

PURIFICATION OF GLYCOPROTEINS

FROM HERPES SIMPLEX VIRUS

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MSc (Med) in the Department of Medical Microbiology in the Faculty
of Medicine at the University of Cape Town

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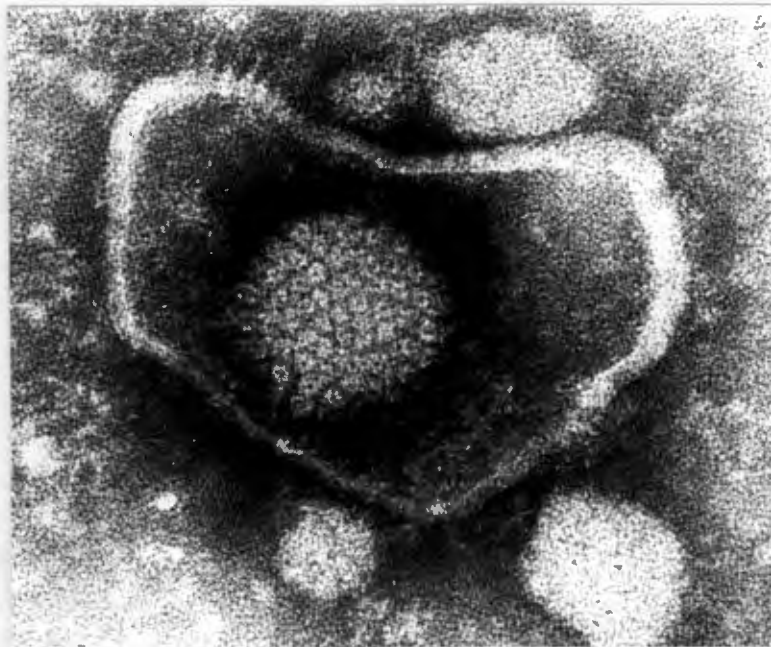
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SUMMARY

The aim of this work was to purify type-specific glycoproteins from herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) for diagnostic use. The most likely candidate for a type-specific glycoprotein of HSV-1 is glycoprotein C (gC), although it has recently been shown to contain some type-common antigenic determinants.

HSV-1 and HSV-2 were produced in BHK-21 cells and labelled with either (^3H)-glucosamine ((^3H)-gln) or a mixture of (^{14}C)-amino acids ((^{14}C)-aa). Analysis of the radiolabelled products by analytical sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography revealed that in the HSV-1 infected cells the radiolabelled components were incorporated into viral specific proteins only, whereas in the HSV-2 infected cells they were incorporated into host cell proteins as well as viral proteins.

Preparative polyacrylamide gel electrophoresis (Prep-PAGE) was used as an initial step in separating HSV-1 infected cell proteins labelled with (^3H)-gln. Two cycles of Prep-PAGE were sufficient to produce solutions containing either glycoprotein B (gB) or glyco-

protein C (gC), which were free of other HSV-1 glycoproteins. However, these solutions still contained a number of non-glycosylated proteins.

Two different techniques were utilized to remove the non-glycosylated proteins from the glycoprotein solutions. Hydroxylapatite (HA-Ultrogel) chromatography in the presence of sodium dodecyl sulphate (SDS-HTP) did not separate the different HSV-1 glycoproteins and was not satisfactory for removing the non-glycosylated proteins.

Gel-bound lectin affinity chromatography using wheat germ lectin and Helix pomatia lectin was not successful in purifying the glycoproteins because the glycoproteins which bound to the lectins could not be eluted under normal conditions.

Difficulties encountered in eluting the HSV-1 glycoproteins from the lectins may have been due to the sodium dodecyl sulphate (SDS) in which the proteins were solubilized. For this reason, the gel-bound lectin affinity chromatography was repeated using HSV-1 membrane proteins solubilized in a non-ionic detergent, Triton X-100. Using material prepared in this way, several HSV-1 glycoproteins were bound by wheat germ lectin and eluted under normal conditions to yield glycoproteins which were purified with respect to non-glycosylated proteins.

DECLARATION

I declare that the work done for this dissertation is my own. It is being submitted for the MSc (Med) degree at the University of Cape Town. It has not been submitted before for any degree or examination at any other University.

RICHARD PETER GRAHAM

16th day of August, 1985

ABBREVIATIONS

A	Absorbance
aa	Amino acid
APS	Ammonium persulphate
ATV	Activated trypsin versene
Ave	Average
BHK-21	Baby hamster kidney cells, clone 21
BSS	Balanced salt solution
°C	Degrees Centigrade
(¹⁴ C)-aa	Amino acids labelled with carbon 14
CBB	Coomassie brilliant blue stain
cm	Centimetre
cm ³	Cubic centimetre
Con A	Concanavalin A
CPE	Cytopathic effect
CPM	Counts per minute
DATD	Diallyltartardiamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
g	Gram

GalNAc	N-acetyl-galactosamine
GlcNAc	N-acetyl-glucosamine
gln	glucosamine
HeLa	Cervical carcinoma cell line
HEp-2	Laryngeal carcinoma cell line
(³ H)-gln	Glucosamine labelled with tritium
HMW	High molecular weight
HPL	<u>Helix pomatia</u> lectin
hr	Hour
HSV	Herpes simplex virus
HSV-1	Herpes simplex virus type-1
HSV-2	Herpes simplex virus type-2
ICP	Infected cell polypeptide
IgG	Immunoglobulin G
K	Kilo (i.e. 1,000)
KB	Epidermoid carcinoma cell line
LMW	Low molecular weight
Log	Logarithm
M	Molar
mA	Milliamperes
MBA	N,N'-methylenebisacrylamide
MCP	Major capsid protein
MEM	Minimum essential medium
min	Minute
ml	Millilitre
mM	Millimolar

MOI	Multiplicity of Infection
m.wt.	Molecular weight
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulphonyl fluoride
Prep-PAGE	Preparative polyacrylamide gel electrophoresis
Rf	Ratio of the distance migrated by a protein to that of phenol red during electrophoresis for the same time
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-HTP	Hydroxylapatite chromatography in the presence of sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Second
TCA	Trichloroacetic acid
TCID ₅₀	50 per cent tissue culture infective dose
TEMED	NNN'N'-Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) methylamine
v/v	Volume in volume
VP	Virion protein
w/v	Weight in volume
WGL	Wheat germ lectin
w/w	Weight in weight
α -MM	α -methyl-D-mannoside
μ Ci	Microcurie (10^{-6} Curie)
μ l	Microlitre (10^{-6} litre)
μ	Micron (10^{-6} metres)

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"You are worthy, our Lord and God, to receive glory and honour and power, for you created all things, and by your will they were created and have their being."

(Revelation 5 : 11)

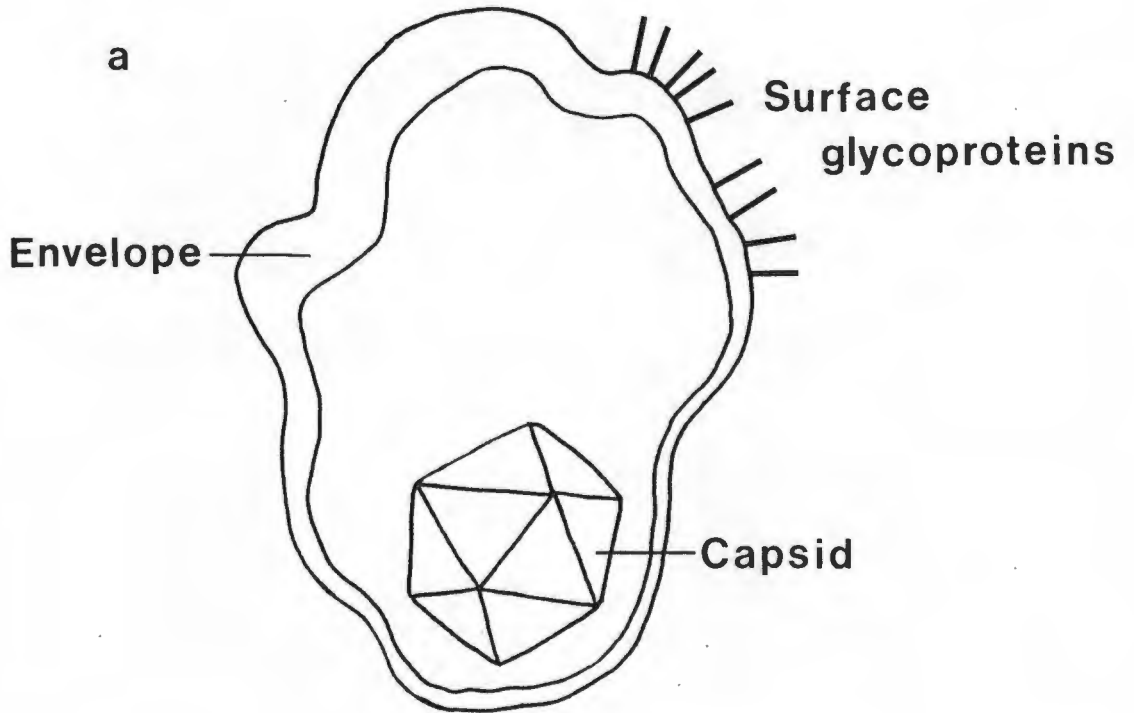
INTRODUCTION

VIRUS STRUCTURE

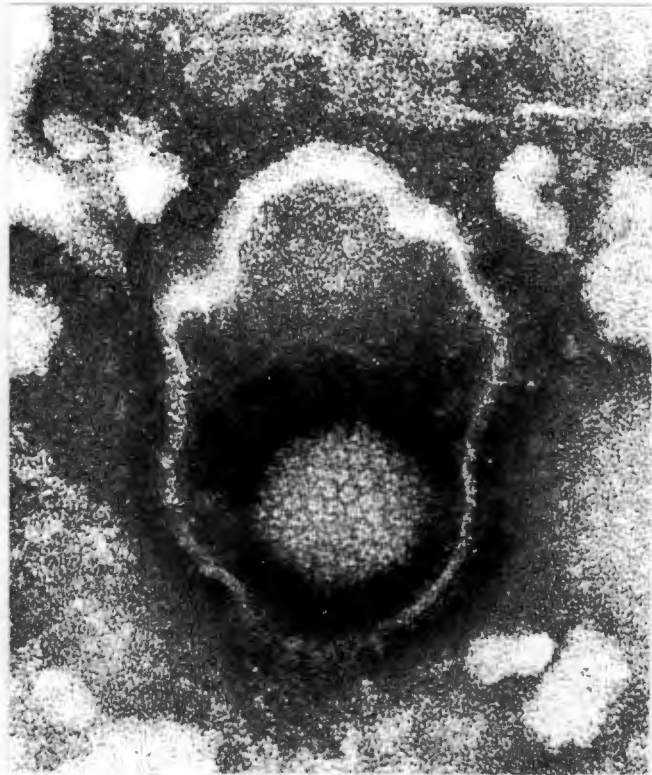
Herpes Simplex virus (HSV) is a member of the Herpesvirus family. The herpesviruses are large DNA viruses characterized by capsids of approximately 100 nm in diameter. Like other herpesviruses, the virions of HSV consist of four major architectural components (Figure 1) (Roizman & Furlong, 1974) :

1. A centrally located DNA core.
2. A capsid containing 162 capsomers arranged in the form of an icosadeltahedron.
3. A tegument, which is the granular zone surrounding the capsid.
4. An envelope consisting of a trilaminar membrane with spikes projecting from its outer surface.

The spikes projecting from the outer surface of the envelope are, in fact, virus specific antigens (Figure 1). These antigens have been shown to be the major, if not exclusive, targets of neutralizing antibodies (Courtney & Benyesh-Melnick, 1974; Honess et al, 1974; Norrild et al, 1978(a)). The same antigens are found on infected cell surfaces (Glorioso & Smith, 1977; Norrild et al, 1978(b)), and their presence early in the infectious cycle, before the production of active virus, makes them potentially important



b



target antigens for the control of virus spread by destruction of infected cells (Shore et al, 1979). These antigens have been shown to be glycoproteins (Spear, 1976; Glorioso & Smith, 1977; Pauli & Ludwig, 1977; Norrild et al, 1979, 1980; Sarmiento et al, 1979; Glorioso et al, 1984).

HSV SPECIFIC PROTEINS

Upon infection of a cell with HSV, the viral DNA finds its way to the cell nucleus and is transcribed. The viral RNA sequences are transported from the nucleus to the cytoplasm, where the proteins are synthesized (Roizman & Furlong, 1974). The viral specified polypeptides may be divided into three sequentially synthesized, co-ordinately regulated groups, designated α , β and γ . The α - group, which consists mainly of non-structural proteins, is produced independently of prior infected cell synthesis mainly between 3-4 hours post-infection. The β -group, consisting of minor structural and non-structural proteins, is produced at the highest rate between 5-7 hours post-infection. The β -group is dependent on both prior α polypeptide synthesis and new RNA synthesis. The γ -group, consisting of major structural polypeptides, is synthesized at increasing rates until at least 12 hours post-infection, and is dependent on prior synthesis of β -polypeptides (Honess & Roizman, 1974).

Using SDS-polyacrylamide gel eletrophoresis (SDS-PAGE) and autoradiography, herpes simplex virus has been shown to induce between

44 and 52 polypeptides in infected cells (Honest & Roizman, 1973; Powell & Courtney, 1975; Marsden, Crombie & Subak-Sharpe, 1976; Morse et al, 1978). Approximately 12 of these polypeptides have been shown to be glycosylated (Heine et al, 1974), although some of these are precursors of the fully glycosylated proteins (Spear, 1976; Baucke & Spear, 1979). The major glycoproteins have been designated gC, gB, gA, gE and gD (Spear, 1976; Baucke & Spear, 1979; Pereira et al, 1980). However, it has been shown that gA and gB are, in fact, different forms of the same polypeptide (Eberle & Courtney, 1980(b); Person et al, 1982; Pereira et al, 1982) (See Chapter 3.1.1 for a discussion of the nomenclature of the HSV-1 glycoproteins). Most, if not all, the glycoproteins fall into the γ -group of polypeptides (Honest & Roizman, 1974).

By the use of antisera and monoclonal antibodies that reacted specifically with the viral glycoproteins of HSV-1, and pulse-chase experiments, polypeptide precursors to the major glycoproteins of HSV-1 have been identified. This work has demonstrated that the major glycoproteins are glycosylated in discrete steps consisting of partially glycosylated intermediates and a fully glycosylated product (Spear, 1976; Cohen et al, 1978; Eisenberg et al, 1979; Baucke & Spear, 1979; Compton & Courtney, 1984(a); 1984(b)).

FUNCTIONS ASSOCIATED WITH THE GLYCOPROTEINS OF HSV

Since viral glycoproteins are the major constituents of the virion surface, and the insertion of viral glycoproteins into the plasma

membrane is the most noticeable change in the plasma membrane of infected cells (Glorioso & Smith, 1977; Pauli & Ludwig, 1977), certain functions have been attributed to the viral glycoproteins. These functions are the adsorption and penetration of the virion into uninfected cells during the initiation of infection, the interaction of infected cells among themselves in cell culture, and the expression of Fc receptors and complement receptors. Mutants which are defective in glycoprotein synthesis and mutants with altered functions which are related to or mediated by viral membrane proteins have been used in determining the functions of HSV glycoproteins.

(a) Virion Infectivity

Events which occur during the initiation of infection are adsorption of virus to the cell surface, fusion of the envelope with the plasma membrane, and dissolution of the fused membrane. Glycoprotein gC is not required for any of these functions since HSV-1 mutants such as HSV-1 (MP), which lack this glycoprotein, are still infectious (Ejercito et al, 1968; Manservigi et al, 1977; Norrild et al, 1979). Glycoprotein gB appears to be required at least for the penetration of virions into the cell because the HSV-1 mutant, HSV-1 (HFEM) tsB5, which does not produce gB at the nonpermissive temperature, adsorbs but does not penetrate into the cell. This is supported by the fact that the addition of polyethylene glycol

(PEG) to the cells after adsorption, significantly increased the infectivity (Sarmiento et al, 1979).

The importance of gB in the initiation of infection is also demonstrated by the fact that antisera raised to gC will only neutralize HSV-1 in the presence of complement (complement-dependent), whereas antisera raised to gB does not require complement for neutralization (complement-independent). This suggests that antibodies to gB bind to sites which are intimately associated with the HSV-1 surface sites which determine virus infectivity (Eberle & Courtney, 1980(a)).

(b) Cell Fusion

The change in the cell-to-cell interactions is demonstrated by the fact that some variants of HSV-1 and HSV-2 cause fusion rather than rounding and clumping (Ejercito et al, 1968; Ruyechan et al, 1979). Gene mapping of several viral mutants causing cell fusion has revealed that there are at least three genetic loci, designated syn 1, syn 2 and syn 3, which determine cell fusion (Ruyechan et al, 1979). Loci syn 1 and syn 2 do not map in the position of the structural genes of any of the major glycoproteins, whereas the syn 3 locus maps within the position assigned to the structural protein of glycoprotein B (gB). Hence, gB appears to play a role in cell fusion as well as viral penetration.

(c) Expression of Receptors

Cells infected with HSV have been shown to express new receptors. One of these receptors has affinity for the Fc region of IgG and another has affinity for the activated C3b component of complement. These receptors have been shown to be composed partially or completely by some of the HSV glycoproteins.

1. Fc receptors. The presence of the Fc receptor on the surface of HSV infected cells was first shown by the adherence of sheep erythrocytes coated with antibody directed to sheep erythrocytes, to HSV infected cells (Watkins, 1964; 1965). This finding has since been confirmed using normal IgG, the purified Fc fragment and soluble immune complexes (Westmoreland & Watkins, 1974; Adler et al, 1978; Nakamura et al, 1978; Dowler & Veltri, 1984).

Glycoprotein E (gE) of HSV-1 and HSV-2 has been purified using affinity columns consisting of rabbit antibodies bound to Sepharose 4B. The affinity of gE for the rabbit antibodies, as well as other properties of the glycoprotein, have shown that gE is the antigen responsible for Fc binding activity (Baucke & Spear, 1979; Para et al, 1982).

2. C3b receptors. Cells infected with HSV-1, but not HSV-2, have been found to express receptors for the C3b component of complement. These receptors are not expressed on cells infected with the mutant strain HSV-1 (MP), which lacks gC, and are blocked by monoclonal antibodies directed against gC, but not any of the other HSV-1 glycoproteins. For these reasons, gC appears to be partially or totally responsible for C3b receptor activity (Friedman et al, 1984).

IMMUNOLOGIC REACTIVITY OF HSV GLYCOPROTEINS

(a) On the surface of intact virions

Humans and experimental animals which have recovered from HSV infections are known to produce antibodies which neutralize the infectivity of HSV (Plummer, 1964; Pauls & Dowdle, 1967). The targets for neutralizing antibodies are believed to be antigenic determinants on the surface of the virion envelope because :

1. The virion envelope is essential for infectivity (Stein et al, 1970; Sarmiento et al, 1979) and is impermeable to micromolecules and electrolytes (Roizman & Furlong, 1974). Hence, neutralizing antibodies do not cause neutralization by attaching to non-envelope proteins.

2. Analysis of purified virions has shown that they contain four major and several minor glycoproteins in the envelope (Glorioso et al, 1983) and labelling of the virion by several methods has shown that the glycoproteins are at the external surface of the virion (Olshevsky & Becker, 1972; Sarmiento et al, 1979; La Thangue et al, 1984).

3. Antibodies raised to the individual HSV glycoproteins also neutralize the virion (Powell et al, 1974; Vestergaard & Norrild, 1989; Eberle & Courtney, 1980(a); Spear 1985).

(b) In the plasma membrane of infected cells

The presence of viral specific determinants on the surface of HSV infected cells has been shown using several different techniques including complement mediated immune cytotoxicity (Glorioso et al, 1983), antibody-dependent cell-mediated cytotoxicity (ADCC) (Norrild, Shore & Nahmias, 1979; Norrild & Pedersen, 1982), immunofluorescence (Hayashi et al, 1973, Glorioso & Smith, 1977; Norrild et al, 1978(b)) and detection of the Fc and C3b receptors (Baucke & Spear, 1979; Friedman et al, 1984). The incorporation of these viral glycoproteins into the plasma membrane of infected cells plays an important role in the destruction of infected cells by antibody dependent immune effector reactions (Shore et al, 1979).

TWO SEROTYPES OF HSV

The existence of two serotypes of HSV, type 1 (HSV-1) and type 2 (HSV-2), has been firmly established on the basis of antigenic and biological differences (Plummer, 1964; Nahmias & Dowdle, 1968; Figuero & Rawls, 1969; Rawls, 1973).

HSV-1 and HSV-2 also differ in the base composition and sequence homology of their DNAs (Ludwig et al, 1972) and the use of restriction endonucleases has demonstrated that the electrophoretic profiles of different HSV-1 strains could be readily distinguished from the corresponding profiles of HSV-2 strains (Hayward et al, 1975; Buchman et al, 1978; 1979). There are also differences in the electrophoretic mobilities of many of the structural and non-structural polypeptides of the two serotypes (Morse et al, 1978; Ruyechan et al, 1979).

Despite these differences, HSV-1 and HSV-2 show many similarities. For instance, there is considerable homology between their DNAs (Kieff et al, 1972; Draper et al, 1984; Swain et al, 1985) and most of the major glycoproteins contain type-common as well as type-specific regions (Zezulak & Spear, 1983; Koussoulas et al, 1984; Eisenberg et al, 1985, Spear, 1985).

PROBLEMS ARISING FROM TWO SEROTYPES OF HSV

For a number of years studies have been conducted on the social and environmental factors related to the development of cervical carci-

noma. It was noticed that this cancer has an epidemiologic pattern very similar to that of venereal diseases, and it was suspected that carcinoma of the cervix may be totally or partially caused by an infectious agent which is transmitted during sexual intercourse.

Interest in determining the incidence of type 1 and type 2 HSV antibodies in cervical cancer patients was stimulated by the finding that over 95% of HSV strains recovered from the female genital tract are type 2 (Nahmias et al, 1969) and by the observation that a group of women with known genital herpetic infection had a higher than expected rate of cervical anaplasia (Naib et al, 1969).

However, the immunological cross-reactivity between both the surface and internal antigens of the two virus types, and the extensive immunity of the general population to HSV-1, have been major problems in seroepidemiological studies attempting to define the relationship of HSV-2 to cervical carcinoma (Rawls et al, 1970; Nahmias et al, 1970; Seth et al, 1978). For this reason the need has arisen to develop a type-specific diagnostic test which will be able to distinguish between patients that have been infected with HSV-1, HSV-2 or both HSV-1 and HSV-2.

PRODUCTION OF ANTISERA TO THE MAJOR GLYCOPROTEINS OF HSV-1

The viral surface glycoproteins of HSV have been shown to be the major if not exclusive targets of neutralizing antibodies (Sim &

Watson, 1973; Courtney & Benyesh-Melnick, 1974; Honess et al, 1974; Powell et al, 1974; Norrild et al, 1978(b)). Several procedures have been used to obtain antisera reacting mainly with antigenic determinants specified by individual viral glycoproteins (Table I).

(a) Glycoprotein C (gC)

Spear produced antiserum to gC by injecting a suspension of HSV-1 (F) envelope proteins in Freund's adjuvant into rabbits and adsorbing the resultant antiserum with HSV-1 (MP)-infected cell homogenates. (HSV-1(MP) is a mutant strain lacking gC (Spear, 1976)). The resultant antiserum was not entirely specific for gC.

The VP 2/3 region of HSV-1 which is equivalent to the VP 123 region of HSV-1 (Dreesman et al, 1979; Eberle & Courtney, 1980(a)) and consists of glycoproteins gA/gB and gC, as well as a non-glycosylated protein (Eberle & Courtney, 1980(a)), was isolated by means of SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The appropriate section of PAA gel containing the VP 2/3 region was cut out, macerated and injected with Freund's incomplete adjuvant into rabbits. The resultant antiserum was tested by neutralization against HSV-1 and HSV-2 and was shown to be comparatively but not totally specific for HSV-1 (Powell et al, 1974; Powell & Watson, 1975).

Crossed immunoelectrophoresis of HSV antigens obtained from infected cells solubilized with the non-ionic detergent, Triton X-100, shows Ag-6 (ie gC) in HSV-1 but not HSV-2. Monospecific antiserum was produced against Ag-6 by intracutaneous inoculation of the corresponding immunoprecipitate into rabbits (Vestergaard & Norrild, 1978). The antibodies to Ag-6 were absolutely type-specific for HSV-1 by the reactions in rocket immunoelectrophoresis, but the plaque reduction test showed a slight cross-reactivity with HSV-2.

Antiserum raised to gC, which was purified using preparative PAGE and SDS-hydroxylapatite chromatography, was found to be specific for gC (Eberle & Courtney, 1980(a)).

More recently, monoclonal antibodies to gC have been produced (Pereira et al, 1980; Showalter et al, 1981; Pereira et al, 1982(b); Chan, 1983; Goldstein et al, 1983; La Thangue et al, 1984; Marlin et al, 1985). These monoclonal antibodies were specific for HSV-1.

TABLE 1 - ANTIBODY PREPARATIONS TO INDIVIDUAL HSV-1 SPECIFIED GLYCOPROTEINS (Adapted from Norrild, 1980)

Reactive to Glycoprotein	Method of Preparation	Reference
gC	Adsorption of polyspecific serum with HSV-1(MP) infected cells.	Spear, 1976
	Immunization with the polypeptide gC.	Powell, et al, 1974 Powell & Watson, 1975, Eberle & Courtney, 1980 (a)
	Immunization with immunoprecipitate Ag-6 from crossed immunoelectrophoretic gels.	Vestergaard & Norrild, 1978
	Monoclonal antibodies	1,2,3,6,7,8,10 (a)
gB	Immunization with the immunoprecipitate Ag-11 from cross immunoelectrophoretic gels.	Norrild et al, 1978, 1979 Vestergaard & Norrild, 1979
	Immunization with the polypeptide gA/gB.	Eberle & Courtney, 1980 (a)
	Monoclonal antibodies	2,3,6,9 (a)
gE	Immunization with purified gE.	Baucke & Spear 1979
	Monoclonal antibodies	4 (a)
gD	Immunization with the immunoprecipitate of band II antigen isolated in Ouchterlony gels.	Watson & Wildy, 1969
	Immunization with the polypeptide gD.	Powell & Watson, 1975
	Immunization with the immunoprecipitate Ag-8 from crossed immunoelectrophoretic gels.	Vestergaard & Norrild, 1979
	Immunization with purified protein CP-1	Cohen et al, 1978
	Monoclonal antibodies	1,2,3,4,5,6,8 (a)

(a) Monoclonal antibodies were produced by the following:-

- | | |
|------------------------------|----------------------------|
| 1 - Pereira et al, 1980 | 6 - Chan, 1983 |
| 2 - Showalter et al, 1981 | 7 - Goldstein et al, 1983 |
| 3 - Pereira et al, 1982 | 8 - La Thangue et al, 1984 |
| 4 - Balachandran et al, 1982 | 9 - Kousoulas et al, 1984 |
| 5 - Eisenberg et al, 1982 | 10 - Marlin et al, 1985 |

(b) Glycoprotein B (gB)

Antibodies to Ag-11 of HSV-1 (an alternative designation for gB) have been produced by inoculating rabbits with the immunoprecipitate of Ag-11. HSV-1 infected cells were solubilized in Triton X-100 and the resultant solution was subjected to crossed immunoelectrophoresis against polyspecific antiserum to HSV-1. The immunoprecipitate corresponding to Ag-11 was cut out of the wet, unstained agarose and disrupted by sonication. The disrupted gel was mixed with Freund's incomplete adjuvant and injected intracutaneously into rabbits. The resultant serum was specific for a glycopolypeptide with a molecular weight of 125K which was common to HSV-1 and HSV-2 (Norrild et al, 1978; Vestergaard & Norrild, 1979).

The HSV-1 glycoprotein, gA/gB, has been purified by means of preparative PAGE followed by SDS-HTP chromatography. Antiserum raised to this glycoprotein, in rabbits, was found to be specific for gA/gB and was cross-reactive with HSV-2 (Eberle & Courtney, 1980(a)).

Monoclonal antibodies have also been produced to gB (Showalter et al, 1981; Pereira et al, 1982(b); Chan, 1983; Kousoulas et al, 1984). Some of these antibodies are type specific, but most are type-common.

(c) Glycoprotein E (gE)

Affinity chromatography was used to isolate the Fc-binding glycoprotein of HSV-1, glycoprotein E (gE) (Baucke & Spear, 1979). After elution from the affinity column, gE was purified further by preparative polyacrylamide gel electrophoresis. Antibody produced to purified gE neutralized HSV-1, but only in the presence of complement.

A glycoprotein which is functionally and antigenically related to glycoprotein E of HSV-1 has been isolated from cells infected with HSV-2. This glycoprotein is clearly not identical to gE of HSV-1 because of differences shown in their apparent molecular weights and because of antigenic differences shown by neutralization assays (Para et al, 1982).

Monoclonal antibodies have only been produced to gE of HSV-2 (Balachandran et al, 1982, Pereira et al, 1982(b)), and these are specific for HSV-2.

(d) Glycoprotein D (gD)

Watson and Wildy prepared an antiserum to VP 8/9(gD) of HSV-1. They obtained a fraction giving a single (Band II) precipitin band with antiserum to herpes infected cells in immunodiffusion reactions. Gel containing the single band was cut from the immunodiffusion plates, pulverized in an equal volume of Freund's incomplete adjuvant and injected into rabbits (Watson

& Wildy, 1969). The resultant antiserum was reactive with HSV-1 and HSV-2 (Powell et al, 1974; Powell & Watson, 1975).

Antisera to VP8/9(gD) was also prepared by disrupting virus particles using SDS, urea, and dithiothreitol, and electrophoresis of the structural virus polypeptides on 7% polyacrylamide gels. After electrophoresis, segments of gel containing VP8/9 were pooled and homogenized in Freund's incomplete adjuvant prior to injection into rabbits. The antiserum reacted with HSV-1 and HSV-2 antigens but was more reactive with HSV-1 (Powell & Watson, 1975).

The immunoprecipitate produced to Ag-8 (gD) of HSV-1 by crossed immunoelectrophoresis of Triton X-100 solubilized HSV-1 infected cells against HSV-1 antiserum, was cut from the unstained wet gel. The gel was disrupted by sonication, mixed with Freund's incomplete adjuvant and injected into rabbits (Vestergaard & Norrild, 1979). The antiserum reacted with both HSV-1 and HSV-2, but was more reactive with HSV-1.

The CP-1 antigen (gp52 or gD) of HSV-1 was purified using a six-step procedure involving centrifugation, precipitation with ammonium sulphate, calcium phosphate chromatography, CM Sephadex C25 chromatography, DEAE - cellulose 23 chromatography, and a second step of calcium phosphate chromatography

(Cohen et al, 1978). Antiserum raised in rabbits to CP-1 (anti-CP-1) contained type-common neutralizing antibodies.

Monoclonal antibodies have been produced to glycoprotein gD of HSV-1 and HSV-2 (Pereira et al, 1980; Showalter et al, 1981; Balachandran et al, 1982; Pereira et al, 1982(b); Eisenberg et al, 1982; Chan, 1983; La Thangue, 1984). These monoclonal antibodies have been shown to be both type-specific and type-common, showing that gD contains both type-common and type-specific antigenic determinants (Dietzschold et al, 1984; Eisenberg et al, 1985).

THE NEED FOR A TYPE-SPECIFIC DIAGNOSTIC TEST FOR HSV

As mentioned earlier, the need to develop a type-specific diagnostic test for screening patients for past infection with either HSV-1, HSV-2 or both HSV-1 and HSV-2, arose from seroepidemiological studies attempting to define the relationship of HSV-2 to cervical carcinoma. Such a test for antibodies to HSV-1 or HSV-2 would require the purification of relatively large amounts of antigens containing exclusively or predominantly type-specific antigenic sites. A major obstacle to the development of such a test has been the difficulty in separating glycoprotein antigens of HSV-1 from host cell and other viral proteins, as well as from one another.

PURIFICATION OF GLYCOPROTEIN C (gC) OF HSV-1

Until recently, when studies using cross-reactive monoclonal antibodies and polyclonal antisera specific for gC showed that HSV-1 gC (gC-1) and HSV-2 gC (gC-2) do share limited antigenic homology (Zezulak & Spear, 1983; Zweig et al, 1983(a), 1983(b)), glycoprotein C (gC) of HSV-1 appeared to be the most likely candidate for an HSV-1 specific antigen, since serological tests performed with antisera raised to gC-1 showed that it contained almost exclusively HSV-1 specific sites (Powell et al, 1974, Spear, 1976; Vestergaard & Norrild, 1978; Vestergaard & Grauballe, 1979; Eberle & Courtney, 1980; Goldstein et al, 1983; Schrier et al, 1983). Thus, several techniques have been used in attempts to obtain purified preparations of gC in quantities which are suitable for use in diagnostic procedures. The techniques that have been used are as follows :

(a) Hydroxylapatite chromatography

Fused rocket and crossed immunoelectrophoresis of fractions obtained from hydroxylapatite chromatography of crude HSV-1 antigen showed that a subfraction of Ag-6 (gC) could be separated from the other HSV-1 antigens. The product was used in an enzyme-linked immunosorbent assay (ELISA) and was shown to react only with HSV-1 antisera (Vestergaard & Grauballe, 1979).

An antigen produced by the same method was found to be HSV-1 specific in inducing delayed hypersensitivity (DH) reactions

in mice. Mice immunized with HSV-1 gC did not respond to challenge with HSV-2, nor did mice immunized with HSV-2 respond to challenge with HSV-1 gC (Schrier, Pizer & Moorhead, 1983).

(b) Gel-bound lectin affinity chromatography

When lysates from HSV-1 and HSV-2 infected cells were subjected to affinity chromatography on soybean and Helix pomatia lectins, glycoprotein C (gC) was found to bind to the lectins and was eluted with N-acetyl-galactosamine (GalNAc). Affinity chromatography permitted a high degree of purification of the type-specific glycoprotein with respect to both host cell components and other viral proteins (Olofsson, Jeansson & Lycke, 1981; Svennerholm et al, 1984).

Antigens purified by Helix pomatia lectin affinity chromatography have been used in an enzyme-linked immunosorbent assay (ELISA). The type specificity of the antigens was assessed by double-immunodiffusion precipitation in gel against rabbit HSV-1 and HSV-2 hyperimmune sera, and by ELISA with human reference sera containing antibodies to either type of HSV. The results showed that the antigens were specific for the type-specific antibodies of HSV-1 and HSV-2, respectively (Svennerholm et al, 1984).

(c) Preparative-PAGE and hydroxylapatite chromatography in the presence of sodium dodecyl sulphate

The major glycoprotein region of HSV-1 (VP123) was purified by preparative PAGE (Prep-PAGE) and further fractionated into two major and two minor components by chromatography of the isolated VP123 region on SDS-hydroxylapatite columns. The two major components (gC and gA/gB) were purified free of other polypeptides and used to prepare specific antisera to these glycoproteins (Eberle & Courtney, 1980). No tests were performed to determine the type-specificity of the products.

(d) HSV-1 glycoproteins, gC, gB and gD have each been purified by means of immunoabsorption chromatography using monoclonal antibodies directed against the respective glycoproteins (Eisenberg et al, 1982; Roberts et al, 1985).

(e) Recent advances in recombinant DNA technology now make it possible to produce virtually unlimited quantities of purified viral antigens without resorting to large-scale cultivation of the infectious pathogen. Using this technology, the HSV-1 glycoprotein, gD, has been produced in Escherichia Coli (Weis et al, 1983) and in the genetically engineered mammalian cell line gD 10.2 (Berman et al, 1985). Although, to date, there are no reports of the production of gC by this procedure, it should be the obvious method of the future for producing large

quantities of HSV type-specific antigens for use in diagnostic tests.

THE AIM OF THE PRESENT WORK

The aim of the present work was to develop a procedure for purifying the type-specific proteins of HSV-1 and HSV-2, and to use these antigens in a suitable diagnostic procedure for testing sera for the presence of antibodies to either HSV-1 or HSV-2.

CHAPTER I: PRODUCTION OF HERPES SIMPLEX VIRUS

1.1. INTRODUCTION

1.1.1. Growth of Herpes Simplex Virus

A wide variety of animals can be infected with herpes simplex virus (HSV), experimentally. Rabbits and mice are particularly susceptible, but the virus has also been used to infect many other species of mammals including rats, hamsters, guinea pigs and cotton rats. Embryonated eggs are also susceptible to experimental infection with HSV (Darlington & Granoff, 1973).

Until the development of cell culture techniques (Enders, Weller & Robbins, 1949) HSV production and infectivity experiments were performed using live animals. The development of cell culture techniques at this time was largely due to the discovery and use of antibiotics as well as the production of media which would support cell growth in vitro. As a result of these improved cell culture techniques, and because of the inconvenience of using large numbers of live animals, HSV production and infectivity experiments are now performed almost exclusively in cell culture. The host range of herpes virus infectivity for cell cultures frequently parallels the in vivo host range, although the virus is usually found to

TABLE 1.1 - GROWTH OF HERPES SIMPLEX VIRUS IN CELL CULTURES
 (Taken from Darlington & Granoff, 1973)

CELL DONOR	CELL TYPE	REFERENCE
Human	Human amnion Human fibroblast	Ousterhout & Tamm (1959) Enders (1953)
Rabbit	Rabbit Kidney Rabbit lung	Barski <u>et al</u> (1955) Sosa-Martinez <u>et al</u> (1955) Kaplan (1957) Szanto (1960)
Rat	Rat lung	Szanto (1960)
Mouse	Mouse fibroblasts	Scherer (1953)
Cat	Feline kidney	Crandell (1959)
Pig	Swine kidney	Hancock <u>et al</u> (1959)
Hamster	Hamster kidney Chinese hamster	Watson & Wildy (1963) Hampar & Ellison (1963)
Tortoise	Tortoise kidney	Fauconnier (1963)
Marmoset	Marmoset kidney	Schur & Holmes (1965)
Chick	Chick embryo	Stulberg & Schapira (1953)
Malignant Cells	HeLa KB HEp-2 Rous sarcoma	Stoker (1959) Scherer & Syverton (1954) Hinze & Walker (1961) Kaplan (1957) Nankervis <u>et al</u> (1959)

infect a greater number of cell species than animals (Darlington & Granoff, 1973). A list of cells which support herpes simplex virus replication appears in Table 1.1.

Production of HSV for the purification and study of its membrane glycoproteins has been performed in several different cell types. Some of the more common cell types that have been used in this work are: rabbit cornea cells (Vestergaard, 1979; Vestergaard et al, 1977), HEp-2 and Vero cells (Eberly & Courtney, 1980 & 1981; Spear, 1976) and Baby Hamster Kidney (BHK-21) cells (Jeansson, Elwig & Nelsson, 1979; Cohen, Ponce de Leon & Diggelman, 1980; Olofsson, Jeansson & Lycke, 1981; Svennerholm et al, 1984). In the present work, both HEp-2 and BHK-21 cells were initially evaluated for the production of HSV, and BHK-21 cells were found to be more suitable because they grew better and yielded an adequate amount of virus.

1.1.2. Purification of Herpes Simplex Virus

Analyses of chemical and some biological properties of herpes viruses requires purified virions. However, herpes viruses are notoriously difficult to purify for a number of reasons which stem from several biological features of these viruses :

1. They are unstable and tend to lose their envelopes easily.
2. They have a tendency to aggregate.
3. A large proportion of the progeny virus remains intracellular.
4. On account of the cell associated nature of the virus, cellular debris, and in particular membrane vesicles, often contaminate preparations of purified virus (Gentry & Randall, 1973).

The solution to the problem of purity has depended on improved methods of preparation. Some of the techniques which have been used in attempts to purify enveloped herpes viruses are as follows :

1. Caesium chloride (CsCl) density gradient centrifugation (Spring & Roizman, 1967; Sydiskis, 1969).
2. Centrifugation on cushions of 60% (w/w) sucrose followed by dilution of the virus containing band and pelleting of the virus by high speed centrifugation (Spear, Keller & Roizman, 1970).
3. Sucrose or potassium tartrate density gradient centrifugation (Abodeely, Lawson & Randall, 1970).
4. Density gradient centrifugation in colloidal silica-polyethylene glycol (Klingeborn & Pertoft, 1972).
5. Separation of cell nuclei from infected cell cyto-

plasm followed by centrifugation of the cytoplasm on dextran 10 gradients to separate virions from most cellular contaminants and sub-virion particles. Flotation of the partially purified virions through a discontinuous sucrose gradient removes most of the remaining contaminants (Spear & Roizman, 1972; Strnad & Aurelian, 1976).

6. Centrifugation in density gradients of polyvinylpyrrolidone-coated colloidal silica (Percoll) (Svennerholm et al, 1980).

It appears that the technique of Spear and Roizman (1972), and also used by Strnad & Aurelian (1976), has been the most successful of these techniques in yielding purified herpes virions (Roizman & Furlong, 1974).

In the present work, CsCl density gradient centrifugation of HSV-1 extracted from BHK-21 cells, produced a good yield of infectious virus. The addition of 1% foetal calf serum to the CsCl solution (Roizman & Roane, 1964; Spring & Roizman, 1967; Lancz & Sample, 1985) was sufficient to protect the virus from disassembly and inactivation (Spring & Roizman, 1967). However, an electron microscopic study of the two light scattering bands formed by CsCl density gradient centrifugation revealed that the bands contained a considerable amount of cellular material as well as the viral and sub-viral particles.

On account of the difficulty encountered in obtaining purified herpes simplex virions, and because a large proportion of the HSV specific glycoproteins are incorporated into the membranes of the host cells, (Heine, Spear & Roizman, 1972; Olshevsky & Becker, 1972; Glorioso & Smith, 1977; Pauli & Ludwig, 1977) and would be lost by purification of the virus, it was decided to proceed with the purification of HSV glycoproteins, using a method that did not require prior purification of the virus.

1.2. METHODS AND RESULTS

The first stage in the production of herpes simplex virus was to obtain a suitable host cell line. Initially HEp-2 and BHK-21 cells were evaluated for the production of HSV and BHK-21 cells were favoured. The growth rate of BHK-21 cells in 10% MEM at 37°C was determined to calculate the cell concentration at the time of infection with HSV.

1.2.1. Growth of herpes simplex virus in BHK-21 cell cultures

A. Growth curve of BHK-21 cells in 10% MEM

The growth rate of BHK-21 cells in 10% MEM was studied by growing BHK-21 cell cultures in twelve identical culture flasks (Appendix 2.1.2). Each

flask was seeded with 5 ml of BHK-21 cell suspension containing $2,0 \times 10^5$ cells/ml in 10% MEM, and then incubated at 37°C. At 12 hourly intervals 2 flasks were removed from the incubator and the cells harvested and counted (Appendix 2.1.4).

B. Growth of HSV-1 and HSV-2 in BHK-21 cell cultures at 37°C.

The growth of HSV-1 and HSV-2 in BHK-21 cell cultures at 37°C was studied by inoculating confluent cell monolayers with a range of virus dilutions. A range of virus dilutions was used to investigate the possibility of interference of virus production by defective interfering particles at the concentrations of virus used (Huang & Wagner, 1966; Huang & Baltimore, 1977; Kang et al, 1978).

BHK-21 cell cultures were grown in eight culture flasks. When confluent, they were inoculated with 2 ml of HSV suspension as follows:

	Virus Type	Concentration of Virus in Inoculum TCID ₅₀ /ml
Bottle 1	HSV-1	10 ^{6.5}
2	HSV-1	10 ^{5.5}
3	HSV-1	10 ^{4.5}
4	HSV-1	10 ^{3.5}
Bottle 5	HSV-2	10 ^{5.5}
6	HSV-2	10 ^{4.5}
7	HSV-2	10 ^{3.5}
8	HSV-2	10 ^{2.5}

The virus was allowed to adsorb to the cells for 1 hour at 37°C before adding 20 ml of maintenance medium (Appendix 1.1.3 (d)) to each bottle and continuing incubation at 37°C.

A 1 ml sample of the maintenance medium was removed from each culture flask at various time intervals and titrated on BHK-21 cells (Appendix 2.1.7).

Results

BHK-21 cells grown in 10% MEM at 37°C showed an initial drop in the number of live cells at 12 hours after seeding. After this, the number of live cells increased at a constant rate of approximately 10^6 cells every 12 hours (Figure 1.1). The number of cells per flask reached a maximum at 60 hours after seeding and began to drop thereafter. The results show that there is approximately a 5-fold increase in the number of live cells after 60 hours at 37°C (Table 1.2, Figure 1.1).

The amount of infectious virus released into the growth medium was similar for HSV-1 and HSV-2 (Figure 1.2). At all the dilutions of inoculum used, there was a latent period of between 9 and 15 hours post-infection. After 15 hours post-infection the amount of virus in the medium increased at a steady rate until 56 hours post-infection when the experiment was stopped.

No plateau of virus titre was reached despite the fact that the cells showed 100% cytopathic effect (CPE).

TABLE 1.2 - CELL COUNTS OF BHK-21 CELLS GROWN IN 10% MEM AT 37°C

Time of Incubation	Total Cell Count (Cells/Flask)		
(Hours)	Flask 1	Flask 2	Average
0	1.0 x 10 ⁶	1.0 x 10 ⁶	1.0 x 10 ⁶
12	0.825 x 10 ⁶	0.675 x 10 ⁶	0.75 x 10 ⁶
24	1.23 x 10 ⁶	1.75 x 10 ⁶	1.49 x 10 ⁶
36	2.83 x 10 ⁶	3.05 x 10 ⁶	2.94 x 10 ⁶
48	3.83 x 10 ⁶	4.23 x 10 ⁶	4.03 x 10 ⁶
60	4.65 x 10 ⁶	5.68 x 10 ⁶	5.17 x 10 ⁶
72	5.10 x 10 ⁶	4.83 x 10 ⁶	4.97 x 10 ⁶

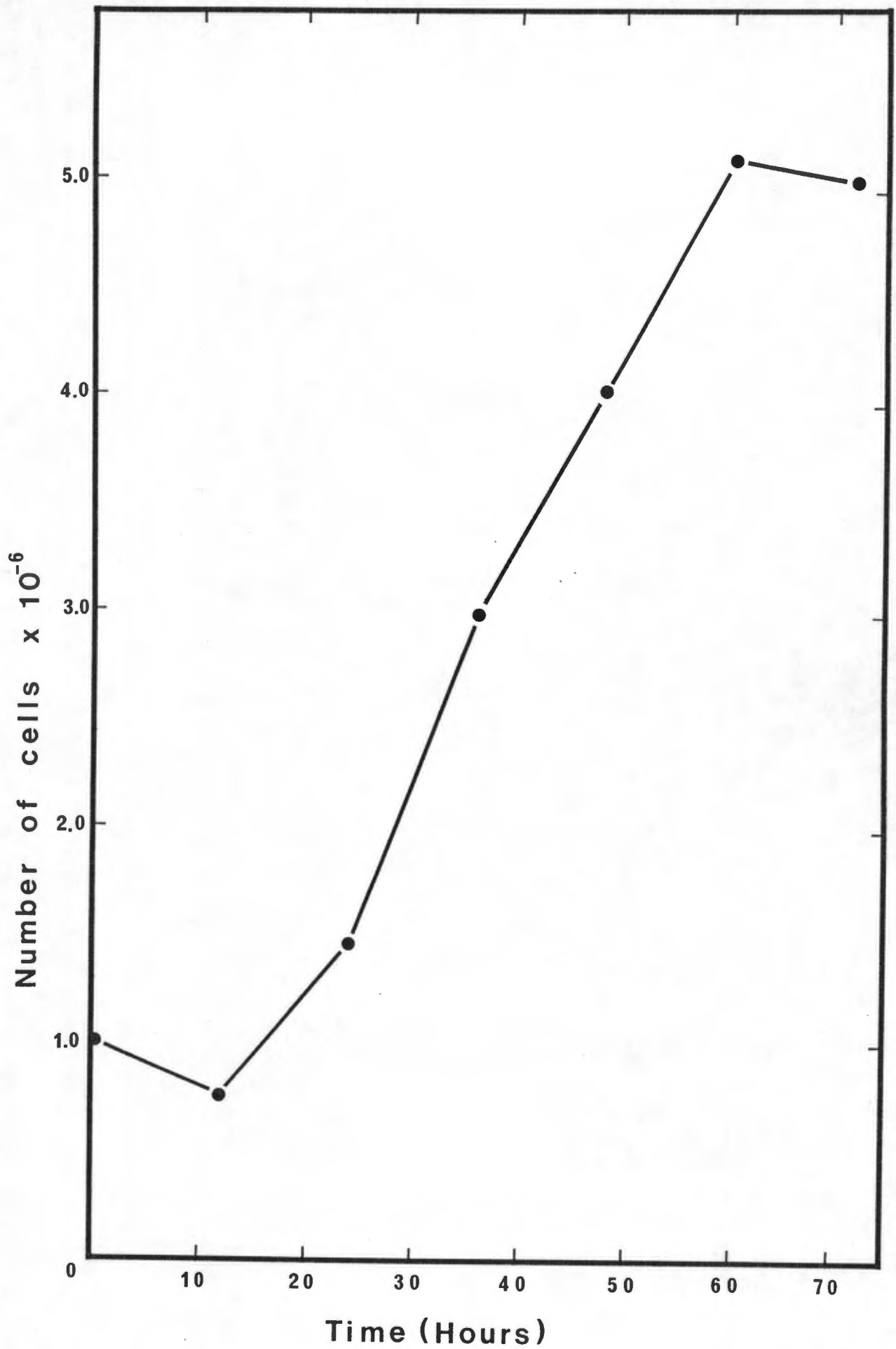


Figure 1.1 Growth curve of BHK-21 cells grown in 10% MEM at 37°C.

