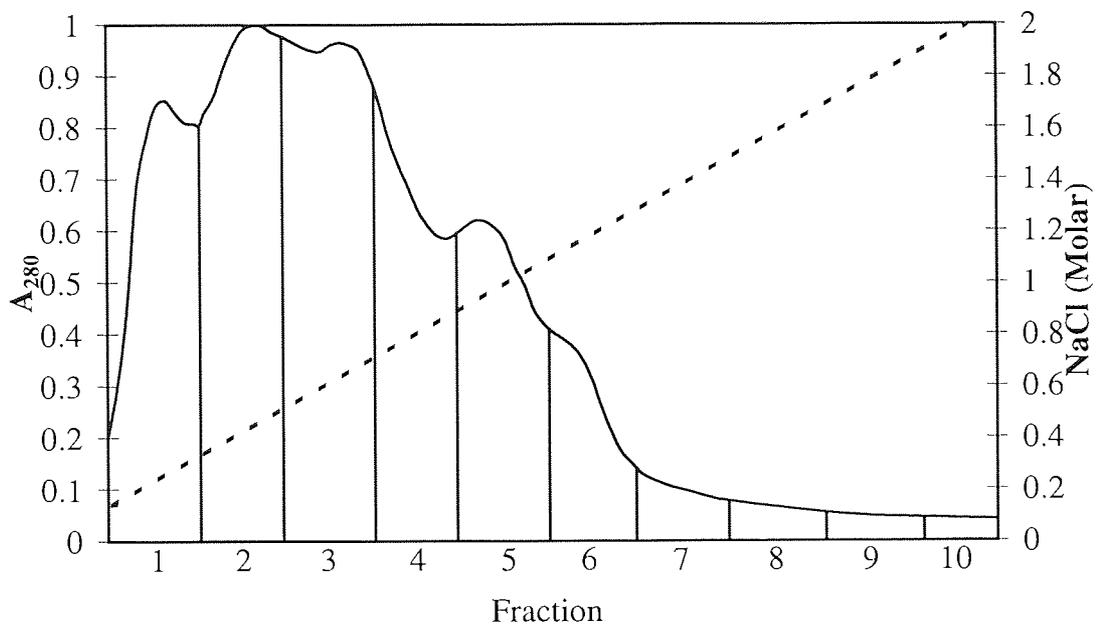
**Figure 6.7** Host cell protein western blot of DISC-HSV gradient eluted from Cellufine Sulphate.

Samples from fractions 1-10 (described in Figure 6.6) were run on SDS-PAGE gels, transferred to a nitrocellulose membrane then incubated with anti-CR2 antibodies as described in Methods. Lanes marked M are molecular weight markers (stained with Ponceau S stain) and lanes marked CR2 are non-infected CR2 cell lysates to determine the cross-reactivity of the antibodies. 1 to 10 represents the fractions collected from the gradient elution.



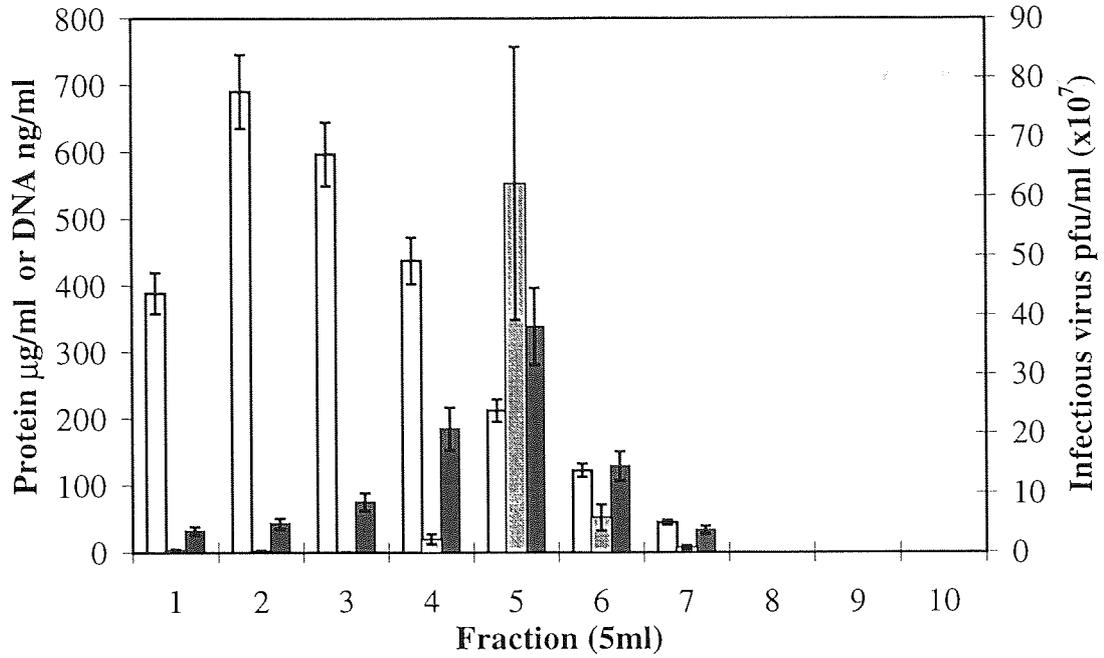
**Figure 6.8** Viral protein western blot of DISC-HSV gradient eluted from Cellufine Sulphate.

Samples from fractions 1-10 (described in Figure 6.6) were run on SDS-PAGE gels, transferred to a nitrocellulose membrane then incubated with anti-HSV2 antibodies as described in Methods. Lanes marked M are molecular weight markers (stained with Ponceau S stain) and lanes marked CR2 are non-infected CR2 cell lysates to determine the cross-reactivity of the antibodies. 1 to 10 represents the fractions collected from the gradient elution.



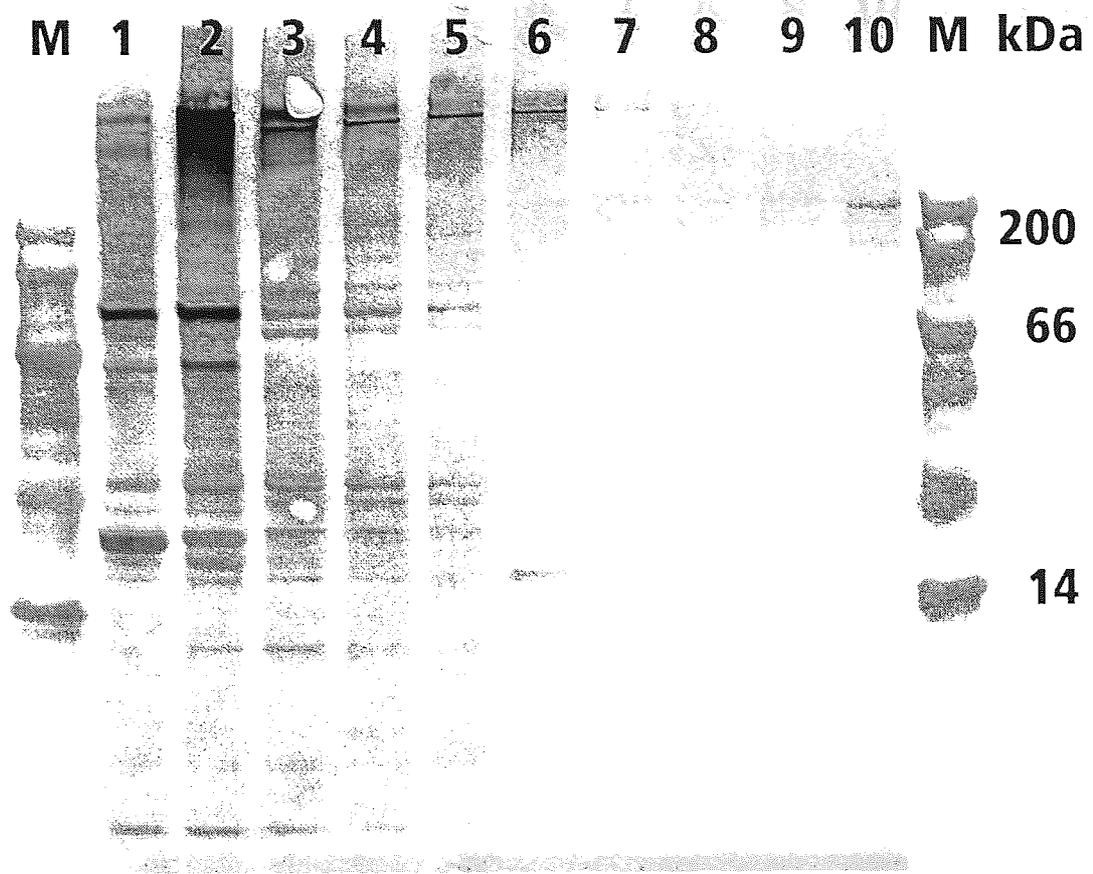
**Figure 6.9 Gradient elution of DISC-HSV from heparin-HP.**

A pre-packed 5 ml heparin-HP (HiTrap) column was loaded with 300 ml of salt harvested virus diluted to 0.14 M NaCl, with 10 mM phosphate, at 1.3 ml/min. Following washing with PBS, the column was gradient eluted (0.14 M - 2 M NaCl, 10 mM phosphate). The A<sub>280</sub> trace (solid line) of the gradient elution is drawn with vertical lines marking the collection of fractions. Also, the conductivity of the column eluate is drawn (dashed line) to indicate the molality of each fraction.



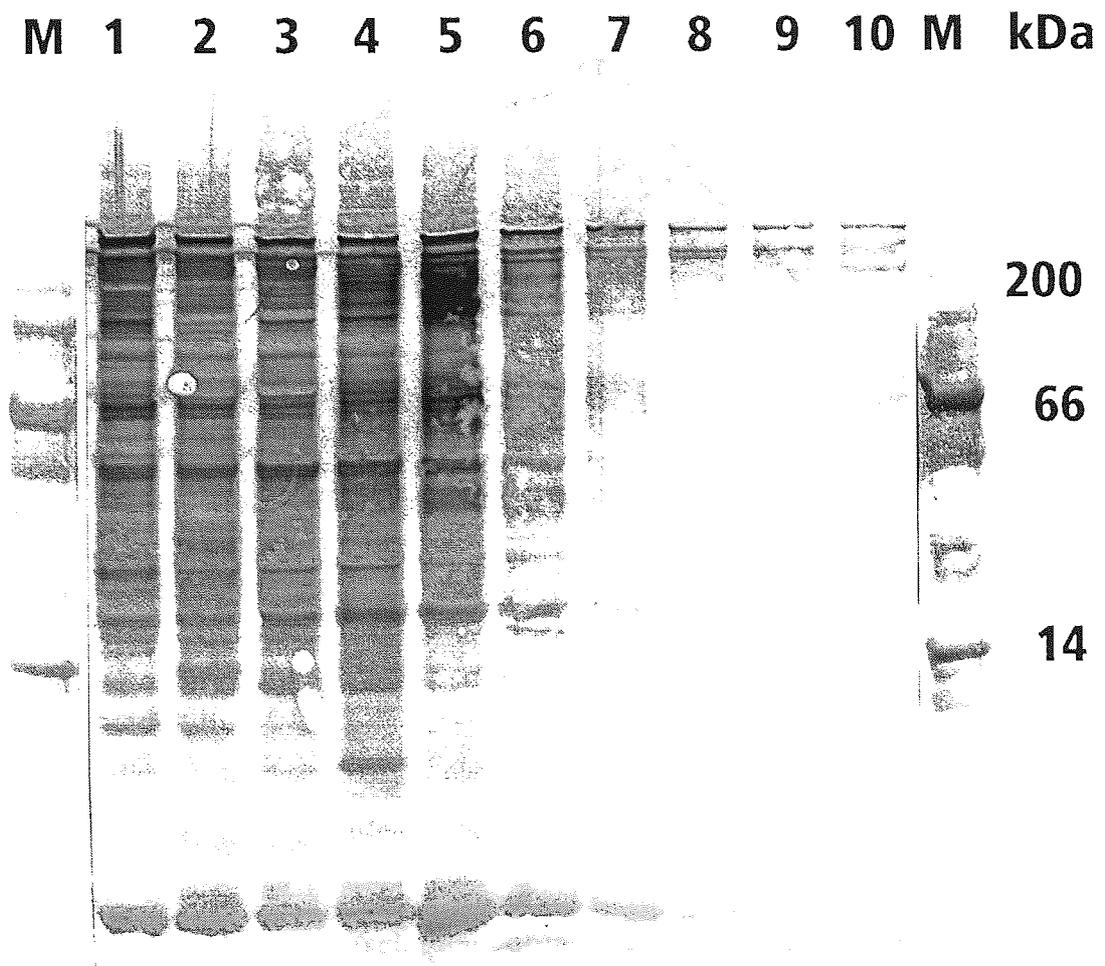
**Figure 6.10 Virus, protein and DNA assays for DISC-HSV gradient eluted from heparin-HP**

A pre-packed 5 ml heparin-HP (HiTrap) column was loaded with 300 ml of salt harvested virus diluted to 0.14 M NaCl, with 10 mM phosphate, at 1.3 ml/min. Following washing with PBS, the column was gradient eluted (0.14 M - 2 M NaCl, 10 mM phosphate). Each fraction was assayed for infectious virus (light grey bars), total protein (clear bars) and CR2-DNA (dark grey bars). Values are plotted for their respective fractions as shown in Figure 6.9.



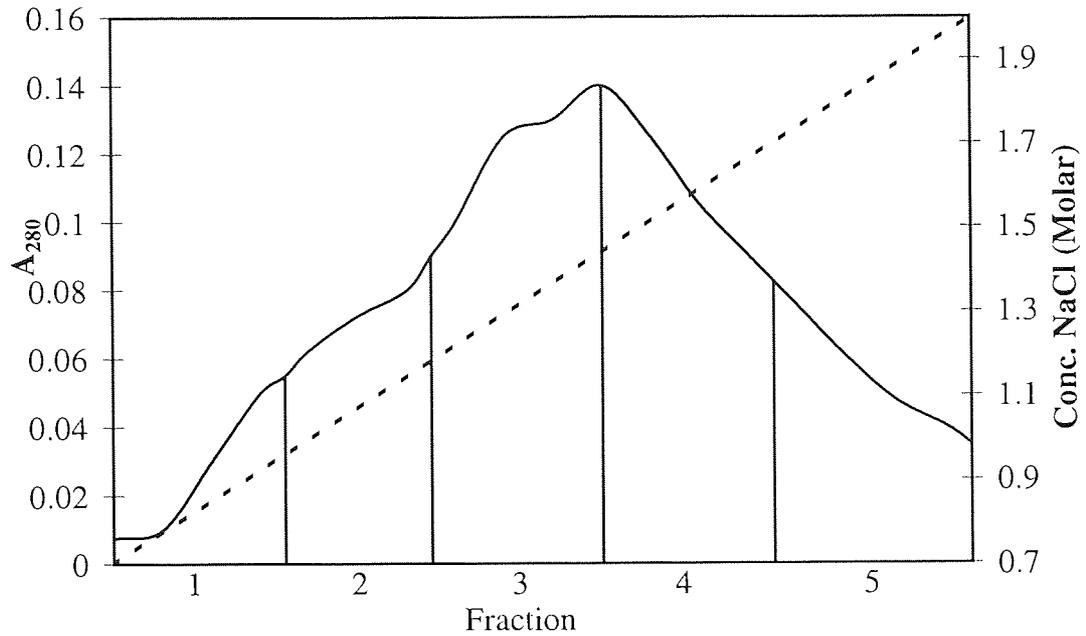
**Figure 6.11** Western blot analysis of eluates from heparin-HP column as shown in Figure 6.9

Samples of fractions 1-10 were reduced in beta-mercaptoethanol and 40  $\mu$ l of each sample run on duplicate SDS-PAGE gels, transferred to nitrocellulose membranes and incubated with anti-CR2 antibodies as described in Methods. Lanes marked M contain molecular weight markers, which were stained with Ponceau S stain. Lanes 1 to 10 represent the fractions collected from the gradient elution.