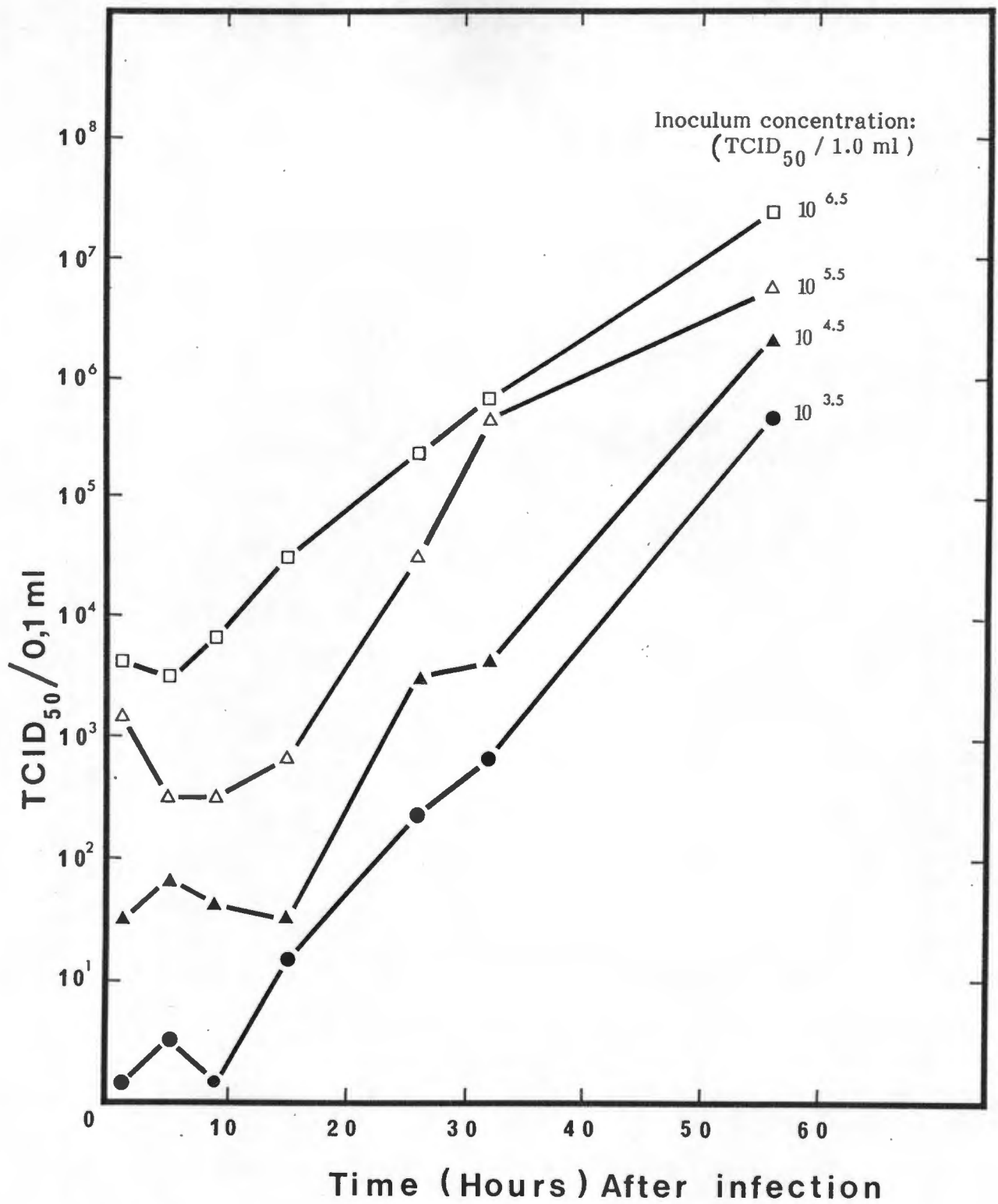


Figure 1.2 (a) Production of infectious HSV-1 in BHK-21 cells.

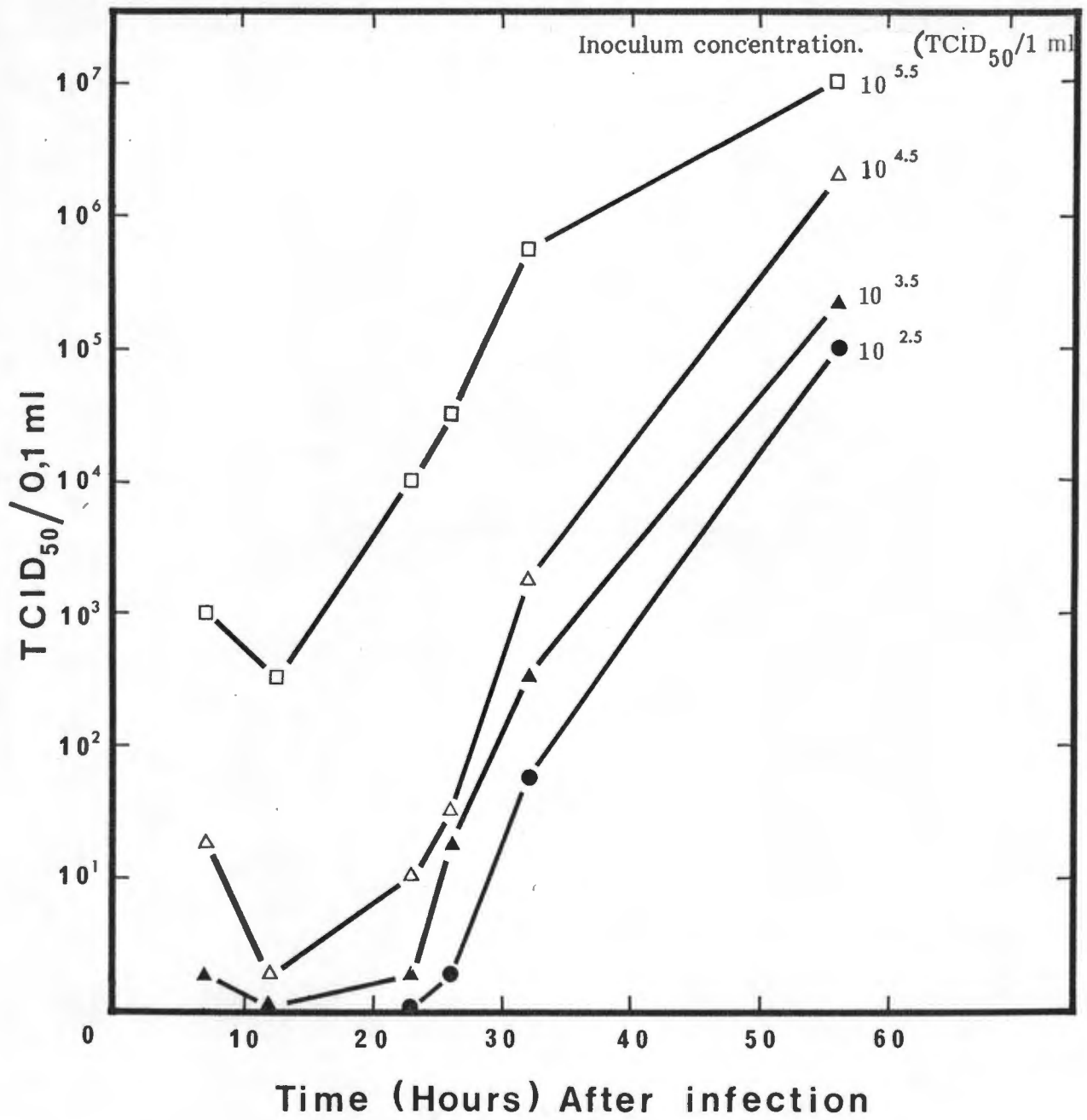


Figure 1.2 (b) Production of infectious HSV-2 in BHK-21 cells.

Discussion

When BHK-21 cell cultures were inoculated with a range of virus dilutions, the amount of extracellular virus in the culture medium only began to increase by 15 hours post-infection. (Figure 1.2). The latent period was shown to be between 9 and 15 hours for HSV-1 and HSV-2. This is in agreement with previous reports (Darlington & Granoff, 1973; Fenner et al, 1974).

No plateau of virus titre in the culture medium was reached during this experiment despite all the cells showing a cytopathic effect. This is compatible with a continual accumulation of infectious virus being released into the culture medium.

Although the amount of virus in the culture medium does not give an accurate indication of the growth of HSV in BHK-21 cells, the results show that because there was no reduction in the yield of infectious virus at any of the concentrations of inoculum used (Figure 1.2), interference due to defective interfering (DI) particles would not be a consideration in the present work.

1.2.2. Test for mycoplasma infection in BHK-21 cell culture

Mycoplasmas are known to be common contaminants of cell cultures which may produce various abnormal changes in cells growing in vitro (Hayflick, 1972; Chanock & Tully, 1980), or they may go undetected because the contaminated culture grows well and appears normal by ordinary light microscopy. The BHK-21 cells used in the present work were investigated for mycoplasma infection as it was realized that mycoplasma infection could interfere with the results obtained. HeLa cells, which are known to be infected with mycoplasma (Macpherson & Allner, 1960; Seman & Dmochowski, 1975), were used as a positive control with which to compare the BHK-21 cells. The test was performed according to the method of Chen (Appendix 2.1.5) which makes use of a DNA-specific fluorescent stain, Hoechst 33258 (Appendix 1.1.5(g)) (Chen, 1977).

H-stain (Hoechst 33258) is known to specifically bind to DNA-protein complexes of cells (Latt, 1973). The stain has a maximum excitation wavelength of 360 nm and emits light in the range of 490-500 nm, giving a bright greenish-yellow fluorescence. Cells that are not infected with mycoplasma show bright greenish-yellow, fluorescent nuclear components (nuclei, micronuclei and chromosomes) which contrast with the dark cytoplasmic surrounding, which cannot be differentiated from the intercellu-

lar spaces. The major DNA components of the cytoplasmic mitochondria are not visible except by using special filters (Chen, 1977). Although the nuclear components of mycoplasma infected and uninfected cells appear the same, the cytoplasm of infected cells is no longer dark but contains discrete patches of fluorescence.

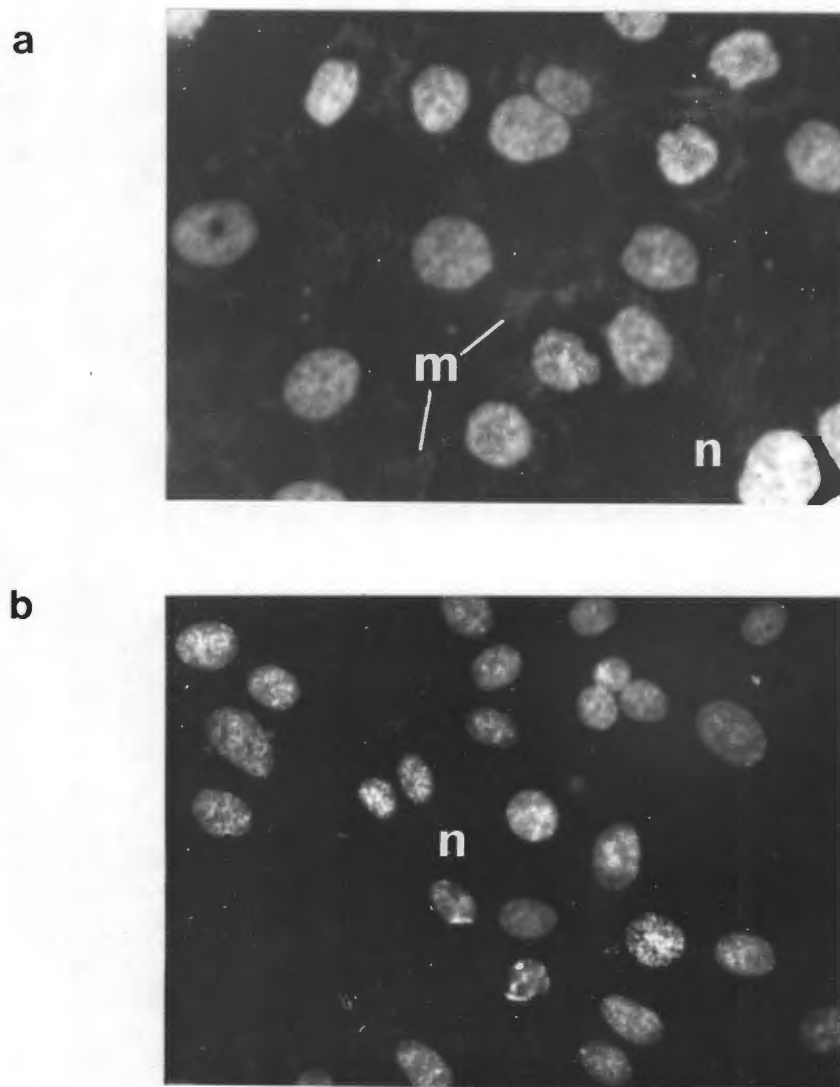


FIGURE 1.3 - (a) HeLa cells, and (b) BHK-21 cells, stained with the DNA-specific fluorescent stain Hoechst 33258.

n = nuclei; m = mycoplasma

Results

Both HeLa cells and BHK-21 cells were stained with H-stain (Appendix 2.1.5). After staining, the nuclei of both cell types fluoresced brightly when illuminated at 360 nm (Figure 1.3). The brightly stained nuclei of the BHK-21 cells contrast with the dark background of the cytoplasm which contains no fluorescent material (Figure 1.3(b)), while the cytoplasm of the HeLa cells contains a considerable amount of fluorescent material dispersed in discrete patches (Figure 1.3(a)). The results of this test proved that the BHK-21 cells were free of mycoplasma infection.

1.2.3. A study of the stability of HSV-1 and HSV-2 when stored in a variety of media at 4°C.

The aim of the present work was to identify and purify type-specific glycoproteins of herpes simplex virus type-1 (HSV-1) and type-2 (HSV-2). As these glycoproteins are known to be constituents of the virion envelope (Spear & Roizman, 1972; Olshevsky & Becker, 1972; Roizman & Furlong, 1974; Sarmiento et al, 1979), it is necessary to purify the herpes virions with intact envelopes. Purification of live virus is a means of ensuring that the envelopes are intact because the virion envelope is essential for infectivity (Stein et al, 1970; Sarmiento et al, 1979). However, the envelope of HSV is particularly

labile (Gentry & Randall, 1973), and so it was necessary to find a suitable medium for preserving the virus in its infectious state during the purification procedure (Chapter 1.2.5).

Water has been used for many years as the suspending medium of choice for herpesvirus (Munk & Ackermann, 1953; Cook & Sears, 1970) and this has been confirmed by more recent tests (Nerurkar et al, 1983). A 1 M Na_2SO_4 solution has also been shown to be a suitable medium for maintaining the infectivity of HSV, particularly at raised temperatures (Wallis & Melnick, 1965). Therefore, distilled water, 1 M Na_2SO_4 solution and the cell growth medium (10% MEM) were tested for their ability to maintain HSV in its infective state when stored at 4°C.

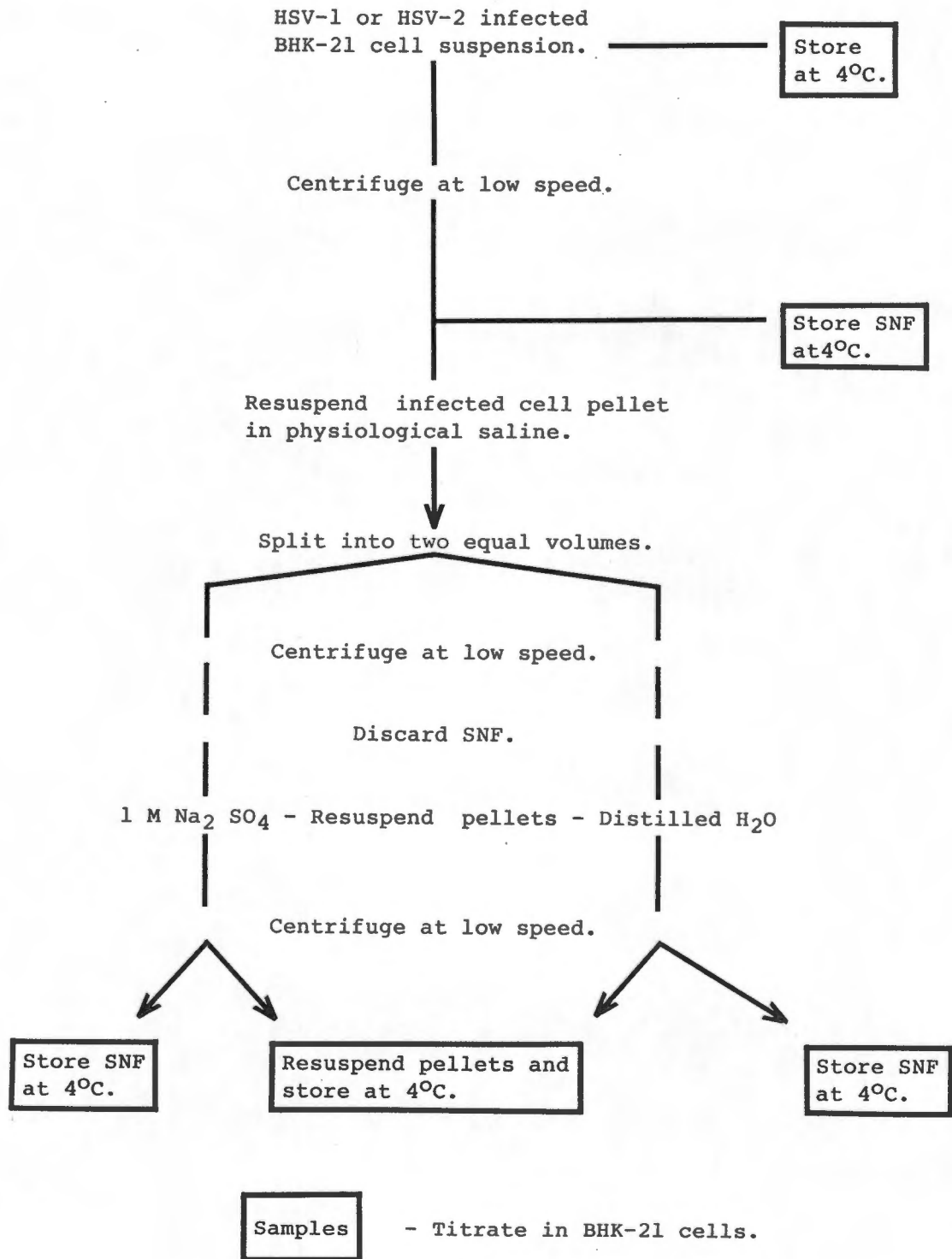
The procedure used is illustrated in the Flow diagram (Figure 1.4) and was as follows:

- (a) BHK-21 cell cultures were infected with HSV-1 and HSV-2 and harvested at 24 hours by freezing and thawing (40 ml of each infected cell suspension).
- (b) The infected cell suspensions were collected and 10 ml of each of HSV-1 infected and HSV-2 infected cell suspensions were removed and stored at 4°C.
- (c) The remaining suspension was centrifuged at 10 K rpm

for 10 min to sediment the cellular material and 10 ml of supernatant fluid was removed from each tube and stored at 4°C.

- (d) The pellets of cellular material were resuspended in 10 ml of physiological saline. Each of these suspensions was divided into two equal amounts (i.e. to give 4 separate suspensions).
- (e) These suspensions were centrifuged at 10 K rpm for 10 min and the supernatant fluids discarded. One of the HSV-1 infected pellets and one of the HSV-2 infected pellets were each resuspended in 10 ml of 1 M Na₂SO₄ solution while the remaining pellet of each were resuspended in 10 ml of distilled H₂O.
- (f) All four of these suspensions were centrifuged at 10 K rpm for 10 min. 5 ml of each supernatant fluid was removed and stored at 4°C.
- (g) The pellets were resuspended in the remaining supernatant fluid and stored at 4°C.
- (h) 0,1 ml aliquots were removed from each of the 12 stored samples at various intervals and titrated on BHK-21 cell cultures (Appendix 2.1.8).

FIGURE 1.4 FLOW DIAGRAM - STABILITY OF HSV IN DIFFERENT MEDIA.



Results

Live HSV could be detected for up to 150 days when the virus was stored in 10% MEM at 4°C (Figure 1.5(a)). However, during this time, the amount of live virus decreased by approximately 99%. Using distilled water and 1 M Na₂ SO₄ solution, live virus could be detected for between 50 and 90 days, with the loss of infectivity varying according to the virus type and the suspending medium (Figure 1.5(b) and (c)).

The effectiveness of these different media for maintaining the infectivity of HSV when stored at 4°C was compared by calculating the survival rate over a seven week period (Table 1.3). A summary of the results in Table 1.3 is given in Table 1.4. These results reveal the following :-

1. The media, in order of their ability to preserve the infectivity of HSV at 4°C, are :-

(a) For HSV-1: Dist. H₂O > 10% MEM > 1 M Na₂ SO₄.

(b) For HSV-2: 1 M Na₂ SO₄ > 10% MEM > Dist. H₂O.

(Table 1.3).

2. The overall ability of each medium to preserve the infectivity of both HSV types was :

Dist. H₂O > 1 M Na₂ SO₄ > 10% MEM (Table 1.4).

3. In 10% MEM and 1 M $\text{Na}_2 \text{SO}_4$, HSV-2 was considerably more stable than HSV-1. In distilled water, HSV-1 was more stable than HSV-2.

4. Using 10% MEM and 1M $\text{Na}_2 \text{SO}_4$, both HSV types were more stable when cell debris was present. The opposite was found to be true for distilled water (Table 1.3).

TABLE 1.3 - APPROXIMATE SURVIVAL RATE OF HSV STORED IN VARIOUS SUSPENDING MEDIA AT 4°C

Virus Sample	Suspending Medium	Starting Conc. of HSV (d)	HSV conc after 7 weeks at 4°C (d)	Approx. Survival Rate (c)
HSV-1 with cells (a)	10% MEM	10 ^{4.35}	10 ^{3.45}	0.133
HSV-1 w-out cells (b)	10% MEM	10 ^{4.25}	10 ^{3.10}	0.07
HSV-2 with cells	10% MEM	10 ^{4.0}	10 ^{3.675}	0.473
HSV-2 without cells	10% MEM	10 ^{3.6}	10 ^{3.125}	0.335
HSV-1 with cells	Dist H ₂ O	10 ^{1.6}	0	0
HSV-1 without cells	Dist H ₂ O	10 ^{1.0}	10 ^{1.0}	1.0
HSV-2 with cells	Dist H ₂ O	10 ^{2.4}	10 ^{1.25}	0.071
HSV-2 without cells	Dist H ₂ O	10 ^{1.5}	10 ^{1.25}	0.562
HSV-1 with cells	1 M Na ₂ SO ₄	10 ^{3.0}	10 ^{1.0}	0.01
HSV-1 without cells	1 M Na ₂ SO ₄	10 ^{2.5}	0	0
HSV-2 with cells	1 M Na ₂ SO ₄	10 ^{3.0}	10 ^{3.0}	1.0
HSV-2 without cells	1 M Na ₂ SO ₄	10 ^{3.0}	10 ^{2.5}	0.316

- a) With cells. BHK-21 cell debris was left in the virus suspension.
- b) Without cells. The virus suspension was centrifuged at 10K rpm for 10 min to sediment the cell debris. The supernatant fluid was retained and stored.
- c) Survival rate = $\frac{\text{HSV concentration after 7 weeks}}{\text{Starting concentration}}$
- (d) Units = TCID₅₀/0.1 ml.

TABLE 1.4 - A SUMMARY OF RESULTS SHOWN IN TABLE 1.3

Virus	Suspending Medium	Average Survival Rate for Each Virus Type (a)	Survival Rate for HSV in Each Medium (b)
HSV 1	10% MEM	0.102	0.253
HSV-2	10% MEM	0.404	
HSV-1	Dist H ₂ O	0.5	0.41
HSV-2	Dist H ₂ O	0.317	
HSV-1	1M Na ₂ SO ₄	0,005	0,332
HSV-2	1M Na ₂ SO ₄	0,658	

(a) Values calculated by averaging the survival rate with cells and the Survival Rate without cells for each virus type in each of the different media (See Table 1.3).

(b) Values obtained by averaging the values in (a).

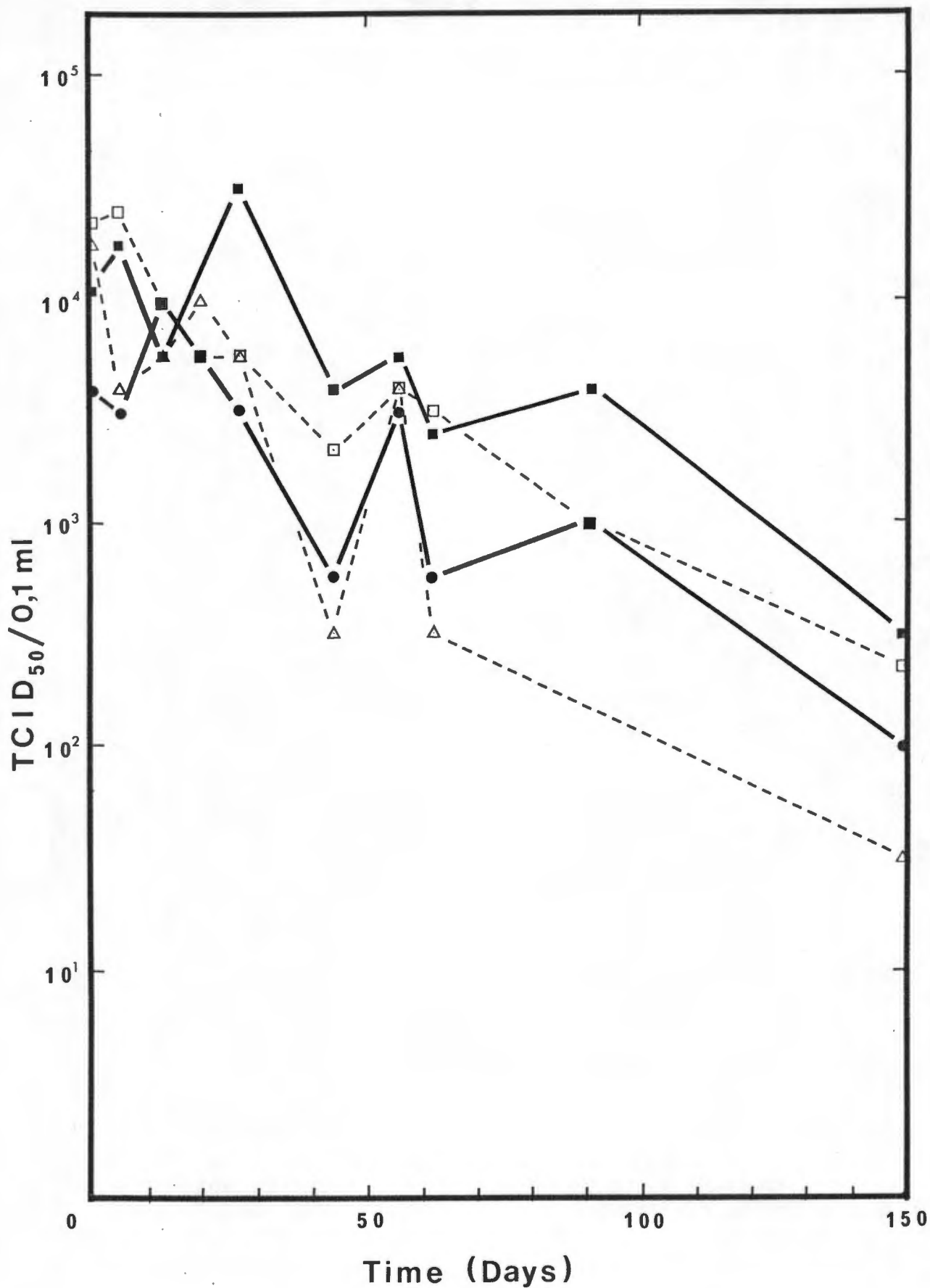


Figure 1.5 (a) Storage of HSV-1 and HSV-2 in 10% MEM at 4°C.

HSV-1 with cells (□- - -□) and without cells (Δ- - -Δ).

HSV-2 with cells (■—■) and without cells (●—●).

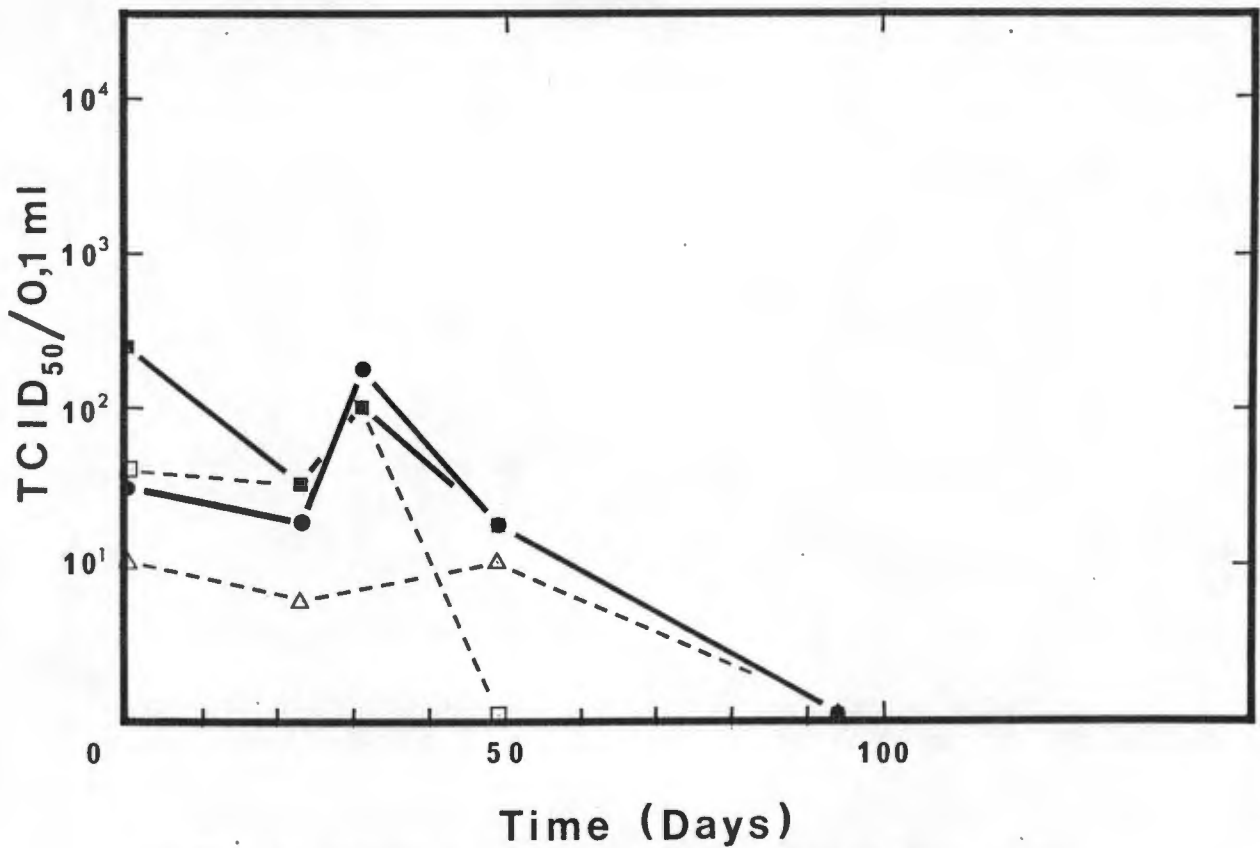


Figure 1.5 (b) Storage of HSV-1 and HSV-2 in distilled water at 4°C.

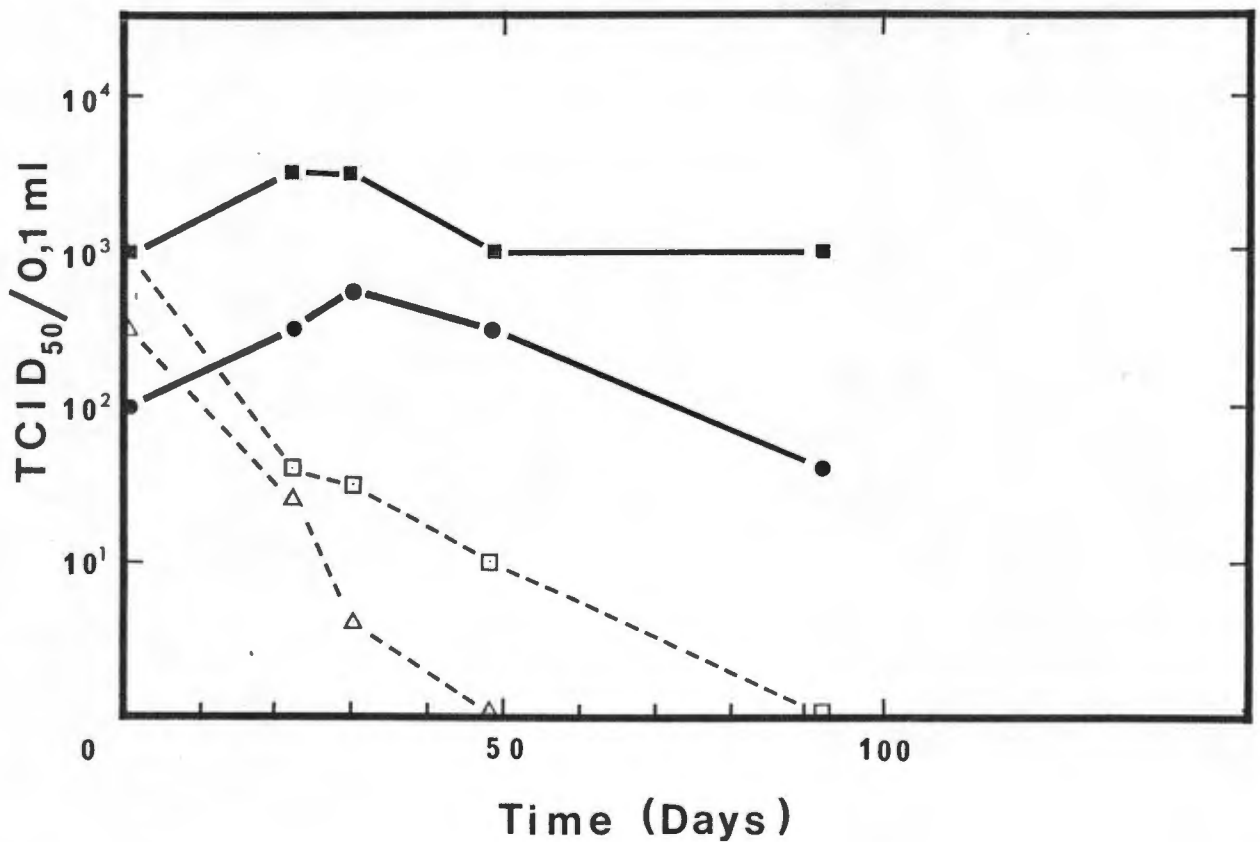
HSV-1 with cells (□--□) and without cells (Δ--Δ).

HSV-2 with cells (■—■) and without cells (●—●).

Figure 1.5 (c) Storage of HSV-1 and HSV-2 in 1M Na₂SO₄ at 4°C.

HSV-1 with cells (□--□) and without cells (Δ--Δ).

HSV-2 with cells (■—■) and without cells (●—●).



Discussion

The results of this experiment demonstrate that the two HSV types were suited to different suspending media for the preservation of their infectivity at 4°C (Figure 1.5(a-c), Table 1.3). However, by taking an average value for the survival rate of both HSV types in each medium, distilled water is found to be the best medium for the storage of HSV at 4°C (Table 1.4). The next best medium was 1 M Na₂ SO₄, with 10% MEM giving the lowest survival rate. However, it is felt that this result cannot be taken as an accurate comparison of the ability of these media to preserve HSV in its infective state because of the difference in starting concentration of virus in each medium. For instance, approximately 100 times more virus was stored in 10% MEM than in the other two media.

A comparison was made of the effect of storing the virus in the presence of cellular material and without it. The reason for this was to determine whether the presence of cellular material caused any protective effect on the virus. The results showed that in 10% MEM and 1 M Na₂ SO₄ solution, both HSV types were more stable in the presence of cellular material than without it, whereas the opposite was true in distilled water. The protective effect of cellular material was to be expected from pre-

vious reports (Gentry & Randall, 1973; Rawls, 1979; Lancz & Sample, 1985). However, the reasons for the observations using distilled water are not clear. A possible explanation is that the "cell free material" did in fact contain a considerable quantity of cellular debris. This is shown in the electron micrographs of the virus taken at a later stage of purification (Figure 1.9).

Conclusion

The three media used in this experiment are all able to preserve the virus in its infective state over quite considerable periods, when stored at 4°C.

1.2.4. Experiment to test different methods for the optimum release of infectious HSV from BHK-21 cells.

Herpes simplex virus is a very cell associated virus which is very difficult to extract from its host cells. Therefore, the first step in purifying the virus was to establish a suitable means of extracting the virus from the host cells without causing excessive damage to the virions. HSV is relatively heat labile, and so, as far as possible, all of the procedures described below were performed at approximately 4°C in an effort to prevent loss of infectivity.

Three different methods of extracting HSV-2 were compared in this experiment. These were:

- 1) Freezing and thawing
- 2) A Dounce homogenizer, and
- 3) Ultrasonication.

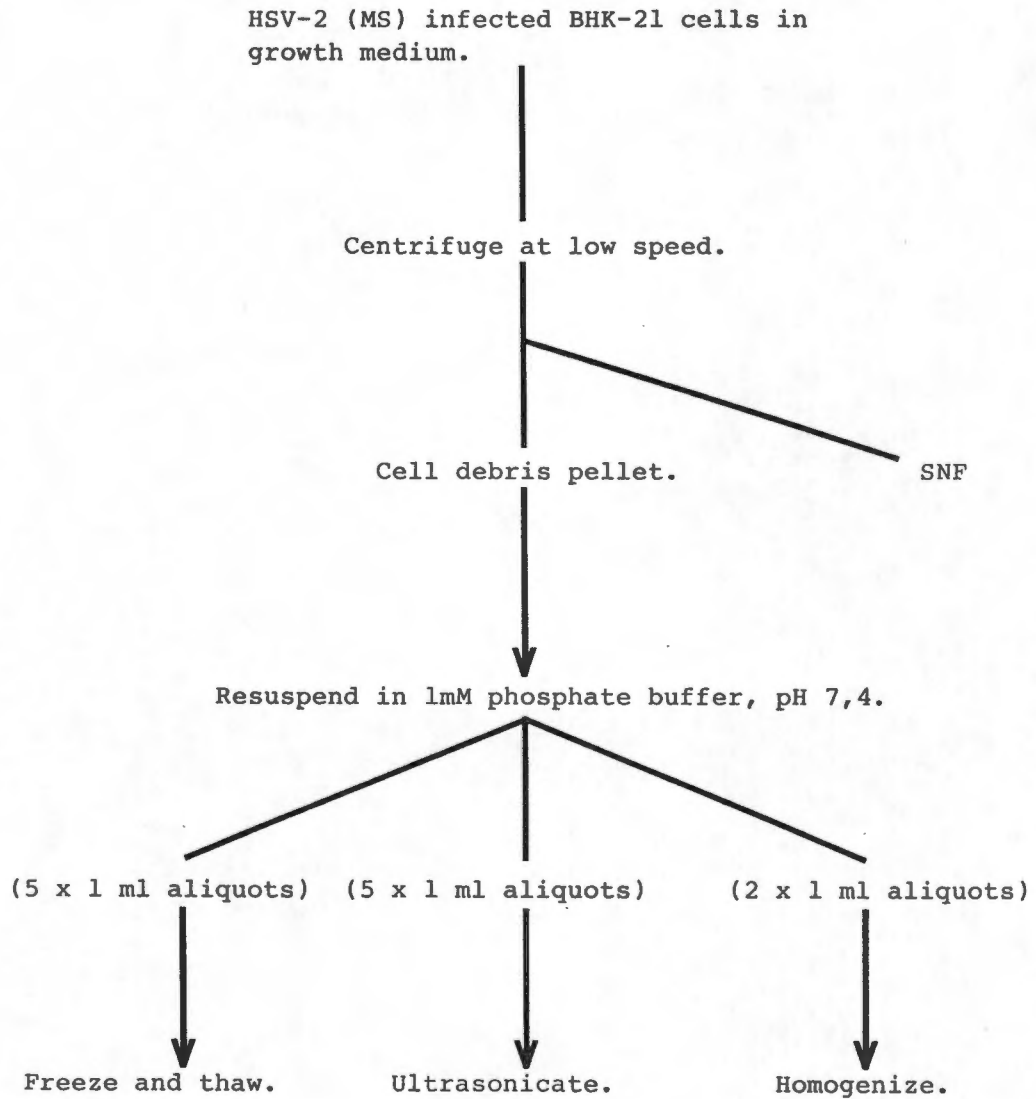
The experimental procedure is illustrated in the flow diagram in Figure 1.6.

Method

- (a) One medical flat bottle of HSV-2 infected BHK-21 cells was harvested at 24 hours post-infection by freezing and thawing. The cell suspension was collected and spun at 10 K rpm for 10 minutes.
- (b) The supernatant fluid was removed and stored at 4°C and the pellet of cellular debris was resuspended in 15 ml of 1mM phosphate buffer, pH 7.4.
- (c) The pellet was mixed thoroughly and then divided into 12 separate aliquots of 1 ml each. These aliquots were numbered 1-12.
- (d) Aliquots 1-5 were frozen and thawed from one to five times, respectively, and then stored at 4°C.
- (e) Aliquots 6-10 were ultrasonicated for 5, 10, 15, 20 and 30 seconds, respectively (Appendix 2.1.9), and then stored at 4°C.

- (f) Aliquots 11 and 12 were homogenized 5 and 10 times respectively and stored at 4°C.
- (g) Each of the aliquots were titrated on BHK-21 cells (Appendix 2.1.8) in order to determine which treatment gave the most efficient release of infectious virus.

FIGURE 1.6 FLOW DIAGRAM - EXTRACTION OF HSV FROM INFECTED CELLS.



Titrate samples (See Figure 1.7 and Table 1.5)

Results

Titration of the products obtained by the different extraction procedures investigated demonstrated that the highest titre of infectious HSV was released by ultrasonication for 10 seconds (Figure 1.7 and Table 1.5). Ultrasonication for 5 seconds and 15 seconds were also shown to give a satisfactory release of virus.

The amount of infectious virus released by freezing and thawing was significantly less than by ultrasonication, and the titre of infectious virus decreased with the number of cycles of freezing and thawing.

Homogenizing the cells gave a yield of infectious virus of the same order as freezing and thawing.

Discussion

Ultrasonication of HSV-2 infected BHK-21 cells was shown to be the most effective means of extracting the virus from infected cells. Under the conditions used in this experiment, ultrasonication for 10 seconds was found to give the optimum yield of infectious virus, while ultrasonication for 5 seconds and 15 seconds gave the next best yields (Table 1.5). It would seem that the time of ultrasonication required for the optimum release of infectious HSV depends on a compromise between the amount

of virus released from the infected cells and the rate at which the virion envelopes are disrupted by the ultrasound. This is because the envelope of HSV, which is easily broken (Cook & Stevens, 1970; Spear & Roizman, 1972; Gentry & Randall, 1973) and which is required for viral infectivity (Stein et al, 1970; Abodeely et al, 1970; Norrild et al, 1978(a)) is likely to be damaged by the physical stresses caused by ultrasonication.

Likewise, the observation that the amount of infectious virus decreased with each cycle of freezing and thawing would indicate that this procedure severely disrupts the virus envelope.

TABLE 1.5 - AMOUNT OF INFECTIOUS HSV RELEASED USING VARIOUS EXTRACTION PROCEDURES (See Figure 1.7).

EXTRACTION PROCEDURE USED	AMOUNT OF HSV RELEASED FROM CELLS (TCID ₅₀ /0.1 ml)
Starting material - infected BHK-21 cells in 10% MEM	10 ^{4.63}
Supernatant fluid after centrifugation at 10 K RPM for 10 min	10 ^{3.5}
Infected cell pellet resuspended in phosphate buffer	10 ^{3.83}
Freeze and thaw x 1 x 2 x 3 x 4 x 5	10 ^{4.17} 10 ^{3.5} 10 ^{3.17} 10 ^{2.5} 10 ^{2.83}
Ultrasonicate x 5 sec x 10 sec x 15 sec x 20 sec x 30 sec	10 ^{4.83} 10 ^{5.17} 10 ^{4.83} 10 ^{2.5} 10 ^{1.5}
Homogenize x 5 x 10	10 ^{3.75} 10 ^{4.17}

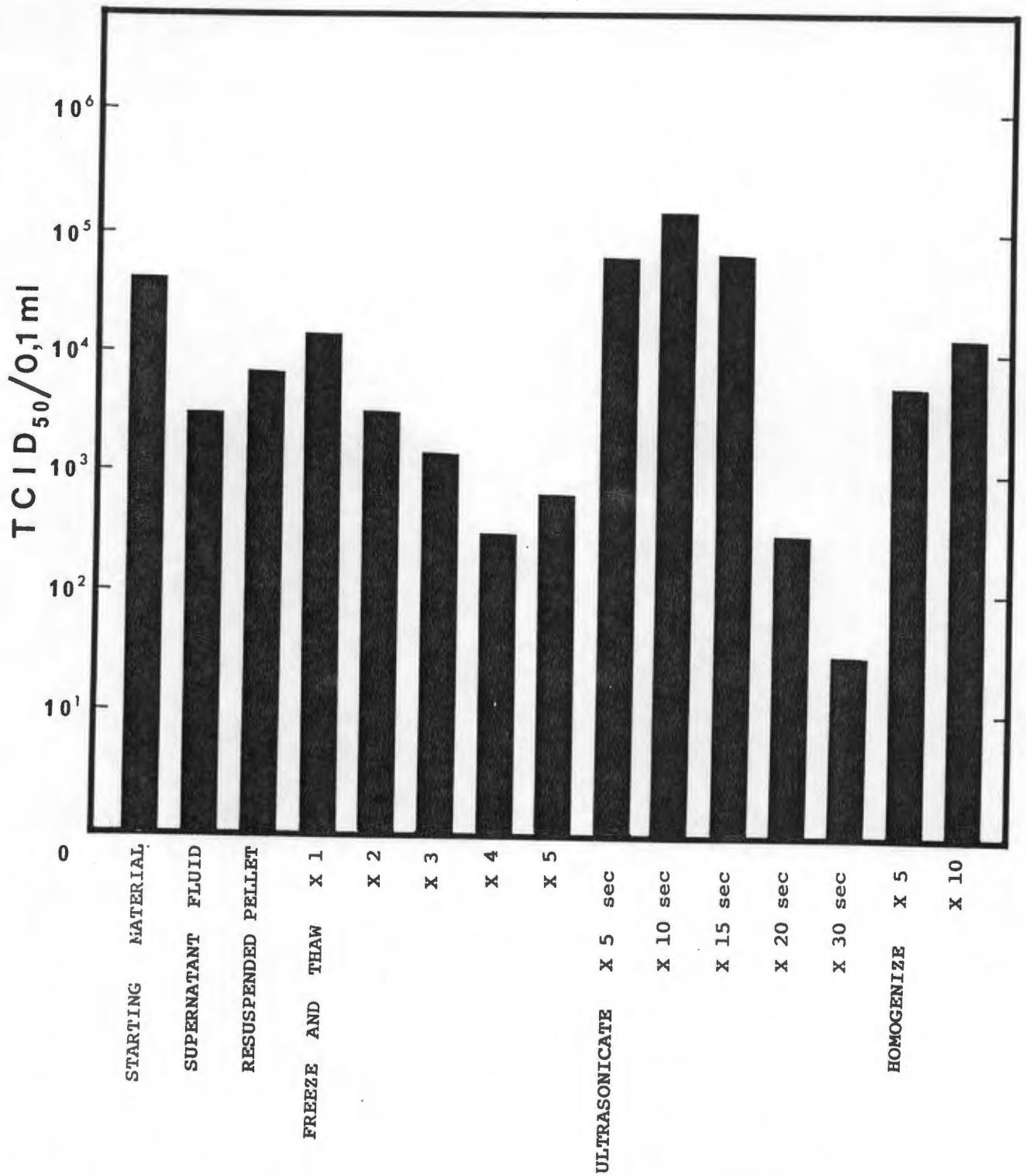


Figure 1.7 Chart showing the amounts of infectious HSV-2 extracted from BHK-21 cells using different procedures.

1.2.5. Herpes simplex virus purification by caesium chloride density gradient centrifugation

At the start of this work it was thought that a pure preparation of herpes simplex virus would be necessary in order to distinguish and purify HSV specific proteins. For this reason, caesium chloride (CsCl) density gradient centrifugation was used to further purify HSV-1 which had been extracted from BHK-21 cells (Chapter 1.2.4).

Method

The procedure for purifying HSV-1 by caesium chloride density gradient centrifugation is described in detail in Appendix 2.1.11. Briefly, HSV-1 infected growth medium was centrifuged at 25 K rpm for 120 minutes to sediment the virus into a cushion of CsCl solution at the bottom of the tube. After centrifugation, the growth medium was discarded and the CsCl cushion was incorporated in equal amounts into two pre-formed CsCl density gradients (Figure 2.1.3). These two density gradients were then centrifuged at 40 K rpm for 20 hours at 4°C. After centrifugation, the two light scattering bands (Figure 1.8) were removed from one of the two gradients using a pasteur pipette. These two bands were centrifuged to form pellets which were stained and viewed in the electron microscope (Appendix 2.1.12). The second density gradient was fractionated into 15 drop fractions and each

fraction was titrated on BHK-21 cells to determine the position of infectious HSV particles (Appendix 2.1.8). The refractive index of each fraction was measured with an Abbé Refractometer, and the density determined by reference to a graph of refractive index against density (Appendix 2.1.10, Figure 2.1.2).