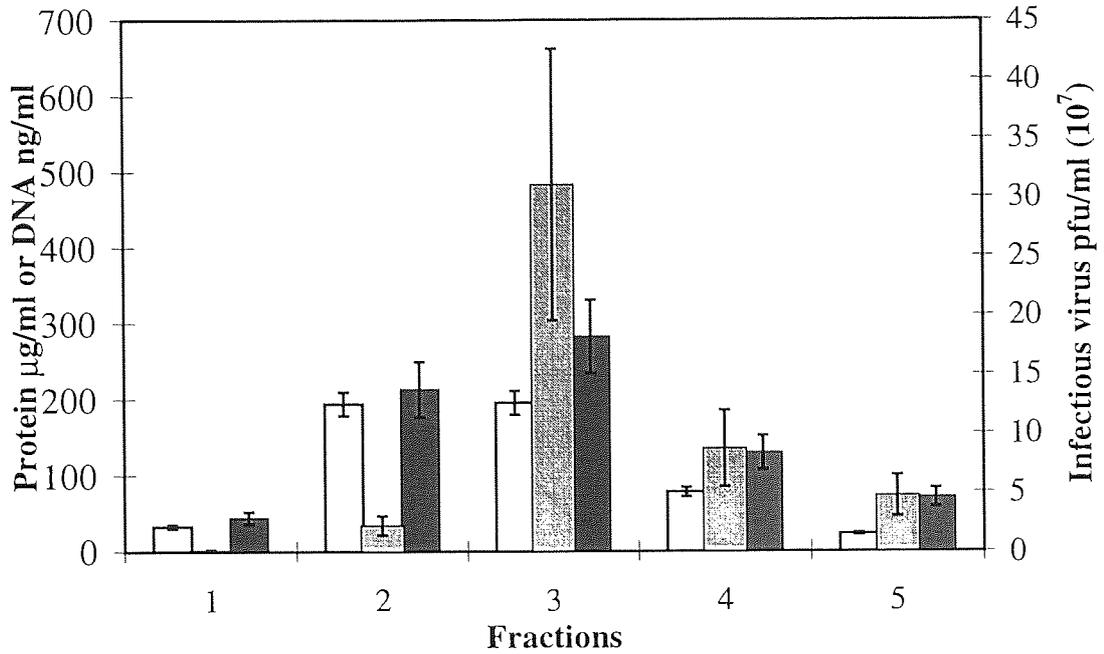
**Figure 6.12** Western blot analysis of eluates from heparin-HP column as shown in Figure 6.9

Samples of fractions 1-10 were reduced in beta-mercaptoethanol and 40  $\mu$ l of each sample run on duplicate SDS-PAGE gels, transferred to nitrocellulose membranes and incubated with anti-HSV antibodies as described in Methods. Lanes marked M contain molecular weight markers, which were stained with Ponceau S stain. Lanes 1 to 10 represent the fractions collected from the gradient elution.



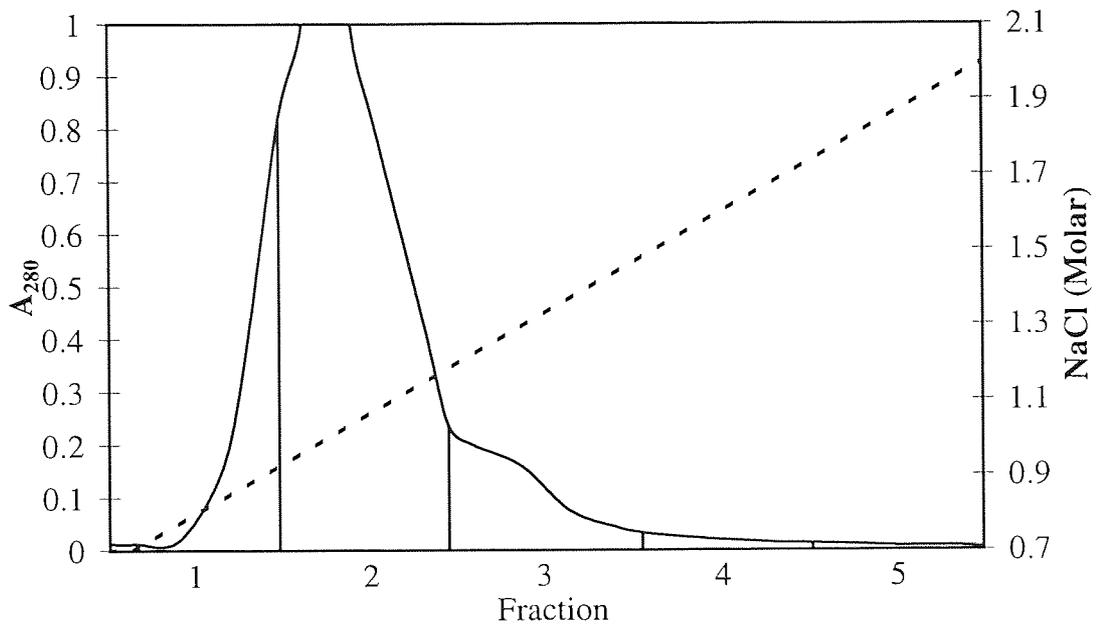
**Figure 6.13 Gradient elution of DISC-HSV, loaded in 0.8M NaCl, from Cellufine-sulphate**

A 5 ml Cellufine-sulphate column was loaded with 130 ml of undiluted salt released virus, 0.8 M NaCl, at 0.7 ml/min. Following washing with 0.7 M sodium chloride, 10 mM phosphate, the column was gradient eluted (0.7 M-2 M NaCl, 10 mM phosphate). The  $A_{280}$  trace (solid line) of the gradient elution is drawn with vertical lines marking the collection of fractions. The conductivity of the column eluate is shown (dashed line) to indicate the molality of each fraction.



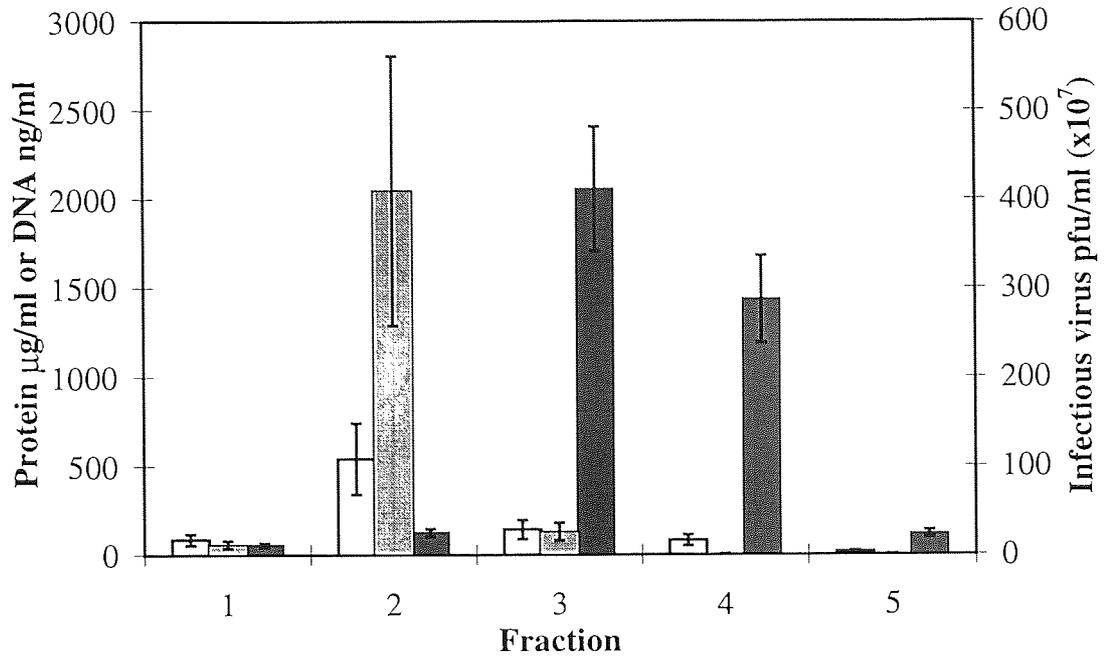
**Figure 6.14** Virus, protein and DNA assays for DISC-HSV, loaded in 0.8 M NaCl, gradient eluted from Cellufine-sulphate

A 5 ml Cellufine-sulphate column was loaded with 130 ml of undiluted salt released virus, 0.8 M NaCl, at 0.7 ml/min. Following washing with 0.7 M sodium chloride, 10 mM phosphate, the column was gradient eluted (0.7 M-2 M NaCl, 10 mM phosphate). Fractions shown in Figure 6.13 were assayed for infectious virus (light grey bars), total protein (clear bars) and CR2-DNA (dark grey bars).



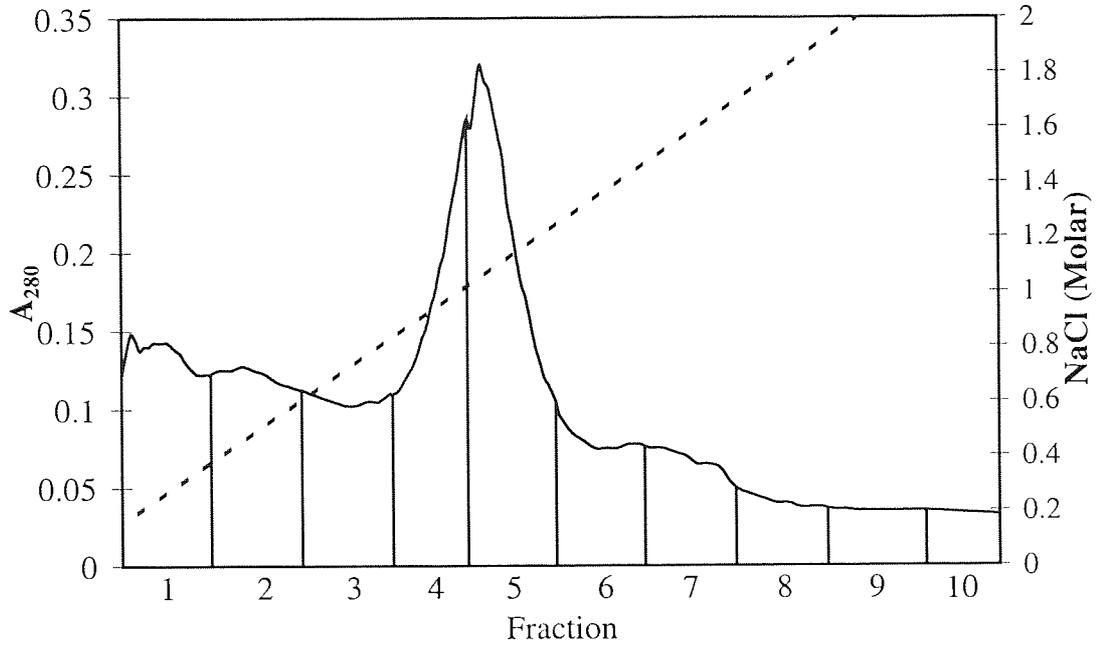
**Figure 6.15 Gradient elution of DISC-HSV, loaded in 0.8 M NaCl, from heparin-HP matrix.**

A pre-packed 5 ml heparin-HP (HiTrap) column was loaded with 150 ml of undiluted salt harvested virus, 0.8 M NaCl, at 0.7 ml/min. Following washing with 0.7 M sodium chloride, 10 mM phosphate, the column was gradient eluted (0.7 M-2 M NaCl, 10 mM phosphate). The A<sub>280</sub> trace (solid line) of the gradient elution is drawn with vertical lines marking the collection of fractions. The conductivity of the column eluate is shown (dashed line) to indicate the molality of each fraction.



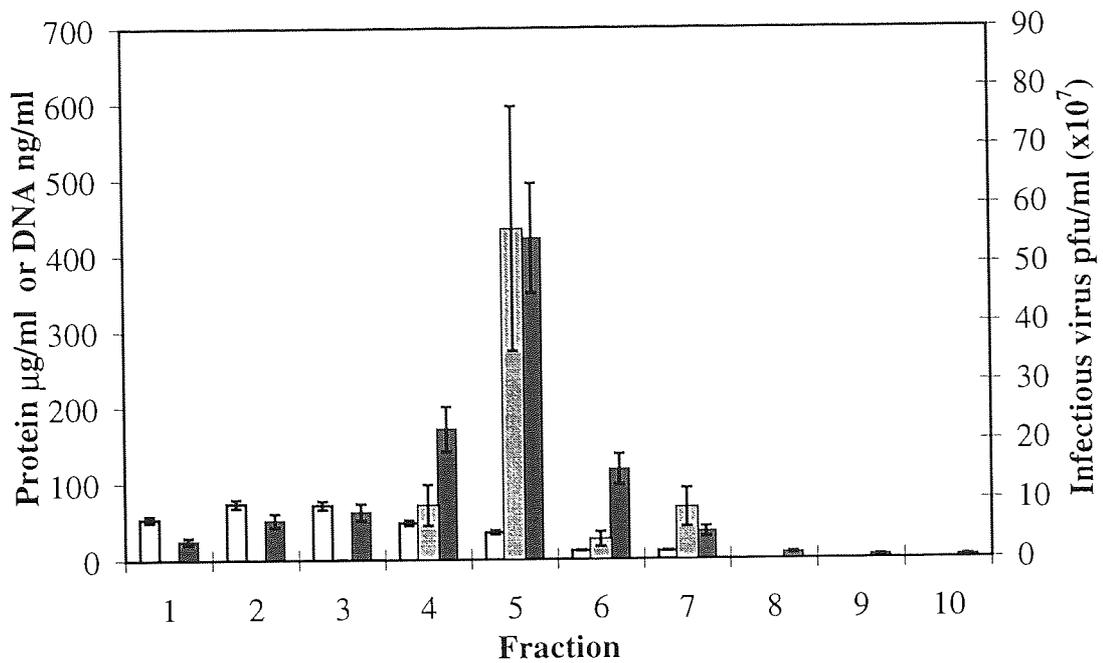
**Figure 6.16 Virus, protein and DNA assays of DISC-HSV, loaded in 0.8 M NaCl, gradient elution from heparin-HP.**

A pre-packed 5 ml heparin-HP (HiTrap) column was loaded with 150 ml of undiluted salt harvested virus, 0.8 M NaCl, at 0.7 ml/min. Following washing with 0.7 M sodium chloride, 10 mM phosphate, the column was gradient eluted (0.7 M-2 M NaCl, 10 mM phosphate). Fractions of the eluate, as shown in figure 6.15, were assayed for infectious virus (grey bars), total protein (clear bars) and CR2-DNA (chequered bars).



**Figure 6.17 Gradient elution of DISC-HSV, heparin harvested, from heparin-HP**

A pre-packed 5 ml heparin-HP (HiTrap) column was loaded with 140 ml of Heparin harvested virus, 50  $\mu\text{g/ml}$  heparin, at 0.7 ml/min. Following washing with PBS the column was gradient eluted (0.14M-2M NaCl, 10mM phosphate). Representation of the  $A_{280}$  trace (solid line) of the gradient elution with vertical lines marking the collection of fractions. Also a representation of the conductivity of the column eluate is drawn (dashed line) to give an indication of the molality of each fraction.



**Figure 6.18** Virus, protein and DNA assays for DISC-HSV, heparin harvested, gradient eluted from heparin-HP

A pre-packed 5ml heparin-HP (HiTrap) column was loaded with 140 ml of Heparin harvested virus, 50 µg/ml heparin, at 0.7 ml/min. Following washing with PBS the column was gradient eluted (0.14M-2M NaCl, 10mM phosphate). Fractions, as shown in figure 6.17, were assayed for virus (grey bars), total protein (clear bars) and CR2-DNA (chequed bars).

Virus Harvest Method	Virus loaded pfu/ml gel	Virus adsorbed pfu/ml gel	Percentage Recovered of Pfu Adsorbed	
			0.7 M NaCl	1.5 M NaCl
Salt diluted to 0.14M NaCl	$2.4 \pm 0.9 \times 10^8$	$2.4 \pm 0.9 \times 10^8$	$4.8 \pm 32$	$21 \pm 28$
Salt	$3.6 \pm 1.3 \times 10^9$	$1.6 \pm 0.6 \times 10^9$	-	$72 \pm 28$
Heparin	$7.0 \pm 1.3 \times 10^8$	$6.25 \pm 2.25 \times 10^8$	$0.03 \pm 15$	$25 \pm 22$

**Table 6.1 Loading capacities and recoveries for DISC-HSV loaded onto Cellufine-sulphate matrix.**

Virus harvest method indicates the load material following clarification by centrifugation and 5µm filtration, as in the methods. Columns were packed to the same bed height as the pre-packed Heparin HiTrap (2.5cm), linear velocity was 3.5mm/min. Where the absorbed pfu is equal to the loaded pfu, breakthrough has not been reached and therefore is not an indication of maximum capacity. Errors for virus titre are typical for the TCID<sub>50</sub> assay.