Results

After CsCl density gradient centrifugation of HSV-1 infected material at 40 K rpm for 20 hours, two light scattering bands were visible when the tubes were viewed under a fluorescent light (Figure 1.8). When the contents of these bands were viewed in the electron microscope (Appendix 2.1.12), the less dense (top) band was shown to contain enveloped virions (Figure 1.9(a)), while the more dense (bottom) band contained HSV related spherical particles (Figure 1.9(b)). However, both of these bands also contained a large amount of cellular material.

Titration of fractions from the second tube showed a peak of HSV infectivity within a density range of between $1,2225 \text{ g/cm}^3$ and $1,2775 \text{ g/cm}^3$ (Figure 1.10).

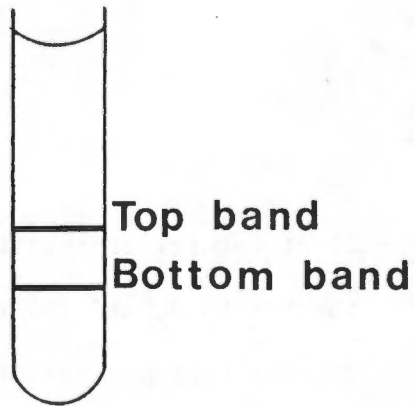
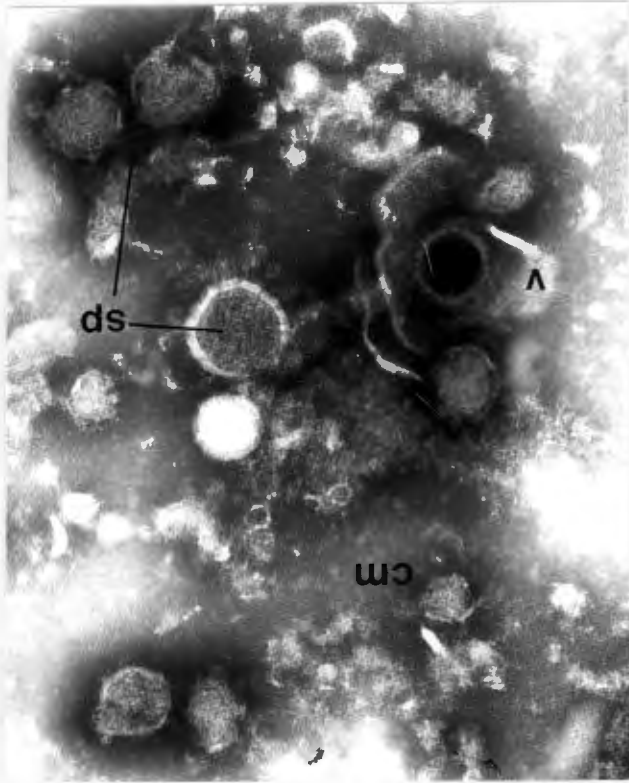


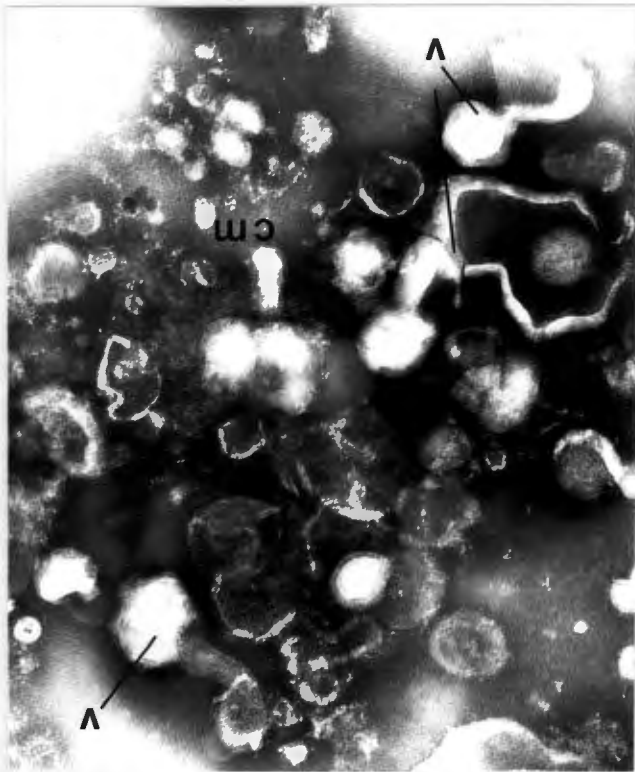
FIGURE 1.8 - Two light scattering bands formed when HSV-1 was centrifuged on CsCl density gradients at 40 K rpm for 20 hours.

FIGURE 1.9 - Electron micrographs of material from (a) the top band, and (b) the bottom band of HSV-1 infected material after CsCl density gradient centrifugation at 40 K rpm for 20 hours (Figure 1.8).

The top band contained enveloped virions (V) and a significant amount of cellular material (cm). The bottom band consisted mainly of HSV related spherical particles (sp) and cellular material (cm). A comparison can be made between a virion (V) contained in the bottom band and the spherical particles. (Magnification x 100,000)



p



a

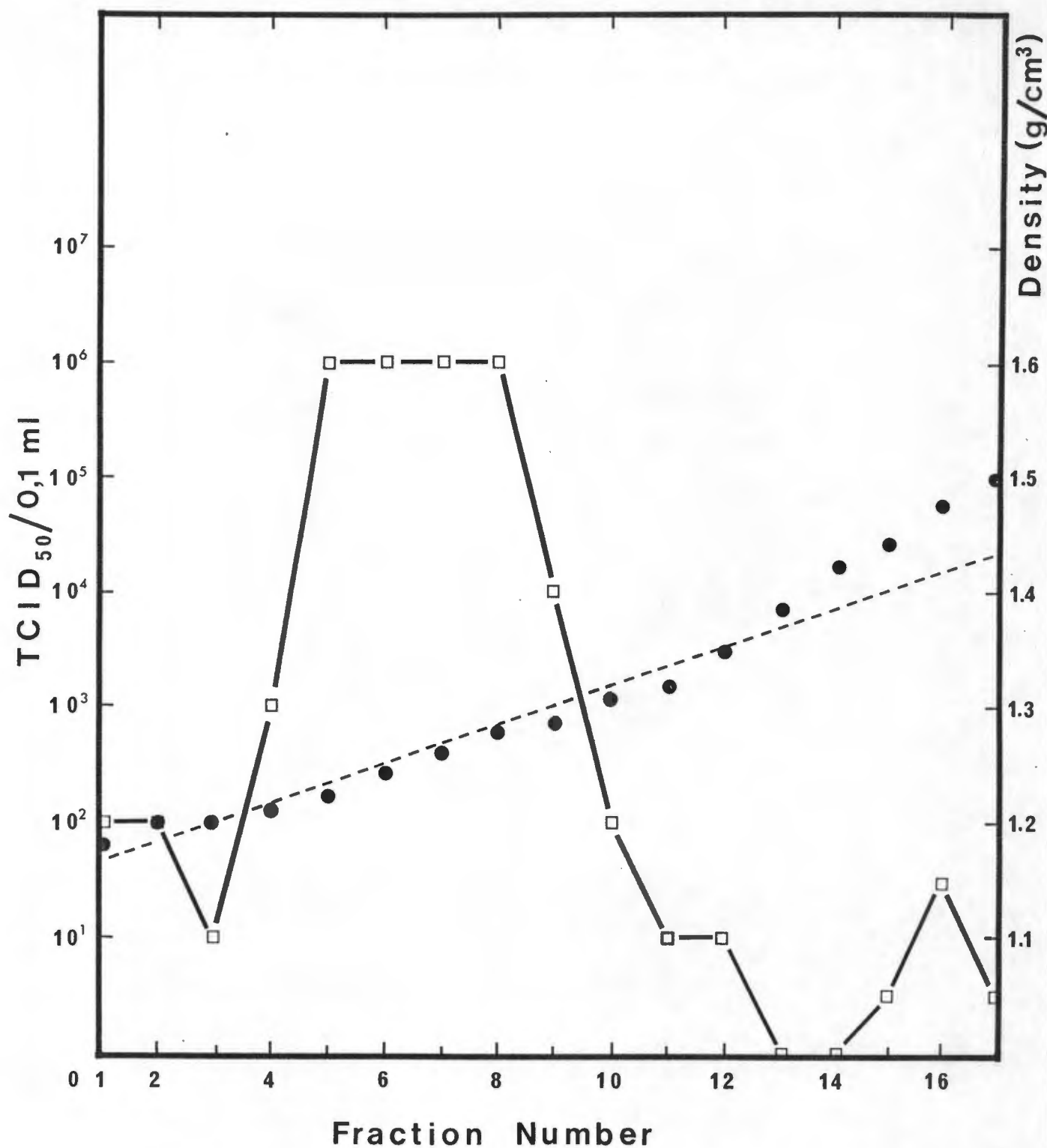


Figure 1.10 HSV-1 extracted from BHK-21 cells was centrifuged on a caesium chloride (CsCl) density gradient (1.2 - 1.5 g/cm³) at 40K rpm for 20 hours. After centrifugation, the CsCl density gradient was fractionated into 17 fractions. Each fraction was titrated for infectious virus (□—□) and the refractive index (RI) of each fraction was read on an Abbé refractometer. The density of the fractions (● ●) was obtained from the graph of RI against Density (Figure 2.1.2).
 (----) - Best line through the density values.

Discussion

Caesium chloride density gradient centrifugation of HSV-1 extracted from BHK-21 cells yielded two bands when viewed under fluorescent light. Electron microscopy revealed that these bands contained enveloped virions and HSV related spherical particles respectively (Figure 1.9). However, both bands also contained a significant amount of host cell material which indicated that this method would not be suitable for purifying HSV.

The HSV related spherical particles found in the more dense (bottom) band (Figure 1.9(b)) were not investigated further but it should be noted that other workers have also reported undefined structures in cells infected with various representatives of the herpes virus group (Abraham & Teftmeyer, 1970). Some of these structures have been described as dense bodies (Cooks & Stevens, 1968), bizarre viral forms (Nii, Morgan & Rose, 1968), and electron-dense spherical particles (Stannard, 1970). Little is known about the composition of these structures.

The peak of HSV infectivity was within a density range of between $1,2225 \text{ g/cm}^3$ and $1,2775 \text{ g/cm}^3$ (Figure 1.10). Previously reported values for the bouyant density of HSV-1 in CsCl lie within the range of $1,234 \text{ g/cm}^3$ to

1,285 g/cm³ (Gentry & Randall, 1973). Spring & Roizman report that CsCl density gradient centrifugation of HSV yielded two bands, with the virion containing band having a bouyant density of 1,27 g/cm³ (Spring & Roizman, 1967).

It has previously been reported that CsCl solution causes disruption of the herpes virions (Spring & Roizman, 1967) and that it is necessary to stabilize the virions by treatment with formaldehyde prior to centrifugation in CsCl. However, in the present work, the virus remained viable with the incorporation of 1% foetal calf serum to the CsCl solution (Roizman & Roane, 1964; Spring & Roizman, 1967; Lancz & Sample, 1985).

1.3 SUMMARY

Baby hamster kidney (BHK-21) cells were chosen as a suitable host for the production of HSV-1 and HSV-2 for the following reasons :

- (a) The cells grew well in 10% MEM at 37°C and were shown to be free of mycoplasma infection. The presence of mycoplasma in the host cells may have interfered with the aims of the present work.
- (b) Both HSV-1 and HSV-2 grew well in BHK-21 cells, with a good yield of infectious virus.

On account of the lability of HSV, it was necessary to find a suitable medium for storing the virus which would minimize the loss of infectivity during the extraction procedures. Distilled water and 1 M Na₂ SO₄ solution were found to be the best media for the preservation of HSV infectivity (Table 1.4). 10% MEM maintained the infectivity of HSV for at least 22 weeks when stored at 4°C (Figure 1.5(a)) and was used for storing HSV during the extraction of virus from infected cells.

Three different methods of extracting virus from infected cells were compared and, of these, ultrasonication was found to be the most effective method (Figure 1.7). A time of 10 seconds was found to give the optimum yield of infectious virus under the conditions tested. This is a compromise be-

tween the maximum release of virus from cells and the minimum disruption of released virions.

CsCl density gradient centrifugation of HSV-1 provided concentrated, live herpes virus with a significant amount of cellular products.

CHAPTER II: RADIOLABELLING AND ANALYSIS OF HSV PROTEINS

2.1 INTRODUCTION

2.1.1 Labelling of Viruses with Radioactive Isotopes

The extreme sensitivity of detection of radioisotopic tracers has been exploited to great advantage by virologists. Since the tracer experiments of Cohen in 1948, viruses have been isotopically labelled to study their composition, sites of synthesis, replication, recombination and assembly.

Viral protein can be isotopically labelled by providing the host cells with labelled amino acids or some other structural component of the protein required (e g labelled glucosamine for glycoproteins) in the growth medium, for uptake into the intracellular pool. The choice of amino acid or structural substance is determined by the amount present in the viral protein under investigation and by its availability in high specific activity (Henry, 1967).

Analysis of purified HSV-1 by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has revealed that between 4 and 22 hours post-infection, at least four major glycoproteins and several minor ones are synthesized (Spear, Keller & Roizman, 1970; Heine et al,

1974). The four major HSV-1 glycoproteins are derived from four antigenically distinct precursor polypeptides which migrate at different rates to their product polypeptides in SDS-PAGE (Spear, 1976).

Several investigators have reported that after about four hours post-infection with HSV, the host cell ceases to produce its own proteins and is used entirely to produce viral proteins (Sydiskis & Roizman, 1968; Spear, Keller & Roizman, 1970; Heine, Spear & Roizman, 1972; Spear, 1976). It has also been reported that the major glycoprotein region of HSV-1 in SDS-PAGE, which is composed of glycoproteins gA/gB and gC (Eberle & Courtney, 1980) is the predominant glycoprotein region in both the virions and in HSV infected cells (Spear, 1975; Spear, 1976; Eberle & Courtney, 1980). Therefore, both because the viral glycoproteins can be specifically labelled and because purification of the virus results in a loss of much of the major glycoprotein region; there is no need to purify the virus before isolating the glycoproteins.

To further ensure the isotopic labelling of the HSV proteins without labelling cellular proteins, BHK-21 cell cultures should be infected with a high multiplicity of infection at confluency to ensure a minimum of cell growth after infection and to ensure that as many cells

as possible would initially be infected. Medium containing the radiolabelled substance should only be supplied at four hours post-infection, by which time the host cells should incorporate all of the label into the viral proteins. Then, in order to eliminate the presence of labelled precursor polypeptides, mentioned earlier, the radioactive medium should be removed from the cell cultures and replaced with normal (unlabelled) medium for the last four hours before harvesting the cells (i e from 24 to 28 hours post-infection).

2.1.2 Analytical sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) is the major technique used for analyzing the proteins from herpes simplex virus (HSV) infected cells in the present work. The technique was originally described by Ornstein & Davis (Ornstein, 1964; Davis, 1964) and is now the most widely used method for determining the molecular weights of proteins. The negatively charged SDS molecules bind extensively to the polypeptide chains of proteins causing them to unfold and dissociate. The molecular weights of the constituent polypeptide chains can be determined by comparing their electrophoretic mobilities on SDS-polyacrylamide (PAA) gels to the mobilities of well characterized marker pro-

teins of known molecular weights. The procedure is simply and rapidly carried out and requires only microgram amounts of proteins.

The relationship between the mobility of a protein and its molecular weight was first observed in an SDS-phosphate buffer system, in which log. molecular weight was found to be inversely related to mobility (Shapiro, Vinuela & Maizel, 1967). This observation has since been confirmed by other workers using continuous and discontinuous buffer systems (Laemmli, 1970; Payne, 1973). Therefore, it is possible to obtain the unknown molecular weight of a protein from its mobility on PAA gel, using a plot of the mobilities of known marker proteins against the logarithm of their molecular weights (Chapter 2.2.1).

For any given PAA gel, the relationship between the mobility of proteins and their molecular weights does not apply within the higher and lower molecular weight ranges. It is, therefore important to use PAA gels of optimum porosity (acrylamide concentration) for an accurate determination of molecular weight. The most suitable acrylamide concentrations for the different molecular weight ranges are : 15% PAA - m.wt. below 40,000; 10% PAA - m.wt. 10,000 - 70,000; 5% PAA - m.wt 25,000 - 250,000 (Payne, 1976).

The use of SDS-PAGE for determining the molecular weights of polypeptides is dependent on a mechanism for SDS-protein interactions which is common to all proteins, and such a mechanism has been demonstrated. It has been shown that a variety of different proteins bind 1,4 grams of SDS per gram of protein when treated with an SDS-monomer concentration above 0,02% ($8 \times 10^{-4}M$) (Pitt-Rivers & Impiombato, 1968; Reynolds & Tanford, 1970). This corresponds to approximately one SDS molecule to two amino acid residues (Payne, 1976).

Protein/SDS complexes appear to behave as rodlike particles in which the length of the particle is proportional to the molecular weight of the polypeptide chain (Reynolds & Tanford, 1970; Fish, Reynolds & Tanford, 1970). However, these are not necessarily fully extended molecules but may contain a high degree of secondary structure.

Glycoproteins have been shown to have a decreased SDS binding ratio which generally leads to decreased electrophoretic mobility and incorrect molecular weight determinations (Pitt-Rivers & Impiombato, 1968). For this reason, the molecular weights of glycoproteins determined by SDS-PAGE are referred to as apparent molecular weights.

The presence of disulphide bonds limits the unfolding of polypeptide chains which may lead to decreased SDS-binding and lower electrophoretic mobilities (Pitt-Rivers & Impiombato, 1968; Reynolds & Tanford, 1970). However, it is possible to break the disulphide bonds by treating proteins with SDS in the presence of 2-mecaptoenthanol as a reducing agent.

2.2 METHODS AND RESULTS

2.2.1 Calibration curves established using SDS-PAGE of mixtures of known molecular weight marker proteins on 7,5% and 10% PAA gels.

In order to analyse and determine the molecular weights of proteins which were separated by analytical SDS-PAGE (Appendix 2.2.1) it was necessary to establish calibration curves by electrophorezing proteins of known molecular weights on both 7,5% and 10% PAA gels, which were the gel concentrations used in the present work. This was done as follows:-

- (a) Mixtures comprised of high molecular weight (HMW) marker proteins or low molecular weight (LMW) marker proteins (Appendix 1.2.1(d)) were treated as specified. 2 μ l, 3 μ l, 4 μ l and 5 μ l amounts of each mixture were electrophoresed separately on both 7,5%

and 10% PAA gels (Appendix 2.2.1) to determine the optimum amount required for a satisfactory result (Figure 2.2).

- (b) After electrophoresis, the gels were fixed (Appendix 2.2.1(d)) and then stained with Coomassie brilliant blue (CBB) stain (Appendix 2.2.2(a)).
- (c) The distances from the beginning of the running gel to each of the protein bands and to the dye marker were measured (Figure 2.1) and the Rf value for each protein was calculated using the equation:-

$$Rf = \frac{\text{Distance travelled by protein (mm)}}{\text{Distance travelled by dye marker (mm)}}$$

- (d) Using the known molecular weights of the marker proteins (Appendix 1.2.3) and the Rf values obtained from the PAA gels, graphs were drawn of log. molecular weight against Rf (Figure 2.3 and 2.4). From these graphs it is possible to determine the molecular weights of unknown proteins electrophoresed on 7,5% or 10% PAA gels, within the molecular weight ranges of these gels.

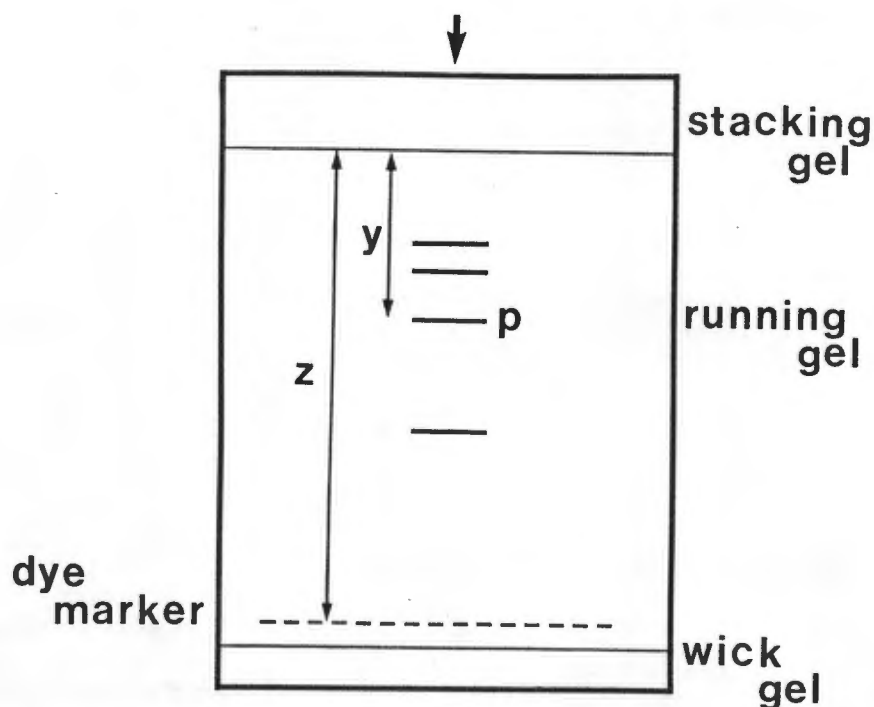


FIGURE 2.1 - Diagram illustrating the measurements required in order to calculate R_f for a particular protein p .

$$R_f = \frac{\text{Distance travelled by protein } (y)}{\text{Distance travelled by dye marker } (z)}$$

Results

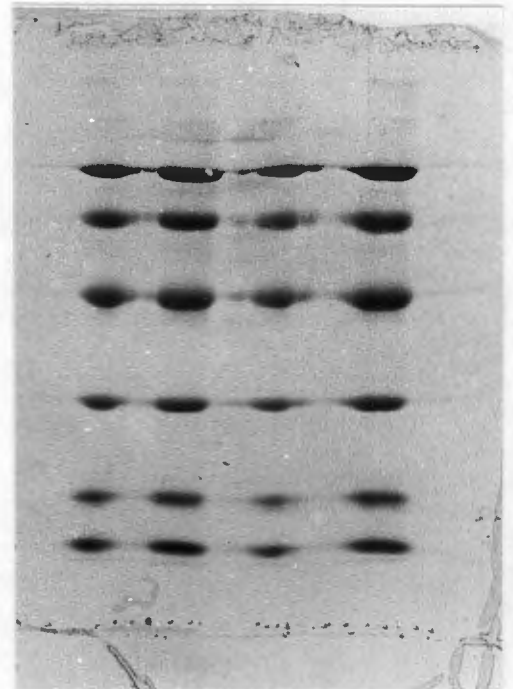
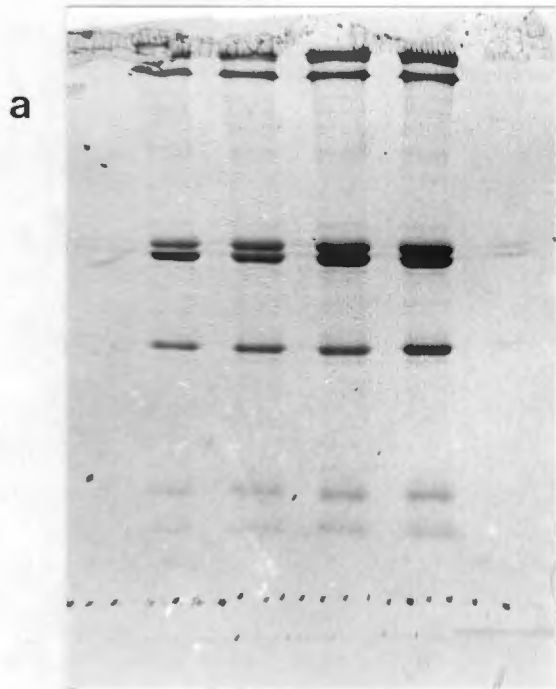
The results of electrophoresing high molecular weight and low molecular weight marker proteins on 7,5% and 10% PAA gels are given in Table 2.1 and 2.2 and Figure 2.3 and 2.4. The coefficient of correlation between the R_f values and the log. molecular weight values was 0,994 in both cases, showing a close correlation between the two parameters (Figure 2.3 and 2.4).



FIGURE 2.2 - High molecular weight (HMW) and low molecular weight (LMW) marker proteins were electrophorezed on (a) 10% PAA gel, and (b) 7,5 PAA gel. The gels were stained with Coomassie brilliant blue stain (Appendix 2.2.2.A).

HMW

LMW



HMW

LMW

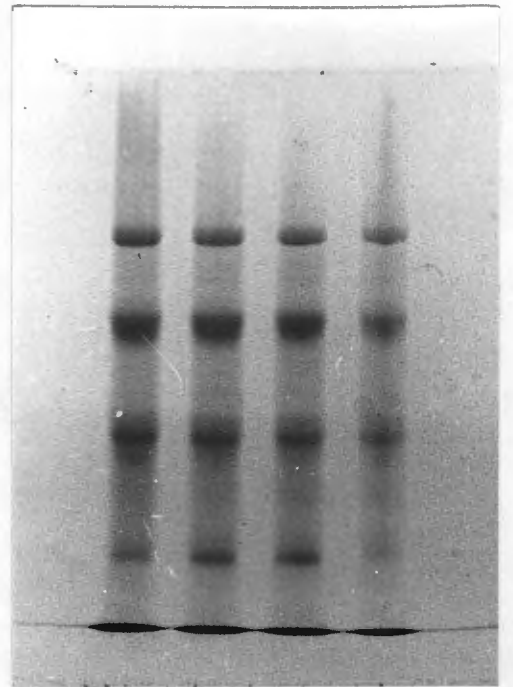
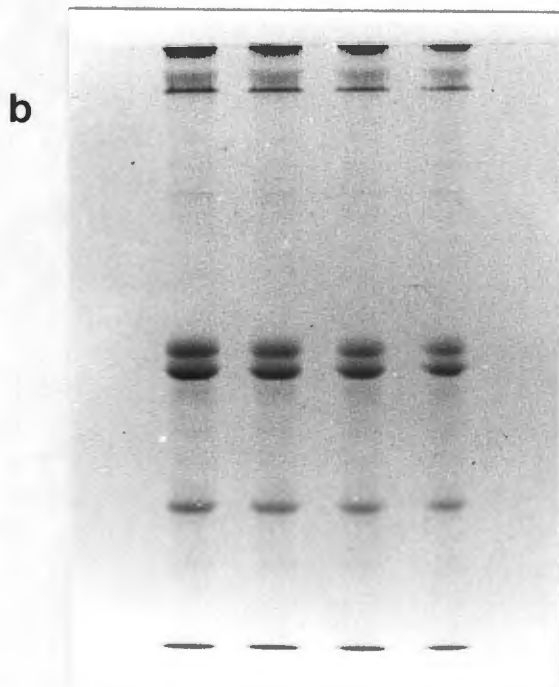


TABLE 2.1 - SDS-PAGE OF PROTEINS WITH KNOWN MOLECULAR WEIGHTS ON 10% PAA GEL

PROTEIN	MOLECULAR WEIGHT (m.wt.)	LOG m.wt.	AVE. DISTANCE MIGRATED (mm) (a)	Rf (b)
Thyroglobulin	330000	5.5185	3.25	0.015
Ferritin (Half Unit)	220000	5.3424	7.75	0.035
Albumin	67000	4.8261	72.50	0.331
Catalase	60000	4.7782	77.50	0.354
Lactate Dehydrogenase	36000	4.5563	114.40	0.522
Ferritin Subunit	18500	4.2672	174.50	0.796
Phosphorylase b	94000	4.9731	43.63	0.182
Albumin	67000	4.8261	63.63	0.266
Ovalbumin	43000	4.6335	98.25	0.411
Carbonic Anhydrase	30000	4.4771	142.50	0.596
Trypsin Inhibitor	20100	4.3032	183.25	0.766
α -Lactalbumin	14400	4.1584	204.75	0.856

(a) Distances measured from scanning densitometer scan of the PAA gels.

(b) $R_f = \frac{\text{Distance travelled by protein}}{\text{Distance travelled by phenol red}}$

TABLE 2.2 - SDS-PAGE OF PROTEINS WITH KNOWN MOLECULAR WEIGHTS ON 7.5% PAA GEL

PROTEIN	MOLECULAR WEIGHT (m.wt.)	LOG m.wt.	AVE. DISTANCE MIGRATED (mm) (a)	Rf
Thyroglobulin	330000	5.5185	- (c)	-
Ferritin (Half Unit)	220000	5.3424	4.85	0.067
Albumin	67000	4.8261	33.50	0.465
Catalase	60000	4.7782	36.0	0.50
Lactate Dehydrogenase	36000	4.5563	51.0	0.708
Ferritin Subunit	18500	4.2672	66.0	0.917
Phosphorylase b	94000	4.9731	19.0	0.279
Albumin	67000	4.8261	28.8	0.424
Ovalbumin	43000	4.6335	41.0	0.603
Carbonic Anhydrase	30000	4.4771	54.0	0.794
Trypsin Inhibitor	20100	4.3032)))) 61.5 (b)	0.904
α -Lactalbumin	14400	4.1584)	

(a) Distances measured directly from PAA gel.

(b) Proteins migrate as a single band.

(c) Distance migrated was too small to measure.

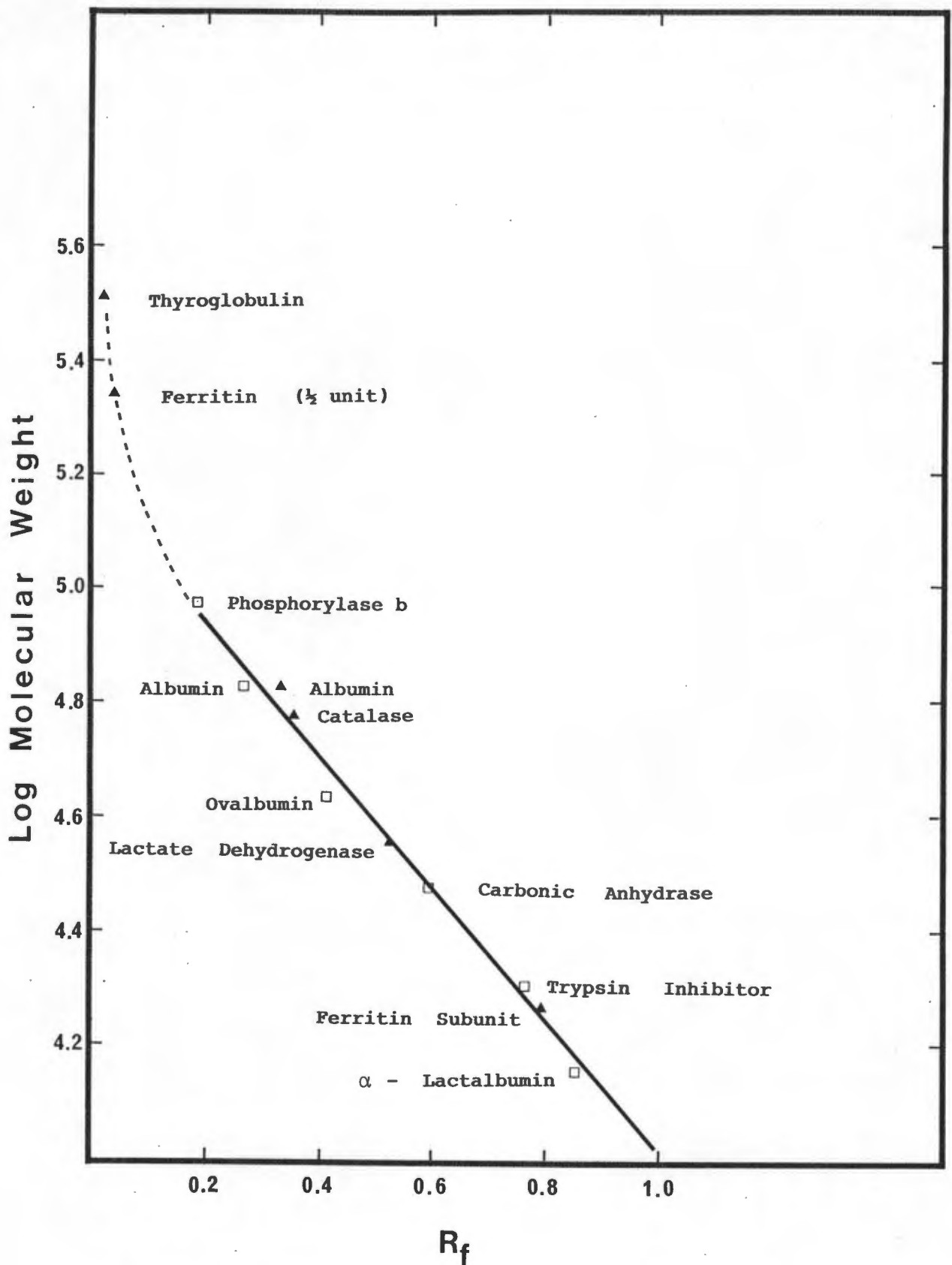


Figure 2.3 Calibration curve established using the HMW and LMW marker protein mixtures on a 10% PAA gel.
Coefficient of correlation = 0.994.

- (▲) - High molecular weight (HMW) protein markers.
- (□) - Low molecular weight (LMW) protein markers.

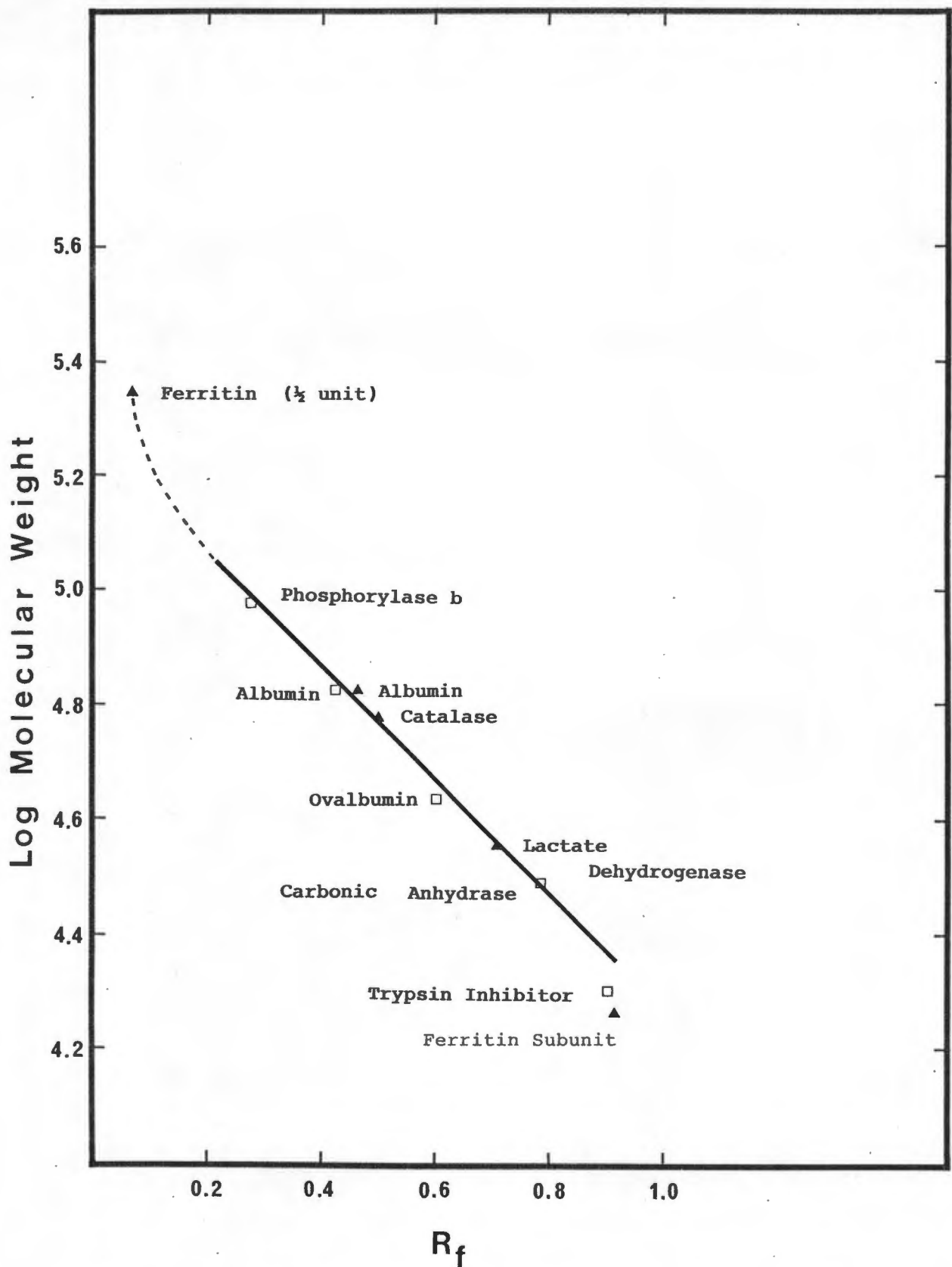


Figure 2.4 Calibration curve established using the HMW and LMW marker protein mixtures on a 7.5% PAA gel. Coefficient of correlation = 0.994.

- (▲) - High molecular weight (HMW) protein markers.
- (□) - Low molecular weight (LMW) protein markers.

2.2.2 Labelling of Herpes Simplex virus Type 1 (HSV-1) and Type 2 (HSV-2) with either (³H) - glucosamine or (¹⁴C) - amino acids.

- (a) BHK-21 cell cultures were grown in culture flasks until the cells were confluent. At confluency, the growth medium was discarded and the cells infected with HSV (either HSV-1 or HSV-2) at a multiplicity of infection of approximately 1,0 TCID₅₀ per cell. The inoculum was distributed over the entire cell monolayer so that as many cells as possible would be infected. The cell cultures were then incubated at 37°C for one hour to allow adsorption of virus to the cells.
- (b) After one hour, maintenance medium was added to the flasks for a further three hours at 37°C.
- (c) At four hours post-infection the maintenance medium was replaced with an equal volume of maintenance medium supplemented with either (³H)- glucosamine (2,8 µCi/ml) or (¹⁴C)-amino acids (2,8 µCi/ml). Incubation was continued at 37°C.
- (d) At 24 hours post-infection, the medium containing the radioisotope was replaced with an equal volume of normal (i e without radioisotopes) maintenance medium and incubation was continued at 37°C.

- (e) At 28 hours post-infection, the maintenance medium was discarded and the HSV infected cells were washed twice with cold, sterile PBS to remove any remaining medium.
- (f) The infected cell monolayers were then solubilized in LMW sample buffer (Appendix 1.2.1(d)). In order to completely solubilize the proteins, the solution was heated at 100°C for 10 minutes.
- (g) The solubilized material was stored at -70°C in ampoules containing 1 ml each.
- (h) The radiolabelled products were analysed by SDS-PAGE on both 7,5% and 10% PAA gels. After electrophoresis, the PAA gels were stained with either Coomassie brilliant blue stain (CBB) or silver stain (Appendix 2.2.2) and photographed (Figure 2.5 - 2.11). Identical gels, which were electrophorezed in parallel with the stained gels, were impregnated with EN³HANCE (Appendix 2.2.4) and dried (Appendix 2.2.3). Any radiolabelled proteins were identified by exposing Kodak X-Omat MA or Kodak X-Omat AR5 film to the dried gels (Figure 2.5 - 2.11).
- (i) The molecular weights of radiolabelled proteins were determined using calibration curves plotted using molecular weight markers which were electrophorezed simultaneously on the same PAA gels (Figures 2.5 - 2.11) (See Chapter 2.2.1).

Results

The radiolabelled products of HSV-1, HSV-2 and mock infected BHK-21 cells, which had been grown in the presence of either (³H)-glucosamine ((³H)-gln) or (¹⁴C)-amino acids ((¹⁴C)-aa), were analyzed by SDS-PAGE. The products were initially analyzed on 10% PAA gel before changing to 7,5% PAA gel which was more suitable for the molecular weight range of the HSV glycoproteins under investigation (Chapter 2.3).

The electrophoretic patterns produced using SDS-PAGE were similar for all the different radiolabelled products when the PAA gels were stained for protein (Figure 2.5 - 2.11). The most noticeable difference between the HSV infected cells and the uninfected cells was the presence of a characteristic polypeptide band, the major capsid protein (MCP), in both HSV-1 and HSV-2 infected cells, which was not present in the uninfected cells (Fig.2.5 - 2.11). The molecular weight of the MCP was calculated to be 132 K and 138 K for HSV-1 and HSV-2, respectively, on 10% PAA gel (Figure 2.5 & 2.6). On 7,5% PAA gel, the molecular weight of the MCP was calculated to be 153 K and 147 K for HSV-1 and HSV-2, respectively (Figure 2.11). (The discrepancy between the molecular weight values obtained on 10% and on 7.5% PAA gels is discussed in Chapter 2.3). Apart from the MCP, it was difficult to

distinguish any other HSV specific polypeptide bands using protein stain.

Although the products appeared very similar when stained for protein, autoradiography revealed that there were significant differences in the radiolabelled polypeptides of each product (Figure 2.5 - 2.11).

(¹⁴C)-amino acid labelled products:

Autoradiography showed that the electrophoretic patterns of the (¹⁴C)-aa labelled proteins in the three products (i e HSV-1, HSV-2 and mock infected BHK-21 cells) were similar on 10% and 7,5% PAA gels, although better resolution was obtained with 10% PAA gel (Figure 2.9 and 2.11). However, molecular weight determinations of these proteins differed considerably between the 10% and the 7,5% PAA gels (Table 2.3 - See Chapter 2.3).

The HSV-1 infected cells incorporated (¹⁴C)-aa into a limited number (approximately 29) of those polypeptides that are visible using protein stain (approximately 50) (Figure 2.5, 2.9 & 2.11). The molecular weights of the (¹⁴C)-aa labelled polypeptides are given in Table 2.3.

In HSV-2 and mock infected BHK-21 cells, the (¹⁴C)-aa was incorporated into almost all of the polypeptides that

were identified using protein stain (Figure 2.6, 2.7, 2.9 and 2.11). A comparison of the autoradiographs of HSV-2 and mock infected cells shows that many of the (^{14}C)-aa labelled polypeptides coincide, indicating that in the HSV-2 infected cells many host cell specific polypeptides were labelled (See Chapter 2.3).

(^3H)-glucosamine labelled products:

SDS-PAGE of the (^3H)-gln labelled products of HSV-1, HSV-2 and mock infected cells on 10% and 7,5% PAA gels and subsequent autoradiography showed that the electrophoretic patterns of the (^3H)-gln labelled polypeptides were different for each of the three products (Figure 2.5-2.8 and 2.10).

HSV-1 infected cells incorporated (^3H)-gln into four major glycoproteins and approximately five minor glycoproteins (Figure 2.5 and 2.10). HSV-2 infected cells, electrophorezed on 10% PAA gel, incorporated (^3H)-gln into two major glycoproteins and approximately eight minor glycoproteins (Figure 2.6). However, on 7,5% PAA gel, these HSV-2 infected cells appeared to have incorporated the label into several more polypeptides than on the 10% PAA gel (Figure 2.10 and Table 2.4).

The autoradiographs of mock infected cells electrophoresed on 10% and 7,5% PAA gels are noticeably different. On 10% PAA gel, no (^3H)-gln labelled polypeptides were visible by autoradiography (Figure 2.7) while on 7,5% PAA gel, as many as eleven polypeptides were visible (Figure 2.10 and Table 2.4).

A comparison of the molecular weights of the (^3H)-gln labelled polypeptides determined from the 10% and the 7,5% PAA gels is given in Table 2.4. The results show that :

- (a) There were more radiolabelled polypeptides visible on the 7,5% PAA gel than on the 10% PAA gel, and
- (b) The molecular weights determined from the 7,5% PAA gels were higher than those from 10% PAA gels (See Chapter 2.3).

FIGURE 2.5 - HSV-1 infected BHK-21 cells were grown in the presence of either (^3H)-glucosamine ((^3H)-gln) or (^{14}C)-amino acids ((^{14}C)-aa). The radiolabelled cells were solubilized and electrophorezed on 10% PAA gels. After electrophoresis, the gels were stained with Coomassie brilliant blue stain and photographed (a) before being impregnated with EN 3 HANCE, dried and exposed to Kodak-Omat MA X-ray film (b).

FIGURE 2.5 (a) - Coomassie blue

Tracks 1 and 4 - High molecular weight (HMW) protein markers (Appendix 1.2.3(a)).

Track 2 - Labelled with (^3H)-gln.

Track 3 - Labelled with (^{14}C)-aa.

FIGURE 2.5(b) - Autoradiograph

Track 1 - Labelled with (^3H)-gln.

Track 2 - Labelled with (^{14}C)-aa.

Arrow indicates the major capsid protein (MCP)

Solid-Phase Radioimmunoassay of Rubella Virus Immunoglobulin G and Immunoglobulin M Antibodies

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A solid-phase radioimmunoassay method has been developed for the detection of rubella virus-specific immunoglobulin G (IgG) and IgM antibodies in human serum specimens. Purified rubella virus was adsorbed onto polystyrene balls, and antibodies that attached to the virus-treated balls were detected by subsequent binding of ^{125}I -labeled anti-human gamma or anti-human mu immunoglobulins. A total of 77 serum specimens were tested. Binding ratios between positive and negative sera were as high as 22 in the IgG assay but rarely exceeded 3 in the IgM assay. The sensitivity of the IgG assay was found to be 16 to 256 times higher than that of the rubella virus hemagglutination inhibition test. The IgG radioimmunoassay can be readily adopted for routine diagnostic use. The IgM radioimmunoassay, however, due to its lower sensitivity, must be modified before being routinely applied.

The accurate diagnosis of rubella virus infections is a major concern of virus diagnostic laboratories. Virus hemagglutination inhibition (HI) and complement fixation (CF) tests are widely used for this purpose. The results obtained, however, are not always conclusive. One problem occasionally encountered is whether a low HI titer is due to a rubella infection or to incomplete removal of nonspecific hemagglutination inhibitors from the serum (15). Another problem arises from the times serum specimens are collected after appearance of clinical rubella symptoms. If the first specimen is collected later than 4 or 5 days after onset of rash, a significant rise in rubella antibody titers between the first and subsequent samples often cannot be detected (21). This makes diagnosis of a recent infection difficult.

In uncomplicated and postnatal rubella infections, specific immunoglobulin M (IgM) antibodies are detectable for only 3 to 8 weeks after onset of rash (20, 21). Thus, if rubella-specific IgM antibodies can be demonstrated, a recent infection is indicated. Several methods have been proposed for detecting such antibodies, including separation of IgM and IgG by sucrose density gradient centrifugation or column chromatography followed by HI testing of the separated immunoglobulins (9, 11, 32), reduction of IgM with 2-mercaptoethanol with subsequent comparison of HI titers of treated and untreated serum (2), *Staphylococcus aureus* absorption of IgG from serum samples followed by HI testing of the IgM-containing supernatant (1), and de-

tection of IgM antibodies by indirect immunofluorescence (5, 12, 20). Most virus diagnostic laboratories have not, however, found any of these techniques consistently reliable or practical enough for the testing of large numbers of serum specimens.

In contrast, detection of viral antigens and viral antibodies by radioimmunoassay (RIA) techniques is highly sensitive and specific (10, 16, 18, 19, 24-27, 29, 30, 35). Furthermore, a refinement of the RIA technique has involved the detection of class-specific viral antibodies (4, 6-8). Recently, a solid-phase RIA for IgM and IgG class antibodies against bovine serum albumin (BSA) in chickens was described by Viljanen et al. (33); BSA was coupled onto a paper disk and antibodies trapped by the antigen were detected by their capacity to bind ^{125}I -labeled anti-chicken mu or anti-chicken gamma immunoglobulin. In the present work, this method was further developed for the assay of virus-specific human antibodies, using polystyrene balls as the solid phase. Presented in this paper is a practical and reliable RIA procedure for detecting rubella virus-specific IgG antibodies in serum specimens. Also included are preliminary results for detection of rubella-specific IgM antibodies using the same methodology.

MATERIALS AND METHODS

Antigen purification. The RA 27/3 strain of rubella virus was grown in suspension cultures of BHK-21/13S cells (13). The cultures were harvested

at 3 days postinfection, and cells were removed by low-speed centrifugation. The supernatant was clarified by centrifugation at $8,000 \times g$ for 15 min, and the pH was immediately adjusted to 8.0. An Amicon 402 apparatus containing an XM-300 membrane was then used at 4°C to concentrate the virus solution approximately 15- to 20-fold. Virus was recovered from the Amicon concentrate by pelleting through 15 ml of 20% sucrose in TSE buffer (0.01 M tris-(hydroxymethyl)aminomethane, 0.15 M NaCl, and 0.002 M ethylenediaminetetraacetate, pH 9.0) onto a 3-ml layer of 60% sucrose in TSE buffer. Centrifugation was for 2 h at 25,000 rpm in a Spinco SW27 rotor at 4°C . Sucrose was removed by dialysis against TSE buffer. The hemagglutination titer of the purified virus varied from 64 to 512, and the protein content, determined by the method of Lowry et al. (22), was 300 to 1,000 $\mu\text{g}/\text{ml}$.

Serum specimens. A series of serum specimens was obtained from six army trainees who had developed an acute clinical rubella infection with significant increases in HI and CF antibody titers. A total of 26 specimens was collected. Other sera consisted of 51 specimens selected from our virus diagnostic laboratory. Included were 32 specimens that were negative in the rubella HI test and 19 specimens that had HI titers of 128 or less. All serum specimens were stored at -20°C until used.

^{125}I -labeled anti-human gamma and anti-human mu immunoglobulins. Human IgG and IgM were purified as described by Viljanen et al. (33). Briefly, human serum immunoglobulins were precipitated with sodium sulfate and chromatographed on Sephadex G-200. IgM fractions were twice recycled on Sephadex G-200. IgG fractions were further purified by diethylaminoethyl-cellulose chromatography. In immunoelectrophoresis and immunodiffusion tests against anti-human sera, the IgG and IgM preparations each gave only one precipitation arc. These preparations were then used to immunize sheep. The immune sera obtained were purified by the use of immunoabsorbents (33). The final preparations of anti-human gamma and anti-human mu immunoglobulins were free of other serum proteins and antibodies.