Virus Harvest Method	Loaded Material		1.5 M NaCl Eluate	
	$\mu\text{g protein} / 10^7 \text{ pfu}$	$\text{ng DNA} / 10^7 \text{ pfu}$	$\mu\text{g protein} / 10^7 \text{ pfu}$	$\text{ng DNA} / 10^7 \text{ pfu}$
Salt diluted to 0.14M NaCl	65.8±11.2	11.6±3.1	24.5±4.2	5.14±1.4
Salt	34.1±5.8	11.74±3.2	5.2±0.9	3.12±0.8
Heparin	15.3±2.6	17.06±4.6	2.7±0.5	4.64±1.3

**Table 6.2 Purity of DISC HSV virus eluted from Cellufine-sulphate matrix**

Samples described in Table 6.1 were assayed for total protein and host cell DNA (see methods) and specific purity calculated using values for virus titre given in Table 6.1. Errors given are the cumulative errors of a typical TCID<sub>50</sub> assay and protein or DNA assay.

Virus Harvest Method	Virus loaded pfu/ml gel	Virus absorbed pfu/ml gel	Percentage Recovered of Pfu adsorbed	
			0.7 M NaCl	1.5 M NaCl
Salt diluted to 0.14M NaCl	3.3±1.2 x10 <sup>9</sup>	2.8±1.1 x10 <sup>9</sup>	2.2±21	89.3±24
Salt	1.8±0.7 x10 <sup>9</sup>	1.8±0.7 x10 <sup>9</sup>	-	100±77
Heparin	5±1.8 x10 <sup>9</sup>	5±1.8 x10 <sup>9</sup>	1.1±15	90.8±20

**Table 6.3 Loading capacities and recoveries for DISC HSV loaded onto heparin-HP matrix**

Virus harvest method indicates the load material following clarification by centrifugation and 5µm filtration, as in the methods. All chromatography was using pre-packed Heparin HiTrap columns, flow rates were 0.7 ml/min (3.5mm/min). Pfu absorbed was calculated by subtracting the titre of the flow through from the total loaded; eluted fractions were collected by monitoring the A<sub>280</sub> and collecting whole peaks. Where the absorbed pfu is equal to the loaded pfu, breakthrough has not been reached and therefore is not an indication of maximum capacity. Errors for virus titre are typical for the TCID<sub>50</sub> assay.

Virus Harvest Method	Loaded Material		1.5 M NaCl Eluate	
	$\mu\text{g protein} / 10^7 \text{pfu}$	$\text{ng DNA} / 10^7 \text{pfu}$	$\mu\text{g protein} / 10^7 \text{pfu}$	$\text{ng DNA} / 10^7 \text{pfu}$
Salt diluted to 0.14M NaCl	18.2 $\pm$ 3.3	45.9 $\pm$ 12.4	3.9 $\pm$ 1.0	4.38 $\pm$ 1.18
Salt	86.2 $\pm$ 15.5	43.1 $\pm$ 11.6	1.3 $\pm$ 0.2	4.85 $\pm$ 1.8
Heparin	4.3 $\pm$ 0.8	5.91 $\pm$ 1.6	0.46 $\pm$ 0.08	1.2 $\pm$ 0.3

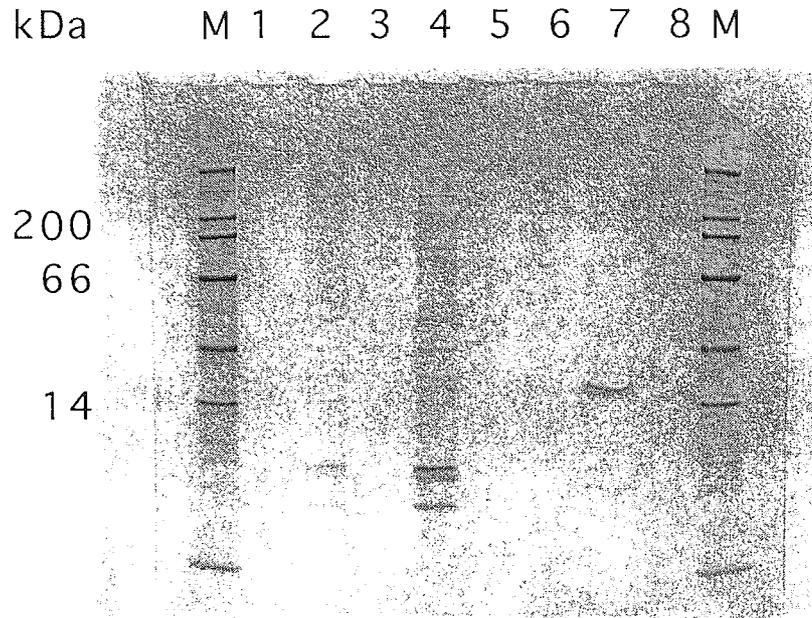
**Table 6.4 Purity of DISC-HSV virus eluted from Heparin -HP matrix**

Samples described in Table 6.3 were assayed for total protein and host cell DNA (see methods) and specific purity calculated using values for virus titre given in Table 6.3. Errors given are the cumulative errors of a typical TCID<sub>50</sub> assay and protein or DNA assay.

<b>Purification Method</b>	<b>Typical final titre (x10<sup>7</sup>) pfu/ml</b>	<b>Typical percent recovery</b>	<b>Typical <math>\mu</math>g protein /10<sup>7</sup> pfu</b>	<b>Typical ng DNA /10<sup>7</sup> pfu</b>
<b>Sucrose Gradient</b>	<b>0.55±0.2</b>	<b>&lt;10</b>	<b>6.5±1.1</b>	<b>2.4±0.6</b>
<b>Bionebulisation* filtration diafiltration</b>	<b>5±2.0</b>	<b>1-35</b>	<b>230±40</b>	<b>0.63±0.16</b>
<b>Heparin release Heparin Chromatography</b>	<b>1000±370</b>	<b>75±20</b>	<b>0.5±0.1</b>	<b>1.2±0.3</b>

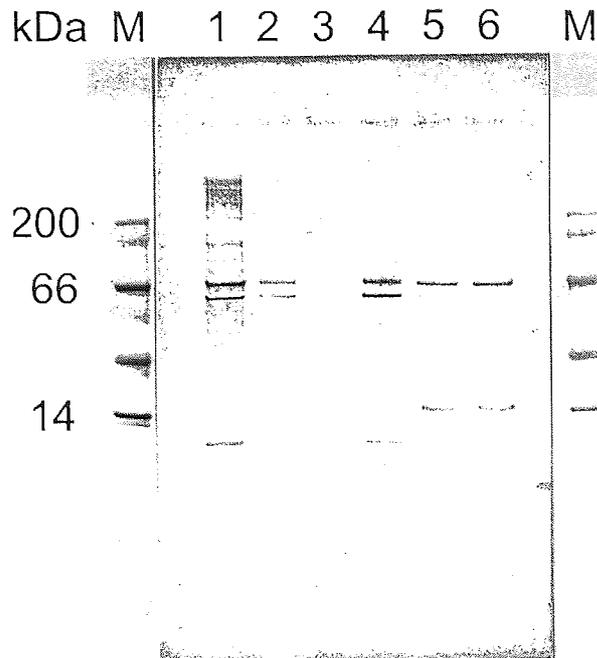
**Table 6.5 Comparison of DISC-HSV recovery and purity between processes**

Purification method describes the method by which the virus was purified. Results for sucrose gradient purification, as described in the introduction, were obtained from Cantab. Bionebulisation filtration diafiltration was the original scale up method developed by Cantab Pharmaceuticals. Data again was obtained from production runs carried out at Cantab Pharmaceuticals. Hypotonic saline harvested virus was filtered using a 0.8  $\mu$ m filter, then DNA digested with Benzonase<sup>TM</sup> endonuclease. Following a diafiltration step with PBS, the virus was formulated in human serum albumin and frozen at -80°C. Heparin release heparin chromatography was virus harvested using the heparin method and loaded onto a 5 ml pre-packed heparin-HP column, following clarification with a 5  $\mu$ m filter, then eluted with 1.5 M NaCl following a wash step of 0.7 M NaCl.



**Figure 6.19** Coomassie stained SDS-PAGE gel of purified viral proteins.

DISC HSV harvested with 0.8 M NaCl and purified on a 5 ml heparin-HP column (lane 2) was digested with octylglucoside (lane 3). Viral capsids were removed by centrifugation at 100,000 g for 1 hour (lane 4). Supernatant (lane 5) was then reloaded onto a 1 ml heparin-HP column, washed with 0.7 M NaCl (lane 6) then eluted with 1.5 M NaCl (lane 7). A single band appears in the 1.5 M NaCl eluate at about 20 kDa which has been concentrated from the supernatant and is probably the protein which binds the virus to the heparin column.



**Figure 6.20** Viral protein western blot of digested and purified DISC HSV.

DISC HSV harvested with 0.8 M NaCl and purified on a 5 ml heparin-HP column (lane 1) was digested with octylglycoside (lane 2). Viral capsids were removed by centrifugation at 100,000 *g* for 1 hour (lane 3). Supernatant (lane 4) was then reloaded onto a 1 ml heparin-HP column, washed with 0.7 M NaCl (lane 5) then eluted with 1.5 M NaCl (lane 6). Lack of protein in the viral capsid pellet was due to pellet being hard to dissolve. Two bands appear in both the 0.7 M and the 1.5 M NaCl washes, one at 66 kDa and one at 20 kDa.

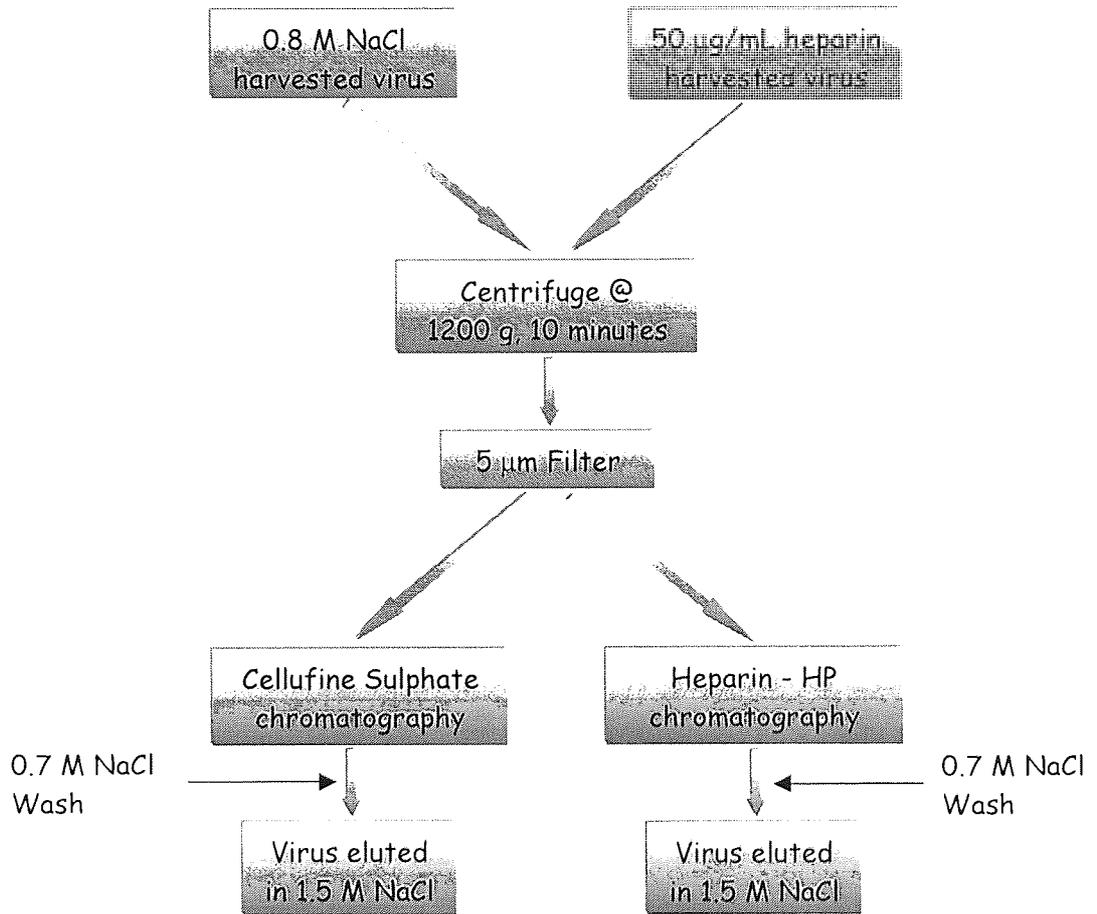


Figure 6.21 Flow diagram of virus purification using either salt or heparin harvesting methods

## 7 General Conclusions and future Work

It was clear from the beginning of this work that standard laboratory methods for growing cells, production of virus and its purification were not going to be practicable for a large scale process. Although the initial growth of cells and infection parameters were conducted in flat flasks, it was not intended for this to be used in the final process. Work conducted at Cantab Pharmaceuticals was already investigating the potential for using a microcarrier system for growing cells and production of DISC HSV (Zecchini *et al.* 1999). Instead of repeating work being conducted, it was decided to use the flask system for the small scale investigation of maximal productivity of CR2 cells for DISC HSV. This would also enable a quick passage and infection regime to be set up to produce sufficient virus for the studies of harvest and purification techniques. Results suggested that a range of conditions could be used to grow and infect cells but the regime chosen gave a passage and infection cycle that could be accommodated in a week and was robust enough to give consistent harvest titres. This regime did not concentrate on the requirements of scale up since other factors at scale become important. Passaging cells would be a trade off between time taken to reach the required cell density and cost of more frequent passages. Similar arguments can be made for the infection and harvest regimes. Infecting at a high MOI may yield slightly higher titres in slightly less time but would require the manufacture of large stocks of virus. The parameters that were chosen for the studies of harvest methods would therefore not necessarily be used in the large scale production of the virus.

Conventional methods of harvesting cell associated virus relied on the recovery of infected cells and their rupture to release infectious particles. A range of these methods was studied to find out their effectiveness on harvesting the DISC HSV virus. Conclusions were quickly drawn that none of these methods were particularly useful. It was important at this stage not just to find a method that released lots of infectious virus, such as sonication, but also to establish a method that would enable efficient purification. The contaminant load that sonication produced resulted in a low

purity harvest and recovery of high yields in purification would be difficult. Another aspect of a successful harvest method that is crucial is the ability of the method to be scaled-up. Although these investigations were conducted on virus grown in flasks a successful method would be required to work on virus grown in a bioreactor. Methods such as scraping of infected cells would therefore be unusable and Dounce homogenisation would be difficult due to the microcarrier content of the harvested cells. These considerations narrowed the list of existing methods that could be tested; even methods such as freeze thaw on a large scale would be time consuming and expensive. For these reasons a novel method of harvesting cell associated virus was sought, which led to the investigation of heparin as a potential agent that may separate virus from cells.

The use of heparin in virus binding studies led to the hypothesis that virus may be associating with cell surface heparan sulphate. There has been no previous work investigating the dissociation of virus from cells during harvesting, only during the initial infection stage where virus can be removed from cells after they have attached and before they have entered the cells. The results in Chapter 5 demonstrate the effective separation of infectious DISC HSV from CR2 cells as a novel method of harvesting cell associated virus. Since heparin was used at low concentrations and in an isotonic solution the levels of contaminants were low; yields were also very high and comparable to sonication. One of the main advantages of this method was the ability to use it on cells cultured in bioreactors and it is therefore scaleable. It also does not require the commissioning of large scale-up apparatus, since it is a chemical method, which makes the process of scale-up cost effective. It has also been demonstrated that dextran sulphate, a synthetic analogue of heparin, can be used to the same effect if heparin is not a suitable material to use due to its animal origin, giving a method that can be used for pharmaceutical manufacture.