The anti-immunoglobulins were labeled with ^{125}I -isotope according to the method of Hunter and Greenwood (17), with modifications according to Viljanen et al. (33). Specific activities ranged from 30 to 100 $\mu\text{Ci}/\mu\text{g}$. After iodination, the anti-immunoglobulin preparations were supplemented with 1% normal sheep serum. Degradation products resulting from radioactive decay were removed weekly by chromatography on Sephadex G-200. Peak fractions were pooled and stored at 4°C until required. Under these conditions, the labeled anti-immunoglobulins were found to remain active for 4 to 8 weeks.

RIA procedure. Purified rubella virus was adsorbed onto polystyrene balls (6.4 mm in diameter; Precision Plastic Ball Co., Chicago, Ill.) by incubating balls submerged in an antigen solution at room temperature for 16 h. The protein concentration of the rubella antigen solution was 110 $\mu\text{g}/\text{ml}$, unless otherwise indicated. Phosphate-buffered saline

(PBS), pH 7.35, was used as diluent. After incubation, the balls were air dried and stored at 4°C .

Fourfold serial dilutions of serum specimens were pipetted in 0.2-ml volumes into 4-ml disposable plastic tubes (Nunc Products, Roskilde, Denmark). The serum samples were diluted with PBS, pH 7.35, containing 1% BSA, fraction V (1% BSA-PBS). A polystyrene ball with adsorbed rubella antigen was then added to each tube. Buffer blanks (balls incubated with 1% BSA-PBS and no serum) and a titration of rubella antibody-positive and -negative control sera were included in each test.

After incubating at 37°C for 1 h, the serum dilutions were aspirated off and the balls were washed twice with 5 ml of tap water. A 0.2-ml volume of ^{125}I -labeled anti-human gamma or mu immunoglobulin was then added to each tube. The labeled antibody preparations were diluted to 30,000 cpm/0.2 ml with Eagle minimum essential medium supplemented with 0.5% lactalbumin hydrolysate, 10% heat-inactivated calf serum, and antibiotics as described by Rosenthal et al. (26). After incubation at 37°C for 1 h, the radioactive solutions were aspirated off and the polystyrene balls were washed as described above. The balls were then placed in clean plastic tubes, and bound radioactivity was counted in an LKB Wallac 1280 gamma counter.

CF and HI tests. Rubella antigen was prepared for the CF test according to the alkaline extraction procedure described by Halonen et al. (13). Sever's microtechnique (28) was used, with veronal-buffered saline containing 0.1% gelatin used as diluent. The optimal dilution of antigen was estimated according to the standardized CF technique (3). Standardization of erythrocyte suspensions was done as in the HI test (31). For the HI test, rubella hemagglutinin was prepared in BHK-21/13S cells maintained in a medium containing BSA and no serum (14). HI tests were performed by microtechnique according to the modified test used at the Center for Disease Control, Atlanta, Ga. (31). CF and HI titers are expressed as reciprocals, except in Tables 1 and 2, where \log_2 values are used.

RESULTS

RIA solid phase. Although data presented in this report were obtained from experiments in which polystyrene balls were used, first attempts to establish a rubella RIA technique involved polyvinyl microtiter plates as the solid phase. When rubella virus was simply air dried in the wells of polyvinyl plates, there was a visible loss of antigen during washings. Fixation of the antigen by absolute methanol treatment for 5 min at room temperature reduced such losses. With fixed antigen, however, non-specific adsorption of serum immunoglobulins was extensive and could not be reduced by treatment of the fixed antigen with blocking agents such as normal sheep serum, 1 or 4% BSA, or 0.1% gelatin (23). Such nonspecific adsorption of immunoglobulins resulted in high

background counts per minute, which made evaluation of test results difficult. Pretreatment of the plates with blocking agents before antigen fixation was also tested without beneficial results.

When rubella virus antigen was methanol fixed in wells of polystyrene microtiter plates, subsequent adsorption of nonspecific immunoglobulins was reduced approximately 80% compared with the polyvinyl plates. The disadvantage of using polystyrene plates as the RIA solid phase, however, was that the individual wells were difficult to cut out for radioactive counting. Preliminary tests were then conducted using more easily handled polystyrene balls, to which rubella antigen had been adsorbed. The RIA results obtained were identical to those with the polystyrene plates; consequently, for practical purposes, polystyrene balls were chosen as the RIA solid phase.

At this point it is relevant to note that polystyrene balls have been used as the solid phase of a RIA method developed to detect hepatitis B antigen and antibody (M. Goldfield, personal communication). In addition, Abbott Laboratories (Chicago, Ill.) presently has available hepatitis B antigen and antibody RIA kits that also utilize polystyrene balls as the solid phase. When the polystyrene balls used in this study were compared with those kindly provided by Abbott Laboratories, no significant differences were noted.

Antigen adsorption onto polystyrene balls. Optimal RIA results were obtained when the polystyrene balls were incubated in a rubella virus antigen solution containing 110 μg of protein per ml (Fig. 1). Using an antigen concentration of 330 μg of protein per ml did not significantly enhance the specificity or sensitivity of the assays. With lower antigen concentrations of 27.5 or 6.9 μg of protein per ml, however, assay sensitivity was decreased. Methanol fixation of the antigen on the balls was also tried without obtaining any improvement in the RIA results. Altering the pH of the antigen diluent did have some effect, since using a pH of 8.35 or 9.0, rather than the usual 7.35, did increase the binding of specific immunoglobulins. Unfortunately, the binding of nonspecific immunoglobulins also increased in the same proportion.

When polystyrene balls were incubated at pH 7.35 in an antigen solution containing 110 μg of protein per ml, approximately 20% of the total protein became adsorbed. At the same time, the rubella hemagglutination titer of the antigen solution decreased between 70 and 80%, indicating that there was some preferential adsorption of the virus hemagglutinin. It was found

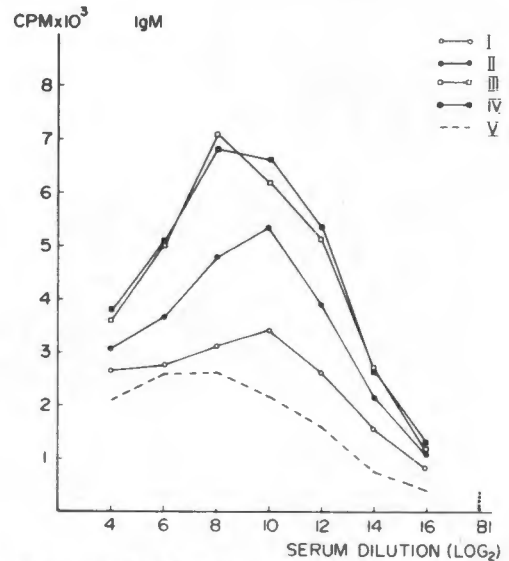
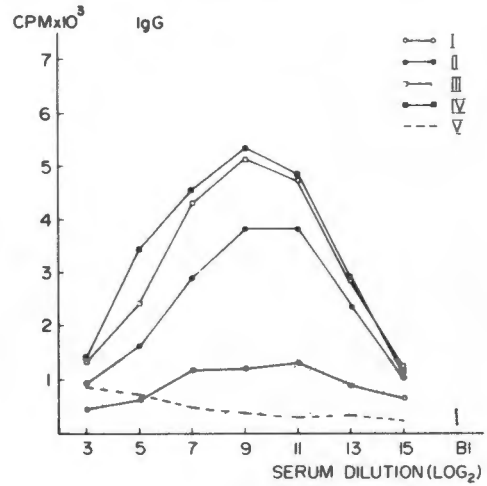


FIG. 1. Effect of protein concentration on adsorption of purified rubella virus antigen on polystyrene balls. Balls adsorbed at each rubella virus protein concentration were incubated with dilutions of a rubella antibody-positive serum and tested for binding of rubella-specific IgG antibodies (upper) or IgM antibodies (lower). I, 6.9 μg of protein per ml; II, 27.5 μg of protein per ml; III, 110 μg of protein per ml; IV, 330 μg of protein per ml; V, 0 μg of protein per ml. In each panel, buffer blanks are designated as Bl.

that an antigen solution could be used for adsorption only once, since the sensitivity of the assays decreased markedly after a second adsorption with fresh balls.

Incubation with serum dilutions. Best assay results were obtained when antigen-adsorbed balls were incubated with serum specimens

that had been diluted with 1% BSA-PBS, pH 7.35. Differences were actually found to be small, however, when the pH of the diluent was between 6.0 and 9.0. Various incubation times and temperatures were also tested. For both the IgG and IgM assays, increasing the incubation time or temperature resulted in greater binding of immunoglobulins from both rubella antibody-positive and -negative sera. For the IgG assay, the greatest difference between positive and negative sera was obtained by overnight incubation at room temperature. In the IgM assay, however, nonspecific immunoglobulin binding increased to such an extent with longer incubations that best results were obtained from a 1-h incubation at 37°C. For practical reasons, a 1-h incubation at 37°C was used for both assays.

Incubation with ¹²⁵I-labeled anti-human immunoglobulins. Dilutions containing 15,000, 30,000, 60,000, and 120,000 cpm/0.2 ml were tested. The binding ratios between rubella antibody-positive and -negative serum samples were almost identical in each case. The dilution containing 120,000 cpm/0.2 ml was used in the experiments in Fig. 1, whereas the dilution containing 30,000 cpm/0.2 ml was used throughout the remainder of this study. Dilution of the labeled anti-immunoglobulins with supplemented Eagle minimum essential medium (see Materials and Methods) was found to be essential, since background counts per minute levels increased greatly if the dilution was done with PBS. The effects of time and temperature on this step of the assays were not tested, since an incubation of 1 h at 37°C was found to be adequate.

Assay results. Background levels of the IgG and IgM assays were determined in three different ways. First, polystyrene balls with adsorbed control antigen (material obtained from supernatants of uninfected cells) were incubated with rubella antibody-positive and -negative control sera. Second, balls with adsorbed rubella antigen were incubated with negative control serum; and, third, balls with no adsorbed antigen were incubated with positive and negative control sera. In each case, the background binding levels of human IgG and IgM were reproducibly low. There was a marked difference, however, between the two assays. In the IgM assay, background levels were two to three times higher than in the IgG assay. Such higher nonspecific binding of IgM from serum samples, compared with that of IgG, has also been observed in other class-specific antibody RIA procedures (4).

Evaluation of assay results was done by means of binding ratios, which were calculated

by dividing the counts per minute of the serum specimen by the counts per minute of the rubella antibody-negative control serum at the same dilution. The highest serum dilution having a binding ratio of 3.0 was chosen as the titer end point in the IgG assay. In the IgM assay, a binding ratio of 1.5 was selected with the proviso the counts per minute of the end point dilution also be 1.5 times the average counts per minute of all the negative control serum dilutions. End point titer values were obtained from the counts per minute versus the dilution curve of each serum specimen. Titer values are expressed as reciprocals, except in the figures and tables, where log₂ values are used.

Intra-assay variation was studied by testing both a rubella antibody-positive and -negative control serum in 10 parallel determinations. The coefficients of variation of the counts per minute at each fourfold dilution were $\pm 7.5\%$ in the IgG assay and $\pm 6.2\%$ in the IgM assay. Variation in the assigned end point titer of a positive control serum was also tested and was found to be twofold in 15 separate IgG and IgM assays.

A comparison of the rubella HI antibody test and the rubella RIA IgG antibody test is shown in Table 1. The 32 serum specimens that were negative in the HI test were also negative in the RIA IgG test. Of the 19 specimens with an HI titer of 128 or less, 18 were highly positive in the RIA IgG test. One serum specimen having an HI titer of 8 was found to be negative in the RIA IgG test. The titer end points of the RIA IgG test were 16 to 256 times higher than the titer end points of the HI test, indicating that the RIA procedure is the more sensitive of the two techniques. The 51 serum specimens used in the experiments of Table 1 were also tested in the RIA IgM test, and all were found to be negative.

The development of rubella-specific RIA IgG and IgM antibodies in a series of serum speci-

TABLE 1. Rubella HI and RIA IgG antibody titers of 51 serum specimens^a

HI titer (log ₂ value)	No. of serum specimens				
	<3 ^b	9	10	11	12
<3	32				
3	1				
4		1	2		
5		4	3	3	1
6			2	1	
7					1

^a The specimens were selected to represent 32 specimens with negative HI results and 19 specimens with relatively low HI titers.

^b RIA IgG titer (log₂ value).

mens collected from a rubella patient is shown in Fig. 2. The early rise and rapid fall of rubella-specific IgM antibodies and the slower rise of rubella-specific IgG antibodies are clearly demonstrated. A comparison of rubella HI, CF, RIA IgG, and RIA IgM antibody titers of 21 serum specimens collected from five rubella patients is shown in Table 2. The rapid development and early decline of IgM antibodies and the slower development of IgG antibodies is also apparent in these sera. Also demonstrated in Table 2 is the fact that both rubella class-specific antibody assays are much more sensitive than the rubella HI and CF tests.

DISCUSSION

The solid-phase RIA methodology described in the present study is both practical and reliable as a test for detecting rubella virus-specific antibodies in serum specimens. A distinct advantage of the procedure is that serum specimens can be tested without any pretreatment,

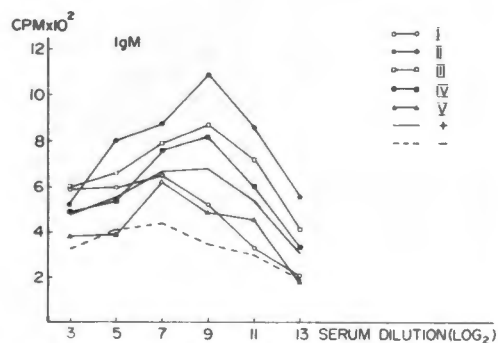
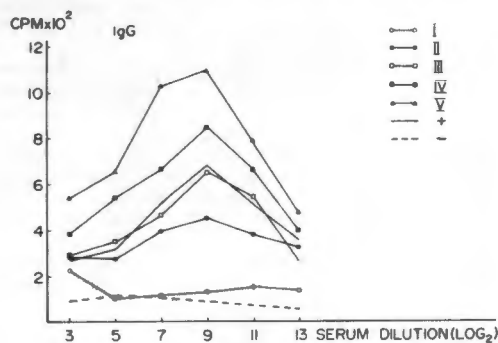


FIG. 2. Development of rubella virus-specific RIA IgG antibodies (upper) and RIA IgM antibodies (lower) in a series of serum specimens from a patient with an acute clinical rubella infection. Serum specimens were obtained at: I, 3 days; II, 10 days; III, 17 days; IV, 24 days; V, 49 days, after onset of rash. (+) Rubella antibody-positive control serum; (-) rubella antibody-negative control serum.

TABLE 2. Rubella HI, CF, RIA IgM, and RIA IgG antibody titers in a series of serum specimens taken from five rubella patients

Patient	Days after onset of rash	Titer (\log_2 value)			
		HI	CF	RIA	
				IgM	IgG
V.V.	0	3	<2	3	3
	7	8	3	14	15
	14	8	4	14	15
	21	6	5	13	15
V.T.	0	<3	<2	<3	<3
	6	9	2	13	17
	12	8	3	13	17
V.S.	68	9	3	<3	17
	1	4	<2	13	<3
	12	9	4	16	14
	14	9	4	15	14
S.S.	21	9	4	15	14
	3	5	<2	6	7
	10	8	4	12	13
	21	9	4	12	13
R.T.	26	8	4	11	13
	37	9	4	<3	14
	1	6	<2	10	<3
	7	9	<2	14	15
	15	9	4	12	15
	26	9	4	10	15

since nonspecific inhibitors of the rubella hemagglutinin do not interfere. Once rubella virus-treated polystyrene balls and radioactively labeled antisera are available, the RIA method is simple to perform and specimens can be evaluated within 1 day. Since the assay results are recorded in printed form as counts per minute, there is no need for visual evaluation, as is the case with the rubella HI test.

Preliminary rubella RIA experiments were conducted with polyvinyl microtiter plates; this material, however, proved to be unsatisfactory as the RIA solid phase. When polystyrene balls were used, a marked improvement in the specificity and sensitivity of the rubella class-specific antibody assays was obtained. End point titers of positive sera in both the RIA IgG and the RIA IgM tests were very high (Tables 1 and 2), and in neither case were false-positive results seen in a test series of 77 specimens.

Positive-to-negative serum-binding ratios in the rubella RIA IgG test were consistently high, occasionally being 20 or more. Binding ratios in the RIA IgM test, however, rarely reached the level of 3.0. The difference between the two assays is due, first, to background levels being two to five times higher in the RIA IgM test and, second, to a slightly lower specific binding in the RIA IgM test compared with the RIA IgG test. In spite of these difficulties, the RIA IgM test clearly demonstrated the early

rise and rapid decline of rubella-specific IgM antibodies after onset of clinical rubella symptoms (Fig. 2, Table 2). The later appearance and subsequent persistence of rubella-specific IgG antibodies in the same serum specimens was also clearly demonstrated by the RIA IgG test.

In recent years, evidence has been accumulating that viruses may play a role in the etiology and/or pathogenesis of certain progressive neurological disorders (for a brief review, see reference 34). A sensitive and reliable assay is a necessary prerequisite for detection of low levels of antiviral antibodies that may be produced in the central nervous system. The rubella RIA IgG test would appear to fulfill these criteria, since it is 16 to 256 times more sensitive than the conventional rubella HI test (Table 1).

In summary, the RIA procedure described herein for the detection of rubella-specific IgG antibodies is feasible for clinical use. The same conclusion, however, cannot be drawn for the RIA detection of rubella-specific IgM antibodies. Although the results presented demonstrate that anti-rubella IgM antibodies can be measured with the described RIA methodology, further work is required to optimize the test conditions. In the present report, antibody levels detected by the RIA procedure are expressed in conventional serum titer values. By selecting a proper serum dilution and comparing its RIA counts per minute with a suitable standard curve, it may be possible to express the specific antibody content of a test serum in terms of micrograms per milliliter, as has been done in the case of chicken antibodies to BSA (33). Experiments to improve the rubella RIA IgM test and to quantitate both the IgG and IgM assays are currently in progress.

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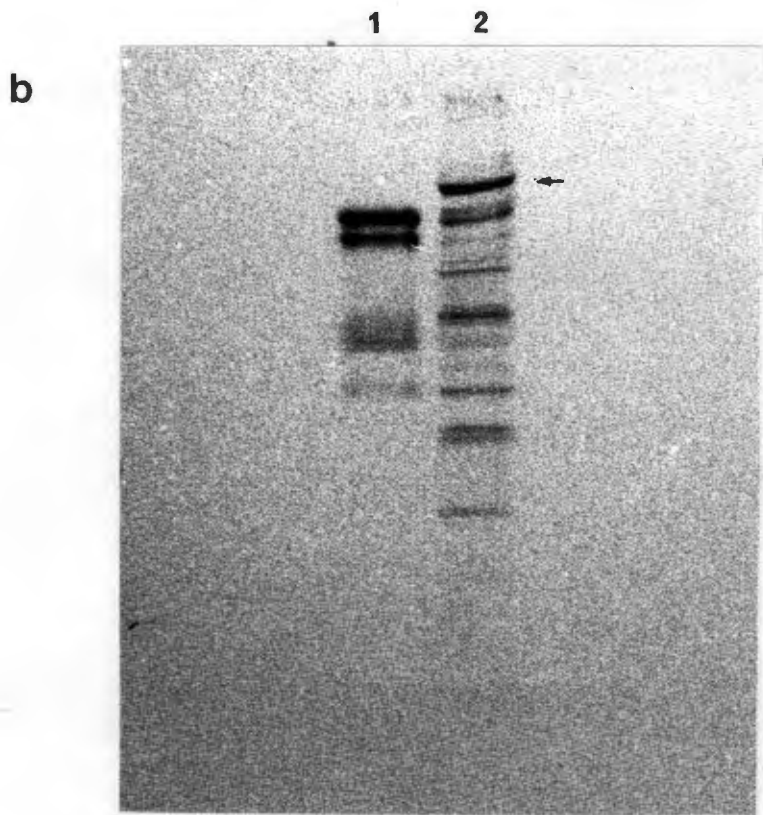
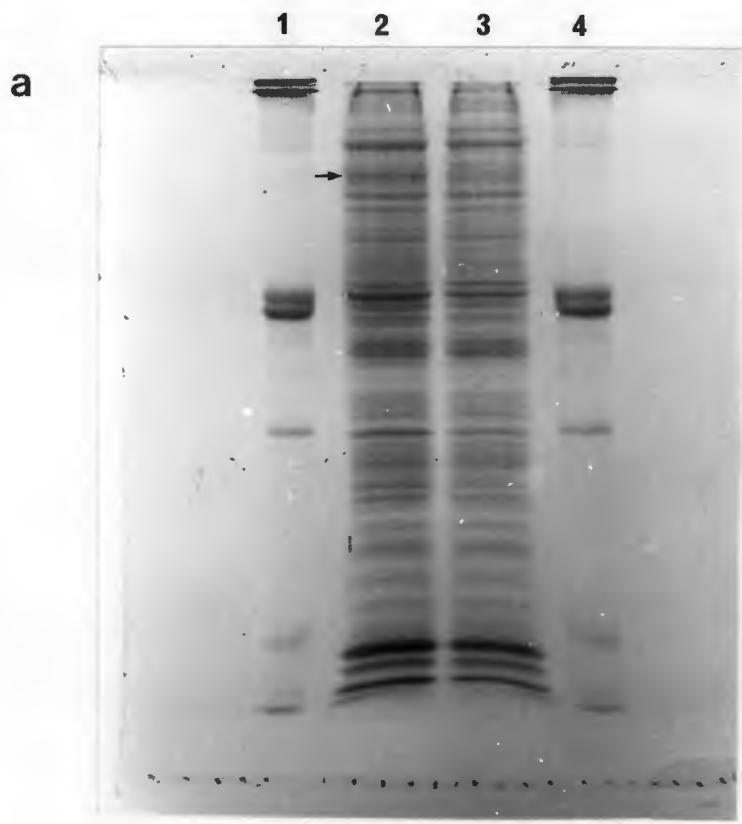


FIGURE 2.6 - HSV-2 infected BHK-21 cells

Treated as described in the legend to Figure
2.5.

FIGURE 2.6(a) - Coomassie blue

Tracks 1 and 4 - HMW protein markers.

Track 2 - Labelled with (^3H)-gln.

Track 3 - Labelled with (^{14}C)-aa.

FIGURE 2.6(b) - Autoradiograph

Track 1 - Labelled with (^3H)-gln.

Track 2 - Labelled with (^{14}C)-aa.

Arrow indicates MCP.

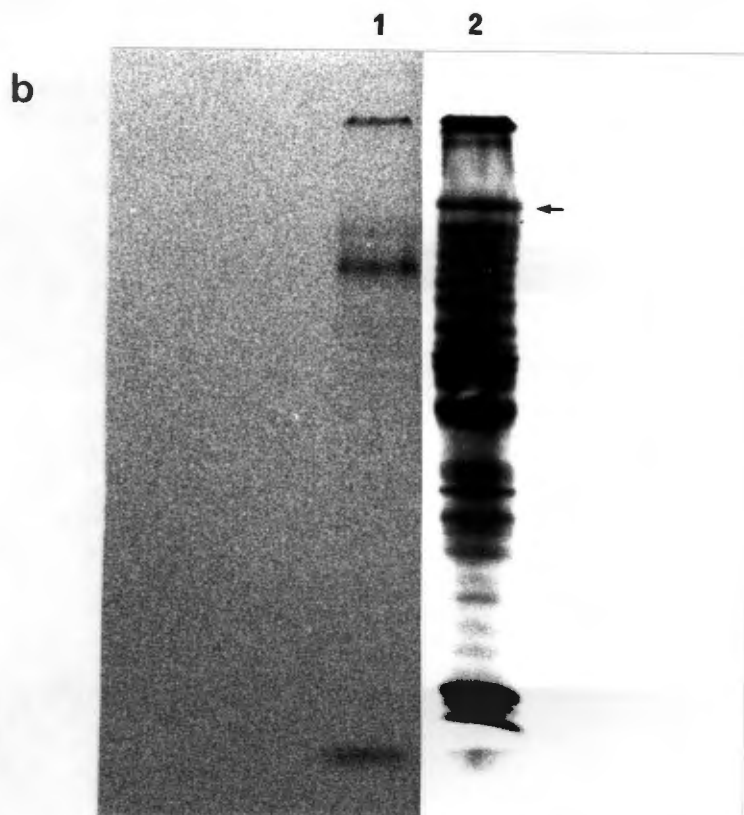
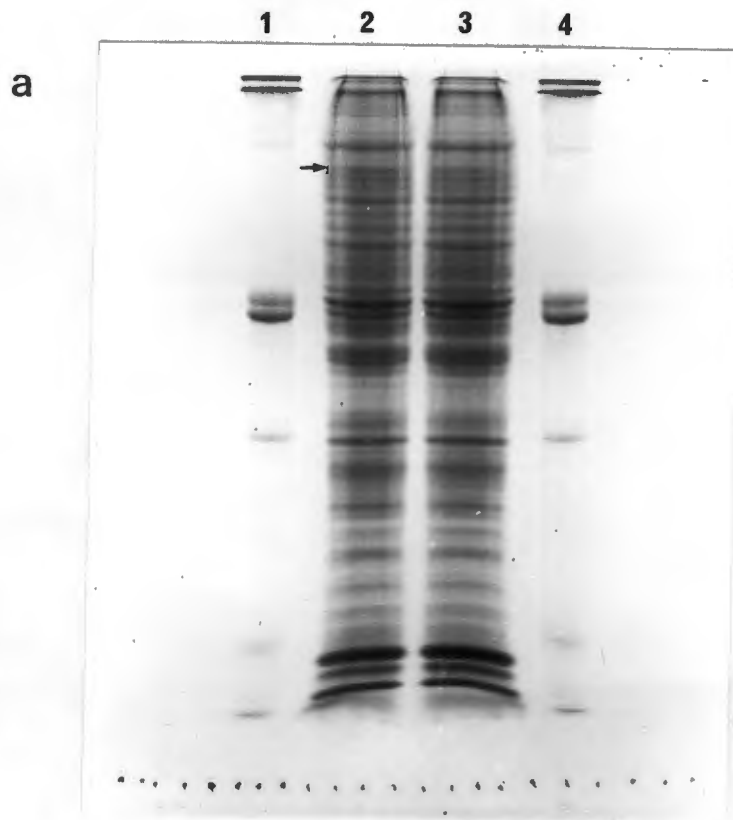


FIGURE 2.7 - Mock infected BHK-21 cells

Treated as described in the legend to Figure

2.5.

FIGURE 2.7(a) - Coomassie blue

Tracks 1 and 4 - HMW protein markers.

Track 2 - Labelled with (^3H)-gln.

Track 3 - Labelled with (^{14}C)-aa.

FIGURE 2.7(b) - Autoradiograph

Track 1 - Labelled with (^3H)-gln.

Track 2 - Labelled with (^{14}C)-aa.

Arrow indicates MCP.

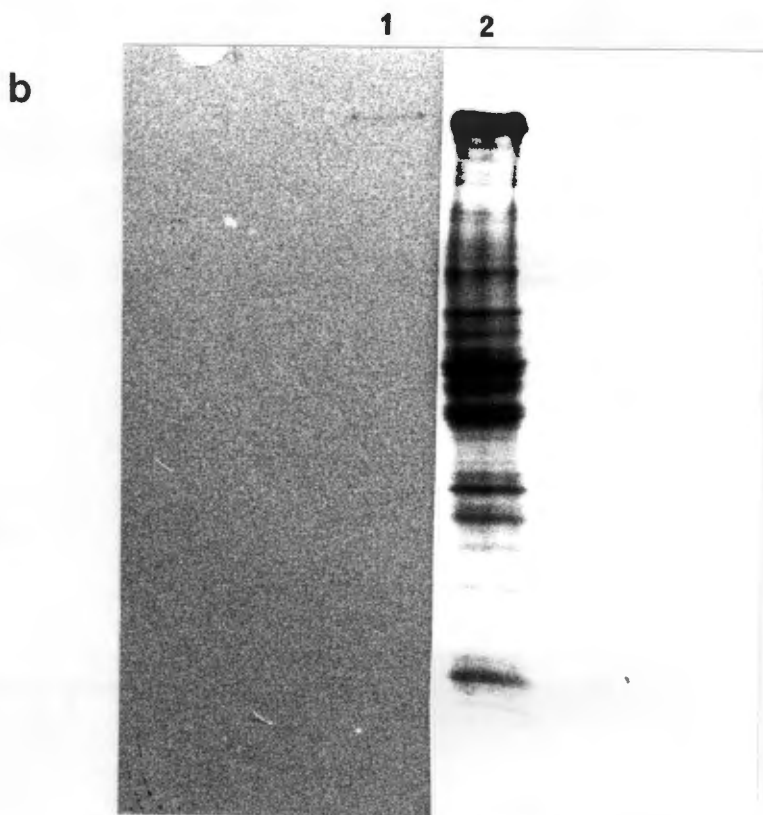
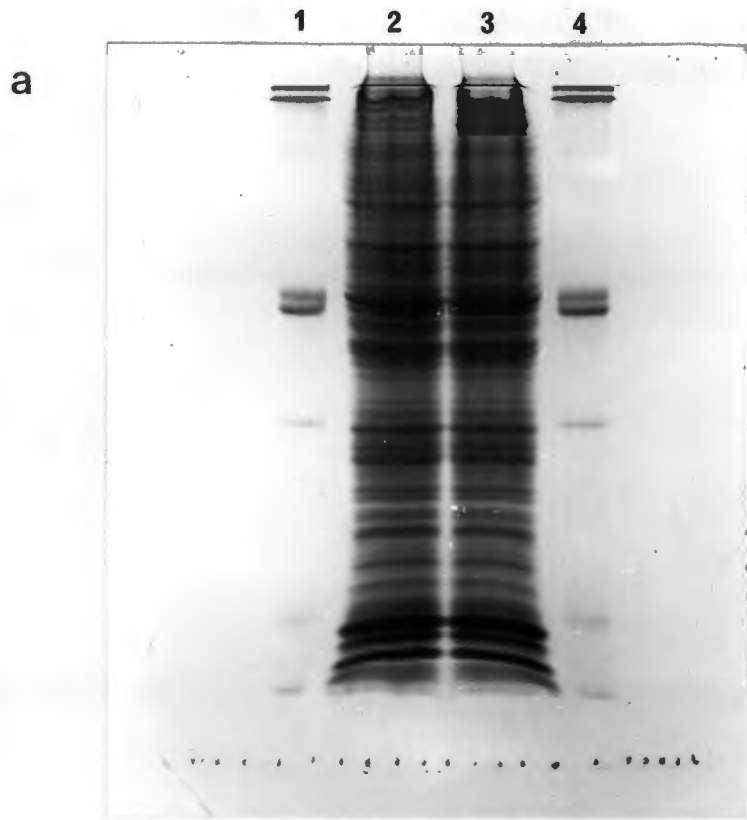


FIGURE 2.8 - HSV-1, HSV-2 and mock-infected BHK-21 cells were grown in the presence of (³H)-gln. The radiolabelled cells were solubilized and electrophorezed on 10% PAA gels. After electrophoresis, the gels were treated as described in the legend to Figure 2.5.

FIGURE 2.8(a) - Coomassie blue
Track 1 - HSV-1 infected cells.
Track 2 - HSV-2 infected cells.
Track 3 - Mock infected cells.
Track 4 - HMW protein markers.

FIGURE 2.8(b) - Autoradiograph
Track 1 - HSV-1 infected cells.
Track 2 - HSV-2 infected cells.
Track 3 - Mock infected cells.

Arrow indicates MCP.

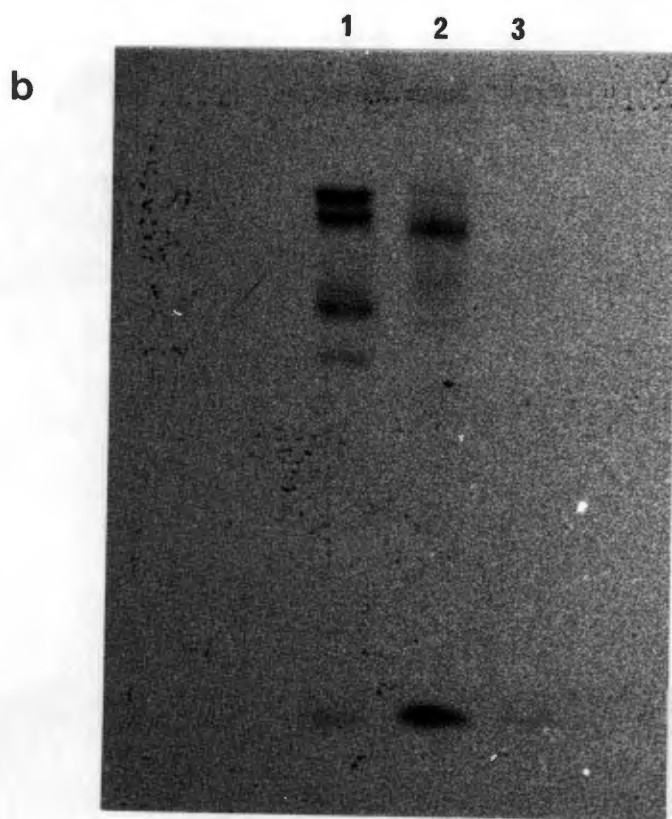
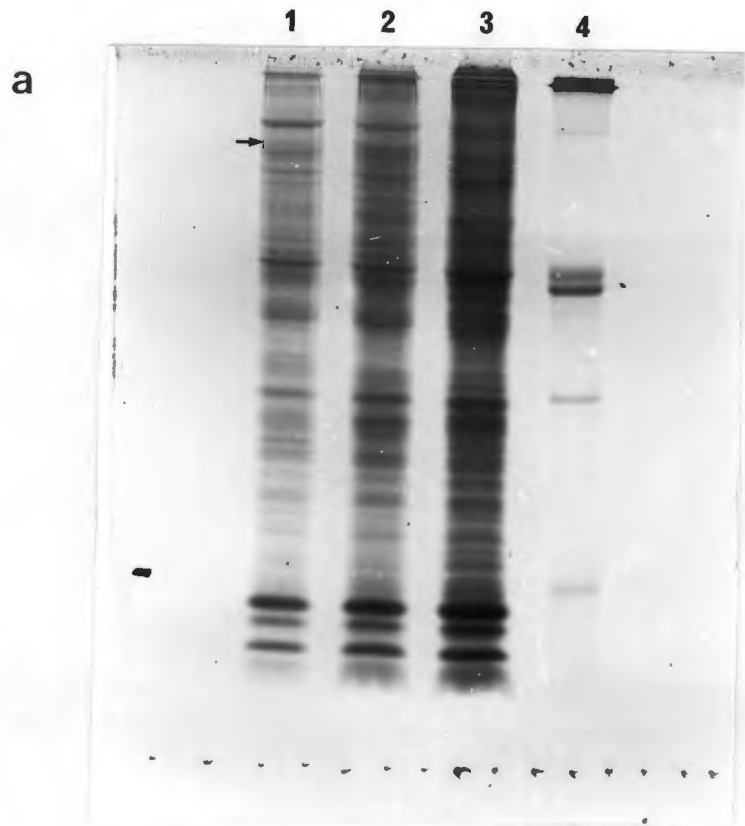


FIGURE 2.9 - HSV-1, HSV-2 and mock infected BHK-21 cells were grown in the presence of (^{14}C)-aa and treated as described in the legend for Figure 2.8.

FIGURE 2.9(a) - Coomassie blue
Track 1 - HSV-1 infected cells.
Track 2 - HSV-2 infected cells.
Track 3 - Mock infected cells.
Track 4 - HMW protein markers.

FIGURE 2.9(b) - Autoradiograph
Track 1 - HSV-1 infected cells.
Track 2 - HSV-2 infected cells.
Track 3 - Mock infected cells.

Arrow indicates MCP.

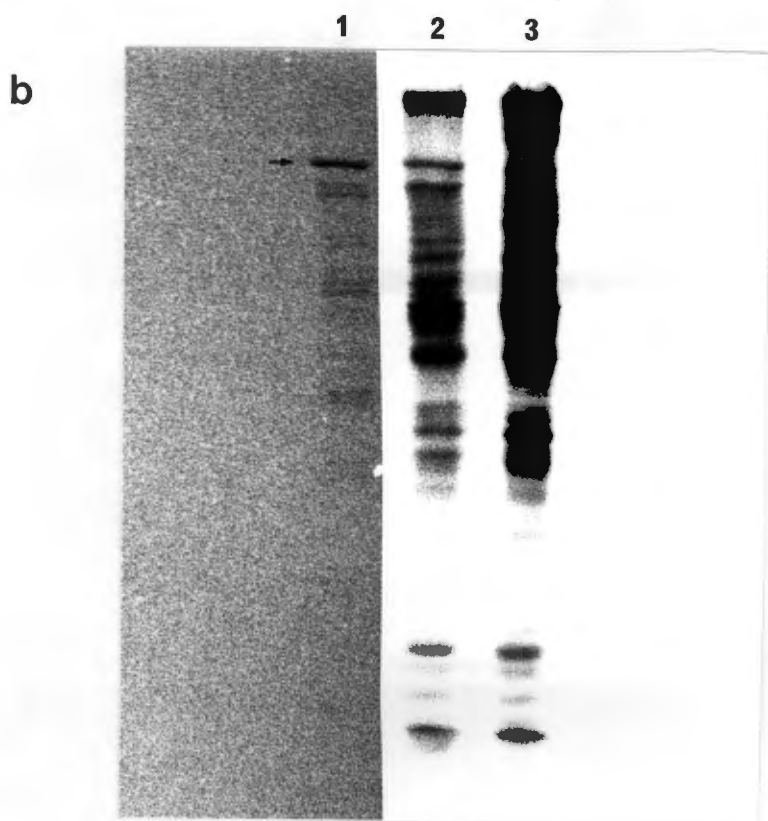
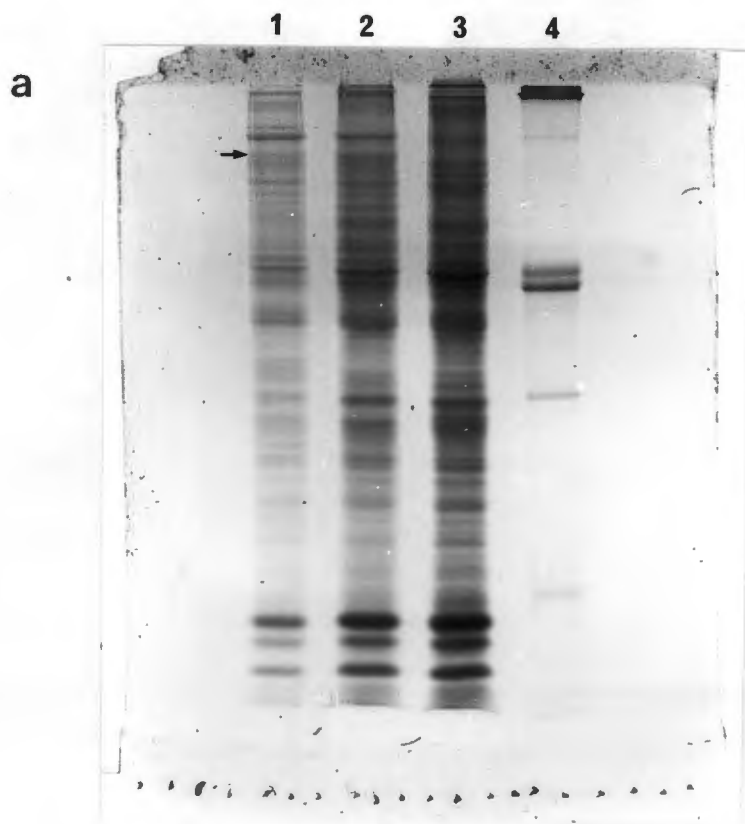


FIGURE 2.10 - HSV-1, HSV-2 and mock infected BHK-21 cells were grown in the presence of (^3H)-gln. The radiolabelled cells were solubilized and electrophorezed on 7,5% PAA gels. After electrophoresis one gel was stained with silver stain (a) while a second gel was impregnated with EN 3 HANCE and exposed to Kodak X-Omat AR5

FIGURE 2.10(a) - Silver stain
Track 1 - HMW protein markers.
Track 2 - HSV-1 infected cells.
Track 3 - HSV-2 infected cells.
Track 4 - Mock infected cells.

FIGURE 2.10(b) - Autoradiograph
Track 1 - HSV-1 infected cells.
Track 2 - HSV-2 infected cells.
Track 3 - Mock infected cells.

Arrow indicates MCP.

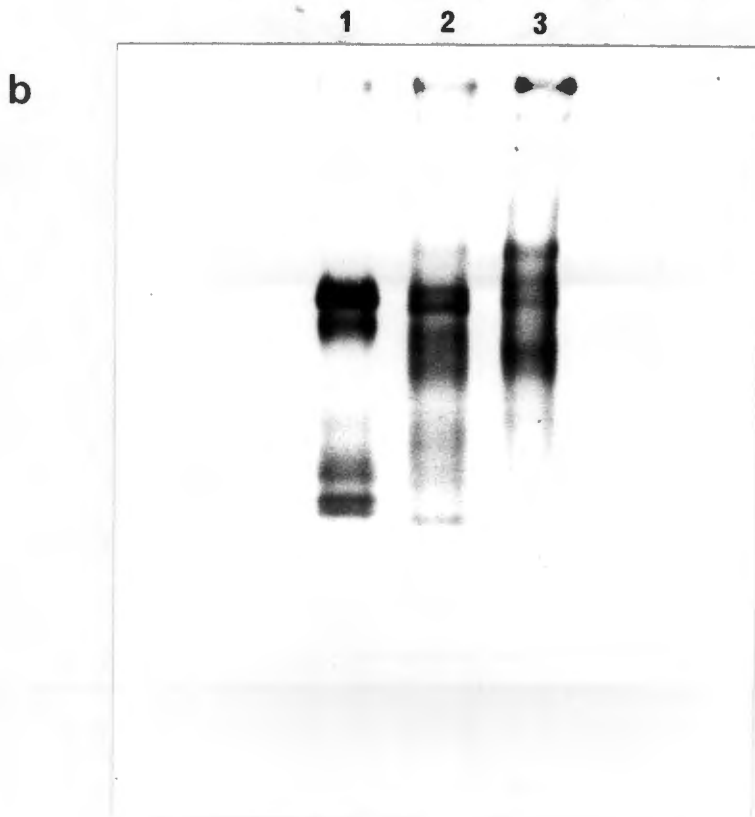
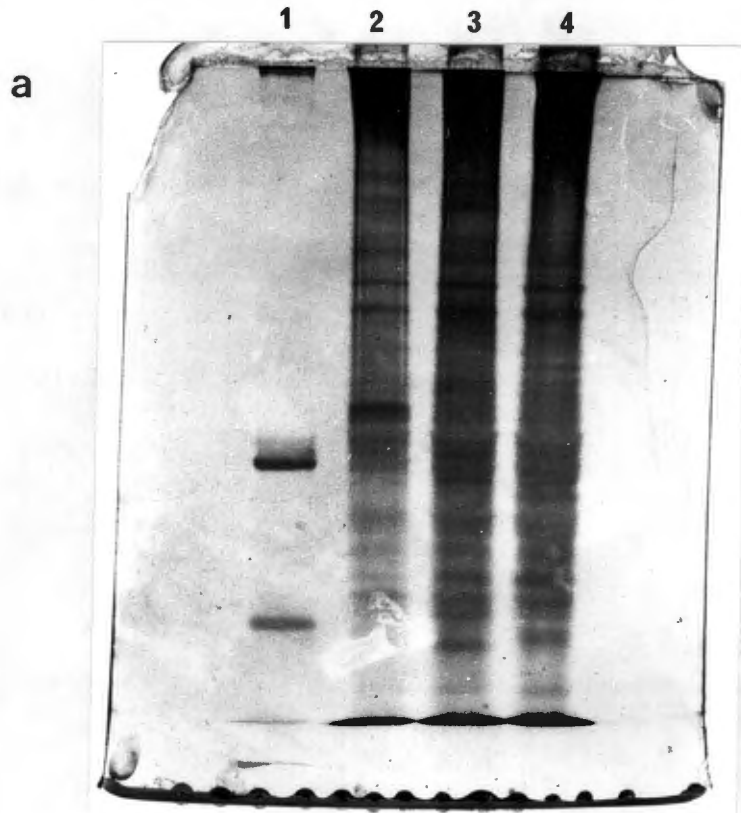


FIGURE 2.11 - HSV-1, HSV-2 and mock infected BHK-21 cells were grown in the presence of (¹⁴C)-aa. The radiolabelled cells were treated as described in the legend to Figure 2.10.

FIGURE 2.11(a) - Silver stain

Track 1 - HMW protein markers.
Track 2 - HSV-1 infected cells.
Track 3 - HSV-2 infected cells.
Track 4 - Mock infected cells.

FIGURE 2.11(b) - Autoradiography

Track 1 - HSV-1 infected cells.
Track 2 - HSV-2 infected cells.
Track 3 - Mock infected cells.

Arrow indicates MCP.

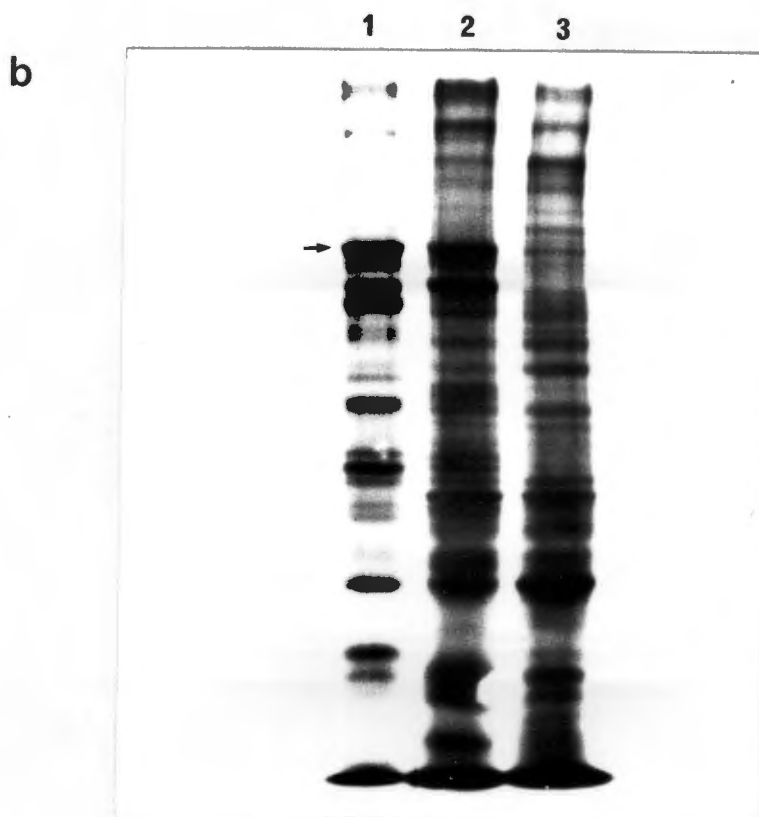
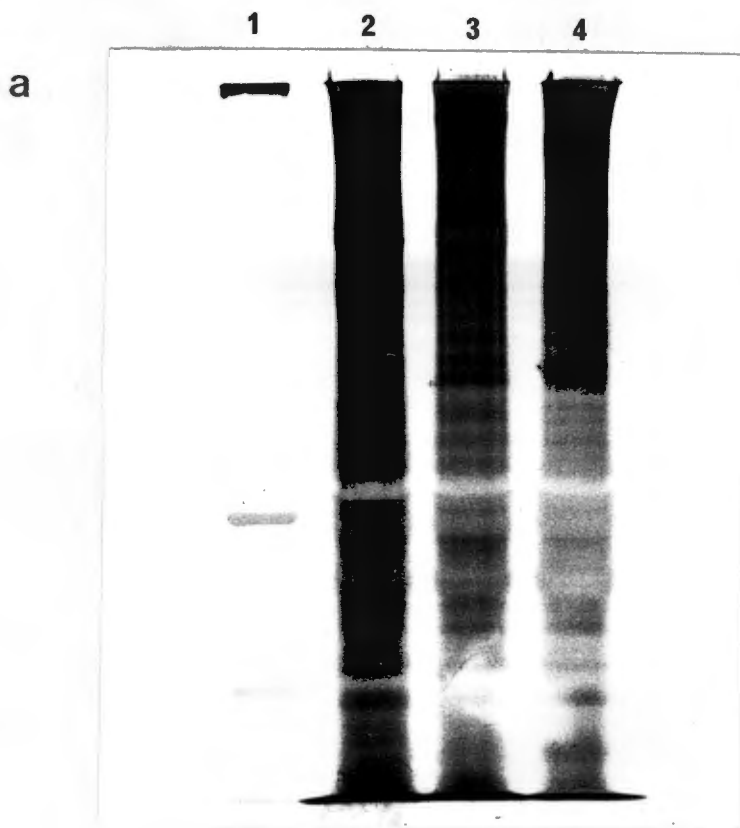


TABLE 2.3 - MOLECULAR WEIGHTS OF HSV-1 PROTEINS LABELLED WITH (^{14}C) -AMINO ACIDS

PROTEIN NUMBER	MOLECULAR WEIGHT $\times 10^{-3}$ (DALTONS)	
	10% PAA (a)	7.5% PAA (b)
1	0 S (g)	0 S
2	0 S	0 S
3	0 S	0 S
4	130.3	164.10
5 (f)	112.2	153.10
6	- (c)	141.30
7	100.7	134.90
8 (gp)	95.5	124.50
9 (gp)	86.1	117.50)(e)
10	-	113.50)
11	82.22	104.70
12	78.52	100.00
13	74.99	92.26
14 (gp)	69.18	83.18
15	65.77	78.52
17 (gp)	61.66	71.61
18 (gp)	58.21	66.07
19 (gp)	56.23	63.83
20 (gp)	51.88	57.54
21	47.86	48.42
22 (gp)	41.02	41.69
23	39.36	38.90
24	36.31	36.31
25	29.51	- (d)
26	22.65	- (d)
27	16.98	- (d)
28	16.41	- (d)
29	14.45	27.86

- (a) Values calculated from the autoradiograph shown in Figure 2.5.
- (b) Values calculated from the autoradiograph shown in Figure 2.11.
- (c) Protein not visible on autoradiograph of 10% PAA gel.
- (d) These protein bands were compressed into a single band, m.wt. 27860.
- (e) Not clearly resolved. Possibly a single protein.
- (f) Major capsid protein (MCP).
- (g) 0 S Off Scale. The molecular weights of these proteins were too high to be determined on 10% and 7,5% PAA gels.
- (gp) Glycosylated protein.

TABLE 2.4 - THE APPARENT MOLECULAR WEIGHTS OF HSV-1, HSV-2 AND MOCK INFECTED BHK-21 CELL PROTEINS LABELLED WITH (3H) GLUCOSAMINE

MOLECULAR WEIGHTS x 10 ⁻³ (DALTONS)					
BHK - 21 CELLS INFECTED WITH :-					
HSV - 1		HSV - 2		MOCK	
10% PAA (a)	7,5% PAA (b)	10% PAA (c)	7,5% PAA (b)	10% PAA (d)	7,5% PAA (b)
		- (e)	0 S (g)	- (e)	0 S
		- (e)	149.60(g)	- (e)	149.60(e)
98.86	127.400	101.20	131.80	- (e)	136.50
89.13	117.400	97.20	124.50	- (e)	125.90
69.98	86.700	91.20	113.50	- (e)	117.50
60.95	76.380	86.10	102.30(h)	- (e)	109.60
58.88	73.280	82.22	82.22	- (e)	102.30
56.23	68.390	66.07	76.74	- (e)	88.10
54.33	66.070	59.57	73.28	- (e)	80.35
48.98)	58.88	55.59	68.30	- (e)	62.37
47.32) (f)	41.21	42.17	54.95(h)	12.59	54.95
		12.74	50.12		

(a) Values calculated from the autoradiograph in Figure 2.5(b).

(b) Values calculated from the autoradiograph in Figure 2.10(b).

(c) Values calculated from the autoradiograph in Figure 2.6(b).

(d) Values calculated from the autoradiograph in Figure 2.7(b).

(e) Not visible on 10% PAA gel - See Chapter 2.3.

(f) Possibly a single band.

(g) Host cell specific protein.

(h) Possibly host cell specific protein

0 S Off-Scale. Protein molecular weight is too high to be determined on 10% or 7.5% PAA gel

2.3 DISCUSSION

Analytical SDS-PAGE was used in conjunction with autoradiography as a means of analyzing the products of HSV-1, HSV-2 and mock infected BHK-21 cells grown in the presence of either (^3H)-glucosamine ((^3H)-gln) or (^{14}C)-amino acids ((^{14}C)-aa). The molecular weights of the polypeptide bands were determined by using calibrated graphs plotted from the molecular weight markers which were electrophoresed with the radiolabelled proteins.

There was little apparent difference between the electrophoretic patterns of HSV infected and uninfected cells when the PAA gels were stained with protein stain (Figure 2.5 -2.11). The most noticeable difference was the presence of the major capsid protein (MCP) in both HSV-1 and HSV-2 infected cells, but not in uninfected cells. The apparent molecular weight of the MCP on 10% PAA gel was determined to be 132 K and 138K, for HSV-1 and HSV-2, respectively (Figure 2.5 & 2.6). The equivalent values on 7,5% PAA gel were 153 K and 147 K, respectively (Figure 2.11). It has previously been reported that the MCP has a molecular weight of approximately 155 K (Spear & Roizman, 1972, Cassai, Sarmiento & Spear, 1975; Cohen et al, 1980), and that the MCP of HSV-1 has a slightly faster electrophoretic mobility than the corresponding protein found in

HSV-2 capsids (Cassai, Sarmiento & Spear, 1975; Cohen et al, 1980).

Throughout the present work, several differences were noticeable between the results obtained for analytical SDS-PAGE using 10% PAA gel and 7,5% PAA gel.

- (a) More proteins were shown by autoradiography with the 7,5% PAA gel than with the 10% PAA gel. This was most noticeable when mock infected cells labelled with (^3H)-gln were electrophorezed (Figure 2.7 and 2.10). The probable reason for this discrepancy was that a more sensitive X-ray film was used for autoradiography of the 7,5% PAA gels (Kodak X-Omat AR5) than was used for the 10% PAA gels (Kodak X-Omat MA).
- (b) The molecular weights of polypeptides determined from 10% PAA gels were considerably lower than those determined from 7,5% PAA gels (Table 2.3). The reason for this is that, with the calibration of gels (Figure 2.3 & 2.4) the relationship between Log. molecular weight and Rf departs from linear at high molecular weights. With more concentrated PAA gels the departure from a linear relationship occurs at lower molecular weights than with less concentrated PAA gels. Some of the proteins of interest in the present work (i.e. MCP, glycoproteins gC and gB) have molecular weights which fall on the non-linear sec-

tion of the calibrated graph for 10% PAA gel (Figure 2.3). As no molecular weight standards were available within the range of 100 K to 200 K, it was not possible to plot the non-linear section of the graph accurately, and so the values within this range are not accurate using 10% PAA gel. For this reason, it was decided to use 7,5% PAA gels which are more suitable for the molecular weight range required in the present work.

(¹⁴C)-Amino Acid Labelled Products

The results obtained for the (¹⁴C)-aa labelled products reveals that the HSV-1 infected cells incorporated (¹⁴C)-aa into approximately 29 polypeptides (Table 2.3). This means that not all of the polypeptides which were visible using protein stain had incorporated the (¹⁴C)-aa label (Figure 2.5). A comparison of the autoradiographs of HSV-1 and mock infected cells shows clearly that the HSV-1 infected cells had not incorporated (¹⁴C)-aa into host cell proteins (Figure 2.11(b) track 1 and track 3). This suggests that the (¹⁴C)-aa label was only incorporated into viral specific polypeptides, which is in agreement with previous reports which state that HSV infection stops the production of host cell proteins (Sydiskis & Roizman, 1968; Spear, Keller & Roizman, 1970; Heine, Spear & Roizman, 1972; Spear, 1976; Fenwick, Morse & Roizman, 1979). Purified virions of HSV-1 have previous-

ly been reported to contain between 23 and 33 polypeptides (Spear & Roizman, 1973; Heine et al, 1974) while cells infected with HSV-1 have been shown to contain approximately 50 infected cell polypeptides. (Hones & Roizman, 1973; Morse et al, 1978). A comparison of the present results with those reported previously is given in Table 2.5.

The HSV-2 infected cells appeared to have incorporated the (^{14}C)-aa into almost all of the polypeptides that were visible using protein stain (Figure 2.6 and 2.9). A comparison of the autoradiographs of HSV-2 and mock infected cells revealed that many of the host cell polypeptides had incorporated the (^{14}C)-aa label in HSV-2 infected cells (Figure 2.11(b) track 2 and track 3). In the light of the fact that HSV-2 strains have been found to inhibit the synthesis of both DNA and protein of the host cell more rapidly than HSV-1 strains (Fenwick et al, 1979) the most probable explanation for the incorporation of (^{14}C)-aa into host cell proteins is that the HSV-2 inoculum did not contain enough virus to produce 100% infection of cells initially (MOI of approximately 1 TCID₅₀/cell). Hence, the uninfected cells would have continued to grow normally for some time after adding the radiolabelled medium, thus labelling host cell polypeptides.

(³H)-Glucosamine Labelled Products

Initial electrophoresis of the (³H)-gln labelled products on 10% PAA gel revealed different electrophoretic patterns for HSV-1 and HSV-2 infected cells by autoradiography, while the mock infected cells appeared to have no labelled glycoproteins (Figure 2.5-2.8, Table 2.4). This suggested that it would be easy to identify the viral specific glycoproteins of HSV-1 and HSV-2 because they would not be confused with host cell glycoproteins. However, electrophoresis of the same products on 7,5% PAA gel showed that (³H)-gln had been incorporated into several mock infected cell polypeptides (Figure 2.10). The reason for this discrepancy has been discussed earlier.

Autoradiography of the (³H)-gln labelled HSV-2 infected cell product after electrophoresis on 7,5% PAA gel, revealed a greater number of labelled polypeptides than had been visible on the 10% PAA gel (Table 2.4). Some of these extra proteins appear to be host cell specific proteins (Figure 2.10, Table 2.4). This is in agreement with the finding that (³H)-gln labelled host cell polypeptides were visible on 7,5% PAA gels but not on 10% PAA gels. (The reason for the presence of labelled host cell proteins in HSV-2 infected cell products has been discussed earlier.) The electrophoretic patterns of (³H)-gln labelled HSV-1 polypeptides were similar on the two types

of gel used, although the 10% PAA gel gave better resolution (Figure 2.5 and 2.10). As mentioned earlier, the molecular weights of these polypeptides, determined from the two types of gel, differed considerably. However, the molecular weight values obtained from the 7,5% PAA gel are comparable with published values (Table 2.6).

Conclusion

1. HSV-1 infected BHK-21 cells were labelled satisfactorily, in that the radioisotopes were only incorporated into viral specific proteins.
2. HSV-2 infected BHK-21 cells were not satisfactorily labelled in that many of the host cell proteins incorporated the radioisotopes. The probable reason for this is that the amount of virus in the inoculum was not sufficient to infect all the cells initially. Hence, the uninfected cells would have continued to grow normally and incorporate the radioisotopes into host cell proteins.

TABLE 2.5 - A COMPARISON OF REPORTED VALUES FOR THE MOLECULAR WEIGHTS OF HSV-1 PROTEINS.

A		B		C		D	
ICP	m.wt	VP	m.wt	VP	m.wt	ICP	m.wt
1	221	1-2	260	1	275	1	0 S
2	205	3	260	2	260	2	0 S
3	194	4	177	3	260	3	0 S
4	170	5	157	4	184	4	164.10
5	151	6	149	5	155	5	153.10
6	146	6.5	130	6	146	6	141,30
7	139	7	129	7	126	7	134,90
7,5	132	8	126	8	126	8	124,50
8	128	8,5	119	9	112	9	117,50
9	122	9	115	10	98	10	113,50
10	117	10	100	11	93	11	104,70
11	114	11	94	12	87	12	100,00
12	111	12	91	13	78	13	92,16
13	109	12,3	88	14	78	14	83,18
14	106	12,6	86	15	71	15	78,52
15	103	13	82	16	65	16	75,86
16	101	14	80	17	59	17	71,61
17	92	14,5	76	18	57	18	66,07
18	88	15	73	19	53	19	63,83
19	78	15,5	70	20	50	20	57,54
20	77	15,8	69	21	44	21	48,52
21	72,5	16	68	22	37	22	41,69
23	71	17	62	23	33	23	38,90
24	68	18	59	24	25	24	36,31
25	64	19E	57			25	29,51
26	61,5	19C	55			26	22,65
27	58	20	51			27	16,98
28	56	21	47			28	16,41
29	55	21h	-				
31	52	22	39				
32	51,5	23	36				
33	50						
34	49,5						
35	46,5						
36	42,5						
37	39						
38	37						
39	36						
40	33,7						
41	32						
43	26,5						
44	24,5						

- A. Results of Morse et al, 1978. The proteins used for m.wt. calibration were β' , β , and α subunits of E.Coli RNA polymerase, bovine serum albumin, and soybean trypsin inhibitor (T_1) (Boehringer Mannheim, Indianapolis, Ind.) with molecular weights of 165 K, 155 K, 39 K, 69 K, and 21 K, respectively.
- B. Results of Heine et al, 1974. Figures taken from Norrild, 1980. Molecular weights determined by electrophoresis on polyacrylamide gels cross-linked with DATD. The proteins used for m.wt. calibration were Myosin, β -Galactosidase, Catalase, Heavy chains, Ovalbumin, Alcohol dehydrogenase, and Lysozyme (Heine et al, 1974).
- C. Results of Spear & Roizman, 1972. The proteins used for m.wt. determination were Myosin, β -Galactosidase, Lactoperoxidase, Bovine Serum albumin, Ovalbumin, and α -Chymotrypsinogen A, with molecular weights of 220 K, 130 K, 92,6 K, 68 K, 43 K and 25,7 K, respectively. PAA gels cross-linked with N, N'-methylenebisacrylamide.
- D. Results of the present work. Taken from Figure 2.11. The proteins used for the m.wt. determination are listed in Appendix 1.2.3. PAA gels were cross-linked with N, N'-methylenebisacrylamide.

m.wt. - molecular weights $\times 10^{-3}$

O S - Off-scale. The molecular weights of these proteins were too high to be determined on 7,5% and 10% PAA gels.

V P - Virion polypeptide - i.e. From purified virions of HSV-1.

I C P - Infected cell polypeptide - i.e. From cells infected with HSV-1.

TABLE 2.6 - A COMPARISON OF THE APPARENT MOLECULAR WEIGHTS OF HSV-1 GLYCOPROTEINS

GLYCOPROTEIN DESIGNATION (a)	APPARENT MOLECULAR WEIGHT $\times 10^{-3}$ (DALTONS)	
	EXPERIMENTAL (b)	LITERATURE (c)
gB	127,4	122
gC	117,5	110
gE	86,7	85
gD	68,4	57

- (a) See Chapter 3.1.1 for a discussion of the designations given to HSV-1 glycoproteins.
- (b) Values derived from the present work using 7,5% PAA gel (Figure 2.10).
- (c) Values obtained from Haarr & Marsden, 1981 and Hope et al, 1982 (See Chapter 3.1.1).

CHAPTER THREE - PURIFICATION OF HERPES SIMPLEX VIRUSTYPE 1 (HSV-1) GLYCOPROTEINS3.1. INTRODUCTION3.1.1. The glycoproteins of HSV-1

HSV-1 has been reported to contain five major glycoproteins; Heine et al, 1974; Spear, 1976; Cohen et al, 1978; Bauke & Spear, 1979). These glycoproteins have been given various designations by different groups of researchers (Table 3.1). However, the nomenclature of Spear has now been agreed upon as the provisional designations by a group of participants at the Herpesvirus Workshop held in 1978 in Cambridge, England (Spear, 1976). In the present work, the major glycoproteins of HSV-1 have been designated, in order of decreasing molecular weight, as gB, gC, gE and gD (Figure 3.1(a)). It should be noted that at no time in the present work was any evidence obtained for two species of glycoprotein comprising the gA/gB glycoprotein as has been previously reported (Spear 1976; Bauke & Spear, 1979; Pereira et al, 1980). Some researchers have designated the glycoproteins gA/gB because of the difficulty of distinguishing between the glycoproteins gA and gB (Eberle & Courtney, 1980 (a)). More recently, Glorioso et al, have stated that HSV encodes

TABLE 3.1 - DESIGNATIONS AND MOLECULAR WEIGHTS OF THE MAJOR HSV-1 GLYCOPROTEINS

Accepted glycoprotein designation (a)	Other designations	Apparent m.wtx10 ⁻³ (Daltons)	Reference
gC		130	Eisenberg <u>et al</u> , 1979
	VP 8	129	Heine <u>et al</u> , 1974
	Ag-6	131	Vestergaard & Grauballe, 1979
	VP 2/3	ND (b)	Powell <u>et al</u> 1974
	VP 2/3	126	Powell & Watson, 1975
	G110	110 (d)	Haarr & Marsden, 1981
gB (c)		126	Spear, 1976 Bauker & Spear, 1979 Eisenberg <u>et al</u> , 1979
	gA (c)	119	Spear 1976
	gA/gB (c)	126	Glorioso <u>et al</u> , 1983
	Ag-11	N.D. (b)	Norrild <u>et al</u> , 1979
	G122	122 (d)	Haarr & Marsden, 1981
gE		65-80	Bauke & Spear, 1979
		67-85 (d)	Hope <u>et al</u> , 1982
gD		59	Eisenberg <u>et al</u> , 1979
	CP-1	59	Cohen <u>et al</u> , 1978
	Ag-8	N.D.(b)	Norrild <u>et al</u> , 1979
	VP 8/9	44-59	Powell & Watson, 1975
	G57	57 (d)	Haarr & Marsden, 1981

(a) The nomenclature accepted as the provisional designations of the HSV-1 glycoproteins at the Herpesvirus Workshop held in Cambridge, England in 1978.

(b) Not done.

(c) gA and gB are now recognized to be different forms of the same protein. The protein is now designated gB (Roberts et al, 1985).

(d) SDS-PAGE on PAA gels cross-linked with MBA.

for at least four antigenically distinct glycopeptides designated gC, gA/gB, gE and gD with corresponding apparent molecular weights of 129 K, 126 K, 80 K and 58 K. Although initial studies indicated that gA and gB were antigenically distinct and separate gene products, recent evidence suggests that gA and gB are different forms of the same polypeptide (Glorioso et al, 1983). Thus, the most recent designation for glycoprotein gA/gB is gB (Roberts et al, 1985) and gA, which is the precursor of gB, is designated pgB.

It is important to note that there has been a large discrepancy between results published by different groups working with the glycoproteins of HSV. This discrepancy chiefly concerns the apparent molecular weights of the HSV-1 glycoproteins, gC and gB. Several groups have reported gC as having a larger apparent molecular weight than gB (Heine et al; Spear, 1976; Bauke & Spear, 1979; Eisenberg et al, 1979; Glorioso et al, 1983, 1984; Roberts et al, 1985), while other groups have reported that the opposite is true (Spear & Roizman, 1972; Heine et al, 1974; Haarr & Marsden, 1981; Marsden et al, 1984). This discrepancy was first reported by Heine et al, and is caused by the use of different cross-linkers in the preparation of polyacrylamide gels (Heine et al, 1974). Therefore, those groups that have used diallyltartardia-

amide (DATD) as a cross-linker, report that gC has a larger apparent molecular weight than gB, while those groups that have used methylenebisacrylamide (MBA) (Appendix 1.2.2(a)) report the opposite. This phenomenon appears to only affect glycosylated proteins (Heine et al, 1974).

MBA was used as the cross-linker in preparing PAA gels for the present work, and so the designation of glycoproteins and comparison of results are taken from those who have used the same PAA gel system (Marsden et al, 1984) (See Figure 3.1).

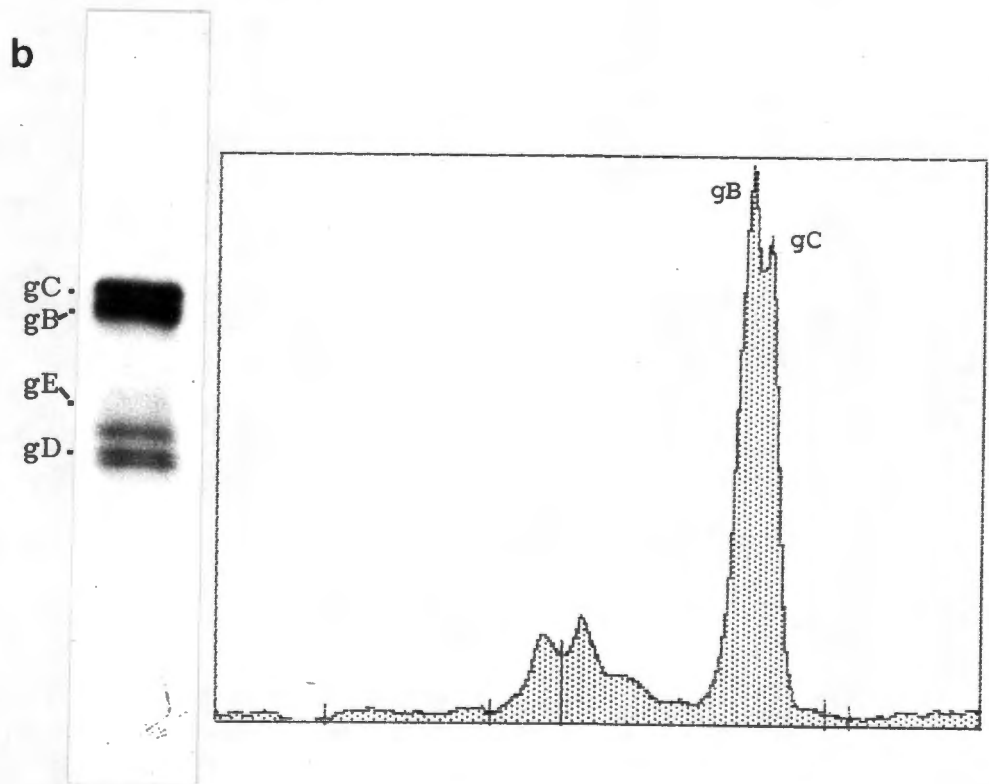
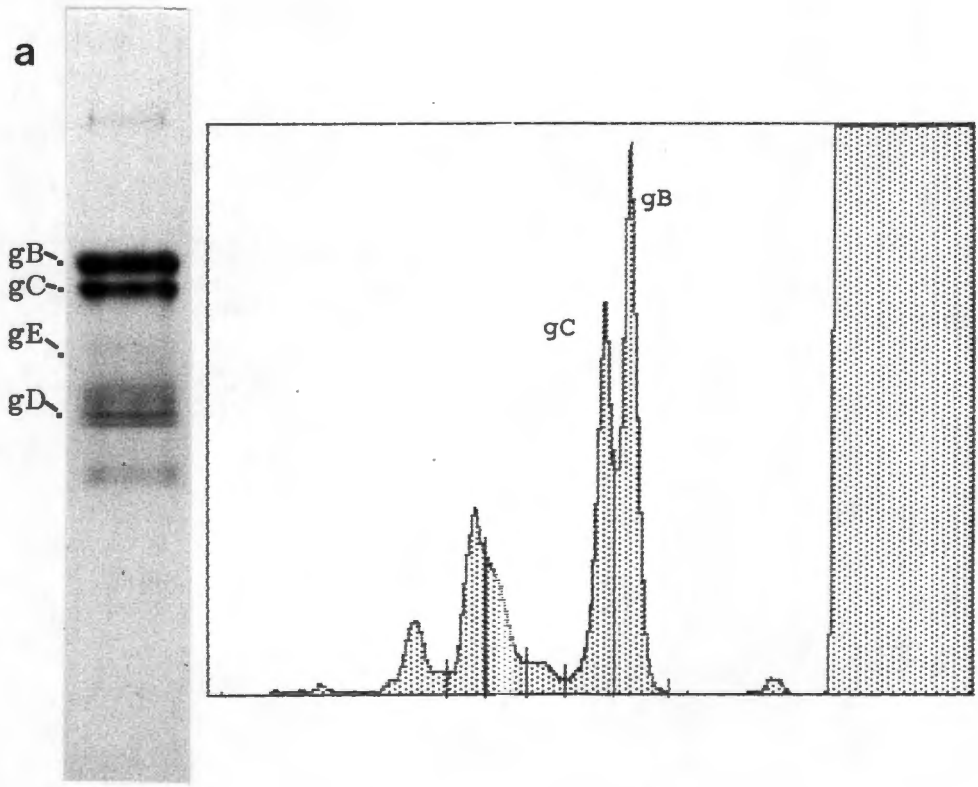
Some of the techniques that have been used for the purification of glycoproteins gC and gB are preparative polyacrylamide gel electrophoresis (Prep-PAGE), hydroxylapatite chromatography in the presence of sodium dodecyl sulphate (SDS-HTP), and gel bound lectin affinity chromatography.

FIGURE 3.1

HSV-1 infected BHK-21 cells, grown in the presence of (3H)-glycosamine, were harvested and solubilized with LMW sample buffer (Chapter 2.2.2). This material was electrophorezed on (a) 10% PAA gel cross-linked with MBA and, (b) 10% PAA gel cross-linked with DATD.

The figure shows the autoradiographs of the resultant gels after electrophoresis, and their corresponding densitometer scans. The densitometer scans show that the more dense band (gB) has a larger apparent molecular weight than the less dense band (gC) in (a), and that the opposite is true in (b). Glycoproteins gC and gB were distinguished according to the radioactive content of their respective bands as shown by the intensity of these bands on the autoradiographs. This method of distinguishing the glycoproteins is similar to that used by Heine et al (1974), but is not entirely satisfactory. The most conclusive method of distinguishing between these proteins would be the use of monoclonal antibodies directed against each protein (Spear, personal communication).

The glycoproteins in (a) are designated according to Hope et al (1982) and Marsden et al (1984), and in (b) are designated according to Bauke & Spear (1979) and Glorioso et al (1983). The designation of glycoproteins in the present work is as shown in (a).



3.1.2. Preparative polyacrylamide gel electrophoresis
(Prep-PAGE)

Prep-PAGE was initially used to isolate a large molecular weight polypeptide of HSV-1 (Courtney & Benyesh-Melnick, 1974) and has since been used for the partial purification of the major glycoprotein regions of HSV-1 and HSV-2 (VP 123 and VP 119, respectively) (Dreesman *et al*, 1979; Eberle & Courtney, 1980(a)). The system is the same as that used for analytical SDS-PAGE (Chapter 2) except that electrophoresis is performed in an apparatus which allows for preparative amounts of material and the collection of proteins eluted from the PAA gel (Figure 2.3.1).

3.1.3. Hydroxylapatite chromatography in the presence of sodium dodecyl sulphate (SDS-HTP)

Some workers have used hydroxylapatite chromatography as a means of selectively isolating the HSV-1 specific glycoprotein, gC (Vestergaard & Grauballe, 1979; Schrier, Pizer & Moorhead, 1983). However, other workers, who used Prep-PAGE as an initial purification step, utilized the technique of hydroxylapatite chromatography in the presence of sodium dodecyl sulphate (Moss & Rosenblum, 1972) to purify the HSV-1 glycoprotein, gC, from other glycosylated and non-glycosylated proteins (Eberle & Courtney, 1980(a)).

This technique involves adsorption of protein-SDS complexes onto hydroxylapatite and elution with 0.1% SDS, 1mM dithiothreitol (DTT) and a gradient of sodium phosphate. A useful feature of the technique is that proteins do not all elute in order of molecular weight. Thus, polypeptide separations on hydroxylapatite are different from those obtained by polyacrylamide gel electrophoresis or gel filtration in SDS (Moss & Rosenblum, 1972). HA Ultrogel (Appendix 1.3.1) which has similar properties to hydroxylapatite was used in the present work.

3.1.4. Gel-bound lectin affinity chromatography

The specificity of lectins for various saccharide residues makes them useful as affinity adsorbents for the fractionation of glycoproteins.

HSV glycoproteins have been characterized by their binding to concanavalin A (Con A), wheat germ, soybean and Helix pomatia lectins (Ponce de Leon, Hessel & Cohen, 1973; Jeansson, Elwing & Nilsson, 1979; Olofsson, Jeansson & Lycke, 1981). Con A and wheat germ lectins have affinity for the internal α -mannose and n-acetyl-D-glucosamine (GlcNAc) respectively, of oligosaccharides, while soybean and Helix pomatia lectins both have affinity for n-acetyl-D-galactosamine (GalNAc).

Con A affinity chromatography was used to selectively remove HSV-induced antigens from extracts of infected BHK cells (Ponce de Leon, Hessel & Cohen, 1973). Two broad groups of viral-induced antigens were isolated from Con A. Group I included two antigens which bound to Con A and were dissociated by α -methyl-D-mannoside (α MM). Group II contained three antigens which bound to Con A by a non-specific or electrolytic mechanism and were only eluted using 2M NaCl.

Wheat germ lectin has been used to purify and concentrate HSV-1 glycoproteins from non-glycosylated proteins (Jeansson, Elwing & Nilsson, 1979). However, this procedure did not differentiate between the different glycoproteins of HSV-1 and four antigens were detected in the glycoprotein eluate when tested against HSV-1 antiserum.

When lysates from HSV-1 and HSV-2 infected cells were subjected to affinity chromatography on soybean and Helix pomatia lectins, one of the virus-specified glycoproteins, probably glycoprotein C, bound to the lectins and was eluted with N-acetyl-galactosamine (GalNAc). The affinity chromatography permitted a high degree of purification of the type specific glycoproteins of HSV-1 and HSV-2 with respect to both host cell components and other viral glycoproteins (Olofsson, Jeansson & Lycke, 1981;

Svennerholm et al, 1984). Antigens purified by Helix pomatia lectin were used in an enzyme-linked immunosorbent assay (ELISA) with human reference sera to both types of HSV. The results correlated completely with the results of virus typing and showed that Helix pomatia lectin affinity chromatography was suitable for purifying type-specific antigens of HSV-1 and HSV-2 (Svennerholm et al, 1984).

- 3.1.5 The aim of the present work was to obtain preparations of the HSV-1 glycoproteins, gC and gB, free from any other viral or host cell proteins.

HSV-1 infected BHK-21 cells were grown in the presence of (³H)-glucosamine and were solubilized in SDS and 2-mercaptoethanol at 28 hours post-infection (Chapter 2.2.2). The first step in the purification of glycoproteins gC and gB was to isolate them from each other and from the other glycoproteins of HSV-1. This was accomplished by two cycles of Prep-PAGE which successfully separated these glycoproteins, but the resultant solutions still contained non-glycosylated proteins.

Therefore, the next step of purification was to remove the non-glycosylated proteins from the glycoprotein solutions. Hydroxylapatite chromatography in the presence of

SDS (Chapter 3.2.2) and gel-bound lectin affinity chromatography (Chapter 3.2.3) were used, but did not successfully purify the glycoproteins.

As the difficulties encountered in purifying the HSV-1 glycoproteins by gel-bound lectin affinity chromatography could have resulted from the use of SDS for solubilizing the infected cell proteins, the technique was repeated using HSV-1 membrane proteins solubilized in Triton X-100 (Chapter 3.2.5). Wheat germ lectin affinity chromatography of this material resulted in the purification of several HSV-1 glycoproteins from the non-glycosylated proteins.

3.2 METHODS AND RESULTS

3.2.1 Preparative Polyacrylamide Gel Electrophoresis (Prep-PAGE)

Polyacrylamide gel electrophoresis (Chapter 2.1.2) can be adapted for preparative purposes by using the apparatus shown in Figure 2.3.1. The Prep-PAGE apparatus is connected to the other instruments shown in the diagram, which allow for the recording and collection of material eluted from the PAA gel. The technique used in Prep-PAGE is essentially the same as that used for analytical SDS-PAGE (Appendix 2.2.1) except that a different apparatus

is used and no "wick" gel is required. The method is described in detail in Appendix 2.3.1.

Prep-PAGE was used for the following purposes:-

A. Prep-PAGE of HSV-1, HSV-2 and mock infected BHK-21 cells labelled with either (¹⁴C)-amino acids or (³H)-glucosamine.

HSV-1, HSV-2 and mock infected BHK-21 cells which had been grown in the presence of either (³H)-glucosamine or (¹⁴C)-amino acids and stored at -70°C (Chapter 2.2.2) were electrophoresed separately in the Prep-PAGE apparatus. A 1,0 ml volume of solubilized material was mixed with 0,3 ml of 100% glycerol and phenol red and applied to the 7,5% PAA gel (Figure 2.3.1). Electrophoresis was performed at constant current (8 mA) and 5 ml fractions were collected at a flow rate of 8.0 ml/hr. Each fraction was assayed for radioactivity (Appendix 2.3.5) and the elution profile of each product was drawn by plotting counts per minute (CPM) against fraction number (Figure 3.2).

B. Separation of HSV-1 glycoproteins by Prep-PAGE

HSV-1 infected BHK-21 cells, which had been grown in the presence of (³H)-glucosamine, solubilized in LMW sample buffer (Appendix 1.2.1(d)) and stored at

-70°C (Chapter 2.2.2) were electrophorezed in the Prep-PAGE apparatus so as to separate the glycoproteins from each other. A 1,0 ml aliquot of the solubilized material was mixed with 0,3 ml of 100% glycerol and phenol red and applied to the PAA gel. The procedure described in (A) was repeated, and from the elution profile that was drawn, the peaks of radioactivity were determined and the constituent fractions were pooled separately (Peaks I, II and III respectively (Figure 3.3)).

The pooled fractions were concentrated by the following procedure:

- (a) Pervaporation to reduce the volume to approximately 10 ml (Appendix 2.3.7).
- (b) Dialysis against the reservoir buffer (Appendix 1.2.1(c)) to reduce the buffer salts.
- (c) Lyophilization (Appendix 2.3.6).
- (d) Resuspension in the required volume of LMW sample buffer (i.e. 1,0 ml) for a repeat cycle of Prep-PAGE electrophoresis.

Peaks I, II and III were then electrophorezed separately in the Prep-PAGE apparatus according to the procedure described in (A). After electrophoresis the fractions constituting the major peak of radio-

activity in each case (Figure 3.4) were pooled and stored at -20°C in the presence of 0.001 M phenylmethylsulphonyl fluoride (PMSF) (Appendix 1.3.6).

RESULTS

A. Prep-PAGE of HSV-1, HSV-2 and mock infected BHK-21 cells labelled with either (^{14}C)-amino acids or (^3H)-glucosamine.

The elution profiles of the six different radiolabelled products (Chapter 2.2.2) after electrophoresis in the Prep-PAGE apparatus are shown in Figure 3.2. These elution profiles record the different radiolabelled polypeptides of each of the products, as has been discussed earlier (Chapter 2.2.2).

B. Separation of HSV-1 glycoproteins by Prep-PAGE

Prep-PAGE of HSV-1 infected BHK-21 cell proteins labelled with (^3H)-glucosamine produced four peaks of radioactivity (Figure 3.3). The first and largest peak of radioactivity eluted with the phenol red and probably contained (^3H)-glucosamine which had not been incorporated into proteins. The three following peaks are named peak I, II and III, respectively (Figure 3.3). Analytical SDS-PAGE showed that peak I consists of several of the intermediate and low molecular weight HSV-1 glycoproteins (Figure

3.6). Peak II consists mainly of the HSV-1 glycoprotein, gC, but also contains some of glycoprotein, gB. Peak III consists mainly of glycoprotein, gB, but also some of glycoprotein, gC (See Chapter 3.1.1 for the designations of these proteins).

Peaks I, II and III were concentrated and individually electrophoresed in the Prep-PAGE apparatus. This second cycle of Prep-PAGE further purified the glycoproteins contained within each peak with respect to the other glycoproteins (Figure 3.4). The fractions within the major peaks of radioactivity were pooled and stored in the presence of 0,001 M PMSF (Appendix 1.3.6) at -20°C until required. Analytical SDS-PAGE of the pooled fractions constituting peaks II and III, showed that after the second cycle of Prep-PAGE they each contained only one species of glycoprotein (Figure 3.7(b)). However, they still contained several non-glycosylated proteins (Figure 3.7(a)).

FIGURE 3.2 - Prep-PAGE ELUTION PROFILES OF HSV-1, HSV-2
AND MOCK INFECTED CELL PROTEINS LABELLED
WITH EITHER (³H)-gln OR (¹⁴C)-aa

HSV-1, HSV-2 and mock infected cells which had been grown in the presence of (³H)-glucosamine or (¹⁴C)-amino acids, were solubilized and electrophorezed in the preparative PAGE apparatus containing a 7,5% PAA gel (Figure 2.3.1). The samples were electrophorezed at constant current (8 mA), and 5 ml fractions of eluant were collected at a flow rate of 8 ml/hr. Fractions were assayed for radioactivity and the elution profiles drawn by plotting counts per minute (CPM) for each fraction.

- (a) - Cells labelled with (³H)-glucosamine.
- (b) - Cells labelled with (¹⁴C)-amino acids.
- (□--□) - HSV-1 infected cells.
- (○--○) - HSV-2 infected cells.
- (▲--▲) - Mock infected cells.

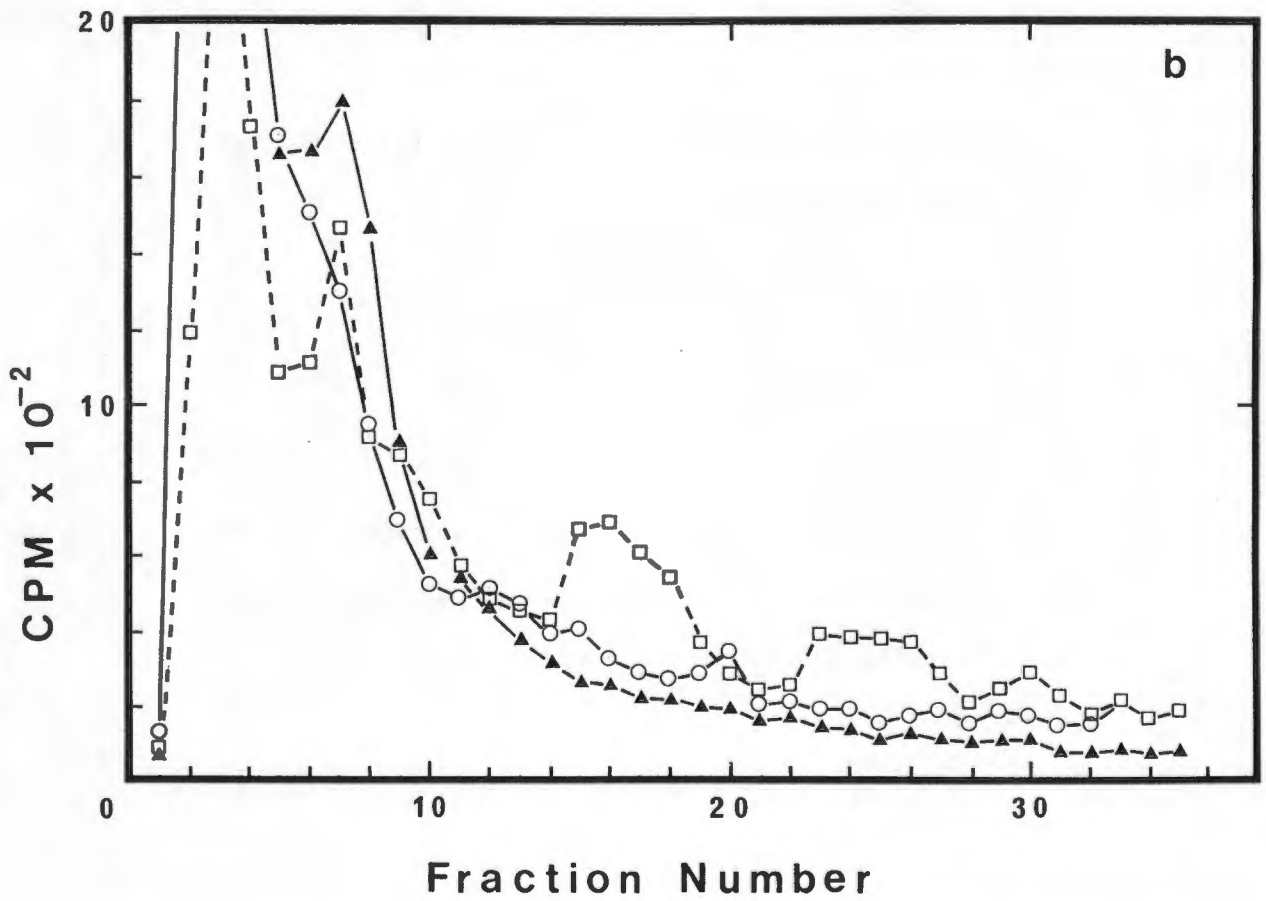
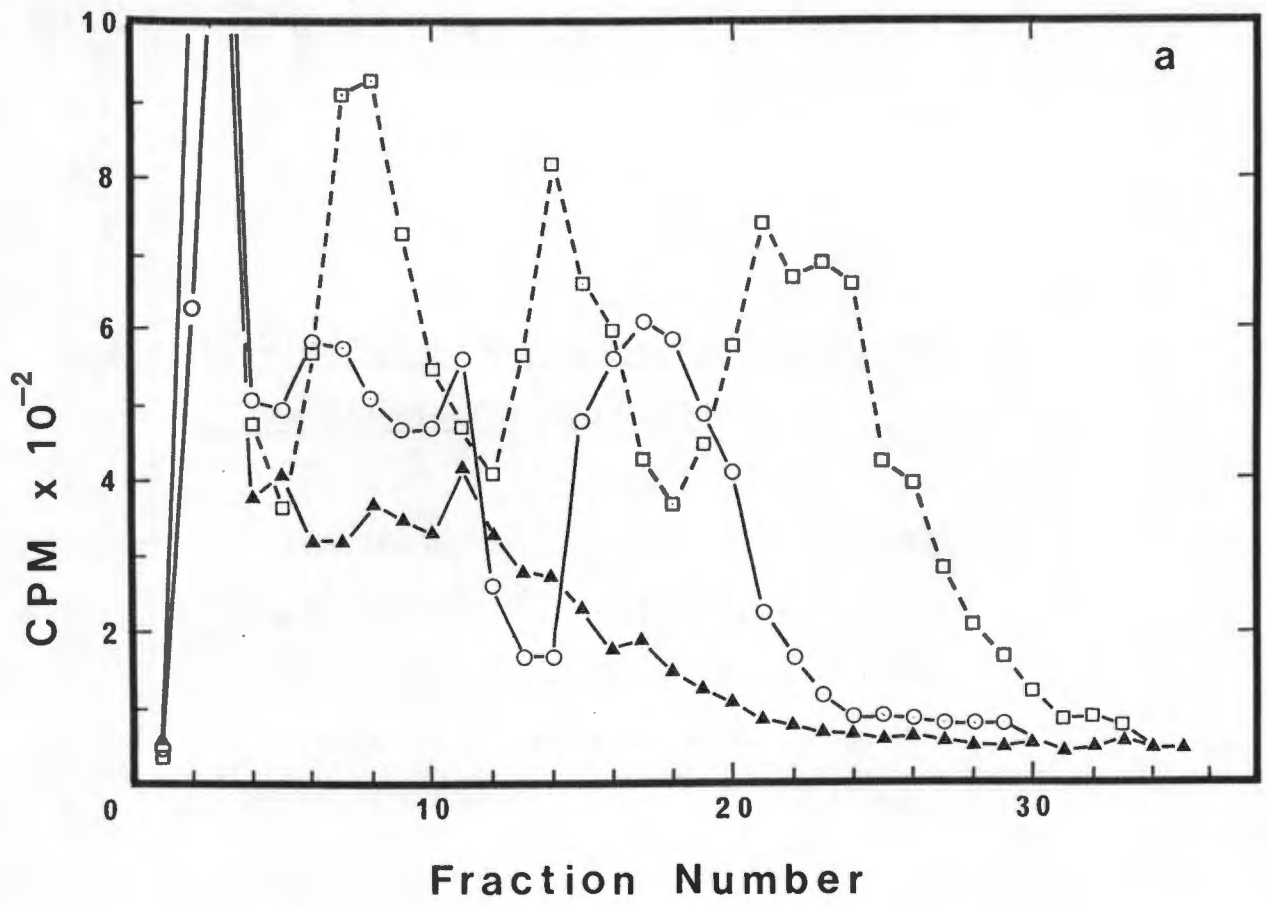


FIGURE 3.3 - Prep-PAGE ELUTION PROFILE OF HSV-1
INFECTED CELL PROTEINS LABELLED
WITH (³H)-gln

HSV-1 infected BHK-21 cells which had been grown in the presence of (³H)-glucosamine were solubilized and electrophorezed in the Prep-PAGE apparatus as described in the legend to Figure 3.2.

Fractions 8, 12 and 21 were analyzed by SDS-PAGE (Figure 3.5).

Fractions within peaks I, II and III were pooled as follows:-

- Peak I - Fractions 5-12
- Peak II - Fractions 13-18
- Peak III - Fractions 19-27

These pooled samples were concentrated and re-electrophorezed (Figure 3.4) as well as being analyzed by SDS-PAGE (Figure 3.6).

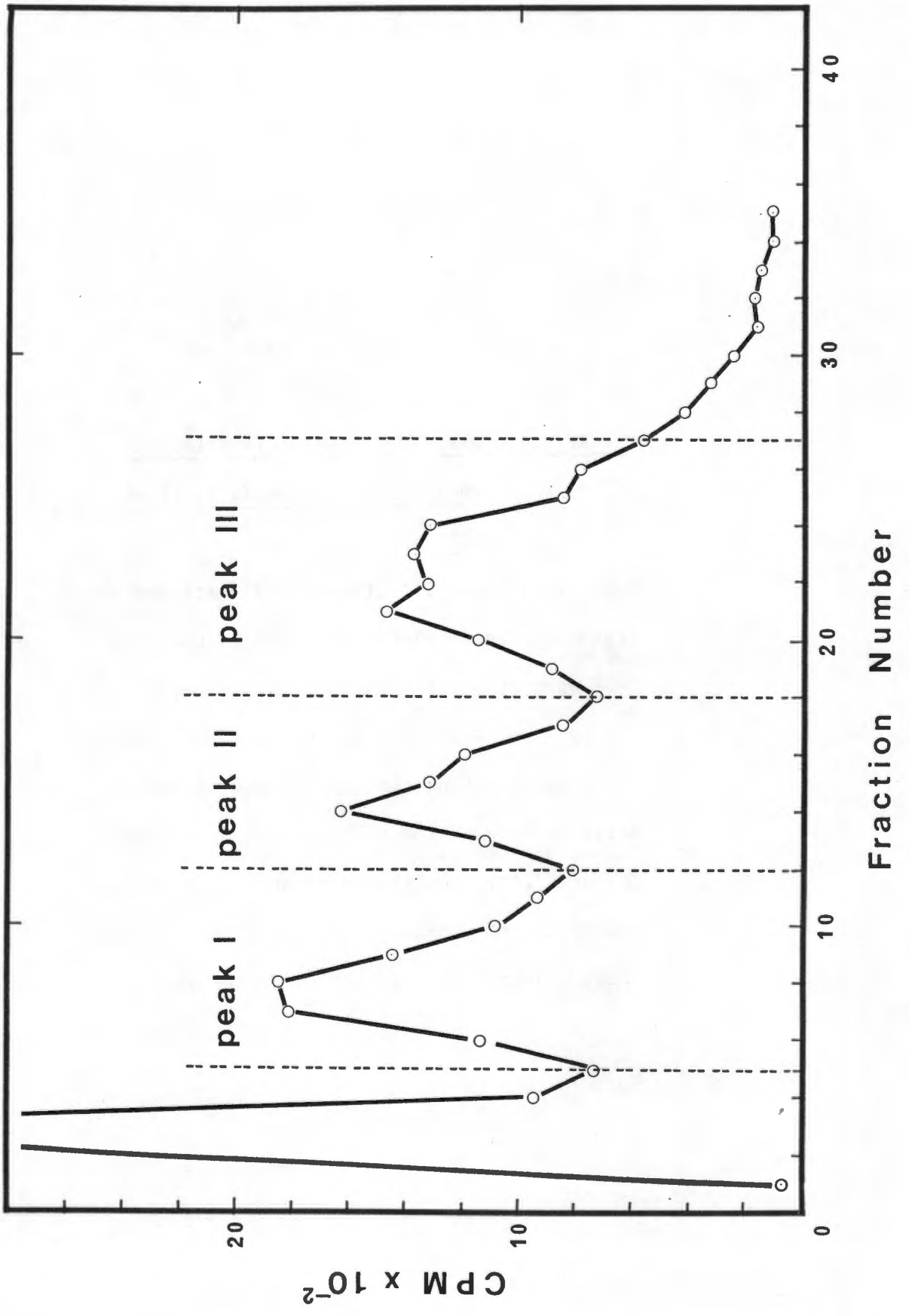


FIGURE 3.4 - Prep-PAGE ELUTION PROFILES OF
PROTEINS WITHIN PEAKS I, II AND
III

Peaks I, II, and III (Figure 3.3) were concentrated and again electrophorezed in the Prep-PAGE apparatus, as described in the legend to Figure 3.2. After electrophoresis the fractions constituting the major peaks of radioactivity were pooled and analyzed by SDS-PAGE (Figure 3.7). The remainder of each peak was stored in the presence of 0,001 M PMSF at -20°C until required for further purification.