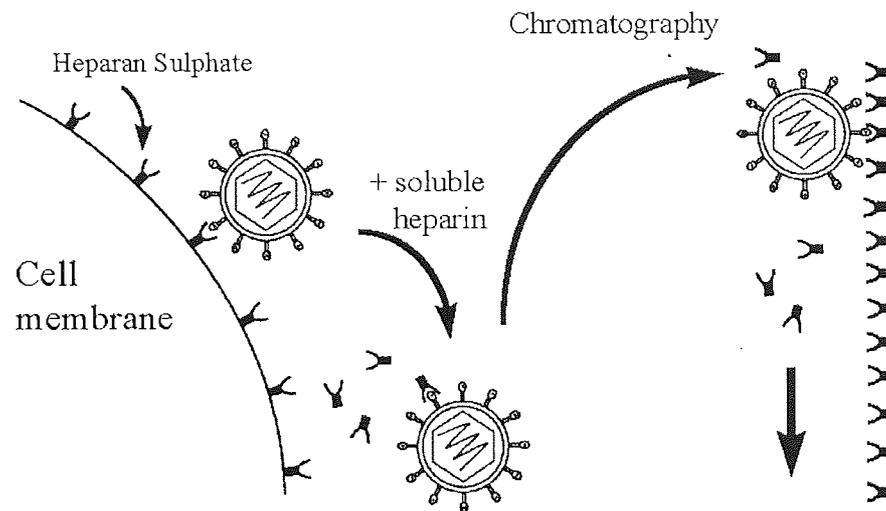
An alternative strategy for harvesting virus was based on this interaction between heparin and the virus. Heparin is a highly charged molecule, possessing approximately 1.5 sulphate groups per sugar unit and the smallest molecules are approximately 30 sugar units long. This makes the molecule negatively charged and

for an interaction to occur the virus must have positively charged regions. Although the interaction may be affinity based, it could not occur if charge were not opposite. A high concentration of sodium chloride was therefore used to separate the virus from the cells and was found to be successful. High salt concentrations had not been used to harvest virus before and had the benefit over the use of heparin or analogues of not being an extra ingredient in the harvesting buffer; this procedure was patented as a novel method of harvesting cell associated herpes viruses (Johnson *et al.* 1997). Both this and the heparin method are being used to harvest DISC HSV from bioreactors at Cantab Pharmaceuticals.

The breakthrough in the harvesting of the virus did not necessarily mean it could be purified. Again the relationship between virus and cell surface heparan sulphate was exploited in the investigation of affinity chromatography as a potential purification process (O’Keeffe *et al.* 1999 and Chapter 6). Various types of heparin matrices were tested for their ability to bind the DISC HSV virus and two different gels were chosen for further investigation, heparin-HP media (Pharmacia) and Cellufine Sulphate (Millipore). Heparin-HP has low molecular weight heparin attached to the surface of agarose beads and Cellufine Sulphate is a carbohydrate based gel and has been activated by sulphating sugar residues making it an analogue of heparin. It was not only discovered that DISC HSV bound to these matrices to a sufficient capacity for a manufacturing process but that the levels of heparin and salt used in the harvesting of the virus were low enough not to interfere with the virus binding to the matrices. Indeed the levels of heparin and salt actually increased the capacity since they prevented non and low specific binding of contaminants, therefore leaving more available ligand for the virus to bind to (see Figure 7.1).

The overall process is therefore vastly different from any pre-existing purification process for herpes viruses. The final specific purity of the affinity purified virus is at least as good or better than virus purified by gradient centrifugation with far greater yields. Other manufacturing scale virus purification processes, reviewed in the introduction, rely on more traditional harvest methods and therefore have more steps involved in the removal of contaminants before polishing steps can be used.

## Schematic representation of virus purification process



**Figure 7.1** Schematic representation of DISC HSV purification process.

*Cell associated DISC HSV can be dissociated with 50 µg/ml heparin in isotonic buffer. Resultant harvest is passed through a heparin-HP column, virus binds and non specific protein passes through. Virus can then be eluted with a salt solution above 1.5 M.*

Although a protein was purified in this study, the knowledge of which of the virus glycoproteins is responsible for the attachment of the virus to the chromatography matrices is still elusive. The technique of digesting the lipid envelope and removing the virus capsids by centrifugation prior to chromatography did work but not enough protein could be purified to enable amino acid sequencing to be conducted.

## **7.1 Further Work**

The process described was mainly conducted at small scale in the laboratory since the production of virus could not be increased at the time this work was conducted. Therefore chromatography was not conducted in greater than 5 ml columns due to the high capacity of the matrix studied. There is a need therefore to scale this chromatography to that required for manufacturing. Flow rates, column dimensions and recoveries of infectious virus all need to be investigated in larger columns.

For this process to be used a more detailed contaminant profile needs to be generated. Here only total protein, CR2 DNA and infectious virus have been studied. Contaminants such as bovine serum albumin (a marker for FBS) and host cell protein need to be studied in case this process does not effect their removal.

The virus in this study was mainly grown in roller bottle cultures and all harvest were conducted in this vessel. The use of microcarriers for the manufacturing process requires the harvesting methods to be optimised in stirred tank vessels. Work on this has recently been conducted with results that closely match those gained from the roller bottle cultures (Wright et al. 1998).

The understanding of the specific interaction between herpes virus and the chromatography resins would provide information on why some resins do not bind the DISC HSV virus. This information would prove useful if different matrices needed to be selected for a manufacturing process.

To complete a manufacturing process the final stage would be to investigate formulation of the virus in order to maintain infectivity at a suitable storage temperature i.e. 4-8°C. Although it has been demonstrated that the virus is stable at these temperatures for days to weeks, the product would have to be stable for far longer time to allow the production of stocks to avoid not keeping production up with demand. Maintaining the stability of the virus would require the investigation of compounds that would prevent the degradation of the viral membrane as well as the surface glycoproteins. Selection of compounds must also take account of whether

they have a direct effect on the virus and whether this will be detrimental to infection. There are a number of possible options as to how the product can be stored. As discussed earlier, holding the product at  $-80^{\circ}\text{C}$  is not viable since this will require clinics to install expensive equipment and also shipping of large quantities of material at this low temperature will be impractical.

If the product was kept at temperatures above  $0^{\circ}\text{C}$  it can either be stored wet or dry. An ideal formulation would be wet and held at  $+2-8^{\circ}\text{C}$ , but wet formulations of viruses have not been reported in the literature. The most common method employed to increase the stability of viruses is lyophilization. Lyophilization is the process of removing water from a sample by sublimation. This is achieved by using low temperatures, to freeze the sample, then gradually increasing the temperature whilst under vacuum to sublime off the water. Stability of greater than two years has been achieved using this method for a Varicella-Zoster virus vaccine (Fang et al. 1996). Also Marburg virus (Frolov et al., 1996) and Yellow fever virus vaccine (Monath 1996) have been demonstrated to have stability on freeze drying. There are many considerations that need to be addressed when investigating potential preserving compounds for freeze drying. For example maintaining pH, isotonicity, size of water crystals during freezing and phase partitioning during freezing. It is common to approach formulation for freeze drying in an empirical fashion. This involves investigating a large variety of preparations that may increase the stability of the virus, during the process and whilst dried, and then selecting the most suitable compounds for further investigation. Recently this time consuming approach has been dramatically shortened by using factorial design software. This approach requires limited input of data then mathematically interpolates and indicates where ideal solutions lie.

If the route of freeze-drying was to be taken then the virus product needs to be placed in the formulation buffer after purification by column chromatography. There are a number of methods that can be used but, on a manufacturing scale, either tangential cross flow or size exclusion chromatography are probably the most suitable methods. Size exclusion chromatography of herpes virus has been demonstrated using an HSV-1 vector (Johnson 1998).

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## 9 Appendix

The appendix includes copies of the following references.