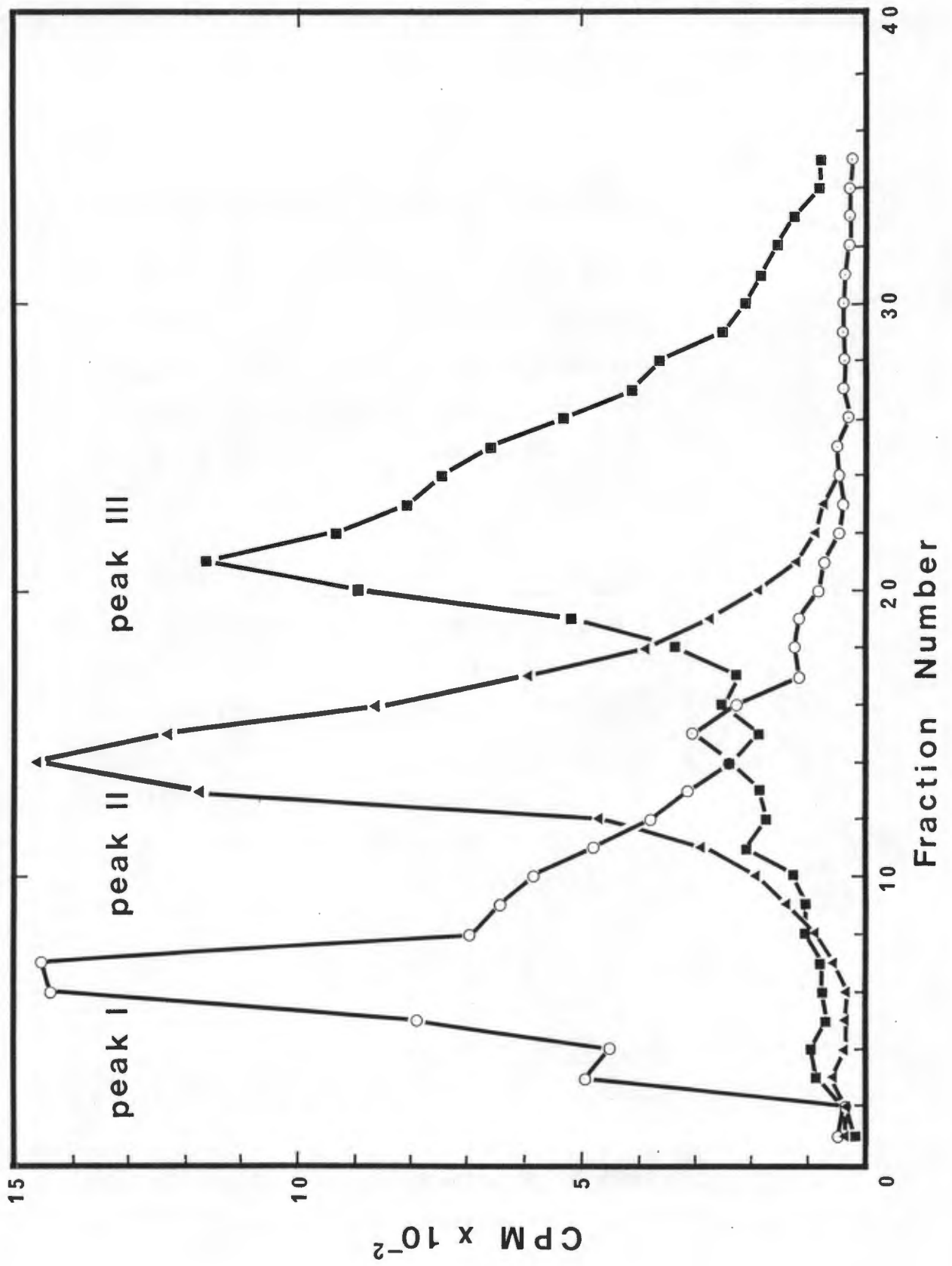


FIGURE 3.5 - ANALYTICAL SDS-PAGE OF FRACTIONS
COLLECTED DURING Prep-PAGE

HSV-1 infected cell proteins labelled with $(^3\text{H})\text{-gln}$ were subjected to electrophoresis in a Prep-PAGE apparatus (Figure 3.3). A fraction from each peak of radioactivity (Fractions 8, 12 and 21) was analyzed by SDS-PAGE and autoradiography.

Figure 3.5(a) - Silver Stain

Track 1 - High molecular weight (HMW) marker proteins.

Track 2 - HSV-1 infected BHK-21 cell proteins labelled with $(^3\text{H})\text{-gln}$. This is the starting material that was applied to the Prep-PAGE apparatus.

Track 3 - Fraction 8

Track 4 - Fraction 12

Track 5 - Fraction 21

Figure 3.5(b) - Autoradiograph

Track 1 - HSV-1 infected cell proteins labelled with $(^3\text{H})\text{-gln}$

Track 2 - Fraction 8

Track 3 - Fraction 12

Track 4 - Fraction 21

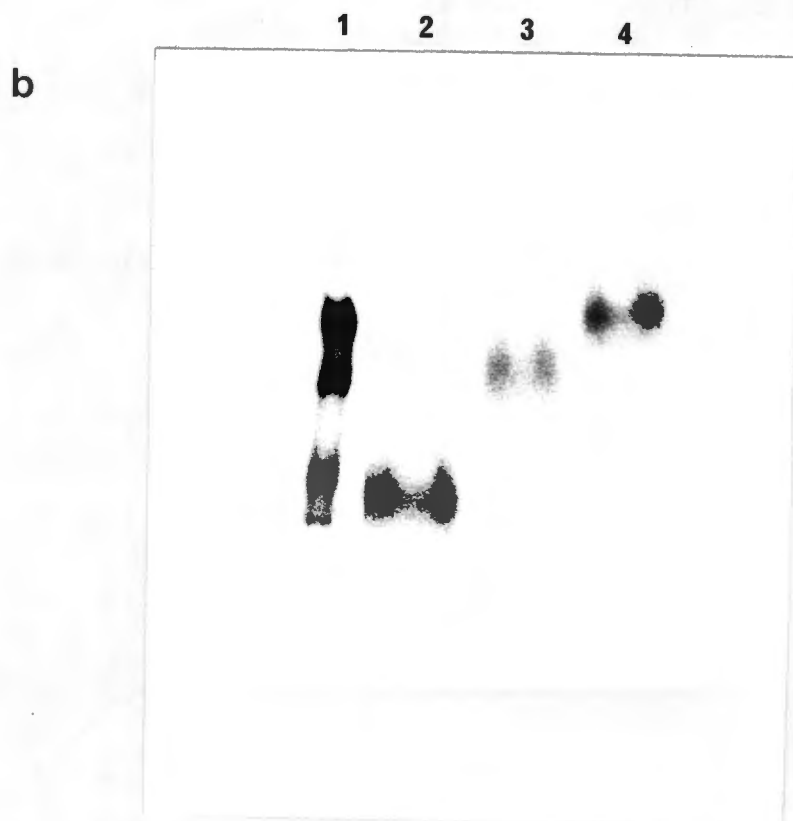
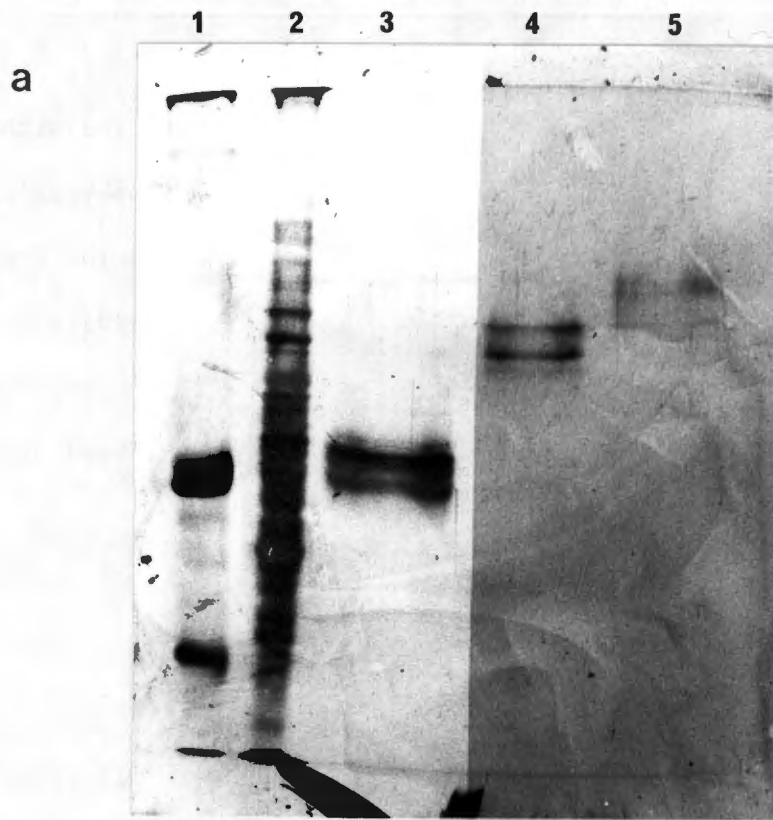


FIGURE 3.6 - ANALYTICAL SDS-PAGE OF PEAKS I, II
and III

HSV-1 infected cell proteins labelled with (^3H)-gln were subjected to electrophoresis in a Prep-PAGE apparatus (Figure 3.3). The fractions within each peak of radioactivity were pooled (Peaks I, II and III) and a sample of each pool was analyzed by SDS-PAGE and autoradiography.

Figure 3.6(a) - Silver Stain

Track 1 - HMW marker proteins.

Track 2 - HSV-1 infected BHK-21 cell proteins
labelled with (^3H)-gln.

Track 3 - Peak I

Track 4 - Peak II

Track 5 - Peak III

Figure 3.6(b) - Autoradiograph

Track 1 - HSV-1 infected BHK-21 cell proteins
labelled with (^3H)-gln

Track 2 - Peak I

Track 3 - Peak II

Track 4 - Peak III

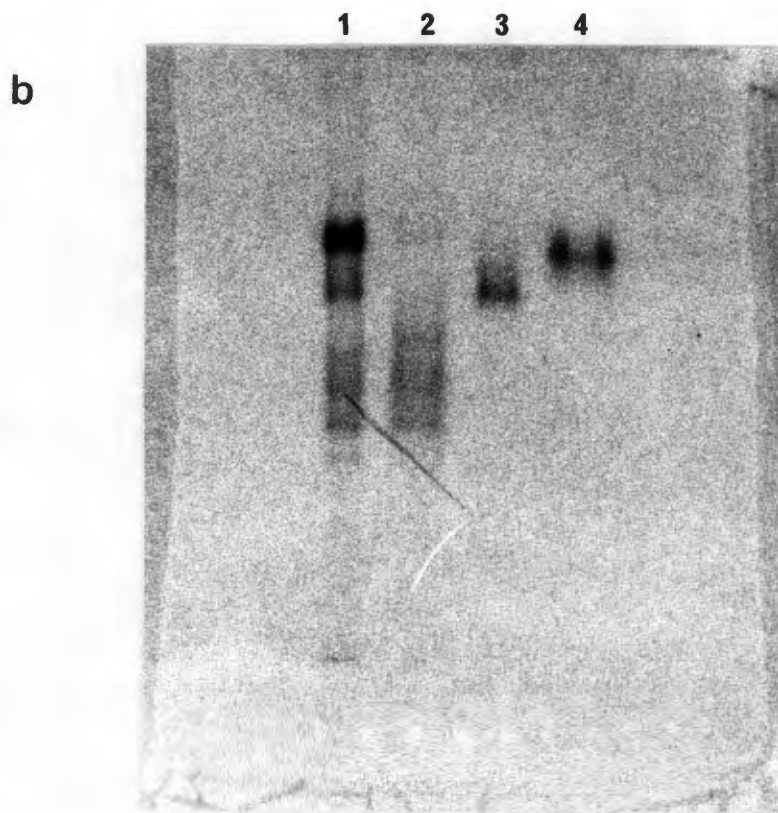
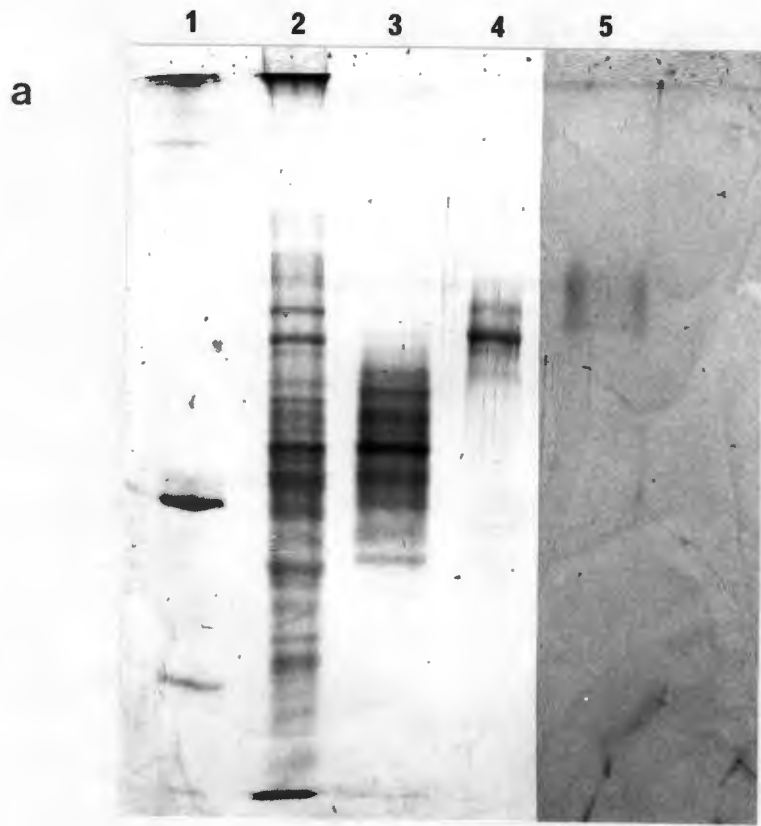


FIGURE 3.7 - ANALYTICAL SDS-PAGE OF PEAKS II
AND III

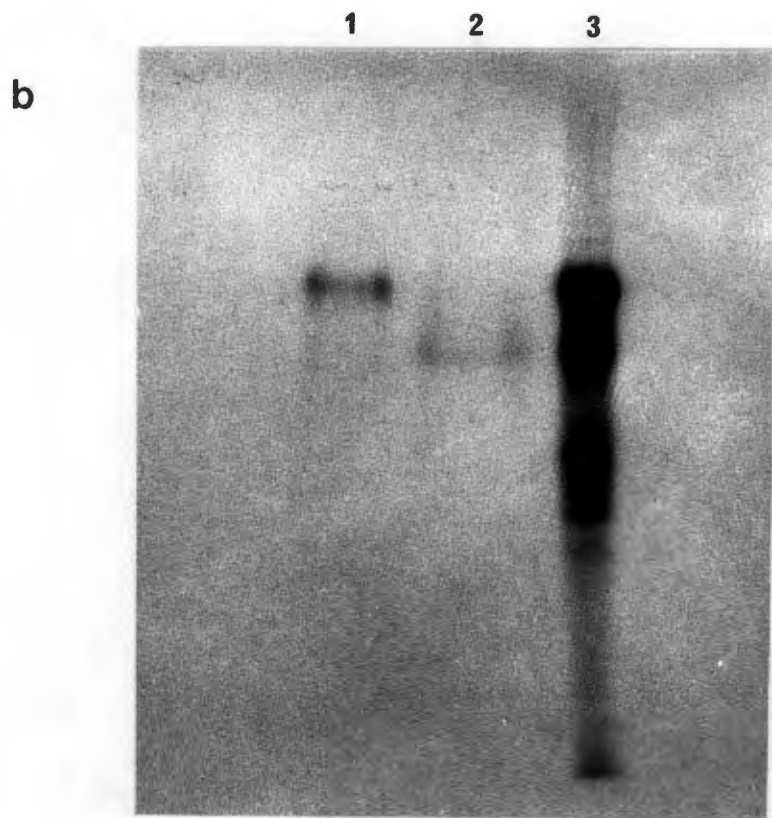
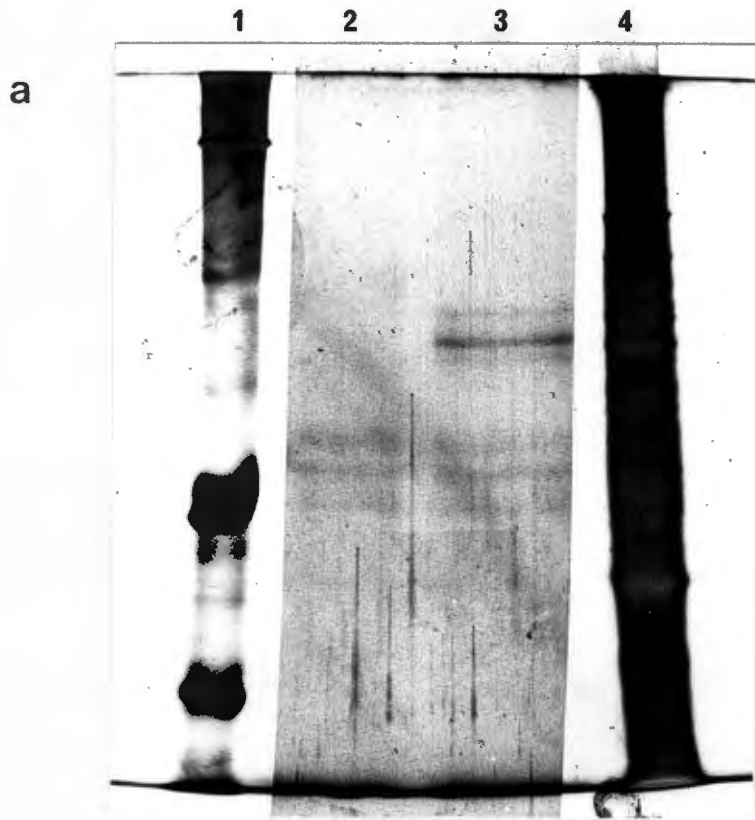
HSV-1 infected cell proteins labelled with (^3H)-glucosamine were electrophoresed in a Prep-PAGE apparatus (Figure 3.3). The fractions within the peaks of radioactivity (peaks I, II and III) were pooled and concentrated. Each peak was then subjected to a second cycle of electrophoresis in the Prep-PAGE apparatus (Figure 3.4). The fractions within each peak of radioactivity were pooled and stored at -20°C in the presence of 0,001 M PMSF. Samples of peak II and peak III were analyzed by SDS-PAGE and autoradiography.

Figure 3.7(a) - Silver Stain

Track 1 - HMW marker proteins
Track 2 - Peak III
Track 3 - Peak II
Track 4 - HSV-1 infected BHK-21 cell proteins
labelled with (^3H)-gln

Figure 3.7(b) - Autoradiograph

Track 1 - Peak III
Track 2 - Peak II
Track 3 - HSV-1 infected BHK-21 cell proteins
labelled with (^3H)-gln



DISCUSSION

Two cycles of Prep-PAGE, using 7,5% PAA gel, were sufficient to produce solutions of the HSV-1 glycoproteins, gC and gB, free of contamination by any of the other HSV-1 glycoproteins (Figure 3.7(b)). However, analytical SDS-PAGE revealed that these solutions contained a significant amount of non-glycosylated proteins (Figure 3.7(a)). As the aim of the present work was to produce pure preparations of glycoproteins, gC and gB, further purification of these solutions was necessary.

3.2.2 Purification of HSV-1 glycoproteins, gC and gB, from non-glycosylated proteins using hydroxylapatite chromatography in the presence of sodium dodecyl sulphate (SDS-HTP)

Hydroxylapatite chromatography in the presence of sodium dodecyl sulphate (SDS-HTP) has previously been used to separate the HSV-1 glycoproteins gA/gB (gB) and gC from each other and from non-glycosylated proteins (Eberle & Courtney, 1980(a)). Therefore, an attempt was made to use SDS-HTP to purify the glycoproteins from non-glycosylated proteins, and also to confirm the separation of gC and gB by this means. In the present work HA Ultrogel (Appendix 1.3.1) was used instead of normal hydroxylapatite. HA Ultrogel is reported to have the same properties as hydroxylapatite but better flow properties on account of being embedded in agarose beads (LKB Catalogue).

The procedure was as follows:-

- (a) The HA Ultrogel column and the samples for chromatography were prepared as described in Appendix 2.3.2.
- (b) 20 ml of HSV-1 glycoprotein, gC solution (Figure 3.4 Peak II) was applied to the HA Ultrogel column at a flow rate of 8 ml/hr.
- (c) The adsorbed proteins were then eluted from the column over a six hour period using a linear gradient of increasing phosphate molarity from 0,1-0,5 M phosphate buffer, pH 6,4, with 0,1% SDS and 1 mM dithiothreitol (DTT) at a flow rate of 15 ml/hr.
- (d) 5 ml fractions were collected and 0,1 ml aliquots of each fraction were assayed for radioactivity (Appendix 2.3.5). The elution profile of counts per minute (CPM) against fraction number was then drawn (Figure 3.8).
- (e) The procedure was repeated using the following samples:-
 - (i) 20 ml of HSV-1 glycoprotein, gB, solution (Figure 3.4 Peak III).
 - (ii) 20 ml of HSV-1 glycoprotein mixture (Figure 3.4 Peak I).
 - (iii) A mixture containing 10 ml of gC solution and 10 ml of gB solution (Figure 3.4 Peak II and Peak III).

RESULTS

SDS-HTP of the major peaks of radioactivity obtained by Prep-PAGE (Peaks I, II and III, Figure 3.4) demonstrated that the glycoproteins contained within these peaks were all eluted from HA Ultrogel over the same range of phosphate molarity - between 0,25 M and 0,39 M phosphate (Figure 3.8).

When a mixture of peak II and peak III was applied to the HA Ultrogel column, the glycoproteins eluted as a single peak of radioactivity (Figure 3.9). Analytical SDS-PAGE of fractions from this peak (Fractions 7, 9, 10 and 12, Figure 3.9) confirmed that glycoproteins gC and gB eluted together and were not separated by this technique (Figure 3.10).

FIGURE 3.8- HYDROXYLAPATITE CHROMATOGRAPHY OF
HSV-1 GLYCOPROTEINS IN THE PRESENCE
OF SDS (SDS-HTP)

HSV-1 glycoproteins were separated by Prep-PAGE into peaks I, II and III (Figure 3.4). 20 ml of each peak was applied separately to an HA Ultrogel column and eluted with a linear gradient of increasing phosphate molarity from 0,1 - 0,5 M phosphate buffer, pH 6,4, with 0,1% SDS and 1 mM DTT (----). 0,1 ml aliquots of each fraction were assayed for radioactivity and the elution profiles of counts per minute (CPM) against fraction number were drawn.

(□—□) - Low molecular weight glycoproteins
(Peak I).

(△—△) - Glycoprotein gC (Peak II).

(■—■) - Glycoprotein gB (Peak III).

Phosphate Molarity

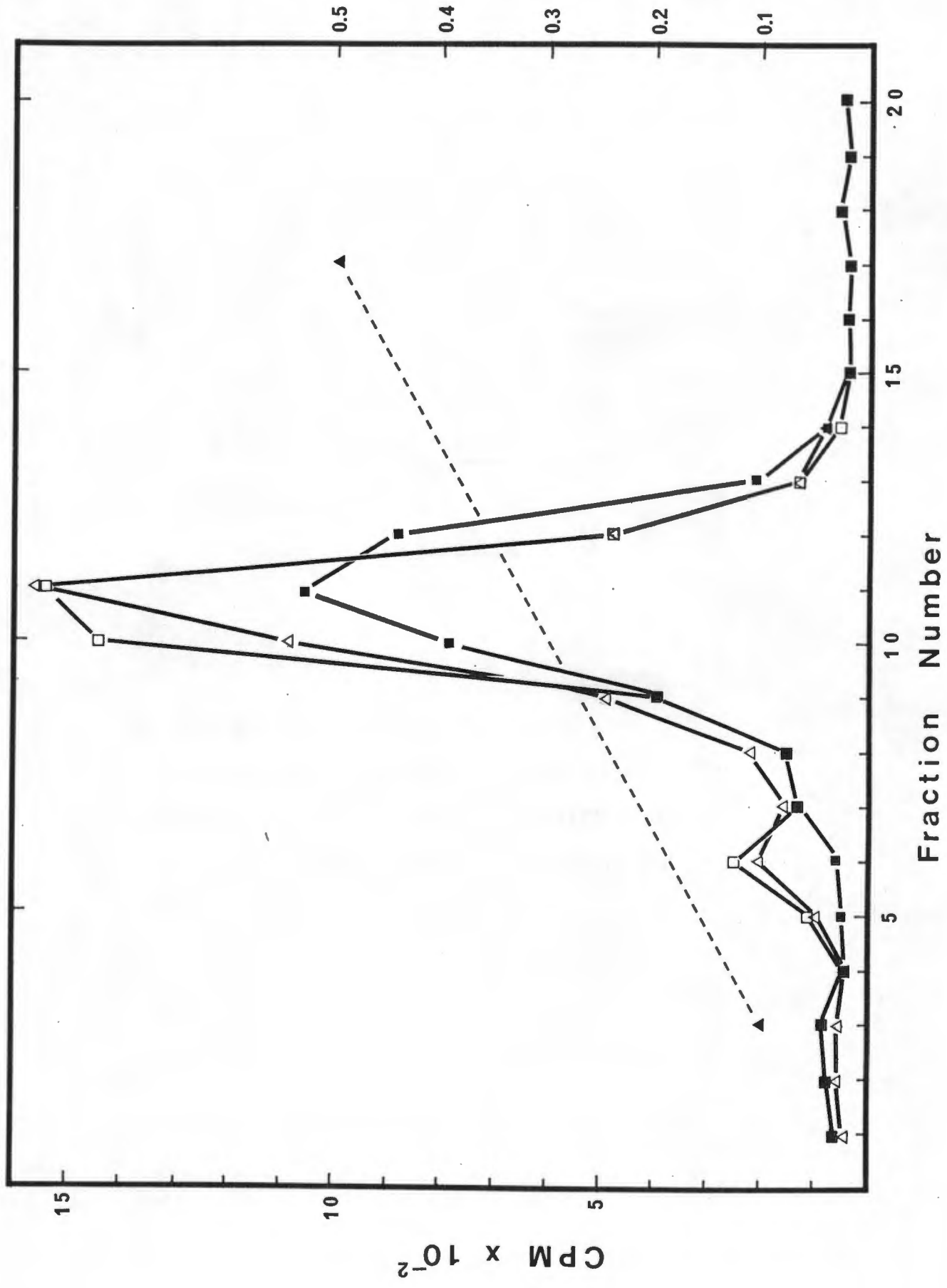
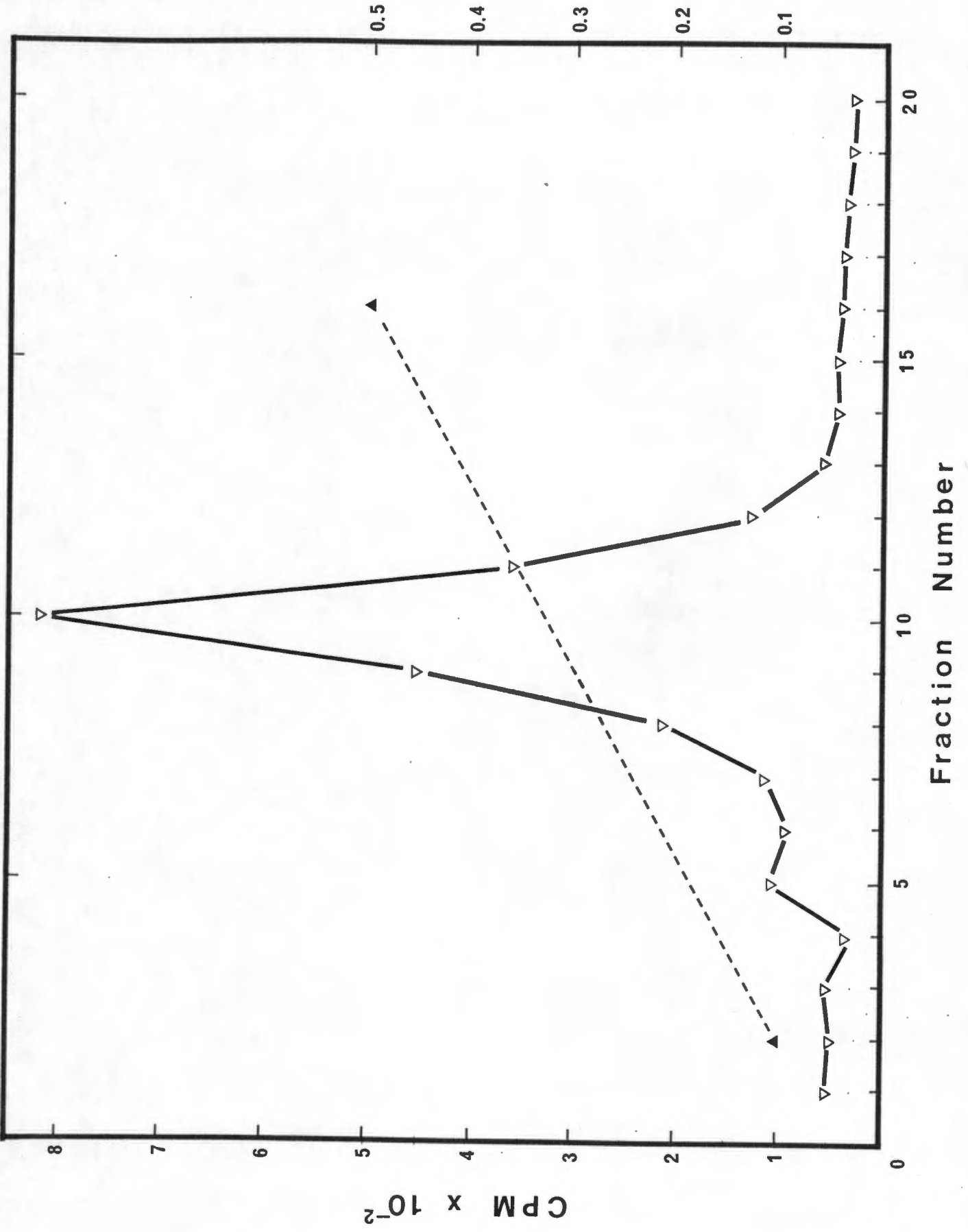


FIGURE 3.9 - SDS-HTP CHROMATOGRAPHY OF A
MIXTURE OF HSV-1 GLYCOPROTEINS,
gC and gB

A mixture of 10 ml of Peak II (gC) and 10 ml of Peak III (gB) (See Figure 3.4) was applied to an HA Ultrogel column and eluted according to the legend in Figure 3.8. Fractions 7, 9, 10 and 12 were analyzed by SDS-PAGE and autoradiography (Figure 3.10).



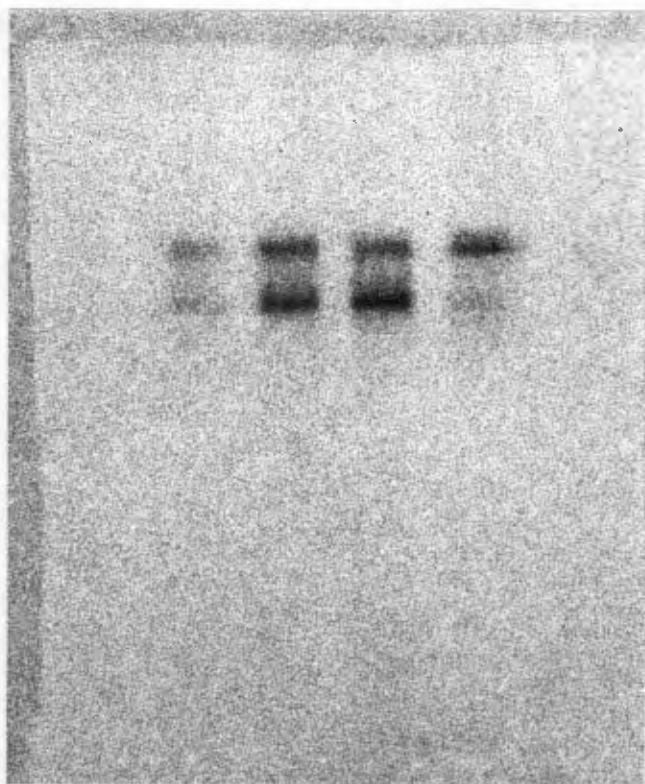


FIGURE 3.10 - AUTORADIOGRAPH OF FRACTIONS ELUTED FROM
HA-ULTROGEL AND ANALYZED BY SDS-PAGE

Fractions 7, 9, 10 and 12 (Figure 3.9) were concentrated and electrophorezed on 7,5% PAA gel. After electrophore-
sis the PAA gel was impregnated with EN³HANCE, dried and
exposed to Kodak X-Omat AR 5 film.

Track 1 - Fraction 7

Track 2 - Fraction 9

Track 3 - Fraction 10

Track 4 - Fraction 12

DISCUSSION

The major glycoprotein region of HSV-1 (VP 123), which consists of a non-glycosylated protein and glycoproteins gA/gB (gB) and gC, has previously been isolated by Prep-PAGE and then further fractionated by SDS-HTP chromatography. The constituent proteins of VP 123 were reported to elute separately from the hydroxylapatite column and within narrow ranges of phosphate molarity. Thus, purified solutions of gC and gA/gB were obtained (Eberle & Courtney, 1980(a)).

The technique of Eberle and Courtney was used to remove non-glycosylated proteins from the glycoprotein solutions of gC and gB. However, the glycoproteins were found to elute over a much wider range of phosphate molarity than had previously been reported, and all the HSV-1 glycoproteins were found to elute within approximately the same phosphate molarity range (Figure 3.8). SDS-HTP chromatography of a mixture of the gC and gB solutions confirmed that these glycoproteins eluted within the same phosphate molarity range (Figure 3.9 and 3.10). These results show that:-

- (a) SDS-HTP chromatography using HA-Ultrogel was not suitable for separating HSV-1 glycoproteins, gC and gB.

(b) Since glycoproteins gC and gB were eluted over such a wide range of phosphate molarity, SDS-HTP would not be suitable for purifying the glycoproteins with respect to non-glycosylated proteins.

3.2.3 Purification of HSV-1 glycoproteins, gC and gB, from non-glycosylated proteins using gel-bound lectin affinity chromatography.

Lectins are proteins which have the ability to react reversibly with specific sugar residues and such proteins have been isolated from a variety of plants and invertebrates. They are not only distinguished by their origins and structures but, most importantly, by their affinities for different carbohydrate moieties (Sharon & Lis, 1972; Lis & Sharon, 1973).

Only the terminal residue of an oligosaccharide is involved in binding with the lectin and the binding reaction is analogous to the interaction between an antibody and an antigen. The bond between the lectin and the specific sugar residue is broken by solutions of high ionic strength or by solutions containing a competitive binding substance (Sharon & Lis, 1972).

A. Wheat germ lectin affinity chromatography

Wheat germ lectin is a protein which has been extracted from wheat germ. The active wheat germ lectin consists of two identical subunits at neutral pH, with a total molecular weight of about 35,000. This dimer dissociates below pH 3.5 to form monomers with a molecular weight of approximately 17,500. Wheat germ lectin contains two binding sites per subunit which are highly specific for N-acetylglucosamine (Nagata & Burger, 1974).

Wheat germ lectin sepharose 6MB (Appendix 1.3.2) has previously been used effectively to obtain relatively pure and concentrated preparations of HSV-1 glycoproteins (Jeansson, Elwing & Nilsson, 1979) and so was chosen as a means of purifying glycoproteins from non-glycosylated proteins.

The HSV-1 glycoproteins gC and gB were separated by means of Prep-PAGE and stored in solution at -20°C (Chapter 3.2.1(B)). Samples of these glycoprotein solutions were prepared for wheat germ lectin (WGL) affinity chromatography by dialyzing overnight against 0,1 M glycine/NaOH, pH 8,8, containing 1% Triton X-100 and 0,02 M NaN_3 . The wheat germ lectin

sepharose 6 MB column was prepared as described in Appendix 2.3.3(A).

A sample containing 20 ml of glycoprotein gC solution (Peak II, Figure 3.4), was applied to the WGL column after dialysis, at a flow rate of 20 ml/hr. The column was then washed with 50 ml of equilibration buffer (Appendix 1.3.5). After washing, the bound proteins were eluted with 20 ml of elution buffer, (equilibration buffer containing 1% Triton X-100 (w/v) and 0,2 M n-acetyl glucosamine). The same flow rate was used throughout. Fractions of 5 ml were collected and 0,1 ml aliquots of each fraction were assayed for radioactivity (Appendix 2.3.5). An elution profile of counts per minute against fraction number was drawn (Figure 3.11).

When it was found that the bound glycoprotein failed to elute in the buffer containing 0,2 M n-acetyl glucosamine (GlcNAC), attempts were made to elute the protein using the following buffers:-

- (a) Equilibration buffer containing 1% Triton X-100, 1 M NaCl and 0,5 M glucosamine.
- (b) Equilibration buffer containing 1% Triton X-100 and 2 M NaCl.

- (c) Equilibration buffer containing 1% Triton X-100, 2 M NaCl and 0,2 M n-acetyl glucosamine.
- (d) 1,4 M KCN containing 0,4 M NaCl, 1% Triton X-100 and 0,2 M n-acetyl glucosamine.

Fractions of 5 ml were collected and assayed for radioactivity. The elution profile of the entire experiment was drawn (Figure 3.11).

The experiment was repeated with 20 ml of glycoprotein gB solution (Peak III, Figure 3.4). An attempt was made to elute the glycoprotein using 20 ml of equilibration buffer containing 0,4 M NaCl, 1% Triton X-100 and 0,2 M n-acetyl glucosamine (Figure 3.12). Another attempt was made to elute the glycoprotein, using the same elution buffer, but containing 0,5 M GlcNAc.

B. Helix pomatia lectin affinity chromatography

Most viral glycoproteins contain N-glycosidic linkages between their oligosaccharide chains and their peptides. However, HSV has been found to possess one glycoprotein which contains alkali-sensitive chains, indicating an O-glycosidic linkage between the oligosaccharide chains and the peptide (Olofsson, Jeansson & Lycke, 1981).

Helix pomatia lectin, which has been isolated from the albumin gland of the snail Helix pomatia (Hammarstrom & Kabat, 1969), is known to bind N-acetylgalactosamine (GalNAc) residues which are commonly found in glycosaccharides O-glycosidically linked to peptides. This property of Helix pomatia lectin was utilized in purifying the one glycoprotein of HSV containing O-glycosidic linkages (Olofsson, Jeansson & Lycke, 1981). This glycoprotein has been identified as glycoprotein C (gC) (Olofsson et al, 1983; Svennerholm et al, 1984).

For this reason, Helix pomatia lectin (HPL) was used as a means of purifying the HSV-1 specific glycoprotein, gC, from contaminating non-glycosylated polypeptides after the glycoproteins had been separated by Prep-PAGE (Chapter 3.2.1(B)).

The Helix pomatia lectin sepharose 6MB column and the glycoprotein samples were prepared for affinity chromatography as described in Appendix 2.3.3(B). After dialysis, 20 ml of glycoprotein, gB, solution (Figure 3.4 Peak III) was applied to the gel bound HPL column at a flow rate of 10 ml/hr. The column was then washed with 20 ml of equilibration buffer

(Appendix 1.3.5) at a rate of 20 ml/hr in order to remove any unadsorbed proteins. The adsorbed proteins were eluted with 20 ml of equilibration buffer containing 1% Triton X-100 and 0,05 M n-acetyl-D-galactosamine (GalNAc), at the same flow rate.

Fractions of 5 ml were collected throughout, and assayed for radioactivity. The elution profile of CPM against fraction number, was drawn (Figure 3.13).

When the radiolabelled protein failed to elute in the presence of 0,05 M GalNAc, an attempt was made to elute using 20 ml of equilibration buffer containing 0,4 M NaCl, 1% Triton X-100 and 0,1 M GalNAc.

RESULTS

A. Wheat germ lectin affinity chromatography

Application of the HSV-1 glycoprotein gC (Figure 3.4 Peak II) to the gel bound WGL column, resulted in the adsorption of all (³H)-gln labelled proteins to the column (Figure 3.11). However, attempts to elute the adsorbed proteins using 0,2 M GlcNAc in the equilibration buffer (according to Jeansson,

Elwing & Nilsson, 1979) proved ineffective, as no radiolabelled material was eluted (Figure 3.11). For this reason, several different buffers were used in attempts to elute the adsorbed proteins from the gel-bound lectin. Buffers containing up to five times the recommended concentration of GlcNAc (Jeansson, Elwing & Nilsson, 1979) failed to elute the adsorbed proteins.

Eventually, a buffer containing the chaotropic CN^- ion (1,4 M KCN + 0,4 M NaCl + 1% Triton X-100 + 0,2 M GlcNAc) was successful in eluting the radiolabeled protein from the column (Figure 3.11). However, analytical SDS-PAGE of the eluant revealed that the protein had been broken down. (Results not shown).

The HSV-1 glycoprotein, gB, (Figure 3.4 Peak III) was also adsorbed to the gel-bound WGL column and attempts to elute the adsorbed protein with 0,2 M GlcNAc in equilibration buffer resulted in a small amount of (^3H)-labelled protein being eluted (Figure 3.12). A further attempt to elute the bound glycoprotein, using an increased concentration of GlcNAc (0,5 M), was not successful.

B. Helix pomatia lectin affinity chromatography

When a solution containing the HSV-1 glycoprotein, gB, (Figure 3.4 Peak III) was applied to a gel-bound HPL column, almost all of the radiolabelled protein was eluted without adsorbing to the lectin (Figure 3.13). A small sample of the HPL Sepharose 6 MB gel was assayed for radioactivity and was found to contain a small amount of radiolabelled protein. The bound glycoprotein was not eluted in the presence of either 0,05 M or 0,1 M GalNac (Figure 3.13).

FIGURE 3.11 - WHEAT GERM LECTIN AFFINITY
CHROMATOGRAPHY OF HSV-1
GLYCOPROTEIN C (gC)

The HSV-1 glycoprotein, gC (Figure 3.4 Peak II) was applied to a column containing 8 ml of wheat germ lectin Sepharose 6 MB. After washing, attempts were made to elute the adsorbed protein using 20 ml of the following buffers:-

1. Equilibration buffer containing 1% Triton X-100 and 0,2 M GlcNAc. (Elute 1)
2. Equilibration buffer containing 1% Triton X-100, 1M NaCl and 0,5 M GlcNAc. (Elute 2)
3. Equilibration buffer containing 1% Triton X-100 and 2 M NaCl. (Elute 3)
4. Equilibration buffer containing 1% Triton X-100, 2 M NaCl and 0,2 M GlcNAc. (Elute 4)
5. 1,4 M KCN + 0,4 M NaCl + 1% Triton X-100 + 0,2 M GlcNAc. (Elute 5)

Fractions of 5 ml were collected throughout and assayed for radioactivity. The figure shows the radioactive counts of each fraction.

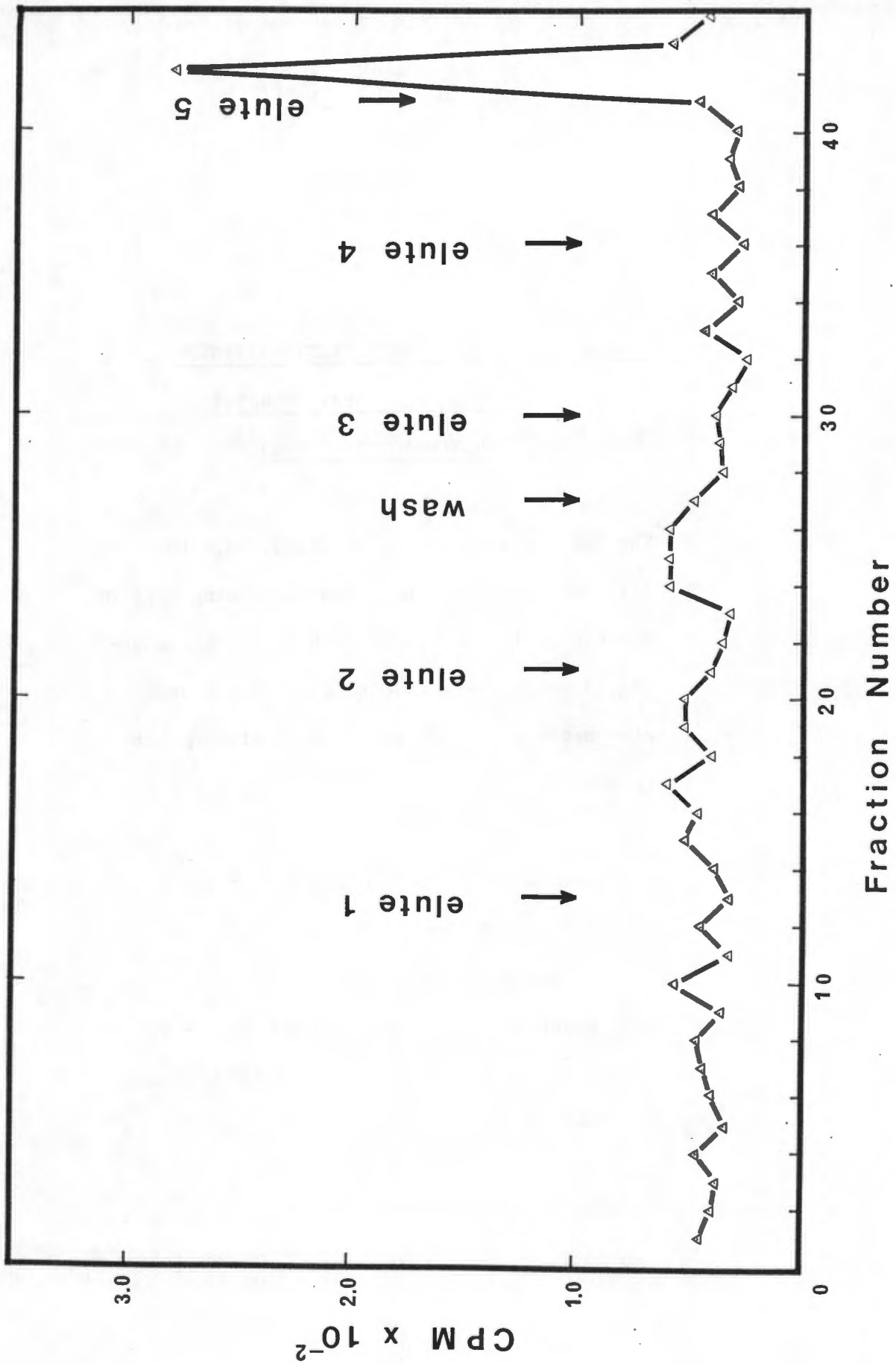


FIGURE 3.12 - WHEAT GERM LECTIN AFFINITY
CHROMATOGRAPHY OF HSV-1
GLYCOPROTEIN B (gB)

The HSV-1 glycoprotein, gB (Figure 3.4 Peak III) was applied to a column containing 8 ml of wheat germ lectin sepharose 6 MB. After washing, attempts were made to elute the bound glycoprotein with 20 ml of the following buffers :

1. Equilibration buffer containing 0,4 M NaCl, 1% Triton X-100 and 0,2 GlcNAc.
(Elute 1).
2. Equilibration buffer containing 0,4 M NaCl, 1% Triton X-100 and 0,5 M GlcNAc.
(Elute 2).

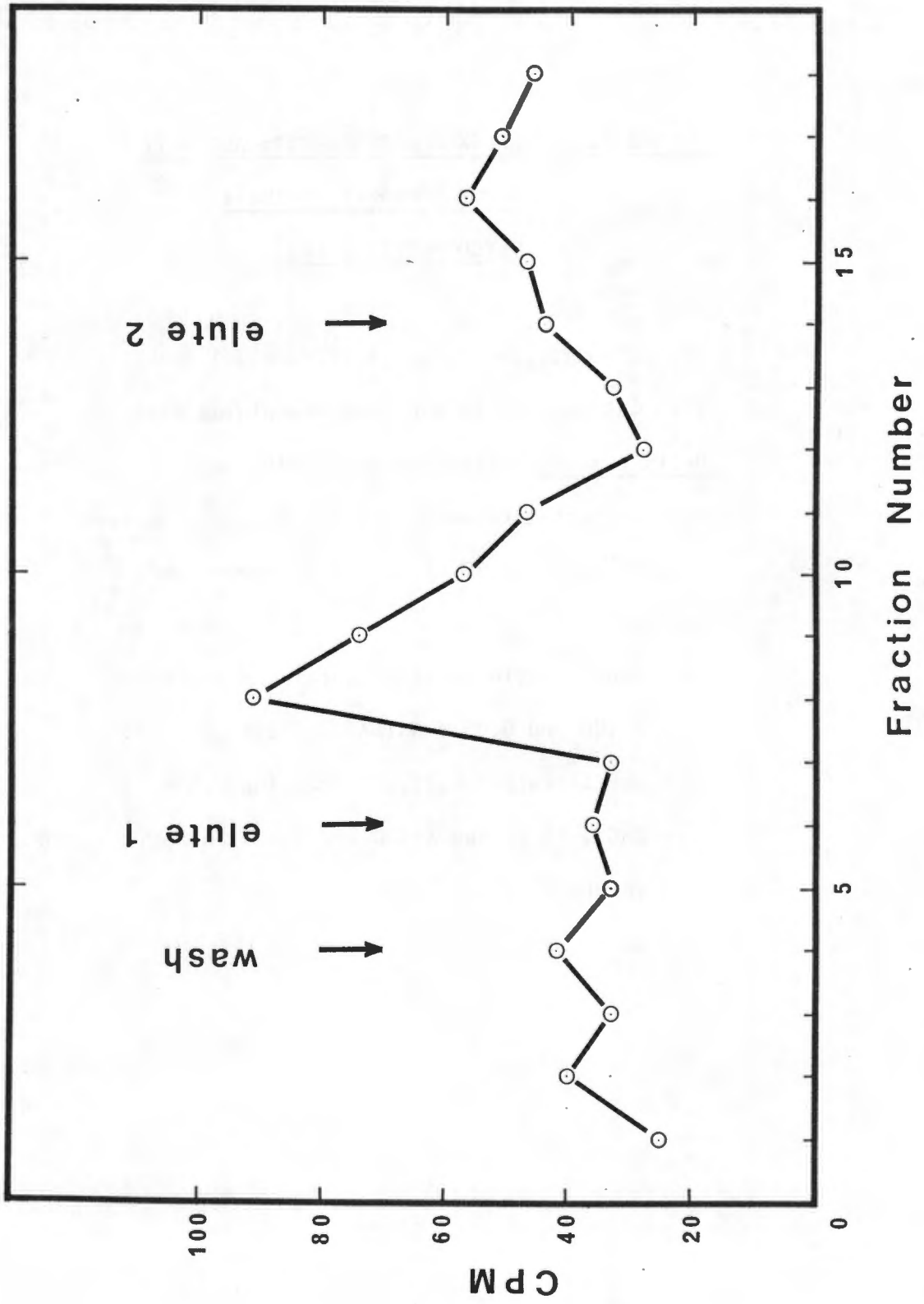
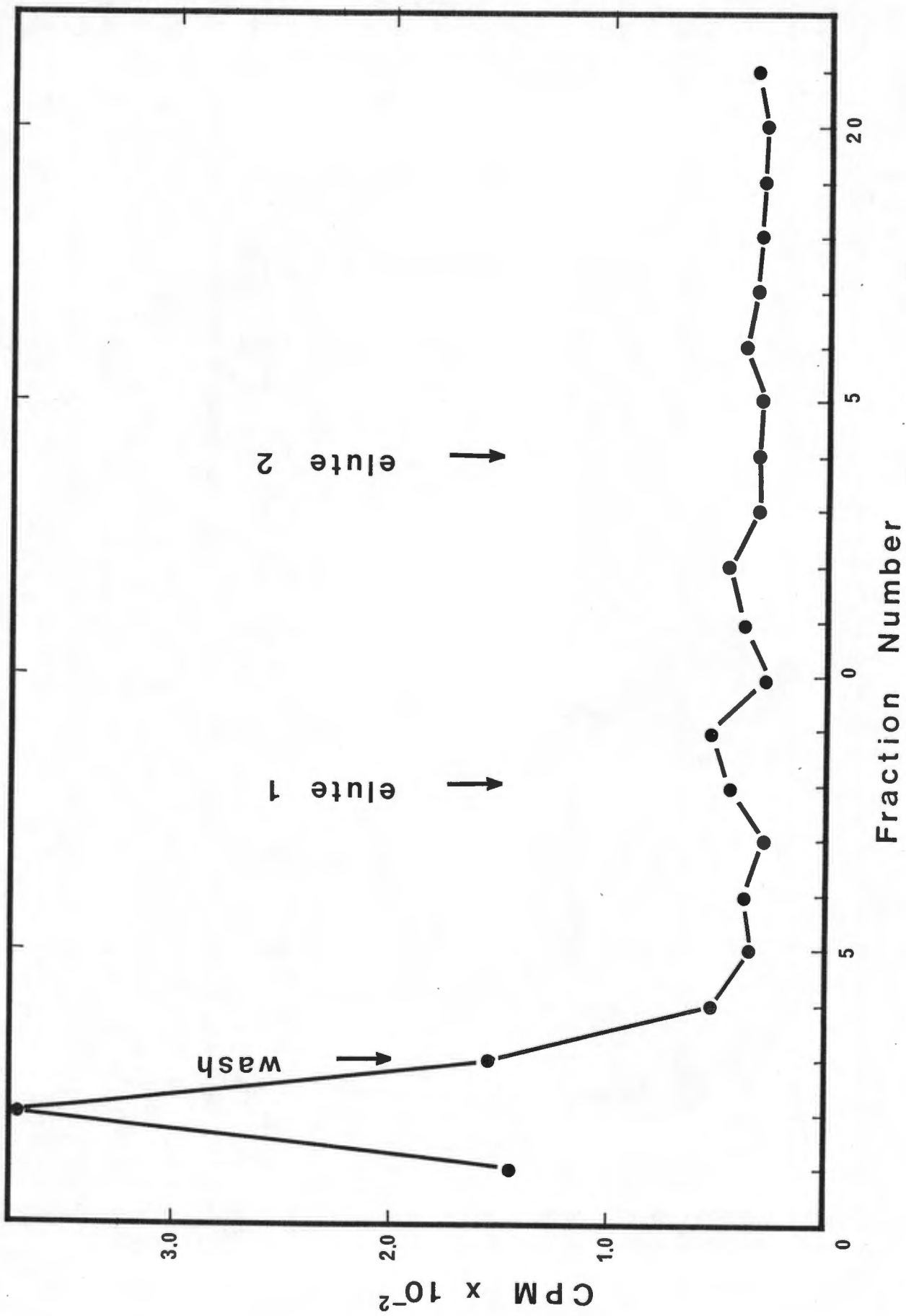


FIGURE 3.13 - HELIX POMATIA LECTIN AFFINITY
CHROMATOGRAPHY OF HSV-1
GLYCOPROTEIN B (gB)

The HSV-1 glycoprotein, gB (Figure 3.4 Peak III) was applied to a column containing 2 ml of Helix pomatia lectin sepharose 6MB. After washing, attempts were made to elute the adsorbed protein with 20 ml of the following buffers:-

1. Equilibration buffer containing 1% Triton X-100 and 0,05 M GalNAc. (Elute 1)
2. Equilibration buffer containing 0,4 M NaCl, 1% Triton X-100 and 0,1 M GalNAc. (Elute 2)



DISCUSSION

Gel-bound lectin affinity chromatography is a technique which is commonly used for purifying glycoproteins, and has been used successfully for purifying some glycoproteins of HSV-1 (Chapter 3.1.4).

Glycoproteins gC and gB of HSV-1, which had been separated and partially purified by Prep-PAGE, were individually applied to a column containing wheat germ lectin sepharose 6MB. Glycoprotein gC bound to the wheat germ lectin column but could only be eluted using very strong conditions (Figure 3.11), and this was unsatisfactory because analytical SDS-PAGE of the eluant revealed that the glycoprotein had been broken up by the treatment (Results not shown). Glycoprotein gB was also bound by the wheat germ lectin and, although some (^3H)-labelled glycoprotein eluted with the elution buffer (Figure 3.12), this was only a small fraction of the total (^3H)-labelled glycoprotein that had been applied to the column. An attempt to elute more of the glycoprotein with a higher concentration of GlcNAc was not successful.

Glycoprotein gB was also applied to a column containing Helix Pomatia lectin sepharose 6MB. Almost all of the radiolabelled glycoprotein eluted without binding to the lectin, and there was no further glycoprotein released by

the elution buffer (Figure 3.13). This is in agreement with previous reports which state that only gC binds to Helix Pomatia lectin (See Chapter 3.1.4).

Glycoprotein gC should also have been applied to the Helix Pomatia lectin column. However, when this work was done, it was believed that gB was, in fact, gC. Subsequently, the phenomenon discussed in Chapter 3.1.1, in which the comparative positions of gB and gC on SDS-PAGE reverse according to the cross-linker used, was realized. Hence, gB was applied to the Helix Pomatia lectin column instead of gC.

The difficulty encountered with eluting the bound glycoproteins from the wheat germ lectin column may have been caused by the use of an anionic detergent - sodium dodecyl sulphate (SDS) - to solubilize the infected cell proteins. The SDS may have formed strong ionic bonds between the glycoproteins and the lectin. To investigate this possibility, the gel-bound lectin affinity chromatography was repeated using HSV-1 membrane proteins solubilized in a non-ionic detergent - Triton X-100 (Chapter 3.2.5).

3.2.4 Experiment to test the activity of the wheat germ lectin

Sepharose 6 MB

As a result of the difficulty encountered in eluting the HSV-1 glycoproteins from the column of wheat germ lectin Sepharose 6MB (Chapter 3.2.3(A)), the column was tested to see whether it responded normally to a glycoprotein which is known to have an affinity for wheat germ lectin and to elute in the presence of N-acetyl-D-glucosamine (GlcNAc). The glycoprotein chosen for this test was ovalbumin, which has previously been used to test the activity of wheat germ lectin (Gombos et al, 1974). The experiment was performed as follows:-

A solution of ovalbumin was prepared in physiological saline with a concentration of 1 mg/ml. A volume of 5 ml was applied to the WGL column at a flow rate of 20 ml/hr. The column was then washed through with 50 ml of equilibration buffer (Appendix 1.3.5). After washing, the ovalbumin was eluted from the column using 25 ml of 0,2 M GlcNAc in equilibration buffer. Fractions of 5 ml were collected throughout the experiment and the absorbance at 280 nm (A_{280}) of each fraction was measured. The elution profile of A_{280} against fraction number was then drawn (Figure 3.14).

RESULTS

Ovalbumin was adsorbed to the wheat germ lectin column and readily eluted in buffer containing 0,2 M GlcNAc.

TABLE 3.2 - THE ABSORBANCE AT 280 nm (A_{280}) OF FRACTIONS ELUTED FROM A WHEAT GERM LECTIN COLUMN WHICH HAD BEEN LOADED WITH OVALBUMIN

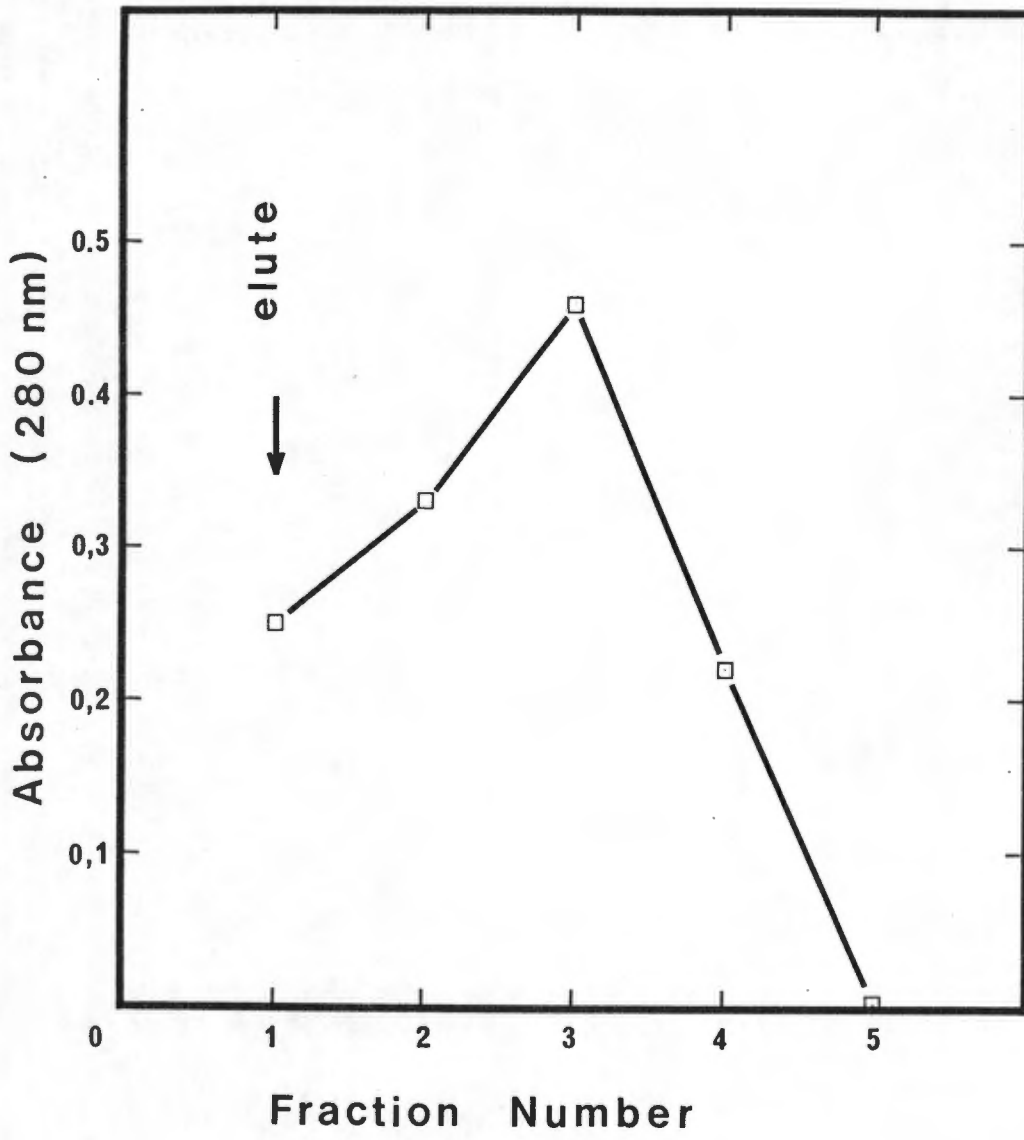
Fraction	A_{280}
Ovalbumin solution containing 1 mg/ml in physiological saline	0.68
Elution buffer containing equilibration buffer + 0,2 M GlcNAc	0
Eluant fraction 1	0.25
fraction 2	0.33
fraction 3	0.46
fraction 4	0.22
fraction 5	0

DISCUSSION

Ovalbumin bound to the wheat germ lectin column and was eluted in the presence of 0,2 M GlcNAc demonstrating that the wheat germ lectin Sepharose 6MB was still active. Therefore, the problems encountered in eluting HSV-1 glycoproteins could not be ascribed to the lectin column.

FIGURE 3.14 - WHEAT GERM LECTIN AFFINITY
CHROMATOGRAPHY OF OVALBUMIN

A solution of ovalbumin containing 1 mg/ml in physiological saline was applied to a wheat germ lectin Sepharose 6 MB column. The column was washed with equilibration buffer and the ovalbumin was eluted using 0,2 M GlcNAc in equilibration buffer. Fractions of 5 ml were collected and the A_{280} was measured. The elution profile of A_{280} against fraction number is shown.



3.2.5 Gel-bound lectin affinity chromatography of HSV-1 membrane proteins solubilized in the non-ionic detergent, Triton X-100

Solutions containing the HSV-1 glycoproteins, gC and gB, respectively, were prepared by solubilizing (^3H)-gln labelled HSV-1 infected BHK-21 cells in a buffer containing 2,5% SDS and 5% 2-mercaptoethanol (Appendix 1.2.1(d)) and isolating the glycoproteins by Prep-PAGE (Chapter 3.2.1). These glycoprotein solutions contained a significant amount of non-glycosylated proteins, and attempts to purify the glycoproteins using gel-bound lectin affinity chromatography were unsuccessful (Chapter 3.2.3).

Both wheat germ lectin Sepharose 6MB and Helix pomatia lectin Sepharose 6MB (Appendix 1.3.2) have previously been used successfully for the purification of HSV-1 glycoproteins (Jeansson, Elwing & Nilsson, 1979; Olofsson, Jeansson & Lycke, 1981; Svennerholm et al, 1984). However, in all cases the HSV-1 proteins were solubilized in the non-ionic detergent, Triton X-100. This would indicate that the failure encountered with gel-bound lectin affinity chromatography thus far, may be due to the use of sodium dodecyl sulphate (SDS) to solubilize the HSV-1 proteins. For this reason, it was decided to repeat the gel-bound lectin affinity chromatography with proteins dissolved in Triton X-100 according to the method of Olofsson, Jeansson & Lycke, 1981.

A. Preparation of HSV-1 membrane proteins for gel-bound lectin affinity chromatography

HSV-1 membrane proteins were prepared according to the method described in Appendix 2.3.4. The product was stored in aliquots of 0,5 ml at -70°C until needed.

B. Helix pomatia lectin affinity chromatography

A volume of 4 ml of a solution of (^3H)-gln labelled HSV-1 membrane proteins (Appendix 2.3.4) was applied to a column containing 2 ml of Helix pomatia lectin Sephase 6MB (Appendix 2.3.3(B)) at a flow rate of 2 ml/hr. The column was washed with 30 ml of equilibration buffer (Appendix 1.3.5) containing 1% Triton X-100 at a flow rate of 50 ml/hr. Bound protein was eluted with 20 ml of 0,1 M GalNAc in equilibration buffer containing 1% Triton X-100 at a flow rate of 5 ml/hr. Fractions of 3 ml were collected throughout and assayed for radioactivity (Appendix 2.3.5) and an elution profile of counts per minute against fraction number was drawn (Figure 3.15). Fractions 3,4 and 5 were analyzed by SDS-PAGE and autoradiography (Figure 3.16).

C. Wheat germ lectin affinity chromatography

A volume of 1,75 ml of a solution of (^3H)-gln labelled HSV-1 membrane proteins (Appendix 2.3.4) was applied to a column containing 8 ml of wheat germ lectin Sepharose 6MB (Appendix 2.3.3(A)). The col-

umn was washed with 30 ml of equilibration buffer containing 1% Triton X-100 and then the bound proteins were eluted with 20 ml of 0,2 M GlcNAc in the same buffer. A flow rate of 8 ml/hr was used throughout and 3 ml fractions were collected. Fractions were assayed for radioactivity and an elution profile of counts per minute against fraction number was drawn (Figure 3.17).

Eluted fractions were analyzed by SDS-PAGE and autoradiography (Figure 3.18).

RESULTS

A. Preparation of HSV-1 membrane proteins for gel-bound lectin affinity chromatography

The procedure used for preparing HSV-1 membrane proteins (Appendix 2.3.4) yielded 10 ml of solution containing approximately 3000 counts per minute (cpm) per 0,1 ml. A comparison of the yield of (³H)-gln labelled proteins obtained by this method with that from whole cells solubilized in SDS (Chapter 2.2.2) showed that a significant amount of the radiolabelled protein was lost during this procedure.

B. Helix pomatia lectin affinity chromatography

The elution profile of (^3H)-gln labelled HSV-1 membrane proteins from a column of Helix pomatia lectin Sepharose 6MB is shown in Figure 3.15. A large proportion of the radioactivity which was applied to the column, eluted without adsorption. When the elution buffer, containing 0,01 M GalNAc, was passed through the column, there was no elution of radio-labelled proteins.

The fractions comprising the peak of radioactivity (Fractions 3,4 and 5; Figure 3.15) were analyzed by SDS-PAGE and autoradiography. This revealed that all of the glycoproteins which were applied to the column (Tracks 1 and 5; Figure 3.16) were eluted immediately without adsorbing to the Helix pomatia lectin (Tracks 2, 3 and 4; Figure 3.16).

C. Wheat germ lectin affinity chromatography

The elution profile of (^3H)-labelled HSV-1 membrane proteins from a column of wheat germ lectin Sepharose 6MB is shown in Figure 3.17. A small proportion of the radioactively labelled protein was eluted during sample application and subsequent washing of the column with the equilibration buffer, while most of the radioactively labelled protein was elu-

ted using the elution buffer containing 0,2 M GlcNAc.

Representative fractions from the small peak of radioactivity (Fractions 2 and 6, Figure 3.17), and from the large peak of radioactivity (Fractions 18, 19 and 23, Figure 3.17) were analyzed by SDS-PAGE and autoradiography (Figure 3.18). The results show that almost all of the protein content of the sample applied to the column was eluted from the column immediately (Figure 3.18(a), Tracks 2 and 3), while very little protein was eluted in the second peak of radioactivity (Figure 3.18(a), Tracks 4, 5 and 6). Conversely, very little radioactive material eluted in the first peak, and this appears to represent one glycoprotein species (Figure 3.18(b), Tracks 2 and 3), while most of the radioactive material eluted in the second peak and represented approximately six different glycoprotein species (Figure 3.18(b), Tracks 4, 5 and 6).

FIGURE 3.15 - HELIX POMATIA LECTIN AFFINITY
CHROMATOGRAPHY OF HSV-1 MEMBRANE
PROTEINS SOLUBILIZED IN TRITON X-
100

(³H)-gln labelled HSV-1 membrane proteins, solubilized in Triton X-100, were applied to a column containing 2 ml of Helix pomatia lectin Sepharose 6MB. The column was washed with equilibration buffer before eluting bound proteins with 0,1 M GalNAc in equilibration buffer. Fractions of 2 ml were collected and assayed for radioactivity. The elution profile of counts per minute against fraction number is shown.

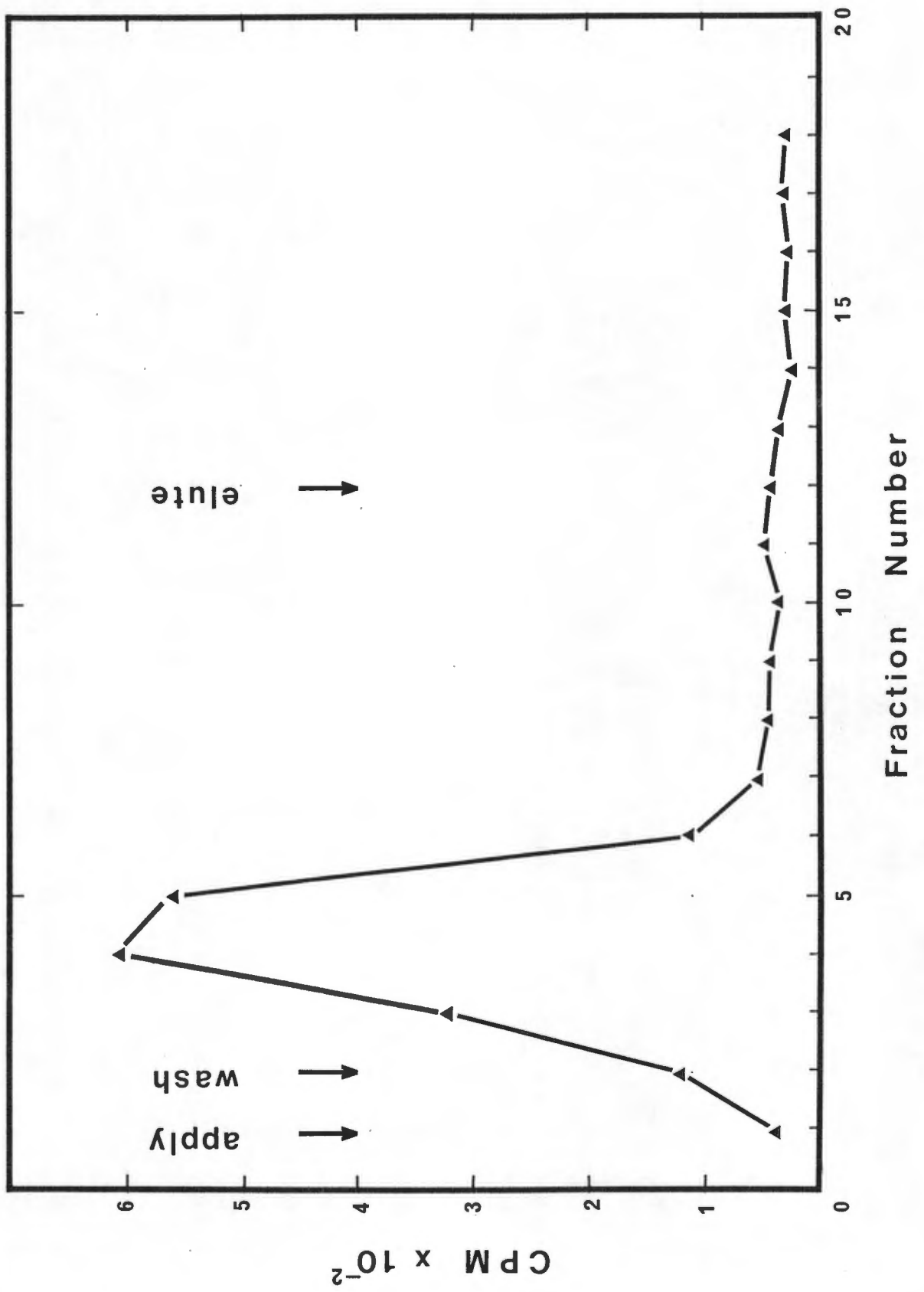




FIGURE 3.16 - AUTORADIOGRAPH OF FRACTIONS ELUTED FROM
HELIX POMATIA LECTIN AND ANALYZED BY SDS-
PAGE

Fractions 3,4 and 5 (Figure 3.15) were analyzed by analytical SDS-PAGE and autoradiography.

Track 1 - (^3H)-gln labelled membrane proteins.

Track 2 - Fraction 5

Track 3 - Fraction 4

Track 4 - Fraction 3

Track 5 - (^3H)-gln labelled membrane proteins.

FIGURE 3.17 - WHEAT GERM LECTIN AFFINITY
CHROMATOGRAPHY OF HSV-1 MEMBRANE
PROTEINS SOLUBILIZED IN TRITON X-
100

(³H)-gln labelled HSV-1 membrane proteins, solubilized in Triton X-100, were applied to a column containing 8 ml of wheat germ lectin Sepharose 6MB. The column was washed with equilibration buffer before eluting bound proteins with 0,2 M GlcNAc in equilibration buffer. Fractions of 1 ml were collected and assayed for radioactivity. The elution profile of counts per minute against fraction number is shown.

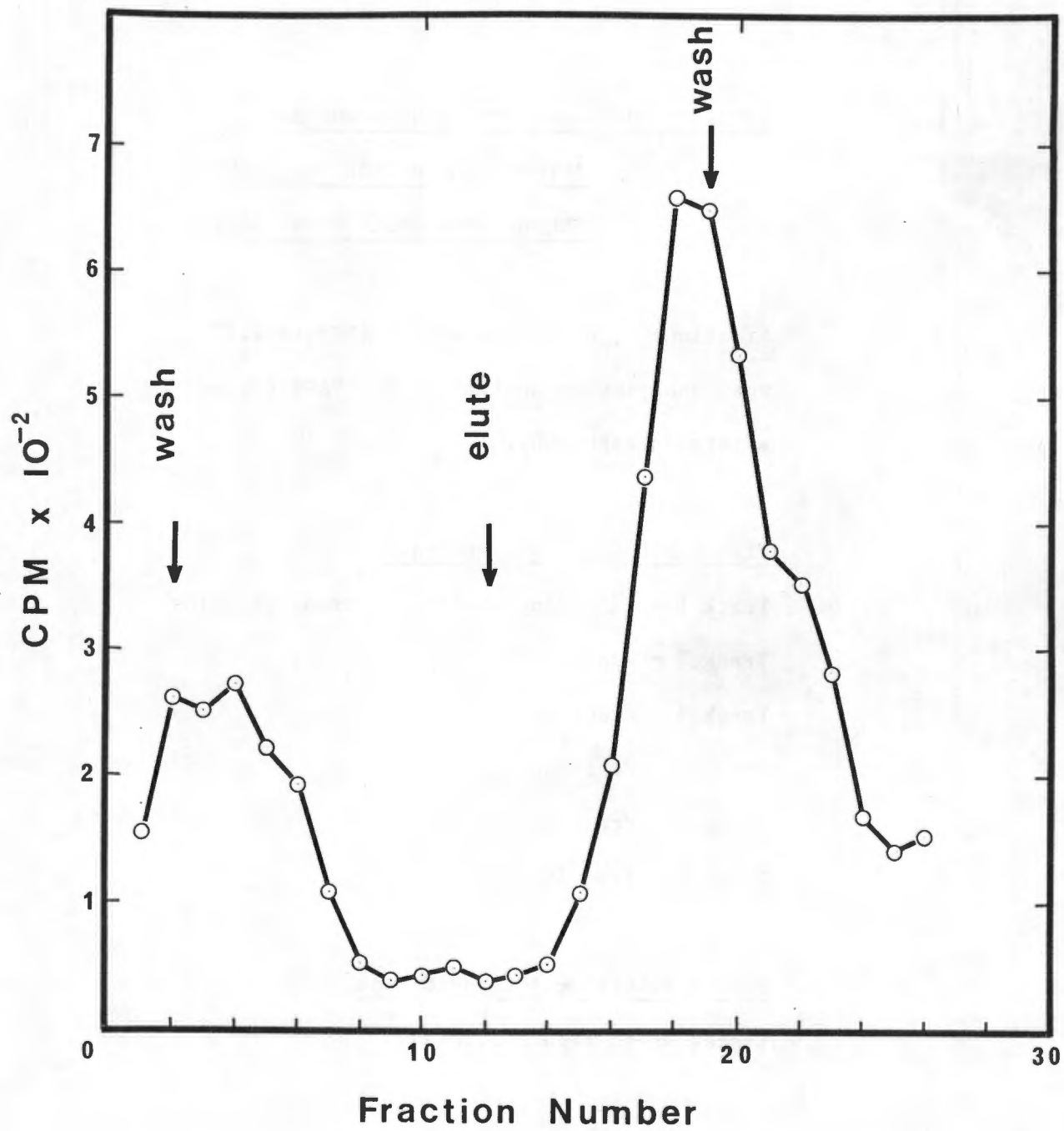


FIGURE 3.18 - ANALYSIS BY SDS-PAGE AND
AUTORADIOGRAPHY OF FRACTIONS
ELUTED FROM WHEAT GERM LECTIN

Fractions 2, 6, 18, 19 and 23 (Figure 3.17)
were analyzed by analytical SDS-PAGE (a) and
autoradiography (b).

Figure 3.18(a) - Silver Stain

Track 1 - (³H)-gln labelled membrane proteins

Track 2 - Fraction 2

Track 3 - Fraction 6

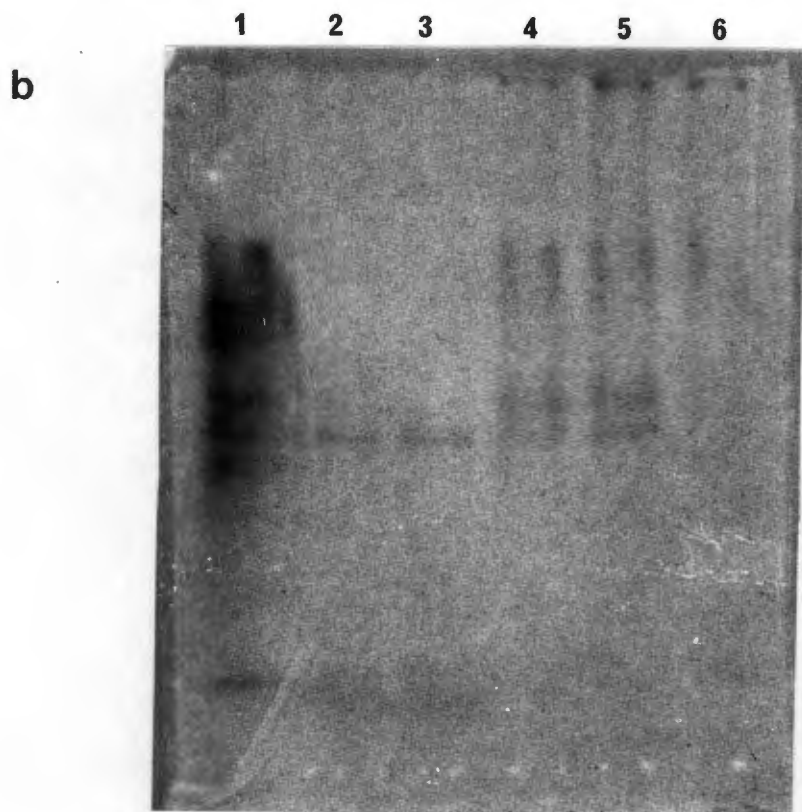
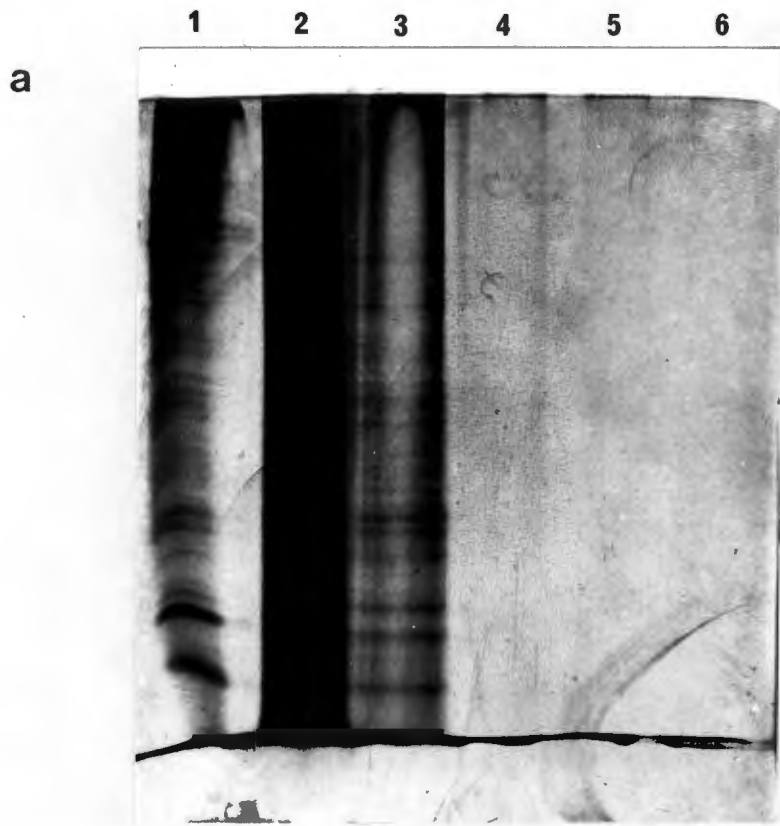
Track 4 - Fraction 18

Track 5 - Fraction 19

Track 6 - Fraction 23

Figure 3.18(b) - Autoradiograph

Tracks as in Figure 3.18(a).



DISCUSSION

Although the HSV-1 membrane proteins were carefully prepared according to the method of Olofsson, Jeansson & Lycke, 1981, the results of Helix pomatia lectin affinity chromatography show conclusively that none of the HSV-1 glycoproteins bound to the lectin column. The reasons for this conclusion are:-

- (a) A large proportion of the radioactivity which was applied to the column eluted without binding (Figure 3.15).
- (b) Analytical SDS-PAGE of the fractions constituting the peak of radioactivity reveals that all of the (³H)-gln labelled proteins which were applied to the column were present in the initial peak of radioactivity (Figure 3.16).
- (c) No (³H)-gln labelled proteins were eluted in the presence of 0,1 M GalNAc (Figure 3.15).
- (d) When a sample of the Helix pomatia lectin Sepharose 6 MB was assayed for radioactivity, no significant radioactivity was present on the gel.

Subsequent to the observation that only glycoprotein C (gC) of HSV-1 has affinity for Helix pomatia lectin (HPL) (Olofsson, Jeansson & Lycke, 1981), it has been reported that approximately 25% of the total gC population has

this affinity. The remainder of the gC population, like the other HSV-1 glycoproteins, is without affinity for HPL (Olofsson et al, 1983). The gC population which bound to the HPL was found to consist of two electrophoretic bands with lower molecular weights than the non-binding gC. For this reason, it was suggested that precursors of gC were responsible for the HPL binding fraction, while the fully glycosylated product lacked this affinity.

However, Olofsson et al concluded that the existence of two populations of gC with different affinities for HPL was the result of two different glycosylation pathways (Olofsson et al, 1983).

In the present work, the HSV-1 infected cells labelled with (³H)-gln were grown in the presence of non-radioactive medium for the last 4 hours before harvesting. This was done to ensure that the radiolabelled proteins would be fully glycosylated and so eliminate the possibility of radiolabelled precursors. Therefore, since no radiolabelled precursors were present during the Helix pomatia lectin affinity chromatography, this may explain why glycoprotein C failed to bind to the HPL. These results may, therefore, be in agreement with the findings of Olofsson et al, and would support the hypothesis that

the two different populations of gC are a result of different levels of glycosylation and not two different glycosylation pathways (Olofsson et al, 1983).

Wheat germ lectin affinity chromatography of the same preparation of HSV-1 membrane proteins revealed that most of the HSV-1 glycoproteins bound to the wheat germ lectin and were eluted by 0,2 M GlcNAc (Figure 3.17). Analytical SDS-PAGE of some of the eluted fractions revealed that all of the non-glycosylated proteins and one glycoprotein were not bound to the lectin and eluted immediately (Figure 3.18, Tracks 2 and 3). Approximately six glycoproteins eluted when 0,2 M GlcNAc was added to the column (Figure 3.18, Tracks 4, 5 and 6).

The observation that one glycoprotein species of HSV-1 was selectively separated from the other glycoproteins on a wheat germ lectin Separose 6MB column, indicates that this technique may be useful in selectively purifying the glycoprotein.

This glycoprotein, which lacks affinity for wheat germ lectin (Figure 3.18, Tracks 2 and 3), appears to co-migrate with a glycoprotein which bound to the column and was eluted in the presence of 0,2 M GlNAc (Figure

3.18(b), Tracks 4, 5 and 6). There are two possible explanations for this observation :

1. They represent the same protein which only binds loosely to wheat germ lectin so that some was bound to the column while the remainder eluted.
2. These are two separate glycoprotein species which have similar electrophoretic mobilities.

If these proteins are in fact different, then the technique of wheat germ lectin affinity chromatography may be a useful means of separating them. This possibility requires further investigation.

CONCLUSION

1. Helix pomatia lectin Sepharose 6MB was not suitable for purifying HSV-1 glycoproteins from non-glycosylated proteins.
2. Helix pomatia lectin Sepharose 6MB did not selectively bind glycoprotein gC of HSV-1, as has been previously reported (Olofsson, Jeansson & Lycke, 1981; Svennerholm et al, 1984).
3. Wheat germ lectin Sepharose 6MB is suitable for purifying HSV-1 glycoproteins from non-glycosylated proteins when the membrane proteins are solubilized in Triton X-100.

4. The use of SDS to solubilize the proteins of HSV-1 infected cells appears to have been responsible for the problems encountered during earlier attempts to purify the glycoproteins of HSV-1 by wheat germ lectin affinity chromatography (Chapter 3.2.3).

3.3 SUMMARY

Preparative-PAGE (Prep-PAGE) was used to provide an initial separation of the HSV-1 glycoproteins. Two cycles of Prep-PAGE were sufficient to purify glycoproteins gC and gB with respect to other HSV-1 glycoproteins, but the solutions still contained non-glycosylated proteins. Two different techniques were used to further purify the glycoprotein solutions:

1. Hydroxylapatite chromatography in the presence of SDS (SDS-HTP) did not separate different HSV-1 glycoproteins and was not suitable for removing the non-glycosylated proteins from the glycoprotein solutions.
2. Gel-bound lectin affinity chromatography using wheat germ lectin and Helix pomatia lectin did not provide a means of purifying the glycoproteins because :
 - (a) The glycoproteins which bound to wheat germ lectin could not be eluted except by using strong buffers containing chaotropic ions, and

- (b) The glycoprotein which was applied to the Helix pomatia lectin did not bind and was eluted with the non-glycosylated proteins.

The difficulties encountered in eluting the glycoproteins from wheat germ lectin may have been due to the SDS in which the proteins had been solubilized. Therefore, the gel-bound lectin affinity chromatography was repeated using HSV-1 membrane proteins solubilized in Triton X-100. Using this material, it was observed that :

- (a) None of the HSV-1 glycoproteins had affinity for Helix pomatia lectin, and
- (b) Several HSV-1 glycoproteins bound to wheat germ lectin and were purified with respect to non-glycosylated proteins.

It still remains to separate glycoprotein C (gC) from the other HSV-1 glycoproteins and Prep-PAGE would be a means of accomplishing this.

APPENDIX I - MATERIALS1.1. PRODUCTION OF HERPES SIMPLEX VIRUS1.1.1. CELLS

Baby Hamster Kidney (BHK-21) cells were provided by the State Vaccine Institute (South Africa) at Passage 73 and were initially obtained by the State Vaccine Institute from Flow Laboratories at Passage 62. The BHK-21 cells were used at Passage levels of between 84 and 95.

1.1.2. VIRUSES

(a) Herpes Simplex virus Type I (HSV-1), strain E₄₇.

This virus was isolated from the liver of a patient with a fatal disseminated herpes infection by the University of Cape Town Diagnostic Virology laboratory in February 1967.

(b) Herpes Simplex Virus Type 2 (HSV-2), strain MS.

This virus was obtained by courtesy of Dr John Stewart, of the Centres for Disease Control, Atlanta, Georgia, USA.

1.1.3. CELL CULTURE MEDIUM

(a) Growth Medium (10% MEM)

The growth medium used was Eagle's minimum essential medium (MEM) (Gibco Europe) with Earle's salts, L-Glutamine and non-essential amino acids. This medium was supplemented with 10% (v/v) foetal calf

serum (Appendix 1.1.3(c)), 1% (v/v) sodium bicarbonate solution (Appendix 1.1.5(b)) and 0,5% (v/v) PSN solution (Appendix 1.1.5(a)). The pH was maintained at approximately 7.0.

(b) Maintenance Medium (4% MEM)

The maintenance medium used was Eagle's MEM with Earle's Salts, L-glutamine and non essential amino acids. The medium was supplemented with 4% (v/v) foetal calf serum, 2% (v/v) sodium bicarbonate solution and 0,5% (v/v) PSN solution.

(c) Serum

Foetal calf serum (FCS) was obtained from the State Vaccine Institute (South Africa) and had been sterilized by filtration and ultraviolet irradiation.

1.1.4. BUFFERS

(a) Phosphate Buffered Saline (After Dulbecco)

To make 1 litre (Ca^{++} and Mg^{++} free).

Use ANALAR grade or equivalent purity reagents.

NaCl	8g
KCl	0.2g
Na_2HPO_4 (Anhydrous)	0.91g
KH_2PO_4	0.12g

Dissolve in about 900 ml of distilled H_2O .

Bring the pH to 7.2 with HCl or NaOH.

Then, bring the volume accurately to 1 litre.

Autoclave at 115°C for 20 minutes in order to sterilize.

(b) Freezing Buffer

10% MEM	91.0 ml
PSN solution	0.5 ml
7,5% Dimethylsulphoxide (DMSO)	7.5 ml
1,5ml HEPES buffer	1.5 ml

(c) Hepes Buffer

To make 100 ml of 1 M stock solution:

Dissolve 23,8 grams Hepes in 80ml of 1/3 N NaOH.

Adjust the pH to 7,2 using 1 N NaOH or 1 N HCl.

Make the volume up to 100 ml with distilled H₂O.

Filter to sterilize.

(d) Trypsin Base (10 x stock)

(Ca⁺⁺ and Mg⁺⁺ free Dulbecco PBS with EDTA and Glucose)

NaCl	80.0g
KCl	2.0g
KH ₂ PO ₄	1.2g
Na ₂ HPO ₄ (anhydrous)	9.1g
Phenol Red (Appendix 1.1.5(c))	25.0ml
Glucose	5.0g

Dissolve the above in about 700 ml distilled H₂O using a magnetic stirrer and adjust the pH to about 7,8.

Dissolve 2,0 gms EDTA in about 100 ml distilled H₂O and add it to the salt solution with stirring.

Adjust the pH to 7,8 with 1 N NaOH and stir until all solids have dissolved completely to give a clear red solution.

Bring the volume to 1000 ml with distilled H₂O.

Membrane filter and dispense in 20 ml amounts.

Store at -20°C.

(e) Trypsin

5% Trypsin Stock (For Activated Trypsin Versene):

Weigh out 5 gm trypsin powder.

Prepare 100 ml of trypsin base (10 ml of 10x concentrated trypsin base diluted with 90 ml of distilled H₂O) and acidify to bright yellow with 1N HCl.

Pour the trypsin base into a glass beaker; sprinkle the trypsin powder onto the surface of the trypsin base.

Allow the trypsin to dissolve slowly, without agitation, at room temperature. Do not stir or shake.

Once dissolved, stir briefly and leave at 4°C overnight.

Next day, stir for 30 minutes on a magnetic stirrer at room temperature.

Centrifuge at 9000 rpm for 45 minutes at 4°C in the Sorvall RC-5 centrifuge.

Taking care not to disturb the deposit, pipette off the clear supernatant fluid into a beaker or bottle.

Sterilize by passing through an 0,2 μ membrane filter.

Dispense in 1 ml amounts

Store at -20°C .

(f) Activated Trypsin Versene (ATV)

Prepare 1x concentration trypsin base by diluting 10 ml of 10x concentrated trypsin base in 90 ml distilled H_2O .

Add 1 ml stock 5% trypsin and 0,5 ml PSN (Appendix 1.1.5(a)).

Adjust pH to 7,8 (purple) with 1 N NaOH.

1.1.5. MISCELLANEOUS SOLUTIONS

(a) PSN Solution

2 bottles penicillin 2 x 10^6 units

2 bottles streptomycin 2 x 1 gram

4 bottles neomycin 4 x 0,5 gram

100 ml physiological saline

Reconstitute the antibiotic powder in each bottle with approximately 4 ml physiological saline. Use a needle and syringe.

Mix well and withdraw the antibiotic with the syringe and add to the remainder of the physiological saline.

Filter to sterilise and dispense in usable amounts.

(b) 5% Sodium Bicarbonate Solution

Weigh out 100 grams NaHCO_3

Dissolve in distilled H_2O in a 2 litre volumetric flask.

When dissolved, add 2 ml of 0,4% (w/v) phenol red.

Make the volume up to 2 litres.

Using dry ice, bubble CO_2 through the solution until it is a brownish pink colour.

Filter to sterilise and dispense in bijou bottles.

The bottles are filled to leave no air space.

Store at 4°C .

(c) Phenol Red

Weigh out 0,4 gm phenol red.

Dissolve in 60 ml $\text{N}/_{20}$ NaOH with the aid of heat.

Do not allow to boil.

Allow to return to room temperature and then make up to 100 ml with distilled H_2O in a volumetric flask.

Filter and dispense in 20 ml amounts.

(d) Trypan Blue

Weigh out 0,5 gms Trypan Blue

Dissolve in 100 ml of normal saline (i e 0,85 gm NaCl in 100 ml H_2O)

Filter through paper.

(e) Physiological Saline

Dissolve 0,85 gm NaCl in 100 ml distilled H_2O .

(f) Fixing Solution

The solution used to fix cells in testing for mycoplasma consists of 25% acetic acid and 75% Methanol (Chen, 1977).

(g) Bisbenzamide Fluorochrome Stain

(i) Stock concentrate (100 ml)

Bisbenzamide fluorochrome stain (Hoechst No 33258) 5 mg.

Hank's balanced salt solution (BSS) without sodium bicarbonate and phenol red 100 mg.

Thiomersal (Merthiolate) 0,01 gm.

Mix these components thoroughly at room temperature with a magnetic stirrer for 30 minutes.

Wrap the bottle in aluminium foil and store in the dark at 2-8°C. The stain is light and heat sensitive.

(ii) Working stain (100 ml)

Add 1 ml of stock concentrate to 100 ml of Hank's BSS for a final concentration of 0,5 g/ml.

Store in a dark bottle wrapped in aluminium foil at 2-8°C. Before use, mix thoroughly with a magnetic stirrer at room temperature for 30 minutes.

Examine periodically for microbial contamination.

Discard when contamination or deterioration occurs.

Membrane filtration diminishes fluorescence.

(h) Citric acid - disodium phosphate buffer for mounting

fluid. (100 ml)

0,1 M Citric acid 22,2 ml

0,2 M Disodium phosphate 27,8 ml

Glycerol 50,0 ml

Final pH 5,5 (Check periodically as pH is critical for optimal fluorescence.)

Store at 2-8°C.

- (i) Maxidens - an inert, water immiscible density gradient displacing liquid (Density = 1.9 g/ml at 25°C) was obtained from Nyegaard and Co, Oslo, Norway.
- (j) D.P.X. mountant - a synthetic mounting fluid. (BDH Chemicals Ltd, Poole, England.)

1.2. RADIOLABELLING AND ANALYSIS OF HSV PROTEINS

1.2.1 BUFFERS

(a) Running gel buffer:

1.5M Tris (hydroxymethyl)methylamine (Tris)/HCl,
pH 8,8 + 0,4% sodium dodecyl sulphate (SDS)

(b) Stacking gel buffer:

0,5 M Tris/HCl, pH 6,8 + 0.4% SDS

(c) Reservoir buffer:

0,025 M Tris + 0,192 M Glycine, pH 8,5 + 0,1% SDS

(d) Sample Buffers:

HMW sample buffer - 10mM Tris/HCl, 1 mM EDTA, pH 8,0
+ 1% SDS + 1% 2-mercaptoethanol

LMW sample buffer - 10mM Tris/HCl, 1mM EDTA, pH 8,0
+ 2,5% SDS + 5,0% 2-mercaptoethanol

1.2.2. MISCELLANEOUS SOLUTIONS

(a) Acrylamide stock solution:

30 grams acrylamide + 0,8 g N, N'-methylenebis-
acrylamide (bis-acrylamide) made up to 100 ml with
distilled H₂O and stored at 4°C in the dark.

(b) Ammonium Persulphate solution:

The ammonium persulphate solution should be made up
fresh each week.

0,1 g Ammonium Persulphate in 1 ml distilled H₂O.
Store at 4°C.

(c) TEMED - NNN'N' - Tetramethylethylenediamine

This is used directly. The solution is stable when kept at 4°C in the dark.

1.2.3. CALIBRATION MARKER PROTEIN SETS FOR MOLECULAR WEIGHT DETERMINATION USING ELECTROPHORESIS.

High molecular weight (HMW) and low molecular weight (LMW) calibration marker protein sets were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The contents of these sets are as follows :

(a) HMW Calibration Set

Protein	Mol.Wt.	Subunit Mol.Wt.
Thyroglobulin	669000	330000
Ferritin	440000	18500 (220000)
Catalase	232000	60000
Lactate Dehydrogenase	140000	36000
Albumin	67000	67000

Treatment - To each ampoule add 100 µl HMW sample buffer (Appendix 1.2.1(d)) and heat at 60°C for 15 minutes.

(b) LMW Calibration Set

Protein	Subunit Mol.Wt.
Phosphorylase b	94000
Albumin	67000
Ovalbumin	43000
Carbonic Anhydrase	30000
Trypsin Inhibitor	20100
α -Lactalbumin	14400

Treatment - To each ampoule add 100 μ l of LMW sample buffer (Appendix 1.2.1(d)) and heat at 100°C for 5 minutes.

1.2.4. RADIOISOTOPES

D - (6 - ^3H) Glucosamine (Specific Activity, 34,6 Ci/mmol), and L - (U - ^{14}C) Amino acid mixture (Specific Activity, 10 mCi/mmol) were obtained from Amersham Corp., Buckinghamshire, England.

Composition of L-(U- ^{14}C) Amino acid mixture :

The following amino acids are present in equimolar proportions:

L-(U-¹⁴C) Alanine
L-(U-¹⁴C) Arginine hydrochloride
L-(U-¹⁴C) Aspartic acid
L-(U-¹⁴C) Glutamic acid
(U-¹⁴C) Glycine
L-(U-¹⁴C) Leucine
L-(U-¹⁴C) isoleucine
L-(U)-¹⁴C) Lysine hydrochloride
L-(U-¹⁴C) Phenylalanine
L-(U-¹⁴C) Proline
L-(U-¹⁴C) Serine
L-(U-¹⁴C) Threonine
L-(U-¹⁴C) Tyrosine hydrochloride
L-(U-¹⁴C) Valine

1.2.5 EN³HANCE

The fluorographic solution EN³HANCE was obtained from New England Nuclear (Boston, Massachusetts).

1.3 PURIFICATION OF HERPES SIMPLEX VIRUS TYPE 1 (HSV-1)

GLYCOPROTEINS

- 1.3.1 HA Ultrogel (LKB Quality Chemicals, Bromma, Sweden).
- 1.3.2 Wheat germ lectin Sepharose 6MB and Helix pomatia lectin Sepharose 6MB were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.
- 1.3.3 Scintillation fluid
Beckman Ready-Solv HP was obtained from Beckman Instruments.
- 1.3.4 Saccharides
n-acetyl-D-glucosamine (GlcNAc) and n-acetyl-D-galactosamine (GalNAc) were obtained from Sigma Chemical Company, St. Louis, U.S.A.
- 1.3.5 Equilibration buffer used for gel-bound lectin affinity chromatography.
0,01 M tris (hydroxymethyl) aminomethane (Tris) - hydrochloride + 0,15 M NaCl + 0,02% (w/v) NaN₃ pH 8.0.
- 1.3.6 Phenylmethylsulphonyl fluoride (PMSF) was obtained from Sigma Chemical company, St. Louis, U.S.A.