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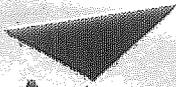
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<p>(21) International Application Number: PCT/GB98/02387 (22) International Filing Date: 7 August 1998 (07.08.98) (30) Priority Data: 9716611.0 7 August 1997 (07.08.97) GB (71) Applicant (for all designated States except US): CANTAB PHARMACEUTICALS RESEARCH LIMITED (GB/GB); 184 Cambridge Science Park, Milton Road, Cambridge CB4 4GN (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): JOHNSTON, Michael, Denis (GB/GB); Jesmond Dene, 9 The Crescent, Impington, Cambridge CB4 4NY (GB). O'KEEFFE, Roderic, Simon (GB/GB); 65 Loompits Way, Saffron Walden, Essex CB11 4DN (GB). (74) Agents: O'BRIEN, Caroline, J. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).</p>	<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GR, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p>	
<p>(54) Title: RECOVERY OF VIRUS FROM CELL CULTURE USING A HYPERTONIC SALT SOLUTION</p>		
<p>(57) Abstract</p> <p>A process of harvesting a herpesvirus from a cell culture infected therewith comprises treating said culture with a hypertonic aqueous salt solution to yield a virus suspension, e.g. to give improved yield of virus for live virus vaccine where otherwise cell-disruption might be used to harvest the virus by disrupting virus-infected cells. The harvesting step can be followed e.g. by nuclease treatment, diafiltration and lyophilisation.</p>		



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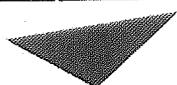
# The Primary Production of an Infectious Recombinant Herpes Simplex Virus Vaccine

R. O'Keeffe,<sup>1</sup> M. D. Johnston,<sup>2</sup> N. K. H. Slater<sup>1</sup>

<sup>1</sup>Department of Chemical Engineering & Applied Chemistry, Aston University, Aston Triangle, Birmingham, United Kingdom; telephone: (44)-121-359-2220; fax: (44)-121-359-4094; e-mail: N.Slater@aston.ac.uk

<sup>2</sup>Cantab Pharmaceuticals Research Ltd., 184 Cambridge Science Park, Milton Road, Cambridge, United Kingdom

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# The Affinity Adsorptive Recovery of an Infectious Herpes Simplex Virus Vaccine

R. S. O'Keeffe,<sup>1</sup> M. D. Johnston,<sup>2</sup> N. K. H. Slater<sup>1</sup>

<sup>1</sup>Department of Chemical Engineering & Applied Chemistry, Aston University, Aston Triangle, Birmingham, UK; telephone: +44-121-359-2220; fax: +44-121-359-4094; e-mail: N.Slater@aston.ac.uk

<sup>2</sup>Cantab Pharmaceuticals Research Ltd., 184 Cambridge Science Park, Milton Road, Cambridge, UK

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INCUBATION WITH HEPARIN AS AN IMPROVED HARVEST METHOD FOR  
HIGH TITRE DISABLED INFECTIOUS SINGLE CYCLE (DISC) HSV-2 FROM  
MICROCARRIER CULTURES

WRIGHT PA<sup>1</sup>, ZECCHINI TA<sup>1</sup>, SMITH RJ<sup>1</sup>, O'KEEFE, RS<sup>2</sup> and  
SLATER NK<sup>2</sup>

<sup>1</sup>Cantab Pharmaceuticals Research Ltd.,  
184 Cambridge Science Park, Cambridge. U.K. CB4 4GN

<sup>2</sup>Dept. of Chemical Engineering and Applied Chemistry  
Aston University, Aston Triangle, Birmingham. U.K. B4 7ET



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## Title – Fractogel chromatography of dH2a00023 –D8d

Run No.- dH2a00023

### Aim

Consistency run using 2x10L USP harvests run using the revised D8 process.

### Methods

Preparation of Fractogel EMS  $\text{SO}_4^-$  (M) (Merck)

- Pour out sufficient slurry for the column (approx. 65% slurry as supplied)
- Leave to settle out, decant supernatant, add equivalent volume of 2 M NaCl then resuspend.
- Repeat above three times.

Packing of VA60 column (Millipore)

- All column components were washed with DO water and flow cells replaced
- Following assembly bottom flow cell was flushed with 2M NaCl to remove air.
- Matrix slurry was poured into the column and top adapter fitted.
- Using 2 bar of air top adapter was lowered onto the slurry, once air had been removed the adapter was fixed in place and matrix packed under flow at  $90 \text{ mLmin}^{-1}$ .
- Once matrix was packed top adapter was lowered to the top of the packed bed.
- Prior to use the column was washed with 5 volumes 0.8 M NaCl at  $40 \text{ mLmin}^{-1}$ .

$A_{280}$  was monitored using Pharmacia UV-1 meter using a 10 mm flow cell and plotted using a Pharmacia chart recorder.

Material used was processed in the run number in title. USP 2000-045, USP 2000-046 were harvested by addition of concentrated dextran sulphate into the reactor, to a final concentration of  $150 \mu\text{g/ml}$ . Harvest was concentrated (20 fold), treated with Benzonase ( $50 \text{ U/ml}$ , 1 hour), diafiltered 10 volumes with 1.2 M NaCl then 5 volumes 0.8 M NaCl prior to loading onto the column at  $40 \text{ mLmin}^{-1}$  (approximately  $90 \text{ cm h}^{-1}$ ).

Once all material had been loaded the column was washed with 5 column volumes of 0.85 M NaCl in 0.01 M phosphate .

DISC HSV was then eluted with 2 M NaCl in 0.01 M phosphate buffer.

Samples flow through and column eluate were assayed for infectious virus by  $\text{TCID}_{50}$  (see figure 2). Samples of eluate were assayed for total protein and BSA (see figure 2).

### Results

### Conclusions



## Title – Fractogel chromatography of dH2a99197 – Technical Transfer Run 16 L D7

### Run No.- dH2a99198

#### Aim

A portion of GW 500 L harvest was split and virus purified using the 16 L D7 protocol, at Cantab and at Beckenham with the aim to achieve similar purity and recovery profiles at the two site for the technical transfer.

#### Methods

For preparation of Fractogel EMD SO<sub>3</sub><sup>-</sup> 650 (M) matrix and packing see page 150 of this lab book. Column was packed at 200 cmh<sup>-1</sup>.

Column used was Millipore VA 60 and all wetted parts were washed with deionised water without detergent use. A<sub>280</sub> was monitored using Pharmacia UV-1 meter using a 10 mm flow cell and plotted using a Pharmacia chart recorder.

Prior to use the column was equilibrated using 3 volumes of 0.8 M NaCl in 0.01 M phosphate buffer (pH 7.4) at 80 cm h<sup>-1</sup>.

Material used was harvested in 100 µg/ml dextran sulphate (GW 419458:02) and processed by concentration, diafiltration into PBS, endonucleased (Benzonase™), diafiltered (10 volumes 1.2 M NaCl in 0.01 M phosphate buffer), diafiltered (5 volumes 0.8 M NaCl in 0.01 M phosphate buffer).

Feed material was pre-filtered prior to loading onto the column using a Millipore Millipak-100 0.45 µm filter using a Verder pump at 900 mlmin<sup>-1</sup> (see results, figure 2, for size and flow rate used).

Feed material was loaded onto the column at approximately 90 cm h<sup>-1</sup> (see figure 2).

Once all material had been loaded the column was washed with 5 column volumes of 0.85 M NaCl in 0.01 M phosphate .

DISC HSV was then eluted with 2 M NaCl in 0.01 M phosphate buffer.

Column eluate was diluted 1:5 with 0.1 M phosphate buffer pH 7.4.

Samples of pre and post filtration, flow through and diluted column eluate were assayed for infectious virus by TCID<sub>50</sub> (see figure 2). Samples of pre-filtration and diluted eluate were assayed for CR2DNA, total protein and BSA (see figure 2).

#### Results

Figure 1 shows the A<sub>280</sub> of this run. Figure 2 shows the results of the TCID<sub>50</sub>, CR2 DNA and BSA assays. 780 ml of processed feed material was filtered through one Millipak-100 0.45 µm filters using a Verder pump at approximately 900 mlmin<sup>-1</sup>. The filter was pre and post flushed with 0.8 M NaCl buffer. The resultant pooled filtrate had a recovery of 82 %. The A<sub>280</sub> trace during loading of the virus onto the Fractogel column (figure 1) does not indicate breakthrough, which was confirmed by the TCID<sub>50</sub> results (figure 2) showing 95 % of feed bound to the column. Overall recovery of 82 % was obtained from the 2 M NaCl eluate. This recovery is at an appropriate level, but the final titre of 1.09x10<sup>7</sup> pfu/ml would not be high enough to obtain a 1x10<sup>7</sup> lyophilised dose. The impurity profile (figure 2 and figure 4) show that final product is within specifications apart from the BSA assay which on FRAC DIL was 16 ng/ml, but is in specifications following DF4 at 6 ng/ml. Figure 3 shows the virus recoveries from both Cantab and GW (BECK05) for this run. GW samples were titred from frozen and Cantab samples were titred both fresh and frozen. When comparing the frozen results the GW recoveries rapidly decline during the benzonase step. Interpretation of the results however is complex since virus disappears and appears through both the GW and Cantab runs. Figure 4 shows the impurity profile for the two runs, which are very similar.

#### Conclusions

Final virus titre for BECK05 and dH2a99199 were both very poor and did not meet the criteria for a successful technical transfer run.