PMSF is a proteinase inhibitor. Prepare a solution of 0,1 M PMSF in n-propanol. Dilute to 0,001 M PMSF in the solution of protein.

APPENDIX 2 - METHODS2.1. PRODUCTION OF HERPES SIMPLEX VIRUS2.1.1. GROWTH OF BABY HAMSTER KIDNEY (BHK-21) CELLS FROM AMPOULES STORED IN LIQUID NITROGEN AT -180°C

- (a) An ampoule containing BHK-21 cells was removed from storage in liquid nitrogen (Appendix 2.1.3) and immediately placed in a beaker of warm water. This was done to thaw the cell suspension rapidly and so reduce cell damage.
- (b) The contents of the ampoule were removed and diluted approximately five-fold with 10% MEM. This cell suspension was centrifuged at 800 rpm for 5 minutes to pellet the cells. The supernatant fluid was discarded and the cells resuspended in another 5 ml of 10% MEM in order to wash away any remaining Dimethylsulphoxide (DMSO) from the cells (See Appendix 2.1.3)
- (c) The cell suspension was centrifuged for another 5 minutes at 800 rpm and then the supernatant discarded. The cells were resuspended in exactly 5,0 ml of 10% MEM and then counted (Appendix 2.1.4).
- (d) Using the cell count, the cell suspension was diluted with 10% MEM to give a cell concentration of 2×10^5 cells/ml. 20 ml of this cell suspension was placed in a medical flat bottle and the bottle then incubated at 37°C.

2.1.2. PASSAGE OF BHK-21 CELLS

- (a) BHK-21 cells were passaged when the cell cultures were confluent. The growth medium was discarded and the cell monolayer was washed with sterile physiological saline to rinse off the remaining growth medium.
- (b) 5 ml of activated trypsin versene (ATV) (Appendix 1.1.4(f)) which had been pre-heated to 37°C, was added to the cell monolayer and distributed evenly over the surface of the cells. The cells were incubated with ATV at 37°C for 20 minutes.
- (c) After the 20 minute incubation the bottle was shaken to dislodge any cells still attached to the growth surface. The cell suspension was collected and centrifuged at 800 rpm for 5 minutes, after which the supernatant fluid was discarded and the cells resuspended in 10 ml of 10% MEM to wash off any remaining ATV and to inactivate the trypsin.
- (d) The cell suspension was again centrifuged at 800 rpm for 5 minutes and the supernatant fluid was discarded. The pellet of cells was resuspended in exactly 10,0 ml of 10% MEM and the cells were counted (Appendix 2.1.4).
- (e) The cell suspension was diluted to a concentration of 2×10^5 cells/ml and 20 ml of this cell suspension was used to seed each new medical flat bottle. The culture bottles were then incubated at 37°C.

2.1.3. STORAGE OF BHK-21 CELLS IN LIQUID NITROGEN at -180°C

- (a) The growth medium was removed from a confluent BHK-21 cell monolayer and the cells were washed with sterile physiological saline to rinse off the remaining growth medium.
- (b) The cells were harvested using ATV. (Appendix 2.1.2(b)) and then washed in 10% MEM.
- (c) After washing, the cell suspension was centrifuged at 800 rpm for 5 minutes and the supernatant fluid was discarded.
- (d) The pellet of cells was resuspended in a total volume of 2 ml of "Freezing Buffer" (Appendix 1.1.4(b)) and the cells were counted (Appendix 2.1.4).
- (e) The suspension of cells in freezing buffer was then diluted to a final concentration of 1×10^6 cells/ml with freezing buffer. This cell suspension was stored in ampoules containing 1 ml each.
- (f) The ampoules were frozen in a controlled rate freezing machine in order to reduce the amount of cell damage, and then stored in liquid nitrogen at -180°C.

2.1.4. CELL COUNTING

- (a) The suspension of cells to be counted is made up to a known volume with growth medium. If the cell concentration is high, then a 1/10 dilution is made by adding 0,1 ml of suspension to 0.9 ml of 10% MEM.
- (b) Dilute the cell suspension 1:1 in Trypan Blue solution (Appendix 1.1.5(d)). Normally, 0.1 ml of each is sufficient.
- (c) Place a clean coverslip over the graduated section of a haemocytometer and apply the trypan blue stained cell suspension to the graduated section so that the surface is just covered.
- (d) Place the haemocytometer under a microscope and count the number of cells in four large squares. (Each large square is subdivided into sixteen squares.)
- (e) From the number of cells in each large square, calculate the average number of cells per large square.
- (f) Assuming that the volume of cell suspension covering a large square is 10^{-4} ml, the cell concentration is calculated as follows:-
- Cell concentration = Average cell count x 2 (dilution with trypan blue) x 10^4 cells/ml.

If the original cell suspension was diluted 1/10, then the result must be multiplied by a factor of 10.

- (g) Multiply this value by the total volume of cell suspension in order to get a total cell count.

2.1.5. TEST FOR MYCOPLASMA CONTAMINATION IN BHK-21 CELL CULTURES

- (a) Grow BHK-21 or HeLa cells on coverslips in tubes. When the cells are confluent, remove the coverslips from the tubes and place them in small bijou bottles. The coverslips must not be allowed to dry as this will cause artifacts.
- (b) Add 5 ml of fixing solution (Appendix 1.1.5(f)) to each bijou bottle. After 5 minutes, remove the fixing solution and replace with a further 5 ml for another 10 minutes.
- (c) Discard the fixing solution and allow the coverslips to air dry.
- (d) Remove the "working stain" (Appendix 1.1.5(g)) from refrigeration at 4°C and mix thoroughly for 30 minutes at room temperature, using a magnetic stirrer.
- (e) Attach each coverslip to a microscope slide using D.P.X Mountant (Appendix 1.1.5(j)). The coverslips should be attached with the cells uppermost.

- (f) Place the slides flat in a container which can be darkened (i.e. a tin) and cover the cells with "working stain". Close the tin and allow the cells to stain for 30 minutes at room temperature.
- (g) After staining, rinse each coverslip three times with distilled water.
- (h) Detach the coverslips from the microscope slides and while still wet, mount them on clean microscope slides using mounting fluid (Appendix 1.1.5(h)). The stained cell monolayer must be positioned between the coverslip and the microscope slide.
- (i) Examine the slides under a fluorescence microscope using incident light at 360 nm.

2.1.6. GROWTH OF HERPES SIMPLEX VIRUS IN BHK-21 CELLS

- (a) Grow BHK-21 cells in cell culture flasks (Appendix 2.1.2). When the cells are confluent, pour off the growth medium and inoculate the cells with 2 ml of the stock virus suspension containing 1,0 TCID₅₀/cell (Appendix 2.1.7). The inoculum should be spread over the entire surface of the cell culture.
- (b) Incubate the culture flask in a horizontal position for 1 hour at 37°C to allow the virus to adsorb to the cells. After the 1 hour incubation period, add another 20 ml of maintenance medium (Appendix 1.1.3(d)) and continue to incubate the cells at 37°C.

(c) Harvest the cells at 24 hours post-infection.

2.1.7. STORAGE OF STOCK HERPES SIMPLEX VIRUS

HSV-1 and HSV-2 were grown in BHK-21 cells. When the cells showed 100% cytopathic effect (CPE) the culture bottles were frozen and thawed to remove cells from surface of the bottle. The suspension of HSV infected BHK-21 cells in culture medium was mixed with 10% skimmed milk solution at a ratio of 1 part skimmed milk to 5 parts culture medium. This suspension was stored at -70°C in ampoules containing 2 ml.

2.1.8. TITRATION OF HERPES SIMPLEX VIRUS IN BHK-21 CELLS

- a) Make up a BHK-21 cell suspension in 10% MEM containing 2×10^5 cells/ml. Place 4 drops of this cell suspension in each well of a flat bottomed microtitre plate.
- b) Seal the top of the microtitre plate with plate sealing tape and incubate the plate at 37°C until the cell cultures are confluent.
- c) When the cell cultures are confluent, remove the growth medium from each well using a pasteur pipette.
- d) Make a 10-fold dilution series in maintenance medium (Appendix 1.1.3(b)) of the HSV-infected material which is to be titrated. Place one drop of each dilution to each of six wells on the microtitre plate as shown in the diagram (Figure 2.1.1).

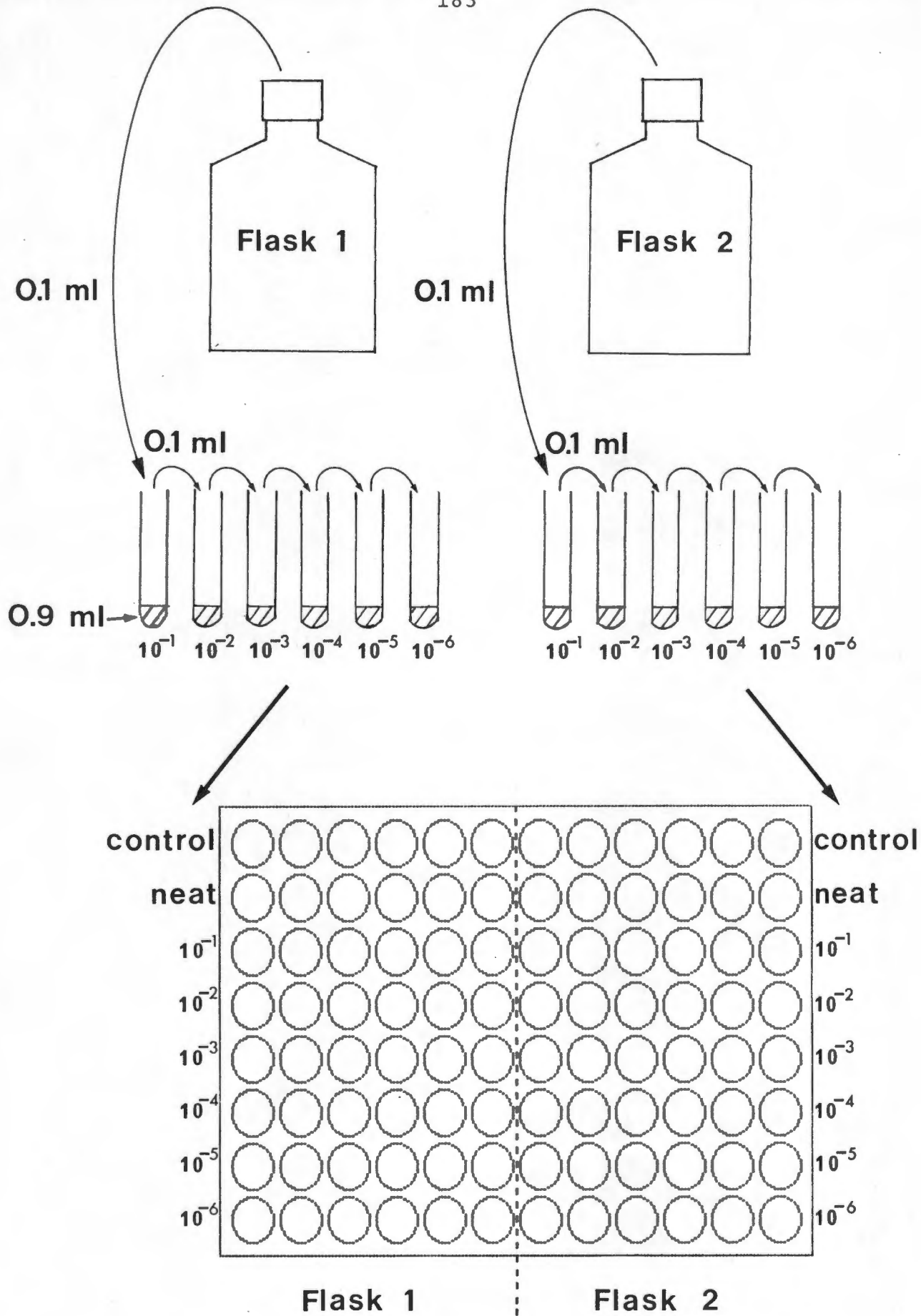


Figure 2.1.1 Diagram illustrating the titration of HSV on BHK-21 cells.

- (e) Seal the plate with plate sealing tape and incubate at 37°C for 1 hour to allow the virus to adsorb to the cells. After 1 hour, add a further three drops of maintenance medium to each well, seal the plate and continue incubation at 37°C.
- (f) Study the microtitre plate under an inverted light microscope every day for 7 days. Note on a record sheet which cell cultures show positive signs of HSV induced cytopathic effect (CPE).
- (g) After 7 days no further infection is likely and the study is terminated. Using the recorded results, calculate the 50% tissue culture infective dose (TCID₅₀) which is the dose at which 50% of the cell cultures are infected.

The TCID₅₀ is calculated by the method of Reed and Muench (Reed & Muench, 1938) as follows:-

Using the example in Table 2.1.1, none of the dilutions gives a 50% infectivity endpoint: this lies between the second and third dilutions. The TCID₅₀ is calculated from the cumulated values, assuming that the proportion of the cultures infected varies linearly with log₁₀ dilution. The interpolated value is given by:-

$$h \times \frac{\% \text{ cultures infected at dilution next above } 50\% - 50\%}{\% \text{ cultures infected at dilution next above } 50\% - \% \text{ cultures infected at dilution next below } 50\%}$$

In this formula h is the log of a dilution step. The interpolated value is then added arithmetically (i.e. with the proper sign) to the log of the total dilution at the step just above 50% of infected cultures (in this case the second dilution). In the example:

$$\text{Interpolated value} = h \frac{100 - 50}{100 - 0} = h \frac{50}{100} = 0,5h \text{ and } h = 1/10$$

$\log h = -1$; the total dilution at the second step is $(1/10)^2 = 10^{-2}$ and $\log \text{ dilution} = -2$. The interpolated value then is $(-1) \times 0,5 = -0,5$. The $\log \text{ TCID}_{50} = -2 + (-0,5) = -2,5$. The TCID_{50} titre is expressed as $= 10^{-2,5}$; i.e. the virus sample contains $10^{2,5} \text{ TCID}_{50}$ doses.

TABLE 2.1.1 - EXAMPLE OF ENDPOINT TITRATION

				CUMULATED VALUES			
DILUTION	INFECTIVITY			TOTAL	TOTAL	INFECTIVITY	
STEP	RATIO	INF	UNINF	INF	UNINF	RATIO	%
1	6/6	6	0	12	0	12/12	100
2	6/6	6	0	6	0	6/6	100
3	0/6	0	6	0	6	0/6	0
4	0/6	0	6	0	12	0/12	0
5	0/6	0	6	0	18	0/18	0
6	0/6	0	6	0	24	0/24	0

INF = Infected

UNINF = Uninfected

2.1.9. DISRUPTION OF HSV INFECTED CELLS BY ULTRASONICATION.

HSV infected cells were disrupted by ultrasonication using the Virsonic Cell Disrupter, Model 16-850 (The Virtis Company, Gardiner, New York.) In all cases, the "intermediate tip" was used at 50% power.

2.1.10. DENSITY DETERMINATION OF CAESIUM CHLORIDE SOLUTIONS

- (a) Samples of caesium chloride (CsCl) containing 0,5g; 1,0g; 2,0g; 3,0g; 4,0g; and 5,0g were weighed out accurately.
- (b) The CsCl samples were made up to 5 ml with buffer (0,01 M Tris, pH 7,4 + 1% foetal calf serum (FCS)) giving solutions of 0,1; 0,2; 0,4; 0,6; 0,8 and 1,0 g/cm³ respectively.
- (c) A clean, dry pycnometer was weighed.
- (d) The pycnometer was filled with distilled water and weighed. (Ensure that the outside of the pycnometer was dry and clean.)
- (e) The room temperature was recorded.
- (f) From the difference in mass between the empty and full pycnometer, the volume of the pycnometer at room temperature was calculated. The density of water at room temperature was obtained from tables.
(Documenta Geigy)
- (g) The pycnometer was emptied and dried before filling with the CsCl solutions, beginning with the most dilute. The pycnometer was reweighed with each of the CsCl solutions.
- (h) The mass of each CsCl solution in the pycnometer was calculated and hence the density determined.

- (i) The refractive indices of the CsCl solutions were read using an Abbé Refractometer.
- (j) The graph of Refractive Index against Density was drawn (Figure 2.1.2).

Temperature (Room Temp)	19°C
Mass of clean and dry Pycnometer	10,6357
Mass of Pycnometer & Distilled Water	12,6643
Mass of Distilled Water	2,0286
Density of water at 19°C	0,998407
Volume of Pycnometer	2,0318

Mass of Pycnometer + CsCl solution

0,1 g/ml.....	12,8248
0,2 "	12,9787
0,4 "	13,2353
0,6 "	13,5848
0,8 "	13,8738
1,0 "	14,1408

Concentration of CsCl (g/ml)	Mass of Pycnometer + CsCl (g)	Mass of CsCl Solution (g)	Density (g/cm ³)	Refrac- tive Index
0	12.6643	2.0286	0.998425	2.3340
0.1	12.8248	2.1891	1.0774	1.3424
0.2	12.9787	2.3430	1.1532	1.3500
0.4	13.2853	2.6496	1.3941	1.3644
0.6	13.5848	2.9491	1.4515	1.3781
0.8	13.8738	3.2381	1.5937	1.3913
1.0	14.1408	3.5051	1.7251	1.4035

See Figure 2.1.2

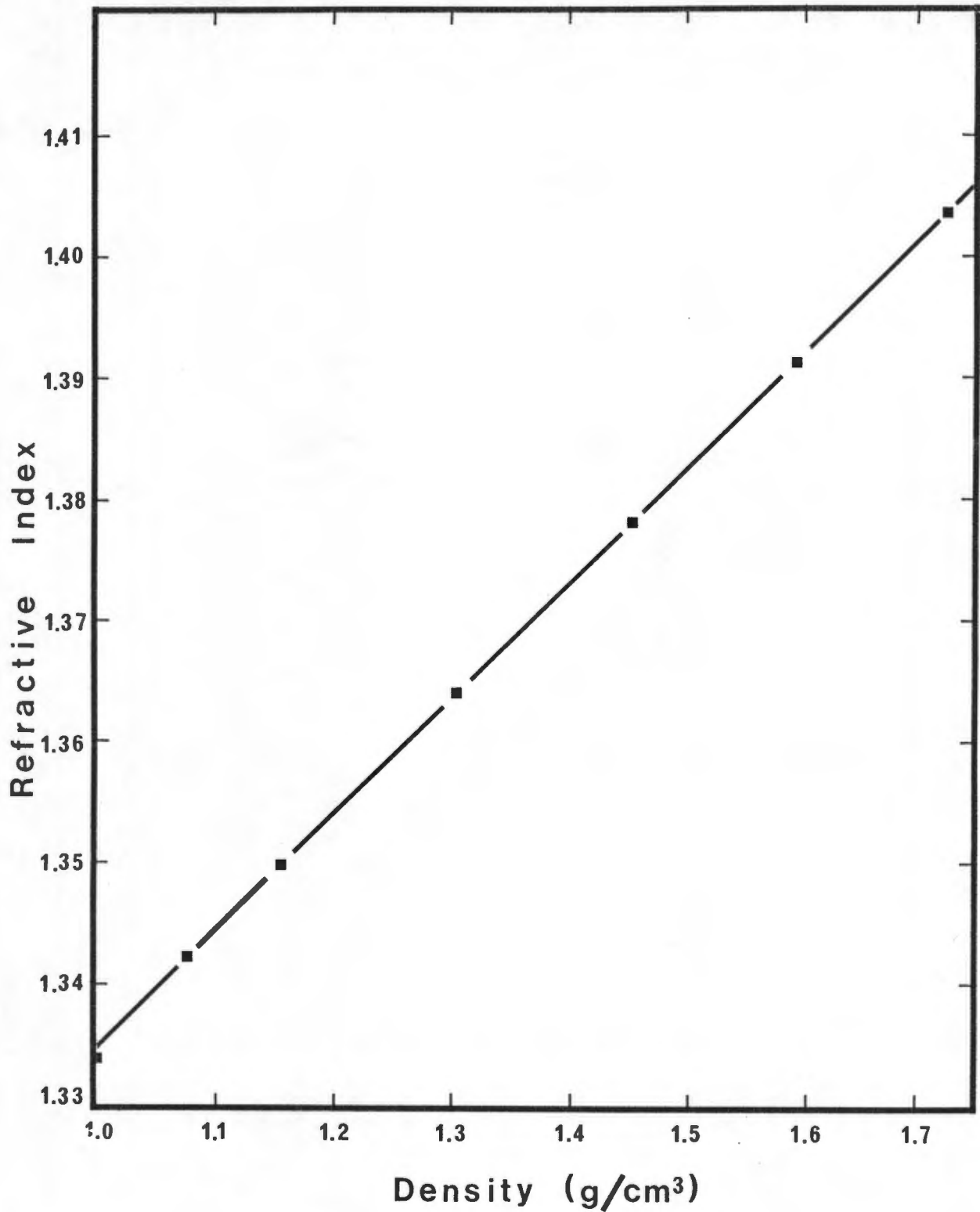


Figure 2.1.2 Graph of refractive index (RI) against density for solutions of caesium chloride (CsCl) in 0.01M Tris, pH 7.4 +1% foetal calf serum.
Coefficient of correlation = 0.9998.

2.1.11. HERPES SIMPLEX VIRUS PURIFICATION BY CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION.

- (a) Herpes simplex virus, type 1 (HSV-1) infected BHK-21 cells were harvested in 20 ml of 10% MEM by freezing and thawing. The infected cell suspension was centrifuged at 10 K rpm for 10 minutes and the supernatant fluid stored at 4°C. The pellet of infected cell debris was resuspended in approximately 3 ml of 10% MEM and then ultrasonicated for 10 seconds (Appendix 2.1.9). The suspension was again centrifuged at 10 K rpm for 10 minutes, after which the supernatant fluid was collected and stored at 4°C and the pellet of cellular material was discarded.
- (b) The HSV was collected from the infected medium by centrifuging the medium at 25 K rpm for 120 minutes at 4°C in a Beckman SW 25.1 rotor. In order to prevent the herpes virions being damaged by pelleting, 4 ml of caesium chloride solution was placed at the bottom of the tube to cushion the sedimenting virus. This cushion had a density of 1,30 g/cm³ in 0,01 M Tris/HCl pH 7,4 buffer + 1% foetal calf serum.
- (c) After centrifugation, the CsCl cushion was carefully removed from the bottom of the tube and inserted into two CsCl density gradients (Figure 2.1.3). The CsCl density gradients were then centrifuged at 40 K rpm for 20 hours at 4°C in a Beckman SW 50.1 rotor.

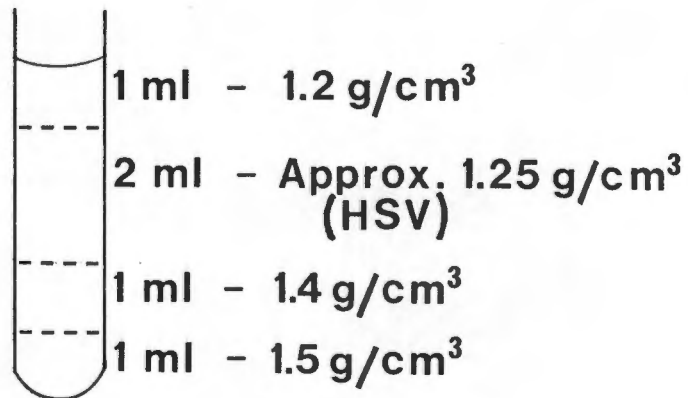


Figure 2.1.3 - Diagram illustrating the formation of CsCl density gradients and showing the position of the HSV containing material before centrifugation.

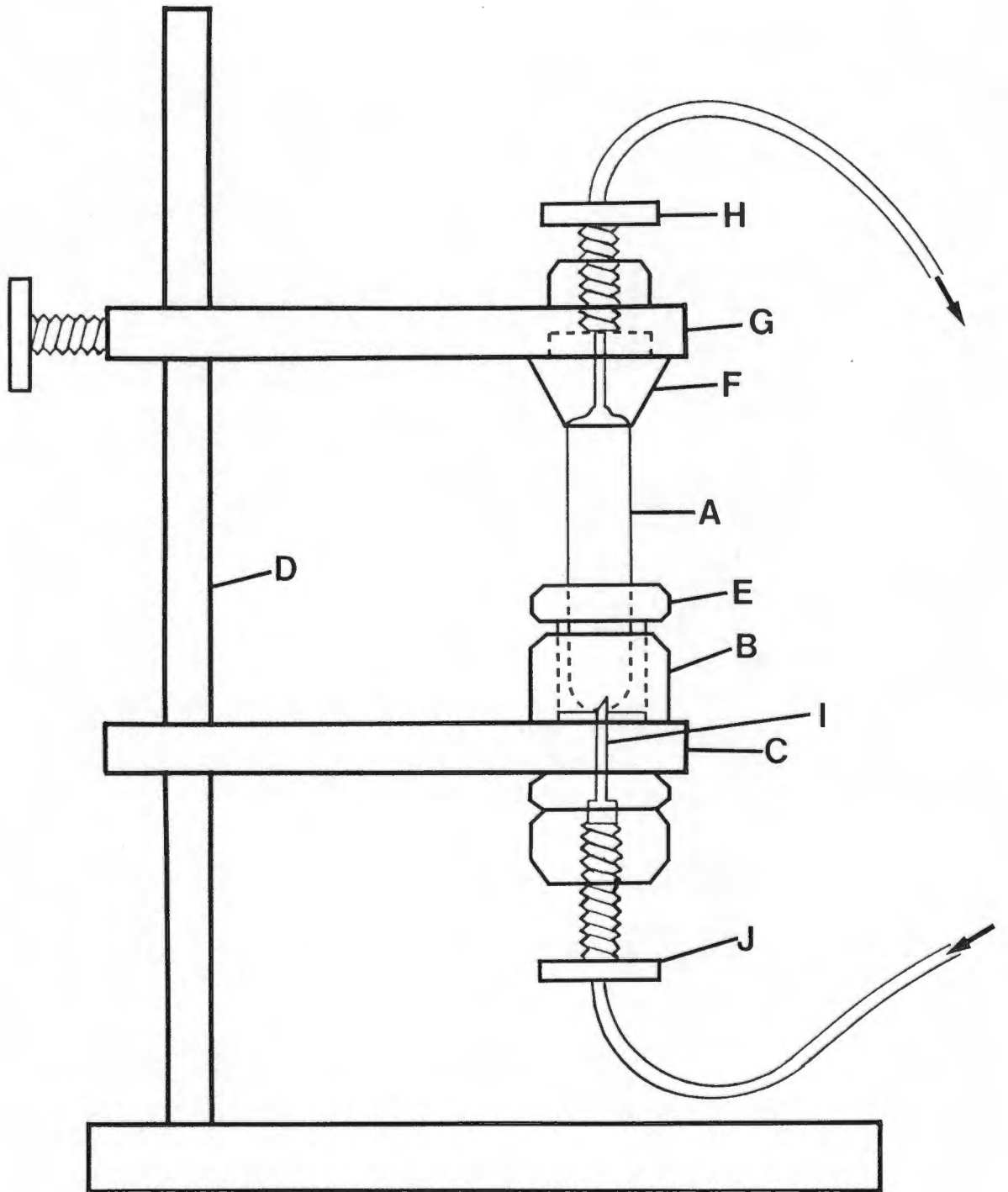
- (d) When the centrifuge tubes were viewed under a fluorescent light after centrifugation, two light scattering bands were visible (Figure 1.8). The two light scattering bands from one of the gradients were carefully removed by hand and diluted to approximately 5 ml in 0,01 M Tris/HCl pH 7,4. These solutions were then centrifuged at 20 K rpm for 60 minutes in a Beckman SW 50.1 rotor. The resultant pellets were studied by electron microscopy. (Appendix 2.1.12) (Figure 1.9)

- (e) The second CsCl density gradient was collected in 15 drop fractions using the apparatus shown in Figure 2.1.4. Each fraction was titrated on BHK-21 cells (Appendix 2.1.8) and the refractive index read on an Abbé Refractometer. The density of CsCl in each fraction was determined from the graph of refractive index against density (Figure 2.1.2).

FIGURE 2.1.4 - UPWARD DISPLACEMENT FRACTIONATOR USED IN
FRACTIONATING CsCl DENSITY GRADIENTS.

Operation

Insert the centrifuge tube (A), containing the material for fractionation, into the cup (B) which is attached to the fixed arm (C) of the stand (D). When using smaller diameter centrifuge tubes, an insert (E) is placed in the cup to accommodate the smaller tube. Place the sealing cone (F) on top of the centrifuge tube and lower the moveable arm (G) onto the sealing cone so as to hold the centrifuge tube firmly between the two arms. Wind in the upper screw (H) so as to apply extra pressure and improve the seal between the sealing cone and the centrifuge tube. Using a peristaltic pump, displace all air from the lower section of tubing by filling the tubing with Maxidens (Appendix 1.1.5(i)) until the Maxidens begins to emerge from the tip of the syringe needle (I). Wind in the lower screw (J) so that the tip of the syringe needle penetrates the centrifuge tube by approximately 2 mm. Slowly pump Maxidens into the centrifuge tube and collect the displaced contents of the centrifuge tube through the upper section of tubing.



2.1.12. ELECTRON MICROSCOPY

In order to study HSV by electron microscopy, the virus is pelleted by centrifuging at 20 K rpm for 60 minutes in a Beckman SW 50.1 rotor. After centrifugation, the supernatant fluid is discarded and the pellet is negatively stained with 2% phosphotungstic acid (PTA), pH 6,0, before examination in a Hitachi 600 electron microscope.

2.2 RADIOLABELLING AND ANALYSIS OF HSV GLYCOPROTEINS

2.2.1 SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

A. Preparation of SDS-Polyacrylamide slab gels.

- (a) Wash the glass plates with water.
- (b) Dry and then wipe with methanol.
- (c) Clean the side pieces (spacers) and place on the larger glass plate.
- (d) Place the smaller plate on top of the spacers, with the clean side inward.
- (e) Fit in a clean comb and ensure that the pieces fit tightly together. Clamp the glass plates together with "bulldog-clips".
- (f) Seal the bottom of the cassette with parafilm
- (g) Place the rubber gasket in position on the stand.
- (h) Prepare the wick gel, running gel and stacking gel ingredients according to Table 2.2.1. Do not add ammonium persulphate (APS) or TEMED (Appendix 1.2.2) at this stage.
- (i) Add TEMED and APS to the wick gel mixture. Remove the comb from the cassette and immediately pour the wick gel mixture into the cassette.
- (j) Remove the parafilm from the cassette and attach the cassette to the stand with the small glass plate facing the buffer reservoir.
- (k) Add TEMED and APS to the running gel mixture. Shake to mix and then pour quickly into the cassette to

approximately 1 cm from the top of the small glass plate.

- (l) Carefully layer distilled water onto the surface of the running gel to seal it from the air which prevents polymerization.
- (m) When running gel has set, remove the water and add TEMED and APS to the stacking gel mixture and pour to the top of the cassette.

TABLE 2.2.1 - COMPOSITION OF PAA GELS OF DIFFERENT
ACRYLAMIDE CONCENTRATION

Ingredients	Wick Gel	Running Gel		Stacking Gel
	15%	7.5%	10%	3,0%
	ml	ml	ml	ml
Acrylamide (30%)	1.0	2.0	2.67	0.5
Running Gel Buffer	0.5	2.0	2.0	-
Stacking Gel Buffer	-	-	-	1.25
Distilled Water	0.5	4.0	3.33	3.25
10% APS	0.025	0.025	0.025	0.025
TEMED	0.025	0.025	0.025	0.025

- (n) Place the comb in position and allow the gel to set.
- (o) To prevent reservoir buffer leaking between the gel cassette and the electrophoresis apparatus, the

surface between the glass plate and the top reservoir should be sealed with 1% agarose gel.

(p) Store the apparatus in a moist container at 4°C until required.

B. Sample Preparation

Samples for analytical SDS-PAGE were added to a mixture of 100% glycerol and phenol red dye so as to give a final glycerol concentration of approximately 25% (v/v). The volume of sample applied to each well was varied according to the protein concentration of the sample.

C. Electrophoresis

Electrophoresis was performed at a constant current of 8 mA per gel.

D. Fixing

After electrophoresis in PAA gel, the proteins must be "fixed" in order to prevent them from being washed out of the gel during the staining procedure. The proteins were fixed by immersing the PAA gel in a solution of 20% (w/v) Trichloroacetic acid (TCA) for a minimum of 30 minutes.

2.2.2. STAINING PROCEDURES FOR PROTEINS IN POLYACRYLAMIDE GELSA. Coomassie Brilliant Blue Stain (CBB)

Stain - 0.05% CBB (w/v) dissolved in a solution containing 30% methanol, 10% acetic acid and 60% distilled water.

Destaining Solution - 30% methanol, 10% acetic acid and 60% distilled water.

Method - After fixing the proteins in 20% TCA, immerse the PAA gel in stain overnight. Then discard the stain and wash the gel with several changes of destaining solution until all of the background stain has been removed from the gel and the background is completely transparent.

B. Silver Stain - Method 1 (Taken from Merril, Goldman and Van Keuren, 1982)

(a) After fixing the proteins in 20% TCA, wash the gel for 30 minutes with a solution containing 10% ethanol and 5% acetic acid (v/v).

(b) Soak the gel for 5 minutes in a solution containing 0,0034 M potassium dichromate and 0.0032 N Nitric acid.

(c) Transfer the gel to a solution containing 0,012 M silver nitrate and soak for at least 2 hours.

- (d) Rinse the gel by agitation in a solution containing 0,28 M sodium carbonate and 0,5 ml formaldehyde (37% commercial formaldehyde) per litre. This step requires at least two changes of solution to prevent precipitated silver salts from adsorbing to the surface of the gel.
 - (e) Development is usually stopped when a slightly yellowish background appears. This is done by immersing the gel in a solution of 1% (v/v) acetic acid.
- C. Silver stain - Method 2 (Taken from Sammons, Adams and Nishizawa, 1981)
- (a) After fixing the proteins, wash the gel extensively (i.e. overnight) in a solution of 10% ethanol and 0,5% acetic acid to completely remove SDS.
 - (b) Immerse the gel in AgNO_3 solution containing 1,9 g/litre for at least 2 hours.
 - (c) Rinse the gel twice in distilled water to remove excess surface silver.
 - (d) Immerse the gel in a reducing solution of 0,75 M NaOH, 7,5 ml/litre formaldehyde (37% commercial formaldehyde) and 87,5 mg/litre NaBH_4 .
 - (e) For the best results, the gel should be removed from the reducing solution within 10 minutes and placed

into a colour enhancing solution of 0,75% Na₂CO₃ for 30 minutes.

- (f) Stop development by immersing the gel in a solution of 1% (v/v) acetic acid.

2.2.3. DRYING OF POLYACRYLAMIDE GELS

The PAA gels should first be soaked for at least 10 minutes in 3% glycerol. The glycerol prevents cracking of the gel during drying. The PAA gel is then wrapped in wet, porous cellophane (Hoefer Scientific Instruments) and dried under vacuum at 80°C, using a slab gel dryer (Hoefer Scientific Instruments).

2.2.4. AUTORADIOGRAPHY

(a) Impregnation with EN³HANCE

After proteins have been fixed in the PAA gel, (Appendix 1.2.3.), the gel is impregnated with EN³HANCE (Appendix 1.2.5) by adding enough EN³HANCE so that the gel is free floating. The gel should remain in EN³HANCE for at least one hour with gentle agitation.

(b) Precipitation

Once the gel has been impregnated with EN³HANCE, discard the used enhancer solution into an appropriate radioactive waste container. An excess of cold water is then added to the gel to precipitate the fluorescent material inside the gel. The gel should

be kept in water for one hour only with gentle agitation, after which the gel should appear uniformly opaque. (Longer times are unnecessary and may be detrimental.)

(c) Drying and Exposure

After the EN³HANCE has been precipitated into the gel, the gel is dried (Appendix 2.2.4). The dried gel is then exposed to X-ray film (either Kodak X-Omat MA or Kodak X-Omat AR5) for an appropriate length of time.

2.3 Purification of Herpes Simplex virus type 1 (HSV-1)
glycoproteins

2.3.1 Preparative Polyacrylamide Gel Electrophoresis (Prep-PAGE)

Preparative polyacrylamine gel electrophoresis (Prep-PAGE) is based on the same technique as analytical SDS-PAGE (Chapter 2.1.2) except that Prep-PAGE is performed in an apparatus which allows for the use of preparative amounts of material and the collection of samples during electrophoresis (Figure 2.3.1). The preparation of the PAA gel for Prep-PAGE is the same as that for analytical SDS-PAGE (Appendix 2.2.1) except that a "wick" gel cannot be used. The method is as follows:-

- (a) Remove the gel tube from the Prep-PAGE apparatus (Figure 2.3.1) and seal the bottom of the gel tube with parafilm.
- (b) Cast a 7,5% PAA running gel (Appendix 2.2.1) in the gel tube (Diameter, 1.3 cm) to a length of 6 cm.
- (c) When the running gel has set, cast a stacking gel (Appendix 2.2.1) above it. The gel tube containing the PAA gel can be stored for up to one week at 4°C

by sealing both ends of the tube with parafilm to prevent the gel dehydrating.

- (d) When the PAA gel is required, remove the parafilm from the gel tube and insert the gel tube into the Prep-PAGE apparatus (Figure 2.3.1). Attach the other instruments to the Prep-PAGE apparatus in order to give the complete set up required for Prep-PAGE as shown in the diagram.
- (e) Fill the buffer reservoirs with the reservoir buffer (Appendix 1.2.1(c)) as shown in the diagram and seal the lower section of the gel tube with dialysis membrane.
- (f) Solubilize the sample for electrophoresis in 1,0 ml of LMW sample buffer (Appendix 1.2.1(d)) and heat at 100°C for 5 minutes. Then add 0,3 ml of 100% glycerol containing phenol red dye.
- (g) Apply the prepared sample to the top of the PAA gel and electrophorese at 8 mA until the required material has been eluted from the bottom of the PAA gel. Fractions of 5 ml. were collected at a flow rate of approximately 8 ml/hour.
- (h) Assay 0,1 ml aliquots of each fraction for radioactivity (Appendix 2.3.5).
- (i) Draw an elution profile of counts per minute (CPM) against fraction number.

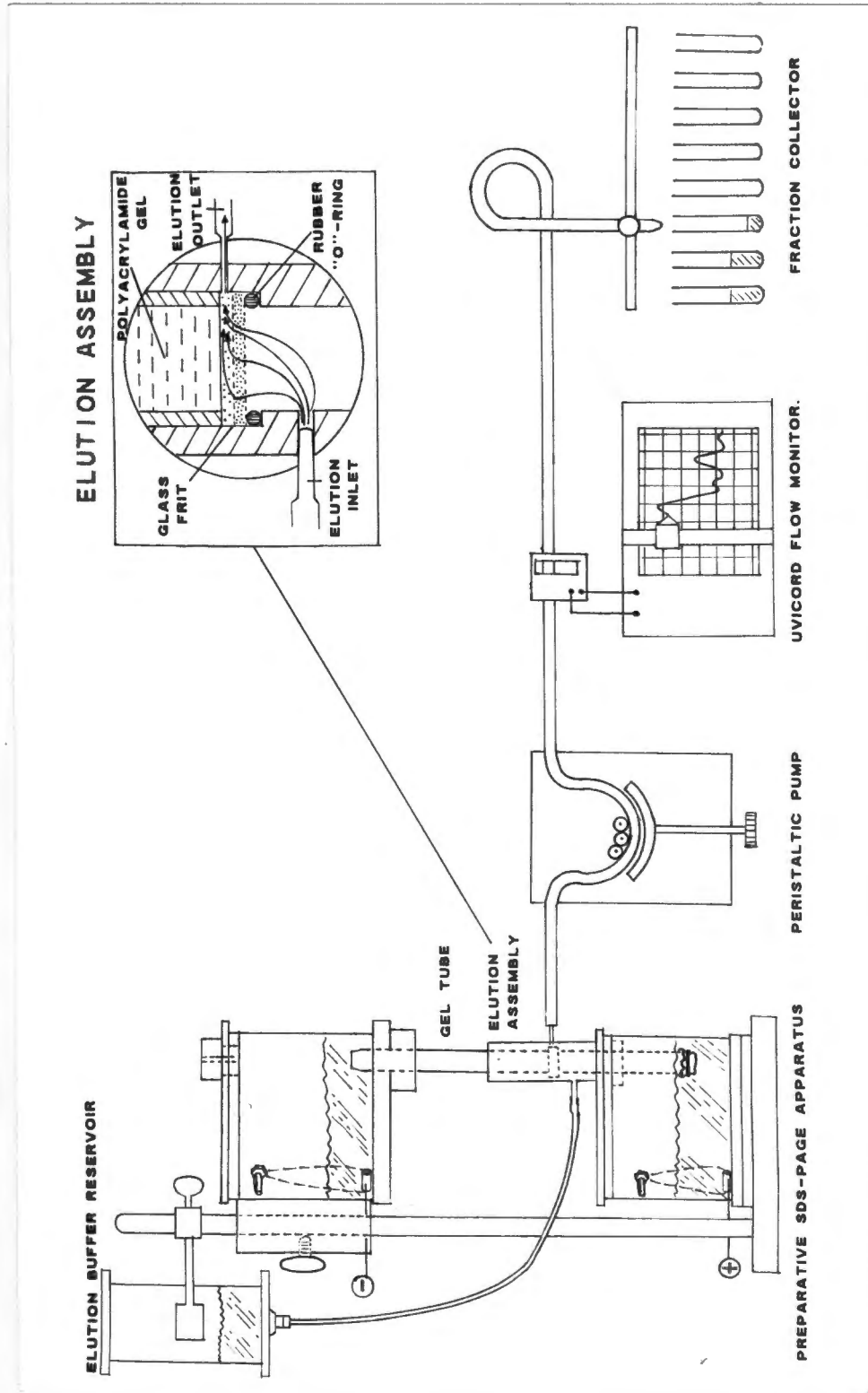


Figure 2.3.1 - Complete system for Preparative Polyacrylamide Gel Electrophoresis (Prep - PAGE).
 (Apparatus is similar to that shown in BRL catalog No. 1013-0)

2.3.2 Hydroxylapatite chromatography in the presence of sodium dodecyl sulphate (SDS-HTP).

Hydroxylapatite chromatography in the presence of sodium dodecyl sulphate (SDS-HTP) was performed according to the method of Eberle & Courtney (Eberle & Courtney, 1980(a)). The only change to the method was the use of HA Ultrogel (Appendix 1.3.1) instead of ordinary hydroxylapatite.

- (a) Pour 10 ml of HA Ultrogel into a column (Diameter 16 mm) and wash the column with 20 ml of 0,01 M phosphate buffer, pH 6,4, containing 0,1% SDS and 1 mM dithiothreitol at a flow rate of 8 ml/hr.
- (b) Samples for SDS-HTP chromatography were dialyzed overnight against 0,01 M phosphate buffer, pH 6,4, containing 0,1% SDS and heated at 100°C for 5 minutes before applying to the SDS-HTP column.
- (c) The material was eluted from the column using a linear gradient of increasing phosphate molarity from 0,1 M to 0,5 M phosphate buffer, pH 6,4, containing 0,1% SDS and 1mM dithiothreitol over a 6 hour period. The phosphate gradient was produced in a Gilson MixoGrad automatic gradient former (Gilson Medical Electronics, Inc.)
- (d) 5 ml fractions were collected at a flow rate of 16 ml/hr.
- (e) 0,1 ml aliquots of each fraction were assayed for

radioactivity (Appendix 2.3.5) and the elution profile of counts per minute against fraction number was drawn.

2.3.3 Gel-bound lectin affinity chromatography

Gel bound lectin affinity chromatography was used as a means to further purify glycoproteins, which had been separated by Prep-PAGE, from non-glycosylated proteins. The techniques used were as follows:-

A. Wheat germ lectin affinity chromatography.

Column preparation: 8 ml of wheat germ lectin Sepharose 6 MB (Appendix 1.3.2) was poured into a column (diameter 9 mm). The gel was then washed with 50 column volumes of equilibration buffer (0,01 M tris (hydroxymethyl) aminomethane (Tris)-hydrochloride - 0,15 M NaCl - 0,02% (wt/vol) NaN₃ (pH 8.0)) in order to remove preservatives.

Sample preparation: Material that had been prepared by Prep-PAGE and stored at -20°C (Chapter 3.2.1(B)) was thawed and dialysed overnight against 0,1 M glycine-sodium hydroxide buffer (pH 8,8) containing 1% Triton X-100. After dialysis, this material was applied to the wheat germ lectin column at a rate of 20 ml/hr.

Elution: After applying the sample to the wheat germ lectin column, the column was washed with a further 50 ml of equilibration buffer, at a flow

rate of 20 ml/hr to wash away any proteins which had not adsorbed to the column. The adsorbed proteins were eluted using 20 ml of 0,2 M N-acetyl-D-glucosamine (GlcNAc) in equilibration buffer with 1% Triton X-100 at a flow rate of 20 ml/hr.

B. Helix pomatia lectin affinity chromatography.

Column preparation: 2 ml of Helix pomatia lectin Sepharose 6 MB (Appendix 1.3.2) was poured into a column and washed with 50 column volumes of equilibration buffer (0,01 M tris (hydroxymethyl) aminomethane (Tris) - hydrochloride - 0,15 M NaCl - 0,02% (wt/vol) NaN₃ (pH 8,0) to remove preservatives.

Sample preparation: As for wheat germ lectin chromatography (Appendix 2.3.3(A)).

Elution: After applying the sample to the Helix pomatia lectin column, the column was washed with a further 20 ml of equilibration buffer at a flow rate of 20 ml/hr to wash away any unadsorbed proteins. Adsorbed proteins were eluted using 20 ml of 0,05 M N-acetyl-D-galactosamine (GalNAc) in equilibration buffer + 1% Triton X-100 at a flow rate of 20 ml/hr.

2.3.4 Preparation of HSV-1 membrane proteins in Triton X-100 for lectin affinity chromatography.

HSV-1 membrane proteins were prepared for lectin affinity chromatography using the method of Olofsson et al, 1981.

The method was as follows:-

- (a) HSV-1 infected BHK-21 cells were grown in the presence of (³H)- glucosamine ((³H)-gln), as described previously (Chapter 2.2.2).
- (b) At 28 hrs post-infection, the infected cells were shaken from the surface of the cell culture flask and the infected cell suspension was centrifuged at 1500 x g for 5 minutes in order to pellet the cells. The supernatant was discarded and the cell pellet stored at -70°C until required.
- (c) The packed cells were thawed and adjusted to 4 times their volume using ice-cold 0,025 M Tris-hydrochloride, pH 8,0. The cell extracts were kept on ice throughout the remainder of the procedure.
- (d) The infected cell suspension was ultrasonicated for three cycles of 5 seconds (Appendix 2.1.9) and the homogenate was centrifuged at 1500 x g for 5 minutes.
- (e) The supernatant was decanted and stored at 4°C and the cell pellet was resuspended in 2 ml of 0,025 M Tris-hydrochloride, pH 8,0 ultrasonicated and centrifuged at 800 rpm for 5 minutes. This procedure was repeated twice and the supernatants were pooled at 4°C.

- (f) The pooled supernatants were centrifuged at 27 000 x g for 1 hr. After centrifugation the supernatant was collected and the pellet discarded.
- (g) The membranes remaining in the supernatant were pelleted by centrifuging at 160 000 x g for 1 hr.
- (h) The pelleted membranes were resuspended in 5 ml of cold 0,1 M glycine - NaOH buffer, pH 8,8 and centrifuged at 160 000 x g for 1 hr.
- (i) The washed membranes were resuspended in 5 ml of cold 0,1 M glycine-NaOH buffer, pH 8,8 containing 1% Triton X-100 (wt/vol).
- (j) The membrane suspension was ultrasonicated for three cycles of 10 seconds and incubated on ice for 10 min. The membrane suspension was then centrifuged at 100 000 x g for 1 hr.
- (k) The supernatant was stored at -70°C until required for affinity chromatography.

2.3.5 Assay for Radioactivity

0,1 ml aliquots of aqueous solutions containing either (³H)-glucosamine or (¹⁴C)-amino acid labelled proteins were placed in scintillation vials. 3,0 ml of scintillation fluid (Appendix 1.3.3) was then added to each vial and mixed thoroughly. The radioactive content of each vial was read in a Beckman LS 8000 Liquid Scintillation System (Beckman Instruments).

2.3.6 Lyophilization (Freeze-drying)

Solutions of proteins can be concentrated by freeze-drying and then resuspending in the required volume of buffer. The method used for freeze-drying was as follows:-

- (a) If the volume of solution was excessive, it could be reduced by pervaporation (Appendix 2.3.7).
- (b) Dialyze the solution against the buffer in which the protein is dissolved in order to remove excess buffer salts.
- (c) Place the protein solution in a suitable container i e a glass-ampoule or test-tube.
- (d) Freeze the sample rapidly in 2-Methoxyethanol which has been pre-cooled with dry-ice to approximately -80°C . While freezing the sample, the container must be rotated constantly so that the sample freezes over as large an area as possible.
- (e) Expose the sample(s) to a vacuum of 0,1 Torr. until they are totally dehydrated.

2.3.7 Pervaporation

Another technique that can be used for concentrating protein solutions is pervaporation.

- (a) Place the protein solution in a dialysis tube and suspend the tube in a fridge or coldroom at 4°C . The solution becomes concentrated as water is evaporated.

- b) When the solution is sufficiently concentrated, dialyze the tube against the original buffer in which the protein is dissolved, in order to remove excess buffer salts.

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ADDENDUM

